

CD30 Is Required for Activation of a Unique Subset of Interleukin-17A-Producing $\gamma\delta$ T Cells in Innate Immunity against *Mycobacterium bovis* Bacillus Calmette-Guérin Infection

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Interleukin-17A (IL-17A)-producing $\gamma\delta$ T cells are known to be activated following *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) infection. Here, we show that CD30, a member of the tumor necrosis factor (TNF) receptor superfamily, is important for activation of IL-17A-producing $\gamma\delta$ T cells after BCG infection. $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells preferentially expressing $V\gamma 6/V\delta 1$ genes were identified as the major source of IL-17A in the peritoneal cavity during the early stage of BCG infection. The number of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells bearing $V\gamma 6$ increased in peritoneal exudate cells (PEC) of wild-type (WT) mice but not in those of CD30 knockout (KO) mice in response to BCG infection. Consistently, CD30 ligand (CD30L) or CD30 expression, predominantly by $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells, was rapidly upregulated after BCG infection. Inhibition of CD30L/CD30 signaling by *in vivo* administration of a soluble CD30 and immunoglobulin fusion protein (CD30-Ig) severely impaired activation of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells in WT mice, while stimulating CD30L/CD30 signaling by *in vivo* administration of agonistic anti-CD30 monoclonal antibody (MAb) restored IL-17A production by $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells in CD30L KO mice after BCG infection. These results suggest that CD30 signaling plays an important role in the activation of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells bearing $V\gamma 6$ at an early stage of BCG infection.

Unlike conventional T cells, which are exported from the thymus as naive cells and acquire effector functions upon antigen (Ag) encounter in the periphery, some subsets of murine $\gamma\delta$ T cells are functionally differentiated into effector cells producing gamma interferon (IFN- γ) or interleukin-17A (IL-17A) within the fetal thymus and are disproportionately distributed in mucosal epithelia such as skin, intestine, uterus, and lung as tissue-associated cells (1–8). IL-17A is a proinflammatory cytokine originally identified from helper CD4⁺ $\alpha\beta$ T cells, Th17 cells, which participate in host defense against various types of pathogens as well as in autoimmune disorders (9–12). Recently, it was found that IL-17A production by $\gamma\delta$ T cells rather than CD4⁺ $\alpha\beta$ T cells plays an important role in the immune response to pulmonary *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) infection as well as in BCG-induced lung granuloma formation (13–15). We have also reported the importance of IL-17A-producing $\gamma\delta$ T cells in other models of infection of mice with *Escherichia coli*, *Candida albicans*, or BCG, which are involved in the neutrophil-mediated clearance of the pathogens or tumor cells (16–18). These findings suggest the hypothesis that a novel host defense mechanism mediated by the “innate” IL-17A-producing $\gamma\delta$ T cells is acting in innate immunity and initiating an inflammatory response following a microbe invasion.

A CD30 ligand (CD30L; CD153) is a 40-kDa type II membrane-associated glycoprotein belonging to the tumor necrosis factor (TNF) superfamily and is expressed on activated and memory CD4⁺ T helper cells in addition to macrophages, dendritic cells (DCs), B cells, and unique CD4⁺ CD3⁻ CD11c⁻ accessory cells (19–22). CD30, the receptor for CD30L, which belongs to the TNF receptor superfamily, was expressed preferentially by activated and memory Th cells but not by resting B or T cells (23–25). CD30L/CD30 signaling was initially thought to be involved in Th2

cell responses and Th2-associated diseases (26–29). However, a number of recent studies indicated that CD30L/CD30 signaling is also linked to Th1 or regulatory T cell responses and their associated diseases (30–35). We have recently found that CD30L/CD30 signaling executed by T cell-T cell interaction plays a novel role in Th1 and Th17 cell differentiation *in vitro* and *in vivo* (36–38). In this regard, CD30L/CD30 signaling may be dispensable for differentiation of a specific Th cell subset in a physiological pathway, while it may be required for the activation and/or amplification of T cells irrespective of the T cell subset in the periphery.

Mycobacterial infection by such bacilli as BCG and *Mycobacterium tuberculosis* has been widely discussed with respect to their adaptive immune response, which mainly depends on IFN- γ production by CD4⁺ Th1 cells (39, 40). Recently, we and others reported that CD30L and CD30 play an important role in acquired immunity against mycobacterial infection by amplifying the Th1 response (32, 36). However, the potential role of CD30L/CD30 signaling in controlling host defense through activation of IL-17A-producing T cells against mycobacterial infection is unknown. In this study, we found that the numbers of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells bearing $V\gamma 6$ significantly decreased

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in peritoneal exudate cells (PEC) of CD30-deficient mice at an early stage of intraperitoneal (i.p.) infection with BCG. Consistently, the expression level of CD30L or CD30 was selectively up-regulated after BCG infection on $V\gamma 1^{-} V\gamma 4^{-} \gamma\delta$ T cells, which are the “innate” source of IL-17A in PEC during the early stage of BCG infection. These findings demonstrate the important role of CD30 signaling in the activation of $V\gamma 1^{-} V\gamma 4^{-} \gamma\delta$ T cells bearing $V\gamma 6$ producing IL-17A in the innate immune response to BCG infection.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) male mice were purchased from Japan KBT Inc. (Shizuoka, Japan). CD30 knockout (KO) (B6.129P2-Tnfrsf8<tm1Mak>/J) mice were purchased from the Jackson Laboratory. The generation and preliminary characterization of CD30L KO (BALB/c background) mice were described previously (41), and those mice were backcrossed for 10 or more generations to B6 mice. All mice were maintained under specific pathogen-free conditions and were offered food and water *ad libitum*. Age- and gender-matched mice were used for all experiments. This study was approved by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University. Experiments were carried out under the control of the Guidelines for Animal Experiments.

Microorganisms. Lyophilized *M. bovis* BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals and dissolved in 7H9 medium (Difco) supplemented with albumin-dextrose-catalase enrichment (Difco). The viable bacterial numbers were determined using a 7H10 (Difco) plate supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco). Small aliquots of BCG suspended in 7H9 medium containing 20% glycerol were stored at -80°C until use. Before use, the bacteria were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 80 and resuspended in PBS. Mice were infected i.p. with 1×10^6 CFU of BCG in 200 μl of PBS.

Bacterial growth. At the indicated time after infection, PEC were subjected to lavage with 1 ml of ice-cold Hanks balanced salt solution (HBSS) and harvested after gentle massage. The spleen and liver were removed and separately placed in homogenizers containing 1 ml of HBSS. For BCG infection, those samples were spread on Middlebrook 7H10 medium enriched with oleic acid-albumin-dextrose-catalase, and colonies were counted after incubation for 3 weeks at 37°C .

Flow cytometry and antibodies. Single cells were isolated from PEC as previously described (7). Briefly, PEC from infected mice were obtained by lavage with 5 ml of HBSS. After washing, PEC were preincubated with an Fc γ receptor-blocking monoclonal antibody (MAb) (CD16/32 [clone 2.4G2]; BD Biosciences, San Diego, CA) for 20 min at 4°C to prevent antibody binding to the Fc receptor. The total cells were then stained with various combinations of MAbs. We purchased the following MAbs: anti-CD11b-fluorescein isothiocyanate (FITC) (clone M1/70; BD Biosciences), anti-NK1.1-phycoerythrin (PE) (clone PK136; BD Biosciences), anti-F4/80-allophycocyanin (APC) (clone BM8; eBioscience, San Diego, CA), anti-Gr1-PE (clone RB6-8C5; BD Biosciences), anti-CD45R-APC (clone RA3-6B2; eBioscience), anti-major histocompatibility complex (MHC) class II-peridinin chlorophyll protein (PerCP)-eFluor 710 (clone M5/114.15.2; eBioscience), anti-CD3e-PE (clone 145-2C11; Biolegend, San Diego, CA), anti-T cell receptor beta (TCR β)-PE (clone H57-597; eBioscience), anti-CD4-PerCP or anti-CD4-APC (clone RM4-5; BD Biosciences), anti-CD8 α -PerCP or anti-CD8 α -FITC (clone RM53-6.7; BD Biosciences), anti-TCR $\gamma\delta$ -APC (clone GL3; Biolegend), anti-TCR $V\gamma 1.1$ -PE or anti-TCR $V\gamma 1.1$ -FITC (clone 2.11; Biolegend), anti-TCR $V\gamma 2$ -PE or anti-TCR $V\gamma 2$ -FITC (clone UC3-10A6; Biolegend), anti-CD30-PE (clone Mcd30.1; BD Biosciences), and anti-CD153-PE (clone RM153; BD Biosciences). The stained cells were acquired and analyzed in a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences). For flow cytometry, neutrophils were defined as CD11b $^{+}$ Gr-1 $^{\text{high}}$ F4/80 $^{\text{low}}$ and macrophages as CD11b $^{+}$ F4/80 $^{\text{high}}$ Gr-1 $^{\text{low}}$ (see Fig. S1 in the supplemental material).

