Th1 Cytokine Responses Fail to Effectively Control *Chlamydia* Lung Infection in ICOS Ligand Knockout Mice

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ICOS ligand (ICOSL) plays an important role in controlling specific aspects of T cell activation, differentiation, and function. Th1-type immune responses have been shown to be critical in host defense against chlamydial infections. To assess the role of ICOSL–ICOS interaction in host defense against chlamydial infection, we compared the immune responses and pathological reactions in ICOSL gene knockout (KO) and wild-type (WT) mice following Chlamydia muridarum lung infection. The results showed that ICOSL KO mice exhibited greater body weight loss, higher pathogen burden, and more severe histopathology in their lung than did WT mice. Cytokine analysis revealed that ICOSL KO mice produced lower levels of Th2 (IL-4 and IL-5) and anti-inflammatory (TGF-β1 and IL-10) cytokines, but higher Th1-related (IFN-γ and IL-12p40/IL-23) and proinflammatory (IL-6 and TNF-α) cytokines. ICOSL KO mice also showed reduced Chlamydia-specific Ab levels in their sera and lung homogenates. In addition, ICOSL KO mice demonstrated significantly lower ICOS expression in T cells and lower Th17 responses than did WT mice. Finally, we showed that ICOS–ICOSL interaction and cell–cell contact are essential for CD4+ T cells to inhibit chlamydial growth in the cultured lung fibroblasts. The data suggest that ICOSL plays a significant role in immunoregulation and protective immunity against Chlamydia infections and that the Th1 skew in cytokine responses per se is not sufficient for effective control of Chlamydia infections. The Journal of Immunology, 2010, 184: 3780–3788.
In the current study, we compared the susceptibility of ICOSL knockout (KO) and wild-type (WT) C57BL/6 mice to C. muridarum lung infection and examined their cytokine patterns and Ab responses following respiratory tract infection. The data revealed that the ICOSL KO mice had higher Th1 cytokine response, but lower Th2 cytokine and humoral immune responses, than did WT mice following C. muridarum infection. The KO mice also showed significantly higher proinflammatory cytokine (IL-6 and TNF-α) but lower immunoinhibitory (TGF-β and IL-10) production. Even with the dramatically higher IFN-γ production than that seen in WT mice, the ICOSL KO mice showed higher C. muridarum growth in the lung and more severe body weight loss and lung histopathological status than did the former. In vitro coculture experiments showed that ICOS–ICOSL interaction was essential for Chlamydia-specific T cells to inhibit intracellular Chlamydia growth in cultured primary lung fibroblasts. Moreover, we showed that the Th17 cell population was lower in ICOSL KO mice than in WT mice both before and after lung infection. The data demonstrate an important role of ICOS–ICOSL interaction in immunity to chlamydial infections.

Materials and Methods

Animals

Female C57BL/6 WT mice (7–10 wk old) were bred at the University of Manitoba (Winnipeg, Manitoba, Canada) breeding facility. Female ICOSL KO C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care, and the protocol was approved by the local ethical committee.

Reagents

Complete RPMI 1640 medium was prepared as previously described (32, 33). Fluorescence-conjugated anti-CD4, anti-ICOS, anti–IL-4, anti–IFN-γ, and anti–IL-17F Abs, matched isotype controls, and Fc-blocker Abs against CD16/32 were purchased from eBioscience (San Diego, CA). Paired Abs for ELISA measurement of IL-4, IL-5, IL-6, IL-10, IL-12p40/IL-23, IFN-γ, TGF-β1, and TNF-α were purchased from eBioscience (San Diego, CA). CD4 microbeads for isolating CD4+ T cells were purchased from Miltenyi Biotec (Auburn, CA). Biotinylated anti-IgG1, anti-IgG2a, and anti-IgA were purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated goat anti-mouse IgG was purchased from Sigma-Aldrich (St. Louis, MO).

Infection of mice

The culture and preparation of C. muridarum were performed as described (32, 33). Briefly, C. muridarum was cultured in HeLa 229 cells in RPMI 1640 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 followed by step gradient centrifugation using 35% Renografin. The purified organisms were resuspended in sucrose-phosphate-glutamic acid buffer and frozen at −80°C until used. The same seed stock of C. muridarum was used throughout the study.

