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Roles of Interleukin-17 in an Experimental *Legionella pneumophila* Pneumonia Model

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Interleukin-17 (IL-17) is a key factor in T helper type 17 (Th17) lineage host responses and plays critical roles in immunological control of a variety of infectious diseases. Although *Legionella pneumophila*, an intracellular bacterium found widely in the environment, often causes a serious and life-threatening pneumonia in humans, the contribution of IL-17 to immune function during *Legionella* pneumonia is unknown. In the present study, we used an experimental *Legionella* pneumonia infection to clarify the role of IL-17 in the resulting immune response. We observed robust production of pulmonary IL-17A and IL-17F (IL-17A/F), peaking on day 1 and declining thereafter. Upregulated production of tumor necrosis factor alpha (TNF-α), IL-6, and IL-1β, but not monocyte chemotactic protein 1 (MCP-1), was observed in *Legionella*-infected bone marrow-derived macrophages from BALB/c mice that had been stimulated with IL-17A or IL-17F. A significant decrease in the production of proinflammatory cytokines IL-6 and TNF-α was observed in IL-17A/F-deficient mice (BALB/c background) infected with *L. pneumophila*. Moreover, we found impaired neutrophil migration and lower numbers of chemokines (KC, LIX, and MIP-2) in IL-17A/F-deficient mice. IL-17A/F-deficient mice also eliminated *L. pneumophila* more slowly and were less likely to survive a lethal challenge. These results demonstrate that IL-17A/F plays a critical role in *L. pneumophila* pneumonia, probably through induction of proinflammatory cytokines and accumulation of neutrophils at the infection site.

Interleukin-17 (IL-17) has recently received considerable attention because of its important role in cross talk between the innate and adaptive immune systems (9). It contributes to host responses to a variety of immunological and inflammatory disorders, such as collagen disease (4), allograft rejection (45), tumor (2), allergy (21), and infectious diseases resulting from infection with *Klebsiella pneumoniae*, *Bordetella pertussis*, *Streptococcus pneumoniae*, and *Candida albicans* (29). IL-17 is secreted by a variety of cells, including T helper type 17 (Th17) cells, natural killer cells, and $\gamma\delta$ T cells, thereby inducing Th17-type host responses characterized by production of proinflammatory cytokines and neutrophil recruitment (46). Specifically, IL-17 upregulates several chemokines, such as macrophage inflammatory protein 1 (MIP-1), MIP-2, and monocyte chemoattractant protein 1 (MCP-1), which, in turn, attract inflammatory cells to the site of infection. IL-17 also promotes granulopoiesis via the induction of granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) and potentiates neutrophilic cytotoxicity and phagocytosis (35). It is reported to enhance the function and survival of recruited macrophages in the airway (36).

Because IL-17 is a strong inducer of neutrophil-chemotactic factors, it has been suggested to play an important role in the pathogenesis, severity, and outcome in pneumonia caused by infection with *Legionella pneumophila*. *L. pneumophila* is a ubiquitous, Gram-negative facultative intracellular bacillus that frequently causes a life-threatening pneumonia, especially in immunocompromised individuals (23, 32). *Legionella* infections usually result from inhalation of contaminated aerosols from environmental sources. Once the bacteria are in the lungs, they predominantly infect and multiply within monocytes and macrophages (13, 24, 28). Mortality rates of up to 50% have been reported, illustrating the fact that *Legionella* pneumonia remains a challenging infectious disease (8, 30, 41).

Cellular immunity and Th1-type cytokine responses are believed to be integral to defense mechanisms against *Legionella* species, as in cases of other intracellular bacterial infections. Th1-type cytokines such as gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-12, play a crucial role in defense against *Legionella* infection (7, 10, 39). However, the roles of IL-17 in pathogenesis of, and host defenses against, *Legionella* pneumonia still require elucidation.

In the present study, we examined the contribution of IL-17 to pathogenesis and lethal sensitivity of *L. pneumophila* pneumonia in mice. We focused our efforts on IL-17A and IL-17F (IL-17A/F), two main factors in the IL-17 protein family that are known to play an important role in antimicrobial host defenses and immunological responses to a variety of infections, including fungi, bacteria, viruses, and parasites (6, 15, 16, 26, 29). IL-17A and IL-17F appear to bind the same receptor complexes, comprising IL-17 receptor A and IL-17 receptor C (42, 54), and therefore appear to have similar biological functions (16). We measured quantities of these chemokines in the lungs of mice that had been infected with

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Legionella pneumonia and examined stimulation of proinflammatory cytokines in response to various levels of IL-17A and IL-17F. We also compared proinflammatory cytokine induction levels, leukocyte numbers, gene expression of CXC chemokines, and total numbers of bacteria in the lungs of wild-type and IL-17A/F-deficient mice (BALB/c background). Finally, we investigated whether survival was lower in IL-17A/F-deficient mice with *Legionella* pneumonia. Cumulatively, our data strongly indicate that IL-17 is a vital part of the host immune response against *Legionella* infections.

