

RESEARCH ARTICLE

Respective IL-17A production by $\gamma\delta$ T and Th17 cells and its implication in host defense against chlamydial lung infection

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The role of IL-17A is important in protection against lung infection with *Chlamydiae*, an obligate intracellular bacterial pathogen. In this study, we explored the producers of IL-17A in chlamydial lung infection and specifically tested the role of major IL-17A producers in protective immunity. We found that $\gamma\delta$ T cells and Th17 cells are the major producers of IL-17A at the early and later stages of chlamydial infection, respectively. Depletion of $\gamma\delta$ T cells *in vivo* at the early postinfection (p.i.) stage, when most $\gamma\delta$ T cells produce IL-17A, failed to alter Th1 responses and bacterial clearance. In contrast, the blockade of IL-17A at the time when IL-17A was mainly produced by Th17 (day 7 p.i.) markedly reduced the Th1 response and increased chlamydial growth. The data suggest that the $\gamma\delta$ T cell is the highest producer of IL-17A in the very early stages of infection, but the protection conferred by IL-17A is mainly mediated by Th17 cells. In addition, we found that depletion of $\gamma\delta$ T cells reduced IL-1 α production by dendritic cells, which was associated with a reduced Th17 response. This finding is helpful to understand the variable role of IL-17A in different infections and to develop preventive and therapeutic approaches against infectious diseases by targeting IL-17A.

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INTRODUCTION

IL-17A is a T-cell-derived pro-inflammatory cytokine that induces the generation, migration and accumulation of neutrophils. It is generally accepted that IL-17 signaling plays an important role in controlling extracellular infections by enhancing chemokine responses, inducing antimicrobial proteins and recruiting inflammatory cells.^{1–4} Although IL-17A is considered less critical for protection against intracellular infections,⁵ recent data suggest a significant contribution of IL-17A in the protective immunity against some intracellular bacterial infections, such as *Francisella tularensis* LVS⁶ and *Chlamydiae*.^{7–12}

$\gamma\delta$ T cells are functionally differentiated within the fetal thymus before being exposed to foreign antigens, and thus are known as naturally occurring effectors.^{13,14} $\gamma\delta$ T cells constitute a small

percentage of the circulating T cells in humans and in mice, but are predominantly distributed in mucosal tissues such as the peritoneal cavity, gut and lung.¹⁴ $\gamma\delta$ T cells are the first to develop and are recruited and expanded in response to various mucosal infections.¹⁴ Although the study of IL-17A production has largely focused on CD4+ $\alpha\beta$ T cells, termed T helper 17 (Th17) cells, a number of recent studies using various experimental mouse models have shown that $\gamma\delta$ T cells are also a major producer of IL-17 following intracellular bacterial infections, including *Mycobacterium tuberculosis*,¹⁵ *Listeria monocytogenes*¹⁶ and *Salmonella enterica enteritidis*.¹⁷ Similarly, $\gamma\delta$ T cells are also found to be a major source of IL-17A in human tuberculosis patients.¹⁸ In addition, other cell types such as CD8+ T cells,¹⁹ NK cells²⁰ and neutrophils²¹ are also reported to be IL-17A producers. Therefore,

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IL-17A production by the innate immune cells may bridge the innate and adaptive immunity, contributing to protection against intracellular infections.

Chlamydia is an intracellular bacterium that mainly infects mucosal epithelial cells and macrophages, causing different types of human and farm animal diseases. Using a respiratory tract infection model of *Chlamydia muridarum* (Cm), we and others have shown that chlamydial lung infection can induce IL-17A production and Th17 cell expansion, which plays an important role in the host defense against the infection.^{7,11} We also found that IL-17A can promote type-1 T-cell immunity through modulating dendritic cell (DC) function,⁷ and that IL-17A can synergize with Th1 cytokines to control *Chlamydia* infections.⁷⁻⁹ However, the sources of IL-17A production in lung chlamydial infection and, more importantly, the contribution of various IL-17A producers in the host defense against chlamydial infection remain unclear. Therefore, we performed this study to specifically address these questions.

In this study, we demonstrate that $\gamma\delta$ T cells and CD4+ T (Th17) cells are the two major producers of IL-17A in the lung at the early and later stages of chlamydial infection, respectively. More importantly, our results indicate that Th17 is the dominant effector of IL-17A-mediated protection against Cm lung infection, although $\gamma\delta$ T cells are the major producer of IL-17A in the very early stages of infection.

MATERIALS AND METHODS

Mice and microorganisms

Female BALB/c mice of 6–8 weeks of age were purchased from Charles River Laboratories (St Constant, Canada) and housed in the animal care facility at the University of Manitoba under pathogen-free conditions. All mice used in experiments were between 6 and 8 weeks old, and matched for sex and age. The research protocol was approved by the institutional ethical committees, and all animal experiments were conducted according to the guidelines of the Canadian Council of Animal Care. Cm, a mouse chlamydial strain, was used for airway infection of the mice. The reproduction and purification of Cm elementary bodies (EBs) were performed as previously described^{22,23} and stored at -80°C until further use. UV-inactivated EBs were used for antigen stimulation in cell culture.

Infection of mice and $\gamma\delta$ T cells depletion

For airway infection, a dose of 1×10^3 inclusion-forming units (IFU) of Cm were given intranasally (i.n.) to the mice under appropriate anesthesia in a 40 μl volume of PBS as described.⁷ For the depletion of airway $\gamma\delta$ T cells, mice were treated i.n. with 10 μg anti-TCR $\gamma\delta$ monoclonal antibody (mAb; clone GL3, BD Pharmingen, San Diego, CA, USA) at 1 day before infection in 40 μl of PBS. Sham-treated control mice were administered i.n. with isotype-matched anti-Hamster IgG mAb (BD Pharmingen) on the same schedule as anti-TCR $\gamma\delta$ mAb delivery. For IL-17A neutralization, $\gamma\delta$ T-cell-depleted mice or isotype control mice were further administered i.n. with 10 μg anti-mouse IL-17A mAb (R&D Systems, Minneapolis, MN, USA) at day 7 postinfection (p.i.). The mice were killed at designated time points p.i. and the immune responses and infection were analyzed.

Determination of pulmonary chlamydial loads and histopathological analysis

The mice were intranasally infected with Cm and were killed at the indicated time points. The lungs were homogenized in sucrose phosphate glutamic acid buffer (SPG). To determine chlamydial growth *in vivo*, lung homogenates were titrated by infection of HeLa cell monolayers as described previously.⁷ For histopathological analysis, the lungs were fixed in 10% formalin. The lung sections were stained with H&E, and the histological changes of the lung were analyzed by light microscopy.