Mice were inoculated intranasally with 1 × 10³ inclusion-forming units (IFUs) of C. muridarum, as described previously (32, 33). Mice were euthanized on days 6, 10, and 14 postinfection. The lungs were aseptically...
isolated and homogenized using a cell grinder in sucrose-phosphate-glu-
tamic acid buffer. Tissue homogenates were centrifuged down at 2000 × g for 30 min at 4°C to remove coarse tissue debris and quantified for chla-
mydial IFUs as described (32). The loads of Chlamydia in the lung were calculated as IFUs (logarithmic scale) in each lung based on dilution titers
of the original lung homogenate inocula to the culture. C. muridarum-specific IgG1 and IgG2a in the sera and IgA in the lung homogenates were measured by ELISA, as described (32, 33). Results are
expressed as ELISA titers, using the endpoint (cutoff at OD 0.5) of the titration curves. Each sample was measured at least three times.

**Histopathology and immunofluorescence**

Lungs isolated from sacrificed mice were immediately frozen in liquid
nitrogen for immunofluorescence staining and/or fixed in 10% neutral
buffered formalin for histological examination, as described previously (33). In brief, formalin-fixed tissues were embedded in paraffin. Sections of 5 μm were cut, stained with H&E, and examined by light microscopy. The ex-
amination was blinded with regard to which samples were derived from
ICOSL KO or WT C57BL/6 mice. For immunofluorescence microscopy, lung sections were cut (at a thickness of 10 μm) from the frozen tissue and were treated with blocking buffer, then incubated with mouse anti-
Chlamydia major outer membrane protein Ab (Chemicon International, Temecula, CA). After incubation, the sections were treated with FITC-
conjugated goat anti-mouse IgG Ab (Sigma-Aldrich); then they were ex-
amined using a fluorescence microscope (Olympus AX70; Olympus, Mel-
ville, NY) equipped with a fluorescence camera (Sensicam; Cooke, Romulus, MI) and investigated using the ImagePlus (Media Cybernetics, Silver Spring, MD) program to detect inclusion bodies inside the lung sections.

**Splenic cell culture**

Mice were euthanized 6, 10, and 14 d postinfection. Single-cell suspensions of
spleen cells were homogenized using a cell grinder and filtered by passing through a 40-μm cell strainer (BD Falcon; BD Biosciences, Franklin Lakes, NJ). RBCs were lysed using ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA); then spleen cells were cultured at 7.5 × 107/ml (2 ml/well), with or without UV-inactivated C. muridarum (1 × 106 IFU/ml), in 24-well plates at 37°C in RPMI 1640 medium containing 10% heat-in-
activated FCS, 1% fetal bovine serum (FBS), as described (32, 33). Culture supernatants were harvested at 72 h and stored at −80°C until they were assayed for cy-
tokines. Murine IL-4, IL-5, IL-6, IL-10, IL-12p40/IL-23, IFN-γ, TGF-B1, and TNF-α in the culture supernatants were measured by ELISA.

**FACS analysis**

Splenocytes were first enriched for CD4+ T cells using CD4 microbeads and MACS LS columns (Miltenyi Biotec) according to the manufacturer’s instructions. The enriched CD4+ cells were further analyzed for intracellular cytokines, as described (36). Briefly, cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 5 h in complete RPMI 1640 medium at 37°C. For the last 4 h of in-
cubation, Brefelding A (BD Biosciences) or monensin (eBioscience) was added to accumulate cytokines intracellularly. The cells (2 × 106) were washed twice and incubated with FcR blocker Abs (anti-CD16/32) for 20 min at 4°C to block nonspecific staining. After surface staining for CD4, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) per the manufacturer’s instructions and stained intracellularly using allopheocyanin-conjugated anti–IFN-γ (XMG 1.2) and anti–IL-4 (BVD6-24G2), or PE-conjugated anti–IL-17F (eBIO18F10), or with corresponding isotope control Abs in per-
meabilization buffer (BD Pharmingen) for 30 min at 4°C. Finally, the cells were washed and resuspended in staining buffer; then they were acquired using a FACS Calibur flow cytometer (BD Biosciences) and analyzed by Cell Quest Pro software. For the staining of cell-surface ICOS, the en-
riched CD4+ cell population was stained with allopheocyanin anti-mouse CD4 (eBioscience) and PE anti-mouse ICOS (eBioscience).