Cell purification. PEC from BCG-infected wild-type (WT) mice were isolated as previously described (7) and prepared in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μl /ml streptomycin, and 50 μM 2-mercaptoethanol (2-ME). After staining with anti-MHC class II-PerCP-eFluor 710 (clone M5/114.15.2; eBioscience), anti-TCR $\gamma\delta$ -PE (clone eBioGL3; eBioscience), and a mixture of anti-TCR $V\gamma 1$ -FITC (clone 2.11; Biolegend) and anti-TCR $V\gamma 4$ -FITC (clone UC3-10A6; Biolegend) MAbs, $\gamma\delta$ T cell subsets in PEC were confirmed by FACSCalibur flow cytometry (BD Biosciences, San Diego, CA). Next, $V\gamma 1^{-} V\gamma 4^{-}$ and $V\gamma 1^{+} V\gamma 4^{+} \gamma\delta$ T cells were sorted respectively by analysis using a fluorescence-activated cell sorter (FACS) Aria II system (BD Biosciences, San Diego, CA) at a purity of more than 97%. In some experiments, $\gamma\delta$ T cells in PEC from WT or CD30 KO mice on day 7 after BCG infection were sorted using a FACS Aria II system at a purity of more than 95%.

Intracellular cytokine staining. PEC were suspended in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μl /ml streptomycin, and 50 μM 2-ME. PEC were stimulated with 25 ng/ml phorbol myristate acetate (PMA) (P-8139; Sigma-Aldrich) and 1 μg /ml ionomycin (I-0634; Sigma-Aldrich) for 5 h at 37°C in 5% CO_2 or left unstimulated. Brefeldin A (BFA) (Sigma-Aldrich) (10 μg /ml) was added for the last 4 h. After incubation, the cells were washed with FACS buffer and surface stained with various combinations of MAbs for 30 min at 4°C . Intracellular staining was then performed according to the manufacturer's instructions (BD Biosciences). Briefly, 100 μl of BD Cytofix/Cytoperm solution (BD Biosciences) was added to the cell suspension with mild mixing and then placed for 20 min at 4°C . The fixed cells were washed twice with 500 μl of BD Perm/Wash buffer (BD Biosciences) and stained intracellularly with anti-IL-17A-PE (clone TC11-18H10; BD Biosciences) and anti-IFN- γ -FITC (clone XMG1.2; BD Biosciences) for 30 min at 4°C . After washing, the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

RNA purification and reverse transcription-PCR (RT-PCR). Total RNA of the sorted $\gamma\delta$ T cells was purified using an RNeasy minikit (Qiagen), and cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer's instructions. For analysis of the $V\gamma$ repertoire, combinations of the following primers were used. The forward primers were as follows: for $V\gamma 1/2$, 5'-ACACAGCTATACATTGGTAC-3'; for $V\gamma 2$, 5'-CGGCAAAAAACAATCAACAG-3'; for $V\gamma 4$, 5'-TGTCCTTGCAACCCCTACCC-3'; for $V\gamma 5$, 5'-TGTCCTTGCAACCCCTACCC-3'; for $V\gamma 6$, 5'-GGAATTCAAAAGAAAACATTGTCT-3'; for $V\gamma 7$, 5'-AAGCTAGAGGGTCTCTGC-3'; for $V\delta 1$, 5'-ATTCAGAAGGCAACAATGAAAG-3'; for $V\delta 2$, 5'-AGTTCCCTGCAGATCCAAGC-3'; for $V\delta 3$, 5'-TTCCTGGCTATTGCCTCTGAC-3'; for $V\delta 4$, 5'-CCGCTTCTGTGACTTCC-3'; for $V\delta 5$, 5'-CAGATCCCTCCAGTTTCATCC-3'; for $V\delta 6$, 5'-TCAAGTCCATCAGCCTTGTC-3'; for $V\delta 7$, 5'-CGCAGAGCTGCAGTGTAAC-3'; and for $V\delta 8$, 5'-AAGGAAGATGGACGATTCAC-3'. The reverse primers were as follows: for C γ , 5'-CTTATGGAGATTTGTTTCAGC-3'; and for C δ , 5'-CGAATTCACAATCTTCT-3'. For β -actin, the primers were 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAACGCAGCTCAACAGTCCG-3'. PCR was performed on a PCR thermal cycler (TaKaRa Corp., Tokyo, Japan).

ELISAs. Supernatants of PEC from the mice at the indicated times after BCG inoculation were obtained by centrifugation at $440 \times g$ for 3 min at 4°C . IL-17A, IFN- γ , IL-6, IL-12 p70, TNF alpha (TNF- α), and IL-23 secretion in the supernatants was measured by using a DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems) according to the manufacturer's instructions.

In vivo treatment of mice with antibodies. Agonistic anti-CD30 MAbs (clone 30.1) were obtained by growing hybridoma cells in Cell Line CL-1000 (BD Biosciences, San Diego, CA) with serum-free medium (medium 101; Nissui Pharmaceutical, Tokyo, Japan). Abs were collected using a HiTrap Protein G HP column (Amersham Biosciences, Piscataway, NJ). The purity of the preparation was confirmed by SDS-PAGE; the concentration of Abs was determined by the Lowry method. The MAbs, diluted to 1 mg/ml in PBS, were stored at -80°C until use. For *in vivo*

activation, 100 μg of agonistic anti-CD30 MAb or isotype control Armenian hamster IgG1 (eBio299Arm; BD Biosciences) was injected i.p. into WT and CD30L KO mice simultaneously with bacterial infection.

Soluble CD30 and immunoglobulin fusion protein (CD30-Ig) was obtained using a previously described method (22). Secreted CD30-Ig protein obtained by growing NIH 3T3 cells transfected with CD30-Ig fusion cDNA expression vector pBMGNeo in serum-free medium (medium 101; Nissui Pharmaceutical) was purified using a HiTrap Protein G HP column (Amersham Biosciences, Piscataway, NJ) and analyzed by SDS-PAGE for purity. CD30-Ig, diluted to 1 mg/ml in PBS, was stored at -80°C until use. For *in vivo* neutralization, 200 μg of CD30-Ig or isotype control mouse IgG1 κ (MG1-45; Biolegend) was injected i.p. into WT mice simultaneously with bacterial infection.

Statistics. Statistical significance was evaluated by Student's *t* test using Prism software (GraphPad, San Diego, CA). *P* values of <0.05 were considered to represent a significant difference.

RESULTS

Bacterial growth, cytokine production, and cell accumulation in PEC of CD30 KO mice after BCG infection. We examined the bacterial growth in PEC, liver, and spleen at an early stage (on days 3, 7, and 14) after i.p. inoculation with 1×10^6 CFU of BCG in CD30 KO mice with a C57BL/6 background. As shown in Fig. 1A, the number of bacteria in PEC in CD30 KO mice was significantly higher than that in WT mice on day 7 after BCG infection, but there was no difference between WT and CD30 KO mice in liver and spleen.

We next monitored cytokine levels in PEC supernatants after infection with BCG. In WT mice, IL-17A secretion increased rapidly and reached a peak on day 7 after infection. The levels of IL-17A secretion decreased markedly in CD30 KO mice on days 3 and 7 after BCG infection compared to those in WT mice (Fig. 1B). IFN- γ secretion progressively increased after infection and appeared to be attenuated until 14 days postinfection (Fig. 1B). We also examined the kinetics of IL-23, which has been reported to promote IL-17A production by $\gamma\delta$ T cells against *E. coli* or *C. albicans* infection (17, 42). However, IL-23 secretion was detected at low levels during the course of BCG infection, and there were no significant differences between CD30 KO and WT mice at those levels (Fig. 1B). In addition, there were also no significant differences between CD30 KO and WT mice in the levels of IL-12 p70, IL-6, and TNF- α in PEC supernatants during the early stage of BCG infection (Fig. 1B).

We also examined the kinetics of infiltration of leukocytes by FACS analysis in PEC on days 3, 7, and 14 after BCG infection. Consistent with increased IL-17A secretion, the number of CD11b⁺ Gr-1^{high} F4/80^{low} cells corresponding to neutrophils increased after BCG infection, and the frequency and absolute number of neutrophils decreased significantly in CD30 KO mice compared to WT mice on days 7 and 14 after infection (Fig. 1C; see also Fig. S1 in the supplemental material). The levels of CD11b⁺ F4/80^{high} Gr-1^{low} cells corresponding to macrophages also decreased in CD30 KO mice on days 7 and 14 after infection (Fig. 1C; see also Fig. S1).

