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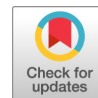

Protective Role for Macrophages in Respiratory *Francisella tularensis* Infection

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ABSTRACT *Francisella tularensis* causes lethal pneumonia following infection of the lungs by targeting macrophages for intracellular replication; however, macrophages stimulated with interferon gamma (IFN-γ) can resist infection *in vitro*. We therefore hypothesized that the protective effect of IFN-γ against *F. tularensis in vivo* requires macrophages receptive to stimulation. We found that the lethality of pulmonary *F. tularensis* LVS infection was exacerbated under conditions of alveolar macrophage depletion and in mice with a macrophage-specific defect in IFN-γ signaling (termed mice with macrophages insensitive to IFN-γ [MIIG mice]). We previously found that treatment with exogenous interleukin 12 (IL-12) protects against *F. tularensis* infection; this protection was lost in MIIG mice. MIIG mice also exhibited reduced neutrophil recruitment to the lungs following infection. Systemic neutrophil depletion was found to render wild-type mice highly sensitive to respiratory *F. tularensis* infection, and depletion beginning at 3 days postinfection led to more pronounced sensitivity than depletion beginning prior to infection. Furthermore, IL-12-mediated protection required NADPH oxidase activity. These results indicate that lung macrophages serve a critical protective role in respiratory *F. tularensis* LVS infection. Macrophages require IFN-γ signaling to mediate protection, which ultimately results in recruitment of neutrophils to further aid in survival from infection.

KEYWORDS interferons, lung defense, lung infection, macrophages, neutrophils, tularemia

Received 25 January 2017 **Returned for modification** 21 February 2017 **Accepted** 28 March 2017

Accepted manuscript posted online 3 April 2017

Citation Steiner DJ, Furuya Y, Jordan MB, Metzger DW. 2017. Protective role for macrophages in respiratory *Francisella tularensis* infection. Infect Immun 85:e00064-17. https://doi.org/10.1128/IAI.00064-17.

Editor Craig R. Roy, Yale University School of Medicine

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The tier 1 biothreat *Francisella tularensis* is a Gram-negative bacterium that is capable of replicating within phagocytes (1–3). In respiratory infection, alveolar macrophages have been reported to be the primary host cell for replication (4–6). It has been suggested that alveolar phagocytes are therefore detrimental to the host during respiratory infection, and a 2005 study reported that depletion of alveolar phagocytes following high challenge doses of LVS resulted in a modestly delayed time to death (7). The course of disease is characterized by a delayed immune response, followed by systemic dissemination and sepsis (8–10). Consequently, the prevailing opinion is that *F. tularensis* evades destruction by innate immunity and subverts myeloid cells, particularly macrophages, for its own benefit.

Despite the proficiency of *F. tularensis* in subverting and exploiting host immunity, it is possible to stimulate innate immunity to successfully counter *F. tularensis* infection. Macrophages significantly contribute to *in vitro* bacterial killing if they are stimulated with interferon gamma (IFN-γ) (2, 11–13). Correspondingly, IFN-γ is known to be required for protection *in vivo* (14). Treatment with exogenous interleukin 12 (IL-12) has been shown to protect mice, and mice lacking either the IL-12p35 or IL-12p40 subunit

are highly susceptible to LVS infection; protection mediated by exogenous IL-12 treatment has been shown to be dependent upon IFN-γ signaling (14). Furthermore, it was recently reported that lethal and sublethal infections recruit myeloid populations with different phenotypic compositions and different levels of maturity (15). This report concluded that mature phagocytic cell populations are essential for protection in sublethal respiratory *F. tularensis* infection.

Although it is increasingly apparent that macrophages and IFN-γ can protect against respiratory *F. tularensis* infection under some circumstances, the exact mechanism of this protection is unclear. IFN-γ-stimulated alveolar macrophages are capable of killing *F. tularensis* in a manner that is not dependent on nitric oxide species (11). IFN-γ-stimulated alveolar macrophages also secrete tumor necrosis factor alpha (TNF-α); while it is not necessary for these cells to control *F. tularensis* LVS *in vitro*, it is required for protection *in vivo* (16). This suggests the involvement of other cell populations, possibly neutrophils, which are recruited to the lung within 3 days of *F. tularensis* infection (4, 13).

Although neutrophils are essential for survival following intravenous or intradermal *F. tularensis* infection, their specific role during lung infection is less clear, and it has in fact been reported that neutrophil depletion does not alter the bacterial burden in mice infected via aerosol challenge (17, 18). In addition, it has been suggested that neutrophils are actually detrimental to the host following pulmonary *Francisella* infection due to induction of overwhelming inflammation (19, 20). However, following protective IL-12 treatment and *F. tularensis* LVS infection, neutrophils are recruited to the lungs 1 day earlier in treated than in untreated mice, and a beneficial role for NADPH oxidase in respiratory *F. tularensis* infection has been observed (13, 21).

We sought to clarify the protective mechanism of macrophages and IFN-γ *in vivo* during respiratory *F. tularensis* infection. Here, we report that during sublethal respiratory *F. tularensis* LVS infection, IFN-γ exerts a protective effect through stimulation of alveolar macrophages and through recruitment of other phagocytes, particularly neutrophils.