MATERIALS AND METHODS

Animals. Specific-pathogen-free 5- to 8-week-old male BALB/c (Charles River Laboratories, Kanagawa, Japan), C57BL/6J (Charles River Laboratories), and A/J (Sankyo Laboratory, Tokyo, Japan) mice were quarantined for 1 week after reception. IL-17A/IL-17F double knockout mice (*Il17a*$^{-/-}$ *Il17f*$^{-/-}$) on a BALB/c genetic background were previously established at the Institute of Medical Science, University of Tokyo (12, 27). All the studies were performed in mice of the BALB/c background. All mice were housed under specific-pathogen-free conditions within the animal care facility at Toho University School of Medicine (Tokyo, Japan) until the day of sacrifice. Experiments were conducted according to our institution's ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments. Animal protocols were approved by the institutional animal care and use committee (approval number 10-52-54).

***L. pneumophila* inoculation.** Clinical isolates of *L. pneumophila* Suzuki, a serogroup 1 strain stocked at Toho University Hospital (38), were prepared as previously reported (37). Animals were anesthetized intramuscularly with ketamine at 7 mg/kg of body weight and xylazine at 15 mg/kg. Their tracheas were exposed, and 30 μl of bacterial suspension was administered via a sterile 26-gauge needle. Skin incisions were closed with surgical staples.

Preparation of BM-Mϕ and their stimulation with *L. pneumophila* and recombinant IL-17. Bone marrow-derived macrophages (BM-Mϕ) were isolated from the femurs of male BALB/c, C57BL/6J, and A/J mice as previously described (3). The cells were cultured in 96-well tissue culture plates (10^5 cells per well) and used for BM-Mϕ experiments. After the medium had been changed, BM-Mϕ were infected with *L. pneumophila* at the indicated multiplicity of infection (MOI) for 2 h. At the end of the infection period (time zero), nonphagocytosed and nonadherent bacteria were removed by two washes with fresh medium. The cells were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2. At the indicated time points, culture supernatants were collected, and the infected macrophages were lysed as previously described (51).

So that we could investigate the effects of elevated IL-17A and IL-17F levels during *Legionella* pneumonia on other inflammatory cytokines, we incubated murine BM-Mϕ for 24 h with increasing doses of IL-17A or IL-17F (1 pg/ml to 10 ng/ml) in the presence or absence of *L. pneumophila* infection (MOI of 1.0). Culture supernatants of five different samples were then collected and centrifuged at 10,000 $\times$ *g* for 5 min. Samples were stored at −40°C until used for enzyme-linked immunosorbent assays (ELISAs) (see below). Recombinant, endotoxin-free mouse IL-17A and IL-17F were obtained from R&D Systems (Minneapolis, MN).

BAL and collection of BALF. At 1, 2, 4, and 7 days after infection, mice were sacrificed by CO_2 asphyxia. Their tracheas were exposed and intubated using a polyethylene catheter with a 1.7-mm outer diameter. Bronchoalveolar lavage (BAL) was performed with 3 ml of phosphate-buffered saline (PBS) containing a complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN). BAL fluid (BALF) samples were pooled for each animal. Leukocyte numbers were counted with a hemocytometer. Differential counts were performed on cytospin preparations (Cytospin 3 centrifuge; Shandon Scientific, Ltd., Astmoor, United Kingdom) stained with May-Giemsa stain. The remaining BALF was stored at −20°C until subsequent use.

Lung harvests for analysis. After the mice had been sacrificed, their chests were opened, and the pulmonary vasculature was perfused with 1 ml of saline via the right ventricle. Whole lungs were removed, and left lungs were homogenized under a vented hood with a homogenizer (IKA Japan K.K., Osaka, Japan) in 1 ml of water containing a complete protease inhibitor cocktail tablet and 0.2 mM phenylmethanesulfonyl fluoride. Portions of homogenates (10 μl) were inoculated onto buffered charcoal-yeast extract agar supplemented with α-ketoglutaric acid (BCYEα) after serial 1:10 dilution in saline. The remaining homogenates (900 μl) were exposed to cell lysis buffer (0.5% Triton X-100, 150 mM NaCl, and 15 mM Tris base) for 30 min at 4°C and then centrifuged at 10,000 $\times$ *g* for 20 min. Supernatants were collected and stored at −20°C until use. Right lungs were treated with RNAlater (Ambion, Austin, TX) and stored at 4°C until use in evaluation of mRNA expression (see below).

