The Journal of Immunology

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J Immunol 2014; 192:3697-3708; Prepublished online 12 March 2014;
doi: 10.4049/jimmunol.1303420
http://www.jimmunol.org/content/192/8/3697

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Staphylococcus aureus Infection of Mice Expands a Population of Memory γδ T Cells That Are Protective against Subsequent Infection

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The development of vaccines against *Staphylococcus aureus* has consistently failed in clinical trials, likely due to inefficient induction of cellular immunity. T cell–derived IL-17 is one of the few known correlates of antistaphylococcoal immunity, conferring protection against *S. aureus* infections through its ability to promote phagocytic cell effector functions. A comprehensive understanding of the discrete T cell subsets critical for site-specific IL-17–mediated bacterial clearance will therefore be necessary to inform the development of vaccines that efficiently target cellular immunity. In this study, we have identified a population of $CD44^+CD27^-$ memory γδ T cells, expanded upon infection of C57BL/6 mice with *S. aureus*, which produce high levels of IL-17 and mediate enhanced bacterial clearance upon reinfection with the bacterium. These cells are comprised largely of the $V\gamma4^+$ subset and accumulate at the site of infection subsequent to an initial $V\gamma1.1^+$ and $V\gamma2^+$ T cell response. Moreover, these $V\gamma4^+$ T cells are retained in the peritoneum and draining mediastinal lymph nodes for a prolonged period following bacterial clearance. In contrast to its critical requirement for γδ T cell activation during the primary infection, IL-1 signaling was dispensable for activation and expansion of memory γδ T cells upon re-exposure to *S. aureus.* Our findings demonstrate that a γδ T cell memory response can be induced upon exposure to *S. aureus,* in a fashion analogous to that associated with classical αβ T cells, and suggest that induction of IL-17–expressing γδ T cells may be an important property of a protective vaccine against *S. aureus*. *The Journal of Immunology*, 2014, 192: 3697–3708.

Staphylococcus aureus is a Gram-positive coccal bacterium comprising part of the normal microbiota of a majority of the healthy human population (1–3). Upon invasive entry, *S. aureus* is capable of causing a wide range of illnesses from minor skin infections such as cellulitis, folliculitis, and impetigo to more life-threatening diseases such as endocarditis, toxic shock syndrome, bacteremia, pneumonia, and sepsis (4). The treatment of staphylococcal infections has become increasingly difficult with the emergence of antibiotic-resistant strains in healthcare-associated settings, most notably methicillin-resistant *S. aureus* (MRSA). Moreover, there is growing concern regarding the emergence of community-acquired MRSA infections in young, immunocompetent individuals outside of the healthcare system (5). Recent studies have reported the emergence of *S. aureus* strains resistant to vanocymin (6), linezolid (7), and daptomycin (8), the last viable treatment options for severe MRSA infections. It is clear that alternative approaches to standard antibiotic therapies are urgently required.

Despite showing promise in preclinical models, *S. aureus* vaccines have to date consistently failed in clinical trials. Their failure may reflect inefficient induction of cellular immunity (9, 10). Recent reports have suggested that T cells play an important protective role against *S. aureus* infections through their ability to promote phagocytic cell effector functions (11). Indeed, using novel adjuvant technology in combination with the *S. aureus* surface protein clumping factor A, we have recently demonstrated vaccine-induced protection against acute systemic *S. aureus* infection in mice in the complete absence of an Ab response (12), supporting the notion that vaccine strategies should target T cell responses. Moreover, a recent report in the field of *Bordetella pertussis* has demonstrated not only the importance of inducing cellular immunity, but also the induction of appropriate subtypes of T cells critical for optimum vaccine-induced immunity (13). Studies involving *S. aureus* infection have demonstrated that Th1/Th17 cells play a protective role in systemic infection (11), whereas γδ T cells were associated with protection in mucosal tissues (14–16) and at surgical site infections (17). A more comprehensive understanding of the specific T cell subsets critical for site-specific bacterial clearance is therefore required to inform the development of vaccines that efficiently target cellular immunity.

There is a growing literature on the importance of IL-17 in antibacterial immunity through its role in neutrophil recruitment (18–22). Patients with hyper-IgE syndrome, who have mutations in the gene encoding STAT3, leading to impaired Th17 cell function, suffer from recurrent and often severe *S. aureus* infections (23, 24). Furthermore, patients with atopic dermatitis are more susceptible to colonization by *S. aureus* (25), due in part to decreased IL-17 responses (26). These and other studies have highlighted the key role

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Received for publication December 23, 2013. Accepted for publication February 11, 2014.

This work was supported by a Wellcome Trust Research Career Development Fellowship (WT086515MA) and a Science Foundation Ireland Investigator Project Grant (12/IP/1532 to R.M.M.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: cRPMI, complete RPMI; HMBPP, hydroxymethyl-but-2-enyl-pyrophosphate; MLN, mediastinal lymph node; MOI, multiplicity of infection; MRSA, methicillin-resistant *Staphylococcus aureus*; PEC, peritoneal exudate cell; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303420

for IL-17 in antistaphylococcal immunity. Therefore, identifying the cellular sources of this cytokine will be vital in the design of novel *S. aureus* vaccines that promote protective cellular immunity.

γδ T cells have recently been identified as a potent source of innate IL-17 and implicated in host protection in murine models of *S. aureus* infection. In a cutaneous infection model, γδ T cell–deficient mice had reduced neutrophil recruitment to the infection site and impaired bacterial clearance (15). γδ T cells were also shown to have a protective role in *S. aureus*–induced pneumonia through their capacity to produce IL-17 (14). We have recently reported that γδ T cells are the dominant source of IL-17 in a surgical site infection model in which IL-17R$^{-/-}$ and TCRδ$^{-/-}$ mice had increased susceptibility to *S. aureus* infection (17). In humans, the number of circulating γδ T cells can increase dramatically upon microbial infection, in some cases reaching >50% of peripheral T cells within days of infection onset (27). Using a humanized chimeric SCID, it was demonstrated that phosphoantigen-activated human Vγ2Vδ2 cells can mediate resistance to murine *S. aureus* infection (28). Traditionally, studies investigating the induction of cellular memory and its induction by vaccines have primarily focused on αβ T cells. A recent report, however, has demonstrated memory responses by γδ T cells in a model of *Listeria* enteric infection (29).

