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In Vivo Regulation of Replicative *Legionella pneumophila* Lung Infection by Endogenous Interleukin-12

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The in vivo role of endogenous interleukin 12 (IL-12) in modulating intrapulmonary growth of *Legionella pneumophila* was assessed by using a murine model of replicative *L. pneumophila* lung infection. Intratracheal inoculation of A/J mice with virulent bacteria (10^6 *L. pneumophila* cells per mouse) resulted in induction of IL-12, which preceded clearance of the bacteria from the lung. Inhibition of endogenous IL-12 activity, via administration of IL-12 neutralizing antiserum, resulted in enhanced intrapulmonary growth of the bacteria within 5 days postinfection (compared to untreated *L. pneumophila*-infected mice). Because IL-12 has previously been shown to modulate the expression of cytokines, including gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-10, which regulate *L. pneumophila* growth, immunomodulatory effects of endogenous IL-12 on intrapulmonary levels of these cytokines during replicative *L. pneumophila* lung infection were subsequently assessed. Results of these experiments demonstrated that TNF-α activity was significantly lower, while protein levels of IFN-γ and IL-10 in the lung were similar, in *L. pneumophila*-infected mice administered IL-12 antiserum, compared to similarly infected untreated mice. Together, these results demonstrate that IL-12 is critical for resolution of replicative *L. pneumophila* lung infection and suggest that regulation of intrapulmonary growth of *L. pneumophila* by endogenous IL-12 is mediated, at least in part, by TNF-α.

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular pathogen of mononuclear phagocytic cells (MPCs) (37, 43, 45). Pulmonary infection usually develops following inhalation of *L. pneumophila*-contaminated water aerosols or microaspiration of contaminated water sources (9). Following inhalation, the bacteria invade and replicate in host MPCs, primarily in alveolar MPCs (34, 36, 37, 43, 45). Intracellular growth of *L. pneumophila* results in eventual lysis of infected MPCs, the release of bacterial progeny, and reinfection of additional pulmonary cells (34, 36). Severe lung damage, mediated by tissue-destructive substances likely derived from both damaged host cells and the bacteria, ensues (20, 21).

Previous studies have demonstrated that resistance to primary replicative *L. pneumophila* lung infection is dependent on the induction of cellular immunity and is mediated in part by cytokines including gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (8, 12, 14, 15, 23, 27, 28, 35, 57). Growth of *L. pneumophila* within permissive MPCs requires iron. IFN-γ limits MPC iron, thereby converting the MPC intracellular environment from one that is permissive to one that is nonpermissive for *L. pneumophila* replication (14, 15). IFN-γ in combination with other cytokines including TNF-α facilitates elimination of *L. pneumophila* from infected MPCs, likely through the induction of effector molecules including nitric oxide (12). In contrast, other cytokines including interleukin 10 (IL-10) facilitate growth of *L. pneumophila* in permissive MPCs, due in part to IL-10-mediated inhibition of TNF-α secretion and IFN-γ-mediated MPC activation (46).

IL-12 is a recently described cytokine with pleiotropic effects on T cells and natural killer (NK) cells which include (i) regulation of expression of cytokines including IFN-γ, TNF-α, and IL-10 by T cells and/or NK cells, (ii) induction of T-cell and/or NK cell proliferation and/or differentiation, and (iii) enhancement of NK cell and T-cell cytotoxic activity (4, 5, 19, 32, 33, 39, 44, 47, 48, 50, 56). While systemic administration of exogenous IL-12 has been demonstrated to increase host resistance to several intracellular pathogens, including *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Plasmodium chabaudi*, in mice (26, 29, 33, 40, 51, 52, 55), the role of endogenous IL-12 in innate immunity to intracellular pathogens including *L. pneumophila* has not been thoroughly investigated. We have recently developed a model of replicative *L. pneumophila* lung infection in A/J mice inoculated intratracheally with virulent bacteria and have used this model system to identify immune responses which mediate host resistance to legionellosis (10–12). Using this murine model of Legionnaires' disease, we assessed the biologic relevance and immunomodulatory role of endogenous IL-12 in innate immunity to replicative *L. pneumophila* lung infection.

