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IL-17A and TNF-α Exert Synergistic Effects on Expression of CXCL5 by Alveolar Type II Cells In Vivo and In Vitro

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CXCL5, a member of the CXC family of chemokines, contributes to neutrophil recruitment during lung inflammation, but its regulation is poorly understood. Because the T cell-derived cytokine IL-17A enhances host defense by triggering production of chemokines, particularly in combination with TNF-α, we hypothesized that IL-17A would enhance TNF-α-induced expression of CXCL5. Intratracheal coadministration of IL-17A and TNF-α in mice induced production of CXCL1, CXCL2, and CXCL5, which was associated with increased neutrophil influx in the lung at 8 and 24 h. The synergistic effects of TNF-α and IL17A were greatly attenuated in Cxcl5−/− mice at 24 h, but not 8 h, after exposure, a time when CXCL5 expression was at its peak in wild-type mice. Bone marrow chimeras produced using Cxcl5−/− donors and recipients demonstrated that lung-resident cells were the source of CXCL5. Using differentiated alveolar epithelial type II (ATII) cells derived from human fetal lung, we found that IL-17A enhanced TNF-α-induced CXCL5 transcription and stabilized TNF-α-induced CXCL5 transcripts. Whereas expression of CXCL5 required activation of NF-κB, IL-17A did not increase TNF-α-induced NF-κB activation. Apical costimulation of IL-17A and TNF-α provoked apical secretion of CXCL5 by human ATII cells in a transwell system, whereas basolateral costimulation led to both apical and basolateral secretion of CXCL5. The observation that human ATII cells secrete CXCL5 in a polarized fashion may represent a mechanism to recruit neutrophils in host defense in a fashion that discriminates the site of initial injury. The Journal of Immunology, 2011, 186: 000–000.

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Abbreviations used in this article: AM, alveolar macrophage; ATII, alveolar type II cell; BALF, bronchoalveolar lavage fluid; BM, bone marrow; CHX, cycloheximide; DCo, dexamethasone plus 8-Br-cAMP plus isobutylmethylxanthine; ELR, glutamic acid-leucine-arginine; ENA-78, epithelial cell-derived neutrophil-activating peptide-78; i.t., intratracheal; KC, keratinocyte chemooattractant; LIX, LPS-induced CXC chemokine; qPCR, quantitative PCR; rm, recombinant mouse; WT, wild-type.

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MIP-2 are mainly expressed by alveolar macrophages. We further generated Cxcl5-deficient mice and demonstrated the nonredundant but opposing roles of Cxcl5 in mediating neutrophil influx to the lung in a localized LPS inhalation model and a severe Escherichia coli pneumonia model (30). Upon LPS inhalation, Cxcl5 expression in bronchoalveolar lavage fluid (BALF) was much more predominant than KC and MIP-2, which contributed to its dominant role in mediating neutrophil influx to the lung. Furthermore, in a model of severe E. coli pneumonia, Cxcl5 release induced high levels of KC and MIP-2 in both BALF and plasma at least partially through inhibiting chemokine scavenging and thereby modifying the inflammatory response. Although our group has described Cxcl5 as a product of ATII cells in mice and rats, and as a critical player in lung inflammation and pulmonary host defense, whether human ATII cells respond similarly is unknown. Furthermore, the stimuli that induce Cxcl5 in the ATII cell are poorly characterized. Because previous reports have indicated that IL-17A in combination with TNF-α increased synergistically the expression of chemokines (16, 17), we hypothesized that IL-17A combined with TNF-α would exert synergistic effects on Cxcl5 expression during lung inflammation. We further tested whether such synergistic effect would be manifested in primary human ATII cells, well known to be critical for development as well as normal homeostasis.

In this study, we first showed the expression of IL-17A, TNF-α, and Cxcl5 in severely inflamed mouse lungs. A synergistic effect on neutrophil influx was induced by intratracheal (i.t.) co-administration of IL-17A and TNF-α, and this was associated with increased expression of Cxcl1 and Cxcl5. In contrast to Cxcl1, Cxcl5 played a dominant role in neutrophil influx stimulated with IL-17A and TNF-α coinoculation at a later phase of influx. In differentiated primary human fetal ATII cells, we demonstrated, for the first time to our knowledge, expression of Cxcl5 by human ATII cells and confirmed the synergistic effect of IL-17A and TNF-α on induction of Cxcl5. The mechanism of this effect was due to both transcription and mRNA stability and required NF-kB activation and new protein synthesis. We also showed, to our knowledge for the first time, a unique polarization both of stimulation and release of Cxcl5 in ATII cells, which may have an important role in acute lung injury during lung inflammation and infection.