Cell preparation

Lung mononuclear cells of mice were prepared and analyzed as described previously.²⁴ Briefly, lung tissues were digested in RPMI 1640 containing 2 mg/ml collagenase type XI (Sigma-Aldrich, St Louis, MO, USA) and 100 g/ml DNase I (Sigma-Aldrich) for 60 min at 37°C and 2 mM EDTA was added during the last 5 min of incubation. After enzymatic digestion, lung leukocytes were further enriched by 35% Percoll (Sigma-Aldrich) centrifugation and followed by lysis of erythrocytes using ACK lysing buffer. Spleen cells were prepared by 2 mg/ml collagenase D (Sigma-Aldrich) digestion. CD11c+ DCs were isolated and purified from splenic single-cell suspensions by using the MACS (Miltenyi Biotec, Auburn, CA, USA) system as previously described.²⁴

Cytokine production assay

Spleen and lung mononuclear cells from Cm-infected- $\gamma\delta$ T-cell-depleted mice or isotype control antibody-treated mice were cultured with UV-sterilized Cm (UV-Cm) to test Cm-driven cytokine production, as described.²⁴ Briefly, spleen and lung single-cell suspensions were cultured at a concentration of 7.5×10^6 cells/well and 5×10^6 cells/well, respectively, in the presence or absence of UV-Cm (1×10^5 IFU/ml) stimulation. For the detection of cytokine production by DCs, the isolated DCs were cultured with UV-Cm in 96-well plates at a density of 5×10^5 cells/well. After 72 h culture, the supernatants were collected for cytokine detection by enzyme-linked immunosorbent assay (ELISA; eBioscience, San Diego, CA, USA) as described.²⁴

FACS analysis

For cell-surface staining, designated cells were incubated with fluorochrome-conjugated mAb for 20 min on ice followed by washing with PBS containing 2% bovine serum albumin (BSA). Conjugated antibodies specific for cell surface markers, CD3, CD4, CD8 and DX5 as well as isotype controls were purchased from eBioscience or Biolegend (San Diego, CA, USA). To determine intracellular cytokine expression, cells were cultured with 50 ng/ml PMA (eBioscience), 1 $\mu\text{g}/\text{ml}$ ionomycin (eBioscience) and 5 $\mu\text{g}/\text{ml}$ brefeldin A (eBioscience) for 5 h at 37°C . The cells were stained first for surface antigens and then fixed with 4% w/v paraformaldehyde in PBS. The cells were washed with permeabilization buffer which contained 0.1% saponin (Sigma), 2% heat-inactivated FCS and 0.1% NaN_3 in PBS followed by incubation with saponin and allophycocyanin-conjugated anti-IFN γ (eBioscience) or anti-IL-17A mAb for 30 min at room temperature. The cells were analyzed by using

an LSR II flow cytometer (BD). The absolute number of $\gamma\delta$ T cells and IL-17A-producing $\gamma\delta$ T, CD4 T, CD8 T and NK cells in the lung was calculated using the total lung mononuclear cell number and the ratio of these cells determined by flow cytometry.

Statistical analysis

Unpaired Student's *t*-test was used to compare the significance of differences between groups, and one-way ANOVA was used for analyzing the significance among multiple groups.

RESULTS

$\gamma\delta$ T and Th17 are the predominant cells producing IL-17A following Cm lung infection

We first identified the major IL-17A-producing cells in the lung following Cm infection, focusing on CD3+ $\gamma\delta$ + T cells ($\gamma\delta$ T cells), CD3+CD4+ T cells (CD4 T cells), CD3+CD8+ T cells (CD8 T cells) and CD3-DX5+ cells (NK cells), which were reportedly IL-17A producers. Lung mononuclear cells obtained from infected mice at different time points after infection were examined for cell surface markers and intracellular IL-17A by flow cytometry. We analyzed the percentage of IL-17A producers of particular cell populations (Figures 1a and b) and also the percentage and total number of the particular type of cells (Figures 1c and d) in the lung. We believe the total number is a better reflection of the particular cell population in the infection. As shown in Figure 1b, $\gamma\delta$ T cells had the highest level (16%) of background IL-17A production among the cells being tested before infection (day 0). More markedly, following Cm lung infection, the number of IL-17A-producing $\gamma\delta$ T cells quickly reached a peak at 24 h (day 1) with 57% of this cell population producing IL-17A (Figures 1b and d), representing 70% of the IL-17A-producing cells in the lung at this time point (Figure 1c). Similar to its quick increase, the IL-17A production by $\gamma\delta$ T cells also dropped quickly, reaching close to the background level at day 4 p.i. (Figure 1d). In contrast, the percentage of IL-17A-producing CD3+CD4+ T cells (Th17) increased slowly after infection, reaching its peak at day 10 p.i.. At day 1 p.i., only 2% of the CD4+ T cells were Th17 cells (Figure 1b), representing ~30% of the total IL-17A-producing cells in the lung (Figure 1c) while at day 10, 6% of the CD4+ T cells were Th17 (Figure 1b), representing 85% of the total IL-17A-producing cells in the lung (Figure 1c). Although IL-17A-producing CD8 T cells also increased slightly following Cm lung infection with a peak at day 10 p.i., the level was very low (<1% of CD8 T cells; Figure 1a). IL-17A-producing NK cells are virtually negligible. These data demonstrate that the $\gamma\delta$ T cell is the major IL-17A producer at the very early stages of infection (day 1), while at the peak of infection (day 10), Th17 is the major producer of IL-17A. Although the percentage of IL-17A producers within CD4+ T cells was never as high as the IL-17A producers within $\gamma\delta$ T cells, the absolute number of Th17 at the later stages of infection was more than three-fold higher than that of the IL-17A producing $\gamma\delta$ T cells. The fold increase in Th17 cells represents the increased IL-17A producers in CD4+ T cells and the increased total number of CD4 T cells in the lung at the later stages of infection. Collectively, the data suggest that $\gamma\delta$ T cell is the major IL-17A producer in the beginning of the infection, while Th17 is

the major IL-17A producer in the later stage of infection when the adaptive immunity plays its role in host defense.

Early IL-17A-producing $\gamma\delta$ T cells largely exist in the lung following infection with Cm

The quick IL-17A production (peak at day 1 p.i.) caused us to wonder about the correlation between $\gamma\delta$ T cell activation/expansion and IL-17A production by these cells. We therefore measured IL-17A-producing cell populations and $\gamma\delta$ T-cell activation following Cm infection. Interestingly, at day 1 p.i., the total number of $\gamma\delta$ T cells did not significantly change compared with CD4 and CD8 T cells (Figures 2a and b), and the expression of CD69, an activation marker, was also minimal (Figure 2c), although it exhibited an upward trend. The absolute number of $\gamma\delta$ T cells in the lung increased, steadily peaking at day 10 p.i. while the expression of CD69 peaked at day 3 and remained high during the experimental period (up to day 14). As the peak of IL-17A production by $\gamma\delta$ T cells occurred at day 1 p.i. (Figure 1) when the $\gamma\delta$ T-cell increase/expansion and activation is minimal (Figure 2), the data suggest that the quick IL-17A-producing $\gamma\delta$ T cells likely exist in the lung that can produce IL-17A without full activation (high CD69 expression) following Cm lung infection. Indeed, although the total number of $\gamma\delta$ T cells more than doubled at the peak of infection (day 10), the percentage of IL-17A-producers was much lower compared with day 1 p.i. (9% versus 59%), thus the total number of IL-17A-producing $\gamma\delta$ T cells was even lower than its baseline. The data also suggest that the newly expanded or recruited $\gamma\delta$ T cells following Cm infection are largely not IL-17A producers.

