

Enhanced Inducible Costimulator Ligand (ICOS-L) Expression on Dendritic Cells in Interleukin-10 Deficiency and Its Impact on T-Cell Subsets in Respiratory Tract Infection

Xiaoling Gao, Lei Zhao, Shuhe Wang, Jie Yang, and Xi Yang

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

An association between inducible costimulator ligand (ICOS-L) expression and interleukin (IL)-10 production by dendritic cells (DCs) has been commonly found in infectious disease. DCs with higher ICOS-L expression and IL-10 production are reportedly more efficient in inducing regulatory T cells (Tregs). Here we use the *Chlamydia muridarum* (Cm) lung infection model in IL-10 knockout (KO) mice to test the relationship between IL-10 production and ICOS-L expression by DCs. We examined ICOS-L expression, the development of T-cell subsets, including Treg, Th17 and Th1 cell, in the background of IL-10 deficiency and its relationship with ICOS-L/ICOS signaling after infection. Surprisingly, we found that the IL-10 KO mice exhibited significantly higher ICOS-L expression by DCs. Moreover, IL-10 KO mice showed lower Tregs but higher Th17 and Th1 responses, but only the Th17 response depended on ICOS signaling. Consistently, most of the Th17 cells were ICOS⁺, whereas most of the Th1 cells were ICOS⁻ in the infected mice. Furthermore, neutralization of IL-17 in IL-10 KO mice significantly exacerbated lung infection. The data suggest that ICOS-L expression on DC may be negatively regulated by IL-10 and that ICOS-L expression on DC in the presence or absence of IL-10 costimulation may promote Treg or Th17 response, without significant impact on Th1.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2013.00035

INTRODUCTION

In addition to T-cell receptor (TCR) engagement, the initiation of optimal T-cell response requires costimulatory signals. The molecular mechanisms underlying the development of different T-cell subsets remain unclear. Inducible costimulator (ICOS) and its ligand (ICOS-L) are recently recognized costimulatory molecules belonging to the CD28 superfamily (1–3). ICOS is not expressed on naive T cells but can be upregulated quickly when the cells are activated (3,4). ICOS can be expressed by different T-cell subsets, but appears more on Th2 and regulatory T cells (5–11). The only ligand

for ICOS is B7RP-1 (ICOS-L), which is found on dendritic cells (DCs), macrophages and some epithelial cells (12). DCs are the most important antigen-presenting cell (APC) in activation and direction of T cells. Expression of surface costimulatory molecules and production of cytokines by DCs are critically important for the modulating effect of DCs on T-cell function and development of T-cell subsets. Akbari *et al.* (13) reported the importance of ICOS–ICOS-L interaction in the development of regulatory T cells (Tregs). They found that both the development and the inhibitory function of regulatory cells depended on the

presence of interleukin (IL)-10 and on ICOS–ICOS-L interactions. More recently, we reported that chlamydial lung infection and systemic mycobacterial infection can generate DCs that exhibit increased surface expression of ICOS-L and increased IL-10 production (14,15). In addition, we found that the DCs, which coexpressed higher ICOS-L and IL-10 after infection, were more efficient in inducing Tregs to allergen exposure (14,15).

A newly identified CD4⁺ T-cell subset (Th17) is characterized by its predominant production of IL-17. Th17 cell is distinct from Th1 and Th2 in its developmental pathway and function. Certain cytokines are particularly important for Th17 response (17–21). There is overlap in the required signaling of the cell surface marker and cytokine environments for Treg and Th17 development (22–27). In particular, ICOS–ICOS-L interaction appears highly associated with both Treg and Th17 responses (22,23). Th17 was initially reported to be pathological in inflammatory autoimmune diseases (28,29)

Address correspondence to Xi Yang, Departments of Medical Microbiology and Immunology, University of Manitoba, Room 523, 730 William Avenue, Winnipeg, Manitoba, R3E 0W3, Canada. Phone: 204-789-3481; Fax: 204-789-3926; E-mail: Xi.Yang@med.umanitoba.ca. Submitted April 16, 2013; Accepted for publication September 25, 2013; Epub (www.molmed.org) ahead of print September 26, 2013.

but was later found to be involved in host defense against extracellular bacterial and fungal infections (rev. in 30). More recently, the involvement of Th17/IL-17 in protective immunity against intracellular bacterial infections was also reported (31–34). In particular, we and others reported that IL-17 is important in host defense against chlamydial lung infection (31,34). Inconsistencies on the role of ICOS–ICOS-L interaction in Th17 responses have been reported (35–38). One study found that ICOS knockout (KO) mice had reduced Th17 cells (37), whereas other studies showed increased Th17 cells in the condition of ICOS or ICOS-L deficiency (36,38).

Chlamydia, a genus of obligate intracellular bacteria, consists of human pathogenic species such as *Chlamydia trachomatis* and *Chlamydia pneumoniae*, causing a diverse spectrum of human diseases involving ocular, genital and respiratory systems (39,40). No human vaccine is available to prevent chlamydial infections. Better understanding of the mechanisms related to protection and pathogenesis of chlamydial infections is essential for rational development of a safe and effective vaccine. *Chlamydia muridarum* (*Cm*), an adapted chlamydial strain in mouse, has been widely used in mouse models for studying the mechanisms of host defense and chlamydial pathogenesis (41). Previous studies have demonstrated that Th1 cells are critically important for host defense against chlamydial infections (42–50). However, we recently found that ICOS-L KO mice showed exacerbated chlamydial infection, even with an enhanced Th1 response (51).

Given the demonstrated involvement of ICOS–ICOS-L signaling in both Treg and Th17 responses and the reported importance of Th1 in host defense against chlamydial infections, we planned to further dissect the condition for the development of these T-cell subsets by using a lung infection model. In addition, considering the reported close association of IL-10 production and ICOS-L expression on DCs, we wanted to specifically test if ICOS-L expression on DCs depends on

IL-10 production. We used IL-10 KO and wild-type (WT) C57BL/6 mice to address these questions. We found that DCs in IL-10 KO mice expressed significantly higher levels of ICOS-L than WT mice after chlamydial lung infection. Moreover, we found that IL-10 deficiency led to reduced Tregs but higher Th17 and Th1 responses. However, only Th17 cells depended on ICOS–ICOS-L signaling, and most of Th17 but not Th1 cells expressed ICOS. Further, blockade of IL-17 in IL-10 KO mice led to enhanced chlamydial infection *in vivo*. The results suggest a negative regulatory role of IL-10 on ICOS-L expression and a key role of ICOS–ICOS-L interaction in Th17 development, which is critical for host defense against an intracellular bacterial infection.

MATERIALS AND METHODS

Mice

Female IL-10 KO mice (IL-10^{-/-}; C57BL/6-*Il-10*^{tm1Cgn}) and ICOS KO mice (ICOS^{-/-}, C57BL/6.129P2-*Icos*^{tm1mak}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Age- and sex-matched WT C57BL/6 mice were purchased from The Jackson Laboratory, Charles River Canada (St. Constant, Canada) or the University of Manitoba breeding facility (Winnipeg, Manitoba, Canada). The mice were hosted under specific-pathogen-free conditions in the University of Manitoba animal facility for at least 2 wks before the experiment started and during the whole experimental period. No difference was noted among the WT mice from different sources after *Cm* infection. Six- to eight-week-old mice were used in the study. All mouse experiments were performed in accordance with the guidelines issued by the Canadian Council on Animal Care. The animal experimental protocol was approved by the ethical committee of University of Manitoba.

Mice Treatment and Quantitation of Chlamydial Growth *In Vivo*

Cm was grown in HeLa 229 cells and purified by discontinuous density gradi-

ent centrifugation as described previously (46). Infectivity of the purified *Cm* elementary bodies was titrated in HeLa cell culture and demonstrated as inclusion-forming units (IFUs) as described (49). The same batch of *Cm* preparation was used throughout the study. IL-10 KO, ICOS KO and WT mice were inoculated intranasally (i.n.) with *Cm* (1,000 IFUs) in 40 μ L sterile, protein-free sucrose-phosphate-glutamic acid buffer as described (46,49). In the designated experiments, IL-17 activity in IL-10 KO mice was neutralized by using monoclonal antibodies (mAbs) as described (34). Briefly, 10 μ g anti-mouse IL-17 mAbs (R&D, Minneapolis, MN, USA) in 40 μ L phosphate-buffered saline (PBS) were administered i.n. to IL-10 KO mice 2 h after inoculation of *Cm* and was repeatedly administered every 48 h until mice were killed at d 7 after infection. The mice were monitored daily for body weight changes. The growth of *Cm* in the lung was determined as described (46,49).

