

## Roles for Tumor Necrosis Factor Alpha and Nitric Oxide in Resistance of Rat Alveolar Macrophages to *Legionella pneumophila*

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*Legionella pneumophila* is an intracellular parasite of alveolar macrophages, and recovery from legionellosis is associated with activation of alveolar macrophages to resist intracellular bacterial replication. Gamma interferon (IFN- $\gamma$ ) is known to activate alveolar macrophages to suppress *L. pneumophila*, but the role of macrophage-derived cytokines in modulating alveolar macrophage resistance is unknown. To test the hypothesis that macrophage-derived mediators contribute to the resistance of alveolar macrophages to *L. pneumophila*, we incubated adherent rat alveolar macrophages with *Escherichia coli* lipopolysaccharide (LPS), recombinant tumor necrosis factor alpha (TNF- $\alpha$ ), recombinant IFN- $\gamma$ , neutralizing anti-TNF- $\alpha$ , and/or N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) for 6 h before challenge with *L. pneumophila*. Monolayers were sonically disrupted and quantitatively cultured on successive days. We also measured bioactive TNF- $\alpha$  release by infected macrophages in the presence or absence of IFN- $\gamma$ . We found that pretreatment of alveolar macrophages with LPS or, to a lesser degree, TNF- $\alpha$ , significantly inhibited intracellular replication of *L. pneumophila*. Both LPS and TNF- $\alpha$  acted synergistically with IFN- $\gamma$  at less than the maximally activating concentration to suppress *L. pneumophila* growth. The independent and coactivating effects of LPS were blocked by anti-TNF- $\alpha$ . Killing of *L. pneumophila* by IFN- $\gamma$  at the maximally activating concentration was inhibited by anti-TNF- $\alpha$ . The synergistic effects of TNF- $\alpha$  or LPS in combination with IFN- $\gamma$  were inhibited by L-NMMA. Infected alveolar macrophages secreted TNF- $\alpha$  in proportion to the bacterial inoculum, and secretion of TNF- $\alpha$  was potentiated by cocultivation with IFN- $\gamma$ . These data indicate that secretion of TNF- $\alpha$  is an important autocrine defense mechanism of alveolar macrophages, serving to potentiate the activating effects of IFN- $\gamma$  through costimulation of nitric oxide synthesis.

*Legionella pneumophila* is the etiologic agent of Legionnaires' disease and a facultative intracellular parasite of alveolar macrophages (43). Host resistance to legionellosis is associated with the development of cell-mediated immunity and the activation of alveolar macrophages to resist intracellular bacterial replication (30, 54, 57). Gamma interferon (IFN- $\gamma$ ) is the major macrophage-activating factor and has been shown to activate human and rodent alveolar macrophages to resist *L. pneumophila* in vitro (44, 55). Evidence has accumulated that macrophage-derived cytokines also play a role in regulating macrophage resistance to infection, but little is known about the effects of autocrine mediators on the antimicrobial defenses of alveolar macrophages. Tumor necrosis factor alpha (TNF- $\alpha$ ) has been shown to activate mononuclear phagocytes to inhibit intracellular parasites (6, 17) and to potentiate the antimicrobial action of IFN- $\gamma$  (13, 22, 24, 37). *L. pneumophila* induces secretion of TNF- $\alpha$  in vitro and in vivo (9), suggesting a potential role for this cytokine in host resistance to legionellosis. We postulated that induction of TNF- $\alpha$  and/or other monokines in infected alveolar macrophages would have an autocrine protective effect on macrophage resistance to intracellular bacterial replication. To test this hypothesis, we used a well-defined rat model with which we have studied factors

regulating the resistance of alveolar macrophages to *L. pneumophila* in vitro and in vivo (54–56).

### MATERIALS AND METHODS

**Bacteria.** *L. pneumophila* Philadelphia 1 (ATCC 33152) was obtained from the American Type Culture Collection, Rockville, Md., and processed as described previously (57). The virulence of this strain has been maintained by annual passage through rats. The organism has been stored at  $-70^{\circ}\text{C}$  as a lung homogenate in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) diluted 1:1 in heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah). Bacteria were prepared for individual experiments as described previously (55). An aliquot was thawed, diluted 1:4,000 into buffered charcoal-yeast extract broth (20), and incubated on a shaking platform for 48 h at  $35^{\circ}\text{C}$ . This suspension then was diluted 1:100 into buffered yeast extract broth and incubated with shaking for 16 to 18 h at  $35^{\circ}\text{C}$ . The bacteria were pelleted by centrifugation at  $1,400 \times g$ , washed twice in phosphate-buffered saline, resuspended in phosphate-buffered saline to an optical density of 0.20 to 0.25 at 540 nm (approximately  $2 \times 10^8$  CFU/ml), and then diluted in RPMI 1640 (RPMI) (Flow Laboratories, McLean, Va.) or Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) as needed. Bacterial suspensions were quantitatively cultured on buffered charcoal-yeast extract agar as described previously (57).

**Animals.** Male Sprague-Dawley rats weighing 180 to 200 g and certified to be free of common pathogens were purchased from Simonsen Laboratories (Gilroy, Calif.). The rats were housed at two or three per cage, allowed free access to rat chow and water, and acclimated to the animal facility for at least 4 days before study. The experiments were approved by the Animal Studies Subcommittee of the Seattle Veterans Affairs Medical Center.

**Alveolar macrophages.** Resident alveolar macrophages were harvested by bronchoalveolar lavage, as described previously (53, 55). Briefly, each rat was anesthetized with intraperitoneal pentobarbital and exsanguinated by cardiac puncture. The trachea was exposed and cannulated, the chest was opened, and the lungs were lavaged with four separate 10-ml volumes of 0.85% sodium chloride containing 0.6 mM EDTA and prewarmed to  $37^{\circ}\text{C}$ . The aliquots were combined, and the cells were pelleted by centrifugation at  $300 \times g$ . The cells were washed twice in cold Hanks' balanced salt solution without calcium or magnesium (GIBCO) and then suspended in RPMI containing 5% heat-inactivated

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fetal calf serum, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml (GIBCO). The cells were counted in a hemacytometer, their viability was measured by the exclusion of trypan blue, and the differential was determined by examination of cytocentrifuge specimens prepared with a modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGaw Park, Ill.). Bronchoalveolar lavage cells prepared in this manner were >95% macrophages and >95% viable.