Depletion of $\gamma\delta$ T cells led to reduced IL-17A production but had no significant effect on *in vivo* chlamydial growth

To investigate the influence of $\gamma\delta$ T cells on general IL-17A production and disease processes, we depleted $\gamma\delta$ T cells from mice by i.n. administration of mAb (GL3) against TCR $\gamma\delta$ before Cm infection. This mAb targets the $\gamma\delta$ TCR and has been successfully used for *in vivo* $\gamma\delta$ T cell depletion in various infection models.²⁵⁻²⁷ We found that more than 95% of $\gamma\delta$ T cells were depleted in the lung in naive (Figure 3a) and Cm-infected (Figure 3b) mice when the antibody was delivered intranasally, without a significant effect on $\alpha\beta$ T-cell population. We then compared the IL-17A production between $\gamma\delta$ T-cell depleted and isotype control antibody-treated mice. We found a significant reduction in IL-17A production by lung mononuclear cells isolated at day 7 and day 14 with UV-Cm stimulation following Cm infection (Figure 3c) and in lung tissue homogenates at day 7 (Figure 3d) in the $\gamma\delta$ T-cell-depleted mice compared with the control mice. The data confirm that $\gamma\delta$ T cells contribute to IL-17A production following Cm infection.

As we and others have previously shown that IL-17A contributes to the host defense against chlamydial lung infection,⁷⁻¹⁴ we then examined the effect of the depletion of $\gamma\delta$ T cells, which significantly contributed to IL-17A production in Cm infection, on the disease process. Body weight monitoring showed a larger scale of body weight loss in the $\gamma\delta$ T-cell-depleted mice (Figure 3e), especially at the later days post infection, but the lung bacterial loads

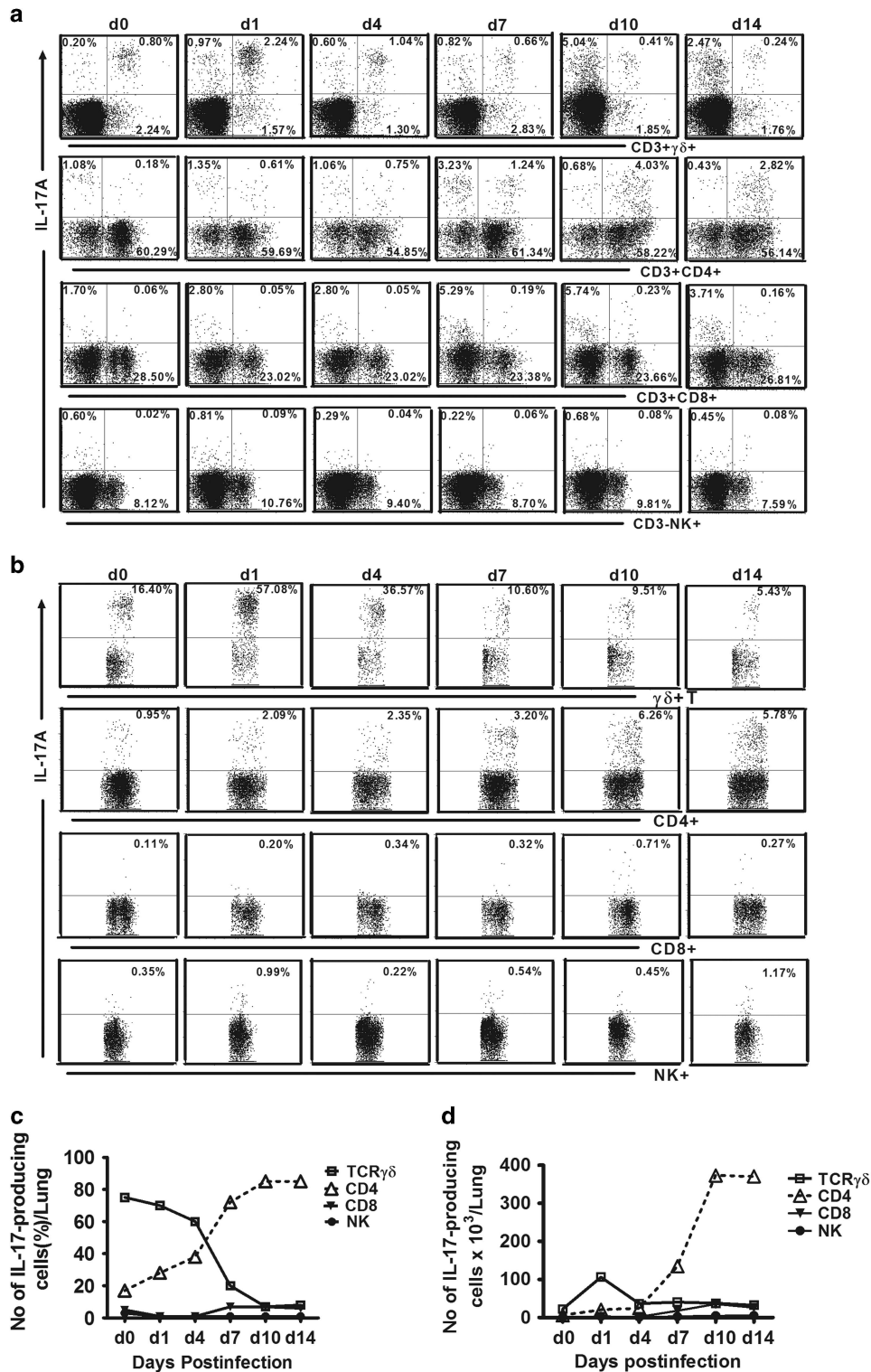


Figure 1 IL-17A producing cells in the lung following Cm infection. BALB/c mice were intranasally infected with Cm (1×10^3 IFUs). Lung mononuclear cells were isolated and intracellular IL-17A, as well as surface markers, were analyzed at various time points by flow cytometry. (a) Percentage of IL-17A-producing-CD3+ $\gamma\delta$ + T, CD3+CD4+ T, CD3+CD8+ T cells and CD3– NK cells in gated lymphocytes. (b) Percentage of IL-17A-producing cells in gated $\gamma\delta$ +CD3+T cells, CD4+CD3+ T cells, CD8+CD3+ T cells and DX5+CD3– cells. (c) Percentage of IL17A producing CD3+ $\gamma\delta$ + T, CD3+CD4+ T, CD3+CD8+ T cells and CD3– NK cells in total IL-17A-producing cells. (d) Absolute number of IL-17A-producing $\gamma\delta$ T, CD4+ T, CD8+ T and NK cells. Three independent experiments with four mice in each group were tested, and a representative experiment is shown.