Lung Mononuclear Cell Preparation

Lung leucocytes were prepared by collagenase XI and DNase digestion of the lung tissue and Percoll gradient isolation (34). Briefly, the lung tissues were minced into small pieces and incubated in digestive buffer (containing 2 mg/mL collagenase type XI and 100 μ g/mL DNase [Sigma-Aldrich, St. Louis, MO, USA]) for 60 min at 37°C. The cell population was purified by centrifugation through a Percoll gradient. Cell suspension was gently mixed with 35% Percoll and centrifuged for 20 min at 750g. The pellet was collected and resuspended in flow cytometry buffer (FAC) buffer (1 \times Dulbecco's phosphate-buffered saline [DPBS], 2% fetal bovine serum [FBS], 0.02% Na₃N₃, 5 mmol/L ethylenediaminetetraacetic acid [EDTA]). The erythrocytes were lysed with ammoniumchloride-potassium (ACK) lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA) followed by two washes in RPMI 1640 with 5% fetal calf serum and resuspended in complete

RPMI 1640 medium (RPMI, 10% FBS, 100 U penicillin).

In Vitro Restimulation Assays and Cytokine Measurement

Mice treated with different approaches were killed at d 7 after infection. Spleen and lungs were aseptically removed. To analyze cytokine production, single-cell suspensions were prepared from spleen and lungs as described previously (53,54). The cells were cultured at a concentration of 7.5×10^6 cells/mL (splenocytes) or 5.0×10^6 cells/mL (lung cells), respectively, in complete culture medium with or without stimulation of ultraviolet-inactivated *Cm* (10^5 IFU/mL). Culture supernatants were harvested at 72 h, and cytokine concentrations in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) by using antibodies purchased from eBioscience (San Diego, CA, USA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To analyze the expression of *Foxp3* retinoic acid-related orphan receptor γ (ROR- γ t) transcripts, the mRNA was prepared from lung tissues by using TRIzol reagent protocol (Invitrogen/Life Technologies, Carlsbad, CA, USA) (52). Briefly, total cellular RNA was extracted from lung tissues using phenol-guanidinium followed by ethanol precipitation. The first-strand cDNA was synthesized from 1.2 μ g RNA by using Moloney leukemia virus (M-MLV) reverse transcriptase (Invitrogen/Life Technologies) and oligo(dT) primer. One microliter of cDNA was used for each PCR. β -Actin was used as a loading control. GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA, USA) was used at 36 cycles for *Foxp3*, forward 5'-AACATCTGGAACCCACGGGCACTA-3', reverse: 5'-GCATTGCTTAGGCTGCGTATGAT-3'; β -actin, forward: 5'-GTGGGCCGCCCTAGGCACCA-3', reverse: 5'-CTCTTTGATGTCACGCACGATTTC-3'. Real-time PCR was conducted on the MJ Mini Thermal Cycler (Bio-Rad, Mississauga, Ontario, Canada) by using iQTM SYBR Green Supermix.

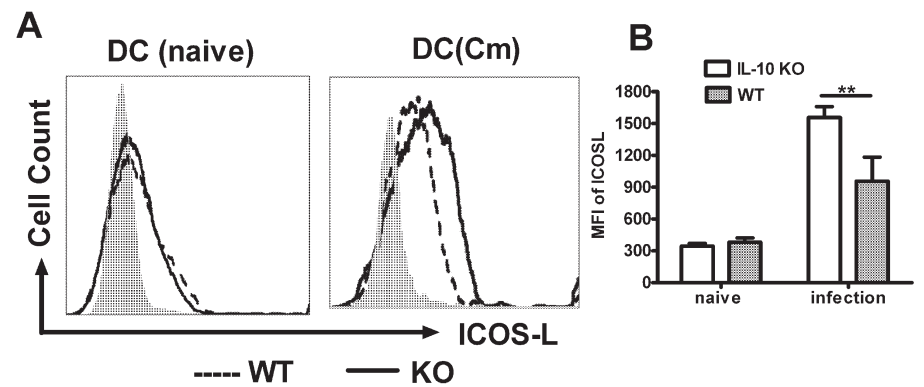


Figure 1. ICOS-L expression on DCs is significantly higher in IL-10 KO than WT mice after *Cm* infection. DCs from IL-10 KO or WT mice with or without infection were isolated from spleens by using CD11c microbeads and LS MACS columns as described in Materials and Methods. DCs were stained with anti-CD11c-FITC and anti-ICOS-L-PE mAbs for 20 min on ice and analyzed by flow cytometry. (A) Representative histogram of ICOS-L expression on DCs. Solid line: DCs from IL-10 KO mice (KO); dashed line: DCs from WT mice (WT); shaded histogram: isotype control. (B) MFI of ICOS-L on DCs of naive and infected WT and IL-10 mice is shown as mean \pm standard deviation (SD) (n = 4). **p < 0.01. One representative experiment of three independent experiments is shown.

DCs and CD4 T-Cell Purification and DC/T-Cell Coculture

C57BL/6 mice were immunized with UV-killed *Cm* (1×10^5) by intraperitoneal injection (IP) and boosted 2 wks later with the same dose. *Cm*-specific CD4⁺ T cells were isolated from the spleen of *Cm*-immunized mice by using a CD4 magnetic cell sorting buffer (MACS)-positive selection column (Miltenyi Biotec, Auburn, CA, USA) as described (54). DCs were isolated from spleen of WT or IL-10 KO mice by using magnetic CD11c columns from MACS. The purities of the purified CD4⁺ cells and DCs (based on CD11c) were >96%. The isolated CD4⁺ T cells were cocultured with purified DCs in the presence of ultraviolet-inactivated *Cm* as described (54). In designated experiments, anti-ICOS-L mAbs (10 μ g/mL, clone HK5.3; eBioscience) were added to the coculture wells to block ICOS-L signal pathway (51). The cells harvested after 48-h DC:CD4⁺ T-cell coculture were washed twice with cold PBS and further analyzed by intracellular cytokine staining.

Flow Cytometry

To evaluate the expression of CD11c and ICOS-L on the surface of DCs, fluorescein

isothiocyanate-conjugated anti-CD11c (anti-CD11c-FITC) and phycoerythrin-conjugated anti-ICOS ligand (anti-ICOS-L-PE) antibodies and corresponding isotype controls were purchased from eBioscience and used for flow cytometric analysis. The freshly isolated DCs were stained by using labeled antibodies as previously described (54). Analyses were performed by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and Cell Express (De Novo, Los Angeles, CA, USA). Standard FACS buffer (1 \times DPBS, 2% FBS, 0.02% Na₂S₂O₃, 5 mmol/L EDTA) was used.

For intracellular cytokine analysis, freshly isolated splenocytes and lung mononuclear cells and cells collected from DC:CD4⁺ T-cell coculture were analyzed as described (54). For splenocytes, erythrocytes were first depleted. For cells from DC:CD4⁺ T-cell coculture, cells were collected after 48 h coculture. All the cells were washed twice and then stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma-Aldrich) and ionomycin (1 μ g/mL; Sigma-Aldrich) and incubated for 6 h in complete RPMI-1640 medium at 37°C. For the last 3 h of incubation, brefeldin A (Sigma-Aldrich) was added to accumulate cytokines intracellu-

RESULTS

DCs from IL-10 KO Mice Show Significantly Higher ICOS-L Expression after *Cm* Infection

We first compared ICOS-L expression on the DCs of IL-10 KO and WT mice before and after *Cm* infection. DCs enriched by MACS columns were double stained with anti-CD11c and anti-ICOS-L mAbs and analyzed by flow cytometry. As shown in Figure 1, DCs from naive IL-10 KO and WT mice showed low and comparable levels of ICOS-L expression, which notably increased after *Cm* lung infection. Rather surprisingly, the ICOS-L levels on the DCs of IL-10 KO mice were higher than those in WT mice (38% versus 21%). Measurement of mean fluorescence intensity (MFI) showed significantly higher density of ICOS-L expression on the DCs of IL-10 KO mice than WT mice. The data suggest that ICOS-L expression on DCs does not rely on IL-10 production and that IL-10 may even inhibit ICOS-L expression.