Materials and Methods

Reagents

E. coli strain (ATCC 25922) was purchased from American Type Culture Collection. Mouse rIL-17A (421-ML/CF), TNF-α (410-MT/CF), human rIL-17A (317-IL), TNF-α (210-TA), mouse Cxcl5/LIX (DY443), and human Cxcl5/ENA-78 ELISA kit (DY254) were obtained from R&D Systems. IsRx phosphorylation inhibitor BAB-11-7082 was obtained from Calbiochem. Stock solutions of the inhibitors were prepared in DMSO. The actinomycin D (A9415) was from Sigma-Aldrich.

Mice

Generation of Cxcl5−/− mice has been described (30). Cxcl5−/− mice (backcrossed with C57BL/6 mice for six generations) and their control C57BL/6 mice were kept in specific pathogen-free conditions in the animal facility of the Children’s Hospital of Philadelphia. All experimental procedures were in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia. Eight- to 12-wk-old and sex- and age-matched mice were used for experiments.

E. coli pneumonia mouse model

The procedures for the E. coli pneumonia (ATCC 25922; American Type Culture Collection) mouse model have been previously described (31). Briefly, the mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and a midventral incision was performed. After isolation of muscles, the trachea was exposed, and each mouse was inoculated with 103 or 104 CFU E. coli in 50 µl in 0.9% saline. The mice were humanely sacrificed at 8 and 24 h postinfection, and BALF was collected.

Intratracheal administration of recombinant mouse IL-17A and/or recombinant mouse TNF-α

C57BL/6 mice were divided into four groups as follows: 1) PBS alone; 2) recombinant mouse (rm)IL-17A (625 ng/mouse) alone; 3) rmTNF-α (500 ng/mouse) alone; and 4) rmIL-17A plus rmTNF-α. All treatments were administered in a volume of 50 µl. Mice were euthanized at 4, 8, and 24 h. Cxcl5−/− mice were compared with C57BL/6 mice receiving rmIL-17A plus rmTNF-α at 8 and 24 h.

Generation of chimeric mice by bone marrow reconstitution

The 6–8-wk-old recipient mice were lethally irradiated in two doses of 800 and 400 rad with an interval of 3 h. Bone marrow (BM) from donor mice was harvested from both the tibiae and femora. After lysis of red cells, ~5 million cells were i.v. injected into recipient mice after lethal irradiation. The BM reconstitution was performed in four groups of mice: 1) BM from wild-type (WT) mice into WT mice; 2) BM from WT mice into Cxcl5−/− mice; 3) BM from Cxcl5−/− mice into WT mice; and 4) BM from Cxcl5−/− mice into Cxcl5−/− mice. Eight weeks after irradiation, mice were used for 106 E. coli i.t. inoculation. Analysis of blood genotypes after reconstitution indicated no admixture of recipient cells (data not shown).

Analysis of leukocytes in BALF

At specified times after treatment, the mice were anesthetized and then sacrificed. BALF was conducted with 0.8-ml aliquots of PBS in a total volume of 3.2 ml containing 100 µM diethylenetriamine pentaaacetic acid. Leukocytes from BALF were quantified using a hemacytometer and also processed by cytospin. Slides were fixed and stained with Diff-Quik staining kit (Sigma-Aldrich), and the neutrophil counts were calculated by multiplying neutrophil percentage by total number of leukocytes in the lavage.

Cell culture

Human fetal lung tissue (16–20 wk gestation) was obtained from Advanced Bioscience Resources (Alameda, CA) under a protocol approved by Children’s Hospital of Philadelphia. Explants of the fetal lung were cultured 4 d in dexamethasone (10 nM) plus 8-bromo-cAMP (0.1 mM) plus isobutylmethylxanthine (0.1 mM) (DCI) at 37°C in 5% CO2 incubator to induce a type II cell phenotype as described (32). A highly enriched population of type II cells was isolated as described (33) and cultured in DCI to maintain the type II phenotype.

