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IL-17–Producing Innate and Pathogen-Specific Tissue Resident Memory γδ T Cells Expand in the Lungs of *Bordetella pertussis*–Infected Mice

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γδ T cells play a role in protective immunity to infection at mucosal surface, but also mediate pathology in certain autoimmune diseases through innate IL-17 production. Recent reports have suggested that γδ T cells can have memory analogous to conventional αβ T cells. In this study we have examined the role of γδ T cells in immunity to the respiratory pathogen *Bordetella pertussis*. γδ T cells, predominantly Vγ4^{-}γ1^{-} cells, produced IL-17 in the lungs as early as 2 h after infection. The bacterial burden during primary infection was significantly enhanced and the induction of antimicrobial peptides was reduced in the absence of early IL-17. A second peak of γδ T cells is detected in the lungs 7–14 d after challenge and these γδ T cells were pathogen specific. γδ T cells, exclusively Vγ4, from the lungs of infected but not naive mice produced IL-17 in response to heat-killed *B. pertussis* in the presence of APC. Furthermore, γδ T cells from the lungs of mice reinfected with *B. pertussis* produced significantly more IL-17 than γδ T cells from infected unprimed mice. γδ T cells with a tissue resident memory T cell phenotype (CD69^{+}CD103^{+}) were expanded in the lungs during infection with *B. pertussis* and proliferated rapidly after rechallenge of convalescent mice. Our findings demonstrate that lung γδ T cells provide an early source of innate IL-17, which promotes antimicrobial peptide production, whereas pathogen-specific Vγ4 cells function in adaptive immunological memory against *B. pertussis*. *The Journal of Immunology*, 2017, 198: 363–374.

A subset of T lymphocytes, γδ T cells share many characteristics with the cells of the innate immune system. They are enriched in mucosal epithelia, where pathogens are first encountered, and their localization allows them to participate in the initiation of the immune response, as well as influencing the subsequent adaptive immune response. γδ T cells can be rapidly activated upon recognition of cognate Ags or in response to cytokines (1). Similar to conventional T cells, γδ T cells perform their function through secretion of soluble mediators or cytotoxicity against target cells. However, their response is much quicker than that of conventional T cells and they recognize a different range of Ags, much of which is self-molecule. Furthermore, it has been suggested that Ag recognition by γδ T cells is B cell-like and does not require Ag processing or presentation. However, human γδ T cells respond to phosphoantigens, which is enhanced in the presence of APCs (2).

Lung γδ T cells play an important role in defenses against mucosal pathogens, such as *Candida albicans,* where they produce IL-17 and control the early influx of neutrophils into the lung (3). In a mouse model of *Mycobacterium tuberculosis* infection, lung γδ T cells promote protective responses by providing a non-redundant early source of IFN-γ (4). In a simian model of *M. tuberculosis* infection, polyclonal Vγ9Vδ2 cells of peripheral origin, but not lung resident cells, were shown to expand in lung tissue and produce IFN-γ in response to phosphoantigen stimulation in vitro (5). During *Bacillus subtilis* infection of mice, Vγ6Vδ1 cells accumulate in lungs and contribute to the clearance of bacteria through IL-17 production and control bacteria-induced fibrosis via secretion of IL-22 (6, 7). IL-17–producing γδ T cells also have a protective role in a bleomycin-induced model of lung injury by promoting coordinated inflammatory response; TCRδ$^{-/-}$ mice had a reduced cellular infiltration to the lungs and showed a delay in the repair process (8).

Immunological memory is a hallmark of the adaptive immune system. Ag-specific T and B cells expand during infection with a pathogen, and contract following its clearance. However, some of these highly specific T and B cells go on to become long-lived memory cells that persist in the body for many years. Higher frequency of Ag-specific T and B cells allows for faster pathogen clearance, often without the infected individual experiencing any of the symptoms of the disease. Recent reports have suggested that memory T cells reside in tissues without recirculating; these tissue-resident memory T (T_{RM}) cells, provide a first line of defense against reinfection (9). The majority of studies on T_{RM} cells in infection have focused on CD8 T_{RM} cells in immunity to viruses (10, 11), with some reports on CD4 T_{RM} cells (11). However, to date there is limited evidence that γδ T cells have memory or that these memory γδ T cells reside in tissues.

Although memory has classically been associated with Ag-specific αβ T cells, and γδ T cells are thought to be mainly involved in innate phase of the immune response and be activated by molecules shared by different classes of pathogens, recent studies in mice have shown that γδ T cells can mount pathogen-specific responses (12, 13). Furthermore, γδ T cells can have phenotypic features of memory αβ T cells, including segregation into subpopulations based on CD44 and CD62L expression and can be divided into CD27^{-} and CD27^{+} γδ T cells, with the majority of

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Abbreviations used in this article: HKBp, heat-killed *Bordetella pertussis*; Lcn2, lipocalin 2; T_{RM}, tissue resident memory T.

$CD27^+$ cells expressing CD62L (14, 15). Furthermore, $CD27^+$ and $CD27^-$ γδ T cells share some characteristics of central and effector memory T cells, respectively. For example, $CD27^+$ have shown a superior proliferation upon TCR stimulation compared with $CD27^-$ γδ T cells (14). In addition, subsets of $CD27^-$ γδ T cells were also found to express the cell surface markers CD69 and CD103, associated with CD4 or CD8 T_{RM} cells (16–18). However, to the best of our knowledge, the expression of T_{RM} cell markers has not been investigated in the context of pathogen-specific γδ T cells. Unlike αβ T cells, a sizeable population of $CD44^{hi}$ γδ T cells is present in naive animals and both $CD27^+$ and $CD27^-$ γδ T cells show an immediate effector function upon TCR engagement. It is therefore unclear whether memory markers commonly used for αβ T cells correlate with the memory phenotype in the γδ T cell compartment.

Infection with the Gram-negative bacteria *Bordetella pertussis* causes whooping cough, a severe respiratory infection in young infants. The incidence of pertussis is increasing and this has been attributed to the waning immunity due to poor immunological memory induced with the current acellular pertussis vaccine. In contrast, previous infection induces long-lived immunity and this has been attributed to better induction of immunological memory. Studies in humans and mouse models have shown that infection induces potent pathogen-specific IFN-γ–producing Th1 cells and IL-17–secreting Th17 cells (19–22), which mediate clearance of bacteria from the respiratory tract (23–26). It has been suggested that γδ T cells may act to limit the early inflammatory response during *B. pertussis* infection (27), but this study did not examine their role in IL-17 production or in adaptive immunity. In this study we provide evidence that, in addition to providing an early source of innate IL-17, a population of γδ T cells that are pathogen-specific and tissue-resident expand in the lungs following reinfection and may therefore function in adaptive immunity to *B. pertussis*.

Materials and Methods

Mice and treatments

All mice were maintained according to European Union regulations and the Irish Health Products Regulatory Authority. Experiments were performed under Health Products Regulatory Authority license with approval from the Trinity College Dublin BioResources Ethics Committee. C57BL/6 wild-type mice were bred in house from established colonies and housed under specific pathogen free conditions. For IL-17 neutralization, mice were injected i.p. with anti–IL-17 Ab (17F3; BioXcell) or the isotype control Ab at 200 μg/mouse 1 d prior and 2 d after infection. For Vγ4 cell depletion, convalescent mice were injected i.p. with anti-Vγ4 Ab (UC3-10A6; BioXcell) or the isotype control Ab (100 μg/mouse) on days −3, −1 and +1 after reinfection.

B. pertussis *respiratory challenge*

Respiratory infection of mice was performed by exposing mice to *B. pertussis* aerosol (BP338 strain; 1×10^9 CFU/ml) for 10 min followed by 10 min rest using PARI TurboBOY SX nebulizer. The course of *B. pertussis* infection was followed by performing CFU counts on lungs from groups of three to four mice at intervals after challenge. The lungs were aseptically removed and homogenized in 1 ml of sterile physiological saline with 1% casein. Undiluted and serially diluted homogenate (100 μl) from individual lungs was plated in duplicate on Bordet-Gengou agar plates and the bacterial colonies were counted after 6 d incubation at 37°C.