Kinetics of $\gamma\delta$ T cells in PEC of CD30 KO mice after BCG infection. $\gamma\delta$ T cells are reported to play a key role in the early immune response to BCG infection (43). In the next experiment, we investigated the kinetics of $\gamma\delta$ T cells in CD30 KO mice at an early stage of BCG infection. As shown in Fig. 2A, the frequency and absolute number of $\gamma\delta$ T cells reached a peak on day 7 after BCG infection, and cell frequency as well as cell number was sig-

nificantly lower in CD30 KO mice than in WT mice on days 3, 7, and 14 after infection (Fig. 2A). In contrast, there was no significant difference between the two PEC preparations in the absolute numbers of $\alpha\beta$ T cells, NK cells, NKT cells, or B cells at an early stage of BCG infection (Fig. 2B).

To further determine which V γ repertoire of $\gamma\delta$ T cells is affected in CD30 KO mice, each subset of V γ from PEC was assessed by staining with available anti-V γ 1 and V γ 4 MAbs. It was found that the V γ 1⁻ V γ 4⁻ subset was the major subset of $\gamma\delta$ T cells distributed in PEC during the early stage of BCG infection and that their frequency and absolute number were significantly lower in CD30 KO mice than in WT mice on days 3, 7, and 14 after BCG infection, while the absolute numbers in the V γ 1⁺ or V γ 4⁺ $\gamma\delta$ T cell subset in CD30 KO mice did not differ from those in WT mice during this early phase of infection (Fig. 2C and D). Therefore, it is suggested that V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells selectively showed a poor response in PEC of CD30 KO mice after BCG infection.

Because of the lack of availability of a V γ 6-specific MAb, we identified the repertoire of V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells expanding in the peritoneal exudate of mice infected with BCG by RT-PCR. After staining with anti-TCR $\gamma\delta$ MAb and a mixture of anti-V γ 1 and anti-V γ 4 MAbs, V γ 1⁻ V γ 4⁻ and V γ 1⁺ V γ 4⁺ $\gamma\delta$ T cell populations were clearly confirmed by flow cytometry and then these two subsets were purified at a purity of more than 97% (Fig. 3A). As shown in Fig. 3B, the V γ 1⁻ V γ 4⁻ subset of $\gamma\delta$ T cells exclusively expressed V γ 2 and V γ 6 of the V γ gene transcripts and V δ 1 of the V δ gene transcripts. In contrast, the V γ 1⁺ V γ 4⁺ subset expressed V γ 1, V γ 2, and V γ 4 of the V γ gene transcripts and V δ 4, V δ 5, and V δ 7 of the V δ gene transcripts. Since V γ 2 transcript is generally expressed in a single $\gamma\delta$ T cell clone expressing other V γ and V γ 6 chains exclusively paired with V δ 1 (3, 44), these observations proved that V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells in peritoneal exudate preferentially expressed the V γ 6 chain paired with V δ 1. Next, total RNA was extracted from $\gamma\delta$ T cells sorted from PEC of mice infected with BCG, and TCR V gene expression was analyzed by RT-PCR. As shown in Fig. 3C, $\gamma\delta$ T cells in the peritoneal exudate of WT mice expressed high levels of TCR V γ 1, V γ 2, V γ 4, and V γ 6 mRNA but lacked expression of TCR V γ 5 or V γ 7 mRNA. Notably, only TCR V γ 6 and V δ 1 expression levels were downregulated in CD30 KO mice after BCG infection. Therefore, these findings further confirmed that CD30 signaling was required for regulating functions of V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells bearing V γ 6 in peritoneal exudate after BCG infection.

Kinetics of IL-17A- or IFN- γ -producing T cells in PEC of CD30 KO mice after BCG infection. We next examined the ability of $\gamma\delta$ T cells or $\alpha\beta$ T cells to produce cytokines after BCG infection by intracellular cytokine FACS analysis. Consistent with our previous report (7), IL-17A-producing $\gamma\delta$ T cells could be detected in PEC of naive WT mice even in the absence of PMA and ionomycin stimulation; the numbers of such "innate" IL-17A-producing $\gamma\delta$ T cells increased immediately after BCG infection and reached a peak on day 7 and then returned to the baseline by day 14 after BCG infection. The frequency and absolute number of the IL-17A⁺ $\gamma\delta$ T cells markedly decreased in CD30 KO mice compared to WT mice on days 3, 7, and 14 after BCG infection (Fig. 4A; see also Fig. S2A in the supplemental material). Such a difference between WT and CD30 KO mice was more obvious in IL-17A⁺ $\gamma\delta$ T cells in response to PMA and ionomycin stimulation (Fig. 4A; see also Fig. S2A). In contrast, very few CD4⁺ $\alpha\beta$ T cells producing IL-17A were detected during the early stage of BCG infection

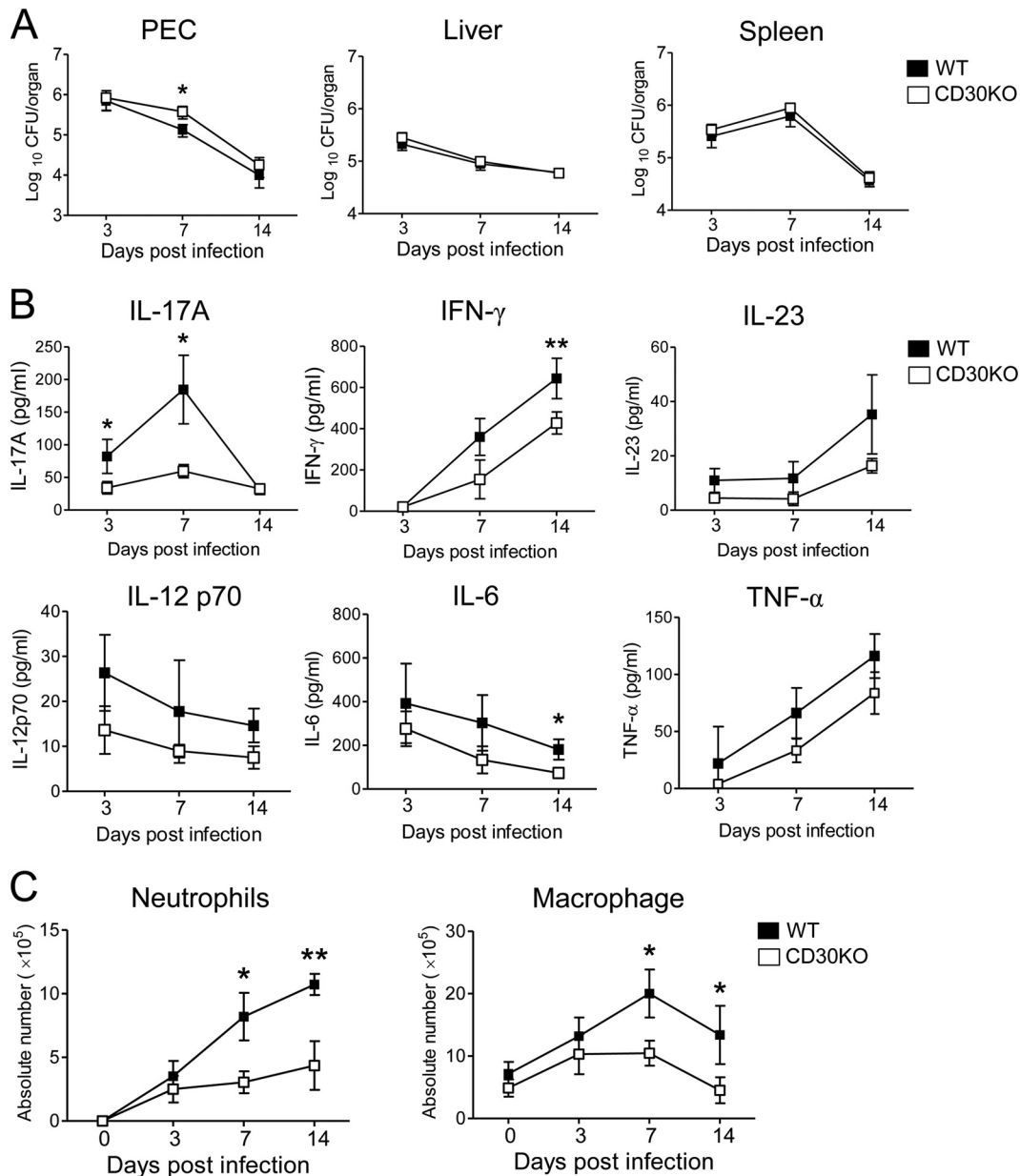


FIG 1 Kinetics of bacterial growth, cytokine production, and cell accumulation in PEC after i.p. infection with BCG. WT (B6) and CD30 KO mice were inoculated i.p. with 1×10^6 CFU of BCG. (A) On days 3, 7, and 14 postinfection, bacterial counts in PEC, liver, and spleen were determined. Data are shown as means and standard deviations (SD) of the results determined for five mice at each time point. Significant differences in *P* values were calculated using Student's *t* test (*, $P < 0.05$). (B) Secretion of IL-17A, IFN- γ , IL-23, IL-12 p70, IL-6, and TNF- α from the supernatants of PEC at the indicated time points after BCG inoculation was measured by ELISA. (C) Kinetics of absolute numbers of neutrophils and macrophages in PEC of infected mice as analyzed by flow cytometry. PEC were harvested on the indicated days after inoculation with BCG and surface stained with various MAbs. The absolute numbers of neutrophils (CD11b⁺ Gr-1^{high} F4/80^{low}) and macrophages (CD11b⁺ F4/80^{high} Gr-1^{low}) were calculated by multiplying the total number of PEC by the percentages of each subset in the PEC. Data in panels B and C are shown as means \pm SD of the results determined for three mice at each time point. Significant differences in *P* values were calculated using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

without further stimulation (Fig. 4A; see also Fig. S2B). These results indicated that IL-17A production in PEC was mainly derived from $\gamma\delta$ T cells during the early stage of BCG infection and that CD30 signaling plays an important role in the activation of IL-17A-producing $\gamma\delta$ T cells in the early response to BCG infection.