Cytokine/chemokine measurements. Cytokines and chemokines were measured with IL-17A, IL-17F, IL-6, TNF-α, MCP-1, and MIP-2 mouse ELISA kits (R&D Systems), according to the manufacturer's protocols.

Gene expression analysis. Total RNA was isolated from lungs using RiboPure kits (Ambion), according to the manufacturer's instructions. Turbo DNA-free kits (Ambion) were used to treat RNA with DNase, after which it was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Data analysis utilizing the SYBR green real-time reverse transcription-PCR (RT-PCR) technique was performed on an ABI Prism 7000 sequence detector system (Applied Biosystems). We used the following PCR primers: keratinocyte-derived chemokine (KC), 5′-GCTGGGATTCACCTCAAGAA-3′ (forward) and 5′-TCTCCGTTACTTGGGGACAC-3′ (reverse); MIP-2, 5′-CGCCCAGACAGAAGTCATAG-3′ (forward) and 5′-TCCTCCTTTCCAGGTCAGTTA-3′ (reverse); LIX, 5′-GGTCCACAGTGCCCTACG-3′ (forward) and 5′-GCGAGTGCATTCCGCTTA-3′ (reverse); IL-12p35, 5′-CACCCTTGCCCTCCTAAACC-3′ (forward) and 5′-CACCTGGCAGGTCCAGAGA-3′ (reverse); IFN-γ, 5′-GGATATCTGGAGGAACTGGCAA-3′ (forward) and 5′-TGATGGCCTGATTGTCTTTCAA-3′ (reverse); IL-4, 5′-CTCATGGAGCTGCAGAGACTCTT-3′ (forward) and 5′-CATTCATGGTGCAGCTTATCGA-3′ (reverse); β-actin, 5′-AGAGGGAAATCGTGCGTGAC (forward) and 5′-CAATAGTGATGACCTGGCCGT (reverse) (31, 33, 49). Relative fold changes in transcript levels were calculated using the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method (22) using the housekeeping gene β-actin as a reference standard for the amount loaded and the quality of the cDNA.

Statistical analysis. All values are expressed as means ± standard error (SE). Data were analyzed with one-way analyses of variance (ANOVAs), *t* tests, and chi-square tests. Survival curves were constructed using the Kaplan-Meier method and were analyzed by log rank tests. Significance was defined as a P value of <0.05.

RESULTS

Production of IL-17A and IL-17F during *Legionella* pneumonia. Levels of IL-17A and IL-17F in lung tissue of BALB/c mice peaked 1 day after inoculation of *L. pneumophila* and then rapidly declined to baseline values over the next 6 days (Fig. 1).

Effects of IL-17A and IL-17F on production of proinflammatory cytokines in macrophages. As shown in Fig. 2, the addition of IL-17A or IL-17F greatly increased *Legionella*-induced production of IL-6, TNF-α, and IL-1β, from 3- to over 10-fold by cultured macrophages from BALB/c mice. These cytokines were maximally induced by different optimal concentrations of IL-17A and IL-17F though increases were significantly different from baseline levels at all doses (1 pg to 10 ng). Similar results in IL-6 and TNF-α production were observed in C57BL/6J and A/J mice (see Fig. S1 in the supplemental material). On the other hand, the proinflammatory mediator response in the un-

