NK T Cell Activation Promotes *Chlamydia trachomatis* Infection In Vivo

Laura Bilienki, Shuhe Wang, Jie Yang, Yijun Fan, Antony George Joyee and Xi Yang

*J Immunol* 2005; 175:3197-3206; doi: 10.4049/jimmunol.175.5.3197

http://www.jimmunol.org/content/175/5/3197

References  This article cites 41 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/175/5/3197.full#ref-list-1

Why *The JI?* Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
NK T Cell Activation Promotes Chlamydia trachomatis Infection In Vivo

Laura Bilenki, Shuhe Wang, Jie Yang, Yijun Fan, Antony George Joyee, and Xi Yang

We used two approaches to examine the role of NK T cells (NKT) in an intracellular bacterial (Chlamydia trachomatis mouse pneumonitis (C. muridarum)) infection. One is to use CD1 gene knockout (KO) mice, which lack NKT, and the other is to activate NKT using α-galactosylceramide (α-GalCer), a natural ligand of these cells. The data showed a promoting effect of NKT activation on Chlamydia lung infection. Specifically, CD1 KO mice exhibited significantly lower levels of body weight loss, less severe pathological change and lower chlamydial in vivo growth than wild-type mice. Immunological analysis showed that CD1 KO mice exhibited significantly lower C. muridarum-specific IL-4 and serum IgE Ab responses as well as more pronounced delayed-type hypersensitivity response compared with wild-type controls. In line with the finding in KO mice, the in vivo stimulation of NKT using α-GalCer enhanced chlamydial growth in vivo, which were correlated with reduced delayed-type hypersensitivity response and increased C. muridarum-driven IL-4/IgE production. Moreover, neutralization of IL-4 activity in the α-GalCer-treated BALB/c mice significantly reduced the promoting effect of α-GalCer treatment on chlamydial growth in vivo. These data provide in vivo evidence for the involvement of NKT in a bacterial pathogenesis and its role in promoting Th2 responses during infection. The Journal of Immunology, 2005, 175: 3197–3206.

Laboratory for Infection and Immunity, Department of Immunology, and Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Received for publication November 2, 2004. Accepted for publication June 9, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by operating grants from the Canadian Institutes for Health Research and the Manitoba Health Research Council. L.B. holds a Canada Graduate Scholar Award (doctoral award) from Canadian Institutes for Health Research. X.Y. is Canada Research Chair in Infection and Immunity.

2 Address correspondence and reprint requests to Dr. Xi Yang, Laboratory for Infection and Immunity, Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Room 523, 730 William Avenue, Winnipeg, Manitoba, Canada R3E 0W3. E-mail address: yangxi@cc.umanitoba.ca

3 Abbreviations used in this paper: NKT, natural killer T cell; C. muridarum, Chlamydia trachomatis mouse pneumonitis; IFU, inclusion-forming unit; KO, gene knockout; α-GalCer, α-galactosylceramide; SPG, sucrose-phosphate-glutamic acid; DTH, delayed-type hypersensitivity.

Copyright © 2005 by The American Association of Immunologists, Inc.
Materials and Methods

Mice
Control BALB/c mice (7–10 wk old) and breeding pairs of CD1 KO mice with BALB/c background (C129-Cd1tmGru, 7–10 wk old) used in the study were bred at the University of Manitoba (Winnipeg, Manitoba, Canada) breeding facility. The breeding pairs of CD1 KO mice, which were originally developed in a 129 background and had been backcrossed to BALB/c 11 times, were purchased from The Jackson Laboratory. Another control strain, 129 mice, was purchased from The Jackson Laboratory. Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care.