Lung and lymph node cell isolation and γδ T cell purification

Mononuclear cells were prepared from the lungs of naive, *B. pertussis* infected or convalescent mice by mechanical disruption of lung tissue with enzymatic digestion. Briefly, lungs were cut into small pieces and digested in with 1 mg/ml Collagenase IV (Roche) and DNAse I (20 U/ml) for 1 h at 37°C and forced through 70 μm cell strainers. For CD27 FACS staining only mechanical disruption of tissue was used to isolate cells. Lymph node single cell suspension was prepared by forcing whole lymph nodes through 70 μm strainers. For cell sorting, lung mononuclear cells were further purified over a 30% Percoll. Lung and lymph node cells were resuspended at 50×10^6 cells/ml in complete RPMI 1640 and blocked with Fcγ Ab. Abs against CD3 (145-2C11) and TCRδ (GL3) were then added. Vγ1 (2.11) and Vγ4 (UC3-10A6) stain was also included when sorting γδ T cell subsets. The cells were sorted on a MoFlo sorter (Beckman Coulter). Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >95%. γδ T cells were cultured in 96-well plates at $0.02–0.04 \times 10^6$ cells/ml.

Detection of Ag-specific γδ T cell responses

Spleen cells from naive C57BL/6 mice were resuspended at 4×10^6 cells/ml in PBS with 20% FCS and irradiated (5 Gy) for 5 min in a γ cell irradiator (Gammacell 3000). Cells were washed and resuspended in complete RPMI 1640. The spleen cells were cultured in 96-well round-bottom plates (Greiner) with heat-killed *B. pertussis* (HKBp) at concentrations indicated in the figures for 2 h at 37°C, 5% CO_2 to allow for Ag uptake and processing. Heat-killed *Staphylococcus aureus* PS80 strain and killed *M. tuberculosis* (1.4 μg/ml) were used as controls. Purified γδ T cell subpopulations ($5–10 \times 10^3$/well) were added and cells incubated at 37°C, 5% CO_2. Alternatively γδ T cells were incubated with 100 μl of supernatants from a 72 h culture of irradiated spleen cells cultured with HKBp or medium only. After 72 h the supernatants were removed and cytokine concentration quantified by ELISA.

FACS analysis

Cells were isolated as above, washed in PBS and incubated with 1/600 diluted LIVE/DEAD Fixable Aqua Dead Cell stain (Aqua-Live/dead; Molecular Probes, Life Technologies) for 30 min at room temperature in the dark. The cells were then washed, blocked with Fcγ block (1 μg/ml; BD Pharmingen) and surface stained for 15 min at room temperature in the dark. For intracellular cytokine staining brefeldin A (5 μg/ml) was added to the collagenase/DNAse during the lung digestion step. After surface staining cells were fixed with 2% paraformaldehyde (Thermo Scientific) and permeabilized with 0.05% saponin/PBS (Sigma). The cells were then stained with intracellular Abs in saponin for 15 min at room temperature. For intranuclear staining Foxp3 buffers were used (eBioscience) and staining performed according to manufacturer's instructions. Samples were acquired on LSRFortessa (BD) or CANTO-II (BD) flow cytometers. FACS data analysis was performed using FlowJo (Treestar) software. The following Abs from eBioscience, BioLegend or BD were used: TCRδ-APC (GL3), TCRδ-BV605 (GL3), Vγ4-FITC (UC3-10A6), Vγ4 PE-Cy7 (UC3-10A6), Vγ1.1-PE (2.11), Vγ1.1-APC (2.11), Vδ4-FITC (GL2), Vδ6.3/2-PE (8F4H7B7), CD27-PE-Cy7 (LG.7F9), CD3-A780 (17A2), CD3-BV650, CD3-PerCP-Cy5.5, CD4-A780 (RM4-5), CD4 BV785 (RM4-5), CD8-A780 (53-6.7), CD8-A700 (53-6.7), CD103-BV786 (M290), CD103-PE (2E7), CD69-FITC ([^{1}H].2F3), CD69 APC ([^{1}H].2F3), CD44-PE-Cy7 (IM7), CD44 BV605 (IM7), IL-17 V450 (TC11-18H10), Ki67 eFluor660 (SolA15), Ki67 V450 (SolA15), and CD62L PerCP-Cy5.5(MEL-14). γδ T cell and CD4 T cell numbers were determined by gating on $CD3^+$ $TCR\delta^+$ and $CD3^+TCR\delta\text{-}CD4^+$ cells, respectively. Gating on $V\gamma4^-V\gamma1^-$ $TCR\delta^+$ T cells was used to identify cells we believe to be Vγ6 γδ T cells. For in vivo circulating cell labeling, mice were injected i.v. through the tail vein with CD45-PE (1.5 μg/mouse in 200 μl PBS) 10 min prior to culling.

RT-PCR

RNA was isolated using Trizol Reagent (Invitrogen) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). When isolating RNA from γδ T cells or their subsets, RNeasy Minikit (QIAGEN) was used according to the manufacturer's instructions. TaqMan polymerase master mix (Applied BioSystems) was used for PCR reaction. All primers were purchased from Applied Biosystems with the exception of GAPDH, which was purchased from Roche.

Statistical analyses

One- or two-way ANOVA was used to test for statistical significance of differences between more than two experimental groups. The Student *t* test was used for analysis when two groups were compared.

Results

Early appearance of innate IL-17–producing γδ T cells in the lungs of B. pertussis*–infected mice*

To examine the role of γδ T cells during primary *B. pertussis* infection, mice were infected by aerosol exposure to a virulent strain of *B. pertussis* and bacterial burden and IL-17 and IFN-γ

production by lung T cells were investigated at different time points after infection. Bacterial numbers in the lungs increased over the first 3 d of infection and then began to decline (Fig. 1A). Bacteria are normally cleared around 35 d after challenge of naive mice (28). IL-17–secreting γδ T cells were detected in the lungs as early as 2 h after *B. pertussis* challenge (Fig. 1B, 1C). The frequency of IL-17$^+$ γδ T cells then declined and increased again on day 7 after infection. There was a small increase in the frequency of IFN-γ-secreting γδ T cells 7 d after respiratory challenge with *B. pertussis* (Fig. 1B, 1C). Significant production of IL-17 and IFN-γ by αβ T cells was only observed 7–21 d after challenge (data not shown).

γδ T cells produce IL-17 in response to stimulation with IL-1β and IL-23, or IL-18 and IL-23 in the absence of TCR engagement (29, 30). Furthermore, IL-17–producing γδ T cells have been shown to highly express the Th17 transcription factor Rorγt (31). We therefore examined the expression of IL-1RI, IL-23R, IL-18R, and Rorγt by lung γδ T cell subsets and their contribution to IL-17 production in the lungs. We found that lung Vγ4$^-$Vγ1$^-$ γδ T cells expressed the highest levels of IL-1RI and IL-23R (Fig. 2A). Vγ4 γδ T cells were also found to highly express IL-23R, whereas Vγ1 γδ T cells expressed the lowest level of this receptor. Lung Vγ4$^-$Vγ1$^-$ and Vγ4 γδ T cells expressed significantly higher levels of Rorγt than Vγ1 γδ T cells (Fig. 2B). We next examined IL-17 production by Vγ subsets of γδ T cells purified from the lungs and stimulated in vitro with IL-1β and IL-23. When compared with Vγ4 and Vγ1, Vγ4$^-$Vγ1$^-$ γδ T cells produced significantly more IL-17 in response to cytokine stimulation, without TCR engagement (Fig. 2C). IL-17 production by Vγ4 cells was also found to be significantly greater than that of Vγ1 cells.

We then investigated IL-17 production by γδ T cell subsets during *B. pertussis* infection. Similar to the in vitro findings, we found that when compared with Vγ4, a greater proportion of Vγ4$^-$Vγ1$^-$ cells produced IL-17 during primary infection with *B. pertussis* (Fig. 2D, 2E). In contrast, we found a very low frequency of IL-17–secreting Vγ1 cell in the lungs of infected mice.

