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SHORT COMMUNICATION

IL-10 Restrains IL-17 to Limit Lung Pathology Characteristics following Pulmonary Infection with *Francisella tularensis* Live Vaccine Strain

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IL-10 production during intracellular bacterial infections is generally thought to be detrimental because of its role in suppressing protective T-helper cell 1 (Th1) responses. *Francisella tularensis* is a facultative intracellular bacterium that activates both Th1 and Th17 protective immune responses. Herein, we report that IL-10–deficient mice (*Il10*$^{-/-}$), despite having increased Th1 and Th17 responses, exhibit increased mortality after pulmonary infection with *F. tularensis* live vaccine strain. We demonstrate that the increased mortality observed in *Il10*$^{-/-}$-infected mice is due to exacerbated IL-17 production that causes increased neutrophil recruitment and associated lung pathology. Thus, although IL-17 is required for protective immunity against pulmonary infection with *F. tularensis* live vaccine strain, its production is tightly regulated by IL-10 to generate efficient induction of protective immunity without mediating pathology. These data suggest a critical role for IL-10 in maintaining the delicate balance between host immunity and pathology during pulmonary infection with *F. tularensis* live vaccine strain. *(Am J Pathol 2013, 183: 1397–1404; http://dx.doi.org/10.1016/j.ajpath.2013.07.008)*

Francisella tularensis, a facultative intracellular bacterium, because of its infectious nature and the severe disease caused by low doses of airborne bacteria, has been classified as a category A select bioterrorism agent.[1] Infection in humans is caused by two main subspecies, *F. tularensis* (type A) and *Francisella holarctica* (type B).[2] An *F. tularensis* live vaccine strain (LVS) has been developed from the *F. tularensis* B strain as an experimental vaccine, but is not licensed for use in humans.[1] *F. tularensis* LVS has been used as a representative attenuated model to address the immune requirements for protection against *Francisella*. By using this model, the importance of IL-12 in driving interferon γ (IFN-γ) and T-helper cell 1 (Th1) responses in immunity to *F. tularensis* LVS infection is well described.[3–5] In contrast, IL-17 is generally thought to play a role in protection against extracellular, but not intracellular, pathogens.[6] However, we and others recently identified a protective role for IL-17 in the induction of cellular immunity to *F. tularensis* LVS pulmonary infection,[7–9] by driving the production of IFN-γ through IL-12 induction.[7] IL-17 is a proinflammatory cytokine also known to induce chemokines, such as keratinocyte chemoattractant, macrophage inflammatory protein 2 (MIP-2), and granulocyte colony-stimulating factor (G-CSF), to mediate granulopoiesis, neutrophil recruitment, and inflammation.[6] Accordingly, the absence of IL-17 during *F. tularensis* LVS pulmonary infection also results in decreased induction of G-CSF and MIP-2, as well as decreased accumulation of neutrophils and lung inflammation.[7] Neutrophil depletion alone does not affect bacterial control after pulmonary infection with *F. tularensis* LVS,[10] suggesting that the role for IL-17 in driving Th1 responses, and not neutrophil

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recruitment, was the primary immune mechanism mediating protection in this model.[7] These data together suggest that both IL-17 and IFN-γ are required for generating protective immunity to pulmonary *F. tularensis* LVS infection.

IL-10 is an anti-inflammatory cytokine best studied for its inhibitory effects on IL-12 production and down-regulation of Th1 responses.[11] Accordingly, IL-10–deficient mice show enhanced protection in models of intracellular bacterial infections, such as *Mycobacterium tuberculosis*[12] and *Listeria monocytogenes*.[13] In addition, in a cutaneous model of *F. tularensis* LVS infection, IL-10–deficient mice exhibit increased protection, and this was reversed when IL-17 was depleted.[14] In contrast to these published studies, in the current study, we report that after pulmonary infection with *F. tularensis* LVS, mice deficient in IL-10 ($Il10^{-/-}$) exhibit increased mortality. We clearly demonstrate that the increased mortality in the $Il10^{-/-}$-infected mice is not associated with loss of protective immunity, because bacterial burden between wild-type and $Il10^{-/-}$ mice is similar, but is caused by exacerbated inflammation and increased lung pathology. We demonstrate that the exacerbated inflammation observed in $Il10^{-/-}$-infected mice is the result of unrestrained IL-17 production and IL-17–dependent recruitment of neutrophils and resulting lung pathology. These data together suggest that, although IL-17 is required for protective immunity against pulmonary infection with *F. tularensis* LVS,[7,9] IL-17 production is tightly regulated by anti-inflammatory cytokines, such as IL-10. Our studies highlight how inflammatory cytokines, such as IL-17, can be beneficial for host protection, but when produced unrestrained, can mediate host pathology.