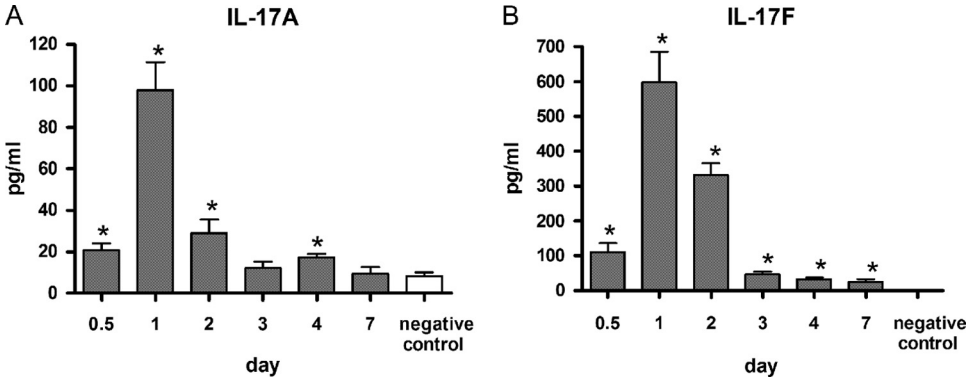

FIG 1 Production of IL-17A and IL-17F in the lung during *L. pneumophila* pneumonia in a mouse model. IL-17A (A) and IL-17F (B) levels are shown as a function of time after the inoculation of approximately 10^5 CFU of *L. pneumophila*. All bars indicate mean ± SE ($n = 6$/group). *, $P < 0.05$ in comparison with a negative control. Similar results were obtained across three repeat experiments.

infected cells was only slightly augmented by stimulation with either IL-17A or IL-17F.

Proinflammatory cytokine production in the lungs of IL-17A/F-deficient mice infected with *Legionella*. In the lungs of wild-type BALB/c mice infected with *Legionella*, there was a sharp increase in IL-6, TNF-α, and IL-1β levels on day 2 (Fig. 3A to C). In contrast, IL-17A/F-deficient mice (BALB/c background) either had significantly lower peaks (TNF-α) (Fig. 3B) or no peak at all (IL-6) (Fig. 3A) on day 2. Corresponding to the data generated by our BM-Mϕ experiments, no significant differences in MCP-1 production were observed between wild-type and IL-17A/F-deficient mice (Fig. 3D).

Leukocyte accumulation and gene expression of chemokines and cytokines. In the week following infection with *Legionella*, wild-type and IL-17A/F-deficient mice (BALB/c background) had similar BALF total cell numbers (Fig. 4A). However, there were significant differences in proportions of specific inflammatory cells. Specifically, on day 2, neutrophils, macrophages, and lymphocytes constituted 71.8, 25.9, and 2.3% of the cells in wild-type BALF and 47.5, 48.3, and 4.2% of cells in BALF of IL-17A/F-deficient mice, respectively.

Correspondingly, gene expression of the pulmonary CXC chemokines KC, LIX, and MIP-2 were lower in IL-17A/F-deficient mice than in wild-type mice (Fig. 4B). In addition, significantly lower MIP-2 levels were observed in the lungs of IL-17A/F-deficient mice (Fig. 4C). Wild-type and IL-17A/F-deficient mice did not have significantly different levels of IL-12, IFN-γ, or IL-4 gene expression (Fig. 4B).