Nuclear run-on assay

For the measurement of gene transcription, nuclear run-on assay was performed following the protocol of Hildebrandt and colleagues (34). Briefly, nuclei were isolated from human fetal ATII cells treated with or without TNF-α (10 ng/ml) in the presence of IL-17 (25 ng/ml) for 24 h. Transcripts that were initiated in the cells were allowed to continue in the presence of NTPs for 15 min at 37°C. RNA was extracted and reverse-transcribed using the Superscript First-Strand System (Invitrogen) according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed using TaqMan Universal PCR Master Mix kit (Applied Biosystems) with the TaqMan Gene Expression Assays (Applied Biosystems).

Cxcl5 mRNA stability

Human alveolar type II cells were cultured with TNF-α (10 ng/ml) in the absence or presence of IL-17A (10 ng/ml) for 10 h. Cells were then washed and incubated with actinomycin D (5 µg/ml) to inhibit further transcription. Total RNA was extracted at 0, 4, 8, and 12 h, and Cxcl5/ENA-78 mRNA was quantified by qPCR. The percentages of the remaining mRNA at different time points as compared with 0 h were calculated.

Luciferase reporter assay

Human ATII cells were seeded onto 96-well plates at a cell density of 5 × 104 cells/well in Waymouth’s medium (11220; Life Technologies) with DCI and incubated at 37°C in 5% CO2 incubator. On day 4, the cells were infected with adenovirus vectors that had NF-kβ-driven firefly luciferase element (3 × 108 PFU/well) and pRL-SV40-driven renilla luciferase element (3 × 106 PFU/well) (Vector Biosciences, Philadelphia, PA).
FIGURE 1. Expression of IL-17A, TNF-α, and CXCL5 in mouse lung in response to E. coli i.t. challenge. C57BL/6 mice were subjected to 10^7 CFU E. coli via i.t. inoculation; BALF was collected at different time points. The protein levels of IL-17A (A), TNF-α (B), and CXCL5 (C) in the BALF were measured by ELISA. Values are mean ± SEM (n = 3–6). *p < 0.05 versus saline control group. **p < 0.01, ***p < 0.001.

Twenty-four hours postinfection, the cells were then treated with IL-17A, TNF-α, or both for 24 h in fresh medium. The cells were assayed for firefly and renilla luciferase activities using Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured by the IVIS Imaging System (Series 100; Caliper Life Sciences). Data was normalized as instructed.

Human ATII cell monolayers on transwell filters

Human ATII cells were cultured on transwell filters (collagen-coated transwell, 12-mm diameter, 0.4 μm pore size; Corning). The human ATII cells were seeded at 2.5 × 10^5 cells/cm^2 in a volume of 500 μl/filter. In the lower compartment, 1.5 ml medium were added, thereby leveling the height of the liquid levels to prevent hydrostatic pressure. Trans-epithelial resistance was measured was measured at >300 Ω/cm^2 prior to stimulation of the cells.

Multiplex 42-bead array assay

The 96-well filter plate was prefilled with 200 μl assay buffer per well, then the buffer was removed using a vacuum manifold. Each standard or control or sample (25 μl) was added into the appropriate wells. Assay buffer was used for background. A total of 25 μl assay buffer was added to the sample wells, and the same amount of cell culture medium was added to the rest of the wells. The premixed beads (25 μl) were loaded into each well and incubated overnight at 4˚C with shaking. The next day, the beads were washed twice by vacuum, and detection Abs were added. After incubation for 1 h at room temperature, 25 μl streptavidin-PE was pipetted into each well. The beads were washed twice and then resuspended in 150 μl sheath fluid. The filter plate was run on Luminex 200.

Statistical analysis

Data are presented as means ± SEM. We used one-way ANOVA or two-way ANOVA to compare datasets.

FIGURE 2. Exogenous administration of IL-17A and TNF-α into the lung exerts synergistic effect on the expressions of ELR^+ CXC chemokines and neutrophil influx

To further explore the role of IL-17A and TNF-α in pulmonary inflammatory responses, we administered IL-17A and TNF-α, alone or in combination, via i.t. instillation to C57BL/6 mice. Lung lavage was performed at 4, 8, and 24 h. IL-17A and TNF-α acted synergistically to promote neutrophil influx to the lung at 8 and 24 h (Fig. 2F). IL-17A and TNF-α together had a synergistic effect on CXCL5 expression at later time points (Fig. 2A). By comparison, the synergistic effect of IL-17A and TNF-α to increase CXCL1 (Fig. 2B) and CXCL2 (Fig. 2C) was evident only at early time points. The combination of two cytokines also synergistically increased G-CSF expression (Fig. 2D) that was detectable at all

