

IL-17-Producing Alveolar Macrophages Mediate Allergic Lung Inflammation Related to Asthma¹

Chuanwang Song,^{2*} Liqiong Luo,^{2*} Zhang Lei,* Bo Li,* Zhihui Liang,[†] Guanghui Liu,[‡] Dong Li,* Guimei Zhang,* Bo Huang,^{3*} and Zuo-Hua Feng^{3*}

IL-17 is a pivotal proinflammatory molecule in asthmatics. However, the cellular source of IL-17 in asthma has not been identified to date. In this study, we report that macrophages rather than Th17 cells are the main producer of IL-17 in allergic inflammation related to asthma. After OVA challenge in a mouse model mimicking allergic asthma, the increased IL-17⁺ cells in the lung were mainly CD11b⁺F4/80⁺ macrophages, instead of T cells or others. Importantly, IL-17⁺ alveolar macrophages (AMs), but not IL-17⁺ interstitial macrophages, were significantly increased after allergen challenge. The increase of IL-17⁺ AMs was not due to the influx of IL-17⁺ macrophages from circulation or other tissues, but ascribed to the activation of AMs by mediator(s) secreted by IgE/OVA-activated mast cells. Depleting alveolar macrophages or neutralizing IL-17 prevented the initiation of OVA-induced asthma-related inflammation by inhibiting the increase of inflammatory cells and inflammatory factors in bronchoalveolar lavage fluid. Th2 cytokine IL-10 could down-regulate IL-17 expression in alveolar macrophages. The increased IL-17 and the decreased IL-10 in bronchoalveolar lavage fluid were further confirmed in asthmatic patients. These findings suggest that IL-17 is mainly produced by macrophages but not Th17 cells in allergic inflammation related to asthma. Mast cell-released mediators up-regulate the expression of IL-17 by macrophages, whereas IL-10 down-regulates IL-17 expression. *The Journal of Immunology*, 2008, 181: 6117–6124.

Allergic asthma is a chronic inflammatory disorder of the lung characterized by a prevailing Th2 immune response and allergic airway inflammation (1). The balance between Th2 response and Th1 response has been thought to determine the allergic airway inflammation. It has been generally accepted that Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 play critical roles in orchestrating and amplifying allergic inflammation in asthma. Th1 cytokine IFN- γ was thought to prevent this process (2, 3), however, recent studies indicate that IFN- γ secreted by Th1 cells may cause severe airway inflammation under certain conditions (4, 5).

In addition to Th1/Th2 cytokines, a critical proinflammatory cytokine IL-17 has attracted great attention recently. IL-17 acts on a broad range of cells to induce the expression of cytokines (IL-6, IL-8, GM-CSF, G-CSF), chemokines, and metalloproteinases (6–9). It also cooperates with TLR ligands, IL-1 β , and TNF- α to enhance inflammatory reactions and stimulate the production of β -defensins and other antimicrobial peptides (10). The important roles of IL-17 in allergic asthma have also been noticed. Although it was speculated that IL-17 may have a negative effect on the established allergic asthma (11), IL-17/IL-17R signaling has been

proved to be critically required to develop allergic asthma (12). The concentration of IL-17 is significantly increased in bronchoalveolar lavage fluid (BALF),⁴ sputum, and blood from patients with asthma (13, 14). The overexpression of IL-17 in lung epithelium causes chemokine production and leukocyte infiltration in vivo (15). IL-17 promotes the recruitment and expansion of neutrophils (16). In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibits the allergen-induced accumulation of neutrophils in the airway (17). These findings suggest that IL-17 plays an important role during the pathophysiological process of allergic asthma. Despite the important role of IL-17 in allergic asthma, the cellular source of IL-17 in allergic asthma has not yet been identified, hindering the further elucidation of the mechanisms underlying the onset of allergic asthma.

Although Th17, the third subset of Th cells, has been considered as the primary producer of IL-17 (18, 19), other cell types can also be the source of IL-17, including CD8⁺ T cells, NK cells, and $\gamma\delta$ T cells (20–23). In this study, we intended to clarify whether Th17 is the main source of IL-17 in allergic inflammation related to asthma. Unexpectedly, we found that IL-17 was mainly produced by CD11b⁺ macrophages, rather than Th17, NK cells, or others. Such macrophage-derived IL-17 was required for the initiation of allergic inflammation in a mouse model mimicking allergic asthma. Allergen-induced allergic inflammation is concomitant with a significant increase of IL-17-producing alveolar macrophages but not interstitial macrophages. This process was negatively regulated by Th2 cytokine IL-10 and positively regulated by mast cell-released mediator(s).

*Department of Biochemistry & Molecular Biology, [†]Department of Immunology, Tongji Medical College, and [‡]Department of Allergy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, The People's Republic of China

Received for publication March 27, 2008. Accepted for publication August 21, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The work was supported by the National Natural Science Foundation of China (No. 30771974 and No. 30772589).

² C.S. and L.L. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Bo Huang or Professor Zuo-Hua Feng, Department of Biochemistry & Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, The People's Republic of China. E-mail addresses: tjhuangbo@hotmail.com or fengzhg@public.wh.hb.cn

⁴ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; BAL, bronchoalveolar lavage; 2-CA, 2-chloroadenosine; DC, dendritic cell; AM, alveolar macrophage; IM, interstitial macrophage; PM, peritoneal macrophage.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/\$2.00

Materials and Methods

Mice

Eight-week-old virgin female BALB/c mice were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College.

Allergic asthma patients and control subjects

Six patients (aged 35 ± 16 years old, range 26–54) with asthmatic symptom were recruited in the Department of Allergy of Tongji Hospital, Tongji Medical College. The diagnosis was based on the asthma guidelines proposed by the American Thoracic Society (24). The atopic status of patients and control subjects was ascertained by skin prick allergen tests and positive plasma-specific IgE assays to house dust mites, cat, dog, mixed cockroaches, and mixed molds. Six age-matched healthy individuals (aged 36 ± 11 years old, range 28–46) were recruited as normal control. All subjects were nonsmokers and free from upper respiratory tract infection for 4 wk preceding the study. This study was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained from all subjects.

Sensitization and allergen challenge in mouse model

Mice were sensitized by i.p. injection of 0.3 ml solution containing 100 μ g of OVA (Sigma-Aldrich) bound to Imject Alum (Pierce) on days 0, 7, and 14. The sensitized mice were challenged by the administration of aerosolized 1% OVA for 25 min on days 17, 18, and 19.

Preparation of BALF

Six days after sensitization or 1 day after the last challenge in mouse model, bronchoalveolar lavage (BAL) was performed four times by instillation of 1 ml of PBS through the tracheal cannula. After lysis of RBC, the cell numbers in BALF were determined using standard hematologic procedures. Cytospin of BALF was prepared and stained with the Wright-Giemsa method (Fisher Scientific). Differential cell counting was performed using standard morphological criteria.