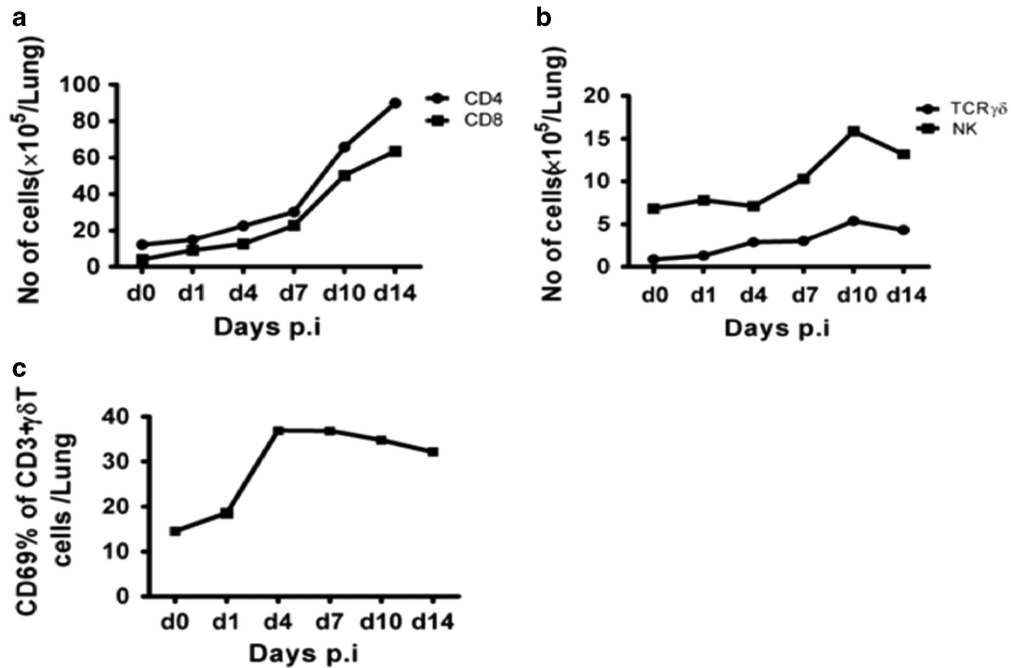


Figure 2 Increase of particular cell populations and activation of $\gamma\delta$ + T cells in the lung following Cm infection. BALB/c mice were intranasally infected with Cm (1×10^3 IFUs) and killed at the indicated time points. The lung mononuclear cells were prepared and stained with PE-Cy7-anti-CD3, FITC-anti-CD4, PE-anti-CD8, PE-anti-TCR $\gamma\delta$ and FITC-anti-DX5 mAbs. The lymphocyte population was analyzed by flow cytometry. The absolute number of CD4+ (CD4+CD3+) T cells and CD8+ (CD8+CD3+) T cells (a), $\gamma\delta$ + (CD3+ $\gamma\delta$ +) T cells and NK (CD3-DX5+) cells (b) were calculated using total lung mononuclear cell number, and the ratio of the particular cells were determined by flow cytometry. Percentage of CD69 expression was evaluated on CD3+ $\gamma\delta$ T cells of the lung during Cm infection (c). Three independent experiments with four mice in each group were tested, and a representative experiment is shown.

(IFUs) were not significantly different between the $\gamma\delta$ T-cell-depleted and isotype control-treated mice at early (day 4), peak (day 7) and later (day 14) stages of infection (Figure 3f). These results suggest that $\gamma\delta$ T cell contributes to IL-17A production and reduces morbidity (measured by less body weight loss), and its role in chlamydial clearance is rather limited.

Depletion of $\gamma\delta$ T cells significantly decreased Th17 but not Th1 following Cm infection

Previous work has shown synergy between the Th17 and Th1 responses in the host defense against chlamydial lung infection, and an enhancing effect of IL-17A on the Th1 response.⁷⁻⁹ Having shown the ample IL-17A production by $\gamma\delta$ T cells and the reduction of general IL-17A production by depletion of this cell type, we then tested if $\gamma\delta$ T cells could influence Th17 cells, that is, IL-17A producing CD3+CD4+ T cells. The results showed that the Th17 response was significantly reduced in the $\gamma\delta$ T-cell-depleted mice, both in percentage and absolute number at day 7 and day 14 p.i. (Figure 4a). Therefore, $\gamma\delta$ T cells do have a promoting effect on the Th17 responses in chlamydial lung infection.

We then further examined the effect of $\gamma\delta$ T-cell depletion on Th1 responses in Cm lung infection. The results showed that there was no significant difference in IFN γ production by cultured lung mononuclear cells (left panel) or lung tissue suspension (right panel) between the $\gamma\delta$ T-cell-depleted and isotype control antibody-treated mice (Figure 4b). Consistently, at the single-cell

level, there was no significant difference in IFN- γ -producing CD4+ T and CD8+ T cells in the lung between the two groups (Figure 4c). The results suggest that $\gamma\delta$ T cells, although producing a large amount of IL-17A at the early stage of infection, have no significant impact on Th1 and Tc1 responses.

Th17, rather than $\gamma\delta$ T cell, is critical for enhancing Th1 response and protective immunity against lung infection

Considering the facts that neutralization of IL-17A has a significant detrimental effect on the Th1 response and chlamydial clearance,⁷ while depletion of $\gamma\delta$ T cells, the major IL-17A producer in early the stage, had no significant impact on chlamydial clearance (Figure 4), we hypothesized that IL-17A-producing $\gamma\delta$ T cells and Th17 had different effects on the Th1 response and host defense. To test this hypothesis, we tried to separately test the role of the $\gamma\delta$ T cell and Th17 by *in vivo* antibody depletion/neutralization. On the basis of the kinetics of IL-17A production by $\gamma\delta$ T cells and Th17 cells observed in Figure 1 ($\gamma\delta$ T cell dominated during the first 3 days while Th17 became dominant from day 7), we reasoned that the early depletion of $\gamma\delta$ T cells (one day before infection) and later neutralization (day 7) of IL-17A, could, respectively examine the function of IL-17A-producing $\gamma\delta$ T cell and Th17 cell. Using this approach, we found that compared with early depletion of $\gamma\delta$ T cells, the late neutralization of IL-17A (day 7) thus blocking Th17 had a much more profound impact on the disease process, demonstrated by more serious body weight loss (Figure 5a),