Next, we examined the kinetics of IFN- γ -producing T cells in PEC after BCG infection. IFN- γ production was hardly detected

by analysis of either $\gamma\delta$ T cells or CD4⁺ $\alpha\beta$ T cells in the absence of PMA/ionomycin stimulation during the first 7 days of BCG infection (Fig. 4B). As BCG infection progressed, the numbers of both $\gamma\delta$ T cells and CD4⁺ $\alpha\beta$ T cells producing IFN- γ increased; the numbers of IFN- γ ⁺ CD4⁺ $\alpha\beta$ T cells were apparently over 10 times greater than those of IFN- γ ⁺ $\gamma\delta$ T cells on day 14 after BCG infection in the absence or presence of PMA/ionomycin stimulation, suggesting that these sharply increased CD4⁺ $\alpha\beta$ T cells serve

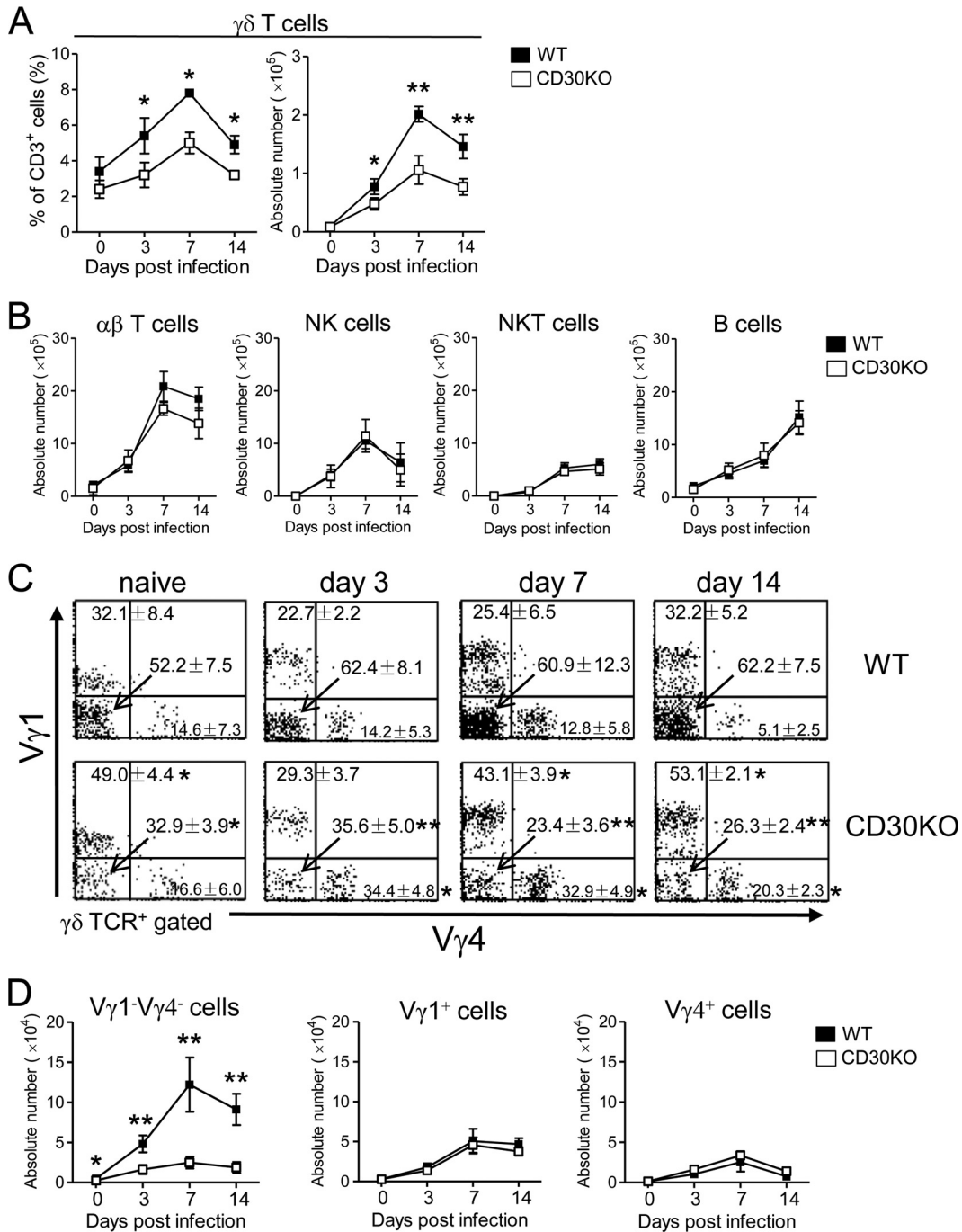


FIG 2 Kinetics of $\gamma\delta$ T cell subsets in PEC of CD30 KO mice during the early stage of BCG infection. WT or CD30 KO mice were inoculated i.p. with 1×10^6 CFU of BCG, and PEC were harvested on days 0, 3, 7, and 14 post-BCG infection. (A) The frequencies and absolute numbers of $\gamma\delta$ T cells in PEC. (B) Absolute numbers of $\alpha\beta$ TCR⁺ cells, NK1.1⁺ CD3⁻ cells, NK1.1⁺ CD3⁺ T cells, and B220⁺ CD3⁻ cells in PEC. (C and D) Frequencies (C) and absolute numbers (D) of V γ 1⁻ V γ 4⁻, V γ 1⁺, and V γ 4⁺ $\gamma\delta$ T cells in PEC. The analysis gate was set on $\gamma\delta$ TCR⁺ cells. The absolute number of each subset of $\gamma\delta$ T cells was calculated by multiplying the total $\gamma\delta$ T cell number by the percentage in each dot plot figure. The numbers on the dot plots are expressed as means and SD of the results determined for at least three mice per group and per day, with data from one representative mouse shown. Student's *t* tests were performed to determine statistical differences between the results from any two groups (*, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

as the main producer of IFN- γ in PEC to participate in the following immune response to BCG infection (Fig. 4B). The frequency and absolute number of IFN- γ ⁺ CD4⁺ $\alpha\beta$ T cells significantly decreased in CD30 KO mice compared to WT mice on day 14 after BCG infection in the absence or presence of PMA/ionomycin

stimulation (Fig. 4B; see also Fig. S2B in the supplemental material). Thus, consistent with our previous report (36), CD30 signaling was also involved in the activation of IFN- γ -producing CD4⁺ $\alpha\beta$ T cells at a relatively late stage of BCG infection.