For human subjects, BAL was performed via a flexible fiber-optic bronchoscope (Olympus BF1T). The upper respiratory tract was anesthetized with 2% lidocaine, and lavaged with 30 ml aliquots of 0.9% warmed saline to a total of 180 ml. The lavage fluid was gently aspirated after the collection of each aliquot. Aliquots of the cell-free BAL were stored at -70°C . Alveolar macrophages were isolated by plating total cells in six-well plates at a density of $1 \times 10^6/\text{ml}$ overnight and removing nonadherent cells. Among them, CD68⁺ cells were more than 95%, confirmed by flow cytometric analysis.

Assay for cytokines

For mouse cytokine detection, BAL was performed by instillation of PBS. The cell-free BALF supernatants of first 0.25-ml aliquot were collected after spinning down the cells. The amounts of IL-4, IL-5, IL-10, IL-13, and IL-17 in the BALF supernatants or IL-17 (IL-17A) and IL-17F in culture supernatants were determined by ELISA kits (R&D Systems). The minimum detectable dose was 2, 7, 4, 1.5, 5, and 4 pg/ml, respectively.

For human cytokine detection, the cell-free supernatants of the first 30-ml aliquot from the lavage were used for IL-10 and IL-17 detection by ELISA kit (R&D Systems).

Isolation of immune cells from lung

Lung tissue of mouse was digested with collagenase and hyaluronidase and minced. After lysis of RBC, the dissociated cells were underlaid with 5 ml of lymphocyte separation solution (Mediatech), centrifuged at 2,200 rpm for 20 min. The mononuclear cells in the middle layer were collected for flow cytometric analysis. The neutrophils were collected from the bottom of the tube.

For lung macrophage isolation, the above cells were incubated in a 6-well plate for 2 h and the adherent cells were harvested as lung macrophages. In certain cases, BALF was used for isolation of alveolar macrophages, and the lung after BAL was used for preparation of interstitial macrophages.

Flow cytometric analysis

The cells from BALF in mouse model were incubated with 1 $\mu\text{g}/10^6$ cells of Fc receptor-blocking Ab (clone 24G2; American Type Culture Collection, Manassas, VA), and then stained with PE-Cy3-conjugated anti-mouse CD3, allophycocyanin-cy5-conjugated anti-mouse CD11b, PE-conjugated

anti-mouse DX5, allophycocyanin-conjugated anti-mouse c-Kit, and PE-conjugated anti-mouse F4/80 Abs. After cellular surface staining, cells were washed, fixed, and permeabilized with Fix-Perm solution for intracellular staining with FITC-conjugated anti-mouse IL-17 Ab (clone eBio17B7, eBioscience). In the same way, the cells from BALF of human subjects were stained with PE-conjugated anti-human F4/80 and FITC-conjugated anti-human IL-17 Abs. All fluorophore-conjugated Abs and the corresponding isotypes were purchased from eBioscience. The stained cells were used for flow cytometric analysis (BD LSR II).

Alveolar macrophage depletion

In mouse model, alveolar macrophages were depleted by administering 2-chloroadenosine (2-CA) (25). Sensitized mice were given the aerosolized 5 mM of 2-CA (Sigma-Aldrich) for 20 min, driven at a rate of 0.75 ml/min. Aerosolized 2-CA was administered twice, 12 h before and 4 h after the first OVA challenge. More than 75% of alveolar macrophages were depleted, evaluated by counting the macrophages in BALF from treated and untreated mice, but other type of cells were not influenced.

Generation of bone marrow-derived mast cells

Bone marrow cells were harvested from femurs of mice and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, and 50 μM 2-ME. The cells were cultured in the presence of IL-3 and SCF (10 ng/ml each, PeproTech), and the nonadherent cells were passaged every 3 days. 4 wk later, 98% of the cells were c-Kit⁺ mast cells, confirmed by flow cytometry.

Histochemistry

The lung of mouse was excised, fixed in 4% buffered formalin for 12 h, and embedded in paraffin. Sections of lung (5 μm thick) were prepared and stained with H&E.

Western blot

Cell lysates and prestained m.w. markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% of Triton X-100) containing 5% nonfat milk, and probed with anti-IL-17 or IL-17F Abs (R&D Systems). After incubation with the secondary Ab conjugated with HRP, membranes were extensively washed, and the immunoreactivity was visualized by ECL according to the manufacturer's protocol (ECL kit, Santa Cruz Biotechnology).

Determination of airway responsiveness

Airway responsiveness was assessed as changes in airway function after challenge with aerosolized methacholine (Sigma-Aldrich) as described previously (26). Briefly, mice were anesthetized, tracheostomized, mechanically ventilated. Ventilation was achieved at 160 breaths per minute at a tidal volume of 0.15 ml with a positive end-expiratory pressure of 2–4 cm H₂O. Lung resistance was continuously computed by fitting flow, volume, and pressure.

Mice were challenged with methacholine aerosol in increasing concentrations (6.25–50 mg/ml in saline). Aerosolized agent was administered for 10 s with a tidal volume of 0.5 ml. From 20 s up to 3 min after each aerosol challenge, the data of lung resistance was continuously collected. Maximum values of lung resistance were selected to express changes in airway function, which was represented as a percentage change from baseline after administration of the saline aerosol.

RT-PCR and real-time RT-PCR

Cells were lysed with TRIzol reagent (Invitrogen) and total RNA was extracted according to manufacturer's instructions. The primers were designed with the Oligo Primer Analysis 4.0 software and the sequences were blasted (<http://www.ncbi.nlm.nih.gov/BLAST/>). One-step RT-PCR kit (Qiagen) was used for RT-PCR. The primer sequences were as follows: IL-17, sense 5'-CTGTGCTCTGATGCTGTTG-3', antisense 5'-ATGTGGTGGTCCAGCTTTC-3'; IL-17F, sense 5'-CTGTTGATGTTGGGACTTG-3', antisense 5'-GTTTCATGGTGCTGCTTCTCCTG-3'; GAPDH, sense 5'-GTGGAGATTGTTGCACAACG-3', antisense 5'-CAGTGGATGCAGGGATGATGTTCTG-3'.