RESULTS

Alveolar macrophage depletion is detrimental to survival in pulmonary *F. tularensis* LVS infection. A previous report indicated a delayed time to death in clodronate-treated mice infected with a lethal dose of *F. tularensis* LVS (7). However, recent evidence suggests that sublethal infection with *F. tularensis* LVS elicits a greater proportion of mature phagocytes, which exhibit a protective effect (15). Reasoning that mature resident phagocytes may play a protective role against low doses of *F. tularensis* LVS, we treated mice intranasally (i.n.) with clodronate liposomes or phosphate-buffered saline (PBS) liposomes to deplete alveolar macrophages. Depletion was confirmed by flow cytometric analysis of bronchoalveolar lavage fluid (BALF) (Fig. 1A). Two days after the final treatment, we infected BALB/c mice i.n. with two sub-50% lethal doses (sub-LD_{50}s) of *F. tularensis* LVS (500 CFU [Fig. 1B] or 100 CFU [Fig. 1C]) and monitored survival for 21 days. These infectious doses were minimally lethal for PBS liposome-treated mice but highly lethal for clodronate liposome-treated mice.

Protection from *F. tularensis* LVS requires macrophage-specific IFN-γ sensitivity. Earlier work demonstrated that IFN-γ, a known stimulator of macrophage cytolytic activity, is essential for survival from respiratory *F. tularensis* infection (14, 22). To determine if macrophages are the primary effectors of IFN-γ-mediated protection, we exploited mice that express a truncated IFN-γ receptor specifically in the $CD68^+$ cell subset, with the result being that IFN-γ signaling is inhibited in macrophages; these mice are termed mice with macrophages insensitive to IFN-γ (MIIG mice) (23). It was found that MIIG C57BL/6 mice had dramatically increased mortality rates compared to wild-type (WT) C57BL/6 mice after infection with 1 LD_{50} or 0.5 LD_{50} of *F. tularensis* LVS (Fig. 2A and B). Bacterial burdens in the lungs, liver, and spleen were also assessed 4 days after i.n. infection, at which time point MIIG mice contained higher numbers of bacterial CFU in the lungs and noticeably (though not statistically significantly) in-

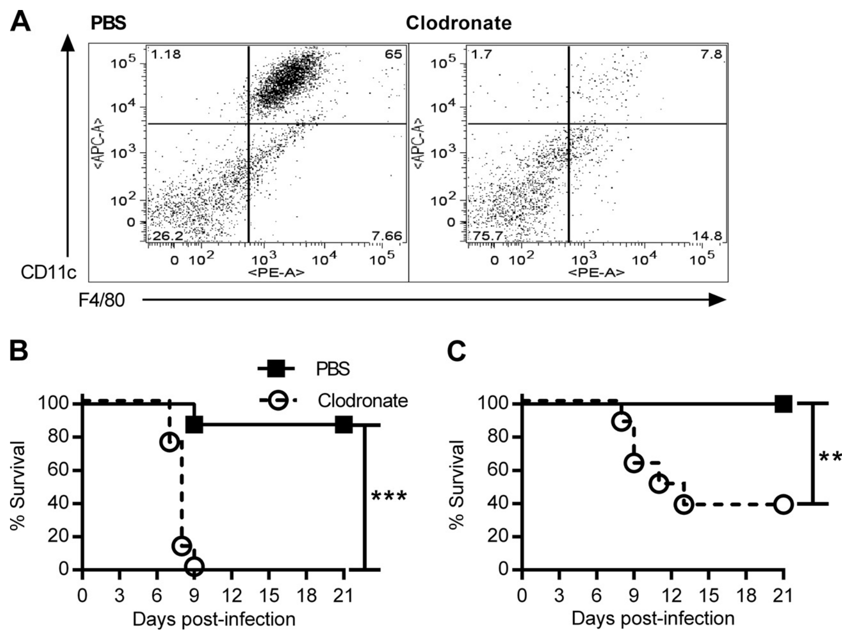

FIG 1 Depletion of alveolar macrophages increases susceptibility to LVS. (A) BALB/c mice were treated i.n. with clodronate liposomes or PBS liposomes on days 2 and 1 prior to infection, and depletion of alveolar macrophages in BALF was confirmed by flow cytometry. (B and C) At 2 days after treatment, mice (8 mice/group) were infected i.n. with 500 CFU (B) or 100 CFU (C) of *F. tularensis* LVS. Survival was monitored for 21 days. *P* values were determined by the log-rank test. **, $P < 0.01$; ***, $P < 0.001$.

creased burdens in the liver and spleen (Fig. 2C to E). These results indicate that protection against pulmonary *F. tularensis* LVS infection specifically requires an IFN-γ-responsive CD68$^+$ population.

Mice can be protected against *F. tularensis* LVS by exogenous IL-12 treatment, and this treatment is known to be dependent upon IFN-γ (14). We therefore investigated whether the protective effects of IL-12 require downstream participation by IFN-γ-stimulated macrophages. To investigate whether IL-12-mediated protection was dependent specifically on IFN-γ stimulation of macrophages, WT and MIIG C57BL/6 mice were treated i.n. with IL-12 or PBS 1 day prior to lethal LVS infection, and survival was monitored. All WT mice treated with PBS succumbed to infection, while all IL-12-treated WT mice survived (Fig. 2F), consistent with our previous results (14). However, IL-12 failed to protect MIIG mice, with 100% lethality being seen within 8 days. NK cells are activated by IL-12 (24) and also during pulmonary LVS infection (25, 26). Thus, we determined whether NK cell activation might be defective in MIIG mice. It was found that NK cells in WT and MIIG mice expressed equivalent amounts of IFN-γ following LVS infection and IL-12 treatment (Fig. 2G). Thus, the loss of IL-12 protective efficacy in MIIG mice was not a result of impeded NK cell activity and was observed only in the presence of IFN-γ-mediated stimulation of macrophages.