Organism
The culture and preparation of C. trachomatis mouse pneumonitis (C. muridarum) were performed as described (25, 26). Briefly, C. muridarum was cultured in HeLa 229 cells in Eagle’s MEM containing 10% FBS and 2 mM L-glutamine for 48 h. For inoculum preparation, infected cells were harvested with sterile glass beads and partially purified by successive 15-min 500 × g and 30-min 30,000 × g centrifugations. The partially purified organisms were resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen at −80°C until used. The same seed stock of C. muridarum was used throughout the study. For the anti-Chlamydia Ab ELISA, the C. muridarum elementary body preparations were further purified by step gradient centrifugation using 35% Renografin (Squibb).

Infection of mice and quantification of chlamydial in vivo growth
Mice were inoculated intranasally with 1 × 10^5 inclusion-forming units (IFU) C. muridarum or PBS. Unless specified, mice were killed on day 11 postinoculation, the peak of infection. The lungs, liver, kidneys, and heart were aseptically isolated and homogenized using a cell grinder in SPG buffer. Tissue suspensions were centrifuged down at 1900 × g for 30 min at 4°C to remove coarse tissues and debris and frozen at −80°C until tested. For C. muridarum quantitation, HeLa 229 cells were grown to confluence in 96-well flat-bottom microtiter plates and washed in 100 μl of HBSS. The monolayers were then inoculated in triplicate with 100 μl of serially diluted organ tissue supernatants from mice infected with C. muridarum. After 2 h of incubation at 37°C, plates were washed, and 200 μl of MEM containing cycloheximide (1.5 μg/ml), gentamicin (20 μg/ml), and vancomycin (25 μg/ml) were added to each well. The plates were incubated for 48 h at 37°C in 5% CO2. After the incubation, the culture medium was removed, and the cell monolayers were fixed with absolute methanol. To identify chlamydial inclusions, plates were incubated with a Chlamydia-specific murine mAb and stained with goat anti-mouse IgG conjugated to HRP and developed with substrate (4-chloro-1-naphthol; Sigma). The number of inclusions was counted under a microscope at ≥200 magnification. Five fields through the midline of each well were counted. The chlamydial levels in each organ were calculated based on dilution titers of C. muridarum-specific IgE as well as total IgE were measured by ELISA using Abs purchased from BD Pharmingen. For the measurement of C. muridarum-specific IgE, serum samples were pretreated by incubating twice with a 50% slurry of protein G-Sepharose (Pharmacia Biotech) to remove IgG (27). This treatment removes >95% IgG from the sera. For C. muridarum-specific IgG1 and IgG2a, sera without protein G-Sepharose treatment were tested. The Abs for IgG1 and IgG2a ELISA were purchased from Southern Biotechnology Associates. Briefly, to determine C. muridarum-specific serum antibodies, ELISA plates were coated overnight with C. muridarum elementary bodies in bicarbonate buffer (0.05 M, pH 9.6). After blocking for 90 min with a 2% BSA, 0.05% Tween 20 solution and extensive washing, serially diluted sera were incubated for 2 h at 37°C. The plates were washed, and biotinylated goat anti-mouse Ab was added and incubated for overnight at 4°C. Alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, BioCan Scientific) was added, and the plates were kept for incubation at room temperature for 45 min. After extensive washing of the plates, p-nitrophenyl phosphate (in 0.5 M Tris, pH 9.8) was added and the reaction was allowed to proceed for 60 min. The plates were read with a microplate reader (Versamax; Molecular Devices) at 405 nm. For measurement of total IgE, ELISA plates were coated with purified anti-mouse IgE capture Ab and detected using biotin anti-mouse IgE Ab as described previously (28).

