Effector Mechanisms Responsible for Gamma Interferon-Mediated Host Resistance to *Legionella pneumophila* Lung Infection: the Role of Endogenous Nitric Oxide Differs in Susceptible and Resistant Murine Hosts

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To facilitate identification of the effector mechanism(s) responsible for gamma interferon (IFN- γ)-mediated host resistance to *Legionella pneumophila*, a murine model of legionellosis in BALB/c mice with a targeted disruption in the IFN- γ gene (gamma knockout [GKO] mice) was developed. Immunocompetent BALB/c mice and GKO mice were inoculated intratracheally with virulent *L. pneumophila* (10^6 bacteria per mouse), and bacterial clearance and the pulmonary inflammatory response were assessed. *L. pneumophila* did not replicate in, and was rapidly cleared from, the lungs of immunocompetent BALB/c mice, demonstrating that immunocompetent BALB/c mice are resistant to replicative *L. pneumophila* pulmonary infections. In contrast, similarly infected GKO mice developed persistent, replicative intrapulmonary *L. pneumophila* infections with extrapulmonary dissemination of the bacteria to the spleen. Histopathologic and flow cytometric analysis of *L. pneumophila*-infected lung tissue demonstrated that while immunocompetent BALB/c mice develop multifocal pneumonitis which resolves, similarly infected GKO mice develop diffuse pneumonitis with persistent neutrophil recruitment into the lung. Intratracheal administration of exogenous IFN- γ to *L. pneumophila*-infected GKO mice facilitated intrapulmonary clearance of the bacteria, confirming the pivotal role of IFN- γ in innate host defenses to *L. pneumophila* lung infection in this murine host. The potential role of endogenous reactive nitrogen intermediates, including nitric oxide (NO), in IFN- γ -mediated resistance to *L. pneumophila* pulmonary infections in immunocompetent BALB/c mice was subsequently assessed. Macrophage inducible nitric oxide synthetase (an enzyme responsible for the production of NO) was induced in alveolar cells from *L. pneumophila*-infected immunocompetent BALB/c mice (with maximal expression at 48 h postinfection) but was not induced in similarly infected GKO mice. However, administration of the NO synthetase inhibitor *N*-monomethyl-L-arginine did not significantly inhibit clearance of *L. pneumophila* from the lung of immunocompetent BALB/c mice (compared with that in similarly infected mice not administered *N*-monomethyl-L-arginine). In contrast, we have previously demonstrated that IFN- γ -induced host resistance to replicative *L. pneumophila* lung infections in a susceptible murine host (A/J mice) is mediated, in part, by endogenous NO. Taken together, these studies identify a differing role of endogenous NO in IFN- γ -mediated resistance to *L. pneumophila* pulmonary infection in susceptible and resistant murine hosts.

Legionnaires' disease, an atypical pneumonia in humans, is caused by *Legionella pneumophila*, a facultative intracellular pathogen of host mononuclear phagocytic cells (MPCs) (25). Persons develop Legionnaires' disease by inhalation of *L. pneumophila*-containing aerosols from contaminated water sources (6). Following inhalation, the bacteria infect and multiply within host MPCs. Intracellular growth of *L. pneumophila* in MPCs results in eventual lysis of these cells, the release of bacterial progeny, and infection of additional pulmonary cells (10, 23, 24). Severe lung damage ensues, mediated by tissue-destructive substances derived from the bacteria and from damaged host cells (10–12).

Previous studies demonstrate that early nonspecific host immune responses, mediated in large part by lymphokines, including gamma interferon (IFN- γ), play a key role in host

defense against *L. pneumophila* infections (2, 7, 26, 28, 34, 38, 46). However, effector mechanisms which are responsible for IFN- γ -mediated early resistance to *L. pneumophila* lung infection have not been thoroughly investigated, in part because of the current lack of an animal model of Legionnaires' disease in a host selectively deficient in IFN- γ . In the current study, we describe the development of an animal model of legionellosis in BALB/c mice with a targeted disruption in the γ -IFN gene (gamma knockout [GKO] mice) and the subsequent use of this murine model to evaluate potential effector mechanisms responsible for IFN- γ -mediated host resistance to *Legionella* infection in vivo. We demonstrate that in contrast to immunocompetent BALB/c mice, which rapidly clear *L. pneumophila* from the lung following intratracheal (i.t.) inoculation with the bacterium (10^6 *L. pneumophila* cells per mouse), similarly infected GKO mice develop persistent replicative *L. pneumophila* lung infections with transient dissemination of the bacteria to the spleen.

In subsequent studies, effector mechanisms responsible for IFN- γ -mediated host resistance to *L. pneumophila* lung infec-

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tion in immunocompetent BALB/c mice were investigated. We demonstrate that IFN- γ -mediated resolution of *L. pneumophila* lung infections in immunocompetent BALB/c mice is temporally correlated with induction of macrophage inducible nitric oxide synthetase (mac-iNOS; an enzyme responsible for the production of reactive nitrogen intermediates, including nitric oxide [NO]), while mac-iNOS is not induced in similarly infected GKO mice. These results suggest that endogenous NO is responsible for IFN- γ -mediated resolution of *L. pneumophila* lung infection in immunocompetent BALB/c mice. However, subsequent studies demonstrated that inhibition of endogenous NO by administration of *N*-monomethyl-L-arginine (NMMA) did not alter resolution of *L. pneumophila* lung infection in immunocompetent BALB/c mice (compared with similarly infected mice not administered NMMA). These results indicate that IFN- γ -mediated resolution of *L. pneumophila* lung infections in immunocompetent BALB/c mice occurs by a NO-independent mechanism. In contrast, we have previously demonstrated that IFN- γ -mediated resolution of a replicative *L. pneumophila* lung infections in a susceptible murine host (A/J mice) is mediated, in part, by endogenous reactive nitrogen intermediates, including NO (8). Taken together, these studies demonstrate that IFN- γ -mediated host resistance to *L. pneumophila* lung infection in susceptible and resistant murine hosts is facilitated by different effector mechanisms.

