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An IL-17F/A Heterodimer Protein Is Produced by Mouse Th17 Cells and Induces Airway Neutrophil Recruitment

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IL-17A and IL-17F are related homodimeric proteins of the IL-17 family produced by Th17 cells. In this study, we show that mouse Th17 cells also produce an IL-17F/A heterodimeric protein. Whereas naive CD4+ T cells differentiating toward the Th17 cell lineage expressed IL-17F/A in higher amounts than IL-17A/A homodimer and in lower amounts than IL-17F/F homodimer, differentiated Th17 cells expressed IL-17F/A in higher amounts than either homodimer. In vitro, IL-17F/A was more potent than IL-17F/F and less potent than IL-17A/A in regulating CXCL1 expression. Neutralization of IL-17F/A with an IL-17A-specific Ab, and not with an IL-17F-specific Ab, reduced the majority of IL-17F/A-induced CXCL1 expression. To study these cytokines in vivo, we established a Th17 cell adoptive transfer model characterized by increased neutrophilia in the airways. An IL-17A-specific Ab completely prevented Th17 cell-induced neutrophilia and CXCL5 expression, whereas Abs specific for IL-17F or IL-22, a cytokine also produced by Th17 cells, had no effects. Direct administration of mouse IL-17A/A or IL-17F/A, and not IL-17F/F or IL-22, into the airways significantly increased neutrophil and chemokine expression. Taken together, our data elucidate the regulation of IL-17F/A heterodimer expression by Th17 cells and demonstrate an in vivo function for this cytokine in airway neutrophilia. The Journal of Immunology, 2007, 179: 7791–7799.

The IL-17 cytokine family consists of six structurally related proteins (IL-17A, B, C, D, E, and F), the functions of which are now being elucidated. The best-characterized molecule of this family is IL-17A. IL-17A is expressed primarily by Th17 cells, a subset of CD4+ T cells (1–5), and signals through a receptor complex that contains IL-17RA and IL-17RC (6, 7). Although these receptor subunits are expressed broadly, IL-17A is believed to act primarily on parenchymal cells such as fibroblasts, epithelial cells, and endothelial cells. Signaling by IL-17A increases matrix metalloproteinase and proinflammatory cytokine expression (reviewed in Refs. 8 and 9). IL-17A also acts to recruit neutrophils to peripheral sites through the induction of chemokines and G-CSF. The expression of IL-17A is enhanced in several pulmonary diseases in which neutrophils are present, including severe asthma, chronic obstructive pulmonary disease, and cystic fibrosis (10–13). As a result, considerable attention has been given to the role of IL-17A in the pathogenesis of airway disease. Administration of IL-17A into the airways induces a significant increase in neutrophils associated with enhanced CXCL1 (KC) and CXCL2 (MIP-2) expression (14, 15). In a model of LPS-driven airway inflammation, neutralization of IL-17A significantly reduces neutrophil numbers (14, 16). These data point to an important role for IL-17A in regulating airway inflammation and neutrophil recruitment.

Of the remaining five IL-17 family members, IL-17F is most closely related to IL-17A. The two molecules share 50% amino acid sequence homology and are syntenic, both located at the A4 region of mouse chromosome 1. Like IL-17A, IL-17F mRNA and protein have been detected in Th17 cells (3, 17). IL-17F exists as a homodimer, adopting a cysteine knot motif formed through the interactions of four cysteines, one of which is responsible for the interchain bonding (18). These cysteines are highly conserved in IL-17A, suggesting that IL-17A has a similar homodimeric structure.

As with structure, IL-17A and IL-17F are proposed to share the same receptor complex (6, 19) and may also share functions. The first reported study of IL-17F activity showed that IL-17F induces TGF-β, lymphotoxin β, and IL-2 from endothelial cells in vitro (20). A subsequent study demonstrated that IL-17F can also induce G-CSF and CXCL1 from primary human epithelial cells (11). Overexpression in mouse airways of human IL-17F using adenoviral vectors or mouse IL-17F using pulmonary gene transfer induces a significant increase in neutrophil numbers and chemokine expression, similar to what has been observed for IL-17A in vivo (21, 22). Although these studies point to potential overlapping functions in mouse airways for IL-17A and IL-17F, the relative potency of these homodimeric proteins in regulating these activities is unknown, as a direct in vivo comparison of mouse IL-17A and IL-17F protein has not been reported.

The high sequence homology between IL-17A and IL-17F, along with the conserved location of their cysteines, suggests that a heterodimer of IL-17A and IL-17F could exist. The coexpression of IL-17A and IL-17F by Th17 cells further supports this possibility. The existence of human IL-17F/A heterodimer has recently
been demonstrated using biochemical and physicochemical methods (23). (Hereafter, we use IL-17A/A and IL-17F/F to specifically refer to the homodimeric proteins, IL-17F/A to refer to the heterodimeric protein, and IL-17A or IL-17F to refer to all proteins containing the given molecule.) Mass spectrometry analysis of natural IL-17F/A heterodimer produced by primary human CD4+ T cells has shown the existence of interchain disulfide-linked peptides containing a peptide from IL-17F and one from IL-17A (23). More recently, mouse IL-17F/A has been detected in conditioned medium from Th17 cells (24). While detected, the regulation of IL-17F/A expression and its in vivo functions have not been evaluated.

