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Legionella pneumophila Infection in Intratracheally Inoculated T Cell-Depleted or -Nondepleted A/J Mice

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The inflammatory response and influence of T cell depletion on the pathogenesis of an experimental Legionella infection were studied. A/J mice were infected with 10⁶ CFU of Legionella pneumophila intratracheally. With this dose all infected animals survived the infection and bacteria were cleared from lung, spleen, liver, and kidney within 10 to 11 days, leaving no residual changes in the affected organs. Inflammatory cells were recruited into the lung on the second day of infection, reaching a maximum on the third day and filling out predominantly the interstitial areas. During the early phase of infection, serum concentrations of IFN-γ, TNF-α, IL-1β, IL-4, and IL-6 but not IL-2 increased dramatically. The cytokine secretion decreased on the third day after infection although bacteria were still present in the lung or even disseminated in different organs. Successful clearance of bacteria from the lung was not observed before recruitment of T cells into the lung. In mice depleted of both CD4⁺ and CD8⁺ T cells, control of infection was impaired and lethality of infection increased. Depletion of either subset left residual antibacterial mechanisms, which, however, were not sufficient to clear the Legionella as rapidly as in undepleted mice. The Journal of Immunology, 1998, 160: 316–321.

Legionella pneumophila is an intracellular bacterial pathogen that causes a serious and often fatal form of pneumonia in humans. Alcohol abuse, smoking, old age, or immunosuppression increase the susceptibility to L. pneumophila infection (1–3).

In vivo studies indicate that L. pneumophila infections result in a humoral and cell-mediated immune response (4–8). Humoral immunity probably plays a role as a second line of defense by reducing intrapulmonary growth of L. pneumophila (9), while cellular immunity in concert with cytokines could be essential for resolution of a primary infection (10). IFN-γ inhibits intracellular Legionella multiplication by down-regulation of transferrin receptors, thus limiting the amount of available iron (11–13). TNF-α contributes to host defense in experimental Legionella infection by factors that are essentially unknown (14). Although cell-mediated host-defense mechanisms seem to be crucial in Legionella infection, kinetics of the cellular response and cytokine release have not been studied in detail.

We show that L. pneumophila infection is indeed ensued by an immediate production of inflammatory cytokines such as IFN-γ, TNF-α, IL-6, and IL-1β, and that the control of infection and clearance of L. pneumophila from the lungs depend on successful recruitment and unimpaired function of CD4⁺ and CD8⁺ T lymphocytes in the lung tissue.

Materials and Methods

Animals

Female pathogen-free A/J mice, 8 to 9 wk of age, were used in all experiments. They were housed and cared for in our animal facility according to standard guidelines.

Bacteria

The L. pneumophila Philadelphia strain 1, serogroup 1, (American Type Culture Collection, Rockville, MD; no. 35133) was cultured for 24 to 36 h on BCYE plates (Merck, Darmstadt, Germany) and harvested with PBS (pH 7.2). The bacteria were washed by centrifugation in sterile saline at 4°C and resuspended to give the appropriate concentration.

Inoculation of animals

Mice were inoculated intratracheally according to a previously described protocol (15). Briefly, the mice were anesthetized by i.p. injection of ketamine (2, 5 mg), and the trachea was isolated. A total of 50 µl of the bacterial suspension in PBS (10⁶ to 10⁸ L. pneumophila) was inoculated directly into the trachea using a 26-gauge needle followed by 10 to 20 µl of air. The skin incision was surgically closed. Control animals were inoculated with PBS only and were sacrificed at different time points.

Quantitation of L. pneumophila in mice

At different time points after inoculation of bacteria, the mice were sacrificed by CO₂ asphyxiation and exsanguinated. The lungs, spleen, liver, and kidneys were subsequently aseptically excised, finely minced, and homogenized in a tissue homogenizer with 5 ml of sterile distilled water. The number of CFU in the organs was determined by a plate dilution method using BCYE agar. After 5 days of incubation at 35°C, 5% CO₂, the colonies were counted and the results were expressed as the number of CFU per organ. Contamination of homogenates was controlled by culturing an aliquot of organ homogenate on Mueller-Hinton blood agar for 3 days.

Histopathologic analysis

The pathologic changes and cell recruitment into the lungs of A/J mice in response to L. pneumophila were assessed by light microscopy. At daily intervals after inoculation, the mice were sacrificed and exsanguinated. The excised lungs were inflated and fixed in 10% neutral formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 µm) were cut and

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stained either with hematoxylin-eosin or with different mAbs using standard immunohistology procedures (anti-monocyte/macrophage Mac1 Ag, clone M 1/70; anti-granulocyte Ag, clone Gr-1; anti-B lymphocyte Ag B220, clone RA3-6B2; anti-CD4 lymphocyte Ag, clone YTS 191.1.2; anti-CD8 lymphocyte Ag, clone YTS 169.4.2; and anti-NK cells, clone DX5). All Abs were obtained from PharMingen (San Diego, CA).

**Cytokine assays**

The serum cytokine concentrations were determined 2 h after inoculation and on days 1, 3, 7, and 11 using commercially available mouse ELISA cytokine kits (Endogen, Cambridge, MA).