MATERIALS AND METHODS

Animal care. Breeding pairs of pathogen-free BALB/c mice heterozygotic for disruption of the IFN- γ gene were generously provided by Genentech, Inc. (South San Francisco, Calif.). These mice were bred to homozygosity for the IFN- γ gene deletion (i.e., GKO mice), as confirmed by PCR using previously described methodology (14). Male and female GKO mice were subsequently used experimentally at 6 to 10 weeks of age. Immunocompetent 6- to 10-week-old pathogen-free BALB/c mice were purchased from Charles River Laboratory (Portage, Mich.). All mice were housed in microisolator cages and were cared for according to standard guidelines.

Bacterial inoculum. A clinical *L. pneumophila* strain, AA100, has been previously described (1). The bacteria were maintained on buffered charcoal-yeast extract (BCYE) agar plates at 37°C (17). For preparation of the i.t. inoculum, the bacteria were quantitated on BCYE agar plates that had been incubated for 48 h and resuspended in phosphate-buffered saline (PBS) at 4×10^7 organisms per ml as previously described (1, 7).

i.t. inoculation of mice. Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* by previously described methodology (7, 8, 41). Mice were anesthetized with ketamine intraperitoneally (2.5 mg per mouse), and tethered, and an incision was made through the skin of the ventral neck. The trachea was isolated, and 25 μ l of the bacterial suspension (10^6 bacteria in PBS) followed by 10 μ l of air was injected directly into the trachea with a 27-gauge needle. The skin incision was closed with a sterile wound clip.

Clearance studies. At specific time points after i.t. inoculation of *L. pneumophila* (0, 24, 72, 120, and 240 h), the mice were humanely sacrificed and the lungs were removed. Lung tissue was finely minced in 10 ml of sterile water and subsequently homogenized (2 min per sample) with a Stomacher (Tekmar, Cincinnati, Ohio) as previously described (3, 7). The tissue homogenates were serially diluted and cultured on BCYE agar containing polymyxin B, cefamandole, and anisomycin (BCYE-PAC) (Baxter) for 72 h (16). CFU per lung homogenate were subsequently determined (3, 7). In selected experiments, the spleens of *L. pneumophila*-infected mice were also harvested and homogenized by the methodology described above. Extrapulmonary dissemination of *L. pneumophila* was assessed by quantitation of the bacteria in the spleen by culture of splenic homogenates on BCYE-PAC.

Histopathology. The pulmonary inflammatory response induced by *L. pneumophila* was assessed by light microscopy as previously described (7). Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse). At specific time points thereafter (24, 72, 120, and 240 h), the mice were humanely euthanized. The lungs were excised, inflated, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 μ m, and stained with hematoxylin and eosin.

Flow cytometry. In vivo recruitment of inflammatory cells into the lung during *L. pneumophila* pulmonary infection was assessed by flow cytometric analysis of whole-lung lysates by previously described methodology (13, 27). Immunocompetent BALB/c mice and GKO mice were inoculated with *L. pneumophila* (10^6 bacteria per mouse). All mice were subsequently processed and analyzed individually. At specific times postinoculation (p.i.) (i.e., 24, 72, 120, and 240 h), the mice were humanely sacrificed. The lungs were excised, minced, and incubated

in RPMI 1640 medium containing 5% fetal calf serum, 1 mg of collagenase A (Boehringer Mannheim, Indianapolis, Ind.) per ml, and DNase (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. The cells were further disaggregated by drawing the resultant lung homogenate repeated through a 10-ml syringe 20 to 30 times prior to pelleting the cell pellet by centrifugation. Erythrocytes were lysed by brief exposure of the cells to sterile water. Recovered cells were subsequently washed once in PBS. To obtain a total cell count, an aliquot of the sample was diluted in 2% paraformaldehyde and counted on a hemocytometer. Another aliquot was stained with modified Wright Giemsa stain (Baxter Health Care Corp., McGaw Park, Ill.). Differential counts were performed under oil immersion; cells were counted as MPCs and polymorphonuclear leukocytes (PMNs) (combined) or as lymphocytes on the basis of standard light microscopic criteria.

Flow cytometry was used to confirm the percentage of MPCs, PMNs, and lymphocytes in the lung cell preparation and further characterize the cells as T cells (CD4⁺ or CD8⁺ cells) or B cells (B220 cells). Briefly, aliquots of lung cells (10^6 cells per 100 μ l of fluorescent antibody staining buffer) were stained with 1 μ g of directly fluorescein isothiocyanate-conjugated monoclonal antibody (rat immunoglobulin G [IgG] RM4-5 [anti-CD4, rat IgG2a], 53-6.7 [anti-CD8a, rat IgG2a], RA3-6b2 [anti-B220, rat IgG2a], and Gr-1 [antigranulocyte, rat IgG2b]; Pharmingen, San Diego, Calif.) or with a fluorescein isothiocyanate-conjugated control monoclonal antibody for 30 min on ice in the dark. The cells were washed in PBS, fixed in 2% paraformaldehyde, and stored at 4°C in the dark. Samples were analyzed on an EPICS Elite flow cytometer with accompanying software (EPICS Div., Coulter Corp., Hialeah, Fla.), examining $\geq 10,000$ events per sample. The small-cell population has previously been shown to be over 90% lymphocytes (as determined by expression of CD4, CD8, and B220), whereas the large-cell population is >95% nonlymphoid cells (i.e., MPCs and PMNs [13]). Absolute numbers of each cell type were obtained by multiplying the percentage times the total number of cells in that lung sample as previously described (13, 27).