In this study, we demonstrate for the first time, to our knowledge, that γδ T cells are the predominant source of IL-17 during *S. aureus*–induced peritonitis. Interestingly, we have identified two waves of γδ T cell infiltration into the peritoneal cavity, characterized by distinct subsets. Initially, a rapid influx of Vγ1.1 and Vγ2 cells (Garman nomenclature) was replaced later by a Vγ4-dominant response. In a novel model of recurrent peritonitis, these Vγ4$^+$ T cells were expanded for a prolonged period and responded more vigorously through IL-17 production during subsequent *S. aureus* infection, which was associated with enhanced protection. Induction of this IL-17 response by memory γδ T cells was not dependent on IL-1 signaling, in contrast to its critical requirement for IL-17 production by γδ T cells in naive mice. Furthermore, transfer of *S. aureus*–primed γδ T cells conferred protection against *S. aureus* infection in naive mice. Our findings demonstrate that a γδ T cell memory response can be induced upon exposure to *S. aureus*, in a fashion analogous to that associated with the classical αβ T cells of adaptive immunity, and suggest that induction of IL-17–expressing γδ T cells may be an important property of a protective vaccine against *S. aureus*.

Materials and Methods

Mice

Age- and sex-matched wild-type (WT) C57BL/6 and IL-1RI$^{-/-}$ (6–8 wk old) were housed under specific pathogen-free conditions at the Trinity College Dublin Bioresources facility. All mice were maintained according to European Union regulations, and experiments were performed under license from the Irish Department of Health and Children and with approval from the Trinity College Dublin Bioresources Ethics Committee.

Bacteria

S. aureus strain PS80 is a capsular polysaccharide 8–expressing strain and has been described previously (30). Staphylococci were cultivated from frozen stocks for 24 h at 37°C on Columbia agar supplemented with 2% NaCl. Bacterial suspensions were prepared in sterile PBS and adjusted to 5×10^9 CFU/ml by measuring the OD of solutions at 600 nm. CFUs were verified by plating serial dilutions of each inoculum onto tryptic soy agar.

S. aureus–*induced peritonitis*

Mice were exposed to *S. aureus* by i.p. injection of 100 μl bacterial suspension (5×10^8 CFU). The recurrent peritonitis model involved repeated exposure to *S. aureus* via i.p. injection on days 0, 7, and 14 before mice were allowed to recover for 21 d. On day 35, the previously exposed, infection-free mice received an i.p. challenge of *S. aureus* (5×10^8 CFU), in addition to a group of naive mice that had not previously been exposed to *S. aureus*. At specific time points postchallenge, mice were sacrificed and systemic infection levels and immune responses assessed. Peritoneal exudate cells (PEC) were isolated from infected mice by lavage of the peritoneal cavity with 2 ml sterile PBS. The lavage fluid was centrifuged, supernatants stored at −20°C for subsequent cytokine analysis, and PEC resuspended in complete RPMI (cRPMI; RPMI 1640: Biosera; 10% FCS: Biosera; 100 mM L-glutamine: Life Technologies; and 100 μg/ml penicillin/streptomycin: Life Technologies). The draining mediastinal lymph nodes (MLN) were isolated and disrupted over 40-μM filters to obtain single-cell suspensions. Erythrocytes were lysed using 0.87% ammonium chloride and cells washed and resuspended in cRPMI. Kidneys, liver, and spleen were homogenized in 3 ml sterile PBS. Total tissue bacterial burden was established by plating serial dilutions of peritoneal lavage or tissue homogenate on tryptic soy agar plates for 24 h at 37°C. Results are expressed as CFU per milliliter.

In vitro coculture of purified γδ T cells with S. aureus–*infected macrophages*

PEC from naive mice were isolated as described above, transferred to a 96-well flat-bottom plate (2×10^5 cells/well), and macrophages allowed to adhere for 1.5 h at 37°C and 5% CO_2 in cRPMI. The media was then aspirated and replaced with RPMI lacking antibiotics. Macrophages were infected with 2×10^6 CFU/well *S. aureus* (multiplicity of infection [MOI] 1:10) for 3 h at 37°C and 5% CO_2. After 3 h, the supernatant was aspirated and replaced with RPMI 1640 supplemented with gentamicin (100 μg/ml). Macrophages were then cocultured with 1×10^5 purified γδ T cells, isolated from naive or previously exposed mice (21 d post–final exposure [i.e., day 35]), for 24 h at 37°C and 5% CO_2. Purified γδ T cells were obtained by negative selection of CD3$^+$ T cells from the peritoneal cavity and the MLN of both naive and *S. aureus*–exposed mice using the murine Pan T Cell Isolation Kit II (Miltenyi Biotec), followed by FACS sorting of γδ T cells using Abs specific to the γδTCR (Beckman Coulter [DakoCytomation] MoFlo Cell Sorter). Some experiments were carried out in the presence or absence of anti–IL-1RI mAb (R&D Systems; 4 or 1 μg/ml) or anti–IL-23R mAb (R&D Systems; 1 μg/ml).

ELISA

ELISAs for IL-1α, IL-1β, IL-17, and IL-23 (R&D Duoset; R&D Systems) were performed on cell culture or peritoneal supernatants, as per the manufacturer's instructions. IL-18 ELISAs were performed with anti–IL-18 Abs and IL-18 protein purchased from MBL International.

Flow cytometry

PEC and MLN cells were incubated in the presence of brefeldin A (Sigma-Aldrich) for 4 h at 37°C and 5% CO_2 to block cytokine secretion. Cells were then incubated with Fcγ block (1 μg/ml) on ice before surface staining with fluorochrome-conjugated Abs against CD3 (BD Biosciences; clone 500A2), γδTCR (BioLegend; clone GL3), IL-1R (BioLegend; clone JAMA147), Vγ1.1 (BioLegend; clone 2.11), Vγ2 (BD Biosciences; clone UC3-10A6), and Vγ3 (BD Biosciences; clone 536). Garman nomenclature is used throughout this study (31). Cells were fixed and permeabilized using the DakoCytomation Intrastain Kit, before intracellular staining with a fluorochrome-conjugated Abs against IL-17A (eBioscience; clone 17B7) and IFN-γ (eBioscience; clone XMG1.2). Flow cytometric data were acquired with a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star). Gates are set on respective fluorescence-minus-ones.

