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In Vivo Regulation of Replicative *Legionella pneumophila* Lung Infection by Endogenous Tumor Necrosis Factor Alpha and Nitric Oxide

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The in vivo role of endogenous tumor necrosis factor alpha (TNF-α) and reactive nitrogen intermediates (RNIs) in modulation of growth of *Legionella pneumophila* in the lung was assessed using a murine model of replicative *L. pneumophila* lung infection. Intratracheal inoculation of mice with *L. pneumophila* resulted in induction of endogenous TNF-α, which preceded clearance of *L. pneumophila* from the lung. Inhibition of endogenous TNF-α activity, via in vivo administration of TNF-α neutralizing antibody, or inhibition of endogenous RNIs, via administration of the nitric oxide (NO) synthetase inhibitor *N*-monomethyl-L-arginine (NMMA), resulted in enhanced growth of *L. pneumophila* in the lung at ≥3 days postinfection (when compared with untreated *L. pneumophila*-infected mice). Because of the similar kinetics of enhanced pulmonary growth of *L. pneumophila* in mice treated in vivo with either anti-TNF-α antibody or NMMA, the immunomodulatory effect of NO on endogenous TNF-α activity in the lung was assessed. Administration of NMMA to *L. pneumophila*-infected mice resulted in a significant decrease in endogenous TNF-α activity in the lung during replicative *L. pneumophila* infections in vivo. However, administration of exogenous TNF-α to NMMA-treated mice failed to significantly enhance clearance of *L. pneumophila* from the lung. Results of these studies indicate that both endogenous NO and TNF-α facilitate resolution of replicative *L. pneumophila* lung infections and that regulation of *L. pneumophila* replication by TNF-α is mediated, at least in part, by NO.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen of mononuclear phagocytic cells (MPCs), primarily alveolar macrophages (27, 32, 35, 36, 47, 51). Susceptibility to replicative *L. pneumophila* lung infections is determined, in large part, by the permissiveness of host MPCs to growth of the bacteria (2, 13, 19, 32, 49, 50). In the susceptible host, resistance to *L. pneumophila* is mediated by the induction of cellular immunity and the production of cytokines, including gamma interferon (IFN-γ) (6, 28, 29, 36, 37, 43, 49). Iron is required for growth of *L. pneumophila* in permissive MPCs. IFN-γ limits the availability of iron in MPCs, in large part through down-regulation of transferrin receptors on the cell surface and by decreasing the expression of intracellular ferritin, thereby inhibiting replication of *L. pneumophila* (10, 11).

Recent in vitro studies indicate that other cytokines, including tumor necrosis factor alpha (TNF-α), also contribute to host resistance to *L. pneumophila* infections (8, 34, 50). Specifically, stimulation of human polymorphonuclear leukocytes (PMNs) with TNF-α results in PMN activation, enhanced PMN bactericidal activity, and decreased viability of extracellular bacteria (8). Similarly, intracellular growth of *L. pneumophila* in human MPCs is directly inhibited by TNF-α (34). Furthermore, stimulation of *L. pneumophila*-infected MPCs with IFN-γ results in enhanced synthesis of TNF-α, and inhibition of endogenous TNF-α production in vitro partially abolishes the inhibitory effect of IFN-γ on intracellular bacterial replication (34). These results indicate that IFN-γ-mediated regulation of *L. pneumophila* replication in permissive MPCs in vitro is regulated, at least in part, by TNF-α.

Stimulation of murine MPCs with IFN-γ, in concert with a cofactor including TNF-α, also results in the induction of nitric oxide synthetase (NOS) and the production of reactive nitrogen intermediates (RNIs), including nitric oxide (NO) (21, 25). NO is a central effector molecule in IFN-γ-mediated bactericidal activity and plays a key role in resistance of cultured murine cells to several intracellular pathogens including *Leishmania*, *Cryptococcus*, *Toxoplasma*, *Mycobacterium*, *Francisella*, and *Plasmodium* species (3, 4, 12, 14, 16, 17, 22–26, 33, 38, 40). While the mechanism(s) by which NO exerts its antimicrobial effects is incompletely understood, a direct toxic effect of NO has been postulated (25). 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, thereby further decreasing MPC intracellular iron (30, 41). Furthermore, recent in vitro studies indicate that NO also regulates the production of cytokines, including TNF-α, by lymphocytes and leukocytes (31, 46, 48). Stimulation of human lymphocytes or neutrophils with NO-generating compounds (e.g., sodium nitroprusside and/or *S*-nitroso-*N*-acetylpenicillamine [31, 46]) results in enhanced synthesis and secretion of TNF-α, thereby potentially altering disease pathogenesis.