Western blot (immunoblot) analysis of alveolar cell lysates for mac-iNOS.

Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse). At specific time points p.i. (0, 24, 48, and 72 h), the mice were humanely euthanized and the lungs were lavaged with 1.6 ml of PBS. An aliquot of the resultant bronchoalveolar lavage sample was diluted in 2% paraformaldehyde and counted on a hemocytometer to obtain a total cell count. Alveolar cells were recovered from the bronchoalveolar lavage sample by centrifugation ($300 \times g$, 5 min, 22°C). The cell pellet was subsequently resuspended in 20 μ l of lysis buffer (i.e., Laemmli sample buffer which contained protease inhibitors [leupeptin, 12 μ g/ml; aprotinin, 12 μ g/ml; phenylmethylsulfonyl fluoride, 1.25 mM], β -mercaptoethanol [5%], and bromophenol blue [0.025%]), heated for 5 min at 95°C, passed several times through a 26-gauge needle to reduce viscosity, and centrifuged ($12,000 \times g$, 5 min, 22°C) to remove intact cells and debris (29). Proteins were size separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE) (29). After completion, the proteins were transferred onto a 0.45- μ m-pore-size nitrocellulose membrane (Gelman, Ann Arbor, Mich.) with 20% methanol-25 mM Tris-192 mM glycine, pH 8.3. The membrane was blocked for 1 h at room temperature (5% nonfat dry milk in 10 mM Tris [pH 7.5]-100 mM NaCl-0.1% Tween 20) and incubated overnight in a 1:1,000 dilution of rabbit anti-mouse inducible nitric oxide synthetase (iNOS) IgG (Transduction Laboratories, Lexington, Ky.). After overnight incubation in the primary antibody, the membrane was washed three times (10 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% Tween 20; 5 min per wash) and incubated for 2 h with a 1:2,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad, Hercules, Calif.). After three washes (10 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% Tween 20; 5 min per wash), the membrane was equilibrated in developing buffer (50 mM Tris [pH 10.0], 3 mM MgCl₂, 0.02% Na₂S₂O₅) and developed in developing buffer containing 100 μ g of nitroblue tetrazolium per ml and 50 μ g of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml.

Identification of cellular sources of mac-iNOS by immunostaining. Cellular sources of mac-iNOS were identified in lung tissue from *L. pneumophila*-infected mice by immunostaining using double labeling techniques (44). Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) and sacrificed at specific time points p.i. for procurement of frozen lung sections (33). Prior to immunostaining, lung samples were fixed in methanol at -20°C for 10 min. Macrophages were subsequently identified in frozen lung sections by staining of the samples with fluorescein-labeled *Griffonia simplicifolia* I lectin (GS-I; Sigma Chemical Co.; 2 μ g/ml [37]), while mac-iNOS was localized by staining of the lung sections with primary anti-iNOS polyclonal rabbit antibody (Transduction Laboratories; 5 μ g/ml), or with a control antibody (rabbit IgG [5 μ g/ml]), followed by addition of a rhodamine-labeled goat anti-rabbit secondary antibody (Sigma Chemical Co.; 1:400 dilution [44]). Under these conditions and with the appropriate filters, macrophages demonstrate green fluorescence, whereas those containing mac-iNOS emit red fluorescence. Stained tissue samples were examined under a microscope equipped with epifluorescence.

Interventional studies. In selected experiments, the effect of administration of exogenous IFN- γ on the resolution of *L. pneumophila* lung infections in GKO mice was determined. GKO mice were coinoculated i.t. with murine recombinant IFN- γ (5×10^4 U per mouse) and with *L. pneumophila* (10^6 bacteria per mouse).

FIG. 1. Effect of targeted disruption of the IFN- γ gene on resolution of *L. pneumophila* lung infection. Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with virulent *L. pneumophila* (10^6 bacteria per mouse). At specific time points thereafter, the mice were sacrificed. The lungs were excised and processed as described in Materials and Methods. *L. pneumophila* was quantitated in whole-lung homogenates by culture. Data represent the mean $\pm$ standard error of the mean CFU per lung from at least six mice per time point. *, significant difference compared with similarly infected immunocompetent mice (Mann-Whitney test, $P < 0.05$).

Results of preliminary studies in our laboratory demonstrated that incubation of *L. pneumophila* (10^6 bacteria) with this concentration of IFN- γ in vitro does not alter *L. pneumophila* viability (data not shown). The mice were subsequently reinoculated with IFN- γ (5×10^4 U per mouse i.t.) at 24 and 48 h p.i. At 72 h p.i., the mice were sacrificed. The lungs were excised and homogenized, and *L. pneumophila* was quantitated by culture of lung homogenates on BCYE-PAC. This concentration of IFN- γ , when administered i.t., has previously been shown to be efficacious in facilitating resolution of *L. pneumophila* lung infection in other rodent models of legionellosis (7, 39).