**Reagents.** Recombinant mouse TNF- $\alpha$  (rmTNF- $\alpha$ ) was purchased from Genzyme (Cambridge, Mass.), aliquoted, and stored at  $-70^{\circ}\text{C}$ . The stated bioactivity of the rmTNF- $\alpha$  was  $4 \times 10^7$  U/mg, and this was confirmed in an L929 cytotoxicity assay, as described below. Polyclonal rabbit anti-mouse TNF- $\alpha$  ( $10^6$  neutralizing units per ml) was obtained from Genzyme as neat antiserum and stored at  $4^{\circ}\text{C}$ . Recombinant murine IFN- $\gamma$  (rmIFN- $\gamma$ ) was a generous gift of Genentech (South San Francisco, Calif.). The rmIFN- $\gamma$  was supplied at a concentration of 1 mg/ml with a stated activity of  $5 \times 10^6$  antiviral units per mg and an endotoxin level of  $<10$  pg/ml by the *Limulus* amoebocyte lysate assay. This material was aliquoted and stored at  $4^{\circ}\text{C}$ . *Escherichia coli* O26:B6 lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, Mo.), suspended at 5 mg/ml in 20 mM EDTA, and dispersed with pulses of ultrasonic oscillation by using a tapered microtip attached to a Branson Sonifier (Branson Ultrasonics, Danbury, Conn.) with the output set at 8. The LPS then was aliquoted and stored at  $-20^{\circ}\text{C}$ . Normal rabbit serum was harvested from a pathogen-free New Zealand White rabbit and stored at  $-70^{\circ}\text{C}$ . *N*<sup>G</sup>-monomethyl-L-arginine monoacetate (L-NMMA) and *N*<sup>G</sup>-monomethyl-D-arginine monoacetate (D-NMMA) were purchased from Calbiochem (La Jolla, Calif.). All of these reagents were diluted as needed in RPMI or DMEM for each experiment.

**Infection of alveolar macrophages.** Adherent alveolar macrophages were infected with *L. pneumophila* as previously described (54, 55). Bronchoalveolar cells from four to eight animals were pooled and diluted to  $10^6$  viable macrophages per ml in RPMI containing 5% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml, and then 0.5-ml portions of this suspension were added to individual wells of 24-well tissue culture plates (Costar, Cambridge, Mass.). After 2 h of incubation at  $37^{\circ}\text{C}$  in humidified air with 5%  $\text{CO}_2$ , the wells were washed six times with prewarmed Hanks' balanced salt solution to remove nonadherent cells and antibiotics. The monolayers and cell-free control wells then were covered with 0.5 ml of antibiotic-free RPMI containing 10% fresh pooled autologous rat serum. DMEM was used in place of RPMI for experiments involving inhibition of arginine-dependent nitric oxide production with NMMA, because of the lower arginine content of DMEM (0.4 mM in DMEM versus 1.15 mM in RPMI). Additional reagents were added in volumes of 10 to 12.5  $\mu$ l each, singly or in combination, to achieve the following concentrations: LPS, 10  $\mu$ g/ml; rmTNF- $\alpha$ , 1, 10, 100, 1,000, or 10,000 U/ml; rmIFN- $\gamma$ , 10 or 250 U/ml; anti-TNF- $\alpha$ , 10,000 neutralizing units per ml (1% antiserum); normal rabbit serum, 1%; L-NMMA, 2 mM; and D-NMMA, 2 mM. The monolayers were incubated for 6 or 20 h and then challenged with approximately  $10^5$  CFU of *L. pneumophila* in a volume of 20  $\mu$ l, for a bacterium/macrophage ratio of approximately 1:2. After 24, 48, and 72 h, triplicate or quadruplicate monolayers representing each condition were ultrasonically disrupted to release intracellular bacteria. A stepped microtip attached to a Branson Sonifier with the output level set at 1 and the duty cycle at 60% was dipped into each well for 10 s. The microtip was dipped in alcohol, flamed, and cooled in sterile water between the sampling of each well. Cell-free control wells containing medium with 10% rat serum were treated similarly and were used to confirm the inoculum at time zero. The suspensions were serially diluted in Mueller-Hinton broth and quantitatively cultured by spreading 0.1-ml aliquots with a bent glass rod onto plates of buffered charcoal-yeast extract agar. The number of colonies was counted after 3 to 4 days of incubation at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

**Measurement of nitrite production.** Alveolar macrophages were prepared as described above and incubated in DMEM with 10% rat serum (medium), medium containing TNF- $\alpha$  (10,000 U/ml) and IFN- $\gamma$  (10 U/ml), medium containing TNF- $\alpha$ , IFN- $\gamma$ , and L-NMMA (2 mM), or medium containing TNF- $\alpha$ , IFN- $\gamma$ , and D-NMMA (2 mM) starting 6 h before infection with *L. pneumophila*. Cell-free control wells contained medium and bacteria. After 24, 48, and 72 h, three wells representing each condition were treated with ultrasonic oscillation and quantitatively cultured as described above. The remaining conditioned medium in each well was stored at  $-70^{\circ}\text{C}$  for later measurement of nitrite concentration by a microplate assay based on the Griess reaction (18). Aliquots (100  $\mu$ l) of thawed conditioned medium or sodium nitrite standard (0.39 to 100  $\mu$ M in DMEM with 10% rat serum) were mixed with 100  $\mu$ l of Griess reagent (1% sulfanilamide and 0.1% naphthylene diamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min at room temperature. The  $A_{550}$  was determined in an MR 700 microplate reader (Dynatech, Alexandria, Va.). The concentrations of nitrite in the unknown samples were calculated from the standard curve. All reagents for this assay were purchased from Sigma.

**Stimulation and measurement of TNF- $\alpha$  release.** Alveolar macrophages were harvested from individual rats, and monolayers of adherent cells representing single animals were prepared as described above. After nonadherent cells and antibiotics were removed by repeated washing, the macrophages were incubated in RPMI with 10% pooled autologous rat serum with *L. pneumophila* ( $10^5$ ,  $10^6$ ,  $10^7$ , or  $10^8$  CFU/ml), rmIFN- $\gamma$  (250 U/ml), *L. pneumophila* plus rmIFN- $\gamma$ , *E. coli* LPS (10  $\mu$ g/ml), or medium alone. Supernatants were harvested after 6, 24, and