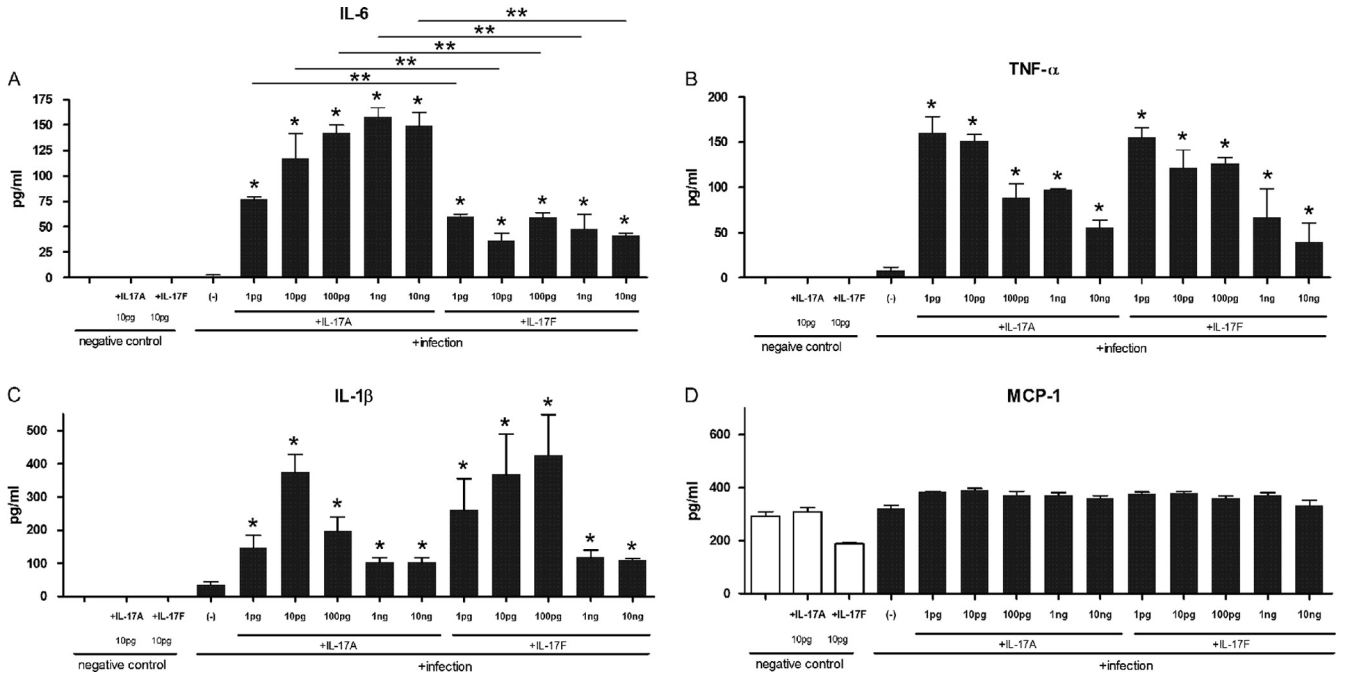

FIG 2 Stimulation of macrophage (BALB/c) proinflammatory cytokines, as indicated on the figure, by IL-17A and IL-17F at 24 h after infection with *L. pneumophila* (10^6 CFU/ml; MOI of 1.0). All bars indicate mean ± SE ($n = 5$/group). *, $P < 0.01$ in a comparison with the infection-only group; **, $P < 0.05$ in a comparison between IL-17A and IL-17F. The results are representative of three independent experiments.

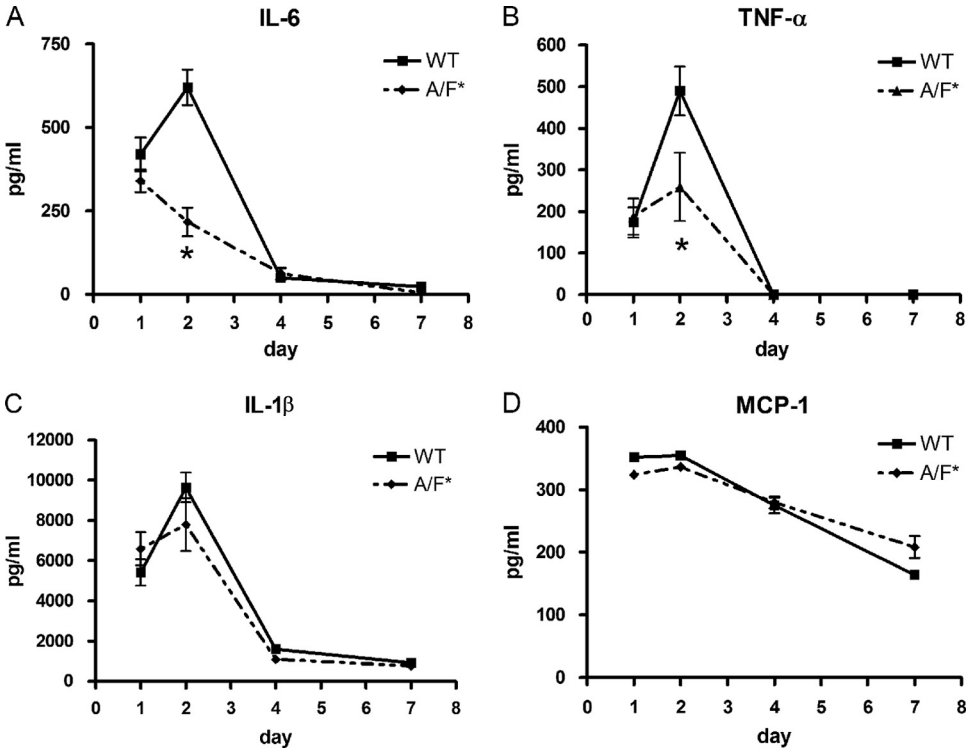

FIG 3 Proinflammatory cytokine production in the lungs of wild-type and IL-17A/F-deficient mice (BALB/c background) during the week following infection with *L. pneumophila*. The production of IL-6, TNF-α, IL-1β, and MCP-1 proteins was examined at 1, 2, 4, and 7 days after infection with *L. pneumophila* (10^5 CFU). All bars indicate mean ± SE ($n = 6$/group). *, $P < 0.05$ in comparisons between *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ (A/F*) and wild-type (WT) mice. Results are representative of two independent experiments.