IL-10 KO Mice Show Reduced Tregs but Increased Th17 and Th1 Responses after *Cm* Lung Infection

We then examined T-cell subsets in the IL-10 KO and WT mice after *Cm* lung infection. The cells isolated from the spleen and lung tissues were analyzed by multi-color staining. CD4 T cells (CD3 ϵ^+ and CD4 $^+$) were gated, and their surface markers and intracellular cytokines were detected. Tregs were identified as CD4 $^+$ Foxp3 $^+$ cells in the infected mice. As shown in Figures 2A and B, in both spleen and the lung, IL-10 KO showed significantly lower Tregs. Analysis of Foxp3 mRNA in the lung tissues by RT-PCR also showed lower expression in IL-10 KO mice. The results suggest that the increase of ICOS-L in the absence of IL-10 failed to enhance Treg response. In contrast, significantly higher percentages of IL-17-producing CD4 $^+$ T cells were found in IL-10 KO mice in the spleen and lung tissues than WT mice (Figures 3D, E). Of note, around 0.5–0.8% of Th17 cells were

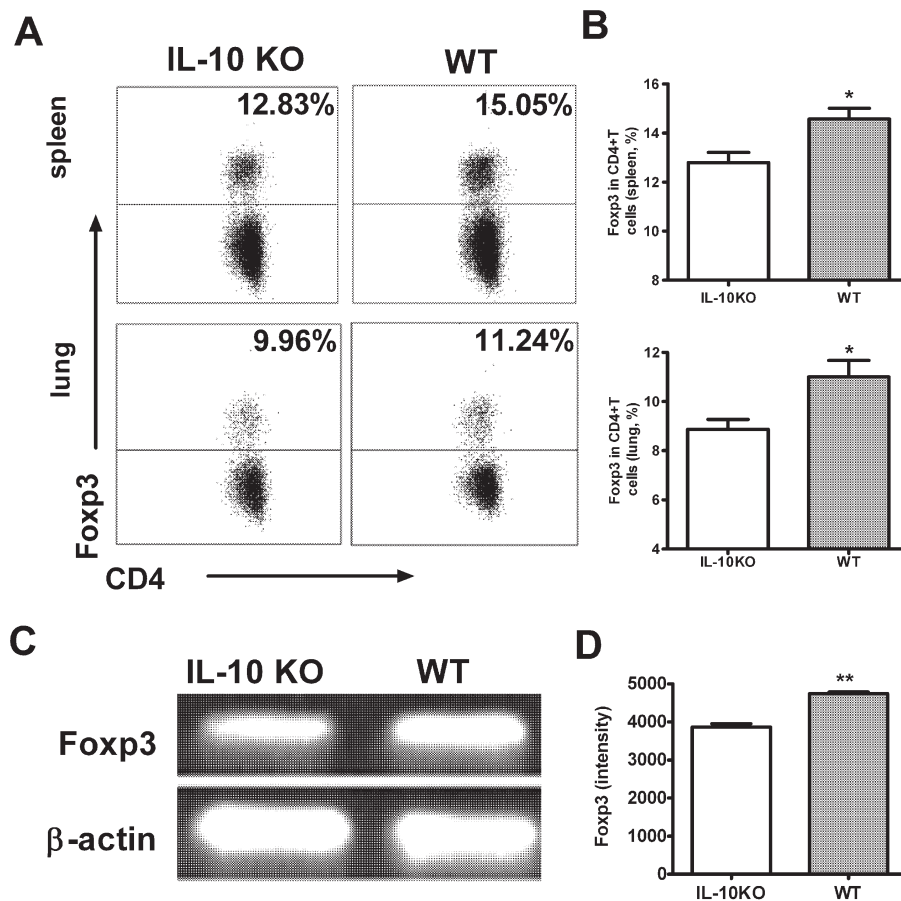


Figure 2. IL-10 KO mice show less Tregs after *Cm* infection. IL-10 KO and WT mice were infected with *Cm* (1×10^3 IFUs) as described in Material and Methods. At d 7 after infection, single cell suspensions of the spleen and lung were stained for expression of CD3 ϵ , CD4 and Foxp3. (A) Representative dot plots of Foxp3 $^+$ CD4 $^+$ cells as a percentage of total T cells are shown. (B) Summary of the frequency of CD4 $^+$ Foxp3 $^+$ T cells in spleen and lung. (C, D) Foxp3 mRNA in the lung tissues was analyzed by RT-PCR in IL-10 KO and WT mice. Data are shown as mean \pm SD ($n = 4$) and are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$.

larly. After the stimulation, cells (2×10^6) were washed twice and incubated with Fc receptor (FcR) block antibodies (anti-CD16/CD32; eBioscience) for 15 min at 4°C to block nonspecific staining. Cell surface markers (CD3 ϵ and CD4) were stained first. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) and stained for intracellular cytokines with allophycocyanin-conjugated anti-IFN γ (anti-interferon- γ) (XMG 1.2) and allophycocyanin-conjugated anti-IL-17 (17B7; eBioscience) or with corresponding isotope control antibodies. Finally, the

cells were washed, resuspended in PBS containing 2% fetal calf serum and analyzed by flow cytometry for intracellular cytokines by gating on CD3 ϵ^+ and CD4 $^+$ or CD8 $^+$ T cells, respectively.

Statistical Analysis

One-way analysis of variance (ANOVA) and Newman-Keuls test or unpaired *t* test were used to determine statistical significance among groups. IFUs of *Cm* were converted to logarithmic values and analyzed using ANOVA. The value of $p < 0.05$ was considered significant.