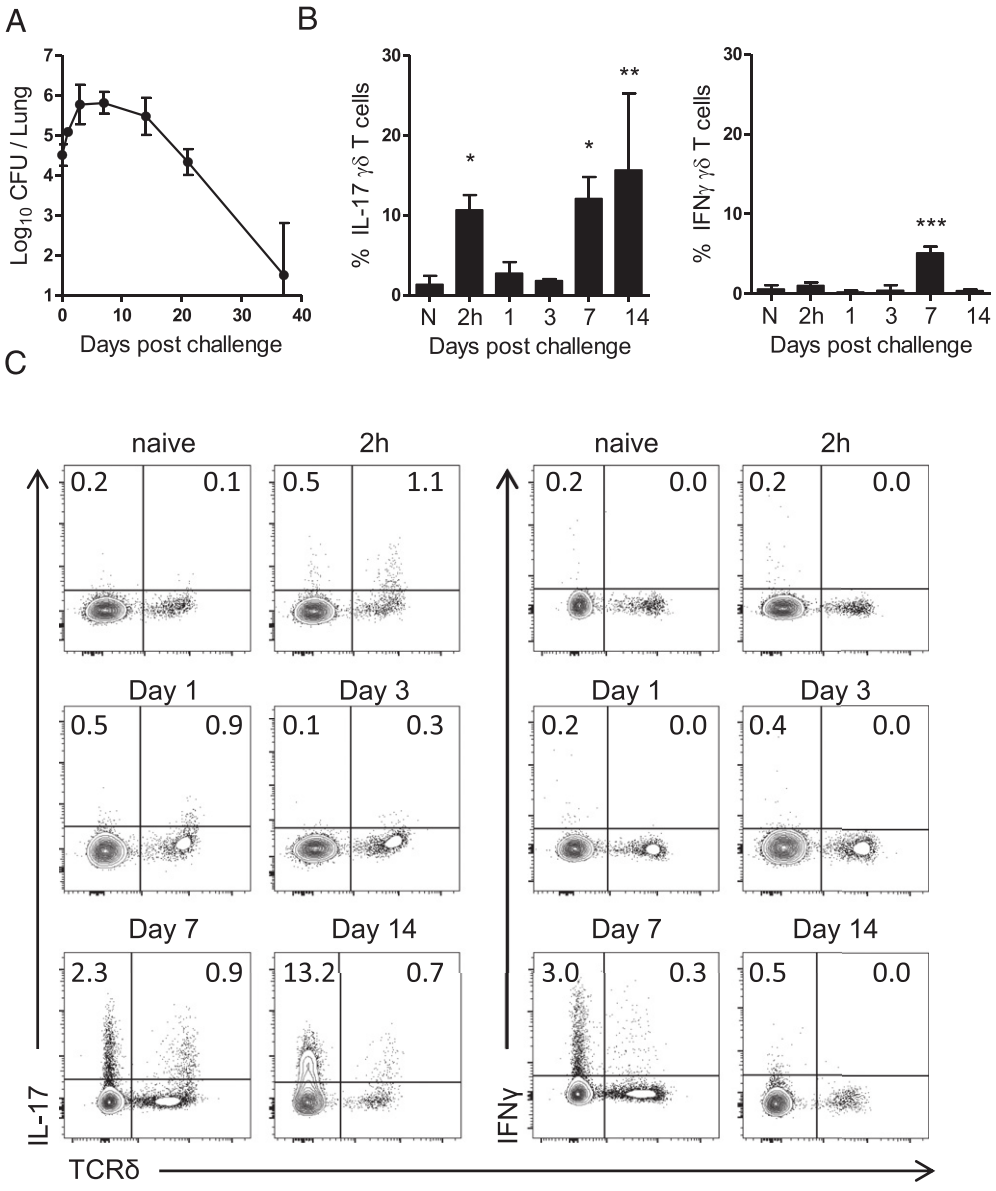

FIGURE 1. IL-17–secreting γδ T cells in the lungs of mice infected with *B. pertussis*. C57BL/6 mice were challenged by aerosol exposure to *B. pertussis*. (**A**) Bacterial burden in the lungs was analyzed by performing CFU counts at intervals after challenge. (**B**) The frequency of IL-17–secreting and IFN-γ–secreting γδ T cells in lungs determined by intracellular cytokine staining and FACS analysis. (**C**) Sample FACS plots. Data in (A) and (B) are mean ± SD (n = 3 or 4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with Dunnett's test.

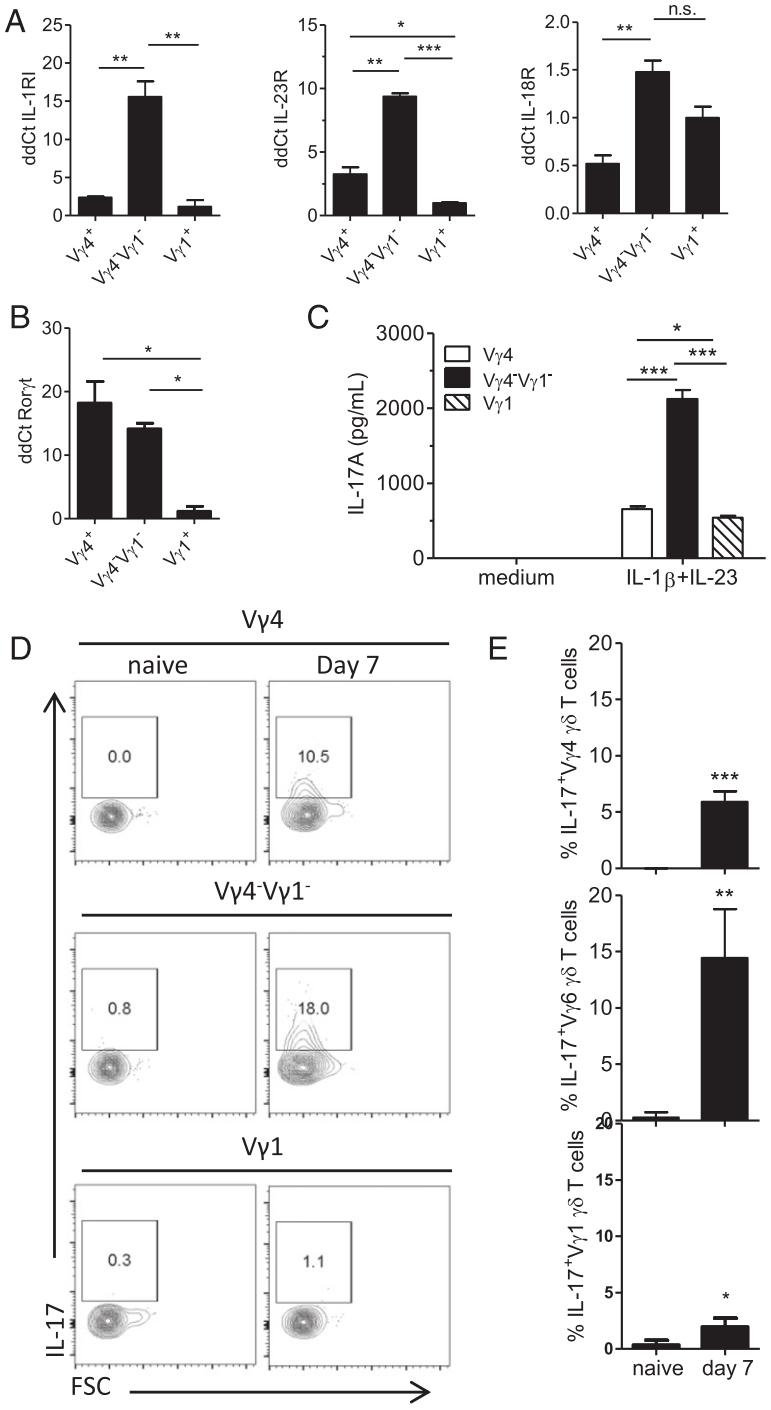

FIGURE 2. Vγ4$^-$Vγ1$^-$ and Vγ4 γδ T cells contribute to innate IL-17 production during *B. pertussis* infection. Vγ1, Vγ4 and Vγ4$^-$Vγ1$^-$ γδ T cells (1×10^5 cells) were FACS purified from the lungs of naive C57BL/6 mice. RNA was isolated and the expression of IL-1RI, IL-23R, IL-18R (**A**), and Rorγt (**B**) was analyzed by RT-PCR relative to unstimulated Vγ1 γδ T cells and normalized to 18s RNA. (**C**) γδ T cells subsets (4×10^4 cells/ml) were purified from the lungs of C57BL/6 mice and stimulated with IL-1β+IL-23 (10 ng/ml) or medium alone and IL-17 production was measured by ELISA. (**D** and **E**) C57BL/6 mice were challenged with virulent *B. pertussis* strain. IL-17 production by γδ T cell subsets was analyzed by FACS. All data are shown as mean ± SD. Data are representative of two (A–C) or three (D and E) independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n.s., not significant by one way ANOVA with Tukey posttest (A–C), by *t* test (E).

These findings suggest that a Vγ4$^-$Vγ1$^-$ subpopulation of γδ T cells is a potent source of innate IL-17 during infection with *B. pertussis.*

To address the role of the IL-17 produced by γδ T cells early in *B. pertussis* infection, IL-17 neutralizing Ab was administered to mice 1 d prior and 2 d after infection. Neutralization of early IL-17 resulted in a significant increase in the bacterial load in the lungs on day 10 after infection (Fig. 3A), with a corresponding significant decrease in the expression of the antimicrobial peptides, lipocalin 2 (Lcn2), CRAMP (encoded by Camp) and S100a8 (Fig. 3B). We also investigated the effect of neutralizing IL-17 on the bacterial burden and antimicrobial peptide expression earlier in infection. The bacterial burden on day 3 was significantly higher in mice treated with anti–IL-17 (Fig. 3C). This reflected a significant decrease in expression of CRAMP (Fig. 3D). S100a8 and Lcn2 expression was not changed (data not shown). The findings suggest that early IL-17 production may help to control early bacterial growth in the lungs through induction of antimicrobial peptide expression.