MATERIALS AND METHODS

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

Preparation of bacteria. *L. pneumophila* serogroup 1, strain AA100, a redesignation of a primary clinical isolate from the Wadsworth Veterans Administration Hospital (Wadsworth, Calif.), was provided by Paul Edelstein. For preparation of the intratracheal inoculum, *L. pneumophila* was quantified on buffered charcoal-yeast extract agar (Becton Dickinson, Cockeysville, Md.) that had been incubated for 48 h and resuspended in phosphate-buffered saline (PBS) at 4×10^7 organisms/ml (10, 25).

Inoculation of A/J mice with *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* as previously described (10, 49). Briefly, the mice were anesthetized with ketamine (2.5 mg/mouse intraperitoneally) 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 (i.e., containing 10^6 *L. pneumophila* cells), followed by 10 μl of air, was injected directly into the trachea with a 26-gauge needle. The skin incision was closed with a sterile wound clip.

Recovery of *L. pneumophila* from infected lung tissue. At specific time points postinoculation (p.i.), the mice were humanely euthanized and the lungs were

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removed. Lung tissue was finely minced in sterile water (10 ml/lung) and subsequently homogenized (2 min/sample) with a Stomacher (Tekmar, Cincinnati, Ohio) (6, 10). Lung homogenates were subsequently serially diluted in sterile water and cultured on buffered charcoal-yeast extract agar containing polymyxin B, cefamandole, and anisomycin (Becton Dickinson) for 72 h (10, 22). The lower limit of detection of *L. pneumophila* with this system is 10^3 CFU per lung.

Collection of lung homogenate supernatant and BALF for cytokine analysis. Lung homogenate supernatant was procured by filtering lung homogenates, prepared as described above, through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.) to remove the bacteria. Alternatively, for collection of bronchoalveolar lavage fluid (BALF), the mice were humanely sacrificed and their lungs were lavaged with 1.6 ml of PBS (6). The resultant lavage fluid was subsequently filtered as described above. Filtered lung homogenates and BALF were stored at −20°C until use for cytokine analysis.

Cytokine analysis. IFN-γ, IL-10, and IL-12 protein levels in lung homogenates and/or BALF were measured by commercially available cytokine-specific murine enzyme-linked immunosorbent assay (ELISA) kits (Intertest-γ, Intertest-IL-10, and Intertest-IL-12X total mouse IL-12, respectively; Genzyme Corp., Cambridge, Mass.) according to the manufacturer's directions. This IL-12 ELISA detects all three forms of IL-12 (i.e., p70 heterodimer, $p40_2$ homodimer, and p40 monomer). TNF-α activity in lung homogenate was measured by a cytotoxicity assay using the WEHI 164 subclone 13 cell line as previously described (24). Briefly, lung homogenate samples were serially diluted directly into 96-well microtiter plates (Costar, Cambridge, Mass.). The WEHI cells were suspended 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, 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 dimethyl-azole tetrazolium bromide (MTT) (5 mg/ml; Sigma, St. Louis, Mo.) was added to the wells and allowed to incubate at 37°C for an additional 4 h. Viable cells (i.e., cells not lysed by TNF-α) metabolize the MTT-tetrazolium to produce dark formazan crystals. The crystals were dissolved in 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. Mice were depleted of endogenous IL-12 by intraperitoneal inoculation with rabbit serum containing neutralizing antibody to IL-12 (0.5 ml/mouse) 2 h prior to intratracheal inoculation with *L. pneumophila*. This antiserum, a generous gift from Steven Kunkel, Department of Pathology, University of Michigan Medical School, Ann Arbor, has previously been shown to be efficacious in neutralizing endogenous intrapulmonary IL-12 in other murine models of lung injury (31). Results of preliminary experiments demonstrated that this concentration of antiserum neutralized ≥95% of endogenous IL-12 activity in lung homogenates of *L. pneumophila*-infected mice for up to 5 days (data not shown). Alternatively, in selected experiments, mice were inoculated with preimmune rabbit antisera (0.5 ml/mouse) prior to intratracheal inoculation with *L. pneumophila*. Results of these preliminary experiments demonstrated that neither recovery of *L. pneumophila* nor cytokine levels in BALF were significantly altered in mice treated with preimmune rabbit serum, compared to similarly infected mice not administered antiserum (data not shown). Consequently, in all subsequent experiments, *L. pneumophila*-infected mice administered anti-IL-12 serum were compared to similarly infected untreated mice.