For real-time RT-PCR assays, 100 ng of total RNA was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μl . Then 2 μl of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad) in duplicate. For sample analysis, the threshold was set based on the exponential phase of products, and CT value for samples was determined. The resulting data were analyzed

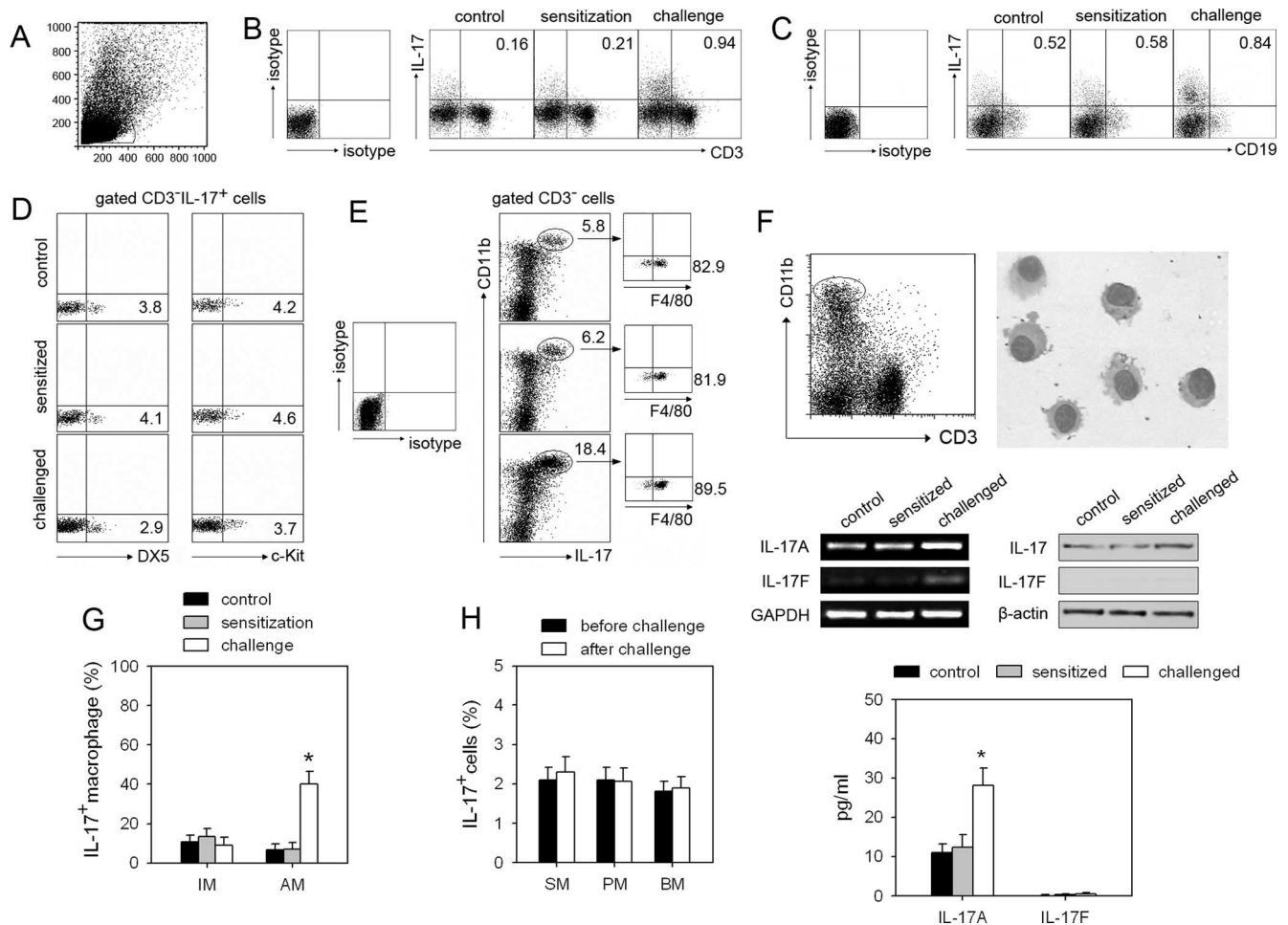


FIGURE 1. CD11b⁺F4/80⁺ macrophages are major source of IL-17 in allergic inflammation in lung. *A–C*, T, B, NK, and mast cells are not the main producer of IL-17 after allergen challenge. The mononuclear cells isolated from the lung tissues of mice were stained for the analysis of CD3, CD19, DX5, c-Kit, and IL-17 by flow cytometry. The data shown are representatives of FACS profiles ($n = 5$). *A*, Low scatter cells were gated and analyzed. *B*, IL-17⁺ T cells (CD3⁺) in mononuclear cells. *C*, IL-17⁺ B cells (CD19⁺) in mononuclear cells. *D*, NK (DX5⁺) and mast cells (c-Kit⁺) in CD3⁺IL-17⁺ cells. The data shown are from the gated CD3⁺IL-17⁺ cells. *E*, Macrophages are a major source of IL-17 after allergen challenge. The above mononuclear cells were stained for the analysis of CD3, CD11b, F4/80, and IL-17. The CD3⁺ cells were gated. Among them, the CD11b⁺IL-17⁺ cells were further gated for the analysis of F4/80. The data shown are representatives of FACS profiles ($n = 5$). *F*, Macrophages express IL-17. The gated CD3⁺CD11b^{high} cells were sorted and used for Wright-Giemsa staining and for the analysis of IL-17 and IL-17F expressions by real-time PCR and Western blot. The cells were also cultured for 48 h and IL-17 and IL-17F in the supernatants were detected by ELISA. *G*, Analysis of IL-17 in AMs and IMs. The percentage of IL-17⁺ macrophages in AMs and IMs was shown ($n = 6$). *, $p < 0.05$, compared with control. *H*, IL-17-producing cells in spleen macrophages (SM), PMs, and blood monocytes (BM). For SM and PM, the results were shown as the percentage of F4/80⁺IL-17⁺ cells in total F4/80⁺ cells. For BM, the result was shown as the percentage of CD14⁺IL-17⁺ cells in total CD14⁺ cells.

with the comparative CT method for relative gene expression quantification against house keeping gene GAPDH.

In vitro treatment of alveolar macrophages

Two $\times 10^6$ mast cells were incubated overnight at 37°C with 1 μ g/ml anti-OVA IgE (Uscnlife Company), washed, and treated with 10 μ g/ml of OVA for 48 h. The untreated mast cells were used as control.

Bone marrow-derived dendritic cells (DCs) were incubated with OVA (0.2 mg/ml) for 45 min at 37°C, washed three times, and then plated in round-bottom 96-well plates at 1×10^4 cells/well. Five $\times 10^4$ T cells isolated from spleen were added to the DCs for 72 h coculture.

Two $\times 10^5$ alveolar macrophages were cultured in 24-well plate in the absence or presence of the supernatants from OVA/IgE-stimulated mast cells or control mast cells, and/or in the presence of IL-4 (20 ng/ml), IL-5 (20 ng/ml), IL-10 (20 ng/ml), or IL-13 (20 ng/ml). Anti-TNF- α Ab (R&D Systems) was used to block TNF- α in the supernatants from OVA/IgE-stimulated mast cells as indicated. The supernatants from OVA/DC-stimulated T cells or control T cells were also used to stimulate macrophages. Twelve hours later, the expression of IL-17 gene was detected by real-time RT-PCR. Forty-eight hours later, the left cells were used for flow cyto-

metric analysis of intracellular IL-17, and IL-17 in supernatants was detected by ELISA as indicated.

