Paradoxically high resistance of natural killer T (NKT) cell-deficient mice to *Legionella pneumophila*: another aspect of NKT cells for modulation of host responses

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In the present study, we examined the roles of natural killer T (NKT) cells in host defence against *Legionella pneumophila* in a mouse model. The survival rate of NKT cell-deficient J α 281 knock-out (KO) mice was significantly higher than that of wild-type mice. There was no bacterial overgrowth in the lungs, but J α 281 KO mice showed enhanced pulmonary clearance at a later stage of infection, compared with their wild-type counterparts. The severity of lung injury in *L. pneumophila*-infected J α 281 KO mice was less, as indicated by lung permeability measurements, such as lung weight and bronchoalveolar lavage fluid albumin concentration. Recruitment of inflammatory cells in the lungs was approximately twofold greater in J α 281 KO mice on day 3. Interestingly, higher values of interleukin (IL)-1 β and IL-18, and increased caspase-1 activity were noted in the lungs of J α 281 KO mice from an early time point (6 h). Exogenous α -galactosylceramide, a ligand of NKT cells, induced IL-12 and gamma interferon at 6 h, but suppressed IL-1 β at later time points in wild-type, whereas no effects were evident in J α 281 KO mice, as expected. Systemic administration of heat-killed *L. pneumophila*, but not *Escherichia coli* LPS, reproduced exaggerated production of IL-1 β in the lungs of J α 281 KO mice. These results demonstrate that NKT cells play a role in host defence against *L. pneumophila*, which is characterized by enhanced lung injury and decreased accumulation of inflammatory cells in the lungs. The regulation of IL-1 β , IL-18 and caspase-1 may be associated with the modulating effect of host responses by NKT cells.

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INTRODUCTION

Legionella pneumophila is a Gram-negative bacillus that causes acute pneumonia, known as Legionnaires' disease.

Abbreviations: BAL, bronchoalveolar lavage; α -GalCer, α -galactosylceramide; KO, knock-out; IFN- γ , gamma interferon; IL, interleukin; NK, natural killer; NKT, natural killer T.

The pneumonia is often serious and life-threatening, and can be nosocomial or community-acquired (Reingold, 1988; Marston *et al.*, 1997). Legionnaires' disease accounts for 2–8% of community-acquired pneumonia cases (Bartlett and Mundy, 1995; Fang *et al.*, 1990), and it is much more frequent among patients admitted to intensive care units (Rello *et al.*, 1993). Unfortunately, high

mortality rates ranging from 10 to 50% have been observed, especially in immunocompromised individuals (el-Ebiary *et al.*, 1997; Pedro-Botet *et al.*, 1998; Tkatch *et al.*, 1998).

Legionella usually infects humans via inhalation of contaminated aerosols from waterborne environmental sources. In lung tissues, the bacteria multiply in several types of host cells, but especially in macrophages (Horwitz & Silverstein, 1980; Nash *et al.*, 1984; Mody *et al.*, 1993). Protective roles of cytokines, such as interleukin (IL)-12, IL-18, tumour necrosis factor alpha, and gamma interferon (IFN- γ) have been reported both in animal models of pneumonia and in human infections (Skerrett & Martin, 1994; Gebran *et al.*, 1994; Brieland *et al.*, 1998, 2000; Salins *et al.*, 2001; Tateda *et al.*, 1998). Recent studies have focused on the mechanisms of bacterial flagellin recognition by the intracellular sensor system, the nucleotide-binding oligomerization domain (NOD), which results in IL-1 β and IL-18 production through activation of the caspase-1 cascade (Franchi *et al.*, 2006; Miao *et al.*, 2006; Molofsky *et al.*, 2006). Infected macrophages respond in several ways, such as apoptosis, autophagy and pyroptosis, depending on the balance between bacterial virulence and host cellular responses (Fink & Cookson, 2005). Pyroptosis is a newly defined state characterized by non-apoptotic, pro-inflammatory programmed cell death. An important hallmark of pyroptosis in *L. pneumophila*-infected macrophages is the caspase-1-dependent production of IL-1 β and IL-18 (Molofsky *et al.*, 2006). Pyroptosis may resist *Legionella* infections by limiting bacterial replication in host cells and enhancing recruitment of inflammatory cells to the site of infection (Fink & Cookson, 2005; Swanson & Molofsky, 2005). The clinical significance of pyroptosis in *L. pneumophila* infections remains unclear.