For analyzing neutrophils in the lung tissues, mice were killed on day 10 postinfection. Preparation of single-cell suspensions of the lung and cell-
surface staining were performed as described (36). Total cells were stained using Abs against markers F4/80, CD11b, and Gr-1 (eBioscience) and analyzed by flow cytometry. Lung neutrophils were identified as F4/80-negative (excluding macrophages) and CD11b+ Gr-1hi cells. For in-
tracellular cytokine staining of CD4 T cells in the lungs, preparations of single-cell suspensions of the lungs were analyzed for IFN-γ and IL-4 production by CD3+ CD4+ cells, as described (36).

**Preparation of primary murine lung fibroblasts**

Naïve WT C57BL/6 or ICOSL KO mice were euthanized, and lungs were removed, minced, and dissociated by the enzymatic action of Dispase (BD Biosciences); then the lung tissue was incubated at 37°C in 5% CO2 for

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**FIGURE 3.** A, More severe lung histopathological status and more C. muridarum inclusions/Ags in the lungs of ICOSL KO mice. Left panel, Photomicrographs of H&E-stained sections show the histopathological status of C. muridarum-infected lungs in ICOSL KO and WT mice on days 6, 10, and 14 postinfection (original magnification ×400). Right panel, Immunofluorescence staining of frozen lung sections for chla-
mydial inclusions. KO mice show more chlamydial inclusions/Ags (green) in their lungs than do WT mice at all three time points tested. B, Higher levels of neutrophils in the lungs of ICOSL KO mice. Mice were killed on day 10 postinfection. Single-cell suspensions of the lung were stained using Abs against markers F4/80, CD11b, and Gr-1 (eBioscience) and analyzed by flow cytometry. For lung neutrophils, F4/80-negative cells (excluding macrophages) were gated and analyzed for CD11bhi Gr-1hi cells.
45 min for two consecutive cycles, accompanied by refreshing the medium (DME, Sigma-Aldrich) at the end of each cycle. Single cells were isolated from the tissue debris, using a 70-μm (DMEM, Sigma-Aldrich) at the end of each cycle. Single cells were passaged every 3 d, and by the fourth passage they were homogeneous monolayers (morphologically consistent with fibroblast-like shape).

Coculture and Transwell experiments

Fibroblasts between the fourth and sixth passages were detached using Trypsin-EDTA solution (Life Technologies, Rockville, MD) and seeded into flat-bottom 96-well plates at 4 × 105 cells/ml DME + 10% FBS containing 2 mM L-glutamine, 20 mM HEPES (Sigma-Aldrich), and 15 μg/ml gentamicin (Sigma-Aldrich). The confluent monolayers formed within 48 h incubation were treated with HBSS-DEAE (Sigma-Aldrich) (30 μg/ml) at room temperature for 30 min, washed with HBSS, then followed by inoculation with C. muridarum elementary bodies (EBs) for 2 h. The inocula were washed and replaced with the above-described medium. CD4+ T cells isolated from mouse spleens were added to the monolayers at a ratio of 1:10, with or without a blocking concentration (10 μg/ml of anti-ICOS Ab (eBioscience). The coculture was allowed to proceed for 48 h. Chlamydial inclusion bodies inside the fibroblasts were measured by specific Ab staining, as described above for HeLa cell cultures. For the Transwell experiment, 5 × 105 CD4+ T cells were added to the upper chamber of a Transwell insert (VWR, West Chester, PA) with a pore size of 1 μm in 50 μl of the complete medium designated above; then they were submerged into the lower chamber containing newly inoculated fibroblast monolayers and incubated for up to 40 h.

Statistical analysis

Results are shown as mean ± SEM, and the data were analyzed using unpaired two-tailed Student t test (GraphPad PRISM 4.00). Each experiment contained five mice in each group, and one representative experiment of at least three independent experiments with similar results is shown. A p value < 0.05 was considered significant in all quantitative experiments.