**Modulation of the immune system**

To characterize the possible role of distinct T cell populations during *Legionella* infection, the mice were depleted in some experiments of either CD4⁺ lymphocytes, CD8⁺ lymphocytes, or both. For depletion we used mAbs (anti-CD4, clone 191.1.2 and anti-CD8, clone 169.4.2) purified from ascites using protein G column (HiTrap; Pharmacia, Uppsala, Sweden). The animals were depleted on day 1 before inoculation and once weekly after infection with 1 mg of IgG/animal of the respective mAb. The control group of animals received an irrelevant but isotype-identical rat anti-mouse Ab (IgG1) at the same time points. The depletion was confirmed by flow cytometry of lymph nodes and spleen cells.

**Statistical analysis**

Differences in survival times were determined using the Mann-Whitney *U* tests, and CFU counts were compared by Student’s *t* test. (16)

**Results**

**Recruitment of inflammatory cells into the lung induced by infection with *L. pneumophila***

Inoculation with 1 × 10⁶ CFU/animal of *L. pneumophila* led to an evenly distributed cellular infiltration of the infected lung 48 h after infection. There was no predilection site for the cellular infiltration. Infiltration was characterized by large, mainly mononuclear cells, which predominantly filled out the interstitial areas. Three days after inoculation, the interalveolar spaces were heavily stuffed with inflammatory cells so that alveolae virtually collapsed (Fig. 1c). We were not able to observe a marked increase of alveolar macrophages or infiltration of mononuclear cells into alveolar spaces at any time point after infection.

On day 7 after inoculation (Fig. 1d) the cellular infiltration resolved and a normal lung histology developed.

The infiltrating cells were further characterized by immunohistology of the lung (Fig. 2). At 24 to 48 h after inoculation of *L. pneumophila*, the infiltrating cells were stained by the Mac1⁺ Ab, which recognizes murine monocyte/macrophages and polymorphonuclear granulocytes. The Mac1⁺ cell infiltration was diffusely distributed showing no predilection to any anatomical compartment of the lung. These cells represent essentially only Mac1⁺ monocyte/macrophages, because a granulocyte-specific Ab (Gr-1) did not stain the cells (data not shown).

Simultaneously with the Mac1⁺ cells, B lymphocytes and NK cells were attracted into lung. B cells segregated nearly exclusively around blood vessels and small bronchioli in the so-called BALI (bronchious associated lymphoid tissue) regions and disappeared at later stages of infection. In contrast, CD4⁺ and CD8⁺ cells could rarely be detected during the first 2 days postinoculation of bacteria into the lung, leaving some of the infiltrating cells during the early stage of infection undefined.

**Systemic production of cytokines during *L. pneumophila* infection of A/J mice**

Serum levels of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, and IL-6 were determined, knowing their importance in the resistance of mice against intracellular bacteria (17). The *L. pneumophila* infection resulted in a rapid up-regulation of systemic levels of all the studied cytokines (Fig. 3) except for IL-2. Pluripotent mediators of the acute phase response such as IL-6 and IL-1β were moderately up-regulated as early as 4 to 6 h postinfection, whereas the other cytokines reached maximal serum levels 24 h after infection. The serum level of cytokines decreased with increasing cell infiltration of the lung returning to the cytokine level of control sham-infected
animals on the third day of infection. An additional wave of TNF-α and IL-4 was seen 7 days after inoculation of bacteria.

**Replication of *L. pneumophila* in different organs of intratracheally inoculated A/J mice**

After intratracheal inoculation, CFU of *L. pneumophila* increased 50 to 100 times in the lung tissue during the first 2 days of infection and then slowly decreased again (Table I). This correlated with the kinetics of cytokines in the serum. On the third day after inoculation, a moderate dissemination of bacteria to spleen (4 × 10⁴ CFU), liver (5 × 10⁵), and kidneys (1 × 10⁴ CFU) was seen. *L. pneumophila* could be found in the lungs and sporadically in the liver until day 7 after inoculation. At all the examined time points after infection we were not able to isolate *L. pneumophila* from the blood.
The influence of T lymphocyte subpopulations on clearance of L. pneumophila

The experiments described above showed that the appearance of CD4⁺ and CD8⁺ T cells correlated with disappearance of L. pneumophila from the lungs. To gain insight into the potential role of T lymphocytes during Legionella infection, we made a selective depletion of CD4⁺, CD8⁺, or both T cell subsets.

Depletion of CD4⁺, CD8⁺, or CD4⁺ and CD8⁺ T lymphocytes leads to a considerable increase of infection lethality (Fig. 4). The kinetics of bacterial replication in the lungs of immunocompromised mice were similar to that in immunocompetent mice, but the peak numbers of bacteria were nearly 10 times higher, reaching 1 to 2 × 10⁸ CFU/lungs 24 h after infection.

The mice with impaired cellular immunity when inoculated intratracheally with 1 × 10⁶ CFU of L. pneumophila could survive but were not able to clear bacteria from the lungs. After having reached the maximal CFU, bacterial concentrations in the lungs decreased slowly and persisted for more than 2 wk after infection (Table II).