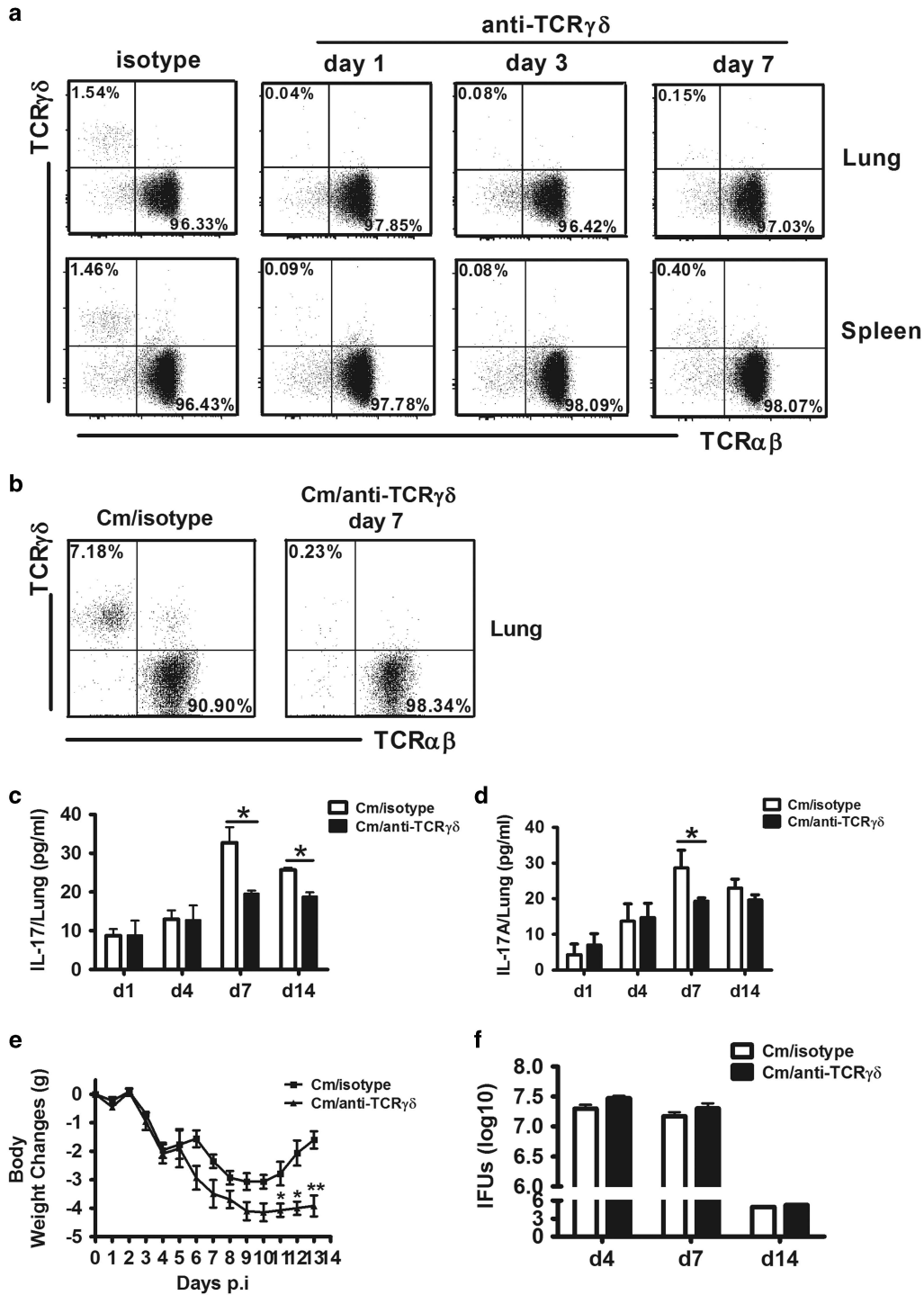


Figure 3 Depletion of $\gamma\delta$ T cells significantly reduced IL-17A production. BALB/c mice were intranasally treated with 10 μ g either anti-TCR $\gamma\delta$ mAb (clone GL3) or isotype-matched anti-Hamster IgG mAb (isotype) 1 day before 1×10^3 IFUs Cm airway infection. (a) Successful depletion of $\gamma\delta$ T cells by the anti-TCR $\gamma\delta$ mAb treatment in naive mice at day 1, day 3 and day 7 after Ab administration. Percentage of TCR $\gamma\delta$ and TCR $\alpha\beta$ in the lung and spleen were analyzed by flow cytometry, gating on CD3+ cells. (b) Percentage of TCR $\gamma\delta$ and TCR $\alpha\beta$ in the lung following isotype control or anti-TCR $\gamma\delta$ mAb administration at day 7 of Cm infection. (c, d) IL-17A production was measured in cell culture supernatants of the lung mononuclear cells (c) and in lung tissue homogenates by ELISA. (d, e) The body weight change in anti-TCR $\gamma\delta$ mAb and isotype control mAb-treated-mice following Cm infection. The original body weights of the two groups of mice were similar. (f) Mice were killed on days 4, 7 and 14 p.i. and the lungs were collected and measured for chlamydial growth *in vivo* as described in the Materials and Methods section. Three independent experiments with four mice in each group were tested. The results are shown as the mean \pm s.d. * $P < 0.05$, ** $P < 0.01$. Note, pooled data of body weight change (e) of 12 mice in each group from three independent experiments are shown.

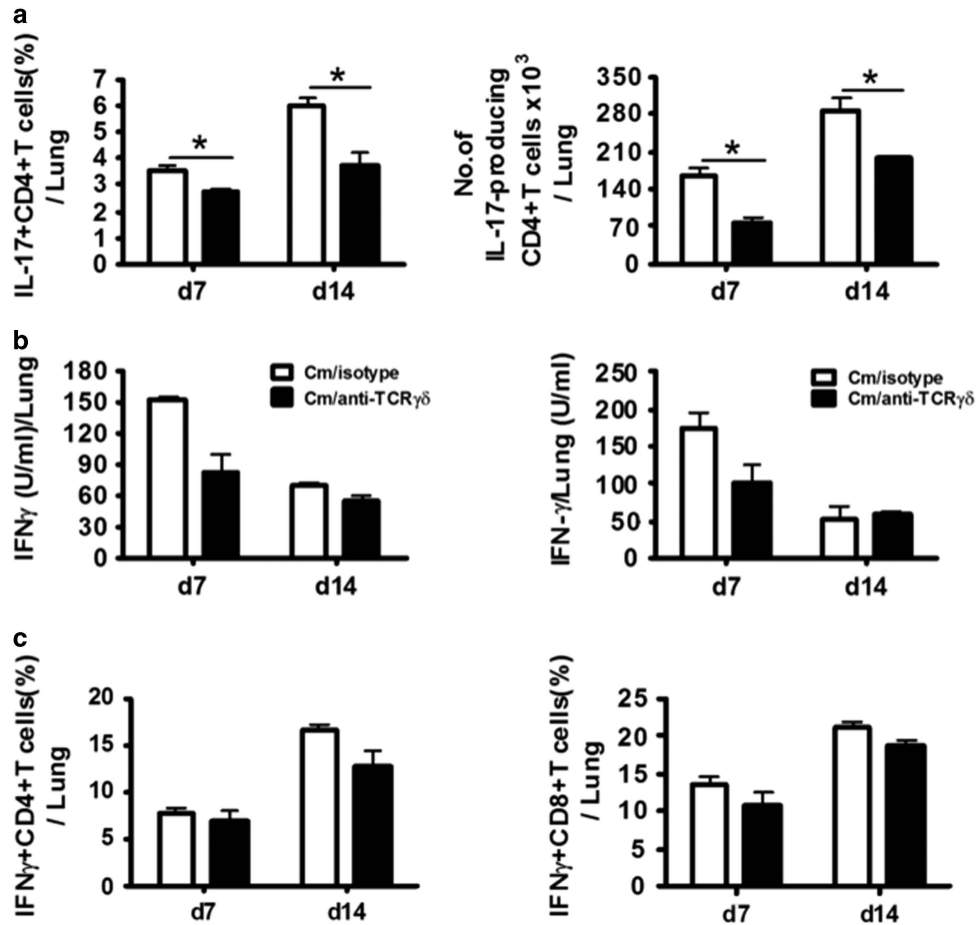


Figure 4 Depletion of $\gamma\delta$ T cells significantly affected Th17 but not Th1 responses during Cm infection. Mice were depleted of $\gamma\delta$ T cells and intranasally infected with Cm, as described in the legend to Figure 3. (a) Percentage of IL-17A-produced CD4+ T cells in the lung tissues were determined by intracellular cytokine staining (left panel) and the absolute number of IL-17A-produced CD4+ T cells in the lung tissues was calculated using total lung mononuclear cell number and the ratio of the IL-17A+CD4 cells determined by flow cytometry (right panel). (b) IFN γ production was measured in the culture supernatants of the lung mononuclear cells in the lung homogenates (right panel) by ELISA at day 7 and day 14 p.i. (c) Percentage of IFN γ -producing CD4+ T cells (left panel) and CD8+ T cells (right panel) in the lung tissue was determined by intracellular cytokine staining. Three independent experiments with four mice in each group were tested, and a representative experiment is shown. The results are shown as the mean \pm s.d. * P <0.05.

bacterial burden (IFU; Figure 5b) and histopathological changes (Figure 5c), semi-quantified by pathological score (Figure 5d). More importantly, the combination of early $\gamma\delta$ T-cell depletion and late IL-17A neutralization did not show an added effect on infection and pathology compared with late neutralization of IL-17A alone. The data suggest that IL-17A production by Th17 cells rather than $\gamma\delta$ T cells is critical for host defense against Cm lung infection. In addition, unlike the minimal changes of the Th1 response by $\gamma\delta$ T depletion (Figure 4), the late neutralization of IL-17A alone significantly reduced the Th1 and Tc1 responses (Figure 6). Again, additional early depletion of $\gamma\delta$ T cells in the mice with late IL-17A neutralization had no effect on the percentage (Figures 6a and b) and absolute number (Figures 6c and d) of Th1 and Tc1 cells. The data suggest that the later developed Th17 response, rather than the earlier IL-17A-producing $\gamma\delta$ T-cell response, significantly contributes to the development of protective Th1 immunity.