Results

ICOSL KO mice showed more weight loss and pathogen burden in the lung and more severe histopathological status

To evaluate the impact of the lack of ICOSL molecules in the host on the process of chlamydial infection and diseases, we compared

Table I. Elevated production of Th1 (IFN-γ and IL-12) and reduced production of Th2 (IL-4 and IL-5) cytokines in ICOSL KO mice compared with WT mice

<table>
<thead>
<tr>
<th>Day</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
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<tr>
<td></td>
<td>IL-4 (pg/ml)</td>
<td>IL-5 (pg/ml)</td>
<td>IL-12p40 (pg/ml)</td>
<td>IFN-γ (U/ml)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>6</td>
<td>27.2 ± 4/56.8 ± 9.3</td>
<td>38.6 ± 5.7/96 ± 13.3</td>
<td>649 ± 189.3/1482 ± 351.4</td>
<td>8.2 ± 3.2/26 ± 5.7</td>
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<tr>
<td>10</td>
<td>12.8 ± 2.9*/21.2 ± 5.3*</td>
<td>19 ± 5.3*/46 ± 8.5*</td>
<td>1608 ± 222.4*/5278 ± 330.2*</td>
<td>30.8 ± 8*/72.6 ± 10.3*</td>
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<tr>
<td>14</td>
<td>41.8 ± 7.5/77 ± 10.2</td>
<td>77 ± 11.5/189.4 ± 24.3</td>
<td>1315 ± 364.8/2956 ± 689.3</td>
<td>9.2 ± 3.4/55.6 ± 10.2</td>
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<td></td>
<td>19 ± 3.7*/39 ± 7.8*</td>
<td>38.2 ± 10.5*/94 ± 15.5*</td>
<td>3173 ± 463.8*/6754 ± 709*</td>
<td>65.2 ± 15.7*/117.4 ± 13.6*</td>
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<td></td>
<td>8.5 ± 7/152.4 ± 22</td>
<td>157.6 ± 21.9/373.2 ± 49</td>
<td>2780 ± 614.5/5858 ± 1316</td>
<td>20.4 ± 5.9/101.4 ± 15</td>
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Mice were infected intranasally with C. muridarum (103 IFU) and euthanized on days 6, 10, and 14 postinfection. Spleen cells were cultured with or without UV-inactivated C. muridarum (103 IFU/ml) for 72 h. Cytokine production was measured by sandwich ELISA, as described in Materials and Methods. The numbers before and after the slashes represent cultures without and with Chlamydia-specific restimulation, respectively. Results are shown as mean cytokine concentration ± SEM in each group of mice (five/group). One of four independent experiments with similar results is shown. IFN-γ, interferon-γ; KO, knockout.
ICOSL KO and WT mice for their body weight changes, pulmonary chlamydial growth, and histological changes by day 14 after intranasal inoculation with a sublethal dose (1 × 10^3 IFU) of C. muridarum. We found that the ICOSL KO mice had significantly more severe body weight loss and much slower body weight recovery than did WT controls (Fig. 1). The general conditions of WT mice (fur ruffling, dehydration, and activity) were also better than those of ICOSL KO mice. In addition, as shown in Fig. 2, chlamydial growth in the lungs of ICOSL KO mice was significantly higher than that of WT controls on all three tested days (6, 10, and 14) postinfection, covering early peak and later stages of infection. Histopathological investigation (Fig. 3A) showed that the WT mice exhibited patchy peribronchial cellular infiltrates composed mainly of mononuclear cells on days 6 and 10, but most (~80%) of them showed only a few small perivascular infiltrates on day 14 postinfection. The remaining 20% showed a lung architecture that was almost normal. In contrast, all ICOSL KO mice showed broad areas of consolidation at these three time points, with even more severe pathological changes at the last two time points than those of day 6 postinfection, showing a progressive inflammation. Furthermore, immunohistochemical staining for chlamydial Ags showed more chlamydial inclusions/Ags in the lung tissues of ICOSL KO mice than of WT mice (Fig. 3A), consistent with the results shown in Fig. 2. In infected ICOSL KO mice, polymorphs were especially numerous, and the bronchi were often seen filled with inflammatory exudates rich in neutrophils. The higher neutrophil infiltration was also confirmed by flow cytometry analysis, which showed a higher percentage of F4/80 negative, CD11b^hi Gr-1^hi cells in the lungs of ICOSL KO mice (Fig. 3B). The data demonstrate a critical role of ICOSL–ICOS interaction in host defense against chlamydial infection.

Lower Ab production in ICOSL KO mice compared with WT controls following Chlamydia infection

Ab levels in infected mice were measured by ELISA at days 6, 10, and 14 following C. muridarum lung infection. The Ab titers for C. muridarum-specific IgG1, IgG2a (in serum), and IgA (in lung homogenates) in ICOSL KO mice were significantly lower than those of WT controls at the three time points (Fig. 4). IgG2a was the predominant subclass of Ab in response to C. muridarum infection, especially in the relatively late stage of infection, and the difference in Ab responses between ICOSL KO mice and WT controls was mainly in the IgG2a subclass. The results indicate that ICOSL KO mice have a profound impairment in Ab response to chlamydial infection.