Results

Expression of IL-17A, TNF-α, and CXCL5 in mouse models of lung inflammation

To investigate the expression patterns of IL-17A, TNF-α, and CXCL5 in pulmonary inflammation, we challenged C57BL/6 mice with 10^7 CFU E. coli via i.t. inoculation. Mice exposed to E. coli challenge demonstrated increased IL-17A protein in BALF 8 h postinoculation that was sustained to 24 h (Fig. 1A). TNF-α protein level was markedly increased 8 h after E. coli inoculation and was decreased to 24 h (Fig. 1B). CXCL5, though increased at 8 h, continued to increase through 24 h postinoculation (Fig. 1C). Thus, expression of CXCL5 is associated with protein production of IL-17A and TNF-α in BALF after E. coli challenge.

Exogenous administration of IL-17A and TNF-α into the lung exerts synergistic effect on the expressions of ELR^+ CXC chemokines and neutrophil influx

To further explore the role of IL-17A and TNF-α in pulmonary inflammatory responses, we administered IL-17A and TNF-α, alone or in combination, via i.t. instillation to C57BL/6 mice. Lung lavage was performed at 4, 8, and 24 h. IL-17A and TNF-α acted synergistically to promote neutrophil influx to the lung at 8 and 24 h (Fig. 2F). IL-17A and TNF-α together had a synergistic effect on CXCL5 expression at later time points (Fig. 2A). By comparison, the synergistic effect of IL-17A and TNF-α to increase CXCL1 (Fig. 2B) and CXCL2 (Fig. 2C) was evident only at early time points. The combination of two cytokines also synergistically increased G-CSF expression (Fig. 2D) that was detectable at all
CXCL5 plays a dominant role in neutrophil influx at later stages. To test this, we hypothesized that CXCL5 in the lung may play a dominant role in neutrophil influx after i.t. administration of IL-17A and TNF-α for 24 h. Because significant enhancement of neutrophil recruitment (Fig. 2A) upon coadministration of IL-17A and TNF-α was associated with upregulated CXCL5 expression (Fig. 2A), we hypothesized that CXCL5 in the lung may play a dominant role in neutrophil influx at later stages. To test this hypothesis, we coadministered IL-17A and TNF-α into Cxcl5−/− and C57BL/6 WT mice. Although CXCL1 expression (Fig. 3B) was higher in the Cxcl5−/− mice than that of the WT mice after 8 h of coadministration of IL-17A and TNF-α, there was no significant difference in neutrophil influx (Fig. 3E) between the two groups. In contrast, in the Cxcl5−/− mice receiving IL-17A and TNF-α for 24 h, the total WBCs and neutrophils in the BALF were significantly reduced as compared with the WT mice (Fig. 3D, 3E). Despite the absence of CXCL5 in the targeted mice (Fig. 3A), CXCL1 (Fig. 3B) and CXCL2 (Fig. 3C) in the BALF were not different from WT animals receiving IL-17A and TNF-α for 24 h. The response to deletion of CXCL5 in the setting of similar amounts of CXCL1 and CXCL2 at this time point indicates that CXCL5 plays a dominant role in neutrophil influx to the lung in response to i.t. coadministration of IL-17A and TNF-α at this later stage.

Pulmonary-resident cells are the primary source of CXCL5 upon E. coli challenge

To determine which cells were responsible for CXCL5 production, we generated BM-reconstituted chimeric mice using WT and Cxcl5−/− mice as both donors and recipients. After lethal irradiation of both WT (denoted as K in the second letter) and Cxcl5−/− (denoted as L in the second letter) recipients (denoted as K in the second letter), these mice were reconstituted with BM of healthy WT (denoted as W in the first letter) or Cxcl5−/− donors (denoted as K in the first letter). After challenge of these mice with i.t. instillation of 10⁵ CFU E. coli, only WT recipient mice showed detectable CXCL5 in BALF, indicating that pulmonary-resident cells, not recruited hematopoietic cells or alveolar macrophages, express CXCL5 in the lung during lung inflammation (Fig. 4). Together with our previous findings that rat ATIII cells expressed CXCL5 in vitro and in vivo upon LPS stimulation (10), these data suggest that ATIII cells may be a major source of CXCL5 in the alveolar space during lung inflammation.