Flow cytometry and peripheral blood differential
Cell surface markers were analyzed by flow cytometry. Single-spleen cell suspensions from naive mice (euthanized) were lysed using NH4Cl. Cells (2 × 10^6 cells) were stained using fluorescent-labeled mAbs purchased from BD Pharmingen. The Abs included PE-anti-mouse CD3 (clone 145-2C11, hamster IgG1, κ), PE-anti-mouse CD4 (clone GK1.5, rat (Lewis) IgG2b, κ), FITC-anti-mouse CD8 (clone 53-6.7, rat (LOU/Ws1/M) IgG2a, κ), FITC-anti-mouse CD19 (clone ID3, rat (Lewis) IgG2a, κ), FITC-anti-mouse pan-NK (clone DX5, rat IgG2a, κ), FITC-anti-mouse TCR β-chain (clone GL3, Armenian hamster IgG2, κ), and corresponding isotype controls. NK cells were stained using PE-mCD9/PBS57 ligand tetramer (generously provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility, Atlanta, GA). After incubation with Ab-tetramer on ice for 30 min in the dark, the cells were fixed using 4% parformaldehyde, washed, resuspended in PBS containing 0.2% BSA, and analyzed using a FACS Calibur flow cytometer and CellQuest program (BD Biosciences). Blood smears were analyzed for leucocyte differential using a Hema 3 Stain Set from Fisher Scientific based on the manufacturer’s instruction.

Lymphocyte proliferation
For cell proliferation measurements, spleens were aseptically excised from naïve mice, and 3.0 × 10^6 cells were either incubated alone or with 4 μg/ml Con A (Sigma-Aldrich) in 96-well cell culture plates for 48 h. After incubation, 1 μCi of [3H]thymidine (Sigma-Aldrich) was added to each well of culture and incubated at 37°C for 6 h. [3H]Thymidine incorporation was analyzed thereafter, using a TopCount NXT microplate scintillation and luminescence counter (Canberra Packard Biosciences).

Treatment with α-GalCer (KRN70000) and anti-IL-4 mAb
α-GalCer (25,33,4R-1-(O-galactopyranosyl)-2-(N-hexaosanoylamino)-1,3,4-octadecanetriol) was synthesized by Pharmaceutical Research Laboratories, Kirin Brewery. The 200-μg/ml stock solution was dissolved to a
CD1 KO mice show reduced morbidity, lower chlamydial in vivo growth, and less pathological changes in the lung

To examine their susceptibility to chlamydial infection, CD1 KO and BALB/c wild-type mice were intranasally infected with a sublethal dose (1 × 10^3 IFU) of *C. muridarum* and monitored for body weight changes and in vivo growth of the organism. As shown in Fig. 1A, the body weight loss was significantly less in CD1 KO mice than in wild-type mice. The overall physical condition of CD1 KO mice was also better (i.e., more activity, less fur ruffling and dehydration) than that of wild-type controls. The chlamydial levels in the lung in CD1 KO mice were close to 100-fold lower than those in control mice (Fig. 2, B and C). The same is true for the chlamydial burdens in the liver. More importantly, although wild-type mice showed detectable levels of *Chlamydia* in the heart, CD1 KO mice consistently exhibited negativity of *Chlamydia* in this organ. Immunofluorescence staining of the lung sections also showed fewer chlamydial inclusions in the lung tissues of CD1 KO mice than in those of BALB/c mice (Fig. 2, C and F). More interestingly, the inclusions in the lung were significantly smaller in CD1 KO mice than in wild-type mice (Fig. 2, C and F). The results indicate higher resistance of CD1 KO mice to chlamydial infection, including the dissemination of the infection.

The histological analysis showed differential changes in the lung in the two types of mice (Fig. 2). BALB/c mice showed severe tissue inflammation with inflammatory exudates and diffused cellular infiltration in the lung, indicative of a significant pathological reaction. The cellular infiltrates were comprised of not only lymphocytes and macrophages but also a significant portion of neutrophils (Fig. 2B). In contrast, in the CD1 KO mice, the inflammatory changes in the lungs were much less severe, characterized by more localized and less dense cellular infiltration with mainly macrophages and lymphocytes (Fig. 2E). Neutrophils were hardly seen in the lung of infected CD1 KO mice. These observations demonstrate that CD1 KO mice have less severe tissue damage during the process of chlamydial infection.