Amplication of the Vγ4 gene in purified Vγ1.1$^-$Vγ2$^-$ cells

Purified subsets of γδ T cells were obtained by negative selection of CD3$^+$ T cells isolated from the peritoneal cavity and the MLN of mice 3 h post–*S. aureus* infection using the murine Pan T Cell Isolation Kit II (Miltenyi Biotec), followed by FACS of Vγ1.1$^+$, Vγ2$^+$, and Vγ3$^+$ γδ T cells using Abs specific for each cell subset (Beckman Coulter [DakoCytomation] MoFlo Cell Sorter). RNA was extracted from purified cells using the TRIzol/chloroform method (Invitrogen) and reverse transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Applied Biosystems), per the manufacturer's instructions. Real-time PCR was performed on a CFX96 Touch Real-Time PCR detection system (Bio-Rad) using the following primer pairs: Vγ1.1, forward 5′-TTCTG-CTGCCTCTGGGTTTTT-3′ and reverse 5′- TCCCTCCTAAGGGTCGT-TGAT-3′; Vγ2, forward 5′-TTGGTACCGGCAAAAAACAAATCA-3′ and

reverse 5′-CAATACACCCTTATGACATCG-3′; Vγ3, forward 5′-TTGCAAGCTCTCTGGGGTTC-3′ and reverse 5′-GGCACAGTAGTACGTGGCTT-3′; Vγ4, forward 5′-GGAAGCAGTCTCACGTCACC-3′ and reverse 5′-CTGCCATGTCCTTGCCTCATA-3′; Vγ5, forward 5′-GATCCAACTTCGTCAGTTCCACAAC-3′ and reverse 5′-AAGGAGACAAAGGTAGGTCCCAGC-3′, and; 18S, forward 5′-CCTGCGGCTTAATTTGACTC-3′ and reverse 5′-AACTAAGAACGGCCATGCAC-3′. The presence of amplified target DNA was determined by 1.5% agarose gel electrophoresis. Target DNA was extracted (High Pure PCR Product Purification Kit; Roche) and sequenced (Source Biosciences, Dublin, Ireland). Sequence alignment to the National Center for Biotechnology Information gene (NG_007033.1) was carried out using ClustalW software (University College Dublin).

Adoptive transfer of S. aureus*–primed γδ T cells*

Purified γδ T cells were obtained by negative selection of $CD3^+$ T cells from the peritoneal cavity of *S. aureus*–exposed mice on day 35 (i.e., 21 d after the final exposure to *S. aureus*) using the murine Pan T Cell Isolation Kit II (Miltenyi Biotec), followed by FACS of γδ T cells using Abs specific to the γδTCR (Beckman Coulter [DakoCytomation] MoFlo Cell Sorter). $CD3^+\gamma\delta^+$ or $CD3^+\gamma\delta^-$ T cells were injected i.p. to naive mice (1×10^5 cells/mouse). At 3 h posttransfer, mice received an i.p. injection of *S. aureus* (5×10^8 CFU). At 72 h postinfection, the peritoneum was lavaged and the kidneys, spleens, and liver isolated to determine bacterial burden as previously described.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad) statistical analysis software. Differences between groups were analyzed by unpaired Student *t* test or a one-way ANOVA with Tukey posttest comparison where indicated. A p value <0.05 was considered significant.

Results

γδ T cells are the major source of IL-17 during S. aureus*–induced peritonitis*

It is widely accepted that IL-17 plays a protective role during *S. aureus* infections (15, 17, 23, 24, 32). An identification of the source of IL-17 at different sites of infection is crucial for the generation of vaccines that will induce protective cellular immunity. Hence, we investigated IL-17 expression in a systemic *S. aureus* infection model induced as a consequence of peritonitis, in which mice received a single i.p. injection of *S. aureus* (5×10^8 CFU) or sterile PBS as a control. IL-17A and IL-1β were rapidly produced upon infection, as measured by ELISA on peritoneal lavage fluid at multiple time points following infection (Fig. 1A). Both IL-17A and IL-1β were maximal at 3 h and had decreased to the levels observed in mice administered PBS by 24 h postinfection. IL-1α, IL-18, or IL-17F were not detected in the peritoneal fluid of infected mice (data not shown). IL-17A will therefore be referred to as IL-17 throughout the remainder of this report.

Intracellular cytokine staining of PEC at 3 h postinfection and cultured with brefeldin A, but without PMA and ionomycin stimulation, revealed that IL-17 was exclusively produced by $CD3^+$ T cells. Moreover, $\gamma\delta^+$ T cells were the primary source of IL-17, whereas few $CD4^+$ and $CD8^+$ T cells expressed IL-17 at this stage (Fig. 1B). IL-17–producing T cells were also detected in the draining MLN during *S. aureus* infection. Again, γδ T cells comprised the major source of IL-17, although significantly fewer cells were cytokine positive in the lymphoid tissues (Fig. 1C). Peritoneal and MLN γδ T cells from PBS-injected mice, cultured with brefeldin A, did not produce IL-17 (Fig. 1B–E). In addition, we did not detect any IFN-γ production by γδ T cells isolated from *S. aureus*–infected mice (Supplemental Fig. 1A).

The frequency of IL-17–producing γδ T cells was significantly elevated in the peritoneal cavity of S. *aureus*–infected mice as early as 1 h postinfection, compared with naive mice (time 0), and continued to increase up to 3 h postinfection (Fig. 1D). By 24 h postinfection, peritoneal γδ T cells had ceased to produce IL-17. In some mice, a second wave of IL-17–producing γδ T cells appeared in the peritoneal cavity by 72 h postinfection (Fig. 1D, Supplemental Fig. 1B), which had subsided by 5 d postinfection (data not shown). This second phase of $IL\text{-}17^+$ γδ T cell accumulation was not detected in the MLN, in which the frequency of these cells was comparable to the PBS-treated controls by 12 h postinfection (Fig. 1E). This biphasic course of IL-17 production may represent the recruitment of an alternative subset of γδ T cells at this stage of the infection or the expansion of a γδ T cell subset resident within the peritoneal cavity.

We and others have previously reported that IL-17 production by γδ T cells during *S. aureus* infection is IL-1β dependent (17, 33). To confirm a role for IL-1 signaling in regulating IL-17 production by γδ T cells in the peritoneal cavity, WT and $IL\text{-}1RI^{-/-}$ mice were infected with *S. aureus* (5×10^8 CFU) via a single i.p. injection. At 3 h postinfection, PEC were isolated and IL-17 production by γδ T cells assessed. The total number of γδ T cells recruited to the peritoneal cavity was comparable in both *S. aureus*–infected WT and $IL\text{-}1RI^{-/-}$ mice (Supplemental Fig. 1C). However, IL-17 production by these γδ T cells was abrogated in the $IL\text{-}1RI^{-/-}$ mice (Fig. 1F, Supplemental Fig. 1D), demonstrating a critical role for IL-1 signaling in the early induction of IL-17 by γδ T cells during *S. aureus*–induced peritonitis. The concentrations of secreted IL-23, another cytokine reported to drive innate production of IL-17 by γδ T cells (34), were generally low following *S. aureus* challenge and did not significantly differ between WT and $IL\text{-}1RI^{-/-}$ mice (data not shown).