Intranasal administration of IL-10

Two hours after each OVA challenge, mice were lightly anesthetized with diethyl ether. In brief, 0.2 μ g IL-10 in 40 μ l PBS was administered into nasal cavities, 20 μ l to each.

Statistics

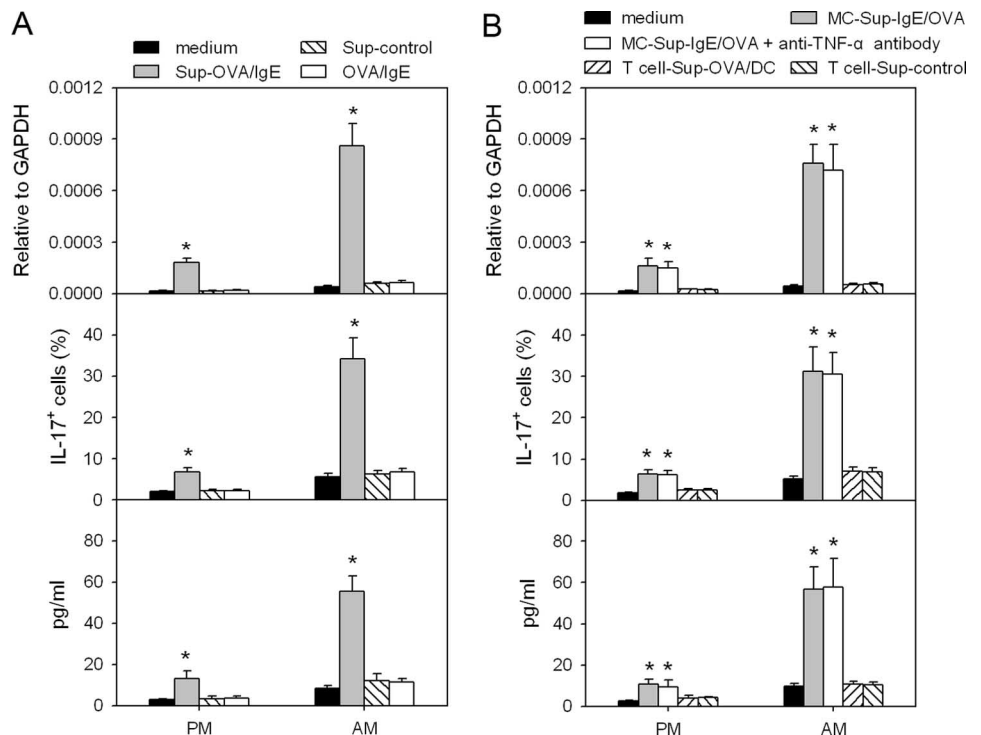
Data were expressed as mean value $\pm$ SD and interpreted by nonparametric ANOVA test. Differences were considered to be statistically significant when $p < 0.05$.

Results

CD11b⁺F4/80⁺ macrophages are major source of IL-17 in murine acute allergic inflammation in lung

To identify whether Th17 is the main producer of IL-17 in allergic asthma, we used a murine model of OVA-induced acute allergic

FIGURE 2. Mast cell-released mediator(s) up-regulates IL-17 expression by macrophages. *A*, The supernatants (Sup) from OVA/IgE-stimulated mast cells or control mast cells were used to stimulate PMs and AMs. Medium and OVA/IgE were used as controls. *B*, The supernatants from OVA/IgE-stimulated mast cells or OVA/DC-stimulated spleen T cells were used to stimulate PMs and AMs. Anti-TNF- α (10 μ g/ml) was used to block TNF- α in the supernatants from OVA/IgE-stimulated mast cells. IL-17 transcripts were detected by real-time RT-PCR 24 h later (*A* and *B*, upper). Forty-eight hours later, the left cells were used for flow cytometric analysis of intracellular IL-17 (*A* and *B*, middle), and IL-17 in the supernatants was detected by ELISA (*A* and *B*, lower). *, $p < 0.05$, compared with medium group.



inflammation in the lung. The expression of IL-17 in mononuclear cells isolated from the lungs of mice was analyzed by flow cytometry. The results from the analysis of low scatter cells (Fig. 1A) showed that IL-17-producing cells in the mononuclear cells was around 6% in both control and OVA-sensitized groups, and reached as high as 15% in the challenge group (Fig. 1B). Surprisingly, CD3⁺IL-17⁺ cells were <1% in mononuclear cells in all of three groups, although they were slightly increased after sensitization and challenge with allergen (Fig. 1B). Similarly, the per-

centage of CD19⁺IL-17⁺ cells in mononuclear cells was also very low, and only slightly changed after sensitization and challenge with allergen (Fig. 1C). These data suggest that neither Th17 cells nor B cells are the main producer of IL-17 in murine lung with allergic inflammation.

We then analyzed the expression of IL-17 in CD3⁺ cells. The result showed that DX5⁺ NK cells and c-Kit⁺ mast cells accounted for only a low percentage in CD3⁺IL-17⁺ cells, and were not increased after allergen challenge (Fig. 1D). There was no