Bacterial numbers in mice challenged with sublethal doses of *L. pneumophila*. Pulmonary bacterial burdens were similar in wild-type and IL-17A/F-deficient mice (BALB/c background) on days 1, 2, and 4 after infection with *L. pneumophila* (10^5 CFU) (Fig. 5). However, on day 7, IL-17A/F-deficient mice had approximately quadruple the number of bacteria found in wild-type mice.

Survival of mice challenged with lethal doses of *L. pneumophila*. When challenged with *Legionella* (~10^7 CFU), wild-type mice had significantly higher survival rates than IL-17A/F-deficient mice (BALB/c background) (69% versus 38% at day 21; $P < 0.05$) (Fig. 6). These results indicate that IL-17A/F activity is critical in preventing lethality of infection with *Legionella* pneumonia.

DISCUSSION

An increasing number of studies have documented the importance of IL-17 in immune responses to a variety of infectious diseases, especially those caused by extracellular pathogens. Our current results are the first to demonstrate the protective functions of IL-17A/F against *L. pneumophila*, an intracellular pneumonia pathogen. Further, our findings suggest that the beneficial effects of IL-17A/F may be associated with robust induction of inflammatory cytokines and recruitment of neutrophils, leading to clearance of *L. pneumophila* from the lungs and decreases in pneumonia-associated mortality.

The addition of IL-17A and IL-17F greatly increased *Legionella*-induced production of IL-6, TNF-α, and IL-1β but not MCP-1. Maximum cytokine inductions were produced by different concentrations of IL-17A and IL-17F, with higher levels of IL-17 tending to lead to suppression. Although the mechanisms behind, and implications of, these results are unknown, significantly stronger induction of IL-6 was observed in the presence of IL-17A than with IL-17F. These data suggest that IL-17-dependent cytokine/chemokine production has fine-tuning mechanisms, including optimal concentrations, IL-17 receptor specificity, intracellular signaling, and a positive/negative feedback loop (48). It is likely that these cytokines positively regulate the Th1 axis (47) and simultaneously induce both Th17-type cellular responses and IL-17 production (44). In contrast, however, our results demonstrate that there is no difference in MCP-1, IFN-γ, IL-12, and IL-4 levels in control and IL-17-deficient mice. Thus, we conclude that IL-17 is likely not associated with induction of these cytokines/chemokines in our model of *Legionella* pneumonia.

Few previous studies have employed IL-17-deficient or IL-17 receptor-deficient mice in order to investigate the cytokine's role in immune responses to pathogenesis. Among IL-17 receptor-deficient mice intranasally challenged with *Klebsiella pneumoniae*, neutrophil recruitment and bacterial clearance were impaired, and lethal sensitivity was higher (50). Likewise, IL-17 receptor-deficient mice with systemic *Candida albicans* infections had higher fungal burdens and impaired influx of neutrophils into the kidneys, leading to higher mortality rates (14). Recent data from a *Listeria monocytogenes* infection model demonstrated that IL-17 produced by γδ T cells plays a critical role in neutrophil recruitment and eradication of bacteria (11, 25). IL-17 has also been shown to have a crucial role in the host defense against *Salmonella enterica* (34), *Toxoplasma gondii* (18), and *Chlamydia trachomatis* (52) as IL-17 blockades or deficiencies during infection led to