Materials and Methods

Animals and Experimental Infection

C57BL/6 (B6) and $Il10^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $Il17^{-/-}$ mice[15] were crossed to the $Il10^{-/-}$ mice, and $Il17^{-/-}/Il\text{-}10^{-/-}$ double-deficient mice were generated in house on the B6 background and were used in accordance with University of Pittsburgh Institutional Animal Care and Use Committee guidelines. *F. tularensis* LVS (BEI Resources, Manassas, VA) were grown in Mueller-Hinton (MH) broth or agar.[5] For pulmonary infections, mice were infected with 1000 colony-forming units (CFUs) of *F. tularensis* LVS. On day 6 after infection, serial dilutions of the homogenized infected lungs were plated on MH agar plates and lung CFUs were determined. In some experiments, survival was monitored in infected B6- and gene-deficient mice. For depletion of neutrophils, mice were treated with 300 μg of Gr1-depleting antibody (clone IA8; BioXCell, West Lebanon, NH) or isotype control antibody (BioXCell) every 48 hours, as previously described.[16] In some experiments, single-cell lung suspensions were prepared, as previously described, and used for flow cytometric analyses.[7]

Histological and Immunofluorescence Data

Lungs from mice were inflated with 10% neutral-buffered formalin and embedded in paraffin. Lung sections were stained with H&E stain (Colorado Histo-Prep, Fort Collins, CO) and processed routinely for light microscopy. Slides were scored by one of the authors (T.D.O.), who was blinded to the sample groups. Briefly, every field in the entire lung was observed with a light microscope and scored for inflammation, as previously described.[17] Scoring was based on the percentage of alveolar tissue with inflammation, according to the following scale: 0, no inflammation; 1, 1% to <25%; 2, 25% to <50%; 3, 50% to <75%; and 4, 75% to 100% inflammation. For immunofluorescence, paraffin was removed from the formalin-fixed lung sections, as previously described,[7] and samples were probed with biotinylated rat, anti-mouse GR1 (Rat AL-21; BD Pharmingen). Slow-fade gold antifade with DAPI (Molecular Probes, Grand Island, NY) was used to counterstain tissues and detect nuclei. Images were obtained with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany) and were recorded with a Zeiss AxioCam digital camera (Carl Zeiss Microscopy).

Determination of Protein Amounts

IL-10, IL-12, and IL-23 protein levels were measured using a Mouse Duoset enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Other cytokine and chemokine protein levels were determined with a mouse Luminex assay (Linco/Millipore, Billerica, MA). Myeloperoxidase (MPO) chlorination and peroxidase activity were determined in lung homogenates using the MPO activity assay kit (Invitrogen, Grand Island, NY).

Flow Cytometry

Single-cell suspensions were stained with fluorochrome-labeled antibodies specific for CD11c (HL3), Gr1 (RB6-8C5), CD11b (M1/70), CD3 (145-2C11), CD4 (RM4-5), NK1.1 (PK136), γδ T cells (GL3), IFN-γ (XMG1.2), IL-10 (JES5-16E3), and IL-17 (TC11-18H10) or relevant isotype control antibodies. For analysis of intracellular cytokines, cells were stimulated with 50 ng/mL phorbol myristate acetate; 750 ng/mL ionomycin (Sigma Aldrich, St. Louis, MO) in the presence of Golgistop (BD Pharmingen, San Jose, CA) was surface stained, permeabilized with Cytofix-Cytoperm solution (BD Pharmingen), and stained for relevant cytokines. Cells were collected in a Becton Dickinson FACS Aria flow cytometer with FACS Diva software version 6.1.2. Cells were gated based on their forward-by-side scatter characteristics, and the frequency of specific cell types was calculated using FlowJo version 7.6.5 (Tree Star Inc., San Carlos, CA). $CD11c^{+}$ cells with low autofluorescence were designated as lung dendritic cells (DCs), and $CD11c^{+}$ cells with high autofluorescence were designated as lung macrophages, as previously described.[16,18,19]