In addition to producing IL-17A and IL-17F, Th17 cells also produce IL-22 (17, 25, 26), an IL-10 family member (27). IL-22 acts on epithelial cells and some fibroblast cells and is proposed to be a regulator of tissue inflammation. IL-22 induces gene expression indicative of an acute phase response (28). Similar to IL-17A and IL-17F, IL-22 by Th17 cells suggests that these cytokines may function together to modulate inflammation. IL-22 can function in synergy with IL-17A or IL-17F on human colonic myofibroblasts and/or human keratinocytes to induce proinflammatory mediators (17, 29).

In this report, we examined the regulation of IL-17F/A expression and quantified the production of IL-17A/A, IL-17F/A, and IL-17F/F by Th17 cells. We compared the activity of IL-17A/A, IL-17F/A, and IL-17F/F in vitro as well as their functions in neutrophil recruitment and chemokine production in vivo. We also established a Th17 cell adoptive transfer model to examine the essential roles of these cytokines in regulating airway inflammation. Taken together, our data show that IL-17F/A is a biologically active protein and demonstrate that IL-17A/A and IL-17F/F, as produced by Th17 cells, regulate the induction of chemokines and the recruitment of neutrophils into the airways.

Materials and Methods

Abs and reagents

Anti-mouse IL-17A Abs (50101, 50104) were obtained from R&D Systems. Anti-mouse IL-17B Abs (RK015-01, RK016-17), anti-mouse IL-22 (Ab-01) and relevant isotype control Abs were generated using methods previously described (17). IL-6, IL-1β, TNF-α, and IL-23 were obtained from R&D Systems. TGF-β and OVA were obtained from Sigma-Aldrich. OVA23,390 was obtained from New England Peptide. Anti-IFN-γ (XMG1.2) and anti-IL-4 (BVD4-1D11) were obtained from BD Pharmingen.

Generation and purification of IL-17A/A, IL-17F/A, and IL-17F/F

Sequences for recombinant proteins were engineered into expression vectors using conventional methods as previously described (17). His-tagged IL-17A and His-tagged IL-17F, produced in Chinese hamster ovary cells, were purified over a Ni-NTA Superflow column (Qiagen). The protein was purified over a Ni-NTA Superflow column (Qiagen). The protein was dialyzed against PBS (pH 7.2), and then characterized by SDS-PAGE gels, Western blot analysis, mass spectrometry, and analytical size exclusion chromatography. The resulting IL-17A/A heterodimer was >99% pure as determined by silver stain analysis. Endotoxin levels for all recombinant proteins were <3 endotoxin units/mg. For Western blot analysis, 35 ng of IL-17A/A, IL-17F/A, and IL-17F/F were loaded. IL-17A was detected by probing with goat anti-mouse IL-17A (AF421NA, 1:2000 dilution; R&D Systems) followed by donkey anti-goat HRP (Jackson ImmunoResearch Laboratories). IL-17F was detected using serum (1:2000 dilution) from rats previously immunized with mouse IL-17F and that tested positive for IL-17F-reactive Abs. Rat Abs were detected with goat anti-rat HRP (Pierce).

In vitro T cell activation

CD4 “CD62L++ naive DO11.10 T cells were purified from spleens of DO11.10 mice using MACS positive and negative selection (Miltenyi Biotec) as previously described (17). In brief, 2 × 105 DO11.10 T cells were activated with 4 × 106 irradiated splenocytes and 1 μg/ml OVA23,390. Cytokines were added at the following concentrations: 1 ng/ml TGF-β, 20 ng/ml IL-6, 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IL-23. For restimulation, cells were harvested on day 7 of primary activation, rested overnight, and restimulated with irradiated splenocytes, 1 μg/ml OVA23,390-5 ng/ml IL-2, and in some cases 10 ng/ml IL-23, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4. Conditioned medium was harvested on day 4 of primary or secondary stimulation. Intracellular cytokine staining was performed by restimulating cells with 50 ng/ml PMA (Sigma-Aldrich) 20 μg/ml ionomycin (Sigma-Aldrich), and GolgiPlug (BD Pharmingen) for 5 h. Cells were surface stained and permeabilized using Cytofix/Cyperm (BD Pharmingen) according to the manufacturer’s directions. Intracellular cytokine staining was performed using anti-IL-17A PE (TC11-18H10) and anti-IL-17F AlexaFluor 647 (RKO15-01). All lymphocytes were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 5 mM HEPES, 10 μM penicillin-streptomycin, and 2.5 μM 2-ME.