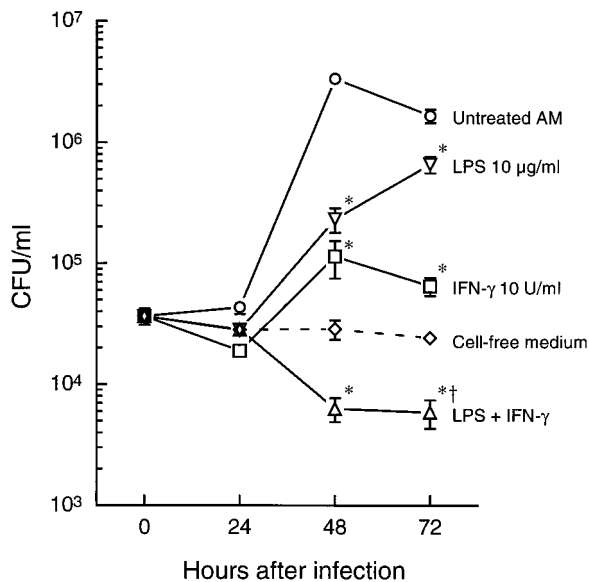


FIG. 1. Effects of LPS and rmIFN- $\gamma$  on the replication of *L. pneumophila* in alveolar macrophages (AM). Adherent AM were incubated in medium alone (untreated) or in medium containing LPS (10  $\mu$ g/ml), rmIFN- $\gamma$  (10 U/ml), or both LPS and rmIFN- $\gamma$  starting 6 h before challenge with *L. pneumophila*. Monolayers were sonically disrupted and quantitatively cultured on successive days. Data are means  $\pm$  SEM for triplicate wells and are representative of at least four experiments. \*,  $P < 0.05$  compared with result for untreated AM; †,  $P < 0.0001$  compared with result for IFN- $\gamma$  or LPS.

48 h of incubation, sterilized by centrifugation through 0.22- $\mu$ m-pore-size filters (Costar), and stored at  $-70^{\circ}\text{C}$ . TNF- $\alpha$  bioactivity in the supernatants was measured as cytotoxicity against L929 cells, as described previously (53). Briefly, L929 cells (American Type Culture Collection) were seeded onto 96-well plates and incubated overnight with serial dilutions of supernatant, with or without anti-mouse TNF- $\alpha$ , or a recombinant human TNF- $\alpha$  standard (Cetus, Emeryville, Calif.), all in the presence of 1  $\mu$ g of actinomycin D (Calbiochem) per ml. The monolayers were washed, and then the remaining target cells were stained with crystal violet and quantitated by absorbance spectroscopy at 550 nm with the Dynatech MR 700 microplate reader. One unit of TNF- $\alpha$  activity was defined as 50% lysis of the target monolayer. That the cytopathic effect of macrophage supernatants was caused by TNF- $\alpha$  was confirmed by its neutralization with anti-mouse TNF- $\alpha$ . The results of individual assays were corrected to those for the human recombinant standard. The endotoxin activities of the bacterial suspension and the *E. coli* LPS were measured in a quantitative *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.) according to the manufacturer's instructions.

**Data analysis.** The data are expressed as means  $\pm$  standard errors of the mean (SEM) of triplicate or quadruplicate monolayers. Statistical comparisons between multiple experimental conditions were made by one-way analysis of variance, with Scheffé's post hoc test to identify significant differences. A  $P$  value of  $<0.05$  was considered significant.

## RESULTS

**LPS stimulates rat alveolar macrophages to resist *L. pneumophila* and potentiates the action of IFN- $\gamma$ .** To study the role of autocrine mediators in the resistance of alveolar macrophages to intracellular infection, adherent cells were stimulated with the potent secretagogue LPS for 6 h before challenge with *L. pneumophila*. As shown in Fig. 1, untreated alveolar macrophages supported a 100-fold increase in bacterial CFU over 48 h in culture, whereas no bacterial replication occurred in culture medium alone. The exponential bacterial growth was significantly inhibited, or at least delayed, in alveolar macrophages pretreated with LPS. This effect was observed consistently in four separate experiments.

Stimulation of alveolar macrophages with LPS also potentiated the effect of low-dose rmIFN- $\gamma$ , resulting in synergistic inhibition of bacterial replication. In the experiment with the

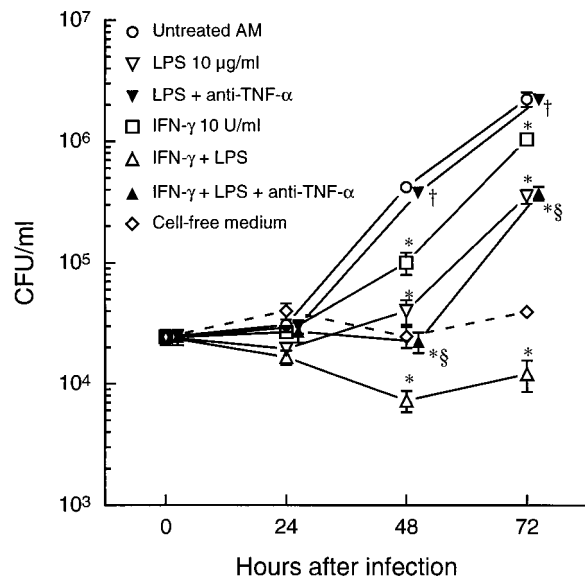


FIG. 2. Antibody to TNF- $\alpha$  blocks the effect of LPS on alveolar macrophage (AM) resistance to *L. pneumophila*. AMs were incubated in medium alone or in medium supplemented with LPS (10  $\mu$ g/ml), rmIFN- $\gamma$  (10 U/ml), or both LPS and rmIFN- $\gamma$  in the presence or absence of polyclonal neutralizing anti-TNF- $\alpha$  starting 6 h before challenge with *L. pneumophila*. Monolayers were disrupted and quantitatively cultured daily. Data are means  $\pm$  SEM for triplicate wells and are representative of four experiments. \*,  $P < 0.01$  compared with result for untreated AMs; †,  $P < 0.001$  compared with result for LPS; §,  $P < 0.05$  compared with result for IFN- $\gamma$  plus LPS.

results illustrated in Fig. 1, LPS-treated alveolar macrophages inhibited the replication of *L. pneumophila* by 1.2 log<sub>10</sub> CFU/ml (16-fold) over the first 48 h of infection, whereas bacterial growth was inhibited by 1.4 log<sub>10</sub> CFU/ml (40-fold) in alveolar macrophages preincubated with rmIFN- $\gamma$  at less than the maximally activating concentration (submaximal rmIFN- $\gamma$ ). Alveolar macrophages pretreated with both LPS and rmIFN- $\gamma$  inhibited the replication of *L. pneumophila* by 2.8 log<sub>10</sub> CFU/ml (631-fold); net bacterial killing was observed under these conditions. This synergistic effect of LPS in combination with rmIFN- $\gamma$  was observed consistently in nine separate experiments. However, the synergistic activity of LPS and rmIFN- $\gamma$  was evident only at low concentrations of rmIFN- $\gamma$ . In a single experiment in which *L. pneumophila* replicated in untreated alveolar macrophages by  $2.2 \pm 0.08$  log<sub>10</sub> CFU/ml over 72 h in culture, a net reduction of  $2.5 \pm 0.23$  log<sub>10</sub> CFU/ml from the initial inoculum was observed in alveolar macrophages pretreated with rmIFN- $\gamma$  250 U/ml, and a reduction of  $1.8 \pm 0.13$  log<sub>10</sub> CFU/ml was observed in alveolar macrophages preincubated with rmIFN- $\gamma$  and LPS. Thus, exposure of alveolar macrophages to gram-negative endotoxin induces increased resistance to intracellular infection and synergistically potentiates the activating effect of submaximal IFN- $\gamma$ .