In additional experiments, the effect of inhibition of endogenous NO on intrapulmonary clearance of *L. pneumophila* in infected immunocompetent BALB/c mice was assessed. Immunocompetent BALB/c mice were anesthetized and implanted intraperitoneally with a micro-osmotic minipump (Alza Corp., Palo Alto, Calif.) which delivered NMMA (Calbiochem, Inc.), a competitive substrate inhibitor of iNOS, at a rate of 2 μ mol/h for 96 h. This concentration of NMMA, administered in vivo, has previously been shown to be efficacious in inhibition of NO production in other rodent models of lung injury (8, 36). Ten minutes following intraperitoneal placement of the pump, the mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse). At 72 h p.i., the mice were sacrificed and *L. pneumophila* CFU in the lung were assessed.

Statistical analysis. The Mann-Whitney test or the Student *t* test was used to compare differences between treatment groups. A *P* value of <0.05 was considered significant.

RESULTS

Resolution of *L. pneumophila* pulmonary infection in immunocompetent BALB/c mice and GKO mice. As shown in Fig. 1, *L. pneumophila* did not replicate in, and was rapidly cleared from, the lungs of immunocompetent BALB/c mice within 120 h p.i. In contrast, *L. pneumophila* replicated in the lungs of similarly infected GKO mice, resulting in a significant increase in the number of *L. pneumophila* CFU being recovered in lung homogenates from GKO mice at 24 h p.i. (Mann-Whitney test, $P < 0.05$). The number of bacteria recovered from the lungs of infected GKO mice reached a maximum at 48 h p.i. and then slowly decreased over the next 72 h p.i. Greater than four times the inoculating dose of *L. pneumophila* was still detectable in lung homogenates of GKO mice at 240 h p.i., demonstrating that GKO mice develop persistent *L. pneumophila* pulmonary infections following i.t. inoculation with the bacterium.

Subsequent experiments were conducted to determine if *L.*

pneumophila-infected GKO mice also developed a disseminated infection involving the spleen. Spleens from *L. pneumophila*-infected immunocompetent BALB/c mice and similarly infected GKO mice were excised and homogenized, and splenic homogenates were cultured on BCYE-PAC for quantitation of *L. pneumophila* CFU. While *L. pneumophila* was not recovered in spleen homogenates from immunocompetent mice at ≥ 72 h p.i., the bacteria were present in low numbers (i.e., 10^3 CFU per spleen homogenate) in 75 and 66% of spleens harvested from *L. pneumophila*-infected GKO mice at 72 and 120 h p.i., respectively (data not shown). *L. pneumophila* could not be cultured from spleen homogenates of infected GKO mice at 240 h p.i.

Clinical signs of disease. *L. pneumophila*-infected immunocompetent BALB/c mice and GKO mice displayed similar non-specific signs of illness by 48 to 72 h p.i., which included lethargy and piloerection. These clinical signs resolved within 120 to 240 h p.i., with no mortality in any of the mice, irrespective of the animals' immunologic status (i.e., immunocompetent versus GKO).

Pathology. No significant lesions were evident grossly in the lungs of immunocompetent BALB/c mice or GKO mice at 24 h p.i. However, at 72 h p.i. there were numerous dark red foci on the lung surface and within the lung parenchyma of lungs of immunocompetent mice representing areas of consolidation. Approximately 40% of the lung parenchyma was involved, with no lobar predilection to the lesions. Lung lesions in GKO mice at 72 h p.i. were similar to those described above for immunocompetent BALB/c mice, except that they were more extensive, involving 60 to 80% of the total lung parenchyma. Lungs from immunocompetent mice and GKO mice sacrificed at 120 h p.i. had lesions similar to those described above for mice at 72 h p.i. Lung lesions in immunocompetent mice and GKO mice at 240 h p.i. were confined primarily to the cranial (BALB/c mice) and hilar (GKO mice) regions of the lung and consisted of scattered tan foci on the lung surface and within the lung parenchyma.

Microscopic lesions were first evident in lung tissue from *L. pneumophila*-infected immunocompetent BALB/c mice and similarly infected GKO mice at 24 h p.i. Pulmonary lesions, which were similar in immunocompetent mice and GKO mice at 24 h p.i., consisted of mild perivascular edema, with multifocal accumulations of PMNs adjacent to vessels and larger bronchi in the hilar region. There were also occasional foci of PMNs at the level of the terminal bronchioles. By 72 h p.i., lungs from both immunocompetent BALB/c mice and similarly infected GKO mice contained patchy areas of PMN accumulation, with no lobar predilection. There were also multifocal areas of mild subpleural inflammation and interstitial alveolar wall thickening in the inflammatory foci. While these lesions were quantitatively similar in both immunocompetent mice and GKO mice, they were more extensive in GKO mice, involving 50 and 80% of the total lung volume in immunocompetent mice and GKO mice, respectively. By 120 h p.i., marked differences were evident between lung lesions in *L. pneumophila*-infected immunocompetent mice and similarly infected GKO mice. While 20% of the total lung in immunocompetent BALB/c mice at 120 h p.i. was pneumonic, 70 to 80% of the total lung in similarly infected GKO mice was affected. Furthermore, in immunocompetent BALB/c mice, there was a mild infiltrate of MPCs and widening of the interstitium in the affected foci. In contrast, in similarly infected GKO mice, the alveolar exudate was characterized by the predominance of PMNs, with relatively few MPCs present. Widespread subpleural inflammation with moderate interstitial thickening as well as mild lymphocyte cuffing of vessels was also evident in the