Enhanced Th1 and proinflammatory cytokines, along with reduced Th2 and TGF-β1 production, in ICOSL KO mice postinfection

To elucidate the role of ICOSL in modulating the pattern of cytokine production, ICOSL KO and WT C57BL/6 control mice were intranasally infected with C. muridarum and were euthanized on days 6, 10, and 14 postinfection. Splenocytes were cultured in the presence or absence of UV-inactivated C. muridarum (10^5/ml) for 72 h, and cytokines in the culture supernatants were measured by ELISA. The results showed that the production of IFN-γ and IL-12p40/IL-23 (Th1-related cytokines) by ICOSL KO mice was significantly higher than that of WT controls at all three time points examined (Table I). In contrast, the production of IL-4 and IL-5 (Th2 cytokines) by ICOSL KO mice was significantly lower than that of WT controls (Table I). Consistently, intracellular cytokine staining showed more IFN-γ-producing but fewer IL-4-producing CD4^+ T cells in ICOSL KO mice than in WT mice in the spleen (Fig 5A) and the lung (Fig. 5B). Moreover, the mean fluorescence intensity was higher for IFN-γ but lower for IL-4 in the CD4^+ T cells of ICOSL KO mice, suggesting differences in production rate per cell (Fig. 5). The data suggest that ICOS–ICOSL interaction plays an important role in determining the pattern of cytokine responses to Chlamydia infection, particularly in the development of Th2 responses.

Considering the observed increased inflammation in the lungs of ICOSL KO mice as shown in Fig. 3, we measured production of two classical proinflammatory cytokines (IL-6 and TNF-α) and production of the most important anti-inflammatory cytokines

\[\text{(FIGURE 5. Dramatically decreased IL-4 but increased IFN-γ production by CD4 T cells isolated from the spleen and lung tissues of ICOSL KO mice, shown by intracellular cytokine staining. Mice were intranasally inoculated with C. muridarum (10^5). Spleen (A) and lung (B) cells were collected at day 10 postinfection. The cytokine production pattern of spleen and lung CD4 T cells was analyzed by intracellular cytokine staining and flow cytometry, as described (36). Representative dot plots show IFN-γ and IL-4 production by CD4 T cells. Analysis was performed on gated CD3^+ CD4^+ cells. The testing on spleen cells and lung cells was performed four times and two times, respectively. The numbers in the left upper corner of each graph represent mean fluorescence intensity, and the ones in the right upper corner represent percentage of positive cells.}\]
CD4+ cells expressed lower levels of ICOS on their surface than naive and infected mice. The results showed that naive ICOSL KO and WT mice for their levels of ICOS expression on T cells, using (35), the impact of ICOSL deficiency on ICOS expression on pre- and postinfection ICOS on their surface compared with those from WT controls experiments with similar results.

Data are representative of three independent experiments with similar results. All density plot SEM in each group of mice (five/group). One of four independent experiments with similar results is shown. *p < 0.05, KO versus WT mice in corresponding conditions.

(TGF-β1 and IL-10) by whole spleen cells postinfection. Consistently, ICOSL KO mice produced significantly higher amounts of IL-6 and TNF-α, but significantly lower amounts of TGF-β1 and IL-10, than did WT controls at all the three time points tested (Table II).

CD4+ T cells from ICOSL KO mice express lower levels of ICOS on their surface compared with those from WT controls pre- and postinfection

Although ICOSL KO mice have been developed for several years (35), the impact of ICOSL deficiency on ICOS expression on T cells has not been reported. We therefore analyzed ICOSL KO and WT mice for their levels of ICOS expression on T cells, using naive and infected mice. The results showed that naive ICOSL KO CD4+ cells expressed lower levels of ICOS on their surface than did those of WT mice (Fig. 6). More importantly, unlike the WT mice, which showed a dramatic increase of ICOS expression on CD4 T cells following C. muridarum infection, the ICOSL KO mice exhibited minimal changes in ICOS expression on these cells postinfection (Fig 6). The data suggest a critical role for ICOS–ICOSL interaction in the expression of ICOS on naive and activated CD4+ T cells, in particular following chlamydial lung infection.