**Anti-TNF- $\alpha$  blocks the antimicrobial effect of LPS and LPS plus rmIFN- $\gamma$ .** To determine whether the protective effect of LPS was caused by the induction of TNF- $\alpha$ , alveolar macrophages were incubated with LPS  $\pm$  IFN- $\gamma$  in the presence of polyclonal rabbit anti-TNF- $\alpha$  or nonimmune rabbit serum for 6 h before challenge with *L. pneumophila*. As shown Fig. 2, bacterial growth in LPS-stimulated alveolar macrophages cocultured with anti-TNF- $\alpha$  was indistinguishable from that in untreated macrophages and was significantly greater than the bacterial replication in macrophages treated with LPS alone. Similarly, the pronounced inhibition of *L. pneumophila* replication in alveolar macrophages synergistically activated by LPS

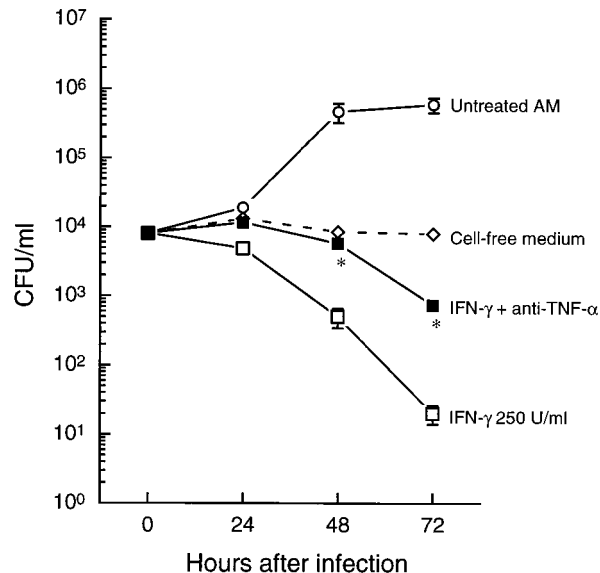


FIG. 3. Antibody to TNF- $\alpha$  inhibits killing of *L. pneumophila* by IFN- $\gamma$ -activated alveolar macrophages (AM). Adherent AM were incubated overnight in medium alone, medium supplemented with rmIFN- $\gamma$  (250 U/ml), or medium containing rmIFN- $\gamma$  and anti-TNF- $\alpha$ . Monolayers then were challenged with *L. pneumophila* and quantitatively cultured each day. Data are means  $\pm$  SEM for triplicate wells and are representative of two experiments. \*,  $P < 0.005$  compared with result for IFN- $\gamma$ .

in combination with submaximal IFN- $\gamma$  was substantially reversed by anti-TNF- $\alpha$ . Nonimmune rabbit serum did not influence the independent or coactivating effects of LPS (not shown). These observations were confirmed in four separate experiments. Thus, anti-TNF- $\alpha$  completely reverses the inhibition of *L. pneumophila* replication in LPS-treated alveolar macrophages and markedly reduces the synergistic effect of LPS in combination with IFN- $\gamma$ . These data suggest that the protective effect of LPS is mediated largely by the secretion of TNF- $\alpha$ .

**Anti-TNF- $\alpha$  partially reverses the killing of *L. pneumophila* by alveolar macrophages activated by rmIFN- $\gamma$ .** We have previously reported that rat alveolar macrophages incubated with  $\geq 100$  U of IFN- $\gamma$  per ml kill *L. pneumophila* (55). To determine whether secretion of TNF- $\alpha$  contributes to the antimicrobial activity induced by rmIFN- $\gamma$ , alveolar macrophages were incubated with a maximally activating concentration of rmIFN- $\gamma$  (250 U/ml) in the presence or absence of anti-TNF- $\alpha$  for 20 h before challenge with *L. pneumophila*. As shown in Fig. 3, anti-TNF- $\alpha$  partially inhibited the net reduction in bacterial CFU per milliliter by rmIFN- $\gamma$ -activated alveolar macrophages. Anti-TNF- $\alpha$  had no effect on the replication of *L. pneumophila* in unstimulated alveolar macrophages in three experiments (data not shown). Thus, the activation of rat alveolar macrophages by rmIFN- $\gamma$  to kill *L. pneumophila* is partially mediated by secretion of TNF- $\alpha$ .

**Recombinant TNF- $\alpha$  inhibits the replication of *L. pneumophila* in rat alveolar macrophages and potentiates the action of rmIFN- $\gamma$ .** In order to test directly the potential role of TNF- $\alpha$  in cellular resistance to infection, alveolar macrophages were incubated with rmTNF- $\alpha$  with or without rmIFN- $\gamma$  for 6 h before challenge with *L. pneumophila*. As shown in Fig. 4, rmTNF- $\alpha$  stimulated alveolar macrophages to inhibit intracellular replication of *L. pneumophila* in a dose-related manner. This effect was small and was evident only at concentrations of rmTNF- $\alpha$  of  $\geq 1,000$  U/ml, but it was reproducible in six sep-

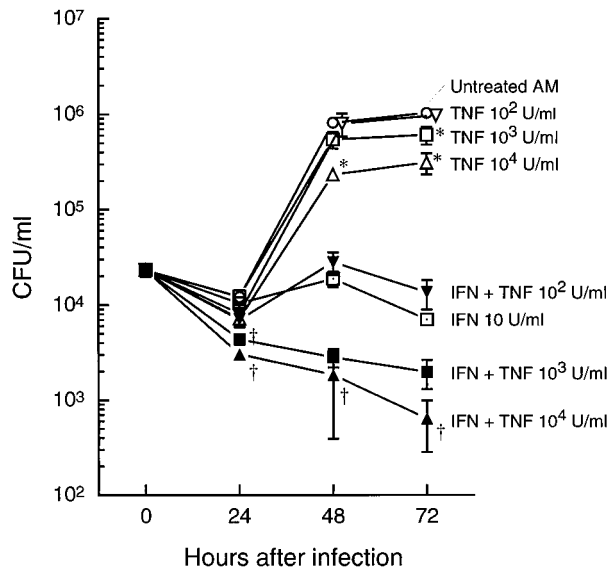


FIG. 4. TNF- $\alpha$  inhibits replication of *L. pneumophila* in alveolar macrophages (AM) and potentiates the action of rmIFN- $\gamma$ . AM were incubated with rmTNF- $\alpha$  (0 to 10,000 U/ml) or with submaximal rmIFN- $\gamma$  (10 U/ml) and rmTNF- $\alpha$  (0 to 10,000 U/ml) starting 6 h before challenge with *L. pneumophila*. Monolayers were sonically disrupted and quantitatively cultured each day. Data are means  $\pm$  SEM for triplicate wells and are representative of at least six separate experiments. \*,  $P < 0.05$  compared with result for untreated AM; †,  $P < 0.05$  compared with result for IFN- $\gamma$  alone.