IL-17A and TNF-α act synergistically to induce CXCL5/ENA-78 expression in differentiated ATIII cells derived from human fetal lung

To determine whether human ATIII cells express CXCL5 in response to TNF-α and IL-17A, epithelial cells isolated from second trimester (16–20 wk gestation) human fetal lung and differentiated in vitro to an ATIII phenotype (33) were exposed to IL-17 and/or TNF-α for 24 h. Consistent with our in vivo data, TNF-α alone increased CXCL5/ENA-78 mRNA (Fig. 5A) and protein (Fig. 5B) to a greater extent than did IL-17A alone in human ATIII cells, but in both cases were dose dependent. The combination of IL-17A and TNF-α (ratio of 1:1) was shown to be synergistic in inducing CXCL5 expression in vitro.
and TNF-α induced a synergistic response on CXCL5/ENA-78 mRNA (Fig. 5A) and immunoreactive protein expression (Fig. 5B).

We then examined the time course of CXCL5/ENA-78 mRNA and protein expression in response to IL-17A and TNF-α compared with TNF-α alone. TNF-α alone induced peak expression (still far below that of TNF-α plus IL-17A) of CXCL5/ENA-78 mRNA by 8–10 h, which then declined toward baseline by 24 h. The addition of IL-17A to TNF-α greatly enhanced CXCL5/ENA-78 mRNA expression, which peaked by 10 h and remained elevated through 24 h (Fig. 5C). By contrast, CXCL5/ENA-78 protein was not detected in the media in either case until 8 h and then increased exponentially between 8 and 24 h, especially in response to the combination of IL-17A and TNF-α (Fig. 5D).

**IL-17A enhances TNF-α–induced CXCL5/ENA-78 gene expression through both transcriptional and posttranscriptional mechanisms**

Gene upregulation is commonly due to the induction of gene transcription, stabilization of mRNA, or the presence of both mechanisms. To investigate the mechanisms of the marked upregulation of CXCL5/ENA-78 expression upon IL-17A and TNF-α costimulation, we performed nuclear run-on assay in human ATII cells treated with IL-17A (25 ng/ml) and/or TNF-α (10 ng/ml) for 24 h to examine the transcription rate. Intact nuclei were isolated, and the RNA transcripts that had been initiated in vivo were allowed to complete in vitro. The relative levels of CXCL5/ENA-78 transcription were normalized to that of GAPDH. As shown in Fig. 6A, IL-17A or TNF-α treatment increased novel CXCL5/ENA-78 mRNA expression by ∼3-fold or 13-fold, respectively. A synergistic increase in CXCL5/ENA-78 transcription of ∼50-fold was observed in the presence of IL-17A plus TNF-α.

The modulation of mRNA stability has emerged as an important means of regulation for a number of proinflammatory genes (35). To understand whether this mechanism contributes to the synergistic effect of IL-17A and TNF-α on CXCL5/ENA-78 message, we tested stability of CXCL5/ENA-78 mRNA in human ATII cells treated with TNF-α (10 ng/ml) in the presence or absence of IL-17A (10 ng/ml) for 10 h. After incubation with actinomycin D, we then examined the time course of CXCL5/ENA-78 mRNA and protein expression in response to IL-17A and TNF-α compared with TNF-α alone. TNF-α alone induced peak expression (still far below that of TNF-α plus IL-17A) of CXCL5/ENA-78 mRNA by 8–10 h, which then declined toward baseline by 24 h. The addition of IL-17A to TNF-α greatly enhanced CXCL5/ENA-78 mRNA expression, which peaked by 10 h and remained elevated through 24 h (Fig. 5C). By contrast, CXCL5/ENA-78 protein was not detected in the media in either case until 8 h and then increased exponentially between 8 and 24 h, especially in response to the combination of IL-17A and TNF-α (Fig. 5D).

**IL-17A and TNF-α act synergistically to induce CXCL5/ENA-78 expression in differentiated ATII cells derived from human fetal lung.**

A. Human ATII cells were stimulated with IL-17A (10, 50 ng/ml) or TNF-α (2, 10 ng/ml) alone or in combination for 24 h. Total RNA was isolated, and CXCL5/ENA-78 mRNA level determined by qPCR and normalized to 18S rRNA (representative result from separate experiments with three donors). B. CXCL5/ENA-78 protein level in cell medium was measured by ELISA. Time course of CXCL5/ENA-78 mRNA (C) and protein (D) expression induced by TNF-α and its augmentation by IL-17A. ATII cells were treated with TNF-α (10 ng/ml) or TNF-α (10 ng/ml) + IL-17A (10 ng/ml). Cell media and cell contents were collected at different time points. Values are mean ± SEM. Data represent results from three independent donors. *p < 0.01 TNF-α + IL-17A–treated cells versus TNF-α–treated cells, ***p < 0.001.