CD4 T cells isolated from infected ICOSL KO mice failed to inhibit chlamydial growth in coculture with infected primary lung fibroblasts

To directly examine the involvement of ICOS–ICOSL interaction in CD4 T cell-mediated inhibition of chlamydial growth in host cells, we cocultured CD4+ T cells isolated from WT and ICOSL KO mice (naive and infected) with the fibroblasts inoculated with C. muridarum EB. As shown in Fig. 7A, CD4 T cells isolated from intranasally infected (iT) WT mice (WT iT) significantly inhibited chlamydial growth in the cultured fibroblasts, whereas the inhibitory effect was virtually abolished by blocking ICOS–ICOSL interaction with anti-ICOS Ab in the coculture. Consistently, the CD4+ T cells isolated from infected ICOSL KO mice (KO iT) and CD4+ T cells from WT naive mice (n iT); WT nT) failed to inhibit chlamydial growth in the host cells in the culture. Similarly, CD4+ T cells from infected WT mice (WT iT) failed to inhibit chlamydial growth in the fibroblasts derived from ICOSL KO mice, although the levels of chlamydial growth in the fibroblasts from WT and ICOSL KO mice were similar.

To test the necessity for cell–cell contact for the inhibitory role of CD4 T cells in chlamydial growth in infected cells, we repeated the coculture experiment by separating the CD4 T cells and chlamydial host cells, using a Transwell system that permits only soluble factors to pass through the membrane with a pore size of 1 μm. As shown in Fig. 7B, CD4 T cells isolated from infected WT mice (WT iT), similar to CD4 T cells from other sources, failed to inhibit chlamydial growth in the fibroblasts. Taken together, these results suggest that ICOS–ICOSL interaction plays an important part in the ability of CD4 T cells to inhibit Chlamydia growth in target cells and that cell–cell contact is essential for the inhibition.

ICOSL KO mice show a reduced Th17 response compared with WT mice postinfection

Because Th17 response has recently been reported to figure significantly in pathological inflammatory reactions (37, 38), we further compared Th17 responses in WT and ICOSL KO mice following C. muridarum infection. ICOSL KO and WT mice were euthanized on days 6, 10, and 14 after intranasal inoculation with C. muridarum, and their CD4+ cells were isolated from the spleens and stained for CD4 and intracellular IL-17F. As shown in Fig. 8, ICOSL KO mice exhibited a decreased Th17 response at all three time points postinfection, compared with WT controls. In addition, naive ICOSL KO mice, compared with WT mice, showed a reduced Th17 population, and the Th17 population expanded

<table>
<thead>
<tr>
<th>Day</th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>TGF-β1 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
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<tr>
<td>6</td>
<td>26.2 ± 8/77 ± 10.9</td>
<td>118.6 ± 36/241.4 ± 33.1</td>
<td>835 ± 186.9/1140 ± 174.4</td>
<td>645.4 ± 132.3/1856 ± 2</td>
</tr>
<tr>
<td>KO: 67.4 ± 13.7*/155.6 ± 21.6*</td>
<td>250.8 ± 37.5*/518.2 ± 79.4*</td>
<td>264.4 ± 81.1*/387 ± 79.8*</td>
<td>203.2 ± 52.4*/763.4 ± 175.8*</td>
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<tr>
<td>10</td>
<td>94.8 ± 28.3/127.6 ± 52.2</td>
<td>238.1 ± 73.33/476.2 ± 55.2</td>
<td>1446 ± 329.9/2039 ± 315.7</td>
<td>1636 ± 224.4/3296 ± 550.4</td>
</tr>
<tr>
<td>KO: 210.2 ± 30.8*/563.4 ± 43.7*</td>
<td>501 ± 68.1*/986.4 ± 122.6*</td>
<td>539.8 ± 160.1*/782.4 ± 148.6*</td>
<td>866.2 ± 177.5*/1304 ± 244.8*</td>
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<tr>
<td>14</td>
<td>192.8 ± 51.6/261 ± 65.7</td>
<td>431 ± 123.3/951.4 ± 105.9</td>
<td>1978 ± 4/4208 ± 524.6</td>
<td>2839 ± 263.7/5944 ± 565.5</td>
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<tr>
<td>KO: 433.8 ± 60.9*/737 ± 93.7*</td>
<td>978 ± 124.5*/1906 ± 221.1*</td>
<td>725.2 ± 169*/1709 ± 294*</td>
<td>1670 ± 342.1*/3479 ± 643.6*</td>
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</table>

The method is the same as the one mentioned for Table I. The numbers before and after the slashes represent cultures without and with Chlamydia-specific restimulation, respectively. Results are shown as mean cytokine concentration ± SEM in each group of mice (five/group). One of four independent experiments with similar results is shown.