CD27$^-$CD44$^+$ Vγ4 γδ T cells expand in the lungs of B. pertussis*–infected mice*

It has been reported that γδ T cells that secrete IFN-γ express CD27, whereas IL-17–secreting γδ T cells lack expression of CD27 (14). Here we found that the number of CD27$^-$ γδ T cells significantly increased in the lungs of *B. pertussis*–infected mice, mirroring the increase in IL-17–producing γδ T cells (Fig. 4A).

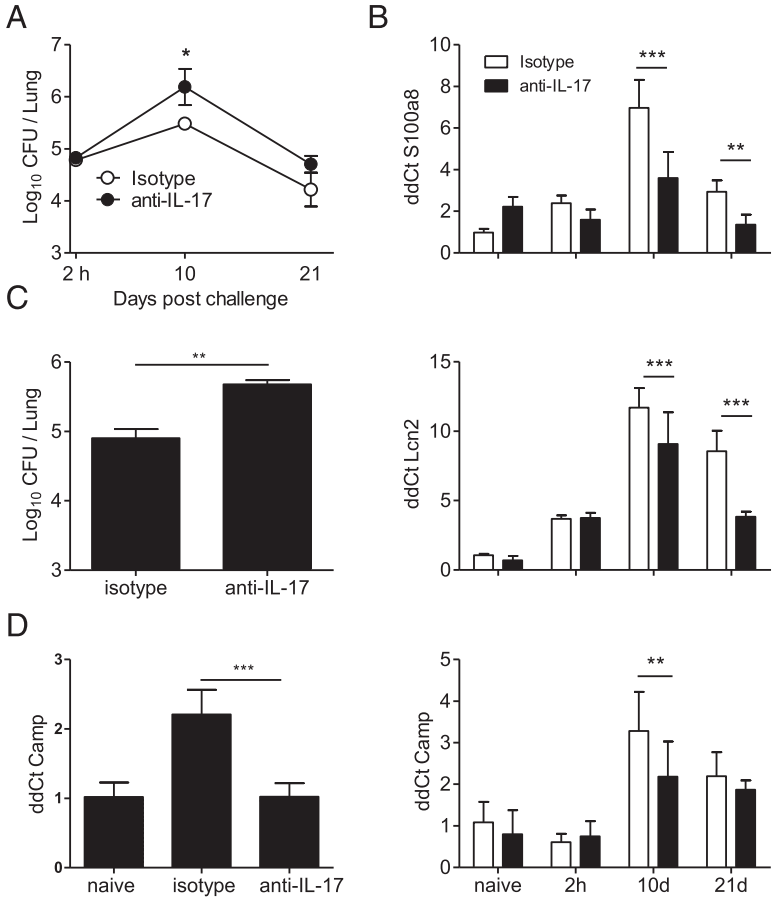

FIGURE 3. Neutralization of early IL-17 results in increased bacterial burden and reduced antimicrobial peptide expression in the lungs of *B. pertussis*–infected mice. C57BL/6 mice were treated with anti–IL-17 or isotype control Ab and challenged with *B. pertussis*. (**A**) Bacterial burden in the lungs was analyzed by performing CFU counts on lungs on days 10 and 21 after challenge. (**B**) Antimicrobial peptide expression was assessed by RT-PCR relative to naive mice and normalized to 18s RNA on lung homogenates. (**C**) Bacterial burden on day 3 after challenge (**D**) Camp expression on day 3 after challenge relative to naive mice and normalized to GAPDH. Results are mean ± SD ($n = 3$ mice). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA with Bonferroni test.

The major γδ T cell subset expanded in the lungs late in infection (days 14 and 21) was Vγ4$^+$ (Fig. 4B), which was largely CD27$^-$ (Fig. 4C). Recent studies have shown that bacterial infection resulted in accumulation of CD27$^-$CD44$^+$ γδ T cells, which exhibited memory responses upon rechallenge (12, 13). We therefore examined CD44 expression on the expanded CD27$^-$ γδ T cells in the lungs of *B. pertussis*–infected mice. We found that the majority of CD27$^-$ γδ T cells were CD44$^+$ and there was a significant increase in this population during the *B. pertussis* infection (Fig. 4D, 4E). An investigation of Vδ chain expression on lung γδ T cell subsets revealed that the proportion of Vγ4Vδ4 cells increased ~2.5-fold following *B. pertussis* infection, whereas the proportion of other subsets remained largely unchanged (Fig. 4F). This suggests that Vγ4Vδ4 γδ T cells account for a significant proportion of expanded CD27$^-$CD44$^+$ γδ T cells in the lungs of infected mice.

Lung Vγ4 cells respond to B. pertussis *in an Ag-specific manner*

We next examined the possibility that the expanded CD27$^-$CD44$^+$ γδ T cells were pathogen-specific memory γδ T cells. γδ T cell subsets were isolated from the lungs of infected or convalescent mice and stimulated in vitro with HKBp in the presence or absence of APC. We found that Vγ4 γδ T cells from infected, but not naive, mice produced IL-17 in response to HKBp in an Ag-dependent manner (Fig. 5A). Neither of the other subsets responded to HKBp, although each of the γδ T cell subtype, especially Vγ4 and Vγ4$^-$Vγ1$^-$, were capable of producing innate IL-17 following stimulation with IL-1β and IL-23. APCs were absolutely required for Ag-specific activation of γδ T cells. IL-17 was detected when Vγ4 γδ T cells were stimulated with HKBp in the presence but not in absence of APCs (Fig. 5B). In addition, Vγ4 T cells from convalescent mice produced IL-17 in response to HKBp but not to unrelated pathogens, *S. aureus* (Fig. 5C), *M. tuberculosis* (Fig. 5D) or *Escherichia coli* (data not shown). To rule out the possibility that γδ T cells were activated in an Ag-nonspecific manner through cytokine production from irradiated APC, purified lung Vγ4 T cells from infected mice were stimulated with supernatants from irradiated spleen cells cultured for 72 h with HKBp. Supernatants from HKBp-conditioned irradiated spleen cells did not induce significant IL-17 production by purified γδ T cells (Fig. 5D), providing further evidence that Ag presentation is required. Collectively, these findings suggest that the responses of *B. pertussis*–induced Vγ4 T cells are *B. pertussis* specific.

Having established that γδ T cells can mount pathogen-specific responses against *B. pertussis*, we examined whether memory γδ T cells were confined to the lung or also present in the periphery. Purified γδ T cell subsets from lungs or lung-draining lymph nodes of convalescent mice were stimulated with HKBp in the presence of APCs. Vγ4 cells isolated from the lungs produced much higher concentrations of IL-17 when compared with Vγ4 cells from lymph nodes, suggesting that memory cells reside preferentially in the lung tissue (Fig. 5E). Our data demonstrate that natural infection with *B. pertussis* results in the expansion of pathogen-specific Vγ4 γδ T cells, which persist in the lungs following the clearance of bacteria.