Studies addressing the role of NO in IFN-γ inhibition of *L. pneumophila* replication in permissive MPCs have been conflicting. Specifically, stimulation of *L. pneumophila*-infected RAW 264.7 cells (a murine macrophage cell line) with IFN-γ both inhibits intracellular bacterial replication and induces NO synthesis. Inhibition of NO production by costimulation of the cells with a competitive inhibitor of NOS (i.e., monomethyl arginine [NMMA]), results in enhanced intracellular growth of

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L. pneumophila, indicating that the inhibitory effects of IFN-γ on *L. pneumophila* replication in RAW 264.7 cells is mediated, at least in part, by NO (45). In contrast, while in vitro stimulation of *L. pneumophila*-infected A/J mouse peritoneal macrophages (which are permissive for *L. pneumophila* replication) with IFN-γ results in both NO production and inhibition of intracellular bacterial replication, addition of NMMA, while inhibiting NO production, does not significantly diminish IFN-γ-mediated anti-*Legionella* activity (20).

Effects of endogenous cytokines, including IFN-γ and TNF-α, and RNIs on replication of *L. pneumophila* in the lung in vivo have not been thoroughly investigated. These studies have been hindered, in part, by the lack of an animal model which is both permissive to replicative *L. pneumophila* lung infections and for which species-specific immunologic reagents are readily available. Recently, our laboratory developed a replicative model of *L. pneumophila* lung infection in A/J mice inoculated intratracheally (i.t.) with virulent *L. pneumophila* (9). Using this model, we previously identified a pivotal role for endogenous IFN-γ in modulating replicative *L. pneumophila* lung infections (9). In the current study, regulation of intrapulmonary growth of *L. pneumophila* in vivo by endogenous TNF-α and NO was investigated. Our results indicate that TNF-α, like IFN-γ, is induced during replicative *L. pneumophila* lung infections in vivo and modulates replication of *L. pneumophila* in the lung. Furthermore, in vivo administration of an inhibitor of NO production (i.e., NMMA) results in a significant enhancement in intrapulmonary growth of *L. pneumophila*, suggesting that endogenous NO also regulates intrapulmonary growth of *L. pneumophila* in the susceptible host. TNF-α activity is significantly decreased in the lungs of NMMA-treated, *L. pneumophila*-infected mice (when compared with that in similarly infected mice not administered NMMA), indicating that endogenous NO has an immunostimulatory effect on TNF-α activity. However, administration of exogenous TNF-α to NMMA-treated, *L. pneumophila*-infected mice does not result in enhanced clearance of *L. pneumophila* from the lung, demonstrating that TNF-α-mediated inhibition of *L. pneumophila* replication in the lung is directly dependent, at least in part, on endogenous NO.

MATERIALS AND METHODS

Animal care. Female pathogen-free 6- to 8-week-old A/J mice (Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. The animals were housed in microisolator cages in Horsefall units and were cared for by standard guidelines.

Bacterial inoculum. *L. pneumophila* AA100, a redesignation of a primary clinical isolate from the Wadsworth Veterans Administration Hospital (Wadsworth, Calif.), was provided by Paul Edelstein. Bacteria were passed three times on buffered charcoal-yeast extract (BCYE) agar (18). For preparation of the i.t. inoculum, *L. pneumophila* was 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, 9).

i.t. inoculation of A/J mice. A/J mice were inoculated i.t. with *L. pneumophila*, using previously described methods (9, 44). Mice were anesthetized with ketamine (2.5 mg per mouse intraperitoneally [i.p.] and tethered, and an incision was made through the skin of the ventral neck. The trachea was isolated, and 25 μl of the bacterial suspension (10^6 *L. pneumophila* organisms) followed by 10 μl of air was injected directly into the trachea using a 27-gauge needle. The skin incision was closed using a sterile wound clip.