FIG. 2. Lung pathology in *L. pneumophila*-infected immunocompetent BALB/c mice (A, C, and E) and in similarly infected GKO mice (B, D, and F) at 240 h p.i. (A) Note the nearly complete resolution of the inflammatory response. (B) Chronic inflammatory response. Note clusters of PMNs (arrowheads). (C) Mild perivascular lymphocyte cuffing. (D) Marked perivascular lymphocyte cuffing. (E) Mild pleuritis. (F) Severe pleuritis. Hematoxylin and eosin stain. Magnification, $\times 330$.

lungs of GKO mice at 120 h p.i. Differences in pulmonary pathology of *L. pneumophila*-infected immunocompetent mice versus similarly infected GKO mice persisted and were most striking at 240 h p.i. As shown in Fig. 2A, at 240 h p.i. the inflammatory response in the lungs of *L. pneumophila*-infected immunocompetent BALB/c mice had, for the most part, resolved. Pulmonary lesions included a few scattered foci of MPCs, mild lymphocyte cuffing (Fig. 2C), and mild interstitial thickening and mild pleuritis (Fig. 2E). In contrast, marked multifocal pneumonia which involved approximately 90% of the total lung was evident in lung tissue of similarly infected GKO mice at 240 h p.i. Many foci of intra-alveolar PMNs were evident (Fig. 2B). Other lesions included moderate thickening of the interstitial space with infiltration by MPCs, marked

perivascular lymphocyte cuffing (Fig. 2D), and severe pleuritis (Fig. 2F).

Quantitation of pulmonary inflammatory cells. As shown in Fig. 3A, the number of inflammatory cells isolated from minced lungs differed markedly between immunocompetent BALB/c mice and GKO mice at ≥ 72 h p.i. Following i.t. inoculation of *L. pneumophila*, the total number of pulmonary inflammatory cells increased up to 72 h p.i. in immunocompetent BALB/c mice and subsequently declined. In contrast, the total number of pulmonary inflammatory cells in *L. pneumophila*-infected GKO mice, while increasing markedly until 72 h p.i., subsequently plateaued, so that at 240 h p.i. there were fourfold more cells in lung digests of *L. pneumophila*-infected GKO mice than in those of similarly infected BALB/c mice or

FIG. 3. Kinetics of inflammatory cell recruitment to the lungs during *L. pneumophila* pulmonary infection. Lungs from immunocompetent BALB/c mice (■) and GKO mice (▨) were minced at various times after i.t. inoculation with *L. pneumophila*. Inflammatory cells were counted by hemocytometer, and differential cell counts were performed on Giemsa-stained cytocentrifuge preparations and confirmed by flow cytometric analysis. (A) Total cell count; (B) MPCs and PMNs; (C) lymphocytes. Results represent the mean $\pm$ standard error of the mean for three animals assayed individually per time point p.i. *, significantly

to uninfected GKO mice. Further analysis of these inflammatory cells demonstrated that there was persistent recruitment of MPCs, PMNs, and lymphocytes into the lungs of *L. pneumophila*-infected GKO mice compared with the lungs of similarly infected immunocompetent BALB/c mice. Specifically, as shown in Fig. 3B, the number of MPCs and PMNs in the lungs of *L. pneumophila*-infected immunocompetent BALB/c mice increased until 72 h p.i. and then declined over the next 168 h p.i. PMNs and MPCs were also recruited into the lungs of *L. pneumophila*-infected GKO mice until 72 h p.i. While this number declined between 72 and 120 h p.i., between 120 and 240 h p.i. there was again recruitment of these cells into the lung, so that at 240 h p.i. the number of PMNs and MPCs in the lungs of *L. pneumophila*-infected GKO mice was significantly enhanced compared with that in similarly infected BALB/c mice ($P < 0.001$). Furthermore, while there was no significant recruitment of lymphocytes into the lungs of *L. pneumophila*-infected BALB/c mice at any time point ($P > 0.05$) (Fig. 3C), there was significant recruitment of lymphocytes into the lungs of *L. pneumophila*-infected GKO mice by 120 h p.i. ($P < 0.001$) (compared with the lungs of uninfected BALB/c mice and GKO mice).

Flow cytometry analysis of pulmonary inflammatory cell phenotypes. Flow cytometric analysis of lung inflammatory cells from *L. pneumophila*-infected immunocompetent BALB/c mice and similarly infected GKO mice at 240 h p.i. (i.e., the time point of maximal cellular influx into the lung in *L. pneumophila*-infected GKO mice and minimal inflammation in similarly infected immunocompetent BALB/c mice) highlights these differences in the pulmonary inflammatory response. Representative laser scatter plots from flow cytometry data from *L. pneumophila*-infected BALB/c mice (Fig. 4A) and similarly infected GKO mice (Fig. 4B) demonstrate that lung homogenates from *L. pneumophila*-infected GKO mice contain considerably more macrophages and granulocytes (i.e., the large-cell population) and lymphocytes (i.e., the small-cell population) than did those of similarly infected immunocompetent BALB/c mice. Further analysis of these recruited cell populations using monoclonal antibodies specific for either PMNs or specific lymphocyte subsets ($CD4^+$ T cells, $CD8^+$ T cells, and B cells) demonstrated that lungs from *L. pneumophila*-infected GKO mice at 240 h p.i. contained significantly more PMNs and B cells than did those of similarly infected immunocompetent BALB/c mice ($P < 0.05$) (Fig. 5). However, recruitment of either $CD4^+$ T cells or $CD8^+$ T cells into the lung was similar in immunocompetent BALB/c mice and GKO mice at this time point. In agreement with previously described histologic analysis of *L. pneumophila*-infected lung tissue, these results confirm that the inflammatory response in the lungs of *L. pneumophila*-infected immunocompetent BALB/c mice resolves within 240 h p.i. while an inflammatory response persists in similarly infected GKO mice, with continued recruitment of both PMNs and lymphocytes (i.e., B cells) into the lung.