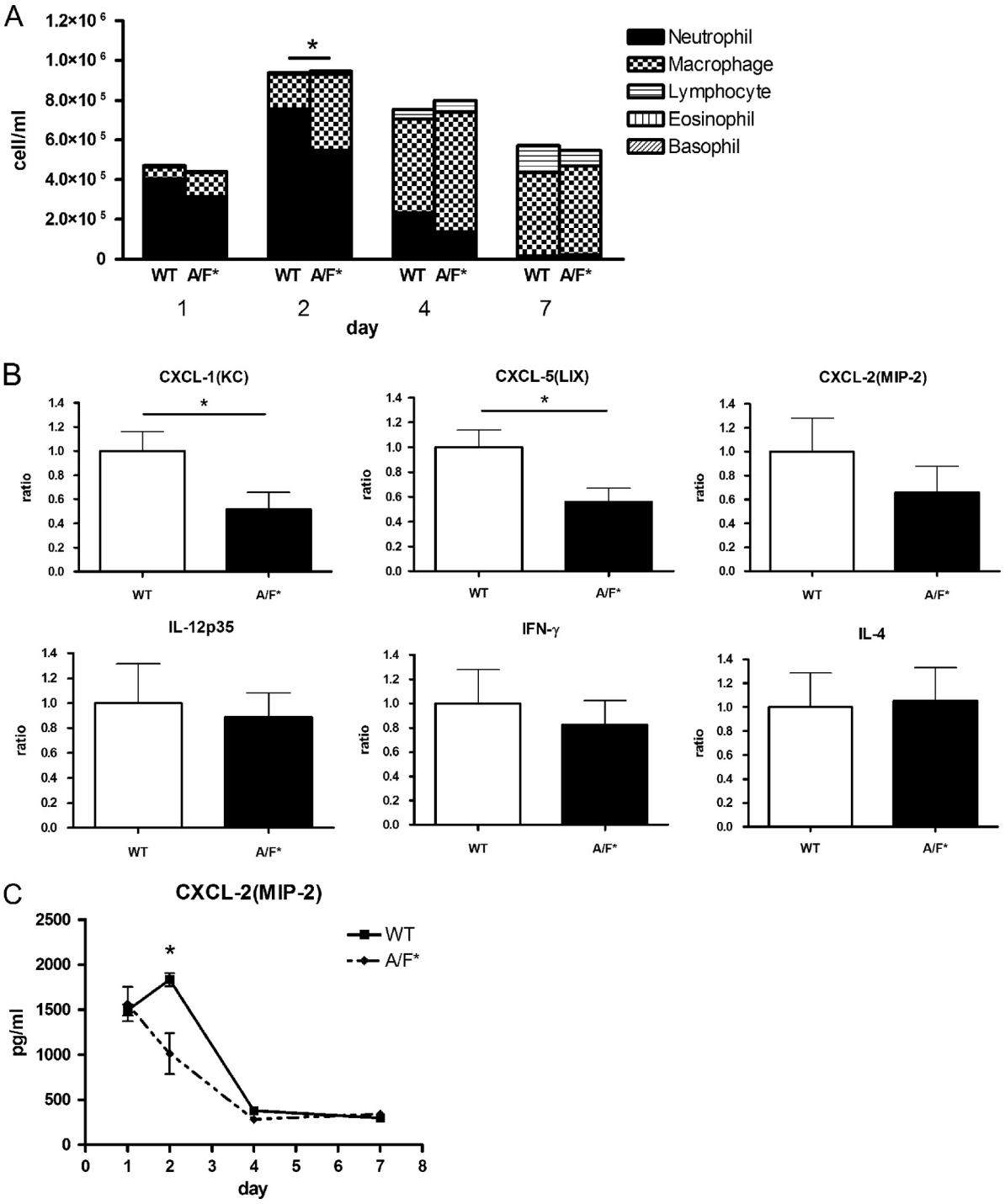

FIG 4 Leukocyte accumulation and gene expression of CXC chemokines in lungs of BALB/c mice infected with *Legionella*. (A) Total cell numbers and proportions of different inflammatory cells in the BALF of wild-type and IL-17A/F-deficient (A/F*) mice (BALB/c background) at 1, 2, 4, and 7 days after infection with 10^5 CFU of *L. pneumophila*. *, $P < 0.05$ for a comparison between the neutrophil numbers of *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ (A/F*) and wild-type (WT) mice. (B) Expression of the chemokines CXCL-1 (KC), CXCL-5 (LIX), CXCL-2 (MIP-2), IL-12p35, IFN-γ, and IL-4 in whole-lung tissue of mice at 48 h after infection with *L. pneumophila*. Data are expressed as fold increase. *, $P < 0.05$ for a comparison between *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ (A/F*) and wild-type (WT) mice. Bars indicate mean ± SE ($n = 5$/group). Results are representative of three independent experiments.

impairment of neutrophil recruitment into the infection site. This is the same pattern observed in the *Legionella* pneumonia model studied here.