**Statistical analysis**

Ab titers (ELISA) were converted to logarithmic values and analyzed using the unpaired Student t test. IFU detection and cytokine production levels were analyzed using the unpaired Student t test.

**Results**

**CD1 KO mice exhibit reduced C. muridarum-specific Th2 cytokine production**

To examine the effect of NKT activation on T cell responses to chlamydial infection, we analyzed the *C. muridarum*-driven cytokine production by spleen and lymph node cells from the infected CD1 KO and BALB/c mice. The results revealed that both spleen and lymph node cells of *C. muridarum*-infected CD1 KO mice produced significantly lower levels of IL-4 and IL-10 than those of the infected wild-type mice. The production of other Th2-like cytokines such as IL-13 and IL-10 also appeared to be in a trend of reduction in the *C. muridarum*-infected CD1 KO mice, although the differences were not statistically significant (Fig. 3). Analysis of Th1-like cytokine showed similar levels of *C. muridarum*-driven IFN-γ production between CD1 KO and wild-type mice. Similarly, the two strains of mice showed similar levels of pro-Th1 (IL-12 and IL-18) and proinflammatory cytokine (TNF-α) production (Fig. 4). Our results demonstrate a role of NKT in promoting Th2 type cytokine responses to chlamydial infection.
CD1 KO mice show significantly lower serum C. muridarum-specific IgE and IgG1 Ab responses

We then measured the levels of total IgE as well as C. muridarum-specific IgE, C. muridarum-specific IgG1 and IgG2a in serum samples from CD1 KO and BALB/c control mice before and after chlamydial infection. After infection, the levels of total IgE as well as C. muridarum-specific IgE were increased in both types of mice, but the levels of IgE Abs were significantly lower (p < 0.05) in the CD1 KO mice than wild-type mice (Fig. 5, A and B). Whereas the C. muridarum-specific serum IgG2a levels were similar between the two groups of mice, the IgG1 levels were significantly lower in the CD1 KO mice than in the wild-type mice (Fig. 5, C and D). The results indicate that the production of Ab isotypes that are regulated by Th2 cytokines, especially IL-4, were significantly affected in NKT-deficient CD1 KO mice.

CD1 KO mice exhibit enhanced DTH response after C. muridarum infection

To examine whether the increased resistance to chlamydial infection observed in CD1 KO mice was correlated with alterations in cell-mediated immune responses, we tested DTH responses in these mice after infection. Although DTH response could be observed in both CD1 KO and wild-type mice, the levels of these responses were markedly stronger in the former than in the latter. Statistically significant enhancement in DTH responses in CD1 KO mice was determined at 48 and 72 h after footpad challenge with heat-inactivated EBs (Fig. 5E). The data suggest that NKT cells play an immunoregulatory role in T cell-mediated immune responses in vivo during chlamydial infection.

Similar difference between CD1 KO mice and wild-type controls is observed in experiments using 129 mice

Because the CD1 KO mice used in the study were originally developed on a 129 background, they might still carry some 129 background genes although they have been backcrossed to BALB/c for 11 generations. Because previous studies have shown that different mouse strains are variable in susceptibility to chlamydial infection (29–31), it is important to exclude the possibility that the differences observed between CD1 KO mice and BALB/c controls shown above are caused by the potential slight differences of the mice in genetic backgrounds. To this end, we also performed some experiments using 129 mice as controls. As shown in Fig. 6, similar to the differences observed between CD1 KO and BALB/c mice, a significantly less body weight loss and Chlamydia growth was observed in CD1 KO mice compared with 129 mice. Again, the IL-4 production in CD1 KO mice was significantly lower than in wild-type (129) mice. The results suggest that the difference observed between CD1 KO mice and wild-type mice is due to the deficiency in NKT rather than the potential slight difference in genetic background between these mice.