ELISAs

To quantitate IL-17A/A homodimers, plates were coated with 2 μg/ml anti-IL-17A (50101) overnight. After plates were blocked with 1% BSA in PBS, samples were incubated in the plate for 2 h at room temperature. A biotinylated version of the same anti-IL-17A Ab (50101) was then used at 1 μg/ml to specifically detect plate-bound IL-17A/A. To quantitate IL-17F/F homodimer, ELISAs were performed following a similar scheme using anti-IL-17F (RKO16-17) as both the capture (2 μg/ml) and detection reagent (1 μg/ml). The limit of detection for IL-17A/A and IL-17F/F ELISAs was 1 and 4 ng/ml, respectively. An IL-17F/A heterodimer ELISA was performed using anti-IL-17A (50101, 2 μg/ml) as the capture Ab and biotinylated goat anti-IL-17F polyclonal Ab (BAF2057, 200 ng/ml; R&D Systems) as the detection reagent. The limit of detection for the IL-17F/A heterodimer ELISA was 40 pg/ml. IL-17F homodimer concentrations was detectable in the homodimer ELISAs. Thus, IL-17F/A was first quantitated. To correct for IL-17F/A cross-reactivity in the homodimer ELISAs, we back-calculated an OD contributed by IL-17F/F for each sample based on a titration of rIL-17F/A. This OD was then subtracted from the experimental OD in the homodimer ELISA and the remaining OD was used to calculate the homodimer concentration. IL-17F/A ELISAs was performed as previously described (17). CXCL1 and CXCL5 were quantitated using DuoSet ELISAs according to the manufacturer’s directions (R&D Systems).

Treatment of MLE-12 cells

In brief, 2.5 × 104 MLE-12 cells (ATCC CRL-2110) were treated with cytokine, or with preincubated combinations of both cytokine and Ab, for 24 h in a 96-well plate. Conditioned medium was harvested at 24 h. MLE-12 cells were grown in HITES (hydrocortisone, insulin, transferrin, estradiol, selenium), 2% FBS, and 2 mM l-glutamine.

Animal experiments

BALB/cByJ and C57-Tg(DO11.10)lolo/J (DO11.10) mice were obtained from The Jackson Laboratory. CD4 “CD62L++ T cells from DO11.10 mice were differentiated to Th17 cells as described above in the presence of TGF-β, IL-6, IL-1β, TNF-α, and IL-23 for 5 days. To establish Th17-mediated airway inflammation, 2.5 × 106 Th17 cells were transferred i.v. into naive BALB/c recipient mice (day 0). Mice were rested for 24 h and then challenged intranasally with 75 μg of OVA daily for 3 consecutive days (days 1, 2, and 3). Control mice either received Th17 cells and intranasal PBS or just intranasal OVA and no cells. For studies with Abs, 300 μg of a given Ab was injected i.p. 1 h before the first OVA challenge on day 1. One hundred micrograms of Ab was also administered intranasally...
The distinct bands revealed that the purified, double-tagged protein contained both distinct protein tags. Western blot analysis on a nonreducing gel the IL-17F/A was achieved by sequential purifications exploiting versions of IL-17A and IL-17F in HEK293 cells. Purification of generated mouse IL-17F/A by overexpressing differentially tagged 17A/A and IL-17F/F have not been reported. To study this, we first IL-17F/A expression by Th17 cells and its expression relative to IL-17A and IL-17F proteins (Fig. 2B).

We next quantitated the amounts of IL-17A/A, IL-17F/A, and IL-17F/F produced by Th17 cells activated under different conditions. Naive DO11.10 T cells were activated with TGF-β and IL-6 and, in some cases, further supplemented with 10 ng/ml TNF-α, IL-1β, or IL-23. IL-17F/F was produced at the greatest abundance, with the IL-17A/F heterodimer having intermediate expression and IL-17A/A being expressed in lowest amounts after 4 days of activation (Fig. 2C, see legend for statistics from three experiments). Although this finding was statistically significant with most but not all of the stimulation conditions tested, we observed this expression pattern consistently in all three experiments. IL-17A/A was below the limit of detection (1 ng/ml) in cells activated with the combination of TGF-β and IL-6 and when this condition was further supplemented with TNF-α or IL-23. Addition of IL-1β, in combination with TGF-β and IL-6, significantly increased IL-17A/A expression by at least 9-fold, IL-17F/A by 5-fold, and IL-17F/F by 3-fold. In contrast, the addition of IL-23 or TNF-α, with TGF-β and IL-6, to naive T cell cultures increased cytokine expression modestly if at all. These data show that IL-1β was more potent than IL-23 or TNF-α when used in combination with TGF-β and IL-6 and significantly enhanced the expression of IL-17A/A, IL-17F/A, and IL-17F/F during the differentiation of naive cells. Our data also show that recently activated naive cells express different IL-17 proteins, with IL-17F/F being expressed in highest amounts, followed by IL-17F/A and then IL-17A/A.

To examine the expression of IL-17A/A, IL-17F/A and IL-17F/F by differentiated Th17 cells, we first activated naive DO11.10 T cells in a primary stimulation culture in the presence of TGF-β, IL-6, TNF-α, and IL-1β with or without IL-23 for 7 days. After harvesting and resting overnight, cells were re-stimulated in the presence of 5 ng/ml IL-2 and OVA323-339 (—) or with IL-2, OVA323-339, 10 ng/ml IL-23, and 10 μg/ml IFN-γ and IL-4-neutralizing Ab (Fig. 2D). Inclusion of IL-23 during the secondary stimulation, and not during the primary stimulation, was required to achieve optimal production of IL-17F/A or IL-17F/F, as has been reported previously for IL-17A/A (5). Our data also showed a consistent trend of IL-17F/F being expressed higher than either homodimer during restimulation in four experiments, although this increase was not always statistically significant (see Fig. 2D legend for p values). Taken together, our data showed that IL-17A/A, IL-17F/A, and IL-17F/F can be produced by naive T cells, stimulated with IL-17-inducing conditions, and optimally by differentiated Th17 cells with the addition of IL-23. In addition, the data also suggested that the pattern of expression of IL-17A/A, IL-17F/A, and IL-17F/F was dependent on the degree of differentiation of the Th17 cell, with IL-17A/A expression relatively limiting with the primary culturing of T cells under Th17 conditions.