Macrophage sensitivity to IFN-γ mediates neutrophil recruitment to the lungs in *F. tularensis* LVS infection. One consequence of IL-12 treatment is recruitment of neutrophils to the lungs up to 24 h earlier than what is normally observed in pulmonary *F. tularensis* LVS infection (13). Although neutrophils have been postulated to be a source of lung tissue damage in *F. tularensis* pulmonary infection (19), they are known to be protective against *F. tularensis* systemic infection (18). We hypothesized that IL-12 treatment would result in IFN-γ-mediated stimulation of macrophages, which in turn would promote neutrophil recruitment to the lungs. We tested this by infecting WT and MIIG C57BL/6 mice i.n. with 1 LD_{50} of *F. tularensis* LVS. Lungs were harvested at 3 and 5 days postinfection, and the numbers of Ly6G-positive (Ly6G$^+$) CD11b$^+$ cells were enumerated by flow cytometry (Fig. 3A). The results showed a statistically significantly lower number of neutrophils in the lungs of MIIG mice throughout the infection

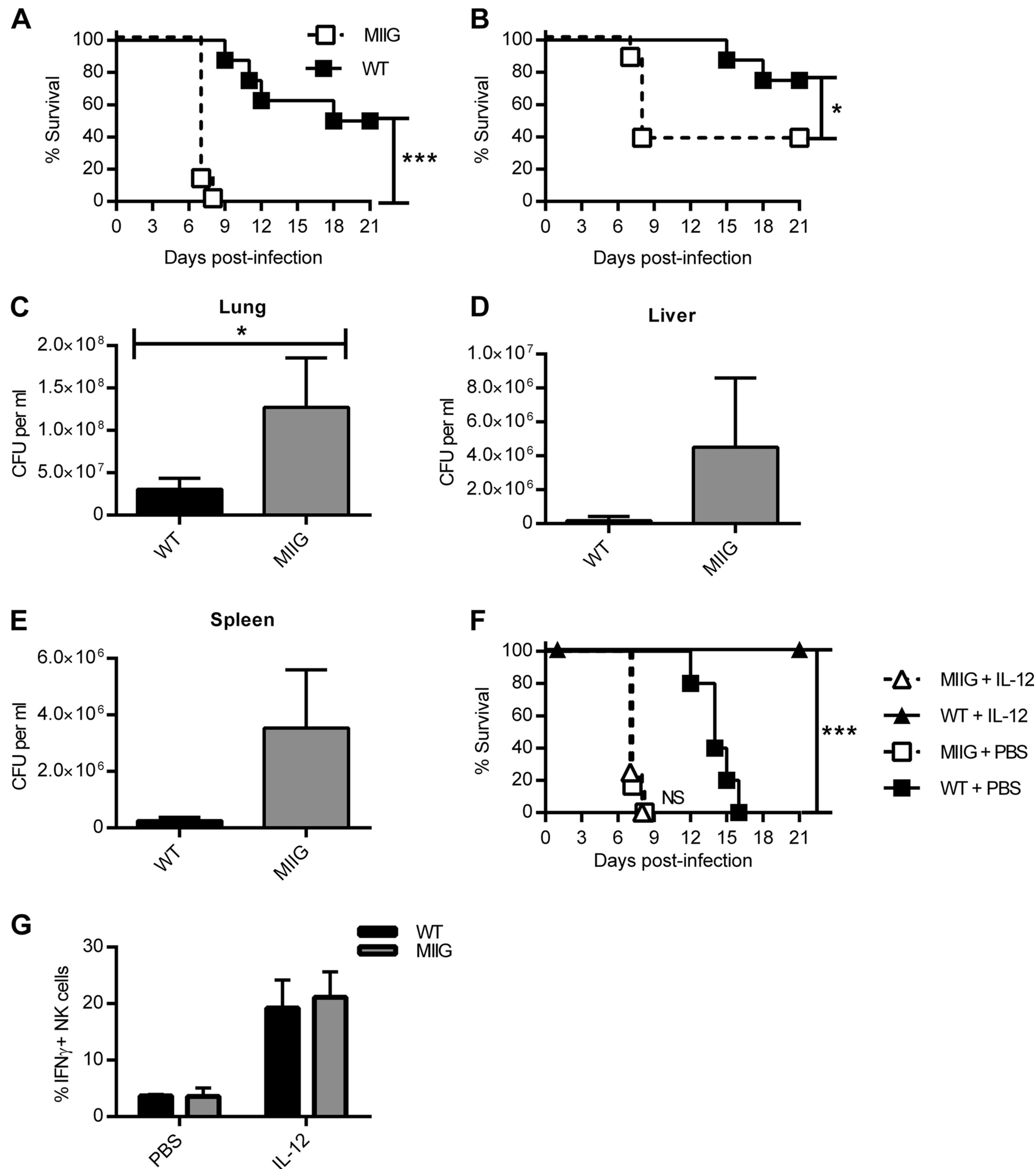

FIG 2 MIIG mice are highly sensitive to LVS respiratory tularemia. MIIG mice contain macrophages which do not respond to IFN-γ stimulation. (A and B) MIIG and WT C57BL/6 mice (8 mice/group) were infected i.n. with 1 LD_{50} (A) or 0.5 LD_{50} (B) of *F. tularensis* LVS. Survival was monitored for 21 days. *P* values were determined by the log-rank test. *, $P < 0.05$; ***, $P < 0.001$. (C to E) MIIG and WT C57BL/6 mice (3 mice/group) were infected i.n. with 1 LD_{50} of *F. tularensis* LVS. Lungs (C), livers (D), and spleens (E) were harvested at 4 days postinfection, and bacterial burdens were assessed. *, $P < 0.05$ by two-tailed *t* test. (F) MIIG and WT C57BL/6 mice (6 to 8 mice/group) were treated i.n. with IL-12 