Depletion of $\gamma\delta$ T cells had significant effect on IL-1 α but not IL-12p40 production by DCs

Our previous studies have demonstrated that IL-17A/Th17 can influence co-stimulatory molecule expression and cytokine production by DC, which may be one of the mechanisms by which IL-17A enhances the Th1 response during Cm lung infection.⁷ To find a clue for the failure of $\gamma\delta$ T depletion in altering Th1 and Tc1 responses, we further tested the effect $\gamma\delta$ T-cell depletion on DC surface markers and cytokines. We analyzed the DCs isolated from Cm-infected, $\gamma\delta$ T-cell-depleted mice and isotype control antibody-treated mice for their surface expression of co-stimulatory molecules, such as CD40, CD80, CD86 and MHC II, and the production of cytokines, such as Th1-promoting cytokines IL-12p40 and IL-12p70, anti-inflammatory cytokines IL-10 and Th17-promoting cytokines IL-23, IL-1 α and IL-6. As shown in Figure 7a, Cm-infected, $\gamma\delta$ T-cell-depleted mice exhibited significantly reduced CD80 expression but a comparable level of CD86, CD40 and MHC II expression compared with

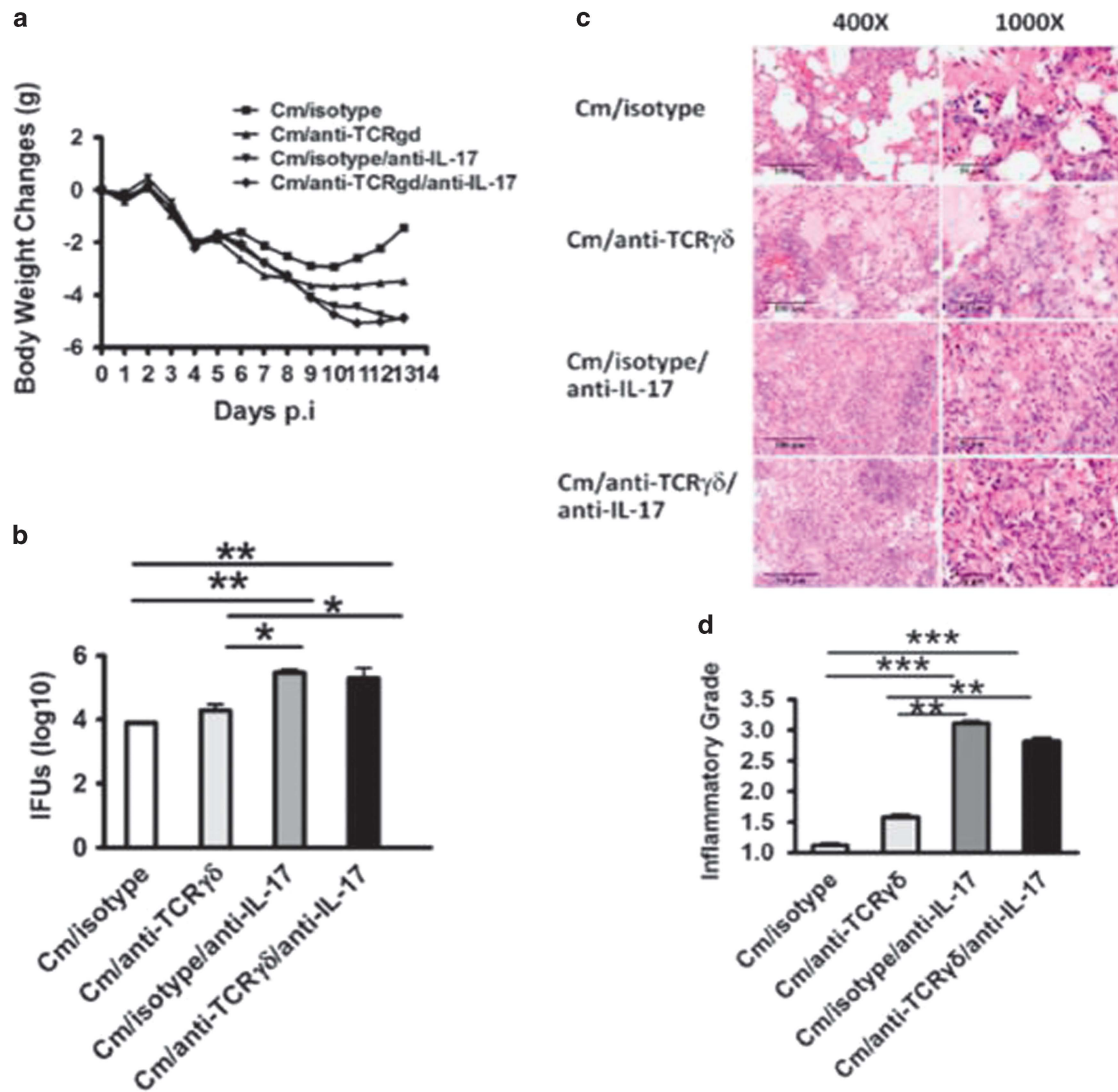


Figure 5 Later neutralization of IL-17A significantly increased infection compared with the early depletion of $\gamma\delta$ T cells. Mice were intranasally infected with 10 μ g anti-mouse $\gamma\delta$ T cell mAb and 10 μ g of H IgG2a isotype control antibody. One day later, two groups of mice were intranasally infected with 1×10^3 IFUs of Cm. At day 7 p.i., two groups of mice were intranasally infected with 10 μ g of anti-mouse IL-17A mAb for the neutralization of airway IL-17A. (a) The mice were monitored daily for body weight change. The original body weights of the four groups of mice were similar. (b) The mice were euthanized on day 13 p.i., and the lungs were collected and measured for chlamydial loads *in vivo* as described in the Materials and Methods section. (c) Histological analysis was performed by staining lung sections with H&E ($\times 400$ and $\times 1000$ magnification under light microscopy). (d) Semi-quantitative analysis of lung inflammation and damage (pathological score). Slides were examined by a blinded pathologist and the inflammatory grades were analyzed as described in the Materials and Methods section. The results are shown as the mean \pm s.d. of four mice for each group in three independent experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

isotype-treated control mice. Interestingly, cytokine analysis showed that depletion of $\gamma\delta$ T cells slightly reduced IL-12p70 production but had no significant effect on IL-12p40 production (Figure 7b). The results also showed that depletion of $\gamma\delta$ T cells had no significant effect on IL-23 and IL-6 production. Interestingly, the depletion of $\gamma\delta$ T cells significantly decreased IL-1 α production but increased IL-10 production by DCs. As IL-1 α signaling is an important pathway in Th17 development, the data fit the findings shown above that depletion of $\gamma\delta$ T cells had a significant effect on Th17 responses.