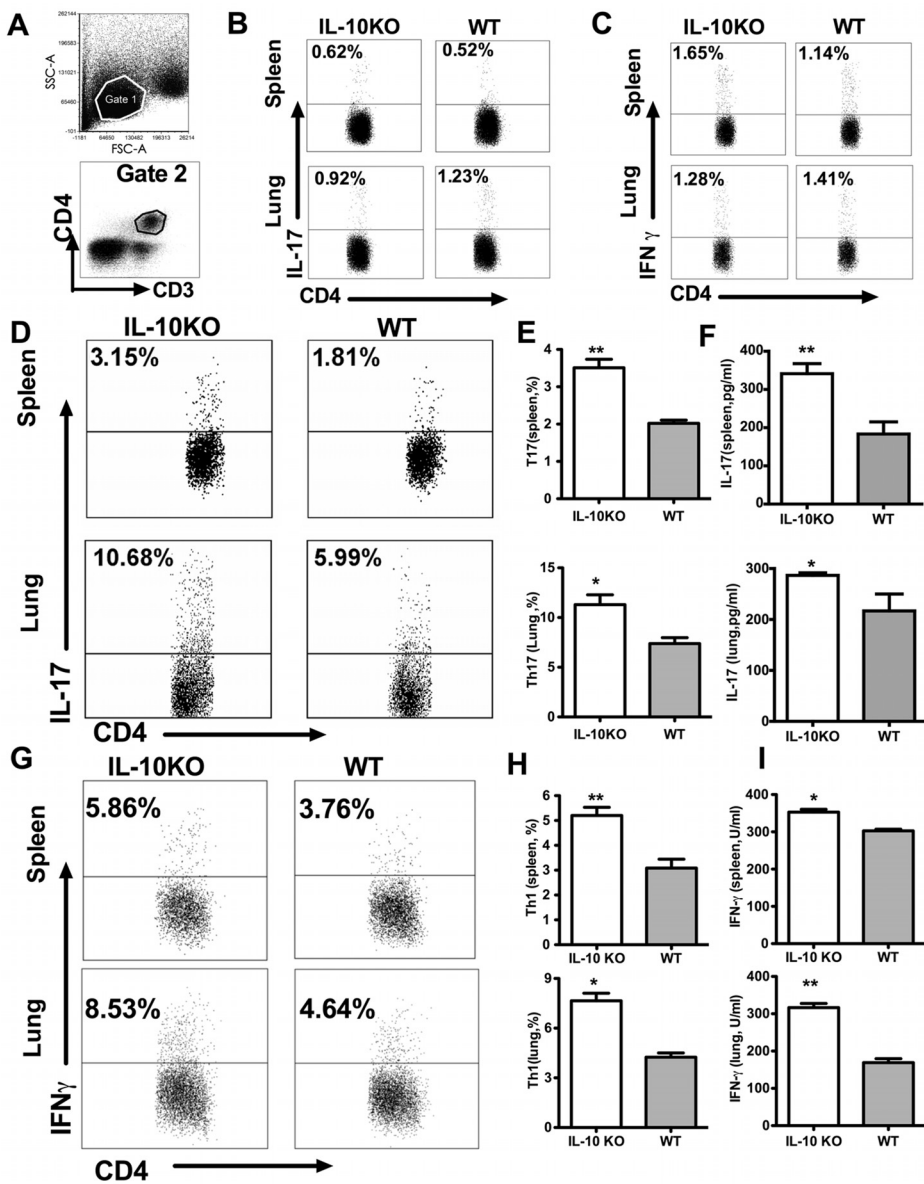


Figure 3. Higher Th17 and Th1 expansion in the spleen and lung tissues of IL-10 KO mice than WT mice after *Cm* infection. Single cell suspensions of spleen and lungs of IL-10 KO and WT mice were prepared before infection (A–C) or at d 7 after intranasal *Cm* infection (D–I), as described in Materials and Methods. Intracellular cytokine staining was performed to detect the percentage of IL-17- and IFN γ -producing CD4⁺ T cells by using fluorescence-conjugated antibodies, including FITC-CD3 ϵ , PE-CD4 and allophycocyanin conjugated IL-17A or IFN γ . (A) Representative gating strategy was shown. Cells were gated on CD3 ϵ ⁺CD4⁺ cells. (B, C) Representative data of intracellular IL-17 and IFN γ staining of CD4⁺ T cells from naive IL-10 KO or WT mice. (D, G) Representative data of intracellular IL-17 and IFN γ staining of CD4⁺ T cells from infected mice. (E, H) Summary of the percentage of Th17 or Th1 cells in the spleen and lungs. (F, I) Spleen (7.5×10^5) and lung cells (5×10^5) were cultured for 72 h, and the culture supernatants were tested for IL-17 and IFN γ protein by ELISA. Data are shown as mean \pm SD (n = 4). *p < 0.05; **p < 0.01. Representative data of three independent experiments are shown.

found in naive mice, which was comparable between IL-10 KO and WT mice (Figures 3B, C). The reading of isotype control antibody staining is 0.01–0.02%. The bulk culture of splenocytes and the cells isolated from lung tissues of IL-10 KO mice also showed significantly higher production of IL-17 (IL-17A), suggesting a higher Th17 response at the population level also (Figure 3F). Similarly, significantly higher IFN γ -producing CD4⁺ T cells (Th1) were found in the spleen and lung tissues (Figures 3G, H) after infection. Consistently, culture of spleen and lung cells showed increased IFN γ levels in the culture supernatants. Collectively, the results demonstrate that the increased ICOS-L expression in IL-10 KO mice is associated with reduced Tregs but increased Th17 and Th1 responses in the lymphoid organ and local tissues following *Cm* lung infection.

Expansion of Th17, but Not Th1, Cells Depends on ICOS–ICOS-L Interaction

The association of higher ICOS-L expression on IL-10 KO DCs and the increased Th17 and Th1 cells suggests that ICOS signaling may contribute to the development and/or expansion of these T-cell subsets. To test this hypothesis, we examined the effect of blocking ICOS–ICOS-L interaction on *Cm*-specific Th17 and Th1 cells by using a DC:CD4⁺ T-cell coculture system. CD4⁺ T cells were isolated from *Cm*-immunized mice and were cultured together with DCs isolated from *Cm*-infected IL-10 KO or WT mice for 48 h. The response of Th17 and Th1 cells in the coculture was determined by staining cell surface CD3 ϵ , CD4 and intracellular IL-17 or IFN γ . As shown in Figures 4A and B, the DCs from IL-10 KO mice, which expressed higher ICOS-L, induced a significantly higher percentage of Th17 cells than WT mice (10.6% versus 4.0%). Importantly, the blockade of ICOS–ICOS-L interaction by anti-ICOS-L mAbs dramatically reduced Th17 responses in the coculture CD4⁺ T cells with DCs from IL-10 KO mice (5.2% versus 10.6%). Similar

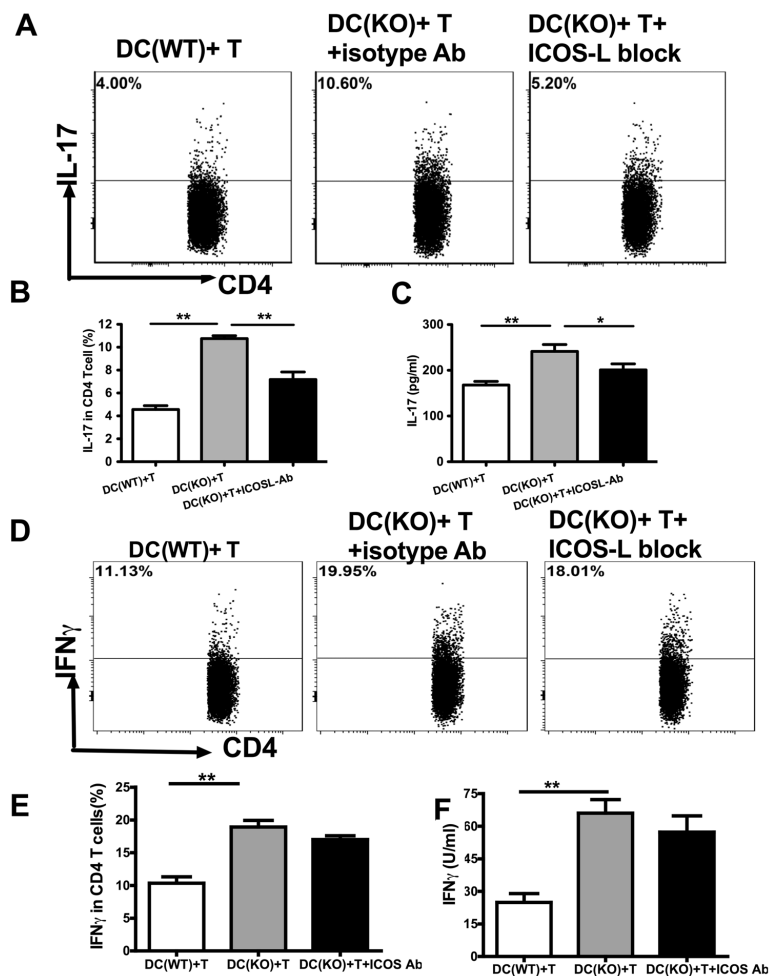


Figure 4. Higher ICOS-L expression is critical for DCs from IL-10 KO mice to promote Th17, but not Th1, response in DC:CD4⁺ T-cell coculture. CD4⁺ T cells isolated from *Cm* immunized mice were cocultured with freshly purified DCs from *Cm*-infected WT (DC(WT)) or IL-10 KO (DC(KO)) mice (DC:CD4⁺ T cells, 5×10^5 : 2×10^6) in the presence of killed *Cm* stimulation with anti ICOS-L mAbs or antibody isotype control. After 48-h cultures, cells were analyzed for surface CD3 ϵ and CD4 and intracellular IL-17 and IFN γ as described in Materials and Methods. Cells were gated on CD3 ϵ ⁺CD4⁺ cells. The percentages of IL-17⁺CD4⁺ T cells or IFN γ ⁺CD4⁺ T cells in total CD4⁺ T cells (CD3 ϵ ⁺CD4⁺) are indicated at the left upper corner. In separated cultures, coculture was carried for 72 h before supernatants were collected, and the supernatants were measured for IL-17 or IFN γ by ELISA. (A) Representative intracellular IL-17 staining in different culture conditions. (B) Summary of the percentages of IL-17⁺CD4⁺ T cells in total CD4⁺ T cells. (C) IL-17A protein levels in the coculture supernatants of each condition. (D) Representative intracellular IFN γ staining in different culture conditions. (E) Summary of the percentages of IFN γ ⁺CD4⁺ T cells in total CD4⁺ T cells. (F) IFN γ protein levels in the coculture supernatants of each condition. Data are shown as mean \pm SD (n = 4). **p* < 0.05; ***p* < 0.01. Representative data of three independent experiments are shown.