CD1 KO and wild-type controls display similar levels of lymphocyte number and function

To exclude the possibility that the altered susceptibility and immune responses observed in CD1 KO mice after C. muridarum infection might be due to the potential alteration of other immune cells in the genetically modified mice, we analyzed the intrinsic levels of T, B, and NK cells in these KO mice. As shown in Table I, the percentages of T cells (CD4+ and CD8+ cells), γδ T cells, NK cells (DX5+ cells), B cells (CD19+ cells) and dendritic cells (CD11c+ cells) in the spleen of CD1 KO mice were similar to those found in the wild-type control mice. Not surprisingly, there were significantly fewer NKT cells in CD1 KO mice than in BALB/c mice. Similar results were obtained in analysis using peripheral blood cells (data not shown). Moreover, we found that the levels of neutrophils and monocytes in the peripheral blood were similar between BALB/c and CD1 KO mice (Table II). Furthermore, we examined the functional integrity of the T lymphocytes from CD1 KO mice. Spleen cells from BALB/c and CD1 KO mice were cultured alone or in the presence of Con A, a polyclonal T activator. After Con A stimulation, spleen cells from CD1 KO mice and wild-type mice showed similar levels of proliferation and production of IL-4 (Table I). These data suggest that, although the CD1 KO mice are deficient in NKT, their major immune cells are normal in amount and function. Therefore, the alteration in host
susceptibility to chlamydial infection observed in CD1 KO mice is unlikely to be due to potential intrinsic changes in immune cells other than NKT.

**Stimulation of NKT activity with α-GalCer enhances chlamydial growth in vivo, which is correlated with enhanced Th2 cytokine/IgE production and reduced DTH responses**

α-GalCer is a natural ligand of type 1 NKT, which can specifically stimulate NKT activity in the context of CD1 in vivo (32, 33). To further confirm the regulatory role of NKT in chlamydial infection, we tested the effect of α-GalCer treatment in wild-type BALB/c and CD1 KO mice on *C. muridarum* infection. BALB/c mice treated with α-GalCer showed substantially enhanced chlamydial growth in the lung (Fig. 7A). In addition, the α-GalCer-treated BALB/c mice displayed significantly increased *C. muridarum*-driven IL-4 and IL-5 production and *C. muridarum*-specific IgE production in comparison with those without the treatment. Moreover, the α-GalCer-treated mice exhibited less pronounced DTH responses after *C. muridarum* infection (Fig. 7B). Interestingly, IFN-γ levels were similar between the *C. muridarum*-infected mice with or without α-GalCer treatment. Similar alteration of immune responses after α-GalCer treatment was observed in the other wild-type control strain, 129 mice (data not shown). As expected, CD1 KO mice failed to respond to α-GalCer treatment in the perspectives of chlamydial growth and IL-4, IgE, and DTH responses, confirming the specificity of α-GalCer on CD1/NKT. Moreover, neutralization of IL-4 activity in vivo in the αGalCer-treated BALB/c mice significantly reduced the promoting effect of α-GalCer treatment on chlamydial growth in vivo (Fig. 7H). Taken together, these observations provide solid evidence that NKT play an important role in modulating the immune responses to *Chlamydia* during a lung infection, thus significantly influencing the process of this infection. In addition, the production of IL-4 by NKT is a key mechanism for the immunomodulating and infection-promoting effect of these cells.
Discussion

In the present in vivo study, we investigated the role of NKT cells in modulating immune responses against \textit{C. muridarum} infection. We found that CD1 KO mice lacking NKT mounted more protective immune responses than the wild-type control (BALB/c and 129) mice after \textit{C. muridarum} infection. This conclusion is supported by the observations that \textit{C. muridarum}-infected CD1 KO mice showed significantly less body weight loss, lower chlamydial in vivo growth, and milder pathological changes during the infection. Moreover, it is supported by the finding that stimulation of NKT using \( /H_{9251}\)-GalCer increased host susceptibility to chlamydial infection. The data indicate that CD1d-restricted NKT cells can modulate the immune response to \textit{Chlamydia} infection and contribute to pathological outcome caused by the infection. Because only classical type 1, but not type 2 CD1-dependent, NKT are responsive to \( /H_{9251}\)-GalCer stimulation (7), the results suggest that the NKT that play immunomodulatory role during chlamydial infection in the lung are type 1 NKT cells.