or PBS 1 day prior to i.n. infection with 1 LD_{100} of *F. tularensis* LVS. Survival was monitored for 21 days. *P* values were determined by the log-rank test. ***, $P < 0.001$; NS, not significant. (G) C57BL/6 and WT mice (3 or 4 mice/group) were treated with IL-12 or PBS prior to infection as described above. At 1 day postinfection, lungs were harvested for analysis of IFN-γ-positive (IFN-γ^+) NK cells by flow cytometry. No significant differences were detected between WT and MIIG mice.

(Fig. 3B). This was supported by a statistically significant difference in the total number of viable cells (Fig. 3C). This suggests that IFN-γ-mediated activation of macrophages results in early neutrophil recruitment into the lungs.

Neutrophils are required for survival in *F. tularensis* LVS infection. Early neutrophil recruitment is observed in a model of IL-12-mediated protection against *F. tularensis* LVS, suggesting a possible beneficial role early in infection (13). Based on the work of others (17), we hypothesized that depletion of neutrophils would hinder control of infection, leading to increased mortality. To assess whether neutrophils influence survival in respiratory *F. tularensis* LVS infection, BALB/c mice were depleted

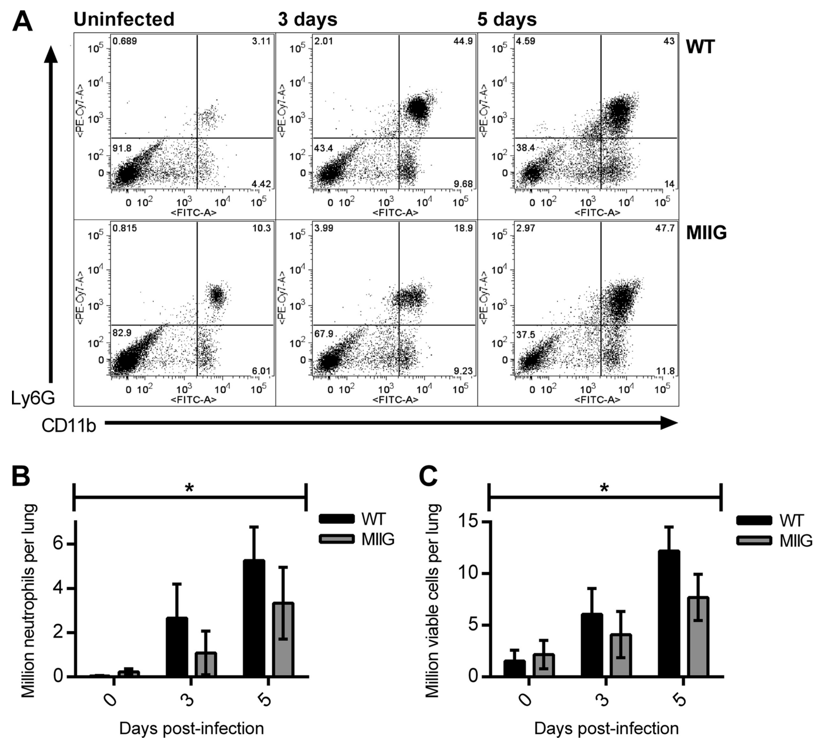

FIG 3 Neutrophil recruitment requires stimulation of CD68$^+$ cells by IFN-γ. (A to C) MIIG and WT C57BL/6 mice were infected i.n. with 1 LD_{50} of *F. tularensis* LVS (5 mice/group, except 3 mice/group for uninfected MIIG mice). At the indicated time points, lung Ly6G$^+$ CD11b$^+$ cells were enumerated by flow cytometry. (A) Representative flow cytometry plots of MIIG and WT mouse lungs after gating on live cells; (B) total lung neutrophils; (C) total viable cells. *, $P < 0.05$ for genotype by two-way ANOVA.