To further analyze the V γ repertoire of IL-17A- or IFN- γ -

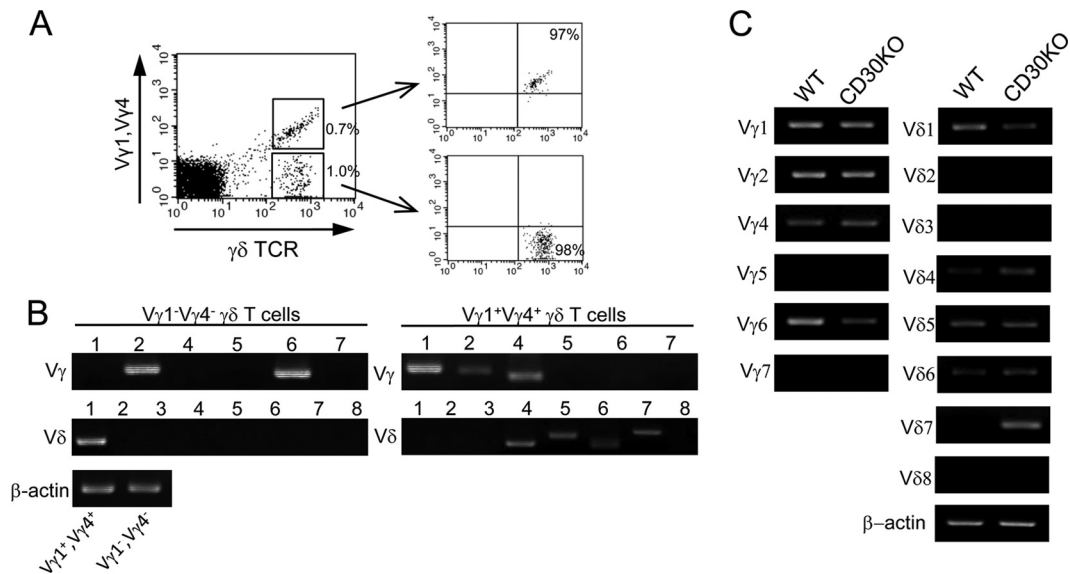


FIG 3 Identification of the TCR $V\gamma$ and $V\delta$ gene repertoire of $\gamma\delta$ T cells by RT-PCR. (A) $\gamma\delta$ T cell subsets in PEC from WT mice ($n = 5$) that had been i.p. infected with BCG 7 days previously were confirmed by FACSCalibur flow cytometry. The numbers on the dot plot (left) indicate the percentages of $V\gamma 1^+ V\gamma 4^-$ or $V\gamma 1^- V\gamma 4^+$ $\gamma\delta$ T cells in PEC. These two subsets of $\gamma\delta$ T cells were then sorted respectively using a FACS Aria II system (purity $> 97\%$). (B) The TCR $V\gamma$ and $V\delta$ gene repertoires of $V\gamma 1^- V\gamma 4^-$ or $V\gamma 1^+ V\gamma 4^+$ $\gamma\delta$ T cells were analyzed by RT-PCR and agarose gel electrophoresis. (C) Total RNA was extracted from $\gamma\delta$ T cells sorted from PEC of WT or CD30 KO mice ($n = 5$) that had been infected with BCG 7 days previously at a purity of more than 95%, and TCR $V\gamma$ and $V\delta$ gene expression levels were analyzed by RT-PCR and agarose gel electrophoresis. mRNA levels were normalized using β -actin mRNA as the reference. Data are representative of three independent experiments.

producing $\gamma\delta$ T cells, PEC isolated from BCG-infected mice were surface stained with anti-TCR $\gamma\delta$ MAb and a mixture of anti-TCR $V\gamma 1$ and $V\gamma 4$ MAbs and then intracellularly stained with anti-IL-17A or anti-IFN- γ . As shown in Fig. 4C, most of the IL-17A-producing $\gamma\delta$ T cells were $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells bearing $V\gamma 6$. Compared to the results seen with WT mice, the frequency and cell number of IL-17A $^+$ $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells in PEC of CD30 KO mice significantly decreased on days 3, 7, and 14 after infection (Fig. 4C and D). Given that $V\gamma 1^+$ $\gamma\delta$ T cells do not produce IL-17A in response to BCG infection (reference 15 and data not shown), the IL-17A $^+$ $V\gamma 1^+ V\gamma 4^+$ $\gamma\delta$ T cells (i.e., the $V\gamma 4^+$ $\gamma\delta$ T cells producing IL-17A) showed a relatively higher frequency in CD30 KO mice in the presence of PMA/ionomycin. However, there was no significant difference between CD30 KO mice and WT mice in the absolute numbers of IL-17A $^+$ $V\gamma 4^+$ $\gamma\delta$ T cells in PEC after BCG infection (data not shown). These results indicated that CD30 signaling selectively promotes the activation of IL-17A-producing $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells bearing $V\gamma 6$ in PEC of mice after BCG infection. Next, by analyzing the $V\gamma$ repertoire of IFN- γ -producing $\gamma\delta$ T cells, we found that not only $V\gamma 1^- V\gamma 4^-$ but also $V\gamma 1^+ V\gamma 4^+$ $\gamma\delta$ T cells in PEC produced IFN- γ upon PMA/ionomycin stimulation and that these two subsets of $\gamma\delta$ T cells producing IFN- γ were significantly reduced in number in CD30 KO mice on day 14 after BCG infection (Fig. 4E and F).

CD8 $^+$ $\alpha\beta$ T cells are also reported to participate in immune responses to *M. tuberculosis* (45). Recently, IL-17-producing CD8 $^+$ $\alpha\beta$ T cells (Tc17 cells) have been identified in mice and their enrichment in the antitumor immune response has been reported (46, 47). Therefore, we examined CD8 $^+$ $\alpha\beta$ T cells in the early phase of BCG infection. As shown in Fig. S3 in the supplemental material, IL-17A $^+$ CD8 $^+$ $\alpha\beta$ T cells were not detected in mice infected with BCG even in the presence of PMA and iono-

mycin stimulation, and there was no significant difference between CD30 KO and WT mice in the percentages and cell numbers of IFN- γ -producing CD8 $^+$ $\alpha\beta$ T cells after BCG infection. Thus, it appears that CD30 signaling may not contribute to the early activation of CD8 $^+$ $\alpha\beta$ T cells after BCG infection.

Taken together, these results indicate that CD30 signaling was primarily involved in the activation of IL-17A-producing $\gamma\delta$ T cells, mainly, $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells bearing $V\gamma 6$, in the early immune response to BCG infection. In addition, it also contributes to the activation of IFN- γ -producing CD4 $^+$ $\alpha\beta$ T cells in the peripheral tissues at a relatively late stage of BCG infection.

Expression of CD30L and CD30 on peritoneal $\gamma\delta$ T cells after BCG infection. To further demonstrate that CD30L/CD30 signaling was selectively involved in the activation of $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells, we examined the expression of CD30L and CD30 on the $\gamma\delta$ T cells of PEC from WT mice after BCG infection. As shown in Fig. 5, CD30L and CD30 were predominantly expressed on $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells presumably bearing $V\gamma 6$ of PEC after BCG infection. The expression levels of both CD30L and CD30 were rapidly upregulated in mice previously infected with BCG compared to those in naive mice and were further enhanced on $\gamma\delta$ T cells after 24 h culture *in vitro* without any stimulation (Fig. 5A and B). It should be noted that the numbers of CD30L-expressing $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells and CD30-expressing $V\gamma 1^- V\gamma 4^-$ $\gamma\delta$ T cells were much greater than those of CD30L $^+$ $V\gamma 1^+$ or $V\gamma 4^+$ T cells and CD30 $^+$ $V\gamma 1^+$ and $V\gamma 4^+$ $\gamma\delta$ T cells, respectively, on specific days after BCG infection (Fig. 5C).

Since CD30L and CD30 are reported to be expressed on cells other than $\gamma\delta$ T cells, we additionally examined their expression on F4/80 $^{\text{high}}$ CD11b $^+$ CD3 $^-$ macrophages, CD11c $^+$ CD3 $^-$ DCs, B220 $^+$ CD3 $^-$ B cells, NK1.1 $^+$ CD3 $^-$ NK cells, NK1.1 $^+$ CD3 $^+$ NKT