**FIGURE 6.** IL-17A and TNF-α synergistically enhance CXCL5/ENA-78 mRNA transcription rate and stabilize CXCL5/ENA-78 mRNA in human fetal ATII cells. A. Nuclei were isolated from human fetal ATII cells treated with or without TNF-α (10 ng/ml) in the presence of IL-17A (25 ng/ml) for 24 h. Transcripts that were initiated in the cells were allowed to continue in vitro. The relative expression levels of novel CXCL5/ENA-78 mRNA was determined by qPCR and normalized to GAPDH. *p < 0.05 versus control, **p < 0.01. B. Human ATII cells were treated with TNF-α (10 ng/ml) or TNF-α + IL-17A (10 ng/ml) for 10 h. Cells were then incubated with actinomycin D to inhibit further transcription. CXCL5/ENA-78 mRNA expression over the next 12 h was quantified by qPCR and normalized to 18S rRNA. Values are mean ± SEM. Data in A and B represent results for three independent donors.
RNA was extracted at various time points and CXCL5/ENA-78 measured using qPCR. In cells treated with TNF-α alone, CXCL5 t_{1/2} was determined to be 3.8 h, whereas in cells treated with both IL-17A and TNF-α, t_{1/2} was much longer (Fig. 6B). Therefore, these data demonstrated that IL-17A enhances TNF-α–induced CXCL5/ENA-78 expression by effects on both increased transcription rates and prolonged stability of mRNA.

**Inhibition of de novo protein synthesis abolishes the synergistic effect of IL-17A and TNF-α on CXCL5/ENA-78 expression**

To determine whether synthesis of an intermediate mediator (such as a transcription factor or RNA-binding protein) might be involved in expression of CXCL5, we pretreated human ATII cells with 5 μg/ml cycloheximide (CHX), a protein synthesis inhibitor. As shown in Fig. 7, the synergistic effect of IL-17A and TNF-α on CXCL5/ENA-78 expression was abolished by treatment with CHX (Fig. 7, black bars), even though CHX by itself increased CXCL5/ENA-78 in control. Overall, these results indicate that IL-17A–enhanced CXCL5/ENA-78 expression was greatly attenuated upon NF-κB inhibition in either stimulation with TNF-α alone or TNF-α plus IL-17A. These data demonstrated that TNF-α, but not IL-17A, activated NF-κB signaling, which was necessary, but not sufficient, for IL-17A enhancement of CXCL5/ENA-78 expression by human ATII cells.

**Human ATII cells secrete CXCL5/ENA-78 in a polarized and regulated fashion**

The alveolar lining layer has developed a highly organized cellular polarity: surfactant components are exclusively secreted to the apical sides; and fibrinogen is mainly secreted basolaterally (38). To understand whether CXCL5/ENA-78 was secreted in a polarized fashion in human ATII cells, a monolayer of cells were plated on transwell filters and stimulated with either IL-17A or TNF-α alone, the combination of both from either the upper or the lower compartments, or both compartments of the transwell (Fig. 8A). After 24 h stimulation, the CXCL5/ENA-78 levels in the supernatants from either the upper or lower chambers were measured by ELISA. Apical stimulation by TNF-α provoked apical secretion of ENA-78, whereas basolateral stimulation by TNF-α led to both apical and basolateral secretion of CXCL5/ENA-78 by human ATII cells. This polarized response was also synergistically enhanced by IL-17A costimulation (Fig. 8B).

**Effect of IL-17A and TNF-α on cytokine and chemokine secretion in human ATII cells**

Because coinfiltration of IL-17A and TNF-α into murine lungs synergistically increased not only CXCL5, but also CXCL1, -2

**FIGURE 7.** Inhibition of de novo protein synthesis abolishes the synergistic effect of IL-17A and TNF-α on CXCL5/ENA-78 mRNA expression. Human ATII cells were pretreated with CHX (5 μg/ml) for 1 h, and then they were stimulated with IL-17A (25 ng/ml) and/or TNF-α (10 μg/ml). Ten hours later, cells were collected, and total RNA was isolated. qPCR were performed to quantify the relative expression of CXCL5/ENA-78 mRNA normalized to 18S rRNA. *p < 0.05 versus control, **p < 0.001, ^NS. Values are mean ± SEM. Data represent results for three independent donors.