Natural killer T (NKT) cells constitute a T-cell subpopulation that expresses natural killer (NK) markers, and they have been reported to play an important role in a variety of autoimmune, allergic, antitumour and antimicrobial immune responses (Godfrey & Kronenberg, 2004; Taniguchi & Nakayama, 2000; Taniguchi *et al.*, 2003; van der Vliet *et al.*, 2004). NKT cells are characterized by an invariant T-cell receptor V α chain, which consists of a V α 14 J α 281 gene segment in mice (Koseki *et al.*, 1991; Exley *et al.*, 1997). Although a natural ligand for these cells remains to be identified, a synthetic glycolipid, α -galactosylceramide (α -GalCer), presented in the context of CD1d induces a prompt production of Th1 and/or Th2 cytokines. Previous reports have demonstrated that NKT cells function as important innate immune effector cells against a variety of pathogens, such as *Leishmania major*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Cryptococcus neoformans* (Ishikawa *et al.*, 2000; Kawakami *et al.*, 2001, 2003; Nieuwenhuis *et al.*, 2002). However, antibody-mediated blockage or gene-disruption of NKT cells has been associated with increased resistance to certain pathogens, such as *Toxoplasma gondii*, *Listeria monocytogenes* and *Chlamydia trachomatis*, which suggest a more complicated

role for NKT cells in host immunological systems (Szalay *et al.*, 1999; Bilenki *et al.*, 2005; Ronet *et al.*, 2005). These data clearly suggest that NKT cell-mediated host responses may be pathogen-dependent, with these cells participating in broad and diverse types of cellular responses.

In the present study, we investigated the roles of NKT cells in *L. pneumophila* challenge, using J α 281 gene-disrupted mice [J α 281 knock-out (KO) mice]. Lethal sensitivity, the bacterial burden and bronchoalveolar lavage (BAL) cell counts in the lungs, in addition to kinetics of cytokines (IL-1 β , IL-10, IL-12, IL-18 and IFN- γ) and caspase-1 activity were compared between J α 281 KO and control (wild-type) mice.

METHODS

Animals. Specific pathogen-free C57BL/6 mice (6- to 8-week-old) were purchased from Charles River Laboratories (Yokohama, Japan). J α 281 KO mice generated at Chiba University (Chiba, Japan) were back-crossed eight times with C57BL/6 mice (Cui *et al.*, 1997). All animals were housed in a pathogen-free environment in the Toho University School of Medicine. The mice were provided sterile food and water.

Bacteria. Clinical isolates of *L. pneumophila* Suzuki, a serogroup 1 strain originally isolated at Toho University Hospital (Tateda *et al.*, 1998), were grown for 3–4 days at 37 °C on buffered charcoal-yeast extract (BCYE) agar supplemented with L-cysteine and ferric nitrates. A single colony was transferred to 3 ml buffered yeast extract broth and incubated overnight at 37 °C with constant shaking. The bacterial suspension was transferred to fresh buffered yeast extract broth as serial fivefold dilutions and incubated overnight at 37 °C with constant shaking. After confirmation of bacterial motility, the concentration of bacteria in the broth was determined at OD₆₀₀. Post-exponential-phase bacteria were used as challenge organisms (Byrne & Swanson, 1998). Heat-killed *L. pneumophila* were prepared by incubating the bacterial suspension in normal saline at 95 °C for 5 min. After the heat treatment, the sterility of the bacterial suspension was confirmed by plating it on BCYE agar. The numbers of viable bacteria in the challenge suspension and the pre-heat treatment suspension were determined by plating and incubating organisms on BCYE agar for 4 days.

Challenge of *L. pneumophila* in mice. Mice were anaesthetized intramuscularly with ketamine (7 mg) and xylazine (15 mg kg⁻¹). The trachea was exposed and 30 μ l bacterial suspension or saline was injected directly into the trachea using a sterile 26-gauge needle. The skin incision was closed with a surgical staple.