Statistical analysis. Student's *t* test was used to compare differences between treatment groups. For comparison of multiple groups to a single control, analysis of variance with post-hoc Tukey analysis was performed. $P < 0.05$ was considered significant.

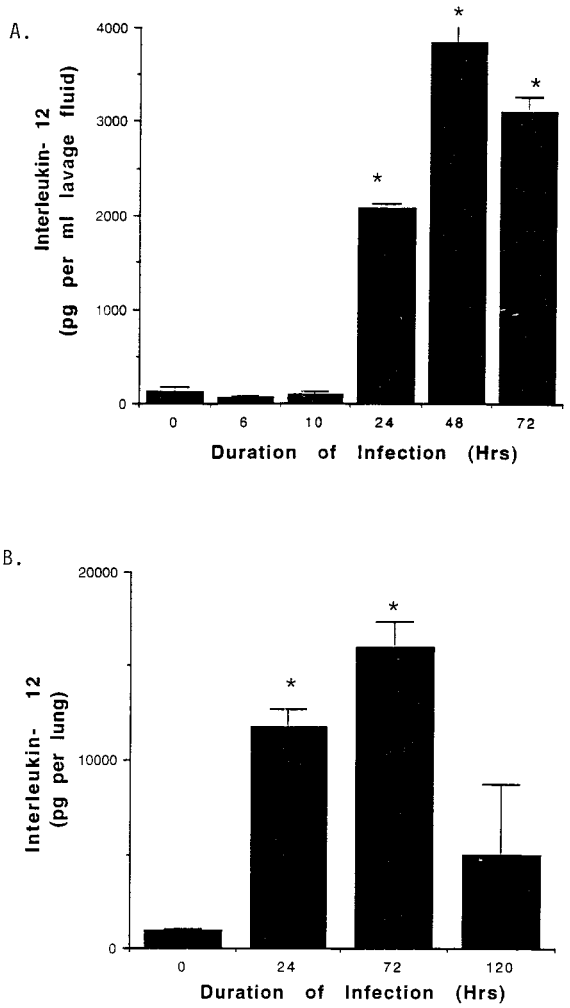

FIG. 1. IL-12 activity in BALF and lung homogenates of A/J mice inoculated intratracheally with *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* as described in Materials and Methods. At specific time points thereafter, the mice were sacrificed and IL-12 was assessed in BALF (A) and whole lung homogenates (B) by ELISA. Results represent the means ± standard errors of the means for three to five animals per time point, *, significantly greater than value for uninfected mice (i.e., 125 pg of IL-12 per ml of BALF; 935 pg of IL-12 per lung) (analysis of variance, $P < 0.05$).

RESULTS

Endogenous IL-12 facilitates resolution of primary replicative *L. pneumophila* lung infection. In initial experiments, induction of intrapulmonary IL-12 during replicative *L. pneumophila* lung infection was assessed. A/J mice were inoculated intratracheally with *L. pneumophila* (10^6 organisms per mouse). At 0 to 120 h p.i., the mice were humanely euthanized and the lungs were either lavaged or homogenized. IL-12 activity in BALF and in whole lung homogenates was subsequently quantified by a murine-specific IL-12 ELISA. As shown in Fig. 1, IL-12 was significantly enhanced in BALF and in whole lung homogenates within 24 h p.i. Furthermore, whole lung homogenates from infected mice contained greater than fivefold more IL-12 than did BALF from similarly infected mice.