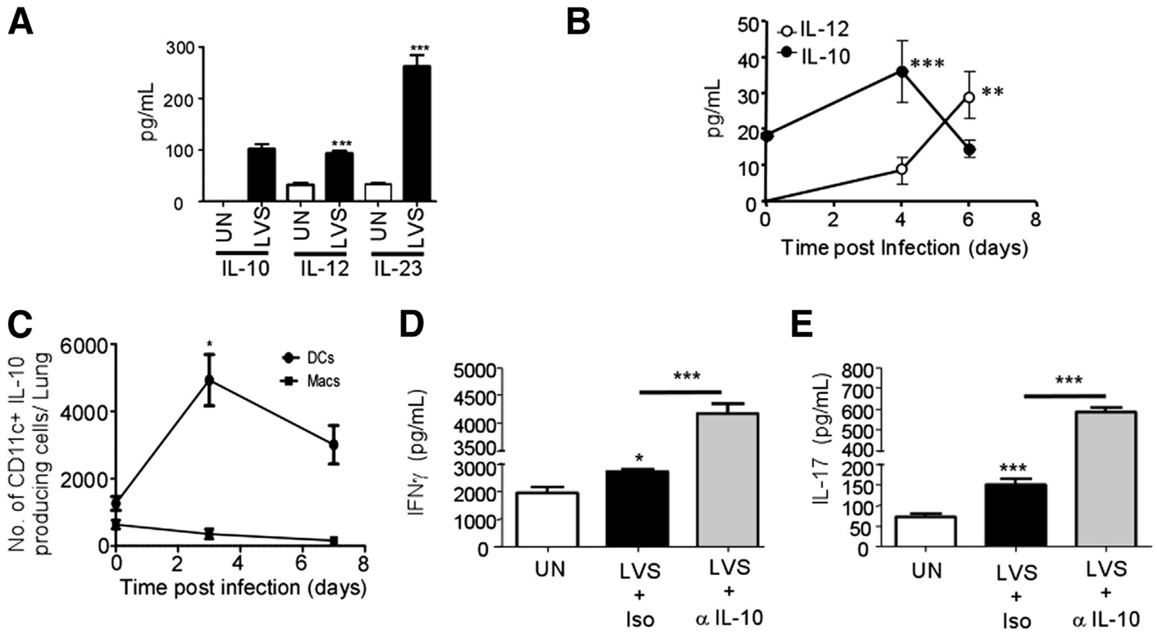

Figure 1 IL-10 is induced after infection with *F. tularensis* live vaccine strain (LVS) infection and restrains Th1 and Th17 responses. **A:** B6 bone marrow dendritic cells (BMDCs) were left uninfected (UN) or infected with *F. tularensis* LVS (multiplicity of infection, 1:100), and IL-10, IL-12, and IL-23 levels were determined in 48-hour culture supernatants by ELISA. **B:** B6 mice were infected with 1000 CFUs *F. tularensis* LVS intratracheally and lung homogenates from 0, 4, and 6 days after infection were assayed for IL-12 and IL-10 levels by ELISA. **C:** Lung suspensions from uninfected and infected mice were assayed for IL-10 production in CD11c$^+$ DCs and macrophages (Macs) by intracellular staining and flow cytometry, and the number of IL-10-producing cells was calculated. **D** and **E:** B6 BMDCS were left untreated (UN), stimulated with *F. tularensis* LVS, and isotype control antibody (LVS + Iso) or with 10 μg/mL anti—IL-10 antibody (LVS + α-IL-10) for 24 hours, after which OT-II T-cell receptor Tg CD4$^+$ T cells were added to the wells, along with 5 μmol/L ovalbumin$_{323\text{-}339}$ peptide. After 6 days in culture, supernatants were assayed for IFN-γ (**D**) or IL-17 (**E**) production. The data points represent the means ± SD of values from three to four samples (**A**, **D**, and **E**) or four to six mice (**B** and **C**). $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. One experiment is representative of two or more.

Generation and Stimulation of BMDCs

Bone marrow dendritic cells (BMDCs) were generated from bone marrow.[7] On day 7, nonadherent cells were infected with *F. tularensis* LVS (multiplicity of infection, 1:100) alone or in combination with 10 μg/mL αIL-10 antibody (clone: JES5-2A5; BioXCell) or isotype control antibody (Rat IgG1; BioXCell) in antibiotic-free Dulbecco's modified Eagle's medium for 48 hours. Naïve CD4$^+$ T cells were isolated from OT-II T-cell receptor αβ Tg mice using magnetic CD4$^+$ beads (GK1.5) (Miltenyi Biotec, Auburn, CA) and were cultured with 1×10^6 cells *F. tularensis* LVS-stimulated or unstimulated BMDCs/mL, with 5 μmol/L ovalbumin$_{323\text{-}339}$ peptide for 6 days, as previously described.[7] Culture supernatants were then analyzed by ELISA.

Statistical Analysis

Differences between the means of two experimental groups were analyzed using the two-tailed Student's *t*-test; a one-way analysis of variance test was used when more than two groups were analyzed. The log-rank test was used for statistical analyses for the survival studies. Differences were considered significant when $P \leq 0.05$.

Results

F. tularensis LVS—Induced IL-10 Production Limits Both Th1 and Th17 Responses

During intracellular bacterial infections, IL-10 expression is detrimental,[12,13] because IL-10—deficient mice (*Il10*$^{-/-}$) show enhanced protection and decreased bacterial burdens. However, the functional role for IL-10 in the context of *F. tularensis* LVS infection has not been completely explored. We found that BMDCs from B6 mice produced IL-10 and other polarizing cytokines, such as IL-12 and IL-23, on infection with *F. tularensis* LVS (Figure 1A). In addition, IL-10 protein was induced early in the lung, whereas IL-12 levels were induced at a later time point after pulmonary *F. tularensis* LVS infection (Figure 1B). Lung IL-23 levels were lower than detectable levels (data not shown). Lung CD11c$^+$ DCs, but not lung macrophages, were one of the primary sources of IL-10 and accumulated at early time points after pulmonary infection (Figure 1C). Both Th1 and Th17 responses are required for protective immunity against *F. tularensis* LVS pulmonary infection.[5,7] To address the role of *F. tularensis* LVS-induced IL-10 on Th1 and Th17 responses, we treated *F. tularensis* LVS-infected B6 BMDCs with IL-10—neutralizing antibody in a DC:T-cell co-culture system and found that IL-10 neutralization resulted in enhanced IFN-γ