changes in Th17 response were found at the population level by measuring IL-17 in the culture supernatants (Figure 4C). In contrast, although higher IFN γ -producing CD4⁺ T cells (Th1) were also

found in the coculture T cells with IL-10 KO DCs than WT DCs (19.95% versus 11.13%), the blockade of ICOS-L had no significant effect on the higher Th1 responses in the coculture with IL-10 KO

mice (Figures 4D, E). A similar finding was found in the culture supernatants (Figure 4F). The results demonstrate that the enhanced Th17 but not Th1 response in IL-10 KO mice depends on ICOS–ICOS-L interaction.

Most *Cm*-Activated Th17 Cells Are ICOS Positive, Whereas Most Th1 Cells Are ICOS Negative

Given the distinction of Th17 and Th1 cells in dependency on ICOS–ICOS-L interaction, we further examined ICOS, the corresponding molecule of ICOS-L, expression on the Th17 and Th1 cells in the naive and *Cm*-infected mice. Spleen and lung mononuclear cells were stained for intracellular IL-17 or IFN γ and surface ICOS in conjunction with CD3 ϵ and CD4. As shown in Figure 5, most (>70%) of the IL-17-producing CD4⁺ T cells were ICOS⁺ in spleen and lung of both IL-10 KO and WT mice before (Figures 5A, B) and after (Figures 5C, D) *Cm* lung infection. Although a small proportion of ICOS[−] Th17 was found (<20%) in the infected mice, these Th17 cells produced significantly less IL-17, as shown by the MFI analysis (Figure 5E). Therefore, the ICOS⁺ Th17 cells were not only the major portion of the Th17 population, but were the higher producers of IL-17. These data, in combination with the observed higher expression of ICOS-L on DCs of IL-10 KO mice, suggest the importance of ICOS signaling in the development of Th17 cells, especially for the higher IL-17 producers. We then examined the expression of ICOS on the IFN γ -producing CD4⁺ T cells (Th1). In sharp contrast to Th17, only a small proportion of Th1 cells in the lung were ICOS positive after *Cm* infection, especially in IL-10 KO mice (Figures 6C, D). Interestingly, most of the Th1 cells in the spleen and lung of naive mice were ICOS⁺ (Figures 6A, B). The infection appeared to selectively enhance ICOS[−] Th1 cells. Indeed, most Th1 cells in the infected lung were ICOS[−] (Figure 6C). Even in the spleen, although it appeared that more Th1 cells were still ICOS⁺ by percentage after infection (Figure 6D), the ICOS⁺ Th1 only increased about twofold,

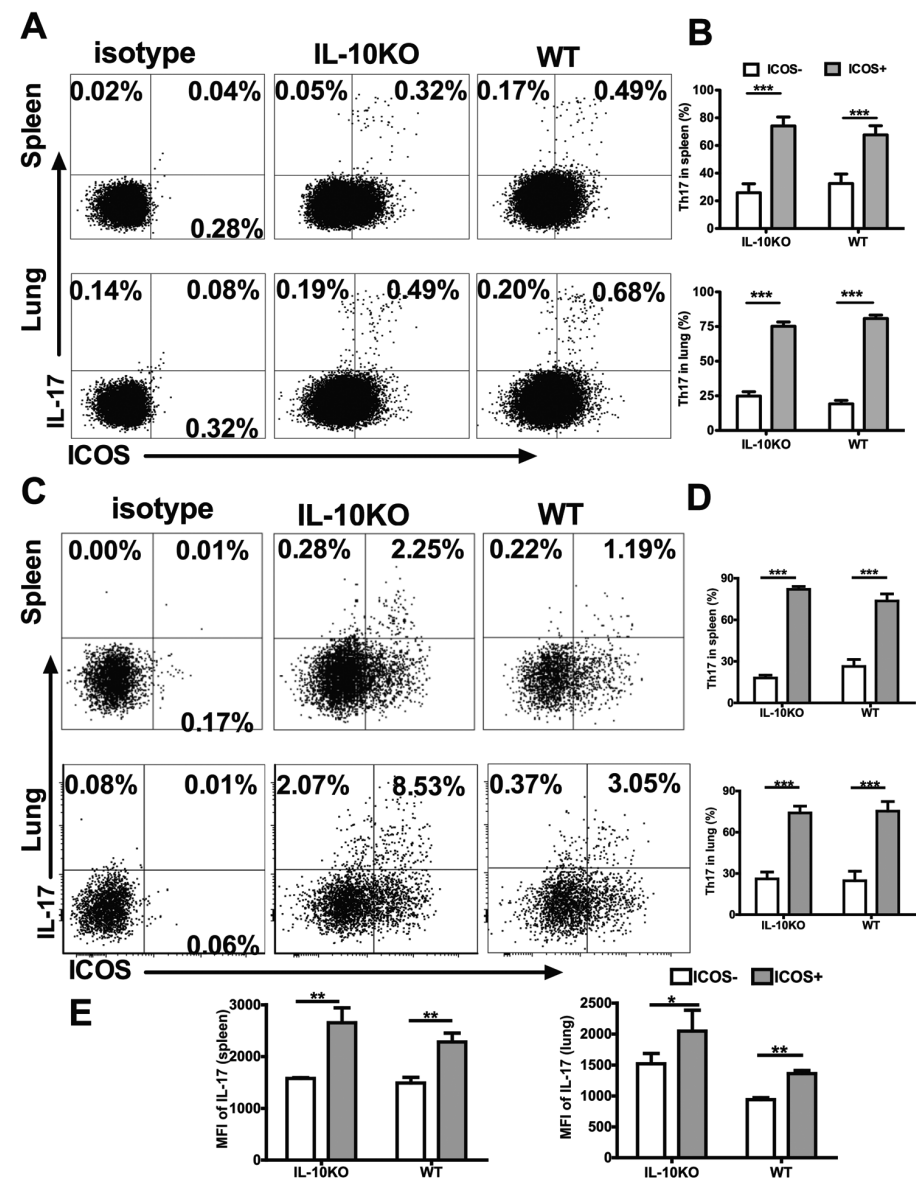


Figure 5. Most Th17 cells are ICOS⁺, and ICOS⁺ Th17 cells produce higher IL-17 than ICOS⁻ Th17 cells. Single spleen cells or lung mononuclear cells isolated from IL-10 KO and WT mice before infection (A, B) or at d 7 (C, D) after *Cm* lung infection (1×10^3 IFUs) were analyzed by four-color staining on surface CD3 ϵ , CD4 and ICOS and intracellular IL-17A. (A) Representative dot plots of ICOS expression on Th17 cells in spleen and lung before *Cm* infection. Cells were gated on CD4⁺CD3 ϵ ⁺ population. (B) Summary of the percentages of ICOS⁺ and ICOS⁻ Th17 cells (CD3 ϵ ⁺CD4⁺IL-17⁺) in total Th17 cells in the uninfected mice. (C) Representative dot plots of ICOS expression on Th17 cells in spleen and lung at d 7 after *Cm* infection. (D) Summary of the percentages of ICOS⁺ and ICOS⁻ Th17 cells (CD3 ϵ ⁺CD4⁺IL-17⁺) in total Th17 cells in the infected mice. (E) The MFI of ICOS⁺ and ICOS⁻ Th17 cells (CD3 ϵ ⁺CD4⁺IL-17⁺) after *Cm* infection. Data are shown as the mean \pm SD for each group (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001. Representative data of three independent experiments are shown.

while the ICOS⁻ Th1 increased about six-fold after infection, if total Th1 cells were considered. Collectively, the data demon-

strate that *Cm* infection preferentially activated ICOS⁺ Th17 cells but ICOS⁻ Th1 cells, consistent with the distinction of

the T-cell subsets on the dependency of ICOS signaling after *Cm* infection.