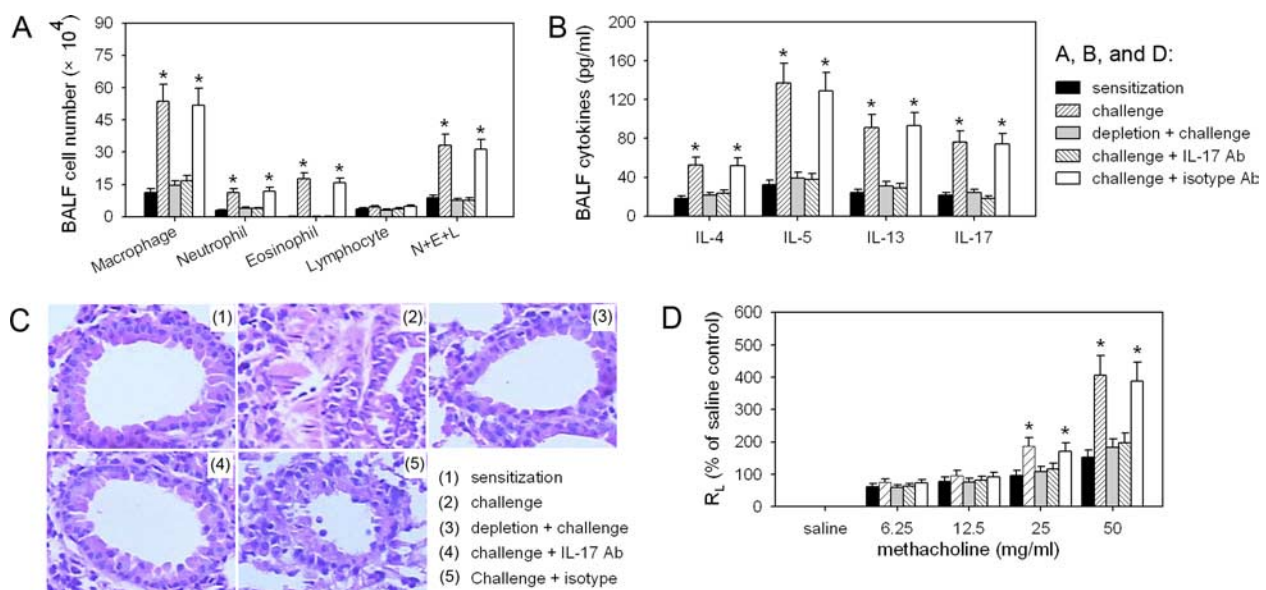


FIGURE 3. Macrophage-derived IL-17 is necessary for the inflammation related to asthma. The sensitized mice were challenged by OVA aerosol with or without macrophage depletion, or challenged by OVA aerosol with the administration of anti-mouse IL-17 mAb or control isotype Ab. The sensitized mice without challenge and further treatment were used as control. The mice were analyzed as follows. *A*, Cells in BALF were counted as described in *Materials and Methods*. *B*, The levels of IL-4, IL-5, IL-13, and IL-17 in the BALF were detected using ELISA kits. *, $p < 0.05$, compared with sensitization group. *C*, Representative photomicrographs of HE staining of lung sections in different group ($n = 5$) were shown. *D*, Airway hyperresponsiveness was measured by airway resistance in response to the inhaled methacholine.

IL-17⁺ cells in neutrophils after sensitization and allergen challenge (data not shown). The increase of IL-17⁺ cells after allergen challenge was mainly contributed by CD3⁺CD11b⁺ cells, and most of CD3⁺CD11b⁺IL-17⁺ cells were F4/80 positive (Fig. 1E). Therefore, we sorted CD3⁺CD11b^{high} cells for further analysis. The results showed that they have macrophage morphological characteristics, and that they indeed produced IL-17 at both the mRNA and protein levels (Fig. 1F). Thus, we identified that the main producer of IL-17 in the lung of mice with allergic inflammation were macrophages, which were strikingly increased after OVA challenge (Fig. 1E).

Next, we analyzed IL-17 expression in alveolar macrophages (AMs) and interstitial macrophages (IMs). The percentages of IL-17⁺ cells in these two populations were low, and not significantly influenced by sensitization with OVA (Fig. 1G). In the challenged mice, the percentage of IL-17⁺ cells in IM was decreased, whereas that in AM was increased by 6-fold (Fig. 1G). We then analyzed spleen macrophages, peritoneal macrophages, and peripheral blood monocytes. A low proportion of cells (around 2%) in each case expressed IL-17, and the expression pattern was not influenced by allergen challenge after sensitization (Fig. 1H). Therefore, the recruitment of peripheral monocyte-macrophage will not increase IL-17⁺ macrophages in the alveolus. The increase of IL-17-producing macrophages in the alveolus after allergen challenge is due to either the activation of AM or the migration of IL-17⁺ IM into the alveolus.

Mast cell-released mediator(s) up-regulates IL-17 expression in murine macrophages

Mast cells play an essential role in the development of airway hyperresponsiveness (27–29). Therefore, we tested whether mast cells may up-regulate IL-17 expression in alveolar macrophages. The bone marrow-derived mast cells were stimulated with OVA/IgE. The supernatants were then used to stimulate peritoneal macrophages (PMs) or AMs from naive mice. IL-17 transcripts were significantly increased in either the PM or AM group in the presence of the supernatant from OVA/IgE-stimulated mast cells (Fig. 2A). However, the direct stimulation of macrophages with OVA/IgE or with the supernatant from control mast cells did not increase IL-17 expression (Fig. 2A). Further analysis revealed that IL-17-producing macrophages and IL-17 in the supernatants were significantly increased after 48-h stimulation with the supernatant of OVA/IgE-stimulated mast cells (Fig. 2A). IL-17 expression of the macrophages stimulated by mast cell-derived mediator(s) was not affected by blocking TNF- α (Fig. 2B), although mast cell-derived TNF- α has been demonstrated to be at the center in the OVA-induced asthma model (30, 31). Furthermore, IL-23, a critical cytokine for IL-17 production was not detectable in the supernatant of IgE/OVA-stimulated mast cells (data not shown). On the other hand, the supernatant from the OVA/DC-stimulated T cells did not induce macrophage to express IL-17 (Fig. 2B). These data indicate that OVA/IgE-activated mast cells release mediator(s) to induce IL-17 expression in alveolar macrophages, which explains the mechanism underlying the increase of IL-17-producing macrophages in the alveolus. The data also indicate that mast cells may stimulate macrophages to express IL-17 by releasing other mediator(s), but not TNF- α or IL-23.

Macrophage-derived IL-17 is necessary for acute allergic inflammation in the lung

Next, we investigated the role of alveolar macrophage-derived IL-17 in the murine acute allergic inflammation model mimicking asthma. Using aerosolized 2-CA, 75% of alveolar macrophages, but not other cell types, were depleted, evaluated by counting the