FIGURE 1. IL-17A and IL-17F proteins are coexpressed by a subset of Th17 cells. CD4⁺CD62L⁻ (naive) DO11.10 T cells were activated with irradiated splenocytes, 1 μg/ml OVA323-339, and either 1 ng/ml TGF-β, 20 ng/ml IL-6, or both. Intracellular cytokine staining was performed on day 4. All plots are gated on CD4⁺ DO11.10 T cells and are representative of three experiments.

All statistical significance values were determined by an unpaired Student’s t test.

Results

IL-17A and IL-17F are coexpressed by mouse Th17 cells

IL-17A protein expression from naive T cells is initiated primarily by the combination of TGF-β and IL-6 (2, 4, 5). To examine the regulation of IL-17F, we activated naive (CD4⁺CD62L⁻) T cells purified from DO11.10 mice with irradiated splenocytes, 1 μg/ml OVA323-339, and either 1 ng/ml TGF-β, 20 ng/ml IL-6, or both. Intracellular cytokine staining for IL-17F was performed on day 4 of activation. Similar to IL-17A, minimal IL-17F expression was detected with either TGF-β or IL-6 alone. The combination of TGF-β and IL-6 induced substantial production of IL-17F protein, with a considerable percentage of cells expressing both IL-17A and IL-17F (Fig. 1). Similar results on the regulation of IL-17F protein expression were recently reported (33). These data demonstrated that TGF-β and IL-6 induced the production of IL-17F and generated a population of Th17 cells that secrete both IL-17A and IL-17F.

IL-17A/A, IL-17F/A, and IL-17F/F are differentially expressed by Th17 cells

Recently, human and mouse IL-17A and IL-17F have been reported to exist as a heterodimer (23, 24). The regulation of IL-17A/F expression by Th17 cells and its expression relative to IL-17A/A and IL-17F/F have not been reported. To study this, we first generated mouse IL-17F/A by overexpressing differentially tagged versions of IL-17A and IL-17F in HEK293 cells. Purification of the IL-17F/A was achieved by sequential purifications exploiting distinct protein tags. Western blot analysis on a nonreducing gel revealed that the purified, double-tagged protein contained both IL-17A and IL-17F in the same bands (Fig. 2A). The distinct bands represented differentially glycosylated species (data not depicted). These data demonstrated the formation of mouse IL-17F/A heterodimers when IL-17A and IL-17F are overexpressed in vitro.

To examine IL-17A/A, IL-17F/A, and IL-17F/F expression by mouse T cells, we first established ELISAs to specifically quantitate each protein. To quantitate homodimers, the same mAb was used as both the capture and detection reagent in a sandwich ELISA. This format allows for a successful sandwich to be formed only by homodimers or higher multimers. To quantitate IL-17F/A heterodimer, a sandwich ELISA was performed using an IL-17A-specific mAb as the capture reagent in combination with an IL-17F-specific polyclonal Ab as the detection reagent. The specificity of these ELISAs was validated using purified rIL-17A/A, IL-17F/A, and IL-17F/F proteins (Fig. 2B).

1 h before each intranasal challenge with OVA on days 1, 2, and 3. Twenty-four hours after the last challenge, the mice were sacrificed and the bronchoalveolar lavage (BAL) was performed using three 0.7-ml washes with PBS. The first of the three lavages was saved for chemokine analysis after the cells were recovered by centrifugation. To determine total cell counts, cells from all three lavages were combined and counted using a CellDyn hematology analyzer (Abbott Diagnostic). Differential cell analysis was performed by counting cytospin slides stained with Hema-3 stain; 200 cells were counted for each slide. Lungs from mice that had not undergone BAL were fixed in 10% neutral-buffered formalin for histological analysis.

Intranasal studies, BALB/cByJ mice were challenged intranasally with 1.5 g of recombinant mouse IL-17A/A, IL-17F/F, IL-17F/A, or IL-22 in 75 μl, either once or daily for 3 consecutive days. 24 h after the last challenge, BAL fluid was harvested and analyzed as described above. All mice were used between 8 and 12 wk of age and were housed in strict accordance to Wyeth Research Institutional Animal Care and Use Committee regulations.

Data analysis

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IL-17F/A heterodimer is a biologically active protein

IL-17A and IL-17F are known to enhance the expression of chemokines by epithelial cells and fibroblasts. To compare the activity of IL-17A/A, IL-17F/A, and IL-17F/F, we treated a mouse lung epithelial cell line, MLE-12, with these cytokines and examined the expression of the neutrophil chemoattractant CXCL1 (KC). IL-17A/A and IL-17F/F both enhanced the expression of CXCL1, although IL-17F/F was 100- to 1000-fold less active than IL-17A/A (Fig. 3A). IL-17F/A heterodimer also enhanced CXCL1 and was consistently less potent than IL-17A/A and more potent than IL-17F/F. These data demonstrated that IL-17F/A is a biologically active protein.