We have previously demonstrated that A/J mice inoculated intratracheally with virulent *L. pneumophila* (10^6 bacteria per mouse) develop replicative *L. pneumophila* lung infections, with logarithmic growth of the bacteria within the first 48 h p.i. followed by gradual clearance of the bacteria from the lung at ≥72 h P.I. (10). Because induction of IL-12 activity in the lung of *L. pneumophila*-infected A/J mice (i.e., at ≥24 h p.i. [Fig. 1]) precedes clearance of the bacteria from the lung (i.e., at ≥72 h p.i.), the potential role of endogenous IL-12 in resistance to primary replicative *L. pneumophila* lung infection was evaluated. Mice were depleted of endogenous IL-12 by administration of rabbit IL-12 antiserum as described in Materials and Methods. At 24, 72, and 120 h p.i., the mice were humanely euthanized, the lungs were excised and homogenized, and *L. pneumophila* CFU were quantified in lung homogenates. As shown in Table 1, while there was no significant difference in recovery of *L. pneumophila* from the lung of mice treated with anti-IL-12 antiserum and similarly infected immunocompetent mice within the first 72 h p.i., significantly more bacteria were recovered in lung homogenates from animals depleted of endogenous IL-12 and sacrificed at 5 days p.i. (compared to similarly infected immunocompetent mice [Student's *t* test, $P < 0.05$]). Furthermore, 10-fold more bacteria were recovered in

TABLE 1. Effect of IL-12 neutralizing antibody on intrapulmonary replication of *L. pneumophila*[a]

Duration of infection (h)	Antibody titer[b]	
	Untreated	Anti-IL-12
24	$(6.00 \pm 2.67) \times 10^7$	$(1.27 \pm 4.68) \times 10^8$
72	$(1.17 \pm 0.43) \times 10^7$	$(2.23 \pm 0.63) \times 10^7$
120	$(4.53 \pm 3.21) \times 10^5$	$(1.37 \pm 0.38) \times 10^{7*}$

[a] A/J mice were administered IL-12 neutralizing antibody as described in Materials and Methods prior to intratracheal inoculation with *L. pneumophila* (10^6 bacteria per mouse). At selected time points thereafter, the mice were euthanized and growth of *L. pneumophila* in the lung was determined and compared to that of untreated mice (i.e., immunocompetent mice inoculated intratracheally with *L. pneumophila*).

[b] Mean ± standard error of the mean for 8 to 14 animals per treatment group. *, significantly greater than value for control mice (Student's *t* test, $P < 0.05$).

lung homogenates of *L. pneumophila*-infected mice treated with IL-12 antiserum at 5 days p.i. compared to the inoculating dose of bacteria (i.e., 10^6 *L. pneumophila* per mouse), suggesting that A/J mice depleted of endogenous IL-12 activity develop persistent replicative intrapulmonary *L. pneumophila* infection. Taken together, these results demonstrate that IL-12 is induced in the lung in response to *L. pneumophila* and plays a key role in innate immunity to the bacteria.

Immunomodulatory activity of endogenous IL-12. Previous in vitro and in vivo studies have demonstrated that growth of *L. pneumophila* in permissive MPCs is modulated by cytokines including IFN-γ, TNF-α, and IL-10 (10, 12, 14, 15, 46). Because IL-12 has previous been shown to modulate the production of these cytokines (44), in subsequent experiments the effect of endogenous IL-12 on intrapulmonary levels of IFN-γ, TNF-α, and IL-10 during replicative *L. pneumophila* lung infection was assessed. A/J mice were administered anti-IL-12 serum and inoculated intratracheally with *L. pneumophila* (10^6 bacteria per mouse) as described in Materials and Methods. At 24, 72, and 120 h p.i., the mice were humanely euthanized, the lungs were excised, and filtered lung homogenates were obtained. TNF-α, IFN-γ, and IL-10 were subsequently quantified in filtered lung homogenates obtained from anti-IL-12-treated and untreated *L. pneumophila*-infected mice by cytokine-specific bioassay or ELISA.