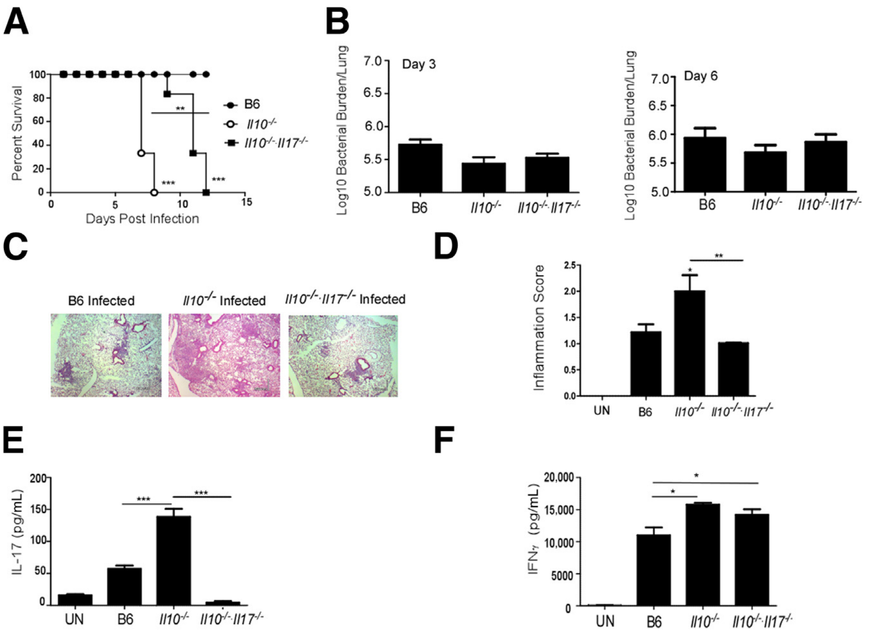

Figure 2 **A:** Dysregulated IL-17 production in *Il10*$^{-/-}$ mice mediates increased mortality and lung pathological characteristics after pulmonary *F. tularensis* LVS infection. B6, *Il10*$^{-/-}$, and *Il10*$^{-/-}$/*Il17*$^{-/-}$ mice were infected with 1000 CFUs *F. tularensis* LVS intratracheally, and the mice that survived were monitored over time. Statistical significance was determined by log-rank test between survival in B6-infected and *Il10*$^{-/-}$ mice, and between B6-infected and *Il10*$^{-/-}$/*Il17*$^{-/-}$-infected mice. **B:** On days 3 and 6 after infection, lung bacterial burden was determined by plating on MH agar plates. **C** and **D:** Day 6, formalin-fixed, paraffin-embedded (FFPE) serial lung sections were stained with H&E(**C**), and sections were scored for percentage of inflammation, as described in *Materials and Methods* (**D**). **E** and **F:** Lung homogenates from infected B6 and gene-deficient mice were assayed for IL-17 (**E**) and IFN-γ (**F**) levels. The data points represent the means ± SD of values from 6 to 10 mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, and $^{***}P \leq 0.0005$. Original magnification, ×10 (**C**). One experiment is representative of two. UN, uninfected.

and IL-17 production by CD4^{+} T cells (Figure 1, D and E, respectively). These data suggest that IL-10, induced after *F. tularensis* LVS infection, may limit both Th1 and Th17 responses.

IL-17—Dependent Inflammation Mediates Increased Mortality in *Il10*$^{-/-}$ Mice after *F. tularensis* LVS Pulmonary Infection

In vitro data suggest that IL-10 may limit both Th1 and Th17 responses, and its presence may be detrimental for protective immunity against *F. tularensis* LVS infection. Unexpectedly, when *Il10*$^{-/-}$ mice were infected intratracheally with *F. tularensis* LVS, they demonstrated decreased survival and increased mortality, when compared with control B6-infected mice (Figure 2A). Interestingly, *Il10*$^{-/-}$-infected mice had similar levels of bacterial burden in the lungs at both early and later time points, suggesting that the decreased survival was not associated with increased bacterial burden (Figure 2B). We also found that *Il10*$^{-/-}$ mice displayed heightened pathology and severe pulmonary inflammation (Figure 2, C and D), suggesting that *Il10*$^{-/-}$-infected mice die as a consequence of increased inflammation rather than loss of protective immunity. *In vitro* data using DC:T-cell co-cultures suggest that IL-10 production restrains both IL-17 and IFN-γ responses in CD4^{+} T cells (Figure 1, D and E). Therefore, we next addressed whether IL-10 also restrains IL-17 and IFN-γ responses *in vivo*, and whether dysregulated expression of these proinflammatory cytokines mediates pathology and inflammation during pulmonary tularemia. We found that, when compared with B6 *F. tularensis* LVS-infected lungs, *Il10*$^{-/-}$-infected lungs expressed significantly higher levels of IL-17 (Figure 2E) and IFN-γ protein (Figure 2F). Both γδ T cells and CD4^{+} T cells are major producers of IL-17 in response to pulmonary infection with *F. tularensis* LVS.[7,9] Accordingly, although numbers of CD4^{+} and γδ T cells producing IL-17 were increased in B6-infected lungs compared with uninfected lungs, their numbers were further enhanced in *Il10*$^{-/-}$-infected lungs (Table 1). These data suggest that, in addition to its known role in restraining Th1 responses,[11] IL-10 also has a role in restraining Th17 responses after pulmonary infection with *F. tularensis* LVS.