**FIGURE 8.** TNF-α, but not IL-17A, activates NF-κB signaling on CXCL5/ENA-78 expression in human ATII cells. A, Human ATII cells were transfected with adenovirus vectors that had NF-κB–driven firefly luciferase element and pRL-SV40–driven renilla luciferase element. Twenty-four hours postinfection, the cells were then treated with IL-17A (25 ng/ml), TNF-α (10 ng/ml), or both for 24 h in fresh medium. The cells were assayed for firefly and renilla luciferase activities using Dual-Luciferase Reporter Assay System. Luciferase activities were measured by the IVIS Imaging. B and C, Human ATII cells were treated with or without phosphorylation IκBα inhibitor (BAY-11-7082) for 30 min and then stimulated with IL-17A and/or TNF-α for 24 h. The CXCL5/ENA-78 mRNA expression was measured by PCR (B) and protein levels were measured by ELISA (C). Values are mean ± SEM. Data represent results for three independent donors. ***p < 0.001 versus BAY-treated group.
The AM has been considered to secrete most of the ELR+ CXC sources of these chemokines are less clear. Although traditionally nature and severity of the resulting inflammatory response. The role in directing this event and, at least in part, determine the pulmonary inflammatory responses through ATII cells.

but it synergized with TNF-\(\alpha\) with IL-17A and/or TNF-\(\alpha\) in media of human ATII cells that were treated technology to measure the protein levels of a variety of cytokines and chemokines in human ATII cells. We used multiplex bead measurement of CXCL5/ENA-78 protein. Values are mean \(\pm\) SEM. Data represent results for five independent donors.

and G-CSF, we further explored whether IL-17A and TNF-\(\alpha\) exerted a synergistic effect on the secretions of other cytokines and chemokines in human ATII cells. We used multiplex bead technology to measure the protein levels of a variety of cytokines and chemokines in media of human ATII cells that were treated with IL-17A and/or TNF-\(\alpha\) for 24 h. As shown in Fig. 10, IL-17A alone induced only minimal cytokine and chemokine secretion, but it synergized with TNF-\(\alpha\) in the induction of cytokine (IL-6, G-CSF, GM-CSF), and chemokine (IL-8, GRO, MCP-1, MIP-1\(\beta\), MCP-3) expression (Fig. 10), suggesting that the synergism of TNF-\(\alpha\) with IL-17A may play an important role in orchestrating pulmonary inflammatory responses through ATII cells.

**Discussion**

During lung inflammation and infection, neutrophils generated in the BM are quickly recruited to inflamed sites to form the first line of lung host defense. ELR+ CXC chemokines play an important role in directing this event and, at least in part, determine the nature and severity of the resulting inflammatory response. The sources of these chemokines are less clear. Although traditionally the AM has been considered to secrete most of the ELR+ CXC chemokines, we have shown murine AM do not express CXCL5 during lung inflammation (10). In the present work, we suggest that the ATII cell contributes to the lung inflammatory response by virtue of its ability to secrete CXCL5 and a variety of other chemokines in response to TNF-\(\alpha\) and IL-17A, a frequently detected combination of cytokines.

The ATII cell has been described for many years, since the original work of Mason and Williams (39), as the defender of the alveolus, based on its ability to secrete surfactants to prevent alveolar collapse. More recently, a body of work indicates that ATII cells participate as well in host defense (7, 8, 40, 41). In this article, we extend the function of ATII cells in inflammation by focusing on a unique member of the ELR+ CXC chemokine family, CXCL5. This chemokine, as we previously demonstrated, is mostly produced by ATII cells during lung inflammation (10) and plays a unique role in chemokine scavenging in blood and regulating neutrophil influx to the lung (30).