Recovery of *L. pneumophila* from infected mice. At specific time points after i.t. inoculation of *L. pneumophila* (0, 24, 72, and 120 h), the mice were humanely sacrificed and the lungs were removed. Lung tissue was finely minced in 10 ml of PBS and subsequently homogenized (2 min per sample) using a Stomacher (Teckmar, Cincinnati, Ohio) as previously described (7, 9). The tissue homogenates were serially diluted and cultured on BCYE agar containing polymyxin B, cefamandole, and anisomycin (BCYE + PAC) (Baxter) for 72 h, and CFU were determined (7, 9, 43).

Collection of lung homogenate supernatant. Lung homogenates (prepared as described above) were centrifuged ($500 \times g$, 10 min, 4°C), and the resultant supernatant was filtered using a 0.22-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.) to remove bacteria. The resultant samples were stored at −20°C until use.

TNF-α analysis using the WEHI assay. The effect of *L. pneumophila* on intrapulmonary TNF activity was measured in lung homogenate supernatant by a cytotoxicity assay using the WEHI 164 subclone 13 cell line as previously described (15). Briefly, lung homogenate samples, procured as described above, were serially diluted directly in 96-well microtiter plates (Costar, Cambridge, Mass.). The WEHI cells were resuspended at 5×10^5 cells per ml in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5 μg of actinomycin D (Calbiochem, Boehring Diagnostics, La Jolla, Calif.) per ml and added to the samples. A standard of human recombinant TNF-α was run in each assay. Samples were incubated overnight at 37°C, after which 20 μl of MTT-tetrazolium (5 mg/ml) (Sigma) was added to the wells and allowed to incubated at 37°C for an additional 4 h. Viable cells (i.e., not lysed by TNF-α) metabolize the MTT-tetrazolium to produce dark blue formazan crystals. The crystals were dissolved with isopropanol-HCl, and the plates were read in a microELISA reader (Bio-Tek Instruments, Inc., Winooski, Vt.) at 550 nm. TNF-α activity was calculated based on the human recombinant TNF-α standard that was run in the same assay.

Interventional studies. To determine the effect of endogenous TNF-α on intrapulmonary growth of *L. pneumophila*, in selected experiments, mice were inoculated i.p. with rabbit serum containing neutralizing antibody to TNF-α or with control antiserum 1 h prior to i.t. inoculation of *L. pneumophila*. Alternatively, to determine the effect of endogenous reactive nitrogen metabolites on *L. pneumophila* replication in the lung, in selected experiments, mice were treated with NMMA, (Calbiochem), a competitive substrate inhibitor of NOS which inhibits production of NO. Briefly, mice were anesthetized as described above and implanted i.p. with a micro-osmotic minipump (Alza Corp., Palo Alto, Calif.) which delivered NMMA at a rate of 2 μmol/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 (42). Ten minutes following i.p. placement of the pump, the mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) and with 20 mM NMMA.

Statistical analysis. The Mann-Whitney test was used to compare differences between treatment groups; $P < 0.05$ was considered significant.

RESULTS

Endogenous TNF-α and resolution of *L. pneumophila* lung infection. In initial experiments, mice were inoculated with 10^6 *L. pneumophila* organisms i.t. At specific time points thereafter, the mice were humanely sacrificed and the effect of *L. pneumophila* infection on intrapulmonary TNF-α activity was assessed by measurement of TNF-α activity in lung homogenate supernatants, using methods described in Materials and Methods. As shown in Fig. 1, TNF-α activity was significantly enhanced in *L. pneumophila*-infected mice within 4 h postinoculation and remained significantly enhanced in lung homogenates from mice until 72 h postinfection.

Results of our previous investigations indicated that i.t. inoculation of *L. pneumophila* (10^6 CFU per mouse) results in a replicative lung infection in A/J mice, with exponential growth of the bacteria 24 to 48 h postinoculation (9). This is followed by a gradual clearance of *L. pneumophila* from the lung at ≥72 h after i.t. inoculation (9). Because induction of TNF-α activity in i.t. inoculated mice (i.e., at 4 to 72 h postinfection) precedes clearance of *L. pneumophila* from the lung (i.e., ≥72 h), the potential role of endogenous TNF-α in resolution of replicative *L. pneumophila* lung infections in A/J mice was evaluated. Mice were inoculated i.p. with rabbit anti-TNF-α antiserum (1/10 dilution) or with control rabbit serum (1/10 dilution) 1 h prior to i.t. inoculation of *L. pneumophila*. (Results of preliminary experiments indicated that administration of TNF-α-neutralizing antibody inhibited 70% of TNF-α activity in lung homogenate supernatant at 24 h postinoculation.) The mice were humanely euthanized at 24, 72, and 120 h postinfection, and *L. pneumophila* was quantitated in lung homogenates by methods described in Materials and Methods. Results of these experiments (Fig. 2) indicate that while there was no significant difference in bacterial recovery from the lungs at 24 h postinfection, irrespective of the treatment protocol, significantly more bacteria were recovered in lung homogenates from animals administered TNF-α-neutralizing antibody and sacrificed