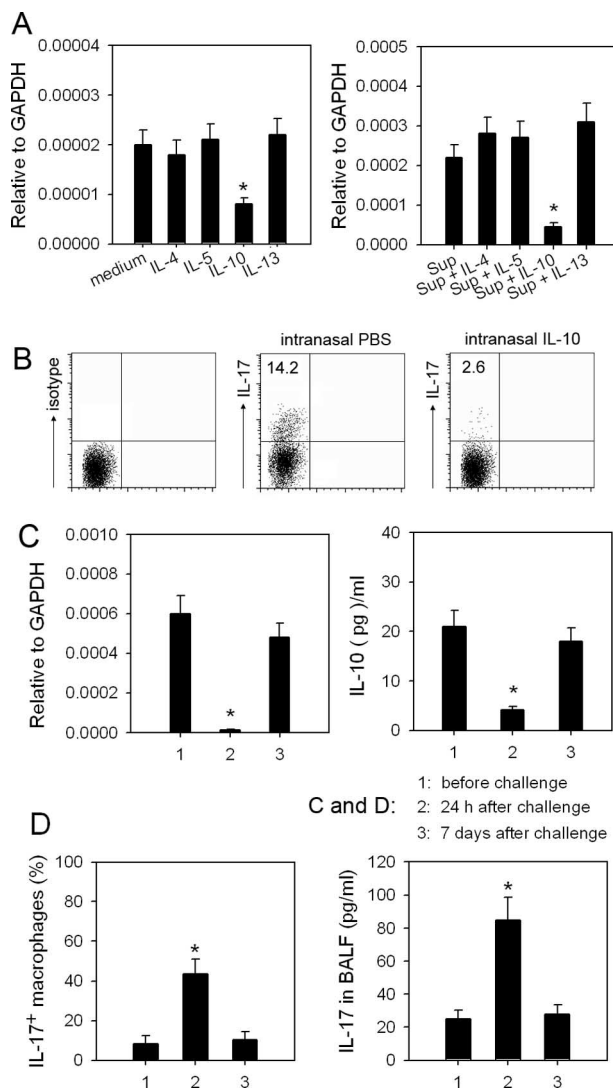


FIGURE 4. IL-10 down-regulates IL-17 expression by macrophages. **A**, IL-10 down-regulates IL-17 expression by AMs. AMs were cultured in the presence of different cytokines, and in the absence (*left*) or presence (*right*) of the supernatants from OVA/IgE-stimulated mast cells (Sup). The expression of IL-17 gene was detected by real-time RT-PCR. **B**, IL-10 suppresses the increase of IL-17-producing AM in vivo. The sensitized mice were challenged by OVA aerosol with the intranasal administration of IL-10. The IL-17-expressing macrophages in BALF were analyzed by flow cytometry. **C** and **D**, Expression of IL-10 in BALF. BALF was prepared from mice before OVA challenge, or 24 h after OVA challenge, or 7 days after OVA challenge. IL-10 expression in the cells from BALF and IL-10 level in BALF were detected by real-time RT-PCR and ELISA, respectively (**C**). IL-17⁺ macrophages in the macrophages from BALF and IL-17 level in BALF were analyzed by flow cytometry and ELISA, respectively (**D**). *, $p < 0.05$, compared with OVA sensitization group (before challenge).

cells in BALF (data not shown). Thus, sensitized mice were challenged by OVA-aerosol with or without macrophage depletion. Alternatively, the sensitized mice without macrophage depletion were treated twice with IL-17-neutralizing Ab (clone eBioMM17F3, IL-17A-specific, eBioscience), 1 h after the first and third OVA-aerosol challenge, respectively. The onset of asthma was well observed in the OVA-challenge group. Either macrophage depletion or treatment with IL-17 neutralizing Ab suppressed the increase of inflammatory cells (Fig. 3A) and cytokine IL-4, IL-5, IL-13, and IL-17 in BALF (Fig. 3B) inhibited the

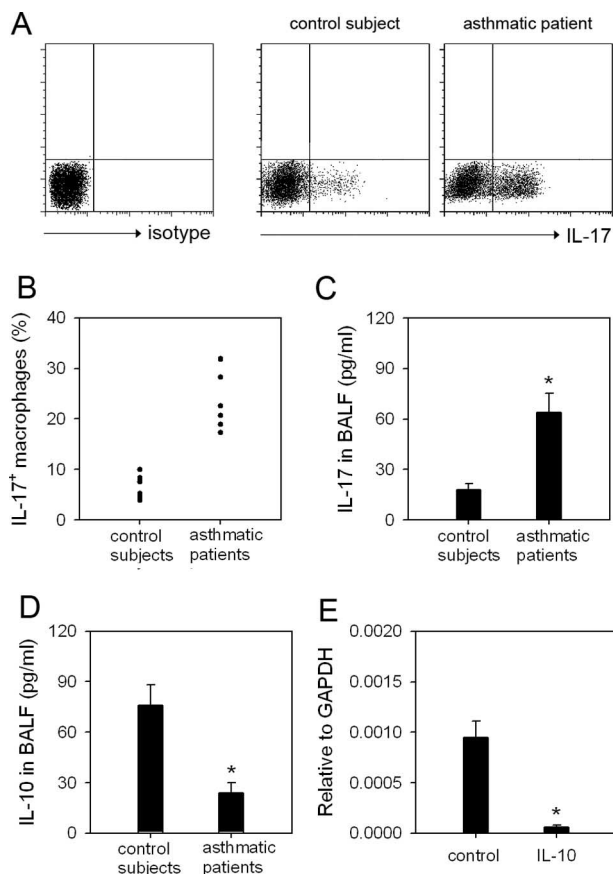


FIGURE 5. IL-17 expression is increased in human asthmatic alveolar macrophages. *A* and *B*, Analysis of IL-17 in alveolar macrophages from asthmatic patients. The alveolar macrophages were isolated from asthmatic patients and normal (control) subjects, and analyzed as described in *Materials and Methods*. The result of flow cytometric analysis (*A*) and the percentage of IL-17⁺ macrophages in AMs (*B*) were shown. *C* and *D*, The levels of IL-17 and IL-10 in the BALF from asthmatic patients. BALF was prepared from asthmatic patients and control subjects. The levels of IL-17 (*C*) and IL-10 (*D*) were detected using ELISA kits. *E*, IL-10 inhibits IL-17 expression by alveolar macrophages. Alveolar macrophages were isolated from asthmatic patients and control subjects as described in *Materials and Methods*. The cells were cultured in the absence or presence of IL-10. The expression of IL-17 gene was detected by real-time PCR. *, $p < 0.05$, compared with control groups.

infiltration of inflammatory cells in the lung (Fig. 3C) and reduced the airway hyperresponsiveness (Fig. 3D). Thus, these data suggest that IL-17-producing macrophages in alveolus are responsible for the initiation of asthma-related inflammation in the lung.

IL-10 down-regulates IL-17 expression by murine macrophages

Given that cytokines IL-4, IL-5, IL-10, and IL-13 play important roles in allergic asthma, in this study, we wondered whether they could regulate IL-17 expression by macrophages. For this purpose, alveolar macrophages were stimulated with these cytokines, respectively. The transcriptional activity of the IL-17 gene was not significantly influenced by IL-4, IL-5, and IL-13, whereas IL-10 significantly down-regulated the expression of IL-17 (Fig. 4A). More convincingly, intranasal administration of IL-10 effectively suppressed the increase of IL-17-producing alveolar macrophages in OVA-challenged mice (Fig. 4B), whereas the other three cytokines did not influence the amount of IL-17-producing macrophages (data not shown). These data suggest that IL-10 is a neg-

ative regulator for IL-17 expression by macrophages in acute allergic inflammation in the lung.

We then further analyzed the expression of IL-10 in BALF after OVA challenge. The result showed that the transcriptional activity of IL-10 gene in the cells from BALF was significantly reduced 24 h after OVA challenge (Fig. 4C). Consistently, IL-10 protein level in BALF was also significantly decreased (Fig. 4C). The expression of IL-10 was recovered 7 days after challenge (Fig. 4C), which was correlated to the decrease of both the percentage of IL-17⁺ AMs and IL-17 level in BALF 7 days after challenge (Fig. 4D). Therefore, the increase of IL-17-producing AMs in allergic asthma may be partially explained by the decrease of IL-10 after the challenge with allergen.