The absence of IL-17 during pulmonary tularemia results in decreased inflammation in the infected lung.[7] Therefore, we hypothesized that excess IL-17, rather than excess IFN-γ, caused the inflammation, and increased the mortality observed in the *Il10*$^{-/-}$-infected mice. To address this, we generated IL-10/IL-17 double-deficient mice (*Il10*$^{-/-}$/*Il17*$^{-/-}$) and infected them with *F. tularensis* LVS via the pulmonary route. We found that absence of IL-17 significantly reversed the increased susceptibility seen in *Il10*$^{-/-}$ mice (Figure 2A). More importantly, we found that the heightened pathological characteristics observed in the lungs of infected *Il10*$^{-/-}$ mice were also reversed in lungs from *Il10*$^{-/-}$/*Il17*$^{-/-}$-infected mice (Figure 2, C and D). We have recently shown that IL-17 is protective and is required to drive IL-12—driven Th1-protective cell immunity against *F. tularensis* LVS.[7] This role for IL-17 is likely to overcome the inhibitory effects of IL-10 on IL-12 production, as demonstrated by us in another intracellular bacterial model (namely, *Mycobacterium bovis* bacillus Calmette-Guerin exposure).[20] In support of a role for IL-17 in driving IFN-γ responses specifically to overcome IL-10—mediated inhibition, significantly higher levels of IFN-γ were found in lungs of both *Il10*$^{-/-}$ and *Il10*$^{-/-}$/*Il17*$^{-/-}$ mice when compared with B6-infected lungs (Figure 2F). In addition, *Il10*$^{-/-}$/*Il17*$^{-/-}$-infected mice showed a similar bacterial burden to B6- and *Il10*$^{-/-}$-infected mice at both early and later time points (Figure 2B). These data together suggest that when IL-10 is absent, IL-17 is not required for generation of Th1 responses or overall protective immunity against pulmonary

Table 1 IL-17 Production on *F. tularensis* LVS Infection

Type of cell	Uninfected lungs	B6-infected lungs	*Il10*$^{-/-}$-infected lungs
CD4$^+$ IL-17$^+$ T cells	$1.8 \times 10^4 \pm 6 \times 10^3$	$2.4 \times 10^5 \pm 9.2 \times 10^{4*}$	$4.2 \times 10^5 \pm 1.5 \times 10^{4\dagger}$
$\gamma\delta^+$ IL-17$^+$ T cells	$1.2 \times 10^4 \pm 6.2 \times 10^3$	$1.7 \times 10^5 \pm 7.4 \times 10^{4*}$	$2.9 \times 10^5 \pm 6.1 \times 10^{4\dagger}$

B6 and *Il10*$^{-/-}$ mice were left uninfected or were infected with 1000 CFUs *F. tularensis* LVS intratracheally. On day 6, single-cell lung suspensions were assayed for IL-17 production by intracellular staining and flow cytometry.

$^*P \leq 0.005$ between B6-infected and uninfected mice.

$^\dagger P \leq 0.05$ between B6-infected and *Il10*$^{-/-}$-infected mice.

F. tularensis LVS infection. Instead, excess IL-17 mediates pathology during pulmonary *F. tularensis* LVS infection.

Dysregulated IL-17 Production Mediates Neutrophil Recruitment and Increased Susceptibility in *Il10*$^{-/-}$ *F. tularensis* LVS-Infected Mice