Cytokine analyses have shed light on the mechanism by which NKT influence host susceptibility to chlamydial infection. Previous animal and human studies in our laboratory (26, 29) and by other researchers (23, 34) have shown that Th2 cytokine responses are associated with susceptibility to chlamydial infection and accompanying pathology. In this study, we further demonstrated that the reduced pathology and decreased bacterial burden in CD1 KO mice were correlated with a reduced Th2 type immune response, especially lower levels of \textit{C. muridarum}-driven IL-4 and IL-5 production. The initial IL-4 production by NKT may be the mechanism by which these cells modulate adaptive immune response and promote host susceptibility to chlamydial infection, because neutralization of IL-4 immediately after \( /H_{9251}\)-GalCer treatment significantly reduced the promoting effect of this NKT ligand on chlamydial growth in vivo (Fig. 7H). The reduction in Th2 cytokines in the CD1 KO mice was associated with a significant reduction in serum total IgE, \textit{C. muridarum}-specific IgE and IgG1 responses. This appears to be the first report regarding \textit{Chlamydia}-specific IgE in mouse models and its correlation with susceptibility to chlamydial infection. A strong link has been established by previous studies between the production of Th2 cytokines, especially IL-4, in response to a given Ag and the subsequent production of the IgE response.
The significant reduction in *C. muridarum*-specific IgE therefore could be explained by the lower levels of *C. muridarum*-specific IL-4 production in the infected CD1 KO mice. It is unlikely that the reduced IL-4 response, IgE and IgG1 Ab levels in CD1 KO mice are due to their potential intrinsic deficiencies, because the level and function of the immune cells in the two types of mice were similar (Table I). However, although *Chlamydia*-specific IgE response was observed in the study, the particular role,

**FIGURE 5.** Significantly lower levels of IgE and IgG1 production and stronger DTH responses in CD1 KO mice than in control BALB/c mice. A and B, The sera collected from mice (four mice/group) before and after (day 11) intranasal *C. muridarum* infection (1000 IFU) were determined for total IgE, *C. muridarum*-specific IgE, IgG1, and IgG2a using ELISA. For detection of *C. muridarum*-specific IgE, the sera were pretreated twice with a 50% slurry of protein G-Sepharose to remove IgG. Untreated samples were tested for *C. muridarum*-specific IgG1 (C) and IgG2a (D). ELISA readings (OD) at proper dilutions are presented as the mean ± SEM. *, *p < 0.05, *C. muridarum*-infected CD1 KO mice vs BALB/c control mice infected with *C. muridarum*. Pooled data for three experiments (16 mice in each group) are presented. For DTH response (E), mice (four mice/group) were intranasally infected with *C. muridarum* (1000 IFU) and challenged with heat-inactivated *C. muridarum* in the footpads at 11 days postinfection. Footpad swelling was measured at 24, 48, and 72 h after dead *C. muridarum* injection. The difference in the thickness of footpads with or without *C. muridarum* injection was used as a measure of DTH responses as described in Material and Methods. Data show the mean ± SD. *, *p < 0.05, comparison between CD1 KO and wild-type mice. One of three independent experiments with similar results is shown.