IL-17 expression is increased in human asthmatic alveolar macrophages

To interpret the above data in human asthmatics, we further analyzed the expression of IL-17 by alveolar macrophages from patients with allergic asthma. The results showed that the percentage of IL-17⁺ cells in AMs and the IL-17 level in the BALF from patients with allergic asthma was much higher than those from control subjects (Fig. 5A–C). However, the IL-10 level in the BALF from patients was much lower than that from control subjects (Fig. 5D). We therefore cultured the alveolar macrophages from patients and treated the cells with IL-10 in vitro. The result showed that the expression of IL-17 was down-regulated by IL-10 (Fig. 5E). These data suggested that IL-10 down-regulates IL-17 expression by human alveolar macrophages.

Discussion

In the present study, we demonstrate that lung macrophages rather than Th17 or other types of immune cells are the main producer of IL-17 in the OVA-induced allergic inflammation in the lung. Moreover, it is alveolar macrophages rather than interstitial macrophages that are the main source of IL-17 in this situation. Either depleting alveolar macrophages or neutralizing IL-17 in lung effectively inhibits OVA-induced allergic inflammation related to asthma, which is consistent with previous reports on the role of IL-17 in OVA-induced airway hypersensitivity response (12).

Enhanced macrophage recruitment during asthmatic process may result in the accumulation of tissue macrophages, which are activated by Th2 cytokines, leading to Th2 polarized reaction and inflammation (32, 33). Thus, the recruited lung macrophages have the potential to promote asthmatic development. However, the inconsistent results have been obtained from different studies. Several reports suggest that macrophages are crucial for down-regulating the initiation and progression of allergic asthma (34–36). Such regulatory role is partly due to the inhibitory effect of macrophages on Th2 by activating Th1 and producing regulatory factors such as NO, PGE₂, and IL-10 (37). On the other hand, other reports suggest the proinflammatory effects of macrophages, since macrophages can promote eosinophilic airway inflammation (38) and can be stimulated by allergen/IgE immune complexes to produce proinflammatory cytokines (39). The difficulty in elucidating the role of lung macrophages in asthma may mainly be ascribed to the heterogeneity and plasticity of macrophages. Consistent with this, our data in this report show that alveolar macrophages, but not interstitial macrophages, play an important role in allergic inflammation related to asthma. Before and after sensitization, IL-17-producing macrophages are kept at a low percentage in alveolar macrophages, but the challenge with allergen increased the percentage by about 6-fold in alveolar macrophages. In contrast, the challenge with allergen did not increase the percentage of IL-17-producing macrophages in lung interstitial macrophages. Taken

together, we propose that lung macrophages may play different roles in allergic asthma, depending on the microenvironment and the progressive stage of allergic asthma. The final proportion of different macrophage subsets may determine the final onset of allergic asthma.

AMs are the predominant immune effector cells resident in the alveolar spaces and conducting airways (40, 41). AMs have a distinctive phenotype compared with other resident macrophages in the body. They express high levels of mannose receptor and scavenger receptor-A, and constitutively secrete large quantities of lysozyme (32, 42). After stimulation, they produce a range of proinflammatory cytokines (43). Therefore, AMs are considered to be responsible for activating inflammatory responses sufficient to eliminate the pathogens (44). However, the role of AM in asthma is largely unexplored, compared with other airway inflammatory cell types (37). In this study, we found that AM promotes asthma-related inflammation in the mouse model by producing proinflammatory cytokine IL-17. The allergen challenge following the sensitization mainly increased the IL-17-producing macrophages in AM rather than in interstitial macrophages. In line with this, both IL-17⁺ cells in alveolar macrophages and IL-17 level in BALF from patients with asthmatic symptom were much higher than those from control subjects. Taken together, these data suggest that AM is an important population of macrophages responsible for the initiation of asthmatic symptoms.

The present study demonstrated that IL-17 expression by alveolar macrophages from mice can be up-regulated by soluble mediators derived from the activated mast cells. Because mast cells are located in the aspiratory tract, alveolar macrophages are easy-to-access mast cell mediators. This may explain why IL-17⁺ AMs are increased after OVA challenge. Meanwhile, this study also demonstrated that IL-17 expression by alveolar macrophages from mouse and asthmatic patients can be negatively regulated by the Th2 cytokine IL-10, even though other Th2 cytokines are considered as an asthmatic mediator. The decrease of IL-10 expression has been found in asthmatic AM (45–47). Our data further show that IL-10 level was significantly reduced in BALFs from both mice and patients with asthmatic symptom, possibly involving the decrease of IL-10 expression in both AM and Th2 cells. Thus, shifting the balance between IL-10 and mast cell-derived mediators in asthma potentiates the increased production of IL-17 by AMs.

Our findings disclose a new role of alveolar macrophage in the pathogenesis of allergic asthma by producing IL-17, and imply a potential strategy for asthma prevention and treatment by blockade of IL-17. However, asthma is an extremely complex disease, involving both innate and adaptive immune responses. The infiltration of different immune cells results in different pathological processes, depending on the immune and inflammatory microenvironment at the moment. Thus, cell populations playing a dominant role in asthma may be different. In this regard, IL-17 may target different cell types and produce different consequences. For instance, if eosinophils are dominant, IL-17 may be a negative regulator of established allergic asthma (11). However, under neutrophil infiltration condition, IL-17 may play a pro-asthmatic role. Therefore, although IL-17 may represent an interesting therapeutic target in asthma, the types of asthma involving different cell populations must be taken into consideration.

Disclosures

The authors have no financial conflict of interest.