In agreement with our previous studies (10, 12), both TNF-α and IFN-γ were significantly induced in the lung of immunocompetent *L. pneumophila*-infected mice within 24 h p.i. compared to uninfected mice (<10 pg of TNF and 170 pg of IFN-γ per lung of uninfected mice) (Fig. 2). In contrast, IL-10 was not significantly induced in the lung of *L. pneumophila*-infected immunocompetent mice at any time point studied compared to uninfected mice (330 pg of IL-10 per lung of uninfected mice). Administration of anti-IL-12 serum to A/J mice prior to intratracheal inoculation with *L. pneumophila* resulted in a significant decrease in intrapulmonary TNF-α activity within 24 h p.i. compared to similarly infected immunocompetent mice (Student's *t* test, $P < 0.05$). In contrast, there was no significant difference between intrapulmonary levels of IFN-γ or IL-10 in *L. pneumophila*-infected mice depleted of endogenous IL-12 compared to similarly infected immunocompetent mice at any time point studied. We have previously demonstrated that TNF-α plays a key role in elimination of *L. pneumophila* from the lung in this murine model system (12). Together, these results suggest that regulation of intrapulmonary growth of *L. pneumophila* by endogenous IL-12 is likely mediated at least in part by TNF-α.

DISCUSSION

In this study, the role of endogenous IL-12 in innate immunity to replicative *L. pneumophila* lung infection was assessed in vivo, using a murine model of Legionnaires' disease in A/J mice inoculated intratracheally with virulent bacteria. We demonstrate that IL-12 is induced in the lung during replicative *L. pneumophila* lung infection and that neutralization of endogenous IL-12 by administration of IL-12 antiserum resulted in impaired ability of A/J mice to resolve a primary replicative *L. pneumophila* lung infection. These results identify a key role of endogenous IL-12 in innate immunity to *L. pneumophila* pulmonary infection.

In subsequent experiments, immunomodulatory effects of endogenous IL-12 on intrapulmonary IFN-γ, TNF-α, and IL-10 during replicative *L. pneumophila* lung infection were assessed. This is of particular interest, as IFN-γ, TNF-α, and IL-10 have previously been shown to regulate growth of the bacteria in permissive MPCs in vivo and/or in vitro (10, 12, 46).