**FIGURE 6.** Similar differences between CD1 KO and wild-type mice were observed in experiments using 129 mice as controls. CD1 KO and 129 mice were intranasally infected with *C. muridarum* (1000 IFU) and monitored for body weight changes. At day 16 after infection, mice were killed, and chlamydial in vivo growth in the lung and organism-driven cytokine production by spleen cells were analyzed using the methods described in Materials and Methods. *, *p < 0.05, 129 vs CD1 KO mice.
Comparison between intrinsic properties of BALB/c and CD1 KO mice
c

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>CD1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>73.7 ± 0.9</td>
<td>77.5 ± 0.7</td>
</tr>
<tr>
<td>CD4+</td>
<td>65.7 ± 1.9</td>
<td>70.8 ± 1.2</td>
</tr>
<tr>
<td>CD8+</td>
<td>10.1 ± 2.0</td>
<td>7.90 ± 1.3</td>
</tr>
<tr>
<td>B cells</td>
<td>21.5 ± 1.4</td>
<td>22.3 ± 0.9</td>
</tr>
<tr>
<td>γδT cells</td>
<td>12.3 ± 0.7</td>
<td>13.2 ± 1.2</td>
</tr>
<tr>
<td>NK cells</td>
<td>17.9 ± 1.4</td>
<td>19.2 ± 2.3</td>
</tr>
<tr>
<td>NKT cells</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Proliferation index</td>
<td>4.1</td>
<td>4.6</td>
</tr>
<tr>
<td>IL-4 production (pg/ml)</td>
<td>880</td>
<td>916</td>
</tr>
</tbody>
</table>

* Blood smears from naive BALB/c and CD1 KO mice were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology.

A Naive CD1 KO mice display similar levels of major immune cells and the T cell function to BALB/c mice. Spleen cells from naive BALB/c and CD1 KO mice were prepared and stained for T, B (CD19), NK (DX5+), dendritic cell (CD1c+), and NKT (CD1 d/PBS57 ligand) cell markers using fluorescence-labeled mAbs/hamster and analyzed by flow cytometry using a FACSCalibur II and CellQuest program. For cellular proliferation measurements, single-spleen cell suspensions were cultured in the presence or absence of Con A stimulation. Con A-stimulated cells were pulsed with [3H]thymidine, and [3H]thymidine incorporation was assessed using a TopCount NXT microplate scintillation and luminescence counter. Stimulation index was calculated based on the folds of counts in Con A-stimulated cells over the control wells; IL-4 levels after polyclonal stimulation in the 72-h culture supernatants were measured by ELISA.

if any, of this Ab in host susceptibility to chlamydial infection remains to be tested. Moreover, Perry et al. (36) reported previously that IL-4 was undetectable in the spleen cell culture after genital tract chlamydial infection. Therefore, whether the finding in IL-4 and IgE production after lung infection is applicable to genital tract chlamydial infection. Our previous study has demonstrated a promoting role of NKT in a bacterial infection (38). Our present study, however, demonstrated an opposite role of NKT in a bacterial infection using both CD1 KO mice and α-GalCer treatment approaches. A recent study also showed a suppressive role of NKT on protective immunity against Toxoplasma gondii, a parasitic infection using an approach of Ab depletion of NKT cells in vivo (39). The role of NKT cells in enhancing Th2-like responses has been demonstrated in mouse allergy models recently (40, 41). It appears that allergens (OVA and ragweed) can activate NKT in vivo, resulting in increased allergen-driven IL-4 production. Considering the finding of increased IL-4 and IgE responses in the chlamydial infection model, Chlamydia may contain components differing from those of some other intracellular bacteria, which preferentially activate IL-4-producing NKT cells, thus enhancing the adaptive organism-specific Th2-type immune response. Further studies on the potential subtypes of NKT and/or the mechanisms by which different types of NKT are activated will provide insight into the basis that determines the promoting or suppressive role of NKT on host defense against infectious diseases.

Collectively, the findings of the present study suggest that NKT may play a crucial role in the regulation of the immune responses to active chlamydial infection. Our data reconfirm that Th2-like immune responses mediate host susceptibility to, and pathological consequences of, chlamydial infection. More importantly, the data suggest that NKT cells may not be always protective but rather may enhance host susceptibility to certain infections. It would be important to further evaluate the role of NKT in various infections and the mechanisms underlying the variability in the role of NKT to derive new preventive and therapeutic strategies for infectious diseases.