Effect of IFN- γ replacement therapy on resolution of *L. pneumophila* lung infection in GKO mice. As shown in Fig. 6, administration of exogenous IFN- γ to *L. pneumophila*-infected GKO mice resulted in a logfold decrease in the number of bacteria recovered from the lung at 72 h p.i. compared with the number in similarly infected GKO mice not administered IFN- γ ($P < 0.005$). Furthermore, the number of bacteria re-

different compared with similarly infected immunocompetent BALB/c mice (unpaired t test; $P < 0.05$); t, significant difference compared with uninfected mice of the same immunologic status (unpaired t test, $P < 0.05$).

FIG. 4. Example of flow cytometric analysis of lung leukocyte populations. Leukocytes were isolated at 10 days p.i. from enzymatically digested lungs of immunocompetent mice (A) or GKO mice (B) infected with *L. pneumophila*. Laser light scatter dot plot of cells (LC, large cells; SC, small cells). The horizontal axis is forward scatter, an indicator of cell size, and the vertical axis is orthogonal scatter, an indicator of cell granularity and surface irregularity. The SC population is over 90% lymphocytes, whereas the LC population is >95% nonlymphoid cells (i.e., MPCs and PMNs).

covered from the lungs of IFN- γ -treated GKO mice was not statistically different from that recovered in lung homogenates from *L. pneumophila*-infected immunocompetent BALB/c mice at 72 h p.i. (data not shown).

Induction of mac-iNOS in the lungs of immunocompetent BALB/c mice and GKO mice during *L. pneumophila* lung infection. Induction of mac-iNOS in alveolar cells from *L. pneumophila*-infected immunocompetent BALB/c mice and similarly infected GKO mice was assessed by Western blot analysis of alveolar cell lysates. Results of initial studies demonstrated that there was no significant difference in the total number of alveolar cells recovered in bronchoalveolar lavage samples from *L. pneumophila*-infected immunocompetent BALB/c mice and similarly infected GKO mice at 0 to 72 h p.i. (data not shown). As shown in Fig. 7, mac-iNOS was not detectable in alveolar cells derived from uninfected immunocompetent BALB/c mice and GKO mice. However, mac-iNOS was in-

duced in alveolar cells during *L. pneumophila* lung infection in immunocompetent BALB/c mice, with maximal expression at 48 h p.i. In contrast, mac-iNOS was not induced in alveolar cells derived from similarly infected GKO mice at any time point studied.

In subsequent studies, immunostaining was used to identify cellular sources of mac-iNOS in lung tissue from *L. pneumophila*-infected mice at 48 h p.i. (This time point was selected on the basis of results of previously discussed Western blot analysis of alveolar cell lysates demonstrating maximal induction of mac-iNOS in infected immunocompetent BALB/c mice at 48 h p.i.) Results of these studies demonstrated that lectin-stained alveolar macrophages in lung tissue from *L. pneumophila*-infected immunocompetent BALB/c mice stained positively for mac-iNOS, while lectin-stained macrophages in lung tissue from similarly infected GKO mice did not stain for mac-iNOS (data not shown). Lectin-stained macrophages from *L. pneu-*

FIG. 5. Pulmonary recruitment of PMNs and lymphocyte subsets at 10 days p.i. Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* as described in Materials and Methods. Leukocytes were subsequently isolated from the lung at 10 days p.i. by mincing and enzymatic digestion. The cells were subsequently stained with fluorescein isothiocyanate-conjugated monoclonal antibodies and analyzed by flow cytometry. Total numbers of positively staining cells were derived by multiplying the total number of leukocytes isolated from the lungs times the percentage of positively staining cells. Data represent the mean $\pm$ standard error of the mean for three mice assayed individually. *, significant difference compared with similarly infected immunocompetent BALB/c mice (unpaired *t* test, *P* < 0.05).

FIG. 6. Effect of exogenous IFN- γ on clearance of *L. pneumophila* from the lungs of infected GKO mice. *L. pneumophila*-infected GKO mice were administered IFN- γ i.t. as described in Materials and Methods. At 72 h p.i. the mice were sacrificed. *L. pneumophila* CFU in the lung were determined in mice administered IFN- γ and compared with those in control mice (i.e., mice inoculated i.t. with *L. pneumophila*). Results represent the mean $\pm$ standard error of the mean for nine animals per treatment group. *, significant difference compared with untreated infected GKO mice (Mann-Whitney test, $P < 0.001$).

mophila-infected immunocompetent BALB/c mice incubated with nonspecific rabbit IgG did not stain with rhodamine-labeled antibody, demonstrating specificity of the mac-iNOS polyclonal primary antibody. These results complement those of the previously described Western blot analysis of alveolar cell lysates from infected immunocompetent BALB/c mice (Fig. 7) and identify specific cells (i.e., alveolar macrophages) as a cellular source of mac-iNOS during *L. pneumophila* lung infections in this resistant murine host.