Intravenous challenge using *Escherichia coli* LPS or heat-killed *L. pneumophila*. *E. coli* 055:B5 LPS (50 μ g) (Difco) suspended in 200 μ l normal saline or 4 $\times$ 10⁸ c.f.u. heat-killed *L. pneumophila* suspended in 200 μ l saline were intravenously injected into mice.

Lung harvesting for analysis. Mice were sacrificed by CO₂ asphyxia at designated time points. Before removal of the lung, the pulmonary vasculature was perfused with 1 ml saline via the right ventricle. After removal, whole lungs were homogenized in 1 ml saline using a tissue homogenizer (Omni International) under a vented hood. Ten microlitre aliquots of each homogenate were inoculated onto BCYE agar after serial 1:10 dilutions in saline. The remainder of each homogenate was incubated on ice for 30 min and then centrifuged at 3000 r.p.m. for 10 min. Supernatant was collected, passed through a 0.45 μ m filter (Kanto Chemical), and stored at -40 °C until use.

Collection of BAL. Mice were sacrificed at designated times after inoculation with bacteria. The trachea was exposed and intubated using a 1.7 mm (outer diameter) polyethylene catheter. BAL was performed by instilling 2 ml RPMI 1640 (Gibco-BRL), and BAL fluid was pooled for each animal. Leukocyte numbers were determined using a haemocytometer. Collected cells were attached to glass slides by cytopins, stained with May-Giemsa, and differential cell counts were performed. The remaining BAL fluid was stored at -40°C until further analysis. In some experiments, albumin concentrations in BAL fluid were determined by a mouse albumin ELISA quantification kit (AKRAL-121; Shibayagi).

ELISA of IL-1 β , IFN- γ , IL-10, IL-12 and IL-18. Levels of IL-1 β , IFN- γ , IL-10, IL-12 and IL-18 cytokines in lung homogenates were measured using ELISA kits (IL-1 β , IFN- γ , IL-10 and IL-12; R & D Systems; IL-18; Medical and Biological Laboratories). The assays were performed according to the manufacturers' instructions, and the level in each sample was determined in duplicate.

Determination of caspase activity. Levels of caspase activity were determined in lung homogenates. Caspase-1 and caspase-3 activities were determined by colorimetric assay (R & D Systems), in which a caspase-specific peptide that conjugated to the colour reporter molecule *p*-nitroanilide was used. The data are expressed as a fold increase, compared to those of control mice ($n=5$).

Treatment with α -GalCer. α -GalCer (Kirin Brewery) was prepared as described previously (Gonzalez-Aseguinolaza *et al.*, 2000). The stock α -GalCer solution ($220\text{ }\mu\text{g ml}^{-1}$ in 0.5% polysorbate 20 in normal saline) was diluted to $10\text{ }\mu\text{g ml}^{-1}$ with PBS. PBS was used as a control vehicle solution. Both the treatment and control solutions ($200\text{ }\mu\text{l}$) were injected intraperitoneally on the day of infection.

Statistical analyses. Statistical significance was determined using the Mann-Whitney test. Survival curves were constructed by the Kaplan-Meier method and were analysed by log rank test. $P<0.05$ was considered as a significant difference.

RESULTS AND DISCUSSION

High resistance of J α 281 KO mice to *L. pneumophila*

C57BL/6 control and J α 281 KO mice were intratracheally challenged with *L. pneumophila* Suzuki strain (3×10^6 c.f.u. per mouse), and survival was observed once a day for 14 days after the exposure (Fig. 1). Wild-type mice started to succumb from day 4, and all 10 mice died by day 8 post-challenge (0/10, 0% survival). In contrast, significantly reduced mortality was observed in J α 281 KO mice, as the survival rate in these mice was 90% (9/10) at the end of observation period ($P<0.05$). Similar results were obtained in three independent experiments. These observations suggested a greater resistance to *L. pneumophila* challenge in J α 281 KO mice, compared with the wild-type controls.