arate experiments. These concentrations of rmTNF- $\alpha$  also potentiated the activation of alveolar macrophages by submaximal rmIFN- $\gamma$  (Fig. 4). As with LPS, the combination of rmTNF- $\alpha$  and rmIFN- $\gamma$  was synergistic: a net reduction in bacterial CFU was observed when alveolar macrophages were costimulated with rmIFN- $\gamma$  and 1,000 or 10,000 U of rmTNF- $\alpha$  per ml. This coactivating effect of TNF- $\alpha$  was documented in seven separate experiments. The rmTNF- $\alpha$  with or without rmIFN- $\gamma$  had no direct effect on the viability of *L. pneumophila* in cell-free medium. The rmTNF- $\alpha$  also did not affect the viability of uninfected alveolar macrophages, judging by the exclusion of trypan blue after 3 days of incubation. The effect of rmTNF- $\alpha$  may be time dependent, as alveolar macrophages preincubated with rmTNF- $\alpha$  (1, 10, 100, or 1,000 U/ml) for 20 h before infection did not exhibit enhanced resistance to *L. pneumophila* (one experiment) (data not shown), and macrophages costimulated with rmTNF- $\alpha$  (100 or 1,000 U/ml) and rmIFN- $\gamma$  (10 U/ml) for 20 h before infection did not demonstrate greater resistance than cells treated with IFN- $\gamma$  alone (one experiment) (data not shown). Thus, rmTNF- $\alpha$  transiently stimulates the resistance of rat alveolar macrophages to *L. pneumophila* and synergistically augments the activating effect of rmIFN- $\gamma$ .

**L-NMMA inhibits the antimicrobial activity of alveolar macrophages stimulated with LPS plus rmIFN- $\gamma$ .** To determine the role of arginine-dependent nitric oxide production in the resistance of activated alveolar macrophages to *L. pneumophila*, we studied the effect of L-NMMA, a competitive inhibitor of nitric oxide synthase, on bacterial replication in alveolar macrophages stimulated with LPS plus rmIFN- $\gamma$  (10 U/ml). As shown in Fig. 5, cocubation with L-NMMA almost completely reversed the marked antibacterial activity of alveolar macrophages pretreated with LPS in combination with submaximal rmIFN- $\gamma$ . This effect of L-NMMA was observed in seven independent experiments. Cocubation with L-NMMA, but not D-NMMA, completely reversed the antibacterial re-

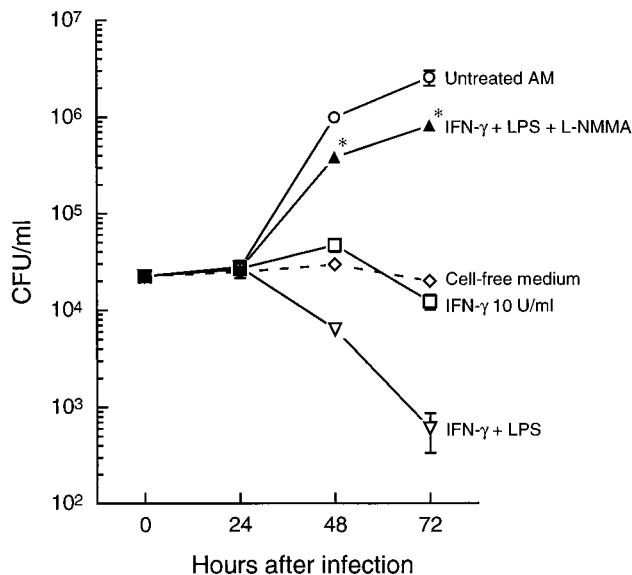


FIG. 5. L-NMMA inhibits LPS-rmIFN- $\gamma$ -induced resistance of alveolar macrophages (AM) to *L. pneumophila*. AM were incubated in medium alone, medium containing rmIFN- $\gamma$  (10 U/ml), medium containing rmIFN- $\gamma$  plus LPS (10  $\mu$ g/ml), or medium containing rmIFN- $\gamma$ , LPS, and L-NMMA (2 mM) starting 6 h before infection. Monolayers were sonically disrupted and quantitatively cultured daily. Data are means  $\pm$  SEM for triplicate wells and are representative of seven experiments. \*,  $P < 0.001$  compared with result for IFN- $\gamma$  plus LPS.

sistance of alveolar macrophages pretreated with LPS alone in the single experiment in which this was tested (not shown). L-NMMA alone did not influence the replication of *L. pneumophila* in unstimulated alveolar macrophages (not shown). Thus, cocubation with L-NMMA largely reverses the synergistic action of LPS in combination with rmIFN- $\gamma$  on the resistance of alveolar macrophages to *L. pneumophila*. The effect of L-NMMA is similar to that of anti-TNF- $\alpha$  and suggests that arginine-dependent nitric oxide production plays a major role in the antimicrobial resistance of rat alveolar macrophages stimulated with LPS in combination with IFN- $\gamma$ .

**L-NMMA inhibits the activation of alveolar macrophages by rmTNF- $\alpha$  and rmIFN- $\gamma$ .** To determine whether induction of nitric oxide also contributes to the antibacterial defenses of alveolar macrophages activated by rmTNF- $\alpha$  in combination with rmIFN- $\gamma$ , alveolar macrophages were stimulated with these cytokines in the presence of the L or D enantiomer of NMMA. Figure 6 shows that the marked resistance to *L. pneumophila* of alveolar macrophages stimulated with rmTNF- $\alpha$  and rmIFN- $\gamma$  was lost in the presence of L-NMMA but not D-NMMA. This effect was documented in five separate experiments. The small effect of rmTNF- $\alpha$  on the replication of *L. pneumophila* in alveolar macrophages was not influenced by cocultivation with L-NMMA in three experiments (data not shown). These data suggest that the combination of rmTNF- $\alpha$  and submaximal rmIFN- $\gamma$  induces rat alveolar macrophages to resist *L. pneumophila* by a pathway partially dependent on L-arginine.