γδ T cells mount superior responses against B. pertussis *upon reinfection*

The hallmarks of memory response are greater magnitude and faster kinetics upon subsequent encounter with the same Ag or infectious agent. To investigate whether the Ag-specific γδ T cells play a role in adaptive immunity to *B. pertussis*, naive

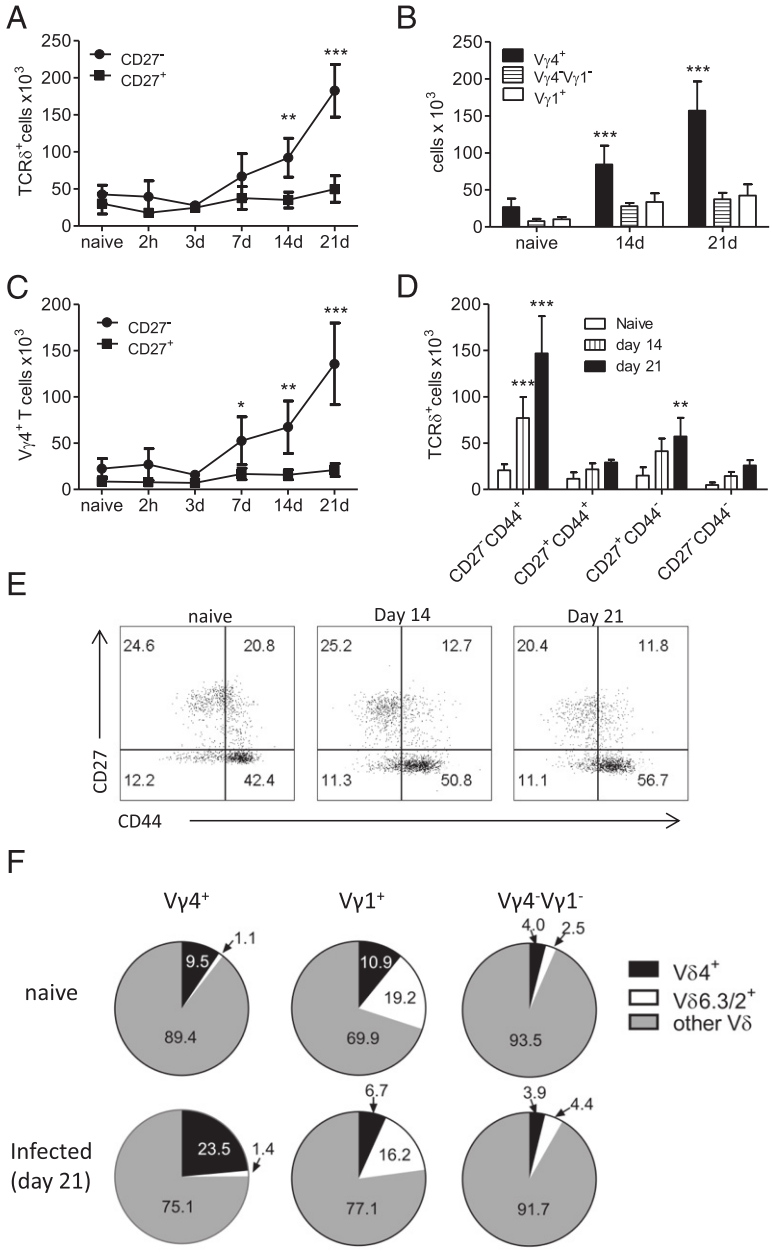

FIGURE 4. CD27$^-$ Vγ4 γδ T cells expand in the lungs of *B. pertussis* infected mice. C57BL/6 mice were challenged with *B. pertussis* and FACS analysis was performed on mononuclear cells from the lungs at intervals after challenge. The results represent the absolute numbers of CD27$^+$ and CD27$^-$ γδ T cells (**A**), the numbers of Vγ1, Vγ4, and Vγ4$^-$Vγ1$^-$ cells (**B**), the numbers of CD27$^+$ and CD27$^-$ Vγ4 cells (**C**), and the numbers of γδ T cells expressing CD27 and CD44 (**D**) with sample FACS plots (**E**). (**F**) Mean percentages of Vδ4$^+$ and Vδ6.3/2$^+$ cells within Vγ4$^+$, Vγ1$^+$ and Vγ4$^-$Vγ1$^-$ γδ T cell subsets in naive and *B. pertussis*–infected (day 21) mice ($n = 4$ mice). Results in (A)–(D) are mean ± SD ($n = 3$ mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA with Bonferroni test.

and convalescent mice were infected with *B. pertussis* and IL-17 production and bacterial clearance were investigated. When compared with naive mice, convalescent mice had significantly lower bacterial counts in the lungs as early as 1 d after *B. pertussis* respiratory challenge and cleared the bacteria completely by day 7 (Fig. 6A). The numbers of IL-17–producing γδ T cells accumulated quickly in the lungs of convalescent mice following reinfection, and peaked 1 d after rechallenge; there were significantly more IL-17–producing γδ T cells 1 d after reinfection of convalescent compared with primary infection of naive mice (Fig. 6B). The number of IL-17–secreting CD4 T cells also increased after reinfection, but here the number continued to increase until at least day 7 (Fig. 6B), when the bacteria had been cleared. Furthermore, when compared with infection of naive mice, reinfection of convalescent mice resulted in a higher frequency of IL-17–secreting Vγ4 and Vγ4$^-$Vγ1$^-$ γδ T cells in the lungs early after *B. pertussis* challenge (Fig. 6C, 6D). These data suggest that memory γδ T cells are induced by infection with *B. pertussis* and can persist in the lungs where they may play a role in resisting reinfection.

γδ T_{RM} cells expand rapidly in the lungs upon reinfection with B. pertussis

To provide evidence that γδ T_{RM} cells are expanded in the lungs during infection with *B. pertussis*, we used an in vivo labeling approach where mice were injected i.v. with fluorescent labeled anti-CD45 Ab 10 min prior to sacrifice. This results in labeling of all circulating lymphocytes, while sparing the cells in the tissues at the time of labeling and allowed us to discriminate between lung-resident and circulating γδ T cells (Fig. 7A). We found that the total number of γδ T cells resident in the lungs increased during infection and remained significantly elevated in convalescent mice following clearance of *B. pertussis* from the respiratory tract (Fig. 7B). Furthermore, the number of resident, but not circulating, γδ T cells expanded significantly upon reinfection with *B. pertussis*.