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FIGURE 6. Lower expression of ICOS on the surface of CD4+ T cells in ICOSL KO mice than in WT mice. Mice were intranasally inoculated with C. muridarum (103), and spleen cells were collected at three different time points, magnetically purified for CD4+ cells, and stained for CD4 and ICOS, as mentioned in Materials and Methods. All density plot charts were based on 103 cells satisfying a gate set on a forward versus side light scatter that defined spleen lymphocytes. The percentages of double positive cells are indicated at the upper right of each chart. The flow cytometric data are representative of three independent experiments with similar results.
The critical role for ICOS–ICOSL interaction in host defense against chlamydial infection is demonstrated. CD4+ T cells from ICOSL KO mice showed lower Th2 (IL-4 and IL-5) and higher Th1 (IFN-γ)-producing cells than did WT mice. The infected ICOSL KO mice had a significantly higher percentage of Th1 (IFN-γ–producing) cells than did WT mice. The data emphasized the critical importance of cell–cell contact at least partly mediated by ICOSL–ICOS interaction between infected cells and activated Th1 cells in T cell-mediated inhibition of chlamydial growth in host cells. This point was supported by an earlier finding that reported that the infected ICOSL KO mice had a significantly higher percentage of Th1 (IFN-γ–producing) cells than did WT mice. The most important implication of the study is that a sole predominant Th1 cytokine response is not efficient for host defense against chlamydial infection. It has been shown by numerous studies that Th1 immune responses are more effective than Th2 responses for fighting against chlamydial infection. However, in this the mice with more serious disease and higher in vivo bacterial loads are the ones with stronger Th1 responses. As seen in Table I, ICOSL KO mice showed lower Th2 (IL-4 and IL-5) and higher Th1-related (IFN-γ) cytokine production than did WT controls at the early, peak and later stages of infection. A similar finding was generated from intracellular cytokine staining (Fig 5), showing that the infected ICOSL KO mice had a significantly higher percentage of Th1 (IFN-γ–producing) cells than did WT mice. The data emphasized the critical importance of cell–cell contact at least partly mediated by ICOSL–ICOS interaction between infected cells and activated Th1 cells in T cell-mediated inhibition of chlamydial growth in host cells. This point was supported by an earlier finding that reported that the Chlamydia-specific T cell line needs direct cell–cell contact to inhibit chlamydial growth in cultured epithelial cells (39).

It might be argued that the failure of the KO mice to show protection against chlamydial infection is a result of their deficiency in Ab responses. Indeed, ICOSL KO mice exhibited a reduced capability to mount strong humoral Chlamydia-specific immune responses, with significantly lower serum IgG1 and IgG2a Abs and IgA Ab in the lung homogenates than found in WT mice (Fig 4). This finding is consistent with involvement of the
ICOS–ICOSL interaction in formation of germinal centers and Ab production previously reported by other researchers (35). Although the role of Ab response in host defense against chlamydial infection has been reported, especially its potential function in blocking organism entry, information on its protective role in primary infection, especially in the early stages of infection, is limited. The data in the current study showed that the level of Ab response at day 6 postinfection in both KO and WT mice was very low (Fig 4), whereas the differences in bacterial loads and body weight changes were already very significant (Fig 2) between the two groups of mice. Therefore, even if the Ab response does have some protective effect, it is unlikely to be the major reason for the difference in resistance to infection observed in our study.