Bacterial burden in the lungs of mice after *L. pneumophila* challenge

After inoculation of the bacteria (1×10^7 c.f.u. per mouse), mice were sacrificed at the indicated time points, and the

Fig. 1. Survival rate of mice after pulmonary challenge with *L. pneumophila*. C57BL/6 control (●) and J α 281 KO mice (○) were challenged intratracheally with *L. pneumophila* Suzuki strain (3×10^6 c.f.u. per mouse), and mortality was observed for 14 days post-infection ($n=10$). Similar results were obtained in three independent experiments. * $P<0.05$ compared with the wild-type mice.

number of viable bacteria in the lungs was determined. There were no differences in bacterial numbers in the lungs of wild-type and J α 281 KO mice for up to 48 h (Fig. 2a). By 72 h, however, there was a modest but significant reduction in bacterial count in the lungs of J α 281 KO mice, compared with the infected wild-type mice ($P<0.05$). *L. pneumophila* c.f.u. in the lungs of J α 281 KO mice continued to decrease thereafter, whereas lung bacterial c.f.u. in wild-type mice could not be determined at time points after 72 h due to the premature deaths of the mice. No *L. pneumophila* c.f.u. was isolated in blood or spleen samples of either strain of mice at any time points examined (data not shown).

Evaluation of lung injury in *Legionella*-challenged mice

To determine the mechanism of enhanced resistance in J α 281 KO mice, changes in markers of lung injury were evaluated on day 3 post-infection. The markers included lung weight (Fig. 2b) and BAL fluid albumin levels (Fig. 2c) in wild-type and J α 281 KO mice. *L. pneumophila* challenge was associated with an increase in the total lung weight divided by the body weight in wild-type and J α 281 KO mice; however, lung weights were significantly less in the latter ($P<0.05$). In addition, albumin concentration in BAL fluid correlated well with the changes in the lung weight, and increases in albumin levels were significantly less prominent in J α 281 KO mice compared with those observed in wild-type mice ($P<0.05$). Collectively, these data demonstrate that the lung injury caused by *Legionella* was less severe in J α 281 KO mice, which may be associated with the survival benefit observed in this mouse strain.

Fig. 2. Bacterial burden and evaluation of lung injury in the lungs of *L. pneumophila*-challenged mice. (a) After intratracheal inoculation of *L. pneumophila*, mice were sacrificed at the indicated time points and the number of bacteria in the lungs were compared between wild-type (black bars) and Jα281 KO (white bars) mice ($n=5$). At 96 h, the bacterial number of wild-type was not determined because mice started to die. (b, c) Total lung weight divided by body weight (b) and albumin concentration in BAL fluid (c) were compared 3 days after bacterial challenge (5×10^6 c.f.u. per mouse) ($n=5$). * $P < 0.05$ compared with wild-type mice.

Leukocyte accumulation in BAL fluid of *Legionella*-challenged mice

To further understand the relative resistance of Jα281 KO mice to *Legionella*, leukocyte accumulation in the air space was examined in mice on days 1 and 3 post-infection (Fig. 3). *L. pneumophila* challenge resulted in a nearly 30-fold increase of total cell number in BAL fluids of wild-type mice on day 3. Importantly, there was a twofold greater number of inflammatory cells observed in Jα281 KO mice than that seen in wild-type mice on day 3. Significantly higher values were observed in all cell types (neutrophils,

macrophages and lymphocytes) in Jα281 KO mice, compared with those in wild-type mice with *L. pneumophila*. These data suggest that more leukocytes accumulated in the lungs of Jα281 KO mice in response to *L. pneumophila*.

Cytokine production in the lungs of *Legionella*-challenged wild-type and Jα281 KO mice and effects of α-GalCer

To further confirm the regulatory role of NKT cells in host defence against *L. pneumophila*, cytokine production (IL-1β, IL-18, IFN-γ, IL-12, IL-10), and bacterial number in the lungs of wild-type and Jα281 KO mice, were determined 6, 12, 24 and 48 h post-infection (Fig. 4). Significantly higher levels of IL-1β and IL-18 were observed in Jα281 KO mice at the early stages, and these levels were maintained for 24 and 48 h, respectively (Fig. 4a, b). In contrast, the production kinetics of IFN-γ, IL-12 and IL-10 were not different between wild-type and Jα281 KO mice, suggesting that there were no changes in Th1/Th2 balance in the absence of NKT cells (Fig. 4c, d, e).