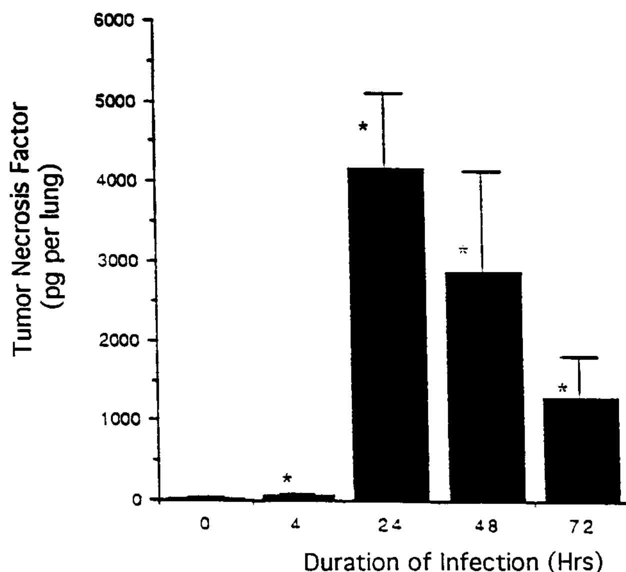

FIG. 1. TNF-α activity in lung homogenates of A/J mice inoculated i.t. with *L. pneumophila*. A/J mice were inoculated i.t. with *L. pneumophila* as described in Materials and Methods. At specific time points thereafter, the mice were sacrificed and TNF-α activity was assessed as previously described. Results represent the means ± standard errors of the means of three separate experiments, 4 to 12 animals per time point. *, significantly greater than for uninfected mice (i.e., 30.3 ± 7.1 pg of TNF-α per lung) (Mann-Whitney test, $P < 0.05$).

at 3 and 5 days postinoculation (when compared with non-antibody-treated, *L. pneumophila*-infected mice; Mann-Whitney test, $P < 0.02$). Administration of control serum had no significant effect on *L. pneumophila* replication in the lung (data not shown). These results indicate that endogenous TNF-α, induced in the lung during replicative *L. pneumophila* lung infections, inhibits intrapulmonary replication of *L. pneumophila* in vivo.

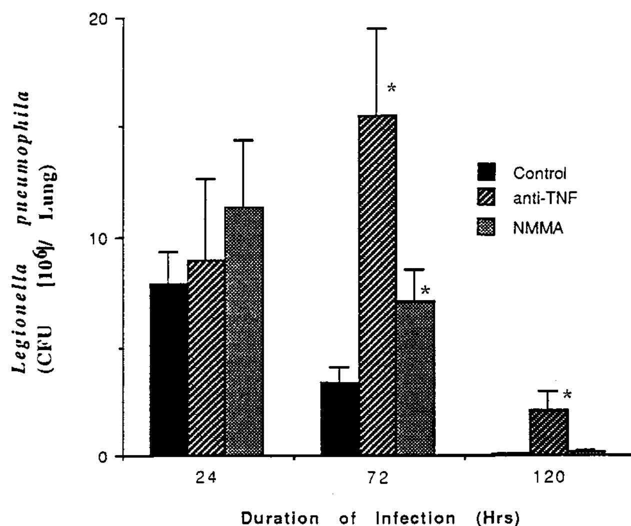

FIG. 2. Effect of TNF-α-neutralizing antibody or NMMA on intrapulmonary replication of *L. pneumophila*. A/J mice were administered TNF-α-neutralizing antibody or NMMA as described in Materials and Methods prior to i.t. inoculation with *L. pneumophila*. At selected time points thereafter, the mice were sacrificed and growth of *L. pneumophila* in the lung was determined in mice administered anti-TNF-α antibody, or NMMA and compared with that in control mice (i.e., mice inoculated i.t. with *L. pneumophila*). Results represent means ± standard errors of the means of three separate experiments, six to nine animals per treatment group. *, significantly greater than for control mice; Mann-Whitney test, $P < 0.02$.