ICOS KO Mice Show Reduced Th17 but Increased Th1 Response after *Cm* Lung Infection

To further confirm the distinction of Th17 and Th1 cells on dependency of ICOS signaling, we examined ICOS KO mice after *Cm* lung infection. We found that Th17 cells in the spleen and lung were significantly reduced in ICOS KO mice compared with WT mice (Figures 7A, B). In contrast, IFN γ -producing Th1 cells were significantly increased in the ICOS KO mice (Figures 7C, D). These results further confirm that the Th17 response, but not the Th1 response, depends on ICOS signaling during *Cm* lung infection.

Blockade of Th17 Function Reverses the Better Protection of IL-10 KO Mice against *Cm* Infection

Having shown significant enhancement of Th17 response in IL-10 KO mice, we further tested whether the enhanced Th17 response was relevant to protection against chlamydial lung infection. WT and IL-10 KO mice were infected i.n. with *Cm*. One group of IL-10 KO mice was treated i.n. with 10 μ g anti-mouse IL-17 mAbs at 2 h after infection and every 48 h thereafter until the mice were killed at d 7 after infection. The chlamydial burden in the lung was examined. As shown in Figure 8, IL-10 KO mice showed significantly lower chlamydial growth than WT mice. However, the neutralization of IL-17 virtually abolished the better protection observed in IL-10 KO mice. The treatment with isotype control antibody has no effect on the disease progression in WT mice (data not shown). These data clearly indicate that the enhanced Th17 response in IL-10 KO mice contributes significantly to the better protection of these mice against *Cm* infection.

DISCUSSION

This study provides clear evidence that ICOS-L expression on DCs does not depend on IL-10 production and that IL-10 may even inhibit ICOS-L expres-

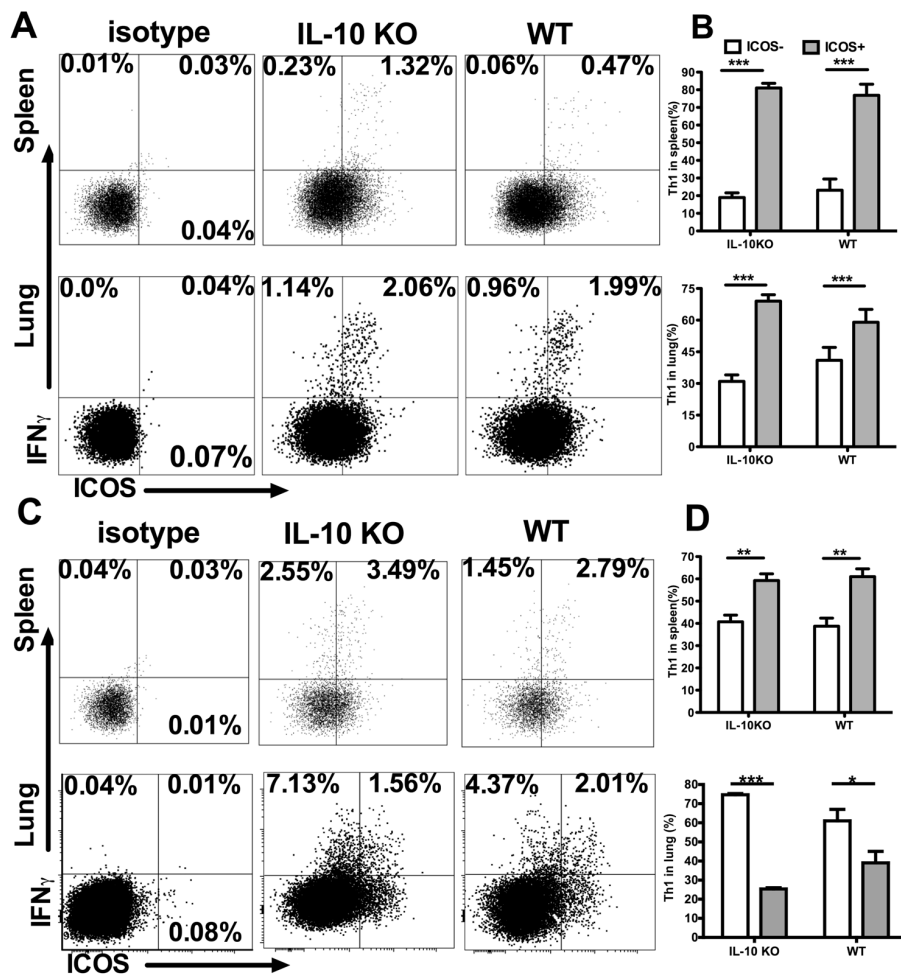


Figure 6. The expression of ICOS on Th1 cells in naive and *Cm*-infected mice. Single spleen or lung cells were prepared from IL-10 KO and WT mice before infection (A, B) or at d 7 after *Cm* infection (C, D). The cells were stained on surface CD3 ϵ , CD4 and ICOS and intracellular IFN γ . (A, C) Representative graph of flow cytometry. The cells were gated on CD3 ϵ ⁺CD4⁺ cells. (B, D) Summary of the percentage of ICOS⁺ and ICOS⁻ cells within IFN γ -producing CD4⁺ T cells (CD3 ϵ ⁺CD4⁺ IFN γ ⁺). Data are shown as the mean \pm SD for each group (n = 4). * p < 0.05, ** p < 0.01; *** p < 0.001. Representative data of three independent experiments are shown.

sion. More importantly, the study demonstrates that ICOS–ICOS-L interaction is critical for Th17, but not Th1, responses in chlamydial lung infection. Notably, this is the first report showing enhanced ICOS-L expression in IL-10 KO mice, which is associated with enhanced protective Th17 response in a respiratory tract infection model.

The finding of the increased ICOS-L expression in IL-10 KO mice is rather surprising but interesting. Given the reported association of ICOS-L expression

by DCs with the induction of Tregs, and immune tolerance, one would expect knockout of IL-10 is likely to reduce the expression of ICOS-L. Indeed, our and others previous studies have shown that the DCs, which are more efficient in inducing Tregs, express higher ICOS-L and produce higher IL-10 (14,15,56,57). However, the data in the present study suggest the possibility that IL-10 may play a negative regulatory role in ICOS-L expression. Therefore, the increase of ICOS-L and IL-10 in tolerogenic DCs is likely

independently regulated. On the other hand, the increased ICOS-L expression, on DCs of IL-10 KO mice, may reflect a compensation effect for the reduction of Treg caused by IL-10 deficiency. The exact mechanism remains unclear, but our data at least suggest that ICOS-L expression on DCs does not depend on, and indeed is reduced by, IL-10 production. It should be pointed out that the method used for DC isolation in this study was based on the expression of CD11c, which could also be expressed in other cells such as macrophages. Although this is a commonly used method for DC isolation, because it mainly isolates higher CD11c cells, which are mostly DCs, the contamination of other cells in the DC preparation cannot be completely excluded.