The absence of IL-17 during pulmonary tularemia results in decreased induction of G-CSF, neutrophil recruitment, and resulting inflammation in the infected lung.[7] Correlating with the increased IL-17 levels in infected *Il10*$^{-/-}$ mice, we also found increased levels of G-CSF and the neutrophil-attracting chemokine, MIP-2, in *Il10*$^{-/-}$-infected lungs (Figure 3, A and B, respectively). This also coincided with increased accumulation of neutrophils in the severely inflamed lungs of *Il10*$^{-/-}$-infected mice (Figure 3C). The activity of MPO, an enzyme that is associated with neutrophil activation and generation of reactive oxygen species, leading to oxidative damage,[21] was also notably enhanced in lungs of infected *Il10*$^{-/-}$ mice (Figure 3D). More importantly, increased numbers of neutrophils also accumulated in day 6–infected lungs of *Il10*$^{-/-}$ mice (Figure 3E). In contrast, we found that absence of IL-17 in *Il10*$^{-/-}$ mice reversed the induction of G-CSF and MIP-2 (Figure 3, A and B) and reduced neutrophil accumulation within lung sections (Figure 3C). This coincided with reversal of inflammation in the lung and decreased expression of MPO and decreased neutrophil accumulation in day 6–infected lungs from *Il10*$^{-/-}$/*Il17*$^{-/-}$ mice, compared with *Il10*$^{-/-}$ mice (Figure 3, D and E). Together, our new data show that exacerbated IL-17 production is pathological during pulmonary infection with *F. tularensis* LVS.

To further confirm that the exacerbated inflammation caused because of increased neutrophil recruitment was mediating the increased mortality in *Il10*$^{-/-}$ mice, we specifically depleted Gr1$^+$ neutrophils using a monoclonal antibody (clone 1A8), which is known to specifically deplete neutrophils without affecting Gr1$^+$ monocyte populations.[22] We treated *F. tularensis* LVS-infected *Il10*$^{-/-}$ mice with 1A8 antibody or isotype control antibody every 48 hours, and found that *Il10*$^{-/-}$ mice depleted of neutrophils exhibited decreased susceptibility (Figure 3F) and decreased lung inflammation (Figure 3, G and H). These data together demonstrate that exacerbated neutrophil recruitment and associated inflammation mediate the increased mortality seen in *Il10*$^{-/-}$ *F. tularensis* LVS-infected mice.

Discussion

Production of IL-10 is largely believed to be detrimental for immunity against intracellular pathogens, such as *M. tuberculosis*[12] and *L. monocytogenes*.[13] In addition, IL-10 also limits the protective efficacy of *M. bovis* bacillus Calmette-Guerin vaccination after *M. tuberculosis* challenge,[20,23] suggesting use of anti–IL-10 as a potential adjuvant to improve Th1 responses and immunity against intracellular pathogens. However, in pathological conditions, such as chronic inflammatory bowel disease,[24] IL-10 deficiency unleashes an inflammatory response, which earlier was thought to be associated with enhanced CD4$^+$ Th1 cells,[25] but was later attributed to inflammatory Th17 cells.[26] In contrast, IL-10 deficiency is associated with enhanced Th17 responses during influenza challenge, and a cutaneous model of *F. tularensis* LVS infection,[14] in which it is associated with better protective outcomes.[27] In this context, the interesting and unexpected finding reported in this study is that unrestrained IL-17, induced in response to pulmonary *F. tularensis* LVS infection in *Il10*$^{-/-}$ mice, mediates neutrophil recruitment and associated pathological characteristics. However, because IL-17 is also required to drive IL-12 production and generate Th1 responses during pulmonary tularemia,[7] our studies together propose the new model that IL-17 is required to drive IL-12 and Th1 responses, likely to overcome the inhibitory effects of IL-10 on IL-12 production. In contrast, in the absence of IL-10, dysregulated and exacerbated production of IL-17 production is pathological and mediates inflammation, suggesting a critical role for IL-10 in maintaining the delicate balance between host immunity and pathological characteristics during pulmonary infection with *F. tularensis* LVS.

Our data show that IL-10 is produced early in the lung after pulmonary *F. tularensis* LVS infection and that lung DCs are one of the primary cells producing IL-10. In addition, data show that infection of BMDCs with *F. tularensis* LVS induces IL-10 production and IL-10 neutralization in DC:T-cell co-cultures increased both IL-17 and IFN-γ production in CD4$^+$ T cells. These data are consistent with a previous study that also showed that BMDCs induced IL-10 mRNA and protein in response to *F. tularensis* LVS infection, in a toll-like receptor-2–dependent manner.[28,29] In addition, *in vitro* studies have demonstrated that *F. tularensis* LVS, cultured in MH broth, induces more robust IL-10 production in DCs, when compared with host-adapted bacteria cultured in brain heart infusion