Effect of NMMA on intrapulmonary replication of *L. pneumophila*. Results of our in vivo studies indicated that IFN-γ and TNF-α, induced during replicative *L. pneumophila* lung infections, inhibit replication of *L. pneumophila* in the lung. Furthermore, results of previous in vitro studies using other intracellular pathogens indicate that these cytokines inhibit bacterial growth of intracellular pathogens in cultured murine cells, in large part through the induction of NOS activity and the production of RNIs, including NO. The potential role of endogenous NO in regulating growth of *L. pneumophila* in the lung in vivo has not been thoroughly investigated. To identify the role of endogenous NO in modulation of *L. pneumophila* replication in the lung, A/J mice were administered NMMA, a competitive inhibitor of NOS, as described in Materials and Methods. The mice were subsequently inoculated i.t. with *L. pneumophila*, and the effect of NMMA on intrapulmonary growth of *L. pneumophila* was determined at 24, 72, and 120 h postinfection. As shown in Fig. 2, there was no significant difference in the number of *L. pneumophila* recovered from the lungs of NMMA-treated versus non-NMMA-treated *L. pneumophila*-infected animals at 24 or 120 h postinfection. In contrast, significantly more *L. pneumophila* organisms were recovered from the lungs of NMMA-treated mice 3 days postinoculation (when compared with untreated *L. pneumophila*-infected animals [$P < 0.01$]). These results suggest that endogenous NO modulates growth of *L. pneumophila* in the lung during the early phase (i.e., within 3 days postinfection) of replicative *L. pneumophila* infections in vivo.

Immunomodulation of endogenous TNF-α activity by NO. Results of experiments described above indicate that in vivo neutralization of either endogenous TNF-α or administration of NMMA facilitates growth of *L. pneumophila* in the lung within 72 h postinoculation. These similar kinetics suggest that endogenous TNF-α and NO may regulate growth of *L. pneumophila* in the lung by a common mechanism. Results of previous in vitro studies using human leukocytes and murine lymphocytes indicate that endogenous NO plays an immunomodulatory role, regulating the production of specific cytokines, including TNF-α (31, 46, 48). Therefore, the immunoregulatory effect of endogenous NO on TNF-α activity in the lung during replicative *L. pneumophila* lung infection was investigated. A/J mice were administered NMMA and inoculated i.t. with *L. pneumophila* as previously described. Twenty-four hours postinfection (the time of maximal TNF-α activity in *L. pneumophila*-infected mice [Fig. 1]), the animals were humanely sacrificed, lung homogenate supernatants were prepared, and TNF-α activity in lung homogenates was assessed. As shown in Fig. 3, 50% less TNF-α activity was recovered in lung homogenates of *L. pneumophila*-infected mice that received NMMA than in those of control mice (i.e., *L. pneumophila*-infected mice that did not receive NMMA [$P < 0.03$]). These results indicate that endogenous NO modulates TNF-α production during replicative *L. pneumophila* lung infections.

Because both NO and TNF-α modulate replication of *L. pneumophila* in the lung, subsequent experiments were designed to identify the relationship between endogenous TNF-α activity, NO production, and intrapulmonary replication of *L. pneumophila*. A/J mice ($n = 5$) were administered recombinant murine TNF-α (Genzyme; 50,000 U i.p.) 2 h prior to administration of NMMA and i.t. inoculation of *L. pneumophila*. This concentration of TNF-α, administered in vivo, has previously been shown to facilitate clearance of *L. pneumophila* from the lung in other rodent models of *L. pneumophila* lung infection