The most interesting finding in the study is the distinction of Th17 and Th1 cells in dependence on ICOS–ICOS-L signaling. Notably, although the functional distinction of different CD4⁺ T-cell subsets have been well documented, the molecular basis for determining the skewing of the different T-cell subsets remains unclear. The present data show that ICOS–ICOS-L interaction was particularly important for Th17 response, but not for Th1 response, in both *in vitro* coculture and *in vivo* infection experiments. Interestingly, IL-10 KO mice showed enhanced response of both Th17 and Th1 cells, but only Th17 response is related to increased ICOS-L expression. The blocked ICOS–ICOS-L interaction significantly reduced Th17, but not Th1, in the DC:CD4⁺ T-cell coculture. The specific role of ICOS–ICOS-L signaling in Th17 response is further confirmed by the fact that ICOS KO mice showed dramatically reduced Th17 cells but not Th1 cells. In addition, the analysis of ICOS expression by CD4 T cells showed that most of the Th17 cells in the spleen and lung of infected mice were ICOS positive (70–80%), whereas most of the Th1 cells in the infected mice were ICOS negative in lung tissues. The results were consistent with our recent finding that ICOS-L KO mice

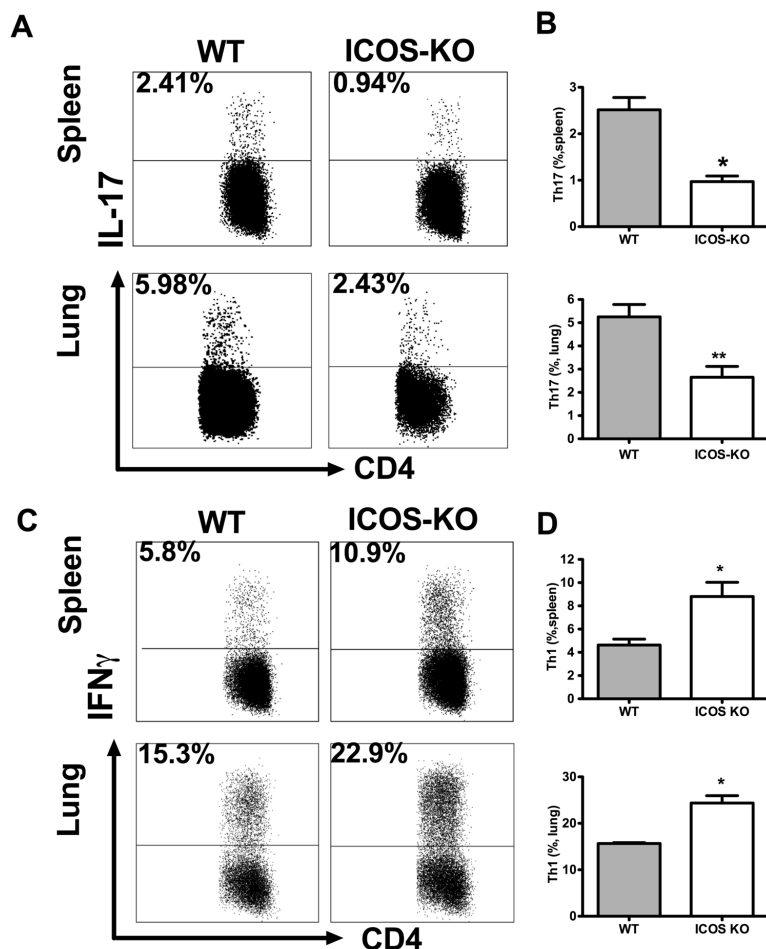


Figure 7. Reduced Th17 but increased Th1 responses in ICOS KO mice compared with WT mice after *Cm* lung infection. ICOS KO or WT mice were infected with *Cm* (1×10^3 IFUs) intranasally, and single cell suspensions of spleen and lung cells were analyzed at d 7 after infection. IL-17 (A, B) or IFN γ -expressing CD4 T cells (C, D) were detected by intracellular cytokine staining. Representative flow cytometric plots (A, C) and summary of the results (B, D) are shown. * $p < 0.05$; ** $p < 0.01$.

mounted significantly lower Th17, but higher Th1, response after chlamydial lung infection (51).

The exact mechanism for ICOS-ICOS-L signaling to enhance Th17 response remains unclear. The importance of ICOS-ICOS-L signaling for Th17 response is reflected not only by its effect on increasing IL-17-producing cells, but by its influence on the capacity of individual Th17 cells to produce IL-17. We not only found that most Th17 cells were ICOS⁺, but also found that the ICOS⁺ Th17 cells were higher producers of IL-17. Although some ICOS⁻ Th17 cell were

found, the data showed that the feature of the ICOS⁻ and ICOS⁺ Th17 cells was different, when measured by their capacity to produce IL-17. Indeed, the MFI of IL-17 in ICOS⁺ Th17 cells was significantly higher than ICOS⁻ Th17 cells in the *Cm*-infected IL-10 KO and WT mice (Figure 5E). It is likely that the higher ICOS-L surface expression (in percentage and MFI) made the IL-10 KO DCs not only more efficient for inducing Th17 cells, but also more efficient for inducing/promoting ICOS⁺ Th17 cells, which are higher IL-17 producers after chlamydial lung infection.

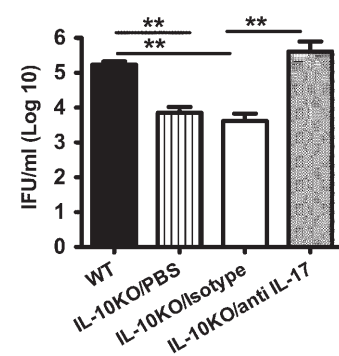


Figure 8. Better protection in IL-10 KO mice after *Cm* infection is virtually abolished by *in vivo* neutralization of IL-17A. WT and IL-10 KO mice were infected i.n. with *Cm* (1×10^3 IFUs). At 2 h after infection, some IL-10 KO mice were administered i.n. with 10 μ g anti-mouse IL-17A mAbs. The same antibody treatment was given at d 2 and 4 after infection. (A) Mice were monitored daily for body weight change. The original body weights of the different groups of mice were similar. Mice were killed on d 7 after infection, and the bacterial loads in the lung were analysis as described in Materials and Methods. Data are shown as the mean \pm SD for each group ($n = 4$). ** $p < 0.01$. Representative data of three independent experiments are shown.

Better protection of IL-10 KO mice in chlamydial infection has been reported previously (46). However, the better protection was attributed to the higher Th1 response in these mice. The present study has confirmed the higher Th1 response in IL-10 KO mice and further demonstrated higher Th17 response in these mice. More importantly we showed that blockade of IL-17 *in vivo* abolished the better protection in IL-10 KO mice. Notably, the role of Th17 cells in host defense against chlamydial lung infection has been recently realized. In particular, we recently reported that IFN γ and IL-17 can work synergistically to inhibit chlamydial growth (58). Therefore, the better protection in IL-10 KO mice is likely the synergistic effect of Th17 and Th1, which are both increased in these mice.

In summary, we found that the IL-10 KO mice exhibited significantly higher

ICOS-L expression by DCs, which is associated with lower Tregs but higher Th17 and Th1 cells. Although both Th17 and Th1 responses are higher in IL-10 KO mice, only the Th17 response is found to depend on ICOS signaling. The data suggest that ICOS-L and IL-10 are discordantly regulated and that the coexistence of ICOS-L with or without IL-10 can determine the polarization of CD4 T cells to Tregs or Th17 cells in bacterial infection.