Effect of NMMA on resolution of *L. pneumophila* lung infection. Results of in vivo studies described above demonstrate that mac-iNOS is induced in *L. pneumophila*-infected immunocompetent BALB/c mice, likely resulting in the production of endogenous NO. To identify the potential role of endogenous NO in IFN- γ -mediated host resistance to *L. pneumophila* lung infections, immunocompetent BALB/c mice were administered NMMA, a competitive substrate inhibitor of iNOS, prior to i.t. inoculation with *L. pneumophila*. This dose of NMMA has previously been shown to inhibit iNOS activity in rodent models of lung injury (8, 36). Resolution of *L. pneumophila* lung infection was subsequently determined at 72 h p.i. as described in Materials and Methods. (This time point p.i. was selected because resolution of *L. pneumophila* lung infections in immunocompetent BALB/c mice begins to occur at 72 h p.i. [Fig. 1].) As shown in Fig. 8, there was no significant difference in the number of *L. pneumophila* cells recovered from the lungs of NMMA-treated versus nontreated *L. pneumophila*-infected immunocompetent mice at 72 h p.i. These results suggest that endogenous NO, generated during the early phase (i.e., within 72 h p.i.) of *L. pneumophila* lung infection in immunocompetent BALB/c mice, is not responsible for IFN- γ -mediated resolution of *L. pneumophila* pulmonary infection in this resistant murine host.

DISCUSSION

In this study, we have developed a murine model of *L. pneumophila* lung infection in BALB/c mice with a targeted

disruption of the IFN- γ gene (i.e., GKO mice) and have used this model to evaluate potential effector mechanisms responsible for IFN- γ -mediated host resistance to *L. pneumophila*. We demonstrate that while immunocompetent BALB/c mice are resistant to replicative *L. pneumophila* lung infections, similarly infected GKO mice develop persistent lung infections with transient extrapulmonary dissemination of the bacteria to the spleen. The pattern of persistent bacterial infection in *L. pneumophila*-infected GKO mice is similar to that previously described in immunocompromised Sprague-Dawley rats induced by corticosteroid administration (40). In GKO mice, as in corticosteroid-treated rats, initial replication of *L. pneumophila* within the lung during the first few days p.i. is followed by a prolonged equilibrium phase, with incomplete elimination of the bacteria from the lung.

Pathologic features of the pneumonia that develops in GKO mice inoculated i.t. with *L. pneumophila*, as demonstrated both microscopically and by flow cytometric analysis of infected lung tissue, differ markedly from those of the pneumonia in similarly infected immunocompetent mice. Both immunocompetent BALB/c mice and GKO mice develop an initial pulmonary inflammatory response (i.e., at <72 h p.i.) which is characterized by an alveolar exudate consisting primarily of PMNs. However, by 240 h p.i. the inflammatory response in the lungs of immunocompetent BALB/c mice is nearly resolved. In contrast, the inflammatory response in similarly infected GKO mice persists, resulting in continued recruitment of PMNs, MPCs, and B lymphocytes into the lungs. The mechanism for persistent recruitment of leukocytes into the lungs of *L. pneumophila*-infected GKO mice has not been thoroughly investigated. However, it is likely that this enhanced recruitment of MPCs into the lungs of GKO mice affords *L. pneumophila* a greater opportunity for intracellular infection and growth.

Previous studies have demonstrated that host susceptibility to replicative *L. pneumophila* lung infections is mediated in large part by the permissiveness of alveolar macrophages to growth of the bacteria (18). Genetic resistance to *L. pneumophila* infection in mice is controlled by a single susceptibility-resistance gene (or a closely linked gene) provisionally designated *Lgn-1* on chromosome 13 (15). In vitro studies have demonstrated that cultured BALB/c peritoneal macrophages are not permissive for *L. pneumophila* replication (47). In the current study, we demonstrate that *L. pneumophila* does not replicate in the lungs of immunocompetent BALB/c mice following i.t. inoculation with virulent bacteria, presumably in large part because of the nonpermissiveness of host alveolar MPCs for growth of the bacteria. In contrast, we demonstrate that *L. pneumophila* does replicate during the initial stages of infection (i.e., at <48 h p.i.) in similarly infected GKO mice. Furthermore, resistance to replicative *L. pneumophila* lung infection in GKO mice is restored by i.t. administration of exogenous IFN- γ . While the mechanism(s) by which *Lgn-1* regulates growth of *L. pneumophila* in vivo and the potential role of IFN- γ in this pathway have not been identified, our results strongly suggest that endogenous IFN- γ contributes towards the genetic resistance of immunocompetent BALB/c mice to replicative *L. pneumophila* infections.

In subsequent experiments, the potential role of endogenous NO in IFN- γ -mediated host resistance to *L. pneumophila* lung infection in immunocompetent BALB/c mice (a resistant host) was investigated. Previous studies have demonstrated that stimulation of murine macrophages with IFN- γ , in concert with a cofactor, including tumor necrosis factor alpha, results in the induction of nitric oxide synthetase and the production of reactive nitrogen intermediates, including NO (21, 22). While results of in vitro studies regarding the potential role of

FIG. 7. mac-iNOS induction in alveolar cell lysates of *L. pneumophila*-infected immunocompetent BALB/c mice or similarly infected GKO mice. Immunocompetent BALB/c mice and GKO mice were inoculated i.t. with *L. pneumophila* as described in Materials and Methods. At specific time points thereafter, the mice were sacrificed, the lungs were lavaged, and alveolar cells were harvested from bronchoalveolar lavage fluid. The cells were subsequently lysed and subjected to SDS-PAGE followed by Western blot with anti-iNOS IgG. (Top) Western blot of mac-iNOS in alveolar cell lysates from *L. pneumophila*-infected GKO mice and immunocompetent mice (three mice per treatment group per time point). (Bottom) Laser densitometry scan of the Western blot analysis shown in the upper panel, demonstrating the mean $\pm$ standard error of the mean mac-iNOS expression in alveolar cell lysates from *L. pneumophila*-infected GKO and immunocompetent mice (three mice per treatment group per time point). Results demonstrate induction of mac-iNOS in alveolar cell lysates from *L. pneumophila*-infected BALB/c mice at 48 h p.i. but not in similarly infected GKO mice.