Differential recruitment of γδ T cell subsets over the course of peritoneal infection

Individual γδ T cell subsets are often associated with tissue-specific homing and functions (35). To characterize the γδ T cell subsets responsible for IL-17 production during *S. aureus*–induced peritonitis, mice were infected with *S. aureus* (5×10^8 CFU) via i.p. injection and PEC and MLN cells isolated at various time points postinfection. Flow cytometric analysis of PEC 1 h post–*S. aureus* infection revealed that the γδ T cell population consisted primarily of $V\gamma1.1^+$ T cells and $V\gamma2^+$ T cells (Fig. 2A, 2B). By 3 h postinfection, a population of $V\gamma1.1^-V\gamma2^-$ cells had also accumulated in the peritoneal cavity. $V\gamma3^+$ cells were almost undetectable in the peritoneal cavity by flow cytometry (0.6 ± 0.2 $V\gamma3^+\gamma\delta^+$). Based on previous studies, therefore, we hypothesized that peritoneal $V\gamma1.1^-V\gamma2^-$ cells were in fact Vγ4 cells, for which no fluorochrome-conjugated Ab is commercially available. To confirm this, we PCR-amplified cDNA from FACS-sorted $V\gamma1.1^-V\gamma2^-$ PEC and MLN cells. Gel electrophoresis of the amplimers indicates that the $V\gamma1.1^-V\gamma2^-$ cells were indeed $V\gamma4^+$ cells (Supplemental Fig. 1E). Furthermore, the translated sequence of the amplified DNA, excised from the gel, aligned with the Vγ4 protein sequence confirming that the $V\gamma1.1^-V\gamma2^-$ cells were $V\gamma4^+$ cells (Supplemental Fig. 1F). These cells are therefore referred to as Vγ4 cells in the figure and throughout the remainder of this study.

At 72 h postinfection, the frequency of $V\gamma4^+$ cells was significantly increased, whereas the frequency of $V\gamma2^+$ cells had significantly decreased in the peritoneal cavity (Fig. 2B). The frequency of $V\gamma1.1^+$ T cells as a proportion of total γδ T cells did not change over the course of acute infection; however, there was a significant increase in the absolute numbers of $V\gamma1.1^+$ and $V\gamma4^+$ T cells at 72 h postinfection (Fig. 2B). $V\gamma3^+$ T cells were not detected in either the peritoneal cavity or the MLN of infected mice (data not shown). These data demonstrate that during the course of *S. aureus* infection in the peritoneal cavity, there is

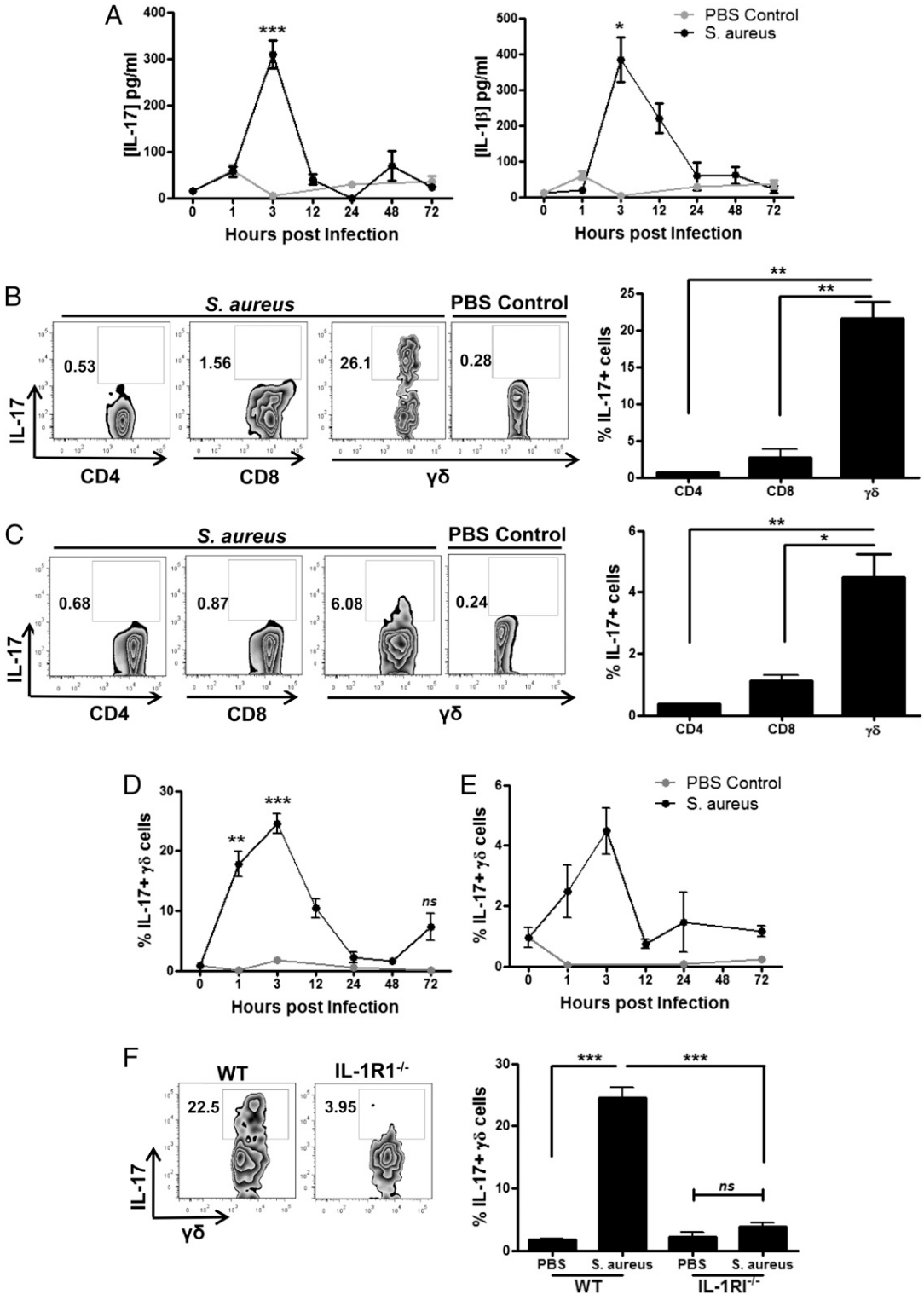

FIGURE 1. γδ T cells are the primary source of IL-17 during *S. aureus*–induced peritonitis. Mice were infected with *S. aureus* (5×10^8 CFU) via i.p. injection. At the indicated times postinfection, the peritoneal cavity was lavaged and the MLN collected. Secreted IL-17A and IL-1β in the peritoneal fluid was measured by ELISA (**A**). Three hours postinfection (**B**, **C**) and at the indicated time points (**D**, **E**), PEC (B, D) and MLN cells (C, E), cultured with brefeldin A but not PMA and ionomycin, were stained for surface CD3, CD4, and CD8, γδTCR, and intracellular IL-17 and analyzed by flow cytometry. WT and IL-1RI$^{-/-}$ mice were infected with *S. aureus* (5×10^8 CFU) via i.p. injection and at 3 h postinfection PEC, cultured with brefeldin A but not PMA and ionomycin, stained for surface CD3 and γδTCR and intracellular IL-17 and analyzed by flow cytometry (**F**). Results expressed as mean ± SEM of $n = 10$ mice/group, with representative FACS plots. Data are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