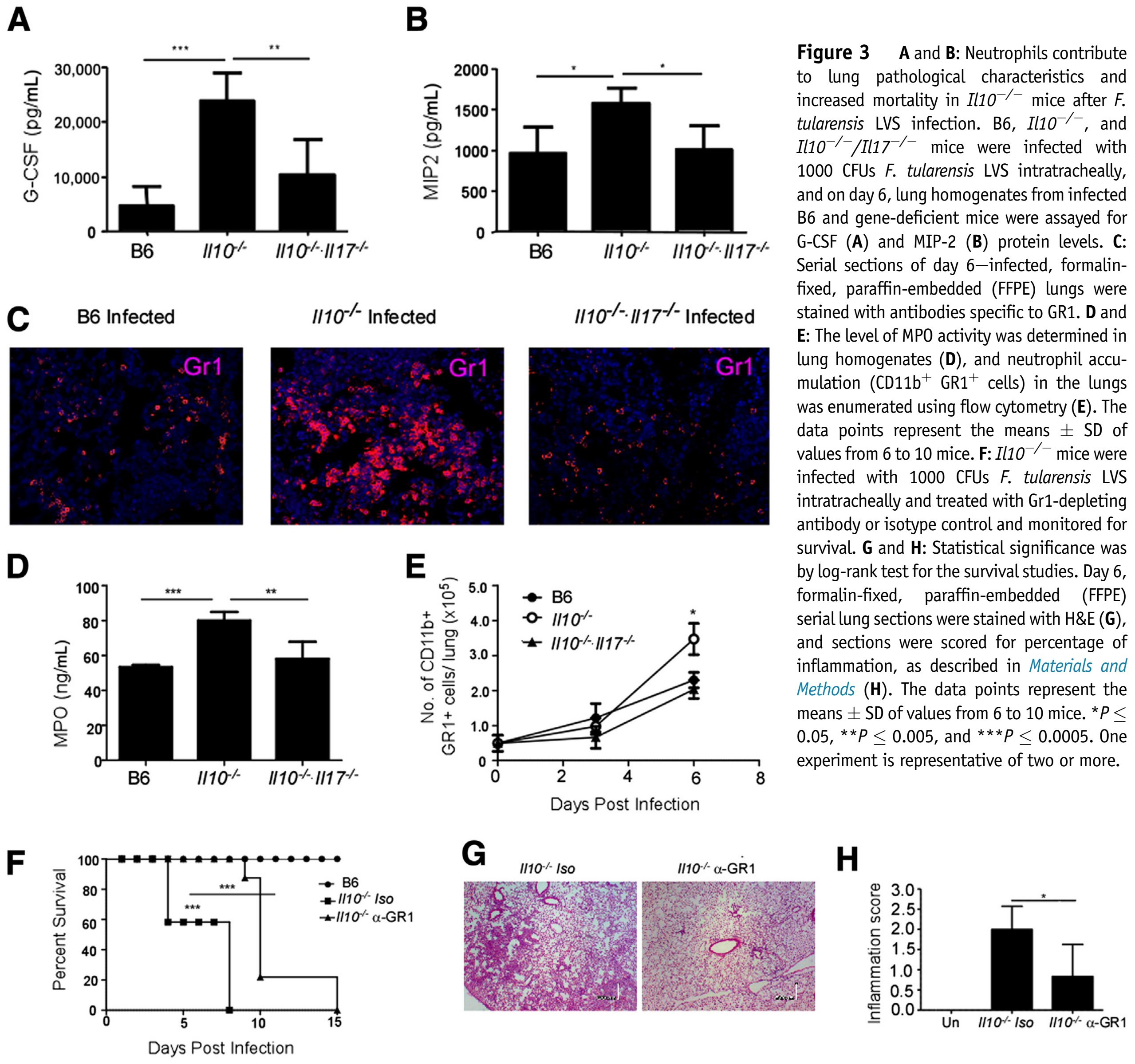

Figure 3 **A** and **B:** Neutrophils contribute to lung pathological characteristics and increased mortality in *Il10*$^{-/-}$ mice after *F. tularensis* LVS infection. B6, *Il10*$^{-/-}$, and *Il10*$^{-/-}$/*Il17*$^{-/-}$ mice were infected with 1000 CFUs *F. tularensis* LVS intratracheally, and on day 6, lung homogenates from infected B6 and gene-deficient mice were assayed for G-CSF (**A**) and MIP-2 (**B**) protein levels. **C:** Serial sections of day 6–infected, formalin-fixed, paraffin-embedded (FFPE) lungs were stained with antibodies specific to GR1. **D** and **E:** The level of MPO activity was determined in lung homogenates (**D**), and neutrophil accumulation (CD11b^{+} GR1^{+} cells) in the lungs was enumerated using flow cytometry (**E**). The data points represent the means ± SD of values from 6 to 10 mice. **F:** *Il10*$^{-/-}$ mice were infected with 1000 CFUs *F. tularensis* LVS intratracheally and treated with Gr1-depleting antibody or isotype control and monitored for survival. **G** and **H:** Statistical significance was by log-rank test for the survival studies. Day 6, formalin-fixed, paraffin-embedded (FFPE) serial lung sections were stained with H&E (**G**), and sections were scored for percentage of inflammation, as described in *Materials and Methods* (**H**). The data points represent the means ± SD of values from 6 to 10 mice. $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. One experiment is representative of two or more.