NO as an effector molecule in IFN- γ -mediated regulation of *L. pneumophila* replication within host MPCs have been conflicting (19, 42), we have recently demonstrated that endogenous NO plays a key role in IFN- γ -mediated resolution of replicative *L. pneumophila* lung infections in a susceptible murine host (A/J mice) (8). While the mechanisms by which NO exerts its antimicrobial effects are incompletely understood, a direct toxic effect of NO has been postulated (22). NO can also complex with iron, resulting in the formation of iron nitrosyl complexes (which are subsequently lost from MPCs), and may facilitate the direct release of iron from ferritin (30, 35). Furthermore, recent in vitro and in vivo studies demonstrate that NO also regulates the production of cytokines, including tumor necrosis factor alpha, by lymphocytes and leukocytes, thereby potentially contributing to disease pathogenesis (8, 31, 43, 45). In the current study, we demonstrate that mac-iNOS (an enzyme responsible for the production of NO) is induced in alveolar macrophages from *L. pneumophila*-infected immunocompetent BALB/c mice but not in similarly infected GKO mice (Fig. 7). Furthermore, induction of mac-iNOS in immu-

nocompetent BALB/c mice (i.e., at 48 h p.i.) preceded clearance of the bacteria from the lung (i.e., at >48 h p.i.) (Fig. 1), suggesting that endogenous NO is responsible, at least in part, for IFN- γ -mediated resistance to *L. pneumophila* lung infections in this resistant murine host. However, inhibition of endogenous NO by administration of NMMA, a competitive substrate inhibitor of iNOS, to *L. pneumophila*-infected BALB/c mice did not result in enhanced growth or recovery of *L. pneumophila* in the lung at 72 h p.i., compared with similarly infected mice not administered NMMA (Fig. 8). Results of these studies demonstrate that in contrast to a susceptible murine host (i.e., A/J mice), IFN- γ -mediated host defense to *L. pneumophila* lung infection in a resistant murine strain (i.e., BALB/c mice) occurs by a NO-independent mechanism.

Previous in vitro studies have identified effects of IFN- γ on MPCs and natural killer cells which may facilitate resolution of *L. pneumophila* lung infections in vivo. With regard to host MPCs, intracellular growth of *L. pneumophila* within MPCs is dependent on iron. Stimulation of MPCs with IFN- γ restricts intracellular iron by decreasing cell surface transferrin recep-

FIG. 8. Effect of NMMA on resolution of *L. pneumophila* lung infections in infected immunocompetent BALB/c mice. BALB/c mice were administered NMMA as described in Materials and Methods prior to i.t. inoculation with *L. pneumophila*. At 72 h p.i., the mice were sacrificed and *L. pneumophila* CFU in the lung were determined and compared with the number in control mice (i.e., mice inoculated i.t. with *L. pneumophila*). Results represent the mean $\pm$ standard error of the mean for two separate experiments, 6 to 10 animals per treatment group. ■, *L. pneumophila*; ▨, *L. pneumophila* plus NMMA.

tors and intracellular ferritin, thereby limiting *L. pneumophila* replication (9, 19, 20). Also, cultured murine macrophages, following stimulation with IFN- γ , secrete enhanced amounts of other cytokines, including tumor necrosis factor alpha. Tumor necrosis factor alpha has previously been shown to inhibit growth of *L. pneumophila* within MPCs as well as enhance PMN bactericidal activity, thereby decreasing the viability of extracellular *L. pneumophila* (4, 32). Stimulation of human natural killer cells with IFN- γ results in enhanced cytolytic activity of these cells to cultured *L. pneumophila*-infected MPCs (5). These results suggest that IFN- γ activated natural killer cells also limit the course of *L. pneumophila* infection in vivo. The potential relationship between each of these mechanisms and the persistence of *L. pneumophila* lung infection in GKO mice remains to be explored.

In summary, we have developed a murine model of Legionnaires' disease in BALB/c mice with a targeted disruption in the IFN- γ gene. To our knowledge, this is the first report of an animal model of *L. pneumophila* lung infection in a murine host which is characterized by a specific defect in the cellular immune response (i.e., genetically deficient in IFN- γ). We anticipate that this animal model of legionellosis in GKO mice will provide a valuable tool in the identification of effector mechanisms which are responsible for IFN- γ -induced early (nonspecific) resistance to *L. pneumophila* pulmonary infections in vivo. In addition, immunocompromised patients frequently develop relapsing *L. pneumophila* infections despite prolonged antibiotic therapy, thereby demonstrating a need for the development of novel antimicrobial agents which are bactericidal for *L. pneumophila*. It is anticipated that this model of legionellosis in GKO mice will be useful in the evaluation of the efficacy of novel antibiotics for the treatment of Legionnaires' disease in this patient population.

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