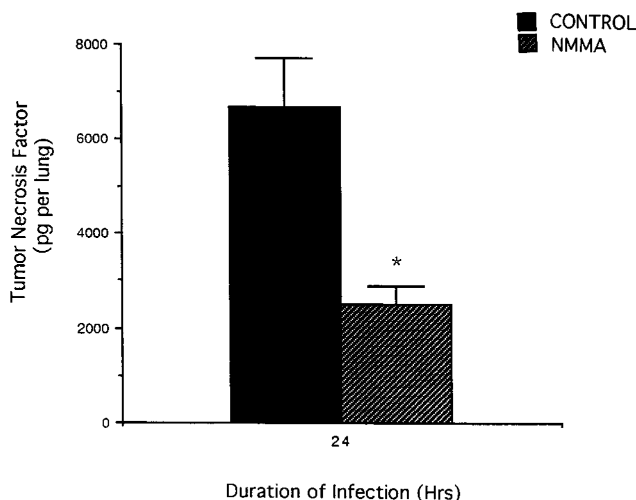

FIG. 3. Effect of NMMA on endogenous TNF-α activity in the lung. A/J mice were administered NMMA as described in Materials and Methods prior to i.t. inoculation of *L. pneumophila*. At 24 h postinfection, the mice were sacrificed. The lungs were excised and homogenized, and TNF-α activity in lung homogenates of NMMA-treated mice was determined and compared with that in control mice (i.e., *L. pneumophila*-infected mice not administered NMMA). Results represent the means ± standard errors of the means of a representative experiment of three experiments, three to four mice per treatment group. *, significantly less than control mice; Mann-Whitney test, $P < 0.03$.

(7). The mice were subsequently inoculated i.t. with *L. pneumophila* as previously described. At 72 h postinfection, (i.e., when growth of *L. pneumophila* in the lung is significantly enhanced in mice treated with NMMA when compared with control mice [Fig. 2]), the mice were humanely sacrificed. The lungs were excised and homogenized, and growth of *L. pneumophila* in the lungs of mice administered TNF-α and NMMA was assessed. Results of these studies (Fig. 4) indicate that growth of *L. pneumophila* in the lungs of NMMA-treated mice

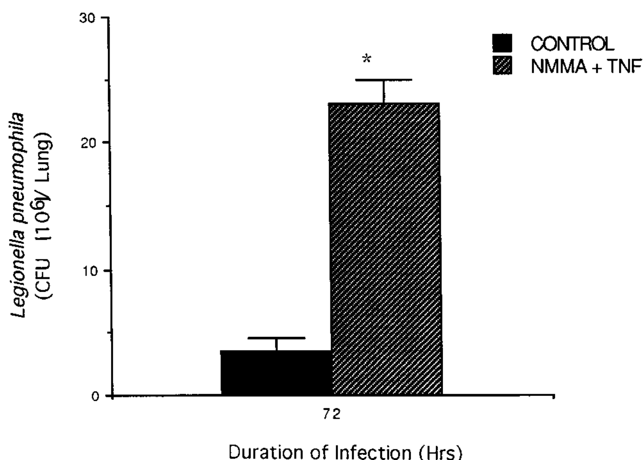

FIG. 4. Effect of exogenous TNF-α on intrapulmonary growth of *L. pneumophila* in NMMA-treated mice. A/J mice were administered murine TNF-α (50,000 U per mouse) prior to administration of NMMA as described in Materials and Methods. The mice were subsequently inoculated i.t. with *L. pneumophila*. At 72 h postinfection, the mice were sacrificed. The lungs were excised and homogenized, and growth of *L. pneumophila* in the lungs of mice treated with NMMA and TNF-α was compared with that in control mice (i.e., mice not administered NMMA or TNF-α). Results represent the means ± standard errors of the means for five mice per treatment group. *, significantly greater than for control mice; Mann-Whitney test, $P < 0.01$.

administered exogenous TNF-α was sevenfold greater than that in control mice (i.e., *L. pneumophila*-infected mice not administered NMMA [$P < 0.01$]). Furthermore, administration of TNF-α to NMMA-treated mice did not facilitate clearance of bacteria from the lung at 72 h postinfection, when compared with clearance of *L. pneumophila* from similarly infected mice treated with NMMA alone (Fig. 2). In a subsequent experiment, mice (four or five mice per group) were implanted i.p. with an Alzet minipump containing either NMMA or NMMA and recombinant murine TNF-α (10,000 U total), thereby resulting in continuous administration of exogenous TNF-α to NMMA-treated mice. The mice were subsequently inoculated i.t. with *L. pneumophila* as previously described. Seventy-two hours later, the mice were sacrificed and *L. pneumophila* was quantitated in whole-lung homogenates. In agreement with results of experiments described above, continuous administration of TNF-α to NMMA-treated, *L. pneumophila*-infected mice failed to facilitate clearance of the bacteria from the lung (when compared with NMMA-treated, *L. pneumophila*-infected mice [data not shown]). These results support the hypothesis that TNF-α-mediated inhibition of *L. pneumophila* growth in the lung is dependent, at least in part, on endogenous NO.