of neutrophils by treatment with anti-Ly6G monoclonal antibody (MAb) 1A8 either prior to infection with 1 LD_{50} of LVS or beginning at 3 days after infection, at a time when neutrophil infiltration is usually most pronounced. Neutrophil depletion significantly increased susceptibility in both cases (Fig. 4A). Neutrophil depletion beginning at 3 days after infection had the greatest effect, with all mice succumbing to infection by day 8. Depletion of neutrophils before infection also suggested reduced survival, although the effects on time to death were less noticeable and one neutrophil-depleted mouse survived the infection. Depletion using the less specific RB6 anti-Gr1 MAb, which also depletes inflammatory monocytes (27, 28), showed similar results, although in this case, anti-Gr1 depletion resulted in 100% lethality following infection regardless of whether cell depletion was performed prior to or 3 days after bacterial challenge (Fig. 4B). For both antibodies, late treatment caused a significant reduction in survival compared to that with early treatment, with P being <0.01 in both cases. The decreased effects of performing depletion prior to bacterial challenge were not due to neutrophil repopulation during infection; flow cytometry analysis showed that the depletion procedure resulted in a reduction in the number of detectable lung neutrophils for at least 6 days in *F. tularensis* LVS-infected mice (Fig. 4C and D). These results indicate that neutrophils exert a protective and not a detrimental role in respiratory infection.

IL-12-mediated protection from *F. tularensis* LVS requires NADPH oxidase. Treatment of mice with IL-12 prior to infection is known to protect mice against intranasal challenge with *F. tularensis* LVS and to reduce the lung bacterial burden (13, 14). IL-12 treatment also results in the earlier recruitment of neutrophils to the lungs following infection (13). We thus hypothesized that the protective effect of IL-12 is due to the early recruitment of neutrophils to the lungs. To test this hypothesis, we examined whether IL-12 enhanced the survival of neutrophil-depleted mice. BALB/c

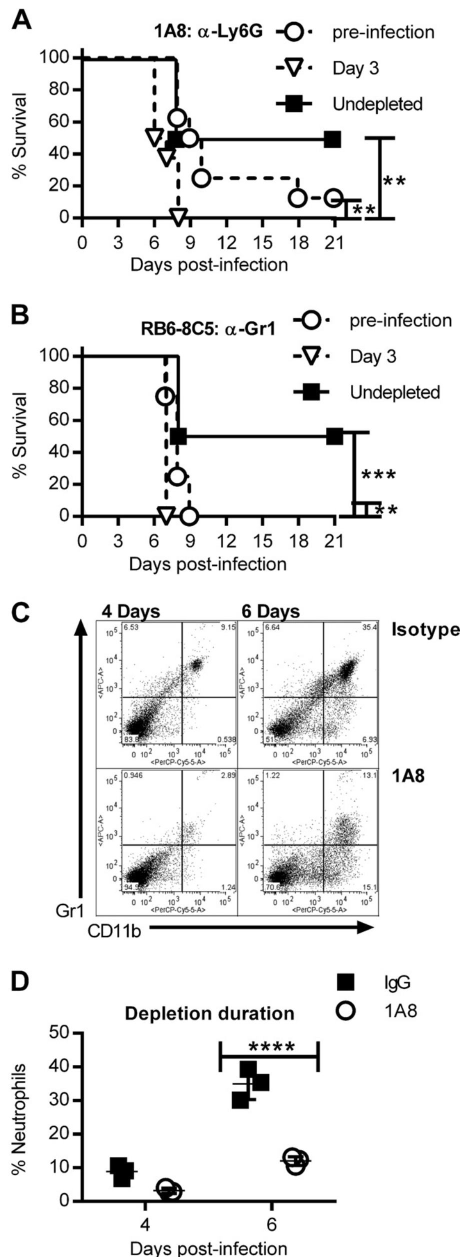

FIG 4 Depletion of neutrophils increases susceptibility to LVS respiratory tularemia. (A and B) BALB/c mice (8 mice/group) were treated i.p. with the neutrophil-depleting 1A8 MAb (A), with the RB6-8C5 MAb (B), or with isotype-matched IgG. Mice were treated either 1 day before and after infection (preinfection) or 3 and 5 days after infection (day 3). The infectious dose was 1 LD_{50}. *P* values for the significance of the differences between the indicated groups were determined by the log-rank test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C and D) BALB/c mice (3 mice/group) were infected i.n. with 1 LD_{50} of *F. tularensis* LVS and given i.p. injections of the 1A8 MAb or an isotype-matched IgG. At the indicated time points, lung Gr1-positive $CD11b^+$ cells were enumerated by flow cytometry. (C) Representative flow cytometry plots of 1A8- and isotype control-treated lungs; (D) quantification of neutrophils as a percentage of live lung cells. ****, $P < 0.001$ by two-way ANOVA with a multiple-comparison test comparing means at each time point.

mice received intraperitoneal (i.p.) injections of 1A8 or an isotype-matched control with or without i.n. IL-12 prior to infection with a lethal dose of *F. tularensis* LVS. Survival was monitored for 21 days. Although neutrophil-depleted mice not treated with IL-12 succumbed to infection, as described above, administration of IL-12 protected both neutrophil-depleted and nondepleted mice (Fig. 5A). These results indicate that despite