a shift in the dominant γδ subsets present. Recruitment of Vγ4$^+$ T cells appeared to be specific to the site of infection, as only the Vγ1.1$^+$ population was expanded in the MLN at 72 h postinfection (Fig. 2C).

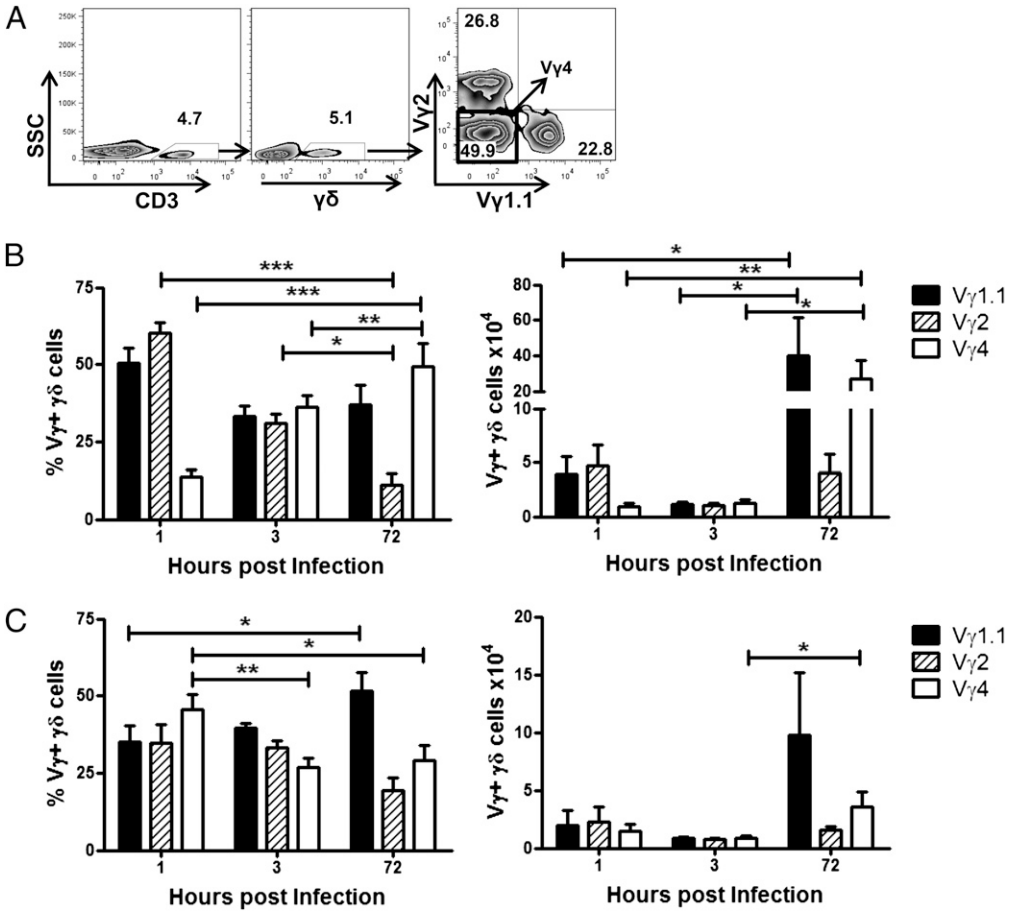

FIGURE 2. Differential recruitment of γδ T cell subsets over the course of peritoneal infection. Mice were infected with *S. aureus* (5×10^8 CFU) via i.p. injection. PEC (**A**, **B**) and MLN cells (**C**) were harvested from mice at the indicated times postinfection, surface stained with fluorochrome-conjugated Abs against CD3, γδTCR, Vγ1.1, Vγ2, and Vγ3, and analyzed by flow cytometry. (A) FACS plots are representative of PEC at 3 h postinfection. (B and C) Results expressed as mean frequency ± SEM of each subset within the $\gamma\delta^+$ gate of $n = 9$–12 mice/group. Data are representative of two to three independent experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

Prior exposure to S. aureus *preferentially expands a population of Vγ4$^+$ T cells capable of enhanced IL-17 production during subsequent infection*

Our results demonstrate that during the course of *S. aureus* infection, there is a shift in the composition of γδ T-cells from primarily Vγ1.1$^+$ and Vγ2$^+$ cells in the acute phase of infection to Vγ1.1$^+$ and Vγ4$^+$ cells during later stages of infection. The Vγ4$^+$ cells may represent a primed γδ subset capable of responding to subsequent *S. aureus* infection. To test this hypothesis, groups of mice were repeatedly exposed to *S. aureus* (5×10^8 CFU i.p. on days 0, 7 and 14) and allowed to clear the infection (<1 log CFU/ml in peritoneal cavity, 21 d post–final exposure). At this stage, flow cytometric analysis revealed the persistence of a significantly higher number of $\gamma\delta^+$ T cells, as well as their frequency among total CD3$^+$ T cells, in the peritoneal cavity of previously exposed mice, compared with naive mice (Fig. 3A). A small but significant increase in both the frequency and absolute number of $\gamma\delta^+$ T cells was also observed in MLN (Fig. 3B). The total number of CD3$^+$ cells did not differ significantly between naive and prior exposed mice at this point (PEC: 0.21 ± 0.04 versus 0.34 ± 0.05 × 10^6; MLN: 1.55 ± 0.06 versus 1.6 ± 0.05 × 10^6 for naive versus previously exposed mice, respectively). Further analysis showed that the composition of the γδ T cell population observed 72 h postinfection in the acute model (Fig. 2B) was maintained with a predominantly Vγ4$^+$ T cell profile found in previously exposed, but currently uninfected, mice (Fig. 3C). Prior exposure to *S. aureus* did not significantly affect the frequency of Vγ1.1$^+$ cells but did result in a significant decrease in the proportions of Vγ2$^+$ cells. Interestingly, a predominance of Vγ4$^+$ T cells was also observed in the MLN at this stage (Fig. 3D). Crucially, in the absence of PMA and ionomycin stimulation, γδ T cells from previously exposed mice did not produce IL-17 (Fig. 3E), and secreted levels of IL-17 protein were undetectable in the peritoneal lavage fluid (data not shown).