DISCUSSION

Intracellular pathogens, including *L. pneumophila*, are normally sequestered and protected from the extracellular environment, making them difficult to eradicate by humoral defense mechanisms and/or by drug therapy. Efficient elimination of *L. pneumophila* from the lung of the susceptible host is likely dependent both on early nonspecific defense mechanisms (including, but not limited to, PMN activation and enhanced bactericidal activity), resulting in decreased uptake and survival of *L. pneumophila* within permissive MPCs, and on the development of cell-mediated immunity and the production of cytokines, including IFN-γ, which facilitate MPC activation. MPC activation changes the intracellular milieu, from a permissive to a nonpermissive environment for *L. pneumophila* replication. Previous studies have identified cytokines, including TNF-α and IFN-γ, which regulate viability and growth of *L. pneumophila* in leukocytes in vitro (5, 6, 8, 10, 11, 29, 34, 37). These cytokines have also been shown to regulate clearance of *L. pneumophila* from the lung in animal models of nonreplicative *L. pneumophila* infections (7, 8, 39). However, the role of these endogenous mediators in regulating growth of *L. pneumophila* in the lung in vivo in the susceptible host (i.e., one that develops replicative *L. pneumophila* lung infections) has not been thoroughly investigated. In a previous study, we identified a pivotal role of endogenous IFN-γ in regulating growth of *L. pneumophila* in the lung in vivo (9). Our current investigation indicates that TNF-α, induced during replicative *L. pneumophila* lung infections, also modulates intrapulmonary replication of *L. pneumophila*. Furthermore, the kinetics of enhanced growth of *L. pneumophila* in the lungs of mice administered either TNF-α-neutralizing antibody or IFN-γ-neutralizing antibody are similar (i.e., both are enhanced at 5 days postinfection), suggesting that endogenous TNF-α and IFN-γ regulate *L. pneumophila* replication in the lung, at least in part, by a common mechanism. While the mechanism has not been definitively identified in vivo, results of previous in vitro studies indicate that stimulation of murine macrophages with cytokines, including IFN-γ and TNF-α, results in the induction of NOS activity and the production of RNIs, including NO, which are bactericidal.

NO has previously been identified as a key effector molecule

in resistance of cultured murine macrophages to several intracellular pathogens (16, 21, 22, 24–26, 33, 38). However, results of previous in vitro investigations regarding the potential effect of NO on growth of *L. pneumophila* in permissive murine macrophages have been conflicting (20, 45). Furthermore, effects of endogenous NO on intrapulmonary growth of *L. pneumophila* in the susceptible host in vivo have not been investigated. Our current study indicates that endogenous NO regulates replication of *L. pneumophila* in the lung. Similarly, our results suggest that NO has an immunostimulatory effect on TNF-α activity in the lung during replicative *L. pneumophila* infections in vivo. However, administration of exogenous TNF-α to NMMA-treated, *L. pneumophila*-infected mice fails to enhance pulmonary clearance of the bacteria. These results demonstrate that TNF-α-induced clearance of *L. pneumophila* from the lung is mediated, at least in part, by endogenous NO. Specifically, while administration of exogenous TNF-α to NMMA-treated mice likely facilitates induction of MPC-derived inducible NOS, NO is not produced, due to the continued presence of the competitive substrate inhibitor of inducible NOS (i.e., NMMA). In the absence of NO production, the macrophage remains permissive for *L. pneumophila* replication, resulting in recovery of *L. pneumophila* from the lungs of mice administered both NMMA and TNF-α.

In summary, we have investigated, through the use of cytokine-specific neutralizing antibodies and enzyme inhibitors, the role of endogenous TNF-α and RNIs in the pathogenesis of replicative *L. pneumophila* lung infections in vivo. Results of these experiments indicate that both endogenous TNF-α and NO modulate intrapulmonary growth of *L. pneumophila* in the susceptible host. Furthermore, while NO modulates the production of TNF-α in the lung during replicative *L. pneumophila* lung infections, TNF-α-induced clearance of the bacteria is mediated, at least in part, by endogenous NO activity. Furthermore, both TNF-α and NO inhibit growth of *L. pneumophila* in the lung during the initial stages (i.e., within 3 to 5 days) of infection, suggesting that these mediators likely play a pivotal role in the host's first line of defense (i.e., nonspecific immune response) to the bacteria, thereby limiting intrapulmonary replication prior to the development of *L. pneumophila*-specific cellular immunity.

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