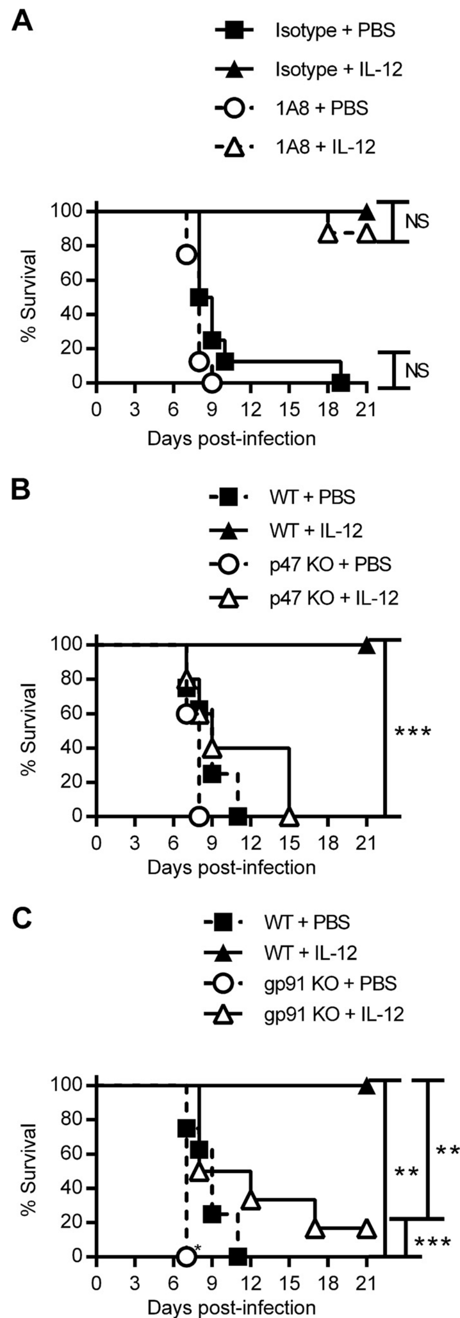

FIG 5 The protective effect of IL-12 treatment requires NADPH oxidase but not neutrophils. (A) BALB/c mice (8 mice/group) were treated i.n. with IL-12 or PBS and given i.p. injections of the 1A8 MAb or an isotype-matched IgG. Mice were then infected i.n. with 1 LD_{100} of *F. tularensis* LVS. Survival was monitored for 21 days. (B and C) Survival between WT C57BL/6 mice (8 mice/group) and $p47^{phox-/-}$ mice (5 mice/group) (B) or $gp91^{phox-/-}$ mice (6 mice/group) (C) was compared after i.n. treatment with IL-12 or PBS 1 day prior to i.n. infection with 1 LD_{100} CFU of *F. tularensis* LVS. Survival was monitored for 21 days. *P* values for the significance of the differences between the indicated groups were determined by the log-rank test. **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. KO, knockout.

the observed protective role of neutrophils, macrophage stimulation in the absence of neutrophils is sufficient to protect against LVS challenge.

The proteins $p47^{phox}$ and $gp91^{phox}$ are components of the NADPH oxidase complex, which is essential for the phagocyte respiratory burst. Mutations in these genes in humans result in chronic granulomatous disease, primarily a defect in neutrophil function (29–32). We infected WT, $p47^{phox-/-}$, and $gp91^{phox-/-}$ C57BL/6 mice with a

lethal dose of *F. tularensis* LVS with or without IL-12 i.n. prior to infection. IL-12 was again highly protective for WT mice but, surprisingly, failed to protect p47$^{phox-/-}$ or gp91$^{phox-/-}$ mice, indicating that the respiratory burst, likely within macrophages, is essential for control of *F. tularensis* infection and plays an important role in IL-12-mediated protection (Fig. 5B and C).

We conclude that alveolar macrophages are essential for protection against sublethal infectious doses of *F. tularensis* LVS and that this protection is mediated at least in part through the recruitment and activation of neutrophils and other phagocytes.

DISCUSSION

Innate immunity can protect against pulmonary *F. tularensis* LVS infection in a manner involving Th1-associated cytokines. Although this protection has been demonstrated both *in vitro* (2, 11–13) and *in vivo* (14, 26), the mechanism of this protection *in vivo* is incompletely understood. Here, we investigated the cellular mechanism contributing to IFN-γ-mediated protection against pulmonary *F. tularensis* LVS infection in mice. Our results demonstrate that alveolar macrophages, long considered detrimental in this infection, are required for protection against sublethal infectious doses. Protection also requires neutrophil recruitment to the infected lungs, and this recruitment is dependent upon IFN-γ stimulation of macrophages.

To test the assumption that intracellular macrophage replication is a key factor in *F. tularensis* virulence, we depleted alveolar macrophages in order to restrict bacterial replication within these cells following pulmonary infection. Liposomal clodronate is phagocytized by macrophages, and the clodronate is then converted to an ATP analogue and causes cell apoptosis (33, 34). Contrary to the concept that macrophage depletion would enhance survival by removing the bacterial replication niche, clodronate depletion actually resulted in decreased survival following LVS infection. We were aware of the possibility that macrophage debris resulting from this process could cause inflammation and potentially explain the increased susceptibility to infection. However, we observed the same heightened sensitivity to LVS infection in MIIG mice, which have a defect in IFN-γ signaling specific to macrophage lineage cells and which do not require cell depletion.