We found a significant reduction of neutrophil numbers in the BALF of IL-17A/F-deficient mice, a pattern that was consistent with our real-time PCR and ELISA results indicating a suppression of CXC chemokines (KC, LIX, and MIP-2). These chemokines, as well as CXCR-2-mediated neutrophil accumulation,

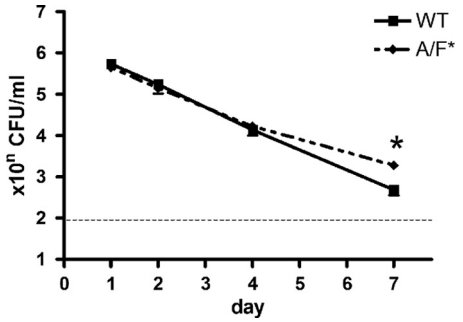

FIG 5 Numbers of bacteria in the lungs of wild-type and IL-17A/F-deficient mice (BALB/c background) infected with *L. pneumophila*. Bacteria were counted at 1, 2, 4, and 7 days after inoculation of 10^5 CFU of *L. pneumophila*. *, $P < 0.01$ for a comparison between *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ (A/F*) and wild-type (WT) mice. Bars indicate mean ± SE ($n = 6$/group). Results are representative of two independent experiments.

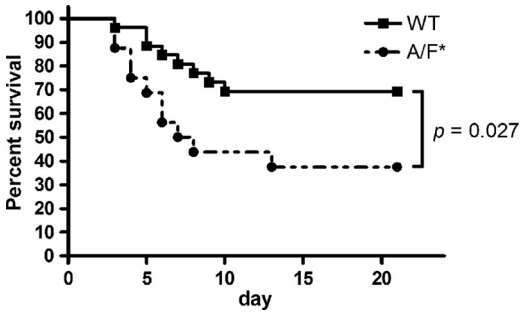

FIG 6 Survival curves for wild-type (WT; $n = 26$) and IL-17A/F-deficient (A/F*; $n = 16$) mice (BALB/c background) infected with *L. pneumophila*. Results are representative of two independent experiments.

have previously been shown to exert their protective effect during *Legionella* infections (40).

Chemokine gene expression in whole-lung tissue could reflect either altered expression of genes or altered cell composition. Modest levels of the latter were observed in the BALF of IL-17-deficient mice. In the future, it will be important to analyze cell composition in whole-lung tissue in addition to examining chemokine gene expression in each cell type (e.g., epithelial cells, neutrophils, and macrophages). The role of epithelial cells, a major source of chemokines in the lung, may be of particular interest.

Previously (37, 39, 40), we have administered the study animals one of two types of bacterial dosage: sublethal (10^5 CFU) or lethal (10^7 CFU). It may be difficult to clearly define our model (true infection, inflammation, or acute damage) because there is no growth of bacteria in the lungs. Thus, in order to appropriately evaluate host responses, it may be necessary to employ an A/J mouse or guinea pig model, in which multiplication of *Legionella* organisms has been observed. Unfortunately, these are not currently available.

There was a 5-day delay between the maximum neutrophil count (day 2) and the decreased pulmonary bacterial burden (day 7). Thus, we cannot definitively conclude that IL-17 is responsible for neutrophil-mediated direct killing of *L. pneumophila*. We have previously reported that neutrophils play a crucial role during primary *L. pneumophila* infection, not via direct killing but by having more immunomodulatory effects (40). In particular, we found that, during the acute phase of infection, neutrophils may be a source of IL-12 in the lungs. This, in turn, drives Th1-type host responses, including IFN-γ induction and the resultant eradication of *L. pneumophila* at the later phase. However, among the IL-17A/F-deficient mice in our current study, we did not observe any variations in the expression of IL-12 and IFN-γ. IL-17 may have been involved in induction of antimicrobial peptides, such as β-defensin-2, lipocalin 2, and members of the S100 family, from colonic, skin, and airway epithelium (1, 5, 17, 20, 53, 54) although the effects of these antibacterial factors on *L. pneumophila* have been less characterized in *in vitro* and *in vivo* pneumonia models.

Th17-driven host defense systems can be positively and/or negatively affected by a variety of factors and cells. In studying the activity of Th17 host systems against infectious diseases, the timing of analysis, infection site, species of the infecting organism, and host specificity will all influence the measured response. Cross talk and synergistic interplay between the Th17 and Th1 domains is plausible as these axes are an integral part of the immune response against several different species of intracellular microorganisms (19, 43).

Cumulatively, our results indicate that IL-17 is an important part of the host immune response to *Legionella* pneumonia as it is upregulated soon after infection, stimulates the production of several important factors in the immune response, and is associated with decreases in intrapulmonary bacteria and with increases in survival rates. Additional research into IL-17 and the Th17 network as a whole is likely to provide new insights into the pathogenesis of the bacteria and novel therapeutic approaches in the treatment of *Legionella* pneumonia.

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