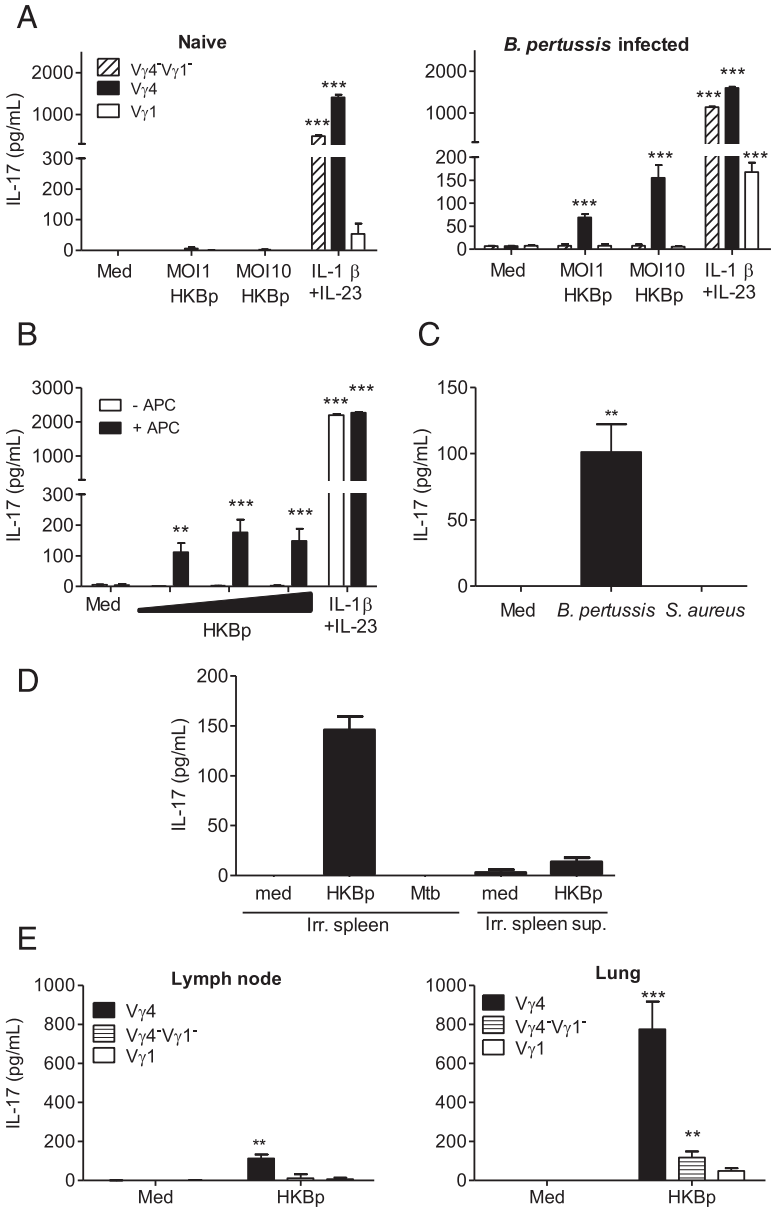

FIGURE 5. *B. pertussis* infection promotes development of pathogen-specific lung-resident memory Vγ4 γδ T cells. (**A**) Vγ1, Vγ4 and Vγ4$^-$Vγ1$^-$ subsets of γδ T cells (2×10^4 cells/ml) were purified from the lungs of naive or *B. pertussis*–infected (day 14) mice and stimulated in vitro with HKBp (equivalent to MOI 1 or 10), IL-1β and IL-23 (10 ng/ml) or medium only. (**B**) Vγ4 γδ T cells (2×10^4 cells/ml) were purified from the lungs of *B. pertussis*–infected mice (day 14) and stimulated with HKBp at MOI 10–100 or medium alone. (**C**) Vγ4 γδ T cells (2×10^4 cells/ml) were purified from the lungs of *B. pertussis* convalescent mice (day 49) and stimulated with HKBp, heat-killed *S. aureus* (MOI 10) or medium only. (**D**) Vγ4 γδ T cells (2×10^4 cells/ml) were purified from lungs of infected mice (day 21) and stimulated with HKBp (1.4 μg/ml, equivalent to MOI 10), *M. tuberculosis* (1.4 μg/ml) or supernatants from irradiated spleen cells incubated for 72 h with HKBp or medium. (**E**) Vγ4, Vγ1, and V4γ$^-$Vγ1$^-$ subsets of γδ T cells (4×10^4 cells/ml) were purified from the lungs or lymph nodes of convalescent (day 49) mice and stimulated with HKBp or medium alone. All stimulations were done in the presence of irradiated spleen cells (2×10^6/ml) as APCs unless otherwise indicated, and the MOI was relative to the spleen cell number. Graphs show mean ± SD ($n = 3$). Data are from individual experiments representative of at least two independent experiments (A–C) or from a single experiment (D and E). $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA with Bonferroni test. MOI, multiplicity of infection.

CD103 and CD69 are markers commonly used to identify CD8$^+$ or CD4$^+$ T_{RM} cells (32). We examined the expression of these markers on γδ T cells from the lungs of naive, *B. pertussis*–infected and convalescent mice. Sizable populations of CD103$^+$ CD69$^+$ and CD103$^-$CD69$^+$ were detected among lung-resident but not circulating γδ T cells in naive mice (Fig. 7C, 7D). The numbers of CD103$^+$CD69$^+$ and CD103$^-$CD69$^+$ γδ T_{RM} cells were slightly elevated in the lungs of convalescent mice, but were significantly increased upon reinfection. This suggests that although the numbers contract following bacterial clearance, γδ T_{RM} cells remain in the lungs following clearance of a primary infection and have the capacity to rapidly expand following secondary challenge.

Ag-specific memory T cells proliferate rapidly following specific Ag recognition. We therefore used the proliferation marker Ki67 to investigate the proliferative activity of circulating and resident Vγ4 γδ T cells in the lungs of *B. pertussis*–infected mice. We found that lung-resident but not circulating Vγ4 γδ T cells express higher levels of Ki67 following infection with *B. pertussis* (Fig. 8A, 8B). The enhanced expression of Ki67 was maintained on lung-resident Vγ4 cells in convalescent mice and was significantly enhanced following reinfection. In contrast, there was no significant increase in Ki67 expression on Vγ4$^-$ γδ T cell in the lungs of convalescent or reinfected mice. The increase in lung-resident Vγ4$^+$ γδ T cells was observed in the lungs when mice were reinfected either 130 or 49 d after primary infection (Fig. 8C, 8D). These findings suggest that γδ T_{RM} cells, especially Vγ4$^+$ T_{RM} cells can persist in the lungs for prolonged periods postinfection.

Discussion

The novel finding of this study is that γδ T cells with a tissue-resident memory phenotype, termed γδ T_{RM} cells, reside in the lungs postinfection with *B. pertussis* and expand significantly after reinfection of convalescent mice. Our results provide evidence that T_{RM} cells are not confined to CD4 and CD8 subpopulations but can be extended to γδ T cells and provide further support for the hypothesis that γδ T cells cross the divide between the innate and adaptive immune systems.

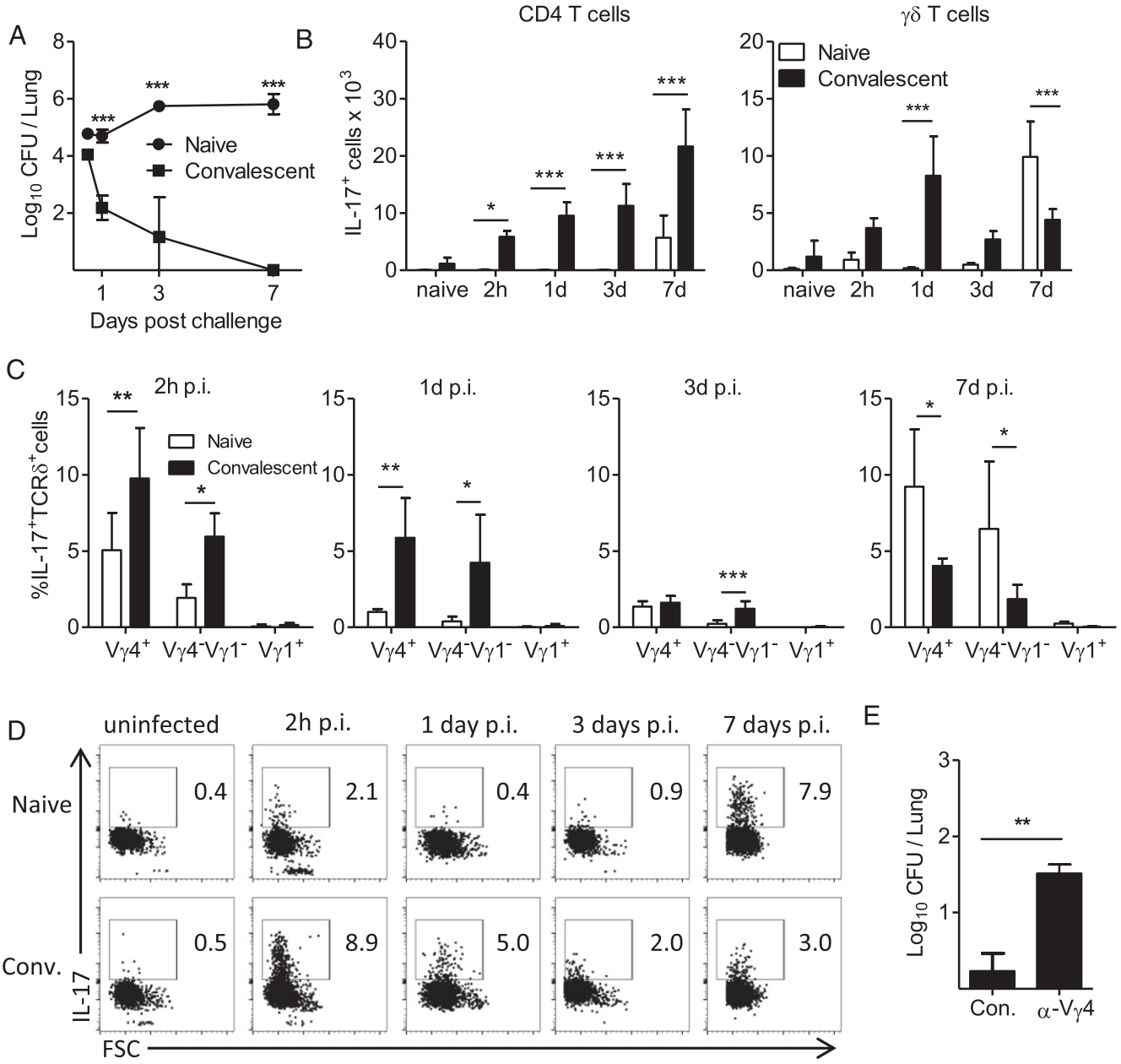

FIGURE 6. IL-17–secreting γδ T cells are activated in the lungs soon after reinfection with *B. pertussis* and contribute to rapid bacterial clearance. Naive and convalescent mice were challenged with *B. pertussis*. (**A**) Bacterial burden in the lungs was analyzed by performing CFU counts on lung homogenates. (**B**) The number of IL-17–producing γδ T cells and CD4 T cells in the lungs was quantified by ICS and FACS analysis. (**C**) The frequency of IL-17–secreting Vγ subsets in the lungs after *B. pertussis* challenge of naive and convalescent mice. (**D**) Representative FACS plots for IL-17–secreting lung γδ T cells. The results are mean ± SD ($n = 4$ mice per group at each time point) and are representative of two independent experiments. (**E**) Convalescent mice (day 63 after primary infection) were treated with anti-Vγ4 or isotype control Ab (days −3, −1 +1) and challenged with *B. pertussis*. Bacterial burden was analyzed by performing CFU counts on lungs on day 2 after infection. Results are mean ± SEM ($n = 3$ mice). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA with Bonferroni test (A–D). $^{**}p < 0.01$ by unpaired t test (E).