It has been reported that depletion of lung cells with liposomal clodronate prior to pulmonary challenge with *F. tularensis* LVS increases the mean time to death (7), a conclusion that is opposite to our findings. However, the authors of that study used a far greater bacterial inoculum in their study (5×10^4 CFU per mouse), which was lethal with or without clodronate treatment, and death was delayed by only 1 to 2 days. It is known that an excessive inflammatory response is a key feature of death from *F. tularensis* infection (8, 9, 19, 20). Although at more physiologically relevant infectious doses macrophages appear to be essential for survival, at higher doses in which control of infection becomes impossible with or without macrophages, the absence of macrophages (and macrophage-derived inflammatory cytokines) may merely delay lethal sepsis. Finally, we have obtained independent confirmatory results regarding the essential protective role of macrophages by use of MIIG mice.

Although neutrophils are known to exert a protective effect against cutaneous and systemic *F. tularensis* infection (17), the role of neutrophils in pneumonic *F. tularensis* infection has also remained somewhat controversial. The survival experiments in our study show unequivocally that neutrophils play a protective role in pneumonic *F. tularensis* LVS infection. This result contrasts with earlier work reporting that depletion of neutrophils using the monoclonal antibody RB6-8C5 prior to aerosol infection with LVS did not affect the bacterial burden in the lungs (18). However, the same work indicated that depletion of neutrophils did significantly increase the bacterial burden in the liver and also resulted in a trend toward an increased burden in the spleen. Other work by the same group indicated that NADPH oxidase, which is involved in the killing of bacteria by neutrophils, extends survival following SchuS4 challenge since gp91$^{phox-/-}$ mice, which are deficient in NADPH oxidase, exhibited a reduced mean time to death after i.n. SchuS4 challenge (21).

IL-12 is expressed in the lungs of *F. tularensis*-infected mice within 48 to 72 h after infection (35). Treatment with exogenous IL-12 prior to i.n. *F. tularensis* LVS infection induces early neutrophil recruitment to the lungs, which is associated with improved control of the bacterial burden relative to that in untreated mice (13). Our present work showed that neutrophil influx was correlated with IFN-γ activation of macrophages. After *F. tularensis* infection, MIIG mice showed statistically significant differences in the amounts of total lung neutrophils relative to WT mice. Recruitment of neutrophils was found to be essential for survival following infection; mice systemically depleted of neutrophils prior to infection or beginning at 3 days after infection showed increased mortality relative to mock-depleted mice. Nevertheless, neutrophil-depleted mice that were treated i.n. with IL-12 to induce IFN-γ secretion in the lungs exhibited protection similar to that seen in nondepleted mice treated with IL-12, which suggests that early stimulation of macrophages with IFN-γ is sufficient for protection in the absence of neutrophils. Transgenic mice with defects in NADPH oxidase activity succumbed to infection regardless of IL-12 treatment. Although in humans neutrophils are believed to be the primary source of NADPH-derived reactive oxygen, it is likely that reactive oxygen species in mice are produced by other phagocytes which are not affected by 1A8 treatment, specifically, macrophages or monocytes (36).

Based on this work, we propose that infection results in increased IL-12 secretion, which induces NK cells, $CD8^+$ T cells, or both, to secrete IFN-γ. While unstimulated macrophages can be targeted by bacteria for replication, macrophages stimulated with IFN-γ are resistant to infection and are capable of killing intracellular bacteria, as shown by *in vitro* experiments (2, 11, 12, 37). *In vivo*, IFN-γ stimulation of macrophages also induces recruitment of neutrophils to the lungs, providing a further level of protection, although neutrophil recruitment is not essential given a sufficient level of macrophage stimulation. The precise mechanism by which IFN-γ-stimulated macrophages mediate protection remains to be elucidated, as do the relative contributions of neutrophils within and outside the lungs. However, an important role for reactive oxygen species in survival is likely.

Taken together, our results challenge the current understanding of alveolar macrophages as a passive or wholly detrimental bacterial reservoir during respiratory *F. tularensis* LVS infection and instead demonstrate that macrophages can play an essential protective role.

MATERIALS AND METHODS

Ethics statement. All animal procedures followed those in the *Guide for the Care and Use of Laboratory Animals* (8th edition) of the National Research Council (38) and were approved by the Institutional Animal Care and Use Committee of Albany Medical College (protocol 12-03011).

Bacteria. The original stock of LVS was obtained from Karen Elkins, FDA, Bethesda, MD. *F. tularensis* was grown in Mueller-Hinton broth (Becton Dickinson) supplemented with IsoVitaleX (Becton Dickinson) or brain heart infusion broth (Becton Dickinson) adjusted to pH 6.8, as described by Hazlett et al. (39). The bacteria were grown to log phase at 37°C and frozen at −80°C.

Mice. BALB/c and C57BL/6 mice were purchased from Taconic, The Jackson Laboratory, and Charles River Laboratories under a contract with the National Cancer Institute. $p47^{phox-/-}$ and $gp91^{phox-/-}$ C57BL/6 mice were purchased from The Jackson Laboratory. MIIG C57BL/6 mice were generated at Cincinnati Children's Hospital Medical Center (23) and were bred and maintained at the Albany Medical College. In experiments using MIIG mice, wild-type (WT) C57BL/6 littermates were used as controls. Mice were anesthetized prior to infection, alveolar macrophage depletion, and/or IL-12 treatment by i.p. injection of a 2% ketamine–0.5% xylazine solution in PBS (both reagents were from Vedco).