Fig. 3. Total BAL cell counts for *L. pneumophila*-challenged mice. Wild-type (black bars) and Jα281 KO (white bars) mice were intratracheally inoculated with *L. pneumophila*. BAL analysis was performed on mice sacrificed on days 1 and 3 ($n=5$). * $P < 0.05$ compared with wild-type mice.

Next, we focused on the influence of NKT cell activation on *L. pneumophila* challenge, especially pertaining to the production of the various cytokines and susceptibility to death. Mice were intraperitoneally pretreated with α-GalCer (2 μg per mouse), which specifically stimulate NKT activity in context of CD1d, prior to the challenge with *L. pneumophila*. Pretreatment of wild-type mice was associated with significant increases of IFN-γ, IL-12 and IL-10 in the early stages, whereas reductions of IL-1β and IL-18 were apparent in the later stages. As expected, Jα281 KO mice failed to respond to α-GalCer treatment, which did not induce any significant cytokine changes. No changes of viable bacterial count in the lungs of wild-type and Jα281 KO mice were observed during these periods, in spite of α-GalCer treatment (Fig. 4f). No enhanced susceptibility to death was apparent following α-

Fig. 4. Effect of *in vivo* stimulation of NKT cells by α -GalCer treatment on host response to *L. pneumophila*. Wild-type and J α 281 KO mice were injected intraperitoneally with α -GalCer (2 μ g per mouse) or PBS on the day of challenge. Each mouse was then challenged intratracheally with *L. pneumophila* (1×10^7 c.f.u. per mouse). Production of IL-1 β (a), IL-18 (b), IFN- γ (c), IL-12 (d) and IL-10 (e), and bacterial burden (f), were examined in the lungs of wild-type mice injected with PBS (black bars), wild-type mice with α -GalCer (grey bars), J α 281 KO mice with PBS (white bars) and J α 281 KO mice with α -GalCer (hatched bars) ($n=5$ per group). * $P<0.05$ compared with those of the corresponding mice with PBS. ** $P<0.05$ compared with those of the wild-type mice with PBS.

GalCer activation of NKT cells (data not shown). These data suggested that absence of NKT cells or NKT cell-derived factors may be associated with an increase in resistance to *L. pneumophila* and a skewed production of cytokines.

Caspase-1 and caspase-3 activity in the lungs of wild-type and J α 281 KO mice

Since significant increases of IL-1 β and IL-18 were observed in the early stages of infection in the lungs of J α 281 KO mice, we next examined the expression of caspase-1, an enzyme required for maturation of IL-1 β and IL-18, and caspase-3, a key enzyme for induction of apoptosis (Fig. 5). Importantly, significantly higher levels of caspase-1 activity were demonstrated at the early time points (6 and 12 h) in the lungs of J α 281 KO mice, whereas no substantial alteration in caspase-3 activity was observed during the observation period.

IL-1 β production in the lungs of wild-type and J α 281 KO mice in response to heat-killed *L. pneumophila*

To further understand the interaction between *L. pneumophila* and NKT cells, we examined whether heat-killed *L.*

pneumophila could trigger IL-1 β production in the lungs of J α 281 KO mice. In preliminary experiments, the intratracheal administration of heat-killed organisms into the lungs induced only a minimal amount of IL-1 β in both mouse strains. Therefore, an intravenous route was used to administer heat-killed bacteria. Wild-type and J α 281 KO mice were injected intravenously with heat-killed *L. pneumophila* (4×10^8 c.f.u. per mouse) (Fig. 6a) or *E. coli* LPS (50 μ g per mouse) as a control (Fig. 6b). The levels of IL-1 β in the lung homogenates were evaluated 2 and 4 h after the injections. As shown in Fig. 6(a), the concentration of IL-1 β was significantly higher in the lungs of J α 281 KO mice than that of wild-type mice when heat-killed *L. pneumophila* was used as a stimulant. In contrast, a trend toward higher levels of IL-1 β was observed in wild-type mice in response to *E. coli* LPS. These data suggest that heat-stable *L. pneumophila* antigen may be responsible, at least partly, for the increased production of IL-1 β in J α 281 KO mice.