IL-17 is a proinflammatory cytokine produced predominately by Th17 cells. Although IL-17 is recognized to participate in host defense and autoimmunity through the induction of proinflammatory gene expression, including CXC chemokines, and subsequently leading recruitment of neutrophils (5), the molecular mechanisms by which this is achieved remain poorly defined. It has been reported that IL-17 treatment is a poor stimulus for gene expression. However, IL-17 can cooperate with other cytokines, particularly TNF-\(\alpha\), to induce a synergistic response (42). We developed a new model to test potential interactions between cytokines. In this model, i.t. instillation of TNF-\(\alpha\) produces a mild inflammatory response, consistent with previous observations in mice (43). The coadministration of IL-17A, however, which to our knowledge has not previously been demonstrated, markedly increased neutrophil accumulation. CXCL1 and -2 were upregulated early, whereas CXCL5 was induced later. Cxcl5–/– mice showed markedly diminished neutrophil influx at later stages, but early neutrophil accumulation was unaffected. These data indicate ELR+ CXC chemokines, despite their similarities, may be responsible for different phases of the inflammatory response. Although the model itself is created through exogenous instillation, it represents conditions that occur in a variety of inflammatory processes.

To study mechanisms of synergism, we used human ATII cells in vitro as a model. We focused on human CXCL5/ENA-78 as a bona fide homolog of murine CXCL5/LIX for several reasons. Although human CXCL5/ENA-78 and human CXCL6/GCP-2 are similarly homologous with murine CXCL5/LIX, the genomic locus of mouse and human CXCL5 are nearly identical, whereas that of human CXCL6 is part of a duplicate region containing pseudogenes. Furthermore, murine CXCL5/LIX and human CXCL5/ENA-78 share functional homology as well, as they both are expressed in platelets (44) and ATII cells (30).

Using human ATII cells, we suggest that the mechanism by which IL-17A synergizes with TNF-\(\alpha\) to induce CXCL5 is apparently complex. IL-17A–induced synergism required new protein synthesis, suggesting that synthesis of new transcription factor(s)
and/or RNA-binding protein(s) was necessary. It was reported that the binding sites for NF-κB, C/EBP, AP-1, and OCT1 were overrepresented in IL-17A target genes by a computational analysis on 18 well-documented IL-17A target promoters; NF-κB was the major transcription factor on IL-17A–induced CXCL5 in this study (45). Activation of the transcription factor NF-κB is critical for the TNF-α–induced inflammatory response (46). In this study, we found that TNF-α induced NF-κB activity; moreover, NF-κB signaling appeared necessary for TNF-α–induced CXCL5/ENA-78 expression in human ATII cells. Hwang et al. (37) demonstrated that IL-17A induced production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-κB–dependent pathways; IL-17A was the potent stimulus in this study. In contrast, in this study, we demonstrated IL-17A was a weak stimulus for CXCL5/ENA-78 expression in human ATII cells; moreover, neither direct IL-17A–induced, or IL-17A–enhanced, CXCL5/ENA-78 gene expression was through an NF-κB signaling pathway. Furthermore, using an Ad-AP-1–luc vector, we examined that AP-1 activity was not induced by either treatment (data not shown). Because C/EBPα, C/EBPβ, and C/EBPδ are expressed in ATII cells (47, 48), and because signaling via the IL-17R activates AP-1 activity was not induced by either treatment (data not shown). Because C/EBPα, C/EBPβ, and C/EBPδ are expressed in ATII cells (47, 48), and because signaling via the IL-17R activates AP-1 activity was not induced by either treatment (data not shown). Because C/EBPα, C/EBPβ, and C/EBPδ are expressed in ATII cells (47, 48), and because signaling via the IL-17R activates AP-1 activity was not induced by either treatment (data not shown). Because C/EBPα, C/EBPβ, and C/EBPδ are expressed in ATII cells (47, 48), and because signaling via the IL-17R activates AP-1 activity was not induced by either treatment (data not shown). Because C/EBPα, C/EBPβ, and C/EBPδ are expressed in ATII cells (47, 48), and because signaling via the IL-17R activates AP-1 activity was not induced by either treatment (data not shown).

Both increased transcription and enhanced stability of the mRNA play a role in the synergistic induction of CXCL5/ENA-78 expression as the 3′ untranslated region of CXCL5/ENA-78 has eleven AUUUAs, which are likely to be involved in regulation of CXCL5/ENA-78 mRNA decay. Regulation of mRNA decay is a potential mechanism by which the duration of the inflammatory response can be limited. However, the ability to prolong the protein expression in the setting of inflammation, and we observed an increase in CXCL5/ENA-78 mRNA stability by IL-17A plus TNF-α. This finding further emphasizes the importance of the role of regulated mRNA stability in the control of proinflammatory gene expression (35).