media.[29] However, these differences are only apparent at early time points after infection,[30] and our studies, both *in vitro* and *in vivo*, were performed over a period of 6 days or more, by which time the bacteria are host adapted. In addition, the observed increased mortality of *Il10*$^{-/-}$ mice after *F. tularensis* LVS infection substantiates that *in vivo* IL-10 is produced and is critical for restraining Th1 and Th17 responses after infection. Accordingly, in this study, increased IFN-γ and IL-17 levels were observed in *Il10*$^{-/-}$ *F. tularensis* LVS-infected lungs. A previous study has demonstrated that *Il10*$^{-/-}$ DCs secrete high levels of IL-12/IL-23 p40 when pulsed overnight with cecal bacterial lysate.[31] Thus, it is possible that the increased expression of these Th1- and Th17-polarizing cytokines and other molecules, such as CD86, in *Il10*$^{-/-}$ DCs[31] drives the increased expression of IL-17 and IFN-γ in CD4^{+} T cells. This finding is consistent with other pulmonary intracellular bacterial infection models, such as pulmonary infection with *M. tuberculosis*, in which *Il10*$^{-/-}$-infected mice demonstrated increased lung IFN-γ and IL-17 levels.[12] However, in contrast to our study, *M. tuberculosis*–infected *Il10*$^{-/-}$ mice demonstrated better bacterial control and no remarkable differences in lung pathology.[12] These data suggest that in some pulmonary infection models, such as *F. tularensis* LVS, IL-10 plays dual roles. On one hand, IL-10 restrains the inflammatory effects of IL-17 and is beneficial, whereas on the other hand, IL-10 restrains IL-12 and IFN-γ responses and is detrimental to host immunity. Thus, our studies further suggest that the indirect role for IL-17 in driving IL-12 production and Th1 responses is to overcome the inhibitory effects of IL-10 on IL-12 and Th1 responses. This hypothesis is consistent with the finding that, although *Il17*$^{-/-}$ mice are more susceptible to pulmonary *F. tularensis* LVS infection and have increased lung bacterial burden and decreased lung IFN-γ levels,[7] this requirement for

IL-17 in driving Th1 immunity is lost when IL-10 is absent. Interestingly, in a cutaneous model of *F. tularensis* LVS infection, increased IL-17 that is induced in the IL-10–deficient mice results in improved survival of mice.[14] Thus, a recent study,[14] along with data shown in this study, demonstrate that, depending on the route of *F. tularensis* LVS infection, excess IL-17 induced in response to infection can drive either protective or pathological responses.

Recent data suggest that *in vitro* infection of human monocytes with the virulent strain *F. tularensis* SCHU S4 induces IL-23 expression,[32] and that pulmonary infection with *F. tularensis* LVS induces lung Th17 cells.[33] Exogenous administration of IL-17 delays the time of death from lethal intranasal *F. tularensis* LVS infection,[9] and rescues IFN-γ production in the *F. tularensis* LVS-infected lung.[7] In addition, IL-17 neutralization enhances bacterial burden in previously immunized mice that were challenged with virulent aerosolized *F. tularensis* SCHU S4.[34] More important, peripheral blood mononuclear cells from *F. tularensis* LVS-immunized human volunteers produce IL-17 in memory $CD4^+$ and $CD8^+$ T cells on stimulation with *Francisella* antigens,[35] suggesting that IL-17 could serve as a good immune correlate for both primary and secondary immunity against tularemia. In this context, it is interesting that prostaglandin E2 is critical for induction of IL-23 and Th17 responses,[36,37] whereas it inhibits IL-12 responses through production of IL-10.[38] In this context, IL-10 restrains IL-17 production and resulting neutrophil recruitment and inflammation during pulmonary infection with *F. tularensis* LVS. This is clearly demonstrated in our study because the increased inflammation, induction of G-CSF and MIP-2, and neutrophil accumulation seen in *Il10*$^{-/-}$-infected lungs was completely reversed in the lungs of *Il10*$^{-/-}$/*Il17*$^{-/-}$-infected mice. More important, we also provide evidence that the increased mortality seen in *Il10*$^{-/-}$-infected mice is the result of exacerbated neutrophilic inflammation, because *Il10*$^{-/-}$ mice depleted of neutrophils result in decreased mortality, improved survival, and decreased inflammation. Previous studies showed that depletion of Gr1 cells (using the RB6-8c5 antibody) in wild-type BALB/c mice infected with aerosolized *F. tularensis* LVS did not affect bacterial burden in the lung.[10] This supports our findings that the improved survival observed in *Il10*$^{-/-}$-infected mice, which underwent neutrophil depletion, was likely due to decreased inflammatory responses observed, rather than a direct role for neutrophils, in protective immunity. The absence of IL-17 in the *Il10*$^{-/-}$ mice does not completely rescue susceptibility and improve survival to levels observed in B6-infected mice, suggesting that the increased IFN-γ responses observed in *Il10*$^{-/-}$-infected lungs may also be playing a secondary role in this model, and should be further explored.

In summary, our data together suggest that, although IL-17 is required for protective immunity against pulmonary infection with *F. tularensis* LVS,[7,9] its production is tightly regulated by anti-inflammatory cytokines, such as IL-10. However, when IL-17 production is dysregulated, it can cause exacerbated inflammation, associated pathological characteristics, and increased mortality after *F. tularensis* LVS infection.

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