To explore the relative contributions of IL-17A and IL-17F in IL-17F/A activity, we first generated neutralizing Abs to IL-17F. We identified two Abs that completely neutralized the activity of up to 200 ng/ml IL-17F/F on MLE cells with 50 μg/ml Ab (Fig. 3B). These anti-IL-17F Abs do not bind or neutralize IL-17A/A and can bind to IL-17F/A heterodimer (Fig. 3, C and D, and data not shown). We also tested an IL-17A-specific Ab (50104) and observed that it can neutralize the effects of IL-17A/A, and not IL-17F/F, on MLE-12 cells (data not shown). We next examined the effects of these Abs on neutralizing IL-17F/A heterodimer. MLE-12 cells were treated with 200 ng/ml IL-17F/A heterodimer in combination with mAbs, used at 80 μg/ml (~100-fold molar excess). The IL-17A-specific Ab significantly reduced the effects of IL-17F/A by 65%, as compared with its isotype control (IgG2a) (Fig. 3E). In contrast, neutralization of IL-17F/A with either IL-17F-specific Abs had no significant effect, as compared with the
Our in vitro data demonstrated that IL-17A/A is more active than IL-17F/F, with IL-17F/A being less potent than IL-17A/A and more potent than IL-17F/F. To evaluate the contributions of these cytokines in vivo, we established a Th17-dependent airway inflammation model. First, naive CD4+CD62L+ T cells from DO11.10 mice were differentiated in vitro with TGF-β, IL-6, IL-1β, TNF-α, and IL-23, with at least 60% of the activated cells expressing IL-17A and/or IL-17F after 5 days in culture (data not shown). Cells were then adoptively transferred into a naive BALB/c host. To induce airway inflammation, mice were subsequently challenged daily with intranasal OVA for 3 consecutive days. Two sets of control mice were used. One control cohort received Th17 cells and was challenged intranasally with PBS, whereas the second cohort did not receive any cells and was challenged intranasally with OVA. In all groups of mice, IL-17A/A and IL-17F/F concentrations in BAL fluid were below the limit of detection (1 and 4 ng/ml, respectively; data not depicted). Since the IL-17F/A-specific assay had a lower limit of detection (40 pg/ml), we were able to detect IL-17F/A heterodimer in the BAL fluid from all groups of mice, observing a 6-fold increase in IL-17F/A heterodimer in mice adoptively transferred with Th17 cells and subsequently exposed to OVA (Fig. 4A). We also detected a significant, 6-fold increase in IL-22, a cytokine recently described to be expressed by Th17 cells (17, 25, 26). The expression of IL-17F/A and IL-22 demonstrated that Th17 cells were present and activated in the airways. We next examined cellular inflammation in this model. Mice receiving Th17 cells and OVA had significantly increased neutrophil and lymphocyte numbers in the BAL fluid as compared with either group of control mice (Fig. 4B). Monocytes and eosinophils were not increased in mice receiving Th17 cells and intranasal OVA. Histological analysis of lung tissue also revealed enhanced peribronchial and perivascular inflammation in mice transfected with Th17 cells and exposed to OVA as compared with control groups (Fig. 4C). Neutrophils were a prominent component of the inflammation, similar to results observed in the BAL fluid. Taken together, these data demonstrated that Th17 cells could induce an airway inflammatory response characterized by the recruitment of neutrophils.

Although Th17 cells can induce airway neutrophilia, it is unknown which cytokine is specifically responsible for these effects. To examine this, we administered neutralizing Abs to IL-17A, IL-17F, or IL-22. Treatment with an IL-17A-specific Ab (50104) significantly reduced the amount of neutrophils to numbers similar to those of control mice (Fig. 5). In contrast, neutralizing Abs to IL-17F (RK015-01 or RK016-17) or IL-22 (Ab-01) did not affect neutrophil numbers (Fig. 5 and data not shown). No significant effects were observed on lymphocyte, eosinophil, or monocyte numbers in mice treated with an Ab specific for IL-17A, IL-17F, or IL-22 (data not shown). Although concentrations of CXCL1...
were not significantly modulated in any of the treatment groups, CXCL5 (LIX), another potent neutrophil chemoattractant (34, 35), was significantly reduced by the IL-17A-specific Ab to concentrations similar to those of control mice. Abs specific for IL-17F or IL-22 did not alter CXCL5 concentrations. These data demonstrated that administration of an IL-17A-specific Ab alone was sufficient to prevent Th17 cell-induced airway neutrophilia.