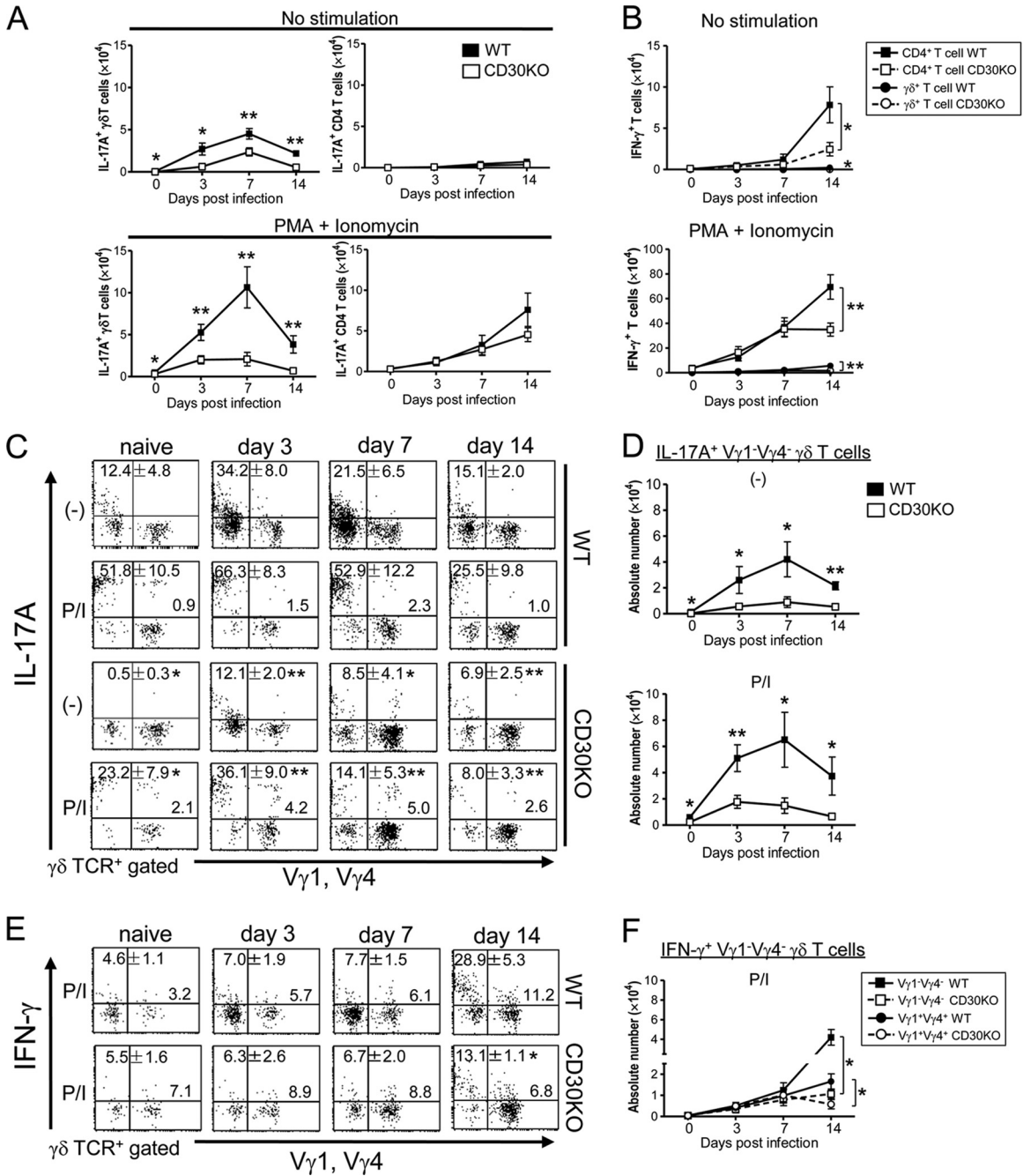


FIG 4 Kinetics of IL-17A- or IFN- γ -producing T cells in PEC of CD30 KO mice during the early stage of BCG infection. WT or CD30 KO mice infected i.p. with 1×10^6 CFU of BCG and PEC were harvested on days 0, 3, 7, and 14 postinfection. Cells were cultured for 5 h without (-) or with PMA/ionomycin (P/I) stimulation, and BFA was added for the last 4 h. After incubation, intracellular flow cytometric analysis was performed on PEC to measure the IL-17A-producing (IL-17A⁺) or IFN- γ -producing (IFN- γ ⁺) T cells. (A) Absolute numbers of IL-17A⁺ $\gamma\delta$ T or IL-17A⁺ CD4 T cells without P/I stimulation (top panels) or with P/I stimulation (bottom panels). (B) Absolute numbers of IFN- γ ⁺ $\gamma\delta$ T or IFN- γ ⁺ CD4 T cells without P/I stimulation (top panel) or with P/I stimulation (bottom panel). (C and D) Frequencies (C) and absolute numbers (D) of IL-17A⁺ V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells without or with P/I stimulation. (E and F) Frequencies (E) and absolute numbers (F) of IFN- γ ⁺ V γ 1⁻ V γ 4⁻ or IFN- γ ⁺ V γ 1⁺ V γ 4⁺ $\gamma\delta$ T cells with P/I stimulation. Data are shown after gating on $\gamma\delta$ TCR⁺ cells. The numbers on the dot plots are expressed as means and SD of the results determined for at least three mice per group and per day, with data from one representative mouse shown. Student's *t* tests were performed to determine statistical differences between two groups (*, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

cells, and CD4⁺/CD8⁺ CD3⁺ T cells in PEC from BCG-infected WT mice, and none of these cells exhibited detectable CD30 expression on cell surfaces (data not shown). Similarly, only a few NK cells, NKT cells, and CD4⁺/CD8⁺ T cells expressed

CD30L. Neither macrophages nor DCs nor B cells from PEC expressed CD30L after BCG infection (data not shown). Taken together, these results strongly suggested that CD30L/CD30 signaling plays an important role in controlling the innate im-

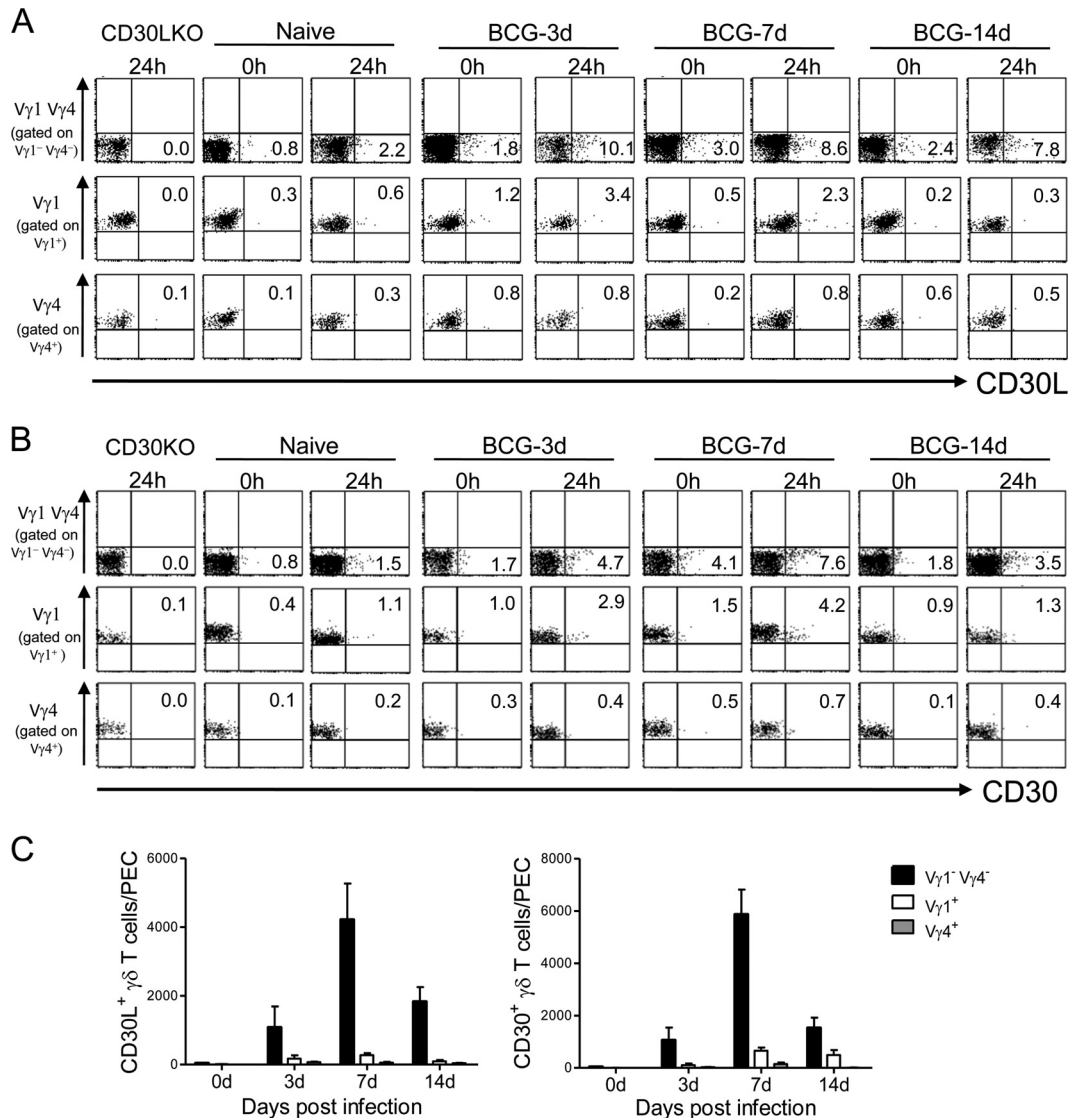


FIG 5 Expression of CD30L and CD30 on $\gamma\delta$ T cells of PEC. Cells were isolated from PEC of naive mice or mice inoculated i.p. with 1×10^6 CFU of BCG. CD30L and CD30 expression on subsets of $\gamma\delta$ T cells before and/or after 24 h of culture in RPMI medium without any stimulation was analyzed by flow cytometry. (A and B) Kinetics of CD30L expression (A) and CD30 expression (B) on the repertoire of $\gamma\delta$ T cells in PEC after BCG infection. Data are expressed as the increase in expression level over that before infection. Each number in a quadrant indicates the percentage of each quadrant. The numbers on the dot plots are expressed as means of the results determined for three mice per group and per day, with data from one representative mouse shown. (C) Absolute numbers of subsets of CD30L⁺ or CD30⁺ $\gamma\delta$ T cells of PEC on indicated day after BCG infection. Data are representative of two independent experiments.

mune response to BCG by the specific V γ 1⁻ V γ 4⁻ subset-bearing V γ 6 $\gamma\delta$ T cells.