**L-NMMA, but not D-NMMA, blocks nitrite production by alveolar macrophages stimulated with rmTNF- $\alpha$  and rmIFN- $\gamma$ .** To confirm that L-NMMA inhibited the production of reactive nitrogen intermediates by alveolar macrophages in our culture system, the conditioned medium from the experiment with the results shown in Fig. 6 was assayed for nitrite by the Griess reaction. As shown in Table 1, L-NMMA completely blocked nitrite production by infected alveolar macro-

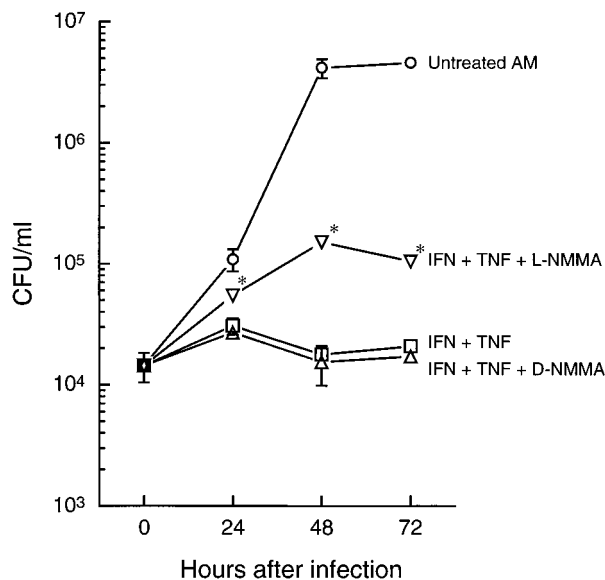


FIG. 6. L-NMMA inhibits rmTNF- $\alpha$ -rmIFN- $\gamma$ -induced resistance of alveolar macrophages (AM) to *L. pneumophila*. AM were incubated in medium alone, medium containing rmIFN- $\gamma$  (10 U/ml) plus TNF- $\alpha$  (10,000 U/ml), medium containing rmIFN- $\gamma$ , rmTNF- $\alpha$ , and L-NMMA (2 mM), or medium containing rmIFN- $\gamma$ , rmTNF- $\alpha$ , and D-NMMA (2 mM) starting 6 h before challenge with *L. pneumophila*. Monolayers were sonically disrupted and cultured daily. Data are means  $\pm$  SEM for triplicate wells and are representative of five experiments. \*,  $P < 0.05$  compared with result for each other condition.

phages stimulated with rmTNF- $\alpha$  and rmIFN- $\gamma$ , whereas D-NMMA had no effect. Nitrite release by unstimulated alveolar macrophages was not detected.

***L. pneumophila* induces TNF- $\alpha$  secretion by alveolar macrophages, and TNF- $\alpha$  release by infected cells is augmented by rmIFN- $\gamma$ .** To determine if alveolar macrophages release TNF- $\alpha$  when challenged with *L. pneumophila* and whether this response is influenced by IFN- $\gamma$ , alveolar macrophages from individual rats were incubated overnight with live *L. pneumophila* in the presence or absence of rmIFN- $\gamma$ , and supernatants were assayed for TNF- $\alpha$  bioactivity. As shown in Table 2, TNF- $\alpha$  was detected in the supernatants of alveolar macrophages stimulated with *L. pneumophila* at bacterial concentrations of  $\geq 10^6$  CFU/ml (approximately five bacilli per macrophage), and TNF- $\alpha$  release was augmented in the presence of IFN- $\gamma$ . Lower inocula of bacteria did not induce measurable TNF- $\alpha$  release (not shown). The *Limulus* amoebocyte lysate test was used to compare the endotoxin activity of the bacterial suspensions with that of *E. coli* LPS. A suspension of live *L. pneumophila* at  $10^8$  CFU/ml was found to have endotoxin activity equivalent to that of 1.4 mg of *E. coli* LPS per ml. Thus, *L. pneumophila* induces secretion of TNF- $\alpha$  by alveolar mac-

TABLE 2. TNF- $\alpha$  release by normal and IFN- $\gamma$ -activated rat alveolar macrophages

Stimulus	TNF- $\alpha$ release (U/ml) <sup>a</sup> with:	
	No IFN- $\gamma$	rmIFN- $\gamma$ (250 U/ml)
None	61.7 $\pm$ 14.4	144.0 $\pm$ 60.6
LPS (10 $\mu$ g/ml)	508.0 $\pm$ 9.0	ND
<i>L. pneumophila</i> ( $10^6$ CFU/ml)	92.7 $\pm$ 16.2	128.0 $\pm$ 25.0
<i>L. pneumophila</i> ( $10^7$ CFU/ml)	167.7 $\pm$ 39.2	339.8 $\pm$ 41.1*
<i>L. pneumophila</i> ( $10^8$ CFU/ml)	1,634.5 $\pm$ 214.9	2,765.5 $\pm$ 652.0

<sup>a</sup> Data are means  $\pm$  SEM;  $n =$  four rats. \*,  $P < 0.05$  compared with result with no IFN- $\gamma$ . ND, not determined.

rophages, and costimulation with IFN- $\gamma$  augments this response. The concentrations of *L. pneumophila* required to induce TNF- $\alpha$  release contain levels of endotoxin activity exceeding 10  $\mu$ g/ml.

## DISCUSSION

We have shown that preincubation of rat alveolar macrophages with *E. coli* endotoxin enhances macrophage resistance to intracellular replication of *L. pneumophila* and potentiates the activating effect of IFN- $\gamma$ . The stimulatory and costimulatory activities of LPS are blocked by coincubation with anti-TNF- $\alpha$  or with L-NMMA, an inhibitor of nitric oxide synthase. Recombinant TNF- $\alpha$  has a small direct stimulatory effect on alveolar macrophage resistance to *L. pneumophila* and potentiates the action of IFN- $\gamma$ . The costimulatory effect of TNF- $\alpha$  is inhibited by L-NMMA. Bioactive TNF- $\alpha$  is released by alveolar macrophages challenged with *L. pneumophila*, and this response is stimulated by coincubation with IFN- $\gamma$ . Collectively, these data suggest that TNF- $\alpha$  is an important autocrine mediator of rat alveolar macrophage resistance to *L. pneumophila* that acts, at least in part, by potentiating nitric oxide release.