Discussion

Intratracheal application of L. pneumophila lead to a pneumonia that was accompanied by multiplication of bacteria, rapid increase of cytokines in serum, and recruitment of inflammatory cells into the pulmonary tissue. The pneumonia resolved spontaneously within 1 wk if the mice were immunocompetent. The infection model used here largely corresponds to that described by Brieland et al. (15). However, in contrast to Brieland et al., who used pulmonary tissue homogenates for immunocytology, we performed immunohistologic studies to get additional information on the distribution patterns of the different cell types.

It was surprising that Legionella more often could be found in the liver than in the spleen. Perhaps this situation corresponds to the clinical presentation of Legionnaires’ disease, with a slight elevation of bilirubin and transaminases. One might speculate that the Kupffer cells could serve as a suitable host for Legionella.

The course of infection can be divided into an early phase, during which a rapid bacterial multiplication and inflammatory response can be observed, and a second phase, beginning on the second or third day after infection with a down-regulation of the unspecific inflammatory response and a decrease in the pulmonary bacterial count. The early cellular response was characterized by an interstitial inflammatory reaction consisting mainly of macrophages, B lymphocytes, and an undefined cell population that was stained by neither granulocyte-, macrophage/monocyte-, nor lymphocyte-specific Abs and that might correspond to the mononuclear/phagocytic cells described by Brieland et al. (15). Additional staining with an NK cell-specific Ab revealed that this population, at least in part, was composed of NK cells. NK cells have also been shown to be the main source of IFN-γ in mice experimentally infected with Yersinia enterocolitica (18). To the best of our knowledge, it has not been published before that NK cells represent an early recruited cell population during experimental Legionella infection.

These cells probably represent a first and effective line of defense against L. pneumophila infection, possibly triggered by Legionella LPS and other toxic bacterial constituents. IFN-γ, secreted by T cells and NK cells (17, 18), might directly contribute to intracellular bacteriostasis or killing, either by down-regulation of transferrin receptors (11) or by endogenous nitric oxide (14). Lack of IFN-γ induced by disruption of the IFN-γ gene impaired clearance of Legionella and led to persistent neutrophil recruitment into the lungs of BALB/c mice (12). As shown by Brieland et al.
TNF-α also helps to control the infection via endogenous nitric oxide. The increase in the number of inflammatory cells might be achieved through the effect of TNF-α, which attracts granulocytes to the site of infection (17). IL-8, mainly produced by endothelial cells, and the monocytic chemoattractant protein 1, produced by pulmonary macrophages and regulated by LPS and inflammatory cytokines (19), also contribute to the increase of inflammatory cells in the lungs. An increase of systemic IL-2 was not detected. This is probably due to the fact that IL-2 is produced by local T cells within the inflammatory region, not affecting the concentration of this cytokine in the bloodstream.

Similar to Brieland et al. (9, 15), we found an increased number of CD4 and CD8 T cells in the second phase of infection, when cytokines and bacterial counts have already declined. Although appearing late in infection, they no doubt significantly contribute to the control of infection during the early phase as well, since depletion of CD4 and/or CD8 cells resulted in an increase of infection lethality. Furthermore, both T cell subpopulations also seem to be relevant for final clearance. This situation is also typical for other intracellular pulmonary pathogens, such as Chlamydia. In a Chlamydia trachomatis pneumonia mice model (20), IFN-γ production decreased when the mice were CD4 cell depleted, while the bacterial burden and lethality increased. The effects of CD8 T cell depletion were similar but less pronounced. Our experiments, however, are suggestive of a slightly more important role for CD8 cells than CD4 cells, since the former are more prominent in the infected lung tissue and bacterial counts in the lungs tended to be lower in CD4- than in CD8-depleted animals.

The two phases of an experimental Legionella infection also reflect the rapid unspecific immune response and the slower developing specific immune response necessary for final eradication of the infection. CD4 and CD8 T cells play a role in both phases of infection, as seen from the increased acute lethality during the first phase and the long bacterial persistence in the second phase of infection in T cell-depleted mice. In the unspecific phase, T cells might contribute to host defense by producing IFN-γ and IL-6, while in the specific phase they support humoral immunity and specific T cell-mediated immunity (17). The initial phase might also have a parallel to the clinical situation of Pontiac fever, in which the flu-like symptoms are characteristic of an overwhelming production of cytokines in response to a massive endotoxin exposure (21).

In this setting of an experimental infection, the immune response was sufficient to successfully combat the infection. In a clinical situation, however, a specific and unspecific immune response might be out of balance, thus giving the bacteria a chance to escape from a pathogen-directed host response. The low endotoxin activity of Legionella LPS (22) might insufficiently trigger the unspecific immune response, and the highly saturated and branched fatty acids of the L. pneumophila LPS molecules might...
help to resist enzymatic degradation of the bacterial cell wall (23). The metalloprotease cleaves and inactivates cytokines and CD4 molecules (25, 26), thus disturbing intercellular communication. In addition, the rapid change of metabolic pathways and a group of so-called early macrophage-induced proteins (26–28) might help bacteria to multiply undetected or to avert effective host-defense mechanisms.

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