In addition to the significantly bigger bacterial loads and body weight losses, the much heavier inflammation in the lung was another characteristic of the ICOSL KO mice. The reasons for this phenomenon are likely multiple. On one hand, the failure to clear the infection by the ICOSL KO mice may lead to a constant stimulation of the innate and adaptive immune system by the bacteria, thus promoting cell recruitment to the local tissues. On the other hand, the difference between the ICOS KO mice and WT mice in producing proinflammatory and anti-inflammatory cytokines also contributes to the difference in inflammation (40–42). Indeed, the ICOSL KO mice were found to produce significantly higher amounts of proinflammatory IL-6 and TNF-α and lower amounts of anti-inflammatory TGF-β1 and IL-10 than did WT controls at all three time points examined, representing the early, peak, and late stages of infection. Although inflammation is a necessary process for controlling infection by innate and adaptive immune cells, exacerbation of inflammation is more detrimental than beneficial. In particular, the dominantly recruited cellular population in KO mice consists of neutrophils, which are not efficient in combatting intracellular pathogens, such as *Chlamydia*. Therefore, the exacerbated inflammation in the KO mice failed to confer efficient protection, especially in the late stage of infection; rather, it caused immunopathological changes in the lung tissues.

A new finding is the lower ICOS expression on CD4 T cells in ICOSL KO mice, both naive mice and those that have been intrasinally infected with *C. muridarum*. Although lower ICOS expression in CD4 T cells in ICOSL KO mice is not surprising, this is the first report, to our knowledge, showing impaired ICOS expression in ICOSL KO mice. Especially considering the facts that much more significant difference in ICOS expression was observed postinfection than preinfection between the two types of mice and that ICOS expression in WT mice constantly increased during infection, the data suggest a positive feedback mechanism for ICOS–ICOSL interaction in the process of infection.

Another interesting observation is the lower Th17 response in ICOSL KO mice following chlamydial infection. The result fits the reports in other model systems showing the particular importance of ICOS–ICOSL interaction in Th17 and Th2 responses (38, 43). Although the role of Th17 was not directly examined in the current study, Th17 likely contributes to protection, based on the fact that ICOSL KO mice showing lower Th17 levels suffered from more serious disease and higher bacterial growth in vivo. This assumption is also supported by our recent report noting that blockade of IL-17 in vivo significantly exacerbated chlamydial infection (44). It is rather surprising that the ICOSL KO mice showed lower Th17 responses, but a heavier neutrophilic inflammation in the lung, because it is commonly accepted that IL-17 is a powerful cytokine in promoting neutrophilic inflammation. The reason for this finding is not clear. However, because ICOSL KO mice showed significantly higher TNF-α and IL-6 production than did WT mice, these cytokines could promote neutrophilic inflammation. In addition, a detectable level of Th17 response still existed in the ICOSL KO mice, which, by itself or in concert with other cytokines/chemokines, might be sufficient for mounting neutrophilic recruitment. Moreover, although IL-6 and Th17 are important cells involving recruitment of polymorphs, especially neutrophils and eosinophils, to sites of inflammation at the beginning of microbe-induced inflammations (45, 46), after provoking polymorph recruitment, Th17 cells, as they expand during the course of infection, can inhibit neutrophilic recruitment to the site of inflammation; in this way, they control overreaction of neutrophilic inflammation to protect against immunopathological damage to tissues. As shown in Fig. 8, ICOSL KO mice have less Th17 response than do WT controls at all three time points postinfection, but the difference was more dramatic in the later stage of infection (day 14). It is possible that the lower level Th17 responses, in combination with other cytokine/chemokine responses, in the early stage in both groups of mice were sufficient to induce beneficial levels of neutrophilic recruitment and infiltration into the lung tissues; however, owing to the failure of the ICOSL KO mice to increase their Th17 levels to inhibit prolonged neutrophilic inflammation at late stages of the infection (day 14, Fig. 8), these mice suffered more serious pathological changes. It is also known that TGF-β1 is the most important cytokine in the development of the Th17 population (37, 38), and as we show in Table II, ICOSL KO mice demonstrated less TGF-β1 production, thus providing a reason for decreased Th17 responses in the KO mice. Fig. 8B also shows that naive ICOSL KO mice carry a smaller Th17 population than do naive WT controls, suggesting a possible function of ICOS–ICOSL interaction in the development and maintenance of the Th17 population under naive physiological steady-state conditions. It has been proposed by other investigators that differentiation of naive CD4 T cells to Th17 cells is mediated by innate immune elements through CD28 and ICOS costimulation and that ICOS–ICOSL interaction is required for production of IL-4 and IL-17, but not of IFN-γ (38, 43, 47, 48). Our data are consistent with these findings, but this study represents the first time that ICOSL KO mice have been used to evaluate Th17 responses pre- and postinfection.

Disclosures
The authors have no financial conflicts of interest.

References