Effects of soluble CD30-Ig on IL-17A-producing $\gamma\delta$ T cells in PEC of WT mice infected with BCG. To further verify that CD30 signaling plays an important role in the activation of IL-17A-producing $\gamma\delta$ T cells after infection, we examined the effect of the inhibition of CD30 signaling caused by administering soluble CD30-Ig *in vivo* on the activation of IL-17A-producing $\gamma\delta$ T cells after BCG infection in WT mice. Mice administered CD30-Ig showed markedly decreased numbers of IL-17A-producing $\gamma\delta$ T cells (Fig. 6A and B), mainly, IL-17A⁺ V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells (Fig. 6C and D), in PEC after BCG infection, while there were no significant changes in the frequency and absolute number of IFN- γ -producing $\gamma\delta$ T cells in CD30-Ig-treated mice (Fig. 6A and B).

These results suggested that *de novo* synthesis of CD30 is important for the activation of the early-appearing $\gamma\delta$ T cells producing IL-17A after BCG infection.

Effects of agonistic anti-CD30 MAB on IL-17A-producing $\gamma\delta$ T cells in PEC of mice infected with BCG. Since CD30L is the exclusive ligand for CD30, CD30L KO mice show similar phenotypes in terms of susceptibility to bacterial infection or inflammatory diseases (38, 41, 48). We found that the appearance of IL-17A-producing $\gamma\delta$ T cells was impaired in PEC of CD30L KO mice on day 7 after BCG infection. Administration of agonistic anti-CD30 MAB not only restored the early appearance of IL-17A-producing $\gamma\delta$ T cells in CD30L KO mice but also stimulated these cells in WT mice (Fig. 7A and B). Furthermore, the frequency and absolute number of IL-17A⁺ V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells significantly

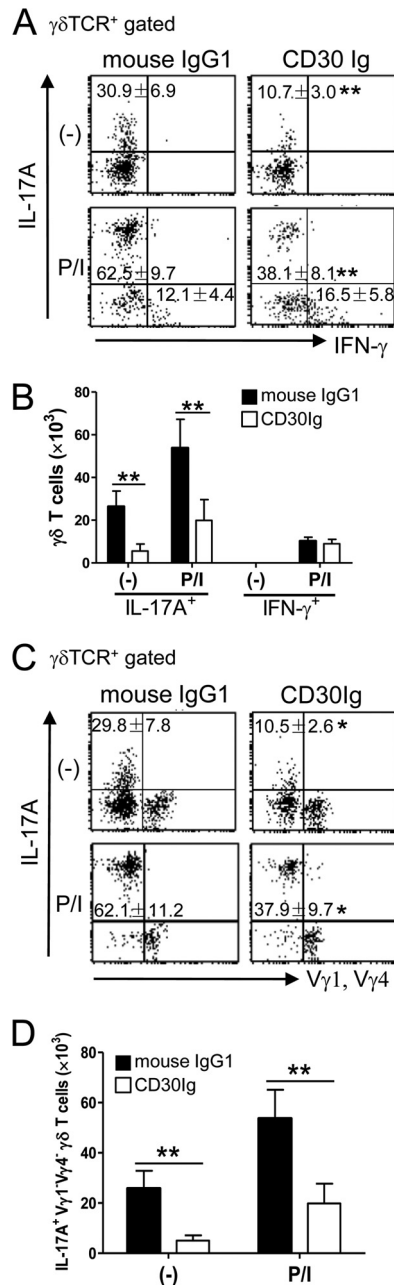


FIG 6 Effects of *in vivo* treatment with CD30-Ig on early activation of IL-17A⁺ $\gamma\delta$ T cells after BCG infection. WT mice were inoculated i.p. with 1×10^6 CFU of BCG and simultaneously treated with CD30-Ig or mouse IgG1 (200 mg/head) as a control. On day 3 postinfection, PEC were harvested and cultured with or without PMA/ionomycin (P/I) stimulation for 5 h, and BFA was added for the last 4 h. After incubation, IL-17A and IFN- γ production of $\gamma\delta$ T cells was measured by flow cytometry. (A and B) Frequencies (A) and absolute numbers (B) of IL-17A⁺ or IFN- γ ⁺ $\gamma\delta$ T cells in PEC. (C and D) Frequencies (C) and absolute numbers (D) of IL-17A⁺ V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells in PEC. Data are shown after gating on $\gamma\delta$ TCR⁺ cells. The numbers on the dot plots are expressed as means and SD of the results determined for five mice per group, with data from one representative mouse shown. Significant differences were calculated by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

increased in both CD30L KO and WT mice administered agonistic anti-CD30 MAb (Fig. 7C and D). There were no differences in the IFN- γ -producing $\gamma\delta$ T cells between agonistic anti-CD30 MAb-treated mice and the control group in WT/CD30L KO mice (Fig. 7A and B). These results strongly prove that CD30L/CD30 signaling selectively contributes to the activation of the V γ 1⁻ V γ 4⁻ subset of $\gamma\delta$ T cells bearing V γ 6, which is the main source of IL-17A during the early stage of BCG infection.

DISCUSSION

IL-17A-producing $\gamma\delta$ T cells are known to be activated at an early stage of BCG infection (13). Here we show that the appearance of IL-17A-producing $\gamma\delta$ T cells preferentially expressing TCR V γ 6/V δ 1 genes was selectively impaired in PEC of CD30 KO mice at an early stage of BCG infection. CD30L or CD30 expression was up-regulated predominantly on a V γ 1⁻ V γ 4⁻ subset of $\gamma\delta$ T cells in PEC after BCG infection. *In vivo* blocking of CD30 signaling by CD30-Ig inhibited the appearance of IL-17A-producing V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells in WT mice, whereas *in vivo* stimulation of CD30 signaling with agonistic anti-CD30 MAb increased the number of IL-17A-producing V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells after infection in CD30L KO mice as well as WT mice. Taken together, IL-17A-producing V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells bearing V γ 6 may be activated in a CD30L/CD30-dependent manner as an innate immunity against BCG infection.

A noteworthy finding in the present study is that CD30 signaling plays an important role in the activation of the subset of IL-17A-producing V γ 1⁻ V γ 4⁻-bearing V γ 6 $\gamma\delta$ T cells in innate immunity against BCG infection. There are several potential mechanisms that may explain the novel role of CD30 signaling in regulating IL-17A response by the unique subset of $\gamma\delta$ T cells in the early response to BCG infection. CD30 is a member of the TNF receptor family that has the potential to induce TNF receptor-associated factor 2 (TRAF-2)-mediated NF- κ B activation and can recruit TRAF-1 (49–51). It probably promotes T cell survival (50, 51). Thus, we speculate that CD30 signaling may enhance $\gamma\delta$ T cell survival after BCG infection. It is widely accepted that Toll-like receptors (TLRs) are responsible for the innate immune response and act by recognizing bacterial products that induce proinflammatory cytokines and chemokine production. Martin et al. (52) recently reported that IL-17A-producing $\gamma\delta$ T cells, which express TLR2 as well as TLR1 and dectin-1, selectively expanded in the early response to *M. tuberculosis* invasion. A previous study has shown that V γ 6⁺ V δ 1⁺ $\gamma\delta$ T cells in PEC express TLR2 mRNA (53). In the present study, we found that expression of CD30L or CD30 was upregulated selectively on a subset of V γ 1⁻ V γ 4⁻-bearing V γ 6 $\gamma\delta$ T cells after BCG infection. Therefore, it is alternatively possible that CD30 signaling in synergy with TLR2 plays an important role in the activation of IL-17A-producing V γ 1⁻ V γ 4⁻-bearing V γ 6 $\gamma\delta$ T cells in the innate immune response to BCG infection. In addition, we previously reported that early IL-23 production and IL-23-triggered Tyk2 signaling were essential for IL-17A production by $\gamma\delta$ T cells after *E. coli* or *C. albicans* infection (17, 42). However, in this study, there was no significant difference between CD30 KO and WT mice in the level of IL-23 secretion in PEC after BCG infection. Moreover, expression of CD30L or CD30 was not detected on either macrophages or DCs. Therefore, impaired IL-17A production in CD30 KO mice may not be caused by impaired accessory functions of macrophages/DCs.