Upon recovery (21 d post–final exposure) mice were rechallenged by i.p. injection of *S. aureus* (5×10^8 CFU), as were a group of naive mice as a control. At specific time points postchallenge, bacterial burden was assessed and PEC and MLN cells isolated to characterize the subsets of γδ T cells responsible for IL-17 production. Bacterial clearance from the peritoneal cavity and peripheral organs was significantly greater in mice previously exposed to *S. aureus* compared with naive mice (Fig. 4A, Supplemental Fig. 2A). Enhanced bacterial clearance was associated with increased IL-17 secretion in the peritoneal cavity of previously exposed mice 1 and 3 h postchallenge (Fig. 4B). Notably, prior exposure to *S. aureus* did not induce the expansion of CD4$^+$IL-17$^+$ (Th17) or CD8$^+$IL-17$^+$ cells in either the peritoneum or the MLN (Fig. 4C, Supplemental Fig. 2B). Conversely, elevated peritoneal IL-17 was associated with a significant increase in the both the frequency and total number of IL-17–producing γδ T cells in the peritoneal cavities of previously exposed mice, compared with naive mice (Fig. 5A, 5B). This effect was not strain specific

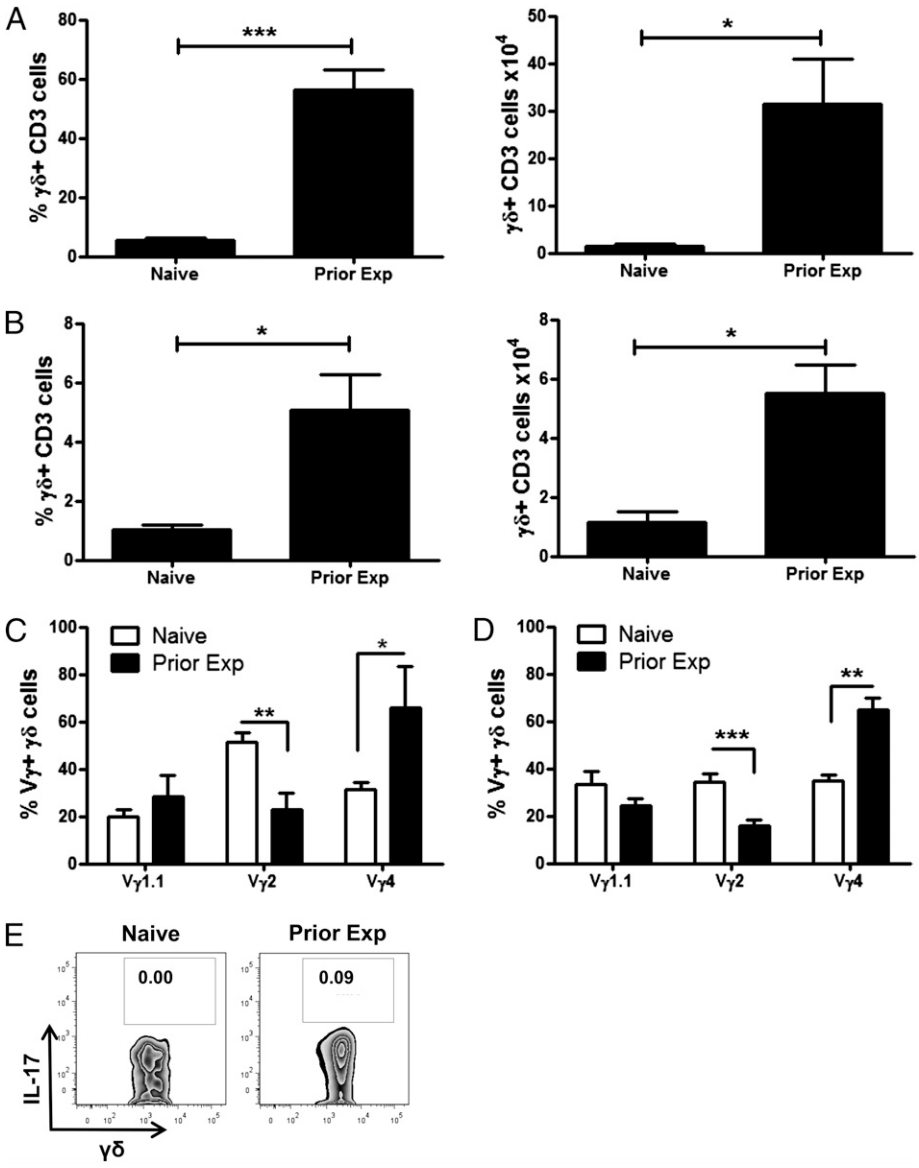

FIGURE 3. Elevated frequency of $V\gamma4^+$ cells in mice previously exposed to *S. aureus.* Groups of mice were exposed to *S. aureus* (5×10^8 CFU) via i.p. injections on days 0, 7, and 14. Following recovery on day 35, isolated PEC and MLN cells were cultured with brefeldin A, but not PMA and ionomycin, stained for surface CD3, γδTCR, Vγ1.1, Vγ2, and Vγ3 and intracellular IL-17, and analyzed by flow cytometry. γδ T cell frequencies among peritoneal (**A**) and MLN $CD3^+$ T cells (**B**) and total numbers are shown. The Vγ subsets comprising the γδ T cell population in the peritoneal cavity (**C**) and MLN (**D**) at this stage were examined. IL-17 expression by γδ T cells from naive or previously exposed mice was compared (**E**). Results expressed as mean ± SEM of $n = 8$ mice/group. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Exp, exposure.

because in mice recurrently infected and subsequently rechallenged with an alternative *S. aureus* strain, SH1000 (36, 37), an increase in IL-17 production by γδ T cells in the peritoneal cavity was also observed (11.1 ± 1.1 versus 31.7 ± 4.7% $IL\text{-}17^+\gamma\delta^+$ cells respectively; $n = 5$/group). Analysis of the individual γδ T cell subsets revealed an increase in IL-17 expression by all subsets at both 1 and 3 h postinfection, particularly IL-17 production by the $V\gamma4^+$ population (Fig. 5C, Supplemental Fig. 2C). A similar increase in IL-17 expression by γδ T cells was observed in the MLN of previously exposed mice (Supplemental Fig. 3A, 3B), including a significant increase in the number of $IL\text{-}17^+V\gamma4^+$ T cells, 3 h postinfection (Supplemental Fig. 3C). A small but significant increase in the frequency and number of IL-17–producing γδ T cells was also detected in the spleens of previously exposed mice 3 h postinfection (Supplemental Fig. 3D).