Infections. Infectious doses were prepared from frozen stocks and diluted to 40 to 50 μl/mouse in PBS. The infectious doses were confirmed by plating on chocolate agar. Six to eight mice were used for the survival experiments and three to five mice per time point were used for the bacterial burden and flow cytometry experiments, except where noted otherwise in the figure legends. BALB/c mice are somewhat more resistant to LVS infection than C57BL/6 mice, and thus, higher challenge doses were required to reach an LD_{50} (26).

Alveolar macrophage depletion. Clodronate liposomes or control PBS liposomes (FormuMax) were administered i.n. in a volume of 50 μl 1 day prior to infection. Mice were anesthetized prior to treatment. Depletion was confirmed by flow cytometry 1 day after treatment. Of note, no weight loss was associated with liposomal clodronate treatment in the absence of infection, indicating that these animals maintained good health.

Bacterial burden analysis. Mice were sacrificed by i.p. injection of pentobarbital (Fort Dodge Laboratories), followed by cervical dislocation. For assessment of the bacterial burdens in lung, liver, and spleen, the organs were harvested and homogenized in 1 ml of PBS using a Minibeadbeater (Biospec Products). Homogenates were centrifuged, and the supernatants were plated on chocolate agar for enumeration.

IL-12 treatment. At 1 day prior to infection, mice were treated i.n. with 0.5 μg of IL-12 in 25 μl PBS with 1% normal mouse serum as a protein carrier, as described previously (40). Treatment with PBS in 1% normal mouse serum was used as a control.

Analysis of IFN-γ expression in NK cells. Mice were treated with IL-12 and infected with *F. tularensis* LVS as described above. At 1 day postinfection, lungs were harvested, digested with collagenase D (Sigma) for 30 min at 37°C, and passed through 40-μm-pore-size nylon filters (BD Falcon). Cells were restimulated *in vitro* with *F. tularensis* at a multiplicity of infection of 100 and IL-12 at a concentration of 5 ng/ml for 1 h at 37°C. Cytokine secretion was inhibited by incubation with the BD GolgiPlug protein transport inhibitor for 1 h at 37°C. Cells were stained with eFluor780-conjugated fixable viability dye (FVD; eBioscience) and phycoerythrin (PE)-conjugated anti-NK1.1 MAb and then fixed and permeabilized with BD Cytofix and stained with allophycocyanin (APC)-conjugated anti-IFN-γ MAb. The fluorescent intensity was determined using a FACSCanto flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo software (TreeStar).

Neutrophil depletion. Neutrophils were depleted using 100 μg of the RB6-8C5 Gr1-specific antibody (Maine Biotechnology and BioXCell) or 500 μg of the 1A8 Ly6G-specific antibody (BioXCell), as described previously (41, 42). One set of mice was depleted at 1 day prior to infection and then at 1 day postinfection, followed by injections of isotype-matched control Ig at 3 days and 5 days postinfection. Another set of mice was depleted beginning at 3 days after infection; these animals were injected with isotype-matched control Ig at 1 day prior to infection and 1 day postinfection, followed by depletion of antibody at 3 days and 5 days postinfection.

To assess of the effects of IL-12 on neutropenic mice, the 1A8 MAb was injected at 1 day prior to infection, 1 day postinfection, and 3 days postinfection. Rat IgG was used as a control in all cases.

Neutrophil expression was assessed by harvesting the lungs 1 day after the last dose of depleting MAb. Lungs were digested in collagenase D (Sigma) for 30 min at 37°C and separated into a suspension of live cells by passage through 40-μm-pore-size nylon filters (BD Falcon). Cell suspensions were stained with APC-conjugated anti-Gr1 MAb and peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD11b MAb. The fluorescent intensity was determined using a FACSCanto flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo software (TreeStar). Cells which were positive for both Ly6G and CD11b were considered to be neutrophils.

Neutrophil recruitment assay. Mice were infected i.n. and sacrificed at several time points after infection, as described in Results. The lungs were harvested, digested in collagenase D (Sigma) for 30 min at 37°C, and separated into a suspension of live cells by passage through 40-μm-pore-size nylon filters (BD Falcon). Cells were stained with eFluor780-conjugated FVD (eBioscience), fluorescein isothiocyanate (FITC)-conjugated anti-CD11b MAb, and PE-Cy7-conjugated anti-Ly6G MAb. The fluorescent intensity was determined using a FACSCanto flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo software (TreeStar). A gate identifying FVD-negative cells as live was drawn, and cells within this gate which were positive for both Ly6G and CD11b were considered to be neutrophils.

Statistical analyses. Survival was analyzed by Mantel-Cox log-rank tests, using the Bonferroni correction for multiple comparisons. Bacterial burdens were analyzed by a 2-tailed *t* test. Comparisons of IFN-γ-positive NK cells and of neutrophil levels between different groups of mice were analyzed by two-way analysis of variance (ANOVA), with Bonferroni's correction for multiple comparisons being used where appropriate. All statistics were determined using GraphPad Prism software. Statistical significance was considered to be a P value of <0.05.

ACKNOWLEDGMENTS

We are indebted to Anthony Hickey, Girish Kirimanjeswara, Sean Roberts, and Sharon Salmon for technical assistance.

This work was supported by NIH grant PO1 A1056320.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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