**IL-17F/A recruits neutrophils in vivo**

In our Th17-dependent airway inflammation model, the expression of IL-17A/A or IL-17F/F in the BAL fluid was below the limit of detection. As a result, we could not show that IL-17A/A or IL-17F/F was being expressed in the airways. However, our evaluation of IL-17A/A, IL-17F/A, and IL-17F/F expression by differentiated Th17 cells in vitro (Fig. 2D) suggested that the homodimer proteins were present in vivo but below detection limit. To directly examine the effects of IL-17A/A and IL-17F/F, we administered 1.5 $\mu$g of recombinant protein into the airways either once (Fig. 6A) or daily for 3 consecutive days (Fig. 6B). We examined neutrophil recruitment and chemokine production in the BAL fluid 24 h after the last administration. IL-17A/A significantly increased neutrophils, CXCL1, and CXCL5 either when

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**FIGURE 4.** Th17 cells induce airway neutrophilia. CD4$^\text{+}$CD62L$^\text{−}$ DO11.10 T cells were differentiated to Th17 with TGF-β, IL-6, IL-1β, TNF-α, and IL-23 for 5 days. On day 5, 2.5 $\times$ 10$^5$ cells were adoptively transferred into naïve BALB/c recipients. Twenty-four hours after transfer, mice were given either Th17 cells and intranasal PBS or just intranasal OVA and no cells. BAL fluid was obtained 24 h after the last OVA dose. A, IL-17F/A and IL-22 concentrations were determined from the BAL fluid. B, Differential cell counts were determined for each group. C, Representative H&E histology of the lung from each group is shown at $\times 40$ magnification with airway lumen (A) and blood vessel (V) annotated. A and B, Data are average ± SEM, $n = 5$–6 mice/group and are representative of at least two experiments.

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**FIGURE 5.** IL-17A is necessary for Th17-induced airway neutrophilia. Neutralizing Abs to IL-17A, IL-17F, and IL-22 or appropriate isotype controls were administered to mice adoptively transferred with Th17 cells and exposed to intranasal OVA. One hour before the first OVA dose, 300 $\mu$g of a given Ab was administered i.p. to neutralize systemic/circulating cytokine. Also, 100 $\mu$g of a given Ab was administered intranasally, 1 h before each OVA dose, to neutralize cytokine in the airways. Control mice were administered intranasal OVA and did not receive any Th17 cells. Neutrophils, CXCL1, and CXCL5 were quantitated from the BAL fluid collected 24 h after the last OVA challenge. Data are average ± SEM, $n = 8$–9 mice/group and are representative of two or three experiments, depending on the Ab.
given once (Fig. 6, A and C) or three times (Fig. 6B). In contrast, IL-17F/F did not significantly enhance neutrophil numbers or CXCL1 (Fig. 6). We did observe a small and significant increase in CXCL5 only when IL-17F/F was given three times (Fig. 6B). Increasing the dose of IL-17F/F by 10-fold (15 μg) did not further enhance neutrophils, CXCL1, or CXCL5 relative to what was observed with 1.5 μg, either when given once or three times (data not shown). We also examined the effects of rIL-22 and did not observe any increase in neutrophils or chemokines when given once (Fig. 6C). We did not detect expression of G-CSF, CXCL-2, MCP-1, IL-6, TNF-α, and IFN-γ in any of these samples (data not depicted). These data are consistent with our in vitro findings, which demonstrated that IL-17F/F has lower activity than IL-17A/A in regulating CXCL1 expression.

We next compared the activity of IL-17F/A heterodimer with IL-17A/A and IL-17F/F in the airways. One dose of 1.5 μg of IL-17F/A induced a significant increase in neutrophils, CXCL1, and CXCL5 (Fig. 6C). Although the induction of neutrophils was similar between IL-17A/A and IL-17F/A (p = 0.76), CXCL1 and CXCL5 expression was 2- to 3-fold less in mice treated with IL-17A/A than IL-17A/A. These findings showed that IL-17F/A heterodimer is a biologically active molecule in vivo and can induce the recruitment of neutrophils.

Discussion
Th17 T cells are a recently described subset of CD4+ effector T cells that express IL-17A, IL-17F, and IL-22 and are proposed to play a major role in inflammation. Previous studies have suggested an overlapping role for IL-17A and IL-17F, with a direct comparison having only been performed with these cytokines in vitro. In this study, we analyzed certain functions of mouse IL-17A and IL-17F, comparing their relative activities in vitro and in vivo. We also showed that Th17 cells produce an IL-17F/A heterodimeric protein and elucidated certain functions and activities. Our data indicate that mouse IL-17A, either as a constituent of IL-17A/A or IL-17F/A, and not IL-17F/F or IL-22, is critical for Th17 cell-mediated airway neutrophil recruitment and chemokine production.