γδ T cells are thought to be mainly involved in the innate phase of the immune response to infection and can be activated both through the TCR or non-specifically in response to cytokines alone (33, 30). TCR engagement is thought to result predominantly in IFN-γ production by $CD27^{+}$ γδ T cells, whereas $CD27^{-}$ cells produce IL-17 in response to innate cues (33). Indeed, the findings of the current study demonstrate that IL-17–secreting γδ T cells, primarily $V\gamma4^{-}V\gamma1^{-}$ cells, are detected in the lungs as early as 2 h after infection. It is possible that these cells are Vγ6 cells, because after Vγ4 and Vγ1, Vγ6 cells are the most abundant γδ T cell population in the lungs of adult mice (34). Neutralization of IL-17 early in infection resulted in a significant, though not a dramatic, reduction in the peak bacterial load. Furthermore, $V\gamma4^{-}V\gamma1^{-}$ γδ T cells had high expression of receptors for IL-1, IL-23 and IL-18 receptor and the IL-17 transcriptional factor Rorγt and produce high concentrations of IL-17 in response to in vitro stimulation with IL-1β and IL-23. $V\gamma4^{-}V\gamma1^{-}$ γδ T cells have been found to secrete the majority of IL-17 during the early stages of bacterial infection in the peritoneal cavity and the liver (35, 36). Furthermore, it has been reported that Vγ6 cells are a key source of IL-17 in a mouse model of bacteria-induced hypersensitivity pneumonitis, where they contribute to bacterial clearance (7). A similar pattern of γδ T cell cytokine production to that seen in the current study was observed in a model *of M. tuberculosis*

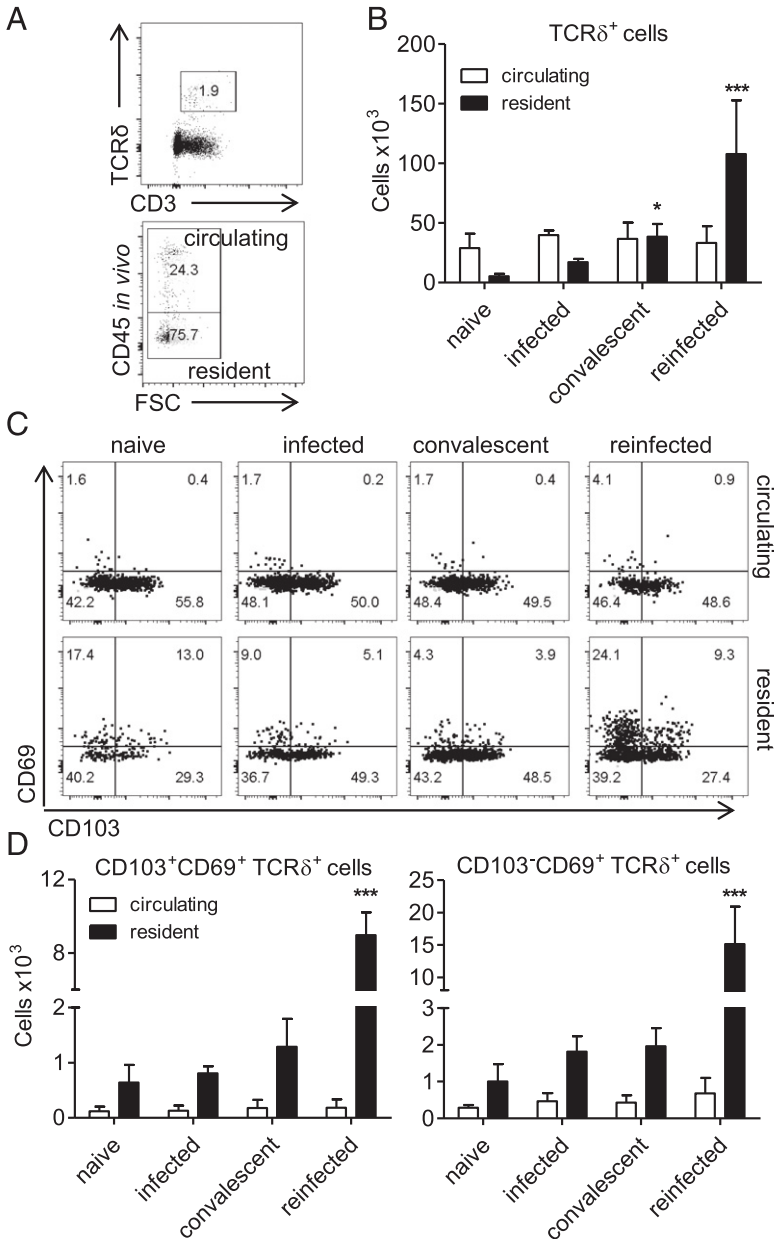

FIGURE 7. CD103$^+$CD69$^+$ and CD103$^+$CD69$^+$ γδ T_{RM} cells rapidly expand in the lungs following reinfection with *B. pertussis*. (**A**) To discriminate lung tissue resident from circulating γδ T cells, mice were injected i.v. with anti-CD45 10 min prior to sacrifice and cells were stained ex vivo with CD3 and TCRδ and FACS analysis performed. (**B**) Using the strategy described in (A), circulating and lung-resident cells were quantified in naive, infected (day 2), convalescent (day 58) or reinfected (2 d after rechallenge of mice initially infected 56 d earlier). Expression of CD103 and CD69 on resident and circulating γδ T cells was analyzed by FACS. Results are presented as sample FACS plots (**C**) and absolute numbers of circulating and resident CD103$^+$CD69$^+$ or CD103$^-$CD69$^+$ γδ T cells (**D**). Results are mean ± SD (n = 4 mice per group) and are representative of three independent experiments. $^*p < 0.05$, $^{***}p < 0.001$ by two-way ANOVA with Bonferroni test.

infection, where Vγ4 and Vγ6 γδ T cells have been shown to promote granuloma formation through production of IL-17 (37).

We have previously reported that IL-17–producing CD4 T cells (Th17 cells) play a key role in natural and vaccine-induce immunity against *B. pertussis* (28, 38). IL-17$^{-/-}$ mice had a significantly increased bacterial burden in the lungs, associated with reduced neutrophil recruitment around the peak of infection (28, 25). It has also been reported that intranasal administration of anti–IL-17 from day 3 of infection lead to a moderate increase in *B. pertussis* counts (26). In the current study, we found that neutralization of early IL-17 immediately before infection resulted in a significant reduction in the expression of antimicrobial peptides Lcn2, CRAMP, and S100a8, which inhibit bacterial growth and attachment. Lcn2, a siderophore-binding antimicrobial protein, is induced in the lungs of *Klebsiella pneumoniae*–infected mice in an IL-1β/IL-17 dependent manner and is crucial for mucosal defenses against this pathogen (39). Similarly, CRAMP, a mouse cathelicidin encoded by Camp, has been shown to play an important role in the control of Gram-negative pneumonia (40). *B. pertussis* is known to be susceptible to a number of antimicrobial peptides, including the human homolog of CRAMP, although some resistance has been observed (41, 42). Furthermore, porcine β-defensin 1 was shown to protect newborn piglets from *B. pertussis* infection (43). The current findings suggest that γδ T cells contribute to early defense against *B. pertussis* at least partially by promoting antimicrobial peptide expression.