DISCUSSION

We demonstrate in the present study that $\gamma\delta$ T and CD4+ T (Th17) cells are mainly responsible for the production of IL-17A in chlamydial lung infection. The dynamics of the two types of cells in producing IL-17A are markedly different, in that $\gamma\delta$ T cells are the major producer of IL-17A in the very early stages of infection, while Th17 cells are the major producer of IL-17A in the later stages. More importantly, based on the kinetics of IL-17A production by the two types of cells, we tested their function in modulating Th1 responses and in the clearance of

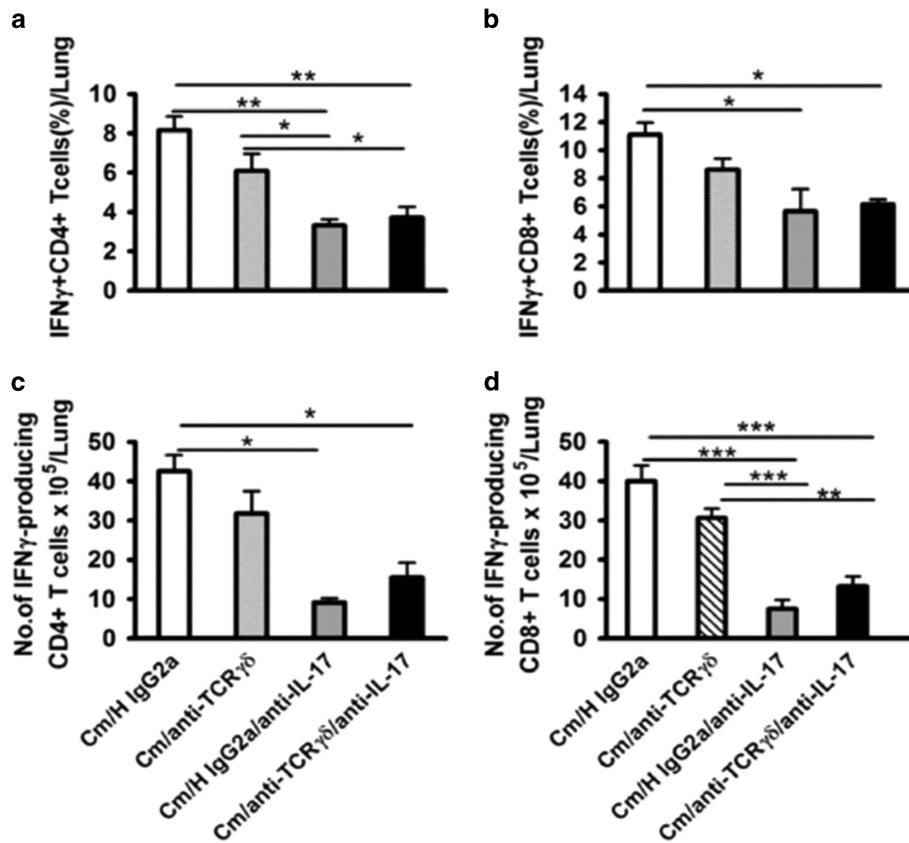


Figure 6 Later-neutralized IL-17A significantly reduced IFN- γ production. Mice were neutralized of IL-17A production with or without depletion of $\gamma\delta$ T cells in Cm-infected mice as described in the legend to Figure 5. Percentage of IFN- γ -producing CD4+ (a) and CD8+ (b) T cells in the lung tissues at day 13 p.i. were determined by intracellular cytokine staining as described in the Materials and Methods section. The absolute number of IFN- γ -producing CD4+ (c) and CD8+ (d) T cells in the lung tissues was calculated as described in the legend to Figure 2. Three independent experiments with four mice in each group were tested. The results are shown as the mean \pm s.d.; * P <0.05; ** P <0.01; *** P <0.001.

bacteria. For the functional analysis, we tested the effect of two antibody treatment approaches on the host defense. One is to deplete $\gamma\delta$ T cells immediately before infection and the other is to block IL-17A at the time point when Th17 becomes the predominant producer of IL-17A while IL-17A producing $\gamma\delta$ T cells have reduced below baseline (day 7). We reason that the former reflects the function of $\gamma\delta$ T cells while the latter approach reflects the function of Th17. The results showed that depletion of $\gamma\delta$ T cells reduced the Th17 response at later time points but had no significant effect on the Th1 response and chlamydial clearance from the lung. In sharp contrast, the blockade of Th17 led to a marked decrease of Th1 and an increase of bacterial growth. Collectively, the data indicate that although $\gamma\delta$ T cells are the major IL-17A producer at the early stage of chlamydial lung infection, Th17 cells are the predominant effector for IL-17A-mediated protection in chlamydial lung infection.

Previous studies have shown that $\gamma\delta$ T cells play complex roles, protective or detrimental, in various infection modalities in experimental animal models. There are at least three possible mechanisms by which $\gamma\delta$ T cells are involved in the immune responses to infection: (1) by regulating innate immune cells by the release of cytokines and chemokines, (2) by lysing infected

targets by the release of cytotoxic molecules contained in granules and (3) by inducing conventional CD4 and CD8 T cells. Depletion of $\gamma\delta$ T cells by using a genetic knockout approach or by treatment with a specific mAb increased infection with a variety of microorganisms such as *Klebsiella*, *Escherichia coli*, *Candidiasis* and *Leishmania major* infections.^{28–37} However, similar depletion of $\gamma\delta$ T cells reduces the infections and diseases caused by *Listeria monocytogenes*, *Candida albicans*, *Eimeria vermiformis* and *Salmonella choleraesuis*.^{38–41} An earlier study by Williams *et al.* reported that $\gamma\delta$ T-cell knockout mice had significantly higher levels of chlamydial growth in the lungs at day 3 and day 7 p.i., but not at the later phase of the infection (day 20).⁴² Perry *et al.*⁴³ reported in a Cm genital tract infection model that $\gamma\delta$ T-cell-deficient mice had similar levels of chlamydial clearance with wild-type mice. Our data in the present study is largely consistent with the results in the previous studies, showing that $\gamma\delta$ T cells can play a supplementary but not essential role in the host defense against chlamydial lung infection. Indeed, although the depletion of $\gamma\delta$ T cells led to decreased IL-17A levels and more serious body weight loss following infection, the bacterial load was not significantly different between the $\gamma\delta$ T-cell-depleted and intact mice. The data further support the critical importance of Th1 in