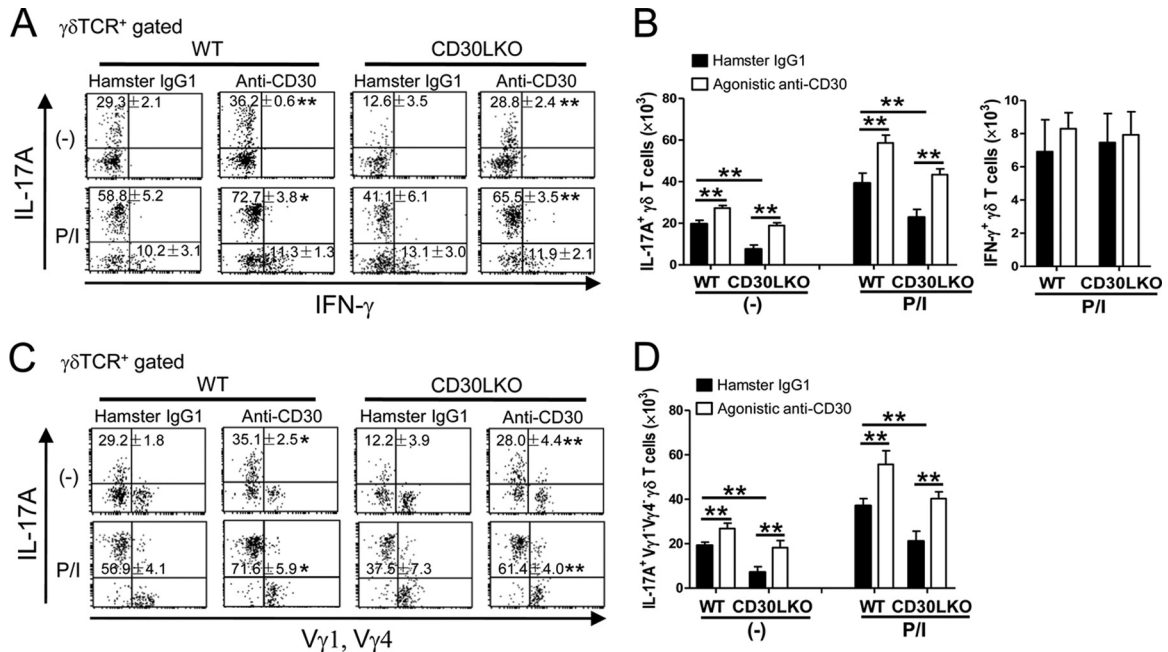


FIG 7 Effects of *in vivo* treatment with agonistic anti-CD30 MAB on early activation of IL-17A⁺ $\gamma\delta$ T cells after BCG infection. WT or CD30L KO mice were infected i.p. with 1×10^6 CFU of BCG and simultaneously treated with agonistic anti-CD30 MAB (clone CD30.1) or hamster IgG1 (100 mg/head) as a control. IL-17A or IFN- γ production of $\gamma\delta$ T cells was measured in PEC on day 3 postinfection by flow cytometry. (A and B) Frequencies (A) and absolute numbers (B) of IL-17A⁺ or IFN- γ ⁺ $\gamma\delta$ T cells in PEC. (C and D) Frequencies (C) and absolute numbers (D) of IL-17A⁺ V γ 1⁺ V γ 4⁻ $\gamma\delta$ T cells in PEC. Data are shown after gating on $\gamma\delta$ TCR⁺ cells. The numbers on the dot plots are expressed as means and SD of the results determined for four mice per group, with data from one representative mouse shown. Significant differences were calculated by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

Here we found that the expression level of CD30L or CD30 on a subset of V γ 1⁻ V γ 4⁻-bearing V γ 6 $\gamma\delta$ T cells was rapidly up-regulated after BCG infection. It remains unknown why the V γ 1⁻ V γ 4⁻ subset selectively expressed CD30L/CD30. We have recently reported that Hes 1 induced by Notch signaling, which is expressed in IL-17A-producing $\gamma\delta$ T cells, can regulate the IL-17A-producing function of $\gamma\delta$ T cells in the peripheral tissues (54). Notch signaling is capable of inducing IL-7R α expression in T cells (55), and mediating IL-7 signaling can promote CD30L expression (56). More recently, IL-17A-producing $\gamma\delta$ T cells were reported to express high levels of IL-7R α on their surface (57). Here we found that the V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells, which preferentially express V γ 6/V δ 1 genes, act as the major IL-17A-producing $\gamma\delta$ T cells predominantly expanded in the early response to BCG infection. Therefore, it is possible that the Notch-Hes/IL-7R α axis may contribute to induce CD30L/CD30 expression preferentially on IL-17A-producing V γ 1⁻ V γ 4⁻ $\gamma\delta$ T cells bearing V γ 6 in response to BCG infection. On the other hand, V γ 6⁺ V δ 1⁺ $\gamma\delta$ T cells induced by infection bear truly invariant TCRs in a nucleotide canonical sequence with no apparent N-region contribution (44, 58, 59). A recent report showed that $\gamma\delta$ T cells can recognize a microbially encoded B cell antigen and induce IL-17A production (60), raising the possibility that V γ 6⁺ V δ 1⁺ $\gamma\delta$ T cells have the capability to recognize some specific self-Ags induced by mycobacterial infection to initiate a swift IL-17 response. At present, whether IL-17A-producing $\gamma\delta$ T cells recognize such a unique Ag with a restricted V γ gene repertoire is still unknown.

In the present study, we observed that $\gamma\delta$ T cells capable of producing IL-17A precede those producing IFN- γ in appearance

following BCG infection. Furthermore, $\gamma\delta$ T cells have the capacity to produce IL-17A without further stimulation, while they required *in vitro* stimulation with PMA/ionomycin for IFN- γ production. It has been recently reported that Ag-naive $\gamma\delta$ T cells produce IL-17A, while Ag-experienced $\gamma\delta$ T cells produce IFN- γ (6). More recently, IL-17A⁺ $\gamma\delta$ T cells were reported to develop earlier than IFN- γ ⁺ $\gamma\delta$ T cells from double-negative thymocytes in ontogeny (61). In this regard, it is possible that $\gamma\delta$ T cells producing IL-17A may precede $\gamma\delta$ T cells producing IFN- γ in appearance following BCG infection, which may recapitulate their ontogeny.

We previously reported that "innate" CD4⁺ $\alpha\beta$ T cells producing IFN- γ are activated and play a role in early protection against *Listeria monocytogenes* (48). However, in the present study, the "innate" CD4⁺ $\alpha\beta$ T cells producing IFN- γ were hardly detected during the first 7 days of BCG infection without further stimulation. Instead, IFN- γ ⁺ CD4⁺ $\alpha\beta$ T cells, here speculated to correspond to *M. tuberculosis*-specific Th1 cells, activated/proliferated in PEC as infection progressed and served as the overwhelming source of IFN- γ to join in the following response to BCG infection. We found here that CD30 signaling was involved in the activation of IFN- γ -producing CD4⁺ $\alpha\beta$ T cells, which is consistent with our previous report on the Ag-specific Th1 response to BCG infection (36). Additionally, we also found that numbers of not only the V γ 1⁻ V γ 4⁻ subset but also the V γ 1⁺ V γ 4⁺ subset of $\gamma\delta$ T cells producing IFN- γ were reduced, though to a very low degree, in CD30-deficient mice on day 14 after BCG infection. Since V γ 1⁺ or V γ 4⁺ $\gamma\delta$ T cells marginally expressed CD30L or CD30 after BCG infection, given previous reports that innate produc-

tion of IL-17A plays an important role in priming of the response of IFN- γ -producing T cells following BCG infection (14, 62), it is possible that the reduction of the numbers of IFN- γ -producing $\gamma\delta$ T cells in CD30 KO mice may have been due to the attenuated production of IL-17A but may not directly link to CD30 ablation. Taking the results together, our report suggests that CD30 signaling-dependent activation of $\gamma\delta$ T cells producing IL-17A may promote a subsequent expansion of IFN- γ -producing T cells that predominantly drives a Th1 response to BCG infection in the peripheral tissues.

It may alternatively be possible that CD30 signaling is necessary for intrathymic differentiation of innate $\gamma\delta$ T cells since basal levels of innate IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells were lower in the peritoneal cavity of naive CD30 KO mice. However, in the present study, inhibition of CD30L/CD30 signaling by administering soluble CD30-Ig *in vivo* markedly decreased the numbers of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells among PEC of WT mice after BCG infection. Furthermore, stimulating CD30L/CD30 signaling by administering agonistic anti-CD30 MAb restored IL-17A production by $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells in CD30L KO mice after BCG infection. These results suggested that *de novo* synthesis of CD30 is important for the activation of the early-appearing $\gamma\delta$ T cells producing IL-17A after BCG infection.

In conclusion, our report suggests that activation of IL-17A-producing $V\gamma 1^- V\gamma 4^- \gamma\delta$ T cells bearing TCR $V\gamma 6$ leading the innate immune response to BCG infection is controlled by a CD30L/CD30-dependent mechanism.

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