References

- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Larche, M., D. S. Robinson, and A. B. Kay. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 111: 450–463.
- Iwamoto, I., H. Nakajima, H. Endo, and S. Yoshida. 1993. Interferon- γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD41 T cells. *J. Exp. Med.* 177: 573–576.
- Hansen, G., G. Berry, R. H. DeKruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103: 175–183.
- Hayashi, N., T. Yoshimoto, K. Izuhara, K. Matsui, T. Tanaka, and K. Nakanishi. 2007. T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyperresponsiveness and lung fibrosis by IFN- γ and IL-13 production. *Proc. Natl. Acad. Sci. USA* 104: 14765–14770.
- Fossiez, F., J. Banchereau, R. Murray, C. Van Kooten, P. Garrone, and S. Lebecq. 1998. Interleukin-17. *Int. Rev. Immunol.* 16: 541–551.
- Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicœur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL- β and TNF- α , by human macrophages. *J. Immunol.* 160: 3513–3521.
- Kao, C. Y., F. Huang, Y. Chen, P. Thai, S. Wachi, C. Kim, L. Tam, and R. Wu. 2005. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF- κ B-dependent signaling pathway. *J. Immunol.* 175: 6676–6685.
- Sylvester, J., A. Liacini, W. Q. Li, and M. Zafarullah. 2004. Interleukin-17 signal transduction pathways implicated in inducing matrix metalloproteinase-3, -13 and aggrecanase-1 genes in articular chondrocytes. *Cell. Signal.* 16: 469–476.
- Kao, C. Y., Y. Chen, P. Thai, S. Wachi, F. Huang, C. Kim, R. W. Harper, and R. Wu. 2004. IL-17 markedly up-regulates -defensin-2 expression in human airway epithelium via JAK and NF- κ B signaling pathways. *J. Immunol.* 173: 3482–3491.
- Schnyder-Canndrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, V. Quesniaux, F. Fossiez, B. Ryffel, and B. Schnyder. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715–2725.
- Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, I. Homma, K. Sekikawa, M. Asano, and Y. Iwakura. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375–387.
- Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Pagé, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430–438.
- Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726–737.
- Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
- Laan, M., Z. H. Cui, H. Hoshino, J. Lötvall, M. Sjöstrand, D. C. Gruenert, B. E. Skoogh, and A. Lindén. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* 162: 2347–2352.
- Hellings, P. W., A. Kasran, Z. Liu, P. Vandekerckhove, A. Wuyts, L. Overbergh, C. Mathieu, and J. L. Ceuppens. 2003. Interleukin-17 orchestrates the granulocyte influx into airways after allergen challenge inhalation in a mouse model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 28: 42–50.
- Weaver, C. T., L. E. Harrington, P. R. Mangan, M. Gavioli, and K. M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24: 677–688.
- Schmidt-Weber, C. B., M. Akdis, and C. A. Akdis. 2007. Th17 cells in the big picture of immunology. *J. Allergy Clin. Immunol.* 120: 247–254.
- Happel, K. I., M. Zheng, E. Young, L. J. Quinton, E. Lockhart, A. J. Ramsay, J. E. Shellito, J. R. Schurr, G. J. Bagby, S. Nelson, and J. K. Kolls. 2003. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J. Immunol.* 170: 4432–4436.
- Ferretti, S., O. Bonneau, G. R. Dubois, C. E. Jones, and A. Trifileff. 2003. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J. Immunol.* 170: 2106–2112.
- Stark, M. A., Y. Huo, T. L. Burcin, M. A. Morris, T. S. Olson, and K. Ley. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22: 285–294.
- Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
- National Heart, Blood and Lung Institute. 1997. Expert Panel Report 2: guidelines for the management of asthma. National Institutes of Health, Bethesda, MD, pp. 97–4051.
- Takemura, Y., Y. Iwasaki, K. Nagata, I. Yokomura, S. Tando, S. Fushiki, and H. Matsubara. 2005. Influence of depletion of alveolar macrophages on apoptosis in *Candida*-induced acute lung injury. *Exp. Lung Res.* 31: 307–321.
- Takeda, K., E. Hamelmann, A. Joetham, L. D. Shultz, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J. Exp. Med.* 186: 449–454.

27. Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192: 455–462.
28. Kobayashi, T., T. Miura, T. Haba, M. Sato, I. Serizawa, H. Nagai, and K. Ishizaka. 2000. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J. Immunol.* 164: 3855–3861.
29. Mayr, S. I., R. I. Zuberi, M. Zhang, J. de Sousa-Hitzler, K. Ngo, Y. Kuwabara, L. Yu, W. P. Fung-Leung, and F. T. Liu. 2002. IgE-dependent mast cell activation potentiates airway responses in murine asthma models. *J. Immunol.* 169: 2061–2068.
30. Nakae, S., L. H. Ho, M. Yu, R. Monteforte, M. Iikura, H. Suto, and S. J. Galli. 2007. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J. Allergy Clin. Immunol.* 120: 48–55.
31. Reuter, S., A. Heinz, M. Sieren, R. Wiewrodt, E. W. Gelfand, M. Stassen, R. Buhl, and C. Taube. 2008. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. *Eur. Respir. J.* 31: 773–782.
32. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23–35.
33. Mantovani, A., A. Sica, and M. Locati. 2005. Macrophage polarization comes of age. *Immunity* 23: 344–346.
34. Vissers, J. L., B. C. van Esch, G. A. Hofman, and A. J. van Oosterhout. 2005. Macrophages induce an allergen-specific and long-term suppression in a mouse asthma model. *Eur. Respir. J.* 26: 1040–1046.
35. Tang, C., M. D. Inman, N. van Rooijen, P. Yang, H. Shen, K. Matsumoto, and P. M. O'Byrne. 2001. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN- γ -dependent mechanism. *J. Immunol.* 166: 1471–1481.
36. Careau, E., and E. Y. Bissonnette. 2004. Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness. *Am. J. Respir. Cell Mol. Biol.* 31: 22–27.
37. Peters-Golden, M. 2004. The alveolar macrophage: the forgotten cell in asthma. *Am. J. Respir. Cell Mol. Biol.* 31: 3–7.
38. Moon, K. A., S. Y. Kim, T. B. Kim, E. S. Yun, C. S. Park, Y. S. Cho, H. B. Moon, and K. Y. Lee. 2007. Allergen-induced CD11b⁺ CD11cint CCR3⁺ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model. *Int. Immunol.* 19: 1371–1381.
39. Hirano, M., R. S. Davis, W. D. Fine, S. Nakamura, K. Shimizu, H. Yagi, K. Kato, R. P. Stephan, and M. D. Cooper. 2007. IgE immune complexes activate macrophages through Fc γ RIV binding. *Nat. Immunol.* 8: 762–771.
40. Lohmann-Matthes, M. L., C. Steinmüller, and G. Franke-Ullmann. 1994. Pulmonary macrophages. *Eur. Respir. J.* 7: 1678–1689.
41. Hocking, W., and D. Golde. 1979. The pulmonary alveolar macrophage. *N. Engl. J. Med.* 301: 580–587.
42. Laskin, D. L., B. Weinberger, and J. D. Laskin. 2001. Functional heterogeneity in liver and lung macrophages. *J. Leukocyte Biol.* 70: 163–170.
43. Grattendick, K., R. Stuart, E. Roberts, J. Lincoln, S. S. Lefkowitz, A. Bollen, N. Moguilevsky, H. Friedman, and D. L. Lefkowitz. 2002. Alveolar macrophage activation by myeloperoxidase: a model for exacerbation of lung inflammation. *Am. J. Respir. Cell Mol. Biol.* 26: 716–722.
44. Zhang, P., W. R. Summer, G. J. Bagby, and S. Nelson. 2000. Innate immunity and pulmonary host defense. *Immunol. Rev.* 173: 39–51.
45. Borish, L., A. Aarons, J. Rumblyrt, P. Cvietusa, J. Negri, and S. Wenzel. 1996. Interleukin-10 regulation in normal subjects and patients with asthma. *J. Allergy Clin. Immunol.* 97: 1288–1296.
46. John, M., S. Lim, J. Seybold, P. Jose, A. Robichaud, B. O'Connor, and P. J. Barnes, and K. F. Chung. 1998. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1 α , granulocyte-macrophage colony-stimulating factor and interferon- γ release from alveolar macrophages in asthma. *Am. J. Respir. Crit. Care Med.* 157: 256–262.
47. Chung, F. 2001. Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon- γ . *Mediat. Inflamm.* 10: 51–59.