Expression of the TNFR family molecule CD27 has been reported to distinguish IFN-γ–producing ($CD27^+$) from IL-17–producing ($CD27^-$) γδ T cells (38). CD27 expression was initially thought to differentiate Ag-experienced ($CD27^+$) γδ T cells from Ag-naive γδ T cells (39, 40). However, a recent study identified a population of memory γδ T cells that expressed high levels of the effector memory marker CD44 and yet were $CD27^-$ and produced high levels of IL-17 (29). In the current study, IL-17–producing γδ T cells present in the peritoneal cavity of S. *aureus*–infected naive and previously exposed mice were also found to express a $CD27^-CD44^{hi}$ phenotype (Fig. 5C, 5D). Taken together, these results suggest that exposure to *S. aureus* results in the generation of a population of primed γδ T cells, predominantly of the Vγ4 subset, that are capable of enhanced IL-17 production and associated bacterial clearance upon subsequent infection with the organism.

Enhanced IL-17 expression by γδ T cells upon secondary exposure to S. aureus *is not a result of increased innate cytokine signaling*

During S. *aureus* infection in naive mice, IL-17 production by peritoneal γδ T cells was critically dependent upon IL-1 signaling (Fig. 1A, 1F). Hence, we investigated the effects of prior exposure to *S. aureus* on innate cytokine production in the peritoneal cavity. Despite significantly enhanced IL-17 secretion upon reinfection of previously exposed mice, compared with infection of naive mice (Fig. 4B), no difference in IL-1β or IL-23 secretion was detected (Supplemental Fig. 4). IL-1α and IL-18, which can also stimulate IL-17 production by γδ T cells (41), were undetectable in the

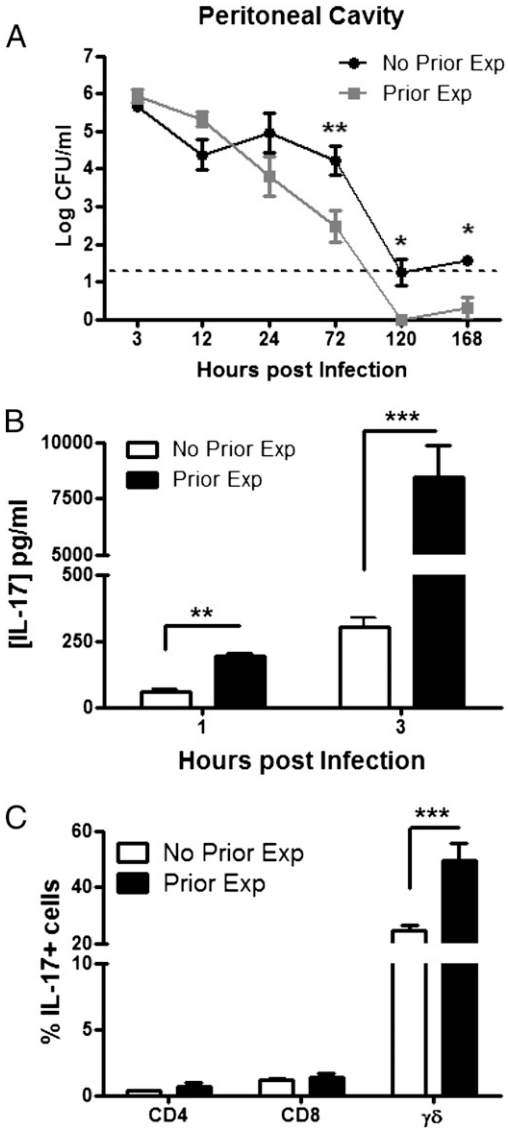

FIGURE 4. Prior exposure (Exp) to *S. aureus* results in protection against subsequent infection and enhanced IL-17 responses. Groups of mice were exposed to *S. aureus* (5×10^8 CFU) via i.p. injections on days 0, 7, and 14. Mice were then rechallenged with *S. aureus* (5×10^8 CFU) on day 35, as were a group of naive controls. At the indicated times postchallenge, the bacterial burden in the peritoneum was assessed (**A**). Results expressed as log CFU/ml of lavage fluid collected from $n = 5$ mice/group/time point. At 1 and 3 h postchallenge, secreted IL-17 in the peritoneal fluid was measured by ELISA (**B**). At 3 h postinfection, isolated PEC were cultured with brefeldin A, but not PMA and ionomycin, stained for surface CD3, CD4, CD8, and γδTCR and intracellular IL-17, and analyzed by flow cytometry (**C**). Results expressed as mean ± SEM of $n =$ 12 mice/group. Data are representative of three independent experiments. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

peritoneal cavities of either naive or prior exposed mice following *S. aureus* infection (data not shown). Microbial colonization has been shown to increase the expression of IL-1RI on the surface of IL-17–producing γδ T cells, leading to increased IL-17 production in response to IL-1β signaling (42). However, we did not detect any difference in the mean fluorescence intensity of IL-1RI expression on peritoneal IL-17$^+$ γδ T cells between previously exposed and naive mice at 1 and 3 h postchallenge (Fig. 6A). A similar pattern was observed in the MLN (data not shown). These findings suggest an alternative mechanism for the enhanced IL-17 expression by *S. aureus*–primed γδ T cells compared with the IL-1–dependent expression observed during acute infection.

To further investigate the mechanisms responsible for enhanced IL-17 production by γδ T cells following prior exposure to *S. aureus*, we cultured γδ T cells from naive or previously exposed mice that were infection free (21 d post–final exposure to *S. aureus*) with peritoneal macrophages infected with *S. aureus* (MOI 1:10). Following 18 h of culture, IL-17 expression by γδ T cells was assessed by flow cytometric analysis and IL-17 secretion into the supernatants measured by ELISA. When compared with γδ T cells from naive mice, γδ T cells isolated from mice previously exposed to *S. aureus* produced significantly more IL-17 when cocultured with *S. aureus*–infected macrophages (Fig. 6B, 6C). *S. aureus*–primed γδ T cells appeared to be exclusively IL-17 producing, as no IFN-γ production was detected in the culture supernatants (data not shown). Moreover, macrophages stimulated with *S. aureus* alone, in the absence of γδ T cells, did not produce any IL-17 (data not shown). To determine the role of innate cytokines in the secondary response of *S. aureus*–primed γδ T cells, blocking Abs to both IL-1RI and the IL-23R were added to the cocultures. Blockade of IL-1RI at the higher concentration of Ab (4 μg/ml) resulted in complete abrogation of IL-17 secretion by γδ T cells from naive mice (Fig. 6B, 6C). Conversely, blockade of IL-1RI, using either concentration of Ab, only partially reduced IL-17 production by previously exposed γδ T cells, and this reduction was not significant (Fig. 6B, 6C). IL-23R blockade had no effect on IL-17 production by either naive or prior-exposed γδ T cells (Fig. 6B). Taken together, our in vitro and in vivo data demonstrate differential requirements for innate IL-1 signaling in the IL-17–mediated response to *S. aureus* infection by primed γδ T cells and those from naive mice.