Table II. Comparison between intrinsic properties of BALB/c, 129, and CD1 KO mice

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>CD1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>70.6 ± 0.96</td>
<td>71.5 ± 0.70</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>15.7 ± 0.41</td>
<td>12.1 ± 0.74</td>
</tr>
<tr>
<td>Monocytes</td>
<td>15.4 ± 0.23</td>
<td>11.0 ± 0.56</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.14 ± 0.32</td>
<td>0.20 ± 0.67</td>
</tr>
</tbody>
</table>

* Blood smears from naive BALB/c and CD1 KO mice were stained using a Hema 3 Stain Set (Fisher Scientific). Differential cells were counted based on cell characteristics and cellular morphology.
FIGURE 7. Effect of in vivo stimulation of NKT by α-GalCer treatment on host response to Chlamydia and the role of IL-4. A–G, Naive CD1 KO and BALB/c mice (four mice/group) were injected i.v. with 4 μg of α-GalCer in PBS or with polysorbate vehicle control. Mice were intranasally infected with C. muridarum (MoPn; 1000 IFU), 2 h after α-GalCer or vehicle injection. Mice were sacrificed on day 11 for analysis of serum IgE, C. muridarum-specific cytokine production by spleen cells and chlamydial growth in the lung using the methods described in Materials and Methods. For DTH responses (B), C. muridarum-infected mice were challenged in the footpads with dead C. muridarum as described in Fig. 5, and the footpad swelling at 72 h post footpad challenge is shown. *, p < 0.05. One representative experiment of three independent experiments is shown. H, Neutralization of IL-4 activity in vivo reduced the promoting effect of α-GalCer treatment on chlamydial in vivo growth. BALB/c mice (three mice/group) received a single i.v. injection of 4 μg of α-GalCer diluted in PBS. Immediately after α-GalCer injection, mice were treated i.p. or not with 0.5 mg of anti-IL-4 Ab (clone 11B11) in PBS. After 1 h, both groups were infected with 1.0 × 10^3 IFU C. muridarum intranasally. Five days postinfection, mice initially treated with anti-IL-4 mAb were reinjected i.p. with this Ab at the same dose. Both groups were sacrificed on day 11 postinfection, and the chlamydial burden in various organs was determined as described in Fig. 1. *, p < 0.001.

Acknowledgments
We thank Dr. Y. Koezuka (Kirin Pharmaceuticals) for kindly providing α-GalCer for our experiments.

Disclosures
The authors have no financial conflict of interest.

References
2. Nieuwenhuis, E. E., T. Matsumoto, M. Exley, R. A. Schleipman, J. Glickman, 3206 NKT PROMOTES CHLAMYDIAL INFECTION
6. Smyth, J. M., K. Y. T. Thia, S. E. A. Street, E. Cretney, J. A. Trapani,
7. Leite-de-Moraes, M. C., A. Hameg, M. Pacilio, Y. Koezuka, M. Taniguchi,
8. Singh, A. K., M. T. Wilson, S. Hong, D. Olivares-Villagomes, C. Du,
13. Apostolou, I., A. Takahama, C. Belmant, T. Kawanou, M. Huerre, G. Marchal,
14. Gonzalez-Aseguinolaza, G., C. Oliveira, M. Tomaska, S. Hong,
17. Nieuwenhuis, E. E., T. Matsumoto, M. Edsky, R. A. Schleipman, J. Glickman,
22. Nieuwenhuis, E. E., T. Matsumoto, M. Edsky, R. A. Schleipman, J. Glickman,
33. Bardin, N., L. Brossay, and M. Kronenberg. 1999. Immunization with α-galactosylceramide polarizes CD1d-reactive T cells towards Th2 cytokine synthe-
36. Yang, X. 2001. Distinct function of Th1 and Th2 type delayed type hypersensi-