In the present study, our findings clearly indicate that NKT cell-deficient J α 281 KO mice are more resistant than control C57BL/6 mice to *L. pneumophila*. In particular, a striking reduction in mortality was observed in J α 281 KO mice, as compared to control mice. These data are in line

Fig. 5. Caspase-1 and caspase-3 activity in the lungs of mice. Wild-type (black bars) and Jα281 KO mice (white bars) were intra-tracheally infected with *L. pneumophila* (1×10^7 c.f.u. per mouse). Caspase-1 (a) and caspase-3 (b) activities in total lung homogenates were determined at the indicated time points after bacterial challenge ($n=5$). Data are expressed as fold increase compared with those of control mice ($n=5$). * $P < 0.05$ compared with wild-type mice.

with the findings of others demonstrating that NKT cells can be either beneficial or detrimental to host anti-bacterial defences, depending upon the inciting pathogen used. Our data further suggest the possibility that NKT cells may contribute to the production of the pyroptosis-related cytokines IL-1β and IL-18, by modulating the caspase-1 cascade.

Previously, we reported that acute lung injury may be a major cause of death in mice with *L. pneumophila* pneumonia (Tateda *et al.*, 2003). As expected, a significant elevation of acute lung injury markers, including lung weight and albumin levels in BAL fluids, was observed in the lungs of mice with *L. pneumophila*. Importantly, Jα281 KO mice demonstrated lower lung weights and BAL albumin levels, indicative of reduced lung injury. The relatively modest differences in *L. pneumophila* c.f.u. between the strains at the 72 h time point is unlikely to account for the more impressive differences in survival and magnitude of lung injury observed in this study.

The mechanisms accounting for the greater resistance of Jα281 KO mice to *L. pneumophila* have not been completely defined. We observed enhanced leukocyte accumulation and a more modest but significant reduction

of bacterial burden in the lungs of Jα281 KO mice on day 3. Although significantly higher levels of IL-1β and IL-18, but not IL-12 and IFN-γ, in the lungs of Jα281 KO mice were noted as early as 6 h post-challenge, the differences were modest and transient. These data suggested involvement of other factors, such as cell accumulating signals, mediators and responses. IL-1β and IL-18 are reported to have several biological roles in inflammatory and immunological events, which may be associated with exaggerated inflammatory cell accumulation in the lungs of Jα281 KO mice. The present data also demonstrated enhanced activation of caspase-1 in Jα281 KO mice at between 6 and 12 h, which may suggest a contribution of NKT cells to the pyroptosis cascade. Mechanisms by which NKT cells modulate the pyroptosis cascade are an ongoing area of investigation.

Accumulating data demonstrated that NKT cells are a critical component for induction of innate immunity against a variety of pathogens. A characteristic feature of NKT cells is the prompt production of Th1 (IL-12, IFN-γ) and/or Th2 (IL-4, IL-10) cytokines in response to invading pathogen, although how the Th1/Th2 cytokine balance is determined at the cellular level is incompletely understood. Moreover, the complexity of NKT cell immunology is illustrated by the fact that blocking of NKT cells by specific antibody or NKT gene-disruption in mice made them more resistant to some organisms. For example, in systemic *Listeria monocytogenes* infection, antibody-mediated inhibition of NKT cells reduced TGF-β2 production, while concomitantly increasing Th1 cytokines (IL-12, IFN-γ), which was associated with amelioration of survival (Szalay *et al.*, 1999). Bilenki and collaborators have reported that NKT cell activation promotes *Chlamydia trachomatis* infection in mice, in which exaggerated IL-4, IL-5 and IL-10 production and skewed Th2-type host responses were observed (Bilenki *et al.*, 2005). In the present study, a shifting of Th1/Th2 balance was not demonstrated in Jα281 KO mice, and in our hands IL-4 was not detected in the lungs of either group of mice during the observation period. In contrast, exogenous α-GalCer treatment induced a prompt response of IL-10, IL-12 and IFN-γ in control mice, but not in Jα281 KO mice, although these alterations were not associated with changes in the number of bacteria in the lungs. These data indicate that NKT cells have a