Alveolar macrophages are the first line of defense against pathogens that reach the distal airspaces of the lung, and augmentation of the antimicrobial resistance of alveolar macrophages by exposure to endotoxin may be important in the early host response to gram-negative bacteria. Our finding that pretreatment with LPS stimulates the resistance of alveolar macrophages to *L. pneumophila* extends observations made with other mononuclear phagocytes to the target cell of an airborne intracellular pathogen. Peritoneal exudate macrophages harvested from A/J mice, which are susceptible to legionellosis, significantly restrict the intracellular replication of *L. pneumophila* when prestimulated with LPS in vitro (3, 21, 26) or in vivo (4). This effect is observed with LPS prepared from a variety of gram-negative bacteria, including *L. pneumophila*, and is associated with the lipid A fraction (3, 21). En-

TABLE 1. Nitrite release by rat alveolar macrophages infected with *L. pneumophila*

Condition(s)	Nitrite release ( $\mu$ M) <sup>a</sup> at:		
	24 h	48 h	72 h
Untreated alveolar macrophages	2.57 $\pm$ 0.10	3.58 $\pm$ 0.18	4.02 $\pm$ 0.01
TNF- $\alpha$ (10,000 U/ml) + IFN- $\gamma$ (10 U/ml)	10.55 $\pm$ 0.12*	20.74 $\pm$ 1.09*	16.96 $\pm$ 0.84*
TNF- $\alpha$ + IFN- $\gamma$ + L-NMMA	2.54 $\pm$ 0.16	2.82 $\pm$ 0.19	3.17 $\pm$ 0.27
TNF- $\alpha$ + IFN- $\gamma$ + D-NMMA	11.51 $\pm$ 0.05*	17.22 $\pm$ 0.38*	17.14 $\pm$ 0.51*
Cell-free medium	2.27 $\pm$ 0.04	2.82 $\pm$ 0.34	2.87 $\pm$ 0.10

<sup>a</sup> Data are means  $\pm$  SEM;  $n =$  three monolayers (supernatants from the experiment with the results shown in Fig. 6). \*,  $P < 0.0001$  compared with results for untreated alveolar macrophages, TNF- $\alpha$  plus IFN- $\gamma$  plus L-NMMA, and cell-free medium.

dotoxin similarly stimulates the resistance of a murine macrophage-like cell line to *L. pneumophila* (34). Indeed, the genetic susceptibility of peritoneal exudate macrophages to parasitism by *L. pneumophila*, which parallels susceptibility to replicative infection in vivo (63), correlates with responsiveness to LPS (64). *L. pneumophila* replicates in peritoneal exudate macrophages harvested from C3H/HeJ mice, which are poorly responsive to LPS, but not in macrophages of C3H/HeN mice (64). Our data extend these observations to the actual target cell of *L. pneumophila*, the alveolar macrophage.

In addition to a direct effect of endotoxin on the resistance of alveolar macrophages to infection, we found that LPS potentiated the effect of submaximal concentrations of IFN- $\gamma$ . The combination of LPS with IFN- $\gamma$  induced marked suppression of the intracellular growth of *L. pneumophila*. Such a coactivating effect of LPS with IFN- $\gamma$  has been described with other models of intracellular infection (2, 14, 35, 49, 51) but has not been reported previously with respect to macrophage resistance to *L. pneumophila*. Our observations also provide the first direct evidence that LPS stimulates the resistance of alveolar macrophages to an intracellular pathogen. Endotoxin has been reported to stimulate the release of monokines and reactive oxygen intermediates by alveolar macrophages (8, 39), but the role of LPS in modulating the antimicrobial activities of alveolar macrophages has not been studied. The effects of endotoxin on the antimicrobial activities of alveolar macrophages may have contributed to our earlier finding that alveolar exudate macrophages harvested after inhalation of live *E. coli* were resistant to *L. pneumophila* in vitro (54). The independent effects of endotoxin on the antibacterial activity of alveolar macrophages may be important in the early containment of infection, but the costimulatory actions of LPS are more dramatic and may contribute to the conversion of alveolar macrophages from target cells to effector cells in the resolution of legionellosis.

We found strong evidence that TNF- $\alpha$  is an important mediator of alveolar macrophage resistance to *L. pneumophila*. First, the independent and costimulatory effects of LPS on alveolar macrophage resistance to *L. pneumophila* appeared to be mediated by secretion of TNF- $\alpha$ . Coincubation with anti-TNF- $\alpha$  completely reversed the protective effect of LPS alone and substantially reversed the effect of LPS in combination with IFN- $\gamma$ . Second, neutralization of TNF- $\alpha$  partially inhibited the killing of *L. pneumophila* by alveolar macrophages stimulated by a maximum concentration of IFN- $\gamma$ . Third, recombinant TNF- $\alpha$  exerted a small, dose-related inhibitory effect on the intracellular replication of *L. pneumophila* and acted synergistically with IFN- $\gamma$ . Finally, bioactive TNF- $\alpha$  was released by alveolar macrophages challenged with *L. pneumophila*, a response potentiated by IFN- $\gamma$ . These observations complement other studies in the literature suggesting that TNF- $\alpha$  plays a role in the resistance of mononuclear phagocytes to *L. pneumophila*. Matsiota-Bernard and colleagues reported that pretreatment of human monocytes with recombinant TNF- $\alpha$  inhibited intracellular replication of *L. pneumophila* (40). Recombinant TNF- $\alpha$  did not potentiate the activating effect of IFN- $\gamma$  in this system, but the concentration of IFN- $\gamma$  that was used may have provided a maximal stimulus. These authors also found that inhibition of endogenous TNF- $\alpha$  induction with pentoxifylline stimulated bacterial multiplication in monocytes and partially reversed the protective effect of IFN- $\gamma$  (40). As we observed with alveolar macrophages, TNF- $\alpha$  release by *L. pneumophila*-infected monocytes is augmented by pretreatment with IFN- $\gamma$  (10, 40). The role of TNF- $\alpha$  in the resistance of murine peritoneal exudate macrophages to *L. pneumophila* is less clear. LPS-induced resistance

of murine peritoneal exudate macrophages is associated with the release of TNF- $\alpha$  (3, 4), and the sensitivity of C3H/HeJ macrophages to *L. pneumophila* is associated with a poor TNF- $\alpha$  response to LPS (7, 64). On the other hand, *L. pneumophila* induces more TNF- $\alpha$  release from peritoneal exudate macrophages of A/J mice, which are susceptible to *L. pneumophila*, than from macrophages harvested from mouse strains that are more resistant to parasitism (5, 63), and exposure of peritoneal macrophages to recombinant TNF- $\alpha$  in vivo does not induce resistance to *L. pneumophila* in vitro (4). However, the contribution of TNF- $\alpha$  to the resistance of activated peritoneal macrophages to *L. pneumophila* has not been tested directly. Recombinant TNF- $\alpha$  also has been reported to stimulate slow killing of *L. pneumophila* by murine peritoneal exudate neutrophils and human neutrophils (10, 11). Thus, induction of TNF- $\alpha$  in infected mononuclear phagocytes may provide an autocrine stimulus to antibacterial resistance and a paracrine stimulus to the antibacterial activity of recruited neutrophils.