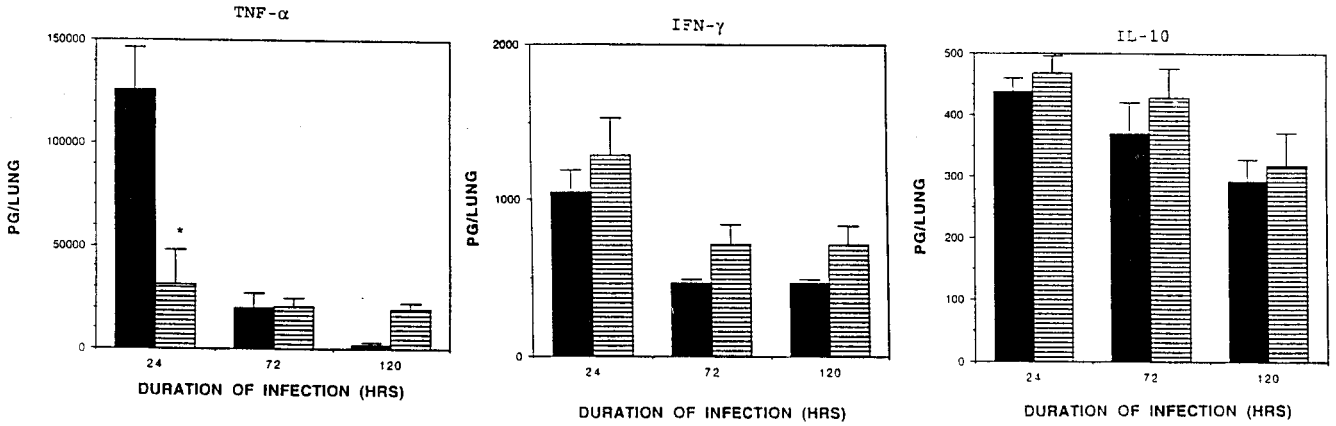

FIG. 2. Effect of IL-12 on endogenous cytokine activity in the lung during replicative *L. pneumophila* lung infection. A/J mice were administered IL-12 antiserum as described in Materials and Methods prior to intratracheal inoculation of *L. pneumophila*. At specific time points p.i., the mice were sacrificed. The lungs were excised, homogenized, and filtered. Levels of TNF-α, IFN-γ, and IL-10 were quantified in filtered lung homogenates of *L. pneumophila*-infected mice depleted of endogenous IL-12 by ELISA or by bioassay and compared to those of similarly infected immunocompetent (i.e., untreated) mice. Results represent the means ± standard errors of the means for five mice per treatment group. ■, untreated mice; ▤, anti-IL-12-treated mice. *, significantly less than value for untreated mice (Student's *t* test, $P < 0.05$).

Results of these studies demonstrated that enhanced growth of *L. pneumophila* in mice depleted of endogenous IL-12 was positively correlated with a significant reduction in TNF-α activity in the lung within 24 h p.i.; however, intrapulmonary levels of neither IFN-γ nor IL-10 were significantly altered by this therapy (Fig. 2). TNF-α has previously been shown to play a key role in resolution of replicative *L. pneumophila* infection, as it enhances polymorphonuclear leukocyte bacteriocidal activity (7), is directly toxic for *L. pneumophila* (42), and in combination with other cytokines, including IFN-γ, induces MPC production of reactive nitrogen intermediates, including nitric oxide, which limit *L. pneumophila* growth and viability (12). Together, these results suggest that regulation of intrapulmonary *L. pneumophila* replication by endogenous IL-12 is likely mediated, at least in part, by TNF-α.

Previous studies have demonstrated that IL-12 is a potent inducer of IFN-γ (41). Furthermore, IL-12-mediated inhibition of other pathogenic microbes, including T. gondii (30), *Histoplasma capsulatum* (1), and *Listeria monocytogenes* (54, 55), has been shown to occur through an IFN-γ-mediated mechanism. Therefore, we were somewhat surprised that neutralization of endogenous IL-12 in *L. pneumophila*-infected A/J mice did not significantly alter intrapulmonary levels in IFN-γ. However, our results demonstrating IFN-γ-independent effects of IL-12 on host immune responses to *L. pneumophila* concur with those of a recent in vitro study by Bermundez et al., who showed that IL-12-induced NK cell-mediated mycobactericidal activity in human MPCs is mediated by a TNF-α-dependent, IFN-γ-independent mechanism (3). Together, these studies suggest that the role of endogenous IL-12 in cytokine networking and host resistance to pathogenic microbes differs with respect to different intracellular pathogens.

While our studies have focused on elucidating immunomodulatory effects of IL-12 on the expression of cytokines which mediate resistance to replicative *L. pneumophila* lung infection, it is likely that endogenous IL-12 also contributes to innate immunity to *L. pneumophila* by cytokine-independent mechanisms. Specifically, costimulation of NK cells and/or T cells with IL-12 and other cytokines, including IL-15 or TNF-α, has been shown to enhance NK cell and T-cell cytotoxicity (2, 13, 16–18, 38, 53). The potential role of cytotoxic T cells and/or NK cells in resistance to primary replicative *L. pneumophila* lung infection remains to be thoroughly explored.

In summary, using a murine model of Legionnaires' disease in A/J mice, we have demonstrated that endogenous IL-12 plays a key role in innate immunity to legionellosis, likely in part by its ability to modulate intrapulmonary activity of other cytokines, including TNF-α. Further studies, which will identify the potential synergy between intrapulmonary IL-12 and other cytokines such as IL-15 in innate immunity to replicative *L. pneumophila* lung infection are warranted and will likely provide a rational approach to immunotherapy for treatment of Legionnaires' disease.

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