Several reports have examined the protein expression of IL-17A. In these previous studies, the reported amounts of IL-17A may include both IL-17A/A homodimer and IL-17F/A heterodimer, depending on the characteristics of the capture and detection Abs. In this study, we purposely examined and quantitated the amount of IL-17A/A, IL-17F/A, and IL-17F/F proteins produced by mouse Th17 cells. Whereas activated naive cells expressed more IL-17F/F than IL-17A/A and more IL-17F/A than IL-17A/A in all conditions tested, differentiated Th17 cells, maintained in IL-23, produced substantial amounts of IL-17F/F, higher than the expression of IL-17A/A and IL-17F/F. These data demonstrate that optimal expression of IL-17F during restimulation of activated Th17 cells requires IL-23, similar to what has previously been shown with IL-17A (5). Furthermore, our data indicate that activated naive cells differentiating toward Th17 express limiting amounts of IL-17A, whereas differentiated Th17 cells no longer have this constraint. Our results suggest that the relative amounts of IL-17A/A, IL-17F/A, and IL-17F/F produced by Th17 cells are regulated depending on the stage of differentiation. These in vitro observations suggest a restriction of IL-17A/A expression in vivo by differentiating Th17 cells during the early phase of the adaptive immune response. The mechanism controlling these expression patterns is not known. Recently, RORγt and STAT3 transcription factors have been identified to be regulators of Th17 differentiation (36, 37). The distinct IL-17A and IL-17F expression profiles we observed may be related to the differential expression of these, or other unidentified, transcription factors in naive cells vs differentiated Th17 cells. There may also be differences between the transcriptional accessibility of the IL-17A and IL-17F loci. Further analysis of the transcription factors and the cis-regulatory elements in the IL-17A and IL-17F loci will provide insights into the regulation of their expression.

The production of these different IL-17 proteins by Th17 cells led us to directly compare the activity of purified mouse IL-17A/A, IL-17A/F, and IL-17F/F in vitro. In these previous studies, the reported amounts of IL-17A may include both IL-17A/A homodimer and IL-17F/A heterodimer, depending on the characteristics of the capture and detection Abs. In this study, we purposely examined and quantitated the amount of IL-17A/A, IL-17F/A, and IL-17F/F proteins produced by mouse Th17 cells. Whereas activated naive cells expressed more IL-17F/F than IL-17A/A and more IL-17F/A than IL-17A/A in all conditions tested, differentiated Th17 cells, maintained in IL-23, produced substantial amounts of IL-17F/F, higher than the expression of IL-17A/A and IL-17F/F. These data demonstrate that optimal expression of IL-17F during restimulation of activated Th17 cells requires IL-23, similar to what has previously been shown with IL-17A (5). Furthermore, our data indicate that activated naive cells differentiating toward Th17 express limiting amounts of IL-17A, whereas differentiated Th17 cells no longer have this constraint. Our results suggest that the relative amounts of IL-17A/A, IL-17F/A, and IL-17F/F produced by Th17 cells are regulated depending on the stage of differentiation. These in vitro observations suggest a restriction of IL-17A/A expression in vivo by differentiating Th17 cells during the early phase of the adaptive immune response. The mechanism controlling these expression patterns is not known. Recently, RORγt and STAT3 transcription factors have been identified to be regulators of Th17 differentiation (36, 37). The distinct IL-17A and IL-17F expression profiles we observed may be related to the differential expression of these, or other unidentified, transcription factors in naive cells vs differentiated Th17 cells. There may also be differences between the transcriptional accessibility of the IL-17A and IL-17F loci. Further analysis of the transcription factors and the cis-regulatory elements in the IL-17A and IL-17F loci will provide insights into the regulation of their expression.

The production of these different IL-17 proteins by Th17 cells led us to directly compare the activity of purified mouse IL-17A/A,