There is accumulating evidence from animal models that TNF- $\alpha$  contributes to the host defense against *L. pneumophila* in vivo. TNF- $\alpha$  is detectable in bronchoalveolar lavage fluid of rodents after pulmonary challenge with *L. pneumophila* (9, 52). That alveolar macrophages are the principal source of this TNF- $\alpha$  is suggested by the observations that TNF- $\alpha$  levels in lavage fluid are diminished if the mice are depleted of macrophages by intraperitoneal administration of dextran sulfate (9) and that bioactive TNF- $\alpha$  is released in vitro by adherent cells in minced lung tissue harvested from infected animals (9). We and others have found that antibody-mediated depletion of endogenous TNF- $\alpha$  impairs pulmonary clearance of *L. pneumophila* (12, 52), and pretreatment with recombinant TNF- $\alpha$  protects mice from lethal intratracheal challenge with *L. pneumophila* (10). Although TNF- $\alpha$  may augment host defenses in several ways (62), our findings suggest that the stimulation of antibacterial mechanisms of alveolar macrophages is one mechanism by which TNF- $\alpha$  promotes resistance to intracellular pathogens of the lung.

TNF- $\alpha$  has been shown to stimulate the antimicrobial activity of mononuclear phagocytes in a variety of in vitro models of infection, but the role of TNF- $\alpha$  in modulating the resistance of alveolar macrophages has received little attention. Recombinant TNF- $\alpha$  alone stimulates human and murine nonpulmonary macrophages to suppress the intracellular replication of protozoa and mycobacteria in some systems (6, 14, 16, 17, 29, 36, 42, 61) but not others (17, 22, 24, 35). Recombinant TNF- $\alpha$  has been found more consistently to act synergistically with IFN- $\gamma$  to augment the antimicrobial resistance of mononuclear phagocytes (13–15, 22, 24, 37, 47, 51), although some investigators have not found this effect (35, 60). Similarly, the activation of macrophages by IFN- $\gamma$  to suppress some intracellular pathogens has been found to be dependent on endogenous secretion of TNF- $\alpha$  (6, 23, 27, 47). The few studies addressing the role of TNF- $\alpha$  in the antimicrobial resistance of alveolar macrophages have been inconclusive. Meylan and colleagues reported that preincubation of human alveolar macrophages with recombinant TNF- $\alpha$  did not influence the intracellular replication of *Mycobacterium tuberculosis* H37Rv (41), but Hirsch et al. found that inhibition of endogenous TNF- $\alpha$  production with neutralizing antisera or pentoxifylline stimulated the replication of *M. tuberculosis* H37Ra in human monocytes and alveolar macrophages (28). Suzuki and coworkers reported that stimulation of human alveolar macrophages with recombinant TNF- $\alpha$  restricted the intracellular replication of *Mycobacterium avium* complex (59), whereas Rose et al. found that neutralizing endogenous TNF- $\alpha$  did not influence the growth

inhibition of *M. avium* complex in alveolar macrophages stimulated with IFN- $\gamma$  in combination with granulocyte-macrophage colony-stimulating factor (50). Polsinelli and colleagues recently found that cocubation with anti-TNF- $\alpha$  did not affect killing of *Francisella tularensis* by IFN- $\gamma$ -activated mouse alveolar macrophages (48). Our data supporting a role for TNF- $\alpha$  in the resistance of rat alveolar macrophages to *L. pneumophila* suggest that the importance of TNF- $\alpha$  in macrophage resistance to infection depends on the target cell and infecting organism.

We found that inhibition of nitric oxide production significantly reduced the growth restriction of *L. pneumophila* in alveolar macrophages stimulated with LPS or TNF- $\alpha$  in combination with IFN- $\gamma$ . This is consistent with the known regulation of calcium-independent nitric oxide synthase in murine macrophages, which is induced by LPS or IFN- $\gamma$  alone and synergistically by IFN- $\gamma$  in combination with LPS or TNF- $\alpha$  (33, 45, 46). A potential role for nitric oxide in the host defense against *L. pneumophila* was first suggested by Summersgill et al., who reported that *L. pneumophila* was sensitive to nitric oxide in a cell-free system and that blockage of nitric oxide production with L-NMMA inhibited IFN- $\gamma$ -induced killing of *L. pneumophila* in murine macrophage-like RAW 264.7 cells (58). In contrast, Gebran et al. reported that inhibition of nitric oxide production did not affect the suppression of *L. pneumophila* replication in mouse peritoneal exudate macrophages activated with IFN- $\gamma$  or LPS (25, 26). Similarly, Kura and coworkers found that L-NMMA did not reverse LPS- or IFN- $\gamma$ -induced resistance of two mouse macrophage-like cell lines to *L. pneumophila* (34). Apart from differences in experimental conditions, these apparently discrepant results suggest that the role of nitric oxide in macrophage resistance to *L. pneumophila* depends on the target cell tested. Indeed, work with other models of intracellular infection has demonstrated that the contribution of nitric oxide to the antimicrobial activity of macrophages depends on both the target cell and the infecting pathogen. Polsinelli et al. found that L-NMMA blocked killing of *Leishmania major* by both peritoneal and alveolar macrophages stimulated by IFN- $\gamma$  but suppressed killing of *F. tularensis* only in activated peritoneal macrophages (48). Evidence supporting a role for nitric oxide in the host defense against *L. pneumophila* in vivo can be found in a recent report by Brieland and colleagues (12), who found that L-NMMA impaired clearance of *L. pneumophila* from the lungs of mice.

Although nitric oxide is an important and broad-spectrum antimicrobial product of activated murine macrophages, its function in human phagocytes is much less clear (1, 19). Inducible, high-output nitric oxide production has been difficult to demonstrate in human macrophages and appears to be regulated differently from the way it is in murine cells (1, 19). Summersgill and colleagues reported that L-NMMA had no effect on the suppression of *L. pneumophila* growth by IFN- $\gamma$ -activated HL-60 cells, a promyelocytic leukemia cell line that assumes macrophage-like characteristics when differentiated with phorbol myristate acetate (58). Whether nitric oxide has a role in the interaction of human alveolar macrophages with *L. pneumophila* is unknown.

Our data make it clear that both TNF- $\alpha$  and nitric oxide are involved in the resistance of activated rat alveolar macrophages to *L. pneumophila*, but they do not establish that these factors are necessarily interdependent, nor do our results exclude the contribution of other antimicrobial pathways. For example, both LPS and TNF- $\alpha$  augment the respiratory burst (8, 18). *L. pneumophila* is sensitive to reactive oxygen intermediates (38), and inhibition of the respiratory burst diminishes

macrophage resistance to *L. pneumophila* in some systems (31, 34) but not others (26, 32, 34).

In summary, the release of TNF- $\alpha$  by infected alveolar macrophages may be an important autocrine defense mechanism against *L. pneumophila*, serving to partially protect alveolar macrophages from intracellular parasitism in the early phase of infection and to potentiate the action of IFN- $\gamma$ , primarily by promoting the induction of nitric oxide. Determination of the importance of TNF- $\alpha$  in the resistance of human alveolar macrophages to *L. pneumophila* will require further investigation.

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