CONCLUSION

ICOS-L expression on DCs may be negatively regulated by IL-10, and the ICOS-L expression of DCs in the presence or absence of IL-10 costimulation may, respectively, promote Treg or Th17 response, without significant impact on Th1, in intracellular bacterial infection.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes of Health Research, Manitoba Health Research Council and Manitoba Institute for Child Health (to X Yang). X Gao was a trainee in the Canadian Institutes of Health Research National Training Program in Allergy and Asthma and a recipient of a Manitoba Health Research Council/Manitoba Institute for Child Health Graduate Studentship. X Yang is the Canada Research Chair in Infection and Immunity. The authors thank Grant McClarty for help editing the manuscript.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Hutloff A, et al. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*. 397:263–6.
- Coyle AJ, et al. (2000) The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity*. 13:95–105.
- Yoshinaga SK, et al. (1999) T-cell co-stimulation through B7RP-1 and ICOS. *Nature*. 402:827–32.
- Sato T, et al. (2004) Hyperexpression of inducible costimulator and its contribution on lamina propria T cells in inflammatory bowel disease. *Gastroenterology*. 126:829–39.
- Dong C, et al. (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature*. 409:97–101.
- McAdam AJ, et al. (2000) Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J. Immunol*. 165:5035–40.
- McAdam AJ, et al. (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature*. 409:102–5.
- Tafari A, et al. (2001) ICOS is essential for effective T-helper-cell responses. *Nature*. 409:105–9.
- Kopf M, et al. (2000) Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. *J. Exp. Med*. 192:53–61.
- Rottman JB, et al. (2001) The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat. Immunol*. 2:605–11.
- Ozkaynak E, et al. (2001) Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat. Immunol*. 2:591–6.
- Subudhi SK, Alegre ML, Fu YX. (2005) The balance of immune responses: costimulation versus coinhibition. *J. Mol. Med. (Berl)*. 83:193–202.
- Akbari O, et al. (2002) Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyper-reactivity. *Nat. Med*. 8:1024–32.
- Gao X, et al. (2012) CD8alpha+ and CD8alpha- DC subsets from BCG-infected mice inhibit allergic Th2-cell responses by enhancing Th1-cell and Treg-cell activity respectively. *Eur. J. Immunol*. 42:165–75.
- Han X, et al. (2006) Chlamydia infection induces ICOS ligand-expressing and IL-10-producing dendritic cells that can inhibit airway inflammation and mucus overproduction elicited by allergen challenge in BALB/c mice. *J. Immunol*. 176:5232–9.
- Jiao L, et al. (2011) NK cells promote type 1 T cell immunity through modulating the function of dendritic cells during intracellular bacterial infection. *J. Immunol*. 187:401–11.
- Dong C. (2008) TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol*. 8:337–48.
- Manel N, Unutmaz D, Littman DR. (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgamma. *Nat. Immunol*. 9:641–9.
- Khader SA, et al. (2007) IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. 2007. *Nat. Immunol*. 8:369–77.
- Das J, et al. (2009) Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J. Exp. Med*. 206:2407–16.
- Korn T, et al. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. 448:484–7.
- Busse M, Krech M, Meyer-Bahlburg A, Hennig C, Hansen G. (2012) ICOS mediates the generation and function of CD4+CD25+Foxp3+ regulatory T cells conveying respiratory tolerance. *J. Immunol*. 189:1975–82.
- Whitehead GS, et al. (2012) IL-35 production by inducible costimulator (ICOS)-positive regulatory T cells reverses established IL-17-dependent allergic airways disease. *J. Allergy Clin. Immunol*. 129:207–15.
- Hiraki S, et al. (2012) Neutralization of interleukin-10 or transforming growth factor-beta decreases the percentages of CD4+ CD25+ Foxp3+ regulatory T cells in septic mice, thereby leading to an improved survival. *Surgery*. 151:313–22.
- Hoffman BE, et al. (2011) Nonredundant roles of IL-10 and TGF-beta in suppression of immune responses to hepatic AAV-factor IX gene transfer. *Mol. Ther*. 19:1263–72.
- Zheng SG, Wang J, Horwitz DA. (2008) Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. *J. Immunol*. 180:7112–6.
- Kimura A, Kishimoto T. (2010) IL-6: regulator of Treg/Th17 balance. *Eur. J. Immunol*. 40:1830–5.
- Langrish CL, et al. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med*. 201:233–40.
- Nakae S, et al. (2003) IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc. Natl. Acad. Sci. U. S. A*. 100:5986–90.
- Curtis MM, Way SS. (2009) Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology*. 126:177–85.
- Zhang X, et al. (2009) A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen *Chlamydia muridarum*. *J. Immunol*. 183:1291–300.
- Umemura M, et al. (2007) IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. *J. Immunol*. 178:3786–96.
- Sieve AN, et al. (2009) A novel IL-17-dependent mechanism of cross protection: respiratory infection with mycoplasma protects against a secondary listeria infection. *Eur. J. Immunol*. 39:426–38.
- Bai H, et al. (2009) IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function. *J. Immunol*. 183:5886–95.
- Park H, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol*. 6:1133–41.

36. Kadkhoda K, *et al.* (2011) ICOS ligand expression is essential for allergic airway hyperresponsiveness. *Int. Immunol.* 23:239–49.
37. Galicia G, *et al.* (2009) ICOS deficiency results in exacerbated IL-17 mediated experimental autoimmune encephalomyelitis. *J. Clin. Immunol.* 29:426–33.
38. Gao X, *et al.* (2012) Anti-chlamydial Th17 responses are controlled by the inducible costimulator partially through phosphoinositide 3-kinase signaling. *PLoS One.* 7:e52657.
39. Ward ME. (1995) The immunobiology and immunopathology of chlamydial infections. *APMIS.* 103:769–96.
40. Schachter J. (1978) Chlamydial infections (third of three parts). *N. Engl. J. Med.* 298:540–9.
41. Morrison RP, Caldwell HD. (2002) Immunity to murine chlamydial genital infection. *Infect. Immun.* 70:2741–51.
42. Igietseme JU, Smith K, Simmons A, Rayford PL. (1995) Effect of gamma-irradiation on the effector function of T lymphocytes in microbial control. *Int. J. Radiat. Biol.* 67:557–64.
43. Cain TK, Rank RG. (1995) Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Infect. Immun.* 63:1784–9.
44. Yang X. (2003) Role of cytokines in *Chlamydia trachomatis* protective immunity and immunopathology. *Curr. Pharm. Des.* 9:67–73.
45. Lu H, *et al.* (2000) *Chlamydia trachomatis* mouse pneumonitis lung infection in IL-18 and IL-12 knockout mice: IL-12 is dominant over IL-18 for protective immunity. *Mol. Med.* 6:604–12.
46. Yang X, Gartner J, Zhu L, Wang S, Brunham RC. (1999) IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following *Chlamydia trachomatis* lung infection. *J. Immunol.* 162:1010–7.
47. Holland MJ, Bailey RL, Hayes LF, Whittle HC, Mabey DC. (1993) Conjunctival scarring in trachoma is associated with depressed cell-mediated immune responses to chlamydial antigens. *J. Infect. Dis.* 168:1528–31.
48. Wang S, Fan Y, Brunham RC, Yang X. (1999) IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. *Eur. J. Immunol.* 29:3782–92.
49. Yang X, HayGlass KT, Brunham RC. (1996) Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of *Chlamydia trachomatis* mouse pneumonitis infection. *J. Immunol.* 156:4338–44.
50. Su H, Caldwell HD. (1995) CD4+ T cells play a significant role in adoptive immunity to *Chlamydia trachomatis* infection of the mouse genital tract. *Infect. Immun.* 63:3302–8.
51. Kadkhoda K, *et al.* (2010) Th1 cytokine responses fail to effectively control *Chlamydia* lung infection in ICOS ligand knockout mice. *J. Immunol.* 184:3780–8.
52. Qiu H, *et al.* (2008) Type I IFNs enhance susceptibility to *Chlamydia muridarum* lung infection by enhancing apoptosis of local macrophages. *J. Immunol.* 181:2092–102.
53. Joyee AG, Uzonna J, Yang X. (2010) Invariant NKT cells preferentially modulate the function of CD8 alpha+ dendritic cell subset in inducing type 1 immunity against infection. *J. Immunol.* 184:2095–106.
54. Joyee AG, *et al.* (2007) Distinct NKT cell subsets are induced by different *Chlamydia* species leading to differential adaptive immunity and host resistance to the infections. *J. Immunol.* 178:1048–58.
55. Ivanov II, *et al.* (2006) The orphan nuclear receptor RORgamma directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 126:1121–33.
56. Ito T, *et al.* (2007) Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J. Exp. Med.* 204:105–15.
57. Martin-Orozco N, *et al.* (2010) Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells. *Cancer Res.* 70:9581–90.
58. Zhang YH, *et al.* (2012) IL-17A synergizes with IFN-gamma to upregulate iNOS and NO production and inhibit chlamydial growth. *PLoS One.* 7:e39214.