**FIGURE 6.** IL-17A/A or IL-17F/A can induce airway neutrophilia in vivo. Mice were administered PBS or 1.5 μg of cytokine intranasally once (A and C) or daily for 3 consecutive days (B). BAL fluid was harvested 24 h after the last dose and neutrophils, CXCL1, and CXCL5 were quantitated. Data are average ± SEM, n = 7, and are representative of two experiments; p values indicate comparisons with PBS group.
IL-17A/A, and IL-17F/F in vitro and in vivo. Although several studies have examined the effects of mouse IL-17A/A, most comparisons of IL-17A/A and IL-17F/F activity have been reported using human cytokines. In these studies, human IL-17A/A was either more potent or equally as potent as IL-17F/F in inducing certain proinflammatory mediators from epithelial cells in vitro (10, 11, 23). Because IL-17A/A and IL-17F/F are glycosylated proteins, a recombinant protein may have different activity depending on whether a prokaryotic or eukaryotic cell produced it. Such differences in cell source of protein may contribute to variability observed between previous reported studies. In this study, we compared eukaryotic-derived mouse IL-17A/A and IL-17F/F and demonstrate that IL-17A/A was considerably more potent than IL-17F/F in inducing neutrophil recruitment in vivo and chemokine production in vitro and in vivo. We also show that IL-17F/F is more potent than IL-17F/F and less potent than IL-17A/A in vitro and in vivo. Our findings agree with and complement the in vitro data of Chang and Dong (24) showing that IL-17A/A was more potent than IL-17F/F on fibroblast cells, with IL-17F/F having intermediate potency. The mechanism for this difference in activity among IL-17A/A, IL-17F/F, and IL-17F/F is still unclear. Human IL-17A/A and IL-17F/F signal through a receptor complex that contains IL-17RA and IL-17RC (6). Mouse IL-17A/A, IL-17F/F, and IL-17F/F signaling is known to require at least IL-17RA (38). If mouse IL-17A/A, IL-17F/F, and IL-17F/F share the same receptor complex(es), then the difference in activity between these cytokines may reflect a difference in binding affinity to their receptors. Alternatively, some evidence exists suggesting that these cytokines may have distinct functions and may not share the same receptor complex(es). Starnes et al. (20) have reported that IL-17F/F can induce IL-2 and TGF-β expression from endothelial cells, cytokines not reported to be induced by IL-17A/A. Furthermore, Starnes et al. (20) report that IL-17F/F inhibits angiogenesis in vitro, whereas IL-17A/A is reported to promote angiogenesis in tumor cells in vivo (39). These data suggest that IL-17A/A and IL-17F/F may not have entirely overlapping functions; IL-17A/A and IL-17F/F may have some distinct roles in inflammation. Although we demonstrate in this study that IL-17A/A is substantially more potent in inducing expression of neutrophil chemoattractants than IL-17F/F, with IL-17F/F having intermediate activity, further direct comparative analysis of the roles of IL-17A/A, IL-17F/F, and IL-17F/F in other biological processes are needed to form a comprehensive understanding of the functions of each cytokine. Whereas previous studies have examined the role of IL-17A/A or IL-17F/F individually in the lung, a function of Th17 cells in regulating airway inflammation has not been reported. In this study, we demonstrate that Th17 cells induce neutrophil influx into the airways, without any effects on eosinophils and monocytes. We show that administration of an IL-17A-specific neutralizing Ab completely prevented Th17-mediated airway neutrophilia. The concomitant reduced expression of CXCL5, with administration of the IL-17A-specific Ab, suggests that this chemokine may be essential for the recruitment of neutrophils by Th17 cells in the lungs. Our findings on the critical role of IL-17A in Th17-mediated airway inflammation complemented our studies showing that direct instillation of IL-17A/A or IL-17F/F induced airway neutrophilia. Our data are also consistent with previous reports demonstrating an essential role for IL-17A in regulating airway inflammation, either using neutralizing Abs to IL-17A (14–16) or IL-17A-deficient mice (40). Since we have shown that an IL-17A-specific Ab can neutralize both IL-17A/A and IL-17F/F in vitro, it is likely that this Ab has blocked both IL-17A/A and IL-17F/F signaling in vivo in this study and, possibly, in previous studies. Further analysis of the relative contributions of IL-17A/A and IL-17F/F with neutralizing Abs specific for each protein will be needed to clarify whether either of these cytokines is indispensable in vivo.

IL-22 has been shown to induce proinflammatory mediators (28–31) by certain cells, similar to ones induced by IL-17A. Our data demonstrate that IL-22 does not regulate airway neutrophilia, indicating that IL-17A and IL-22 do not always have overlapping functions. We also did not observe a role for IL-17F in regulating neutrophil recruitment into the lung, either with direct administration of cytokine or with neutralization in our Th17 cell-mediated airway inflammation model. Other reports have demonstrated that overexpression of human or mouse IL-17F in the airway via adenoviral constructs or pulmonary gene transfer leads to enhanced neutrophil accumulation (21, 22). The amount of IL-17F expressed in these overexpression systems is unknown and may be high. As a result, it may be difficult to fully extend these findings to a more physiological setting. Infection with adenoviral vectors may also induce other factors that can act in concert with IL-17F. We did observe that IL-17F/F induced a small, but significant increase in CXCL5 when administered daily for 3 days. This increase demonstrates that the IL-17F/F protein is active in vivo.

It is possible that IL-17F may be regulating other aspects of lung inflammation. A study on IL-17F polymorphisms in asthmatic patients has identified a variant with a single amino acid substitution (H161R) that is associated with protection from asthma (41). This variant does not induce production of proinflammatory molecules from epithelial cells or the activation of signal transduction pathways. Furthermore, the IL-17F (H161R) protein can block signaling of native IL-17F. The association of IL-17F (H161R) with protection from asthma, along with the ability of this variant to block native IL-17F signaling, suggests that native IL-17F may play a pathogenic role in asthma. Further study of native and the H161R variant of IL-17F may reveal novel functions and their roles in regulating the pathogenesis of asthma.

Taken together, we demonstrate through direct in vitro and in vivo comparisons that IL-17A/A has some distinct in vivo roles from IL-17F/F or IL-22 in the airways. We show that although the expression of IL-17F/F homodimer is higher than IL-17A/A homodimer during initial Th17 differentiation, IL-17A/A is considerably more active than IL-17F/F in regulating chemokine production and inducing airway neutrophilia. Furthermore, we demonstrate that mouse Th17 cells produce a heterodimeric protein composed of both IL-17A and IL-17F. IL-17F/F heterodimer is expressed by differentiated Th17 cells in substantial amounts, comparable to IL-17A/A and IL-17F/F, suggesting an important role for this heterodimeric protein in mediating the functions of Th17 cells. We show that mouse IL-17F/F is a biologically active protein that induces chemokine production and airway neutrophilia. Further analysis of the relative contributions of IL-17A/A, IL-17F/F, and IL-17F/F, as well as IL-22, using Abs specific to each protein will improve our understanding of their functional relationship in mediating tissue inflammation in other organs and disease models. The IL-17F/F heterodimer represents a protein capable of mediating certain functions of Th17 cells and adds another dimension of possible functional cooperativity among cytokines produced in the Th17 lineage.

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Disclosures

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