Adoptive transfer of S. aureus*–primed γδ T cells protects against subsequent infection*

Our results demonstrate an accumulation of Vγ4 T cells in the peritoneal cavity as a result of *S. aureus* exposure. Upon reexposure to the bacterium, these Vγ4 cells contribute substantially to the augmented IL-17 response observed in previously exposed mice, which was associated with increased bacterial clearance (Fig. 4A, Supplemental Fig. 2). To confirm a direct role for primed γδ T cells in protection against *S. aureus* infection, we transferred 1×10^5 *S. aureus*–primed γδ T cells, purified from mice previously exposed to *S. aureus*, 21 d following the final exposure to *S. aureus* (i.e., day 35), to naive syngeneic hosts. Prior to transfer, purified *S. aureus*–exposed γδ T cells primarily consisted of the Vγ4 subset (>90% Vγ4$^+$; data not shown). A total of 1×10^5 γδ T cell–depleted CD3$^+$ T cells (i.e., CD3$^+$γδ$^-$ cells) isolated from the same mice previously exposed to *S. aureus* were transferred to a separate group of naive mice as a control. Three hours posttransfer, mice were challenged by i.p. injection of *S. aureus* (5×10^8 CFU). At 72 h postinfection, the bacterial burden at the local site of infection was assessed, as well as dissemination of the bacteria to peripheral sites.

Transfer of *S. aureus*–primed γδ T cells significantly reduced bacterial burden in the peritoneal cavity (the site of infection), compared with mice that received primed CD3$^+$ T cells depleted of γδ T cells (Fig. 7). Dissemination of bacteria to the kidneys and liver was also reduced in recipients of *S. aureus*–primed γδ T cells, compared with controls. These results demonstrate an important protective role for *S. aureus*–primed γδ T cells, specifically Vγ4$^+$ cells, which display enhanced IL-17 production upon subsequent exposure to the bacterium.

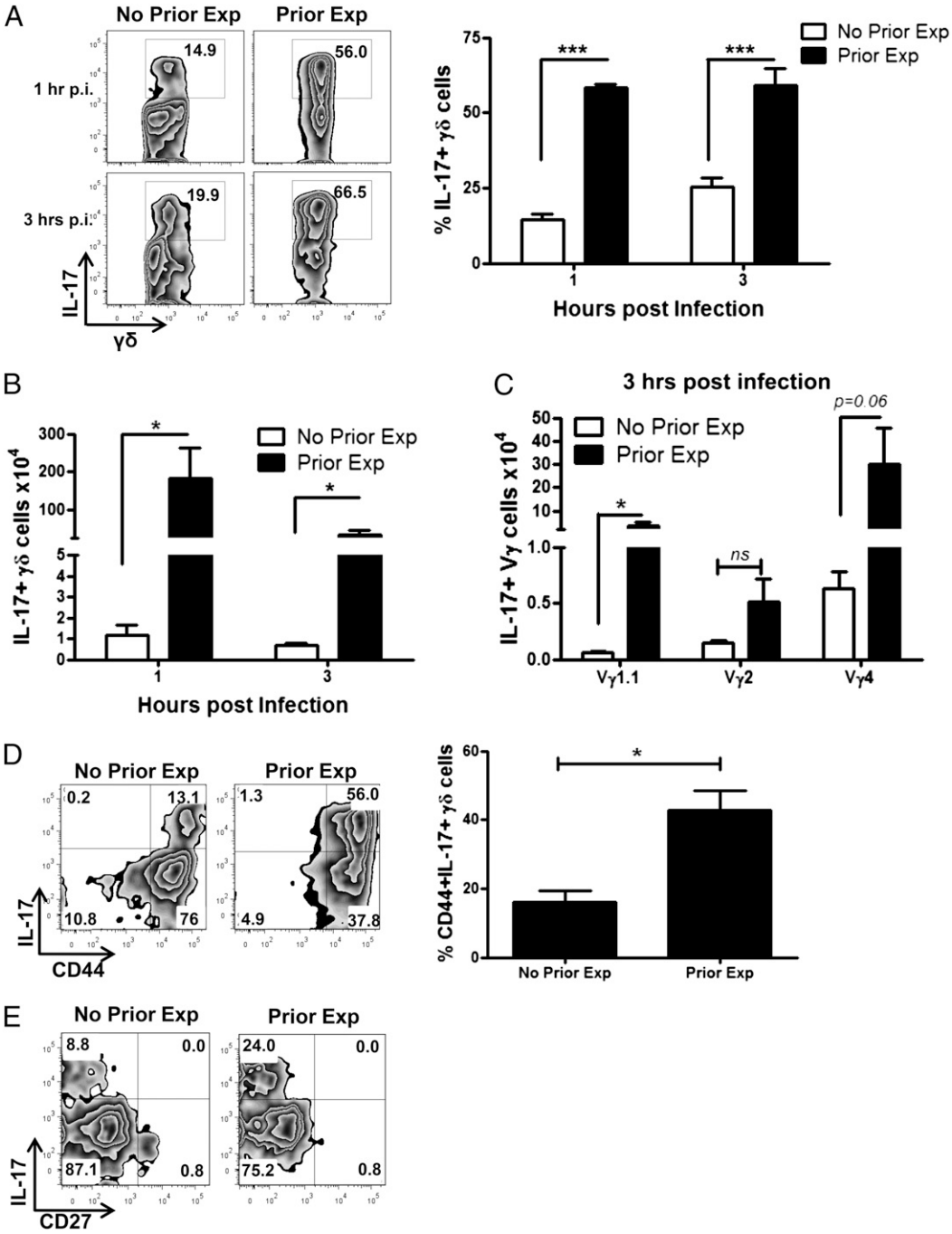

FIGURE 5. Prior exposure (Exp) to *S. aureus* results in an enhanced IL-17 response by predominantly Vγ4