Fig. 6. Levels of IL-1β production in the lungs of mice in response to heat-killed *L. pneumophila*. Wild-type (black bars) and Jα281 KO mice (white bars) were injected intravenously with heat-killed *L. pneumophila* (4×10^8 c.f.u. per mouse) (a) or *E. coli* LPS (50 μg per mouse) (b). The levels of IL-1β in the lung homogenates were evaluated 2 and 4 h after the injection ($n=5$). * $P < 0.05$ compared with wild-type mice.

promoting role in *L. pneumophila* challenge, as they do in *Chlamydia* and *Listeria*. However, the previously proposed biased production of Th1/Th2 cytokines may not explain the resistant mechanism(s) in J α 281 KO mice.

L. pneumophila-mediated activation of NK cells and their robust IFN- γ production dependent on MyD88 expression have been reported to represent the crucial mechanism of *in vivo* control of *L. pneumophila* infection (Sporri *et al.*, 2006). Another study also indicated that NK cells respond *in vivo* and *in vitro* to stimulation by producing IFN- γ and by increased cytolytic activity (Blanchard *et al.*, 1988). In the present study, however, there was no difference in IFN- γ production between wild-type and J α 281 KO mice. Our findings suggest that unlike NK cells, IFN- γ production may not be the key factor in modulating host responses by NKT cells.

The present data may not correctly reflect human *L. pneumophila* infection because there was no growth of bacteria in the lungs. The important fact is that murine macrophages exhibit strain-dependent levels of *in vitro* resistance to *Legionella* replication, ranging from the highly susceptible A/J strain to the resistant C57BL/6 strain (Diez *et al.*, 2003; Gowney & Dietrich, 2000; Wright *et al.*, 2003). Consistent with these data, previously we have reported 10–100-fold replication of *L. pneumophila* in the lungs of A/J mice, but not C57BL/6 mice (Tateda *et al.*, 2001). In the present study, we have used wild-type and J α 281 KO mice in a C57BL/6 background, and as expected no growth of the bacteria was observed in the lungs. In spite of these experimental limitations, several investigators have examined pathogenesis of *L. pneumophila* infection in C57BL/6 background mice (Molofsky *et al.*, 2006; Archer & Roy, 2006). Another important factor to consider with respect to human relevance of this model is the challenge dose of the bacteria. We have used 3×10^6 c.f.u. per mouse for survival experiments. This number is quite similar to that in the previous reports (Zamboni *et al.*, 2006; Archer & Roy, 2006), where 1×10^6 c.f.u. per mouse was used for pulmonary *L. pneumophila* infection in C57BL/6 mice. Importantly, we have obtained substantially the same results in lower challenge dose experiments (3×10^5 c.f.u. per mouse), no increase of bacteria and higher levels of IL-1 β and IL-18 in J α 281 KO mice (data not shown).

Heat-killed *L. pneumophila* was prepared as a crude LPS, and its biological activity compared to that of *E. coli* LPS. The data obtained from these experiments were that heat-killed *L. pneumophila*, but not *E. coli* LPS, reproduced exaggerated IL-1 β in the lungs of J α 281 KO mice at early time points after stimulation. *L. pneumophila* LPS is known to have a specific structure characterized with a longer fatty acid side chain and stronger hydrophobic nature (Knirel *et al.*, 1996; Moll *et al.*, 1997; Girard *et al.*, 2003). We and other investigators have reported that *L. pneumophila* LPS is recognized through TLR-2, but not TLR-4, in macrophages (Archer & Roy, 2006; Akamine *et al.*, 2005; Braedel-Ruoff *et al.*, 2005; Fuse *et al.*, 2007). Given that

macrophages are both a major niche *in vivo* for *L. pneumophila* infection and cellular sources of IL-1 β /IL-18, the present data suggest an important interplay between NKT cells and macrophages. Intracellular and intercellular regulation of the pyroptosis cascade in macrophages, probably involving TLR- and NOD-mediated signalling, may be crucial for host defence systems against an intracellular pathogen such as *L. pneumophila*.

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