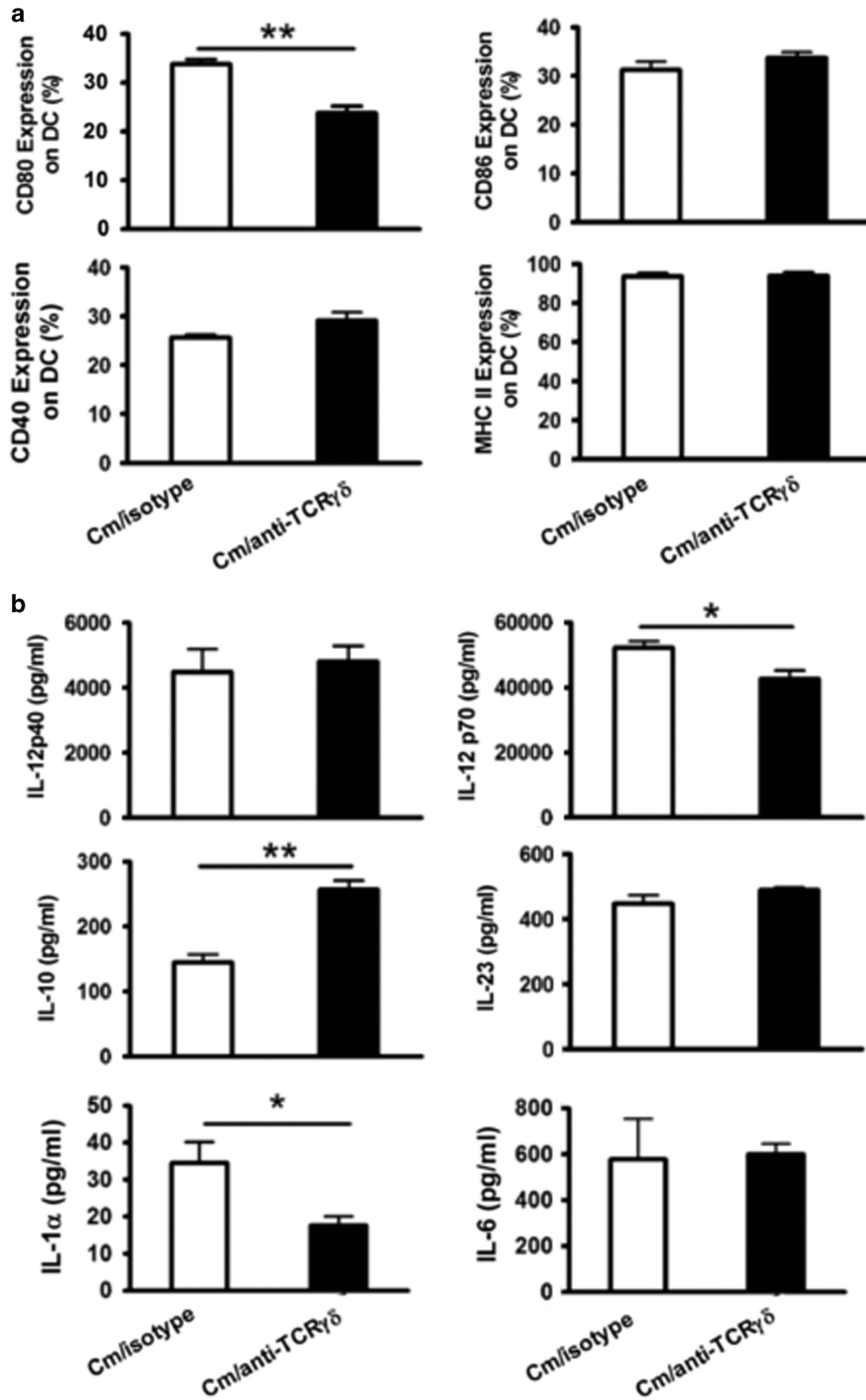


Figure 7 Effect of $\gamma\delta$ T cell depletion on DC surface markers and cytokine production. Mice were treated as described in the legend to Figure 3, and killed on day 7 p.i. Splenic DCs were isolated using MACS CD11c microbeads as described in the Materials and Methods section. Isolated DCs were double stained with allophycocyanin-conjugated anti-CD11c and PE-labeled antibodies specific to surface markers. **(a)** The gated CD11c⁺ DC were analyzed for CD80, CD86, CD40 and MHC II percentages by flow cytometry. **(b)** Freshly purified CD11c⁺ DC were cultured at 5×10^5 cells/well in 96-well plates for 72 h, and IL-12p40, IL-12p70, IL-10, IL-23, IL-1 α and IL-6 levels in the culture supernatants were measured by ELISA. Three independent experiments with four mice in each group were tested, and a representative experiment is shown. The results are shown as the mean \pm s.d.; * $P < 0.05$; ** $P < 0.01$.

protection because the present study showed that depletion of $\gamma\delta$ T cells had no significant impact on the Th1 response, while blockade of IL-17A significantly reduced the Th1 responses and increased infection. Then, the question is why $\gamma\delta$ T cells can promote Th17 responses but have no significant effect on Th1 cell and protection. The reasons are not clear, but it is possible that only a certain level of Th17 is required for enhancing the production of Th1 cytokines and for proper protection. Although the level of IL-17A production by Th17 is reduced in $\gamma\delta$ T-cell-depleted mice, the amount is still sufficient for enhancing Th1 responses and for synergizing with Th1 for enhanced protection as we previously reported.⁸

The lack of any effect on type-1 T-cell responses following the depletion of $\gamma\delta$ T cells is rather unexpected. The dominant role of Th1 cells in the host defense against chlamydial infections has been well documented.^{44,45} Many previous studies have shown that $\gamma\delta$ T cells could promote effector T-cell response, such as Th1 and DTH responses. For example, it was reported that the changes of $\gamma\delta$ T cells in *M. tuberculosis* infection significantly changed the function of protective CD8 cytotoxic T cells. It was likely that the cytokines produced by $\gamma\delta$ T cells promoted CD8 T-cell induction in BCG infection.^{46,47} The discrepancy between our data and others might be related to the type, route and degree of infections. Notably, the increase of IL-17A production by $\gamma\delta$ T cells in chlamydial lung infection is very transient, returning to baseline at 3–4 days following infection. It is interesting that although the $\gamma\delta$ T cell response (absolute number) remained increased up to 2 weeks following infection, very few of the later developed $\gamma\delta$ T cells produced IL-17A, in sharp contrast with the existing $\gamma\delta$ T cells that are largely IL-17A producers. The transient nature of IL-17A production by $\gamma\delta$ T cells may partially explain the difference of this cell type compared with Th17 cells in modulating DC function. Our data showed that although $\gamma\delta$ T-cell depletion led to reduced CD80 expression and increased IL-10 production by DCs, the impact on IL-12 production is minimal, with only a slight reduction of IL-12p70, while no change was observed in IL-12p40 production. Although IL-12p70 is important for the Th1 response, this minor degree of decrease may not translate into a significant reduction of Th1 responses. In contrast, the reduction of IL-1 α is more apparent. As IL-1 α signaling is an important pathway for Th17 development, the finding is consistent with the reduction of Th17 following $\gamma\delta$ T-cell depletion. It should also be mentioned that although the antibody depletion method is efficient in the depletion of $\gamma\delta$ T cells, a small population of residual cells still exists. The residual cell population may still play a role in influencing Th1 cells and other cells.

The assumption that the blockade of IL-17A on day 7 reflects the function of Th17 cells but not $\gamma\delta$ T cells is not without controversy. It is true that, at this time point, some $\gamma\delta$ T cells may still produce IL-17A. However, because the IL-17A production by $\gamma\delta$ T cells returns to baseline or even much below baseline, the effect observed should largely reflect the function of Th17. Due to the lack of Th17 KO mice, this appears the most feasible way at the current stage to address this question.

In conclusion, our data show respective IL-17A production by $\gamma\delta$ T cells and Th17 cells at the early stage and later stage of chlamydial lung infection. The IL-17A production by $\gamma\delta$ T cells

increased very quickly but was very transient in nature, while its production by Th17 increases steadily and maintains a high level. More importantly, our data show that Th17 cells are the major IL-17A producers that promote the Th1 responses and mediate protection in chlamydial infection. The findings in the present study provide new insights into the mechanisms bridging innate and adaptive immunity during lung chlamydial infections, which may have implications in developing effective chlamydial vaccines and in the understanding of host defense mechanisms in other lung infections.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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