In addition to the early IL-17–producing γδ T cells (Vγ4$^-$Vγ1$^-$) in the lungs during *B. pertussis* infection, we observed a second peak of IL-17–producing γδ T cells, primarily Vγ4 cells, in the lungs 7–14 d after challenge. These Vγ4 cells responded specifically to *B. pertussis* Ag but not to unrelated pathogens *S. aureus*, *E. coli* and *M. tuberculosis.* Furthermore, pathogen-specific γδ T cells were only detected in infected or convalescent but not in naive mice, were dependent on APC for specific pathogen activation, and were at a higher frequency in the lungs than in the draining lymph nodes. It has previously been reported that mouse γδ T cells can express CD44 and CD62L, markers of memory T cells (12, 14, 44, 45). We found that γδ T cells in the lungs of mice at the peak of

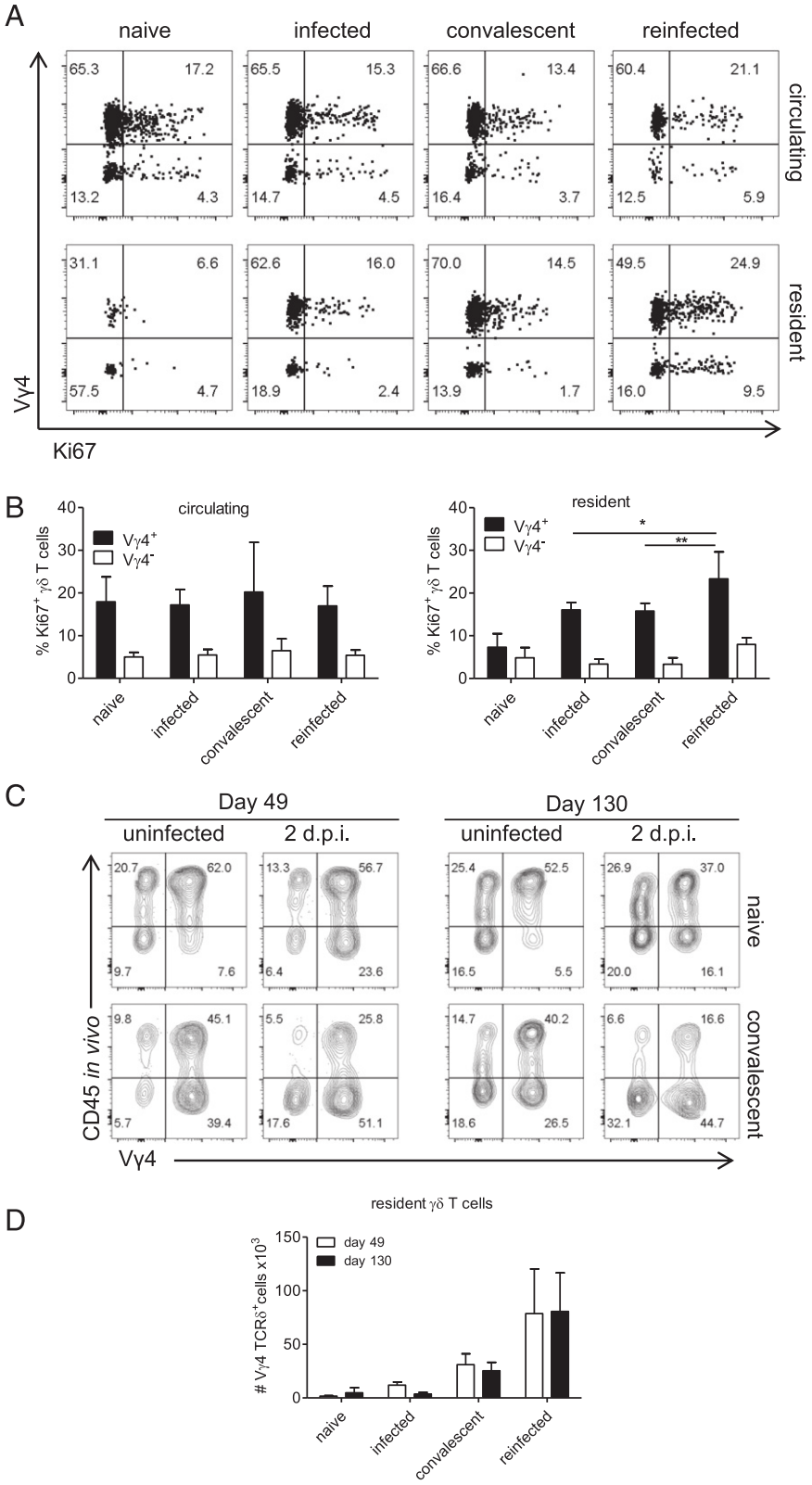

FIGURE 8. Enhanced proliferation of lung resident Vγ4 cells upon reinfection with *B. pertussis*. Naive and convalescent mice were challenged with *B. pertussis*. Mice were injected i.v. with anti-CD45 on day 2 after infection, 10 min prior to sacrifice to distinguish lung resident and circulating γδ T cells. Proliferation and frequency of γδ T cells was analyzed by FACS. (**A**) Representative FACS plots for γδ T cells in the lungs of naive, infected (day 2) convalescent (day 51) or reinfected mice (2 d after rechallenge of mice initially infected 49 d earlier). (**B**) Percentage of proliferating $V\gamma4^+$ and $V\gamma4^-$ cells among resident and circulating γδ T cells from (A). Graphs show mean ± SD (n = 4 mice) from a single experiment representative of two independent experiments, $*p < 0.05$, $**p < 0.01$ by two-way ANOVA with Bonferroni test. (**C**) Representative FACS plots for resident and circulating γδ T cells in the lungs of naive, infected (day 2), convalescent (day 51 or day 132) or reinfected mice (day 2 after rechallenge of mice initially infected 49 and 130 d earlier). (**D**) Numbers of resident $V\gamma4^+$ γδ T cells from (C). Graphs show mean ± SD (n = 4 mice) from a single experiment.

B. pertussis infection or thereafter were $CD44^+CD62L^+CD27^-$, an effector memory phenotype. This is consistent with a report showing a significant increase in the percentage of $CD27^-CD44^{hi}$ Vγ6 γδ T cells in the lamina propria of *L. monocytogenes*–infected mice (12), which persisted in the gut for at least 5 mo and exhibited a rapid recall response upon reinfection. Vγ6 γδ T cells were also shown to significantly expand in the peritoneal cavity of *S. aureus*–infected mice and to exhibit protective pathogen-specific responses upon reinfection (13). More recently, memory-like Vγ4 cells have been shown to have a pathogenic role in an imiquimod model of psoriasis during secondary challenge (46). However, the secondary responses appeared to involve innate immune training rather than classical Ag-specific memory. In another psoriasis model, $CD27^-CD44^+$ Vγ4Vδ4 γδ T cells have been shown to establish a long-lived memory in the

skin and become reactivated upon secondary challenge in a TCR-dependent fashion, although Ag specificity was not established (47).

The current study has demonstrated that memory γδ T cells reside in the lungs and expand rapidly upon secondary challenge, producing greater amounts of IL-17. Furthermore, lung-resident but not circulating γδ T cells expressed CD69 and/or CD103, markers typical for T_{RM} cells. Importantly, these γδ T_{RM} cells expanded rapidly in the lungs following a secondary infection, even when mice were rechallenged 130 d after primary infection. In contrast to our data, Sheridan et al. (12) found that $CD27^{-}CD44^{hi}$ γδ T cells in the gut lacked the expression of CD103. However, αβ T_{RM} cells can be identified as either $CD103^{+}CD69^{+}$ or $CD103^{-}CD69^{+}$ cells (48). On the other hand, memory Vγ4δ4 cells in the Aldara psoriasis model were mainly $CD103^{+}$ (47). None of these studies examined the expression of CD69 on memory γδ T cells. CD69 expression is often used as a surrogate marker for recent T cell activation and γδ T cells upregulate CD69 in a TNF-dependent manner within hours of stimulation (49). In the current study, significant upregulation of CD69 on γδ T cells was detected on day 7 after infection coinciding with the appearance of the adaptive response (data not shown). Furthermore, TNF has been shown to have a role in promoting tissue resident phenotype in αβ T cells characterized by CD103 and CD69 expression (50). Our study demonstrated sizable populations of $CD103^{+}CD69^{+}$ and $CD103^{-}CD69^{+}$ γδ T_{RM} cells in the lungs of *B. pertussis*–infected and, particularly, reinfected mice. $CD44^{+}CD69^{+}$ γδ T cells have been previously shown to expand in the lungs following *Streptococcus pneumoniae* infection and exhibit a lung-homing capacity when transferred into either naive or infected recipients, supporting the idea that CD69 expression is associated with lung-resident γδ T cells (51). Further studies are necessary to better characterize surface marker expression by T_{RM} γδ T cells. Nonetheless, the significant increase in proliferation of lung resident γδ T cells that we observed upon reinfection suggests that high numbers of γδ T_{RM} cells in the lungs following reinfection with *B. pertussis* is due to their local proliferation rather than infiltration from the circulation.

Collectively, our findings demonstrate that γδ T cells contribute to both innate and adaptive immunity to the same pathogen. During *B. pertussis* infection, γδ T cells provide a very early source of IL-17 that promotes antimicrobial peptide production, whereas Vγ4 γδ T cells are recruited to the lungs at the peak of infection and expand locally following reinfection. These pathogen-specific γδ T_{RM} cells contribute to adaptive immunity and immunological memory against *B. pertussis* and may therefore be important target for new pertussis vaccines designed to generate long-term protective immunity against *B. pertussis*.

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Disclosures

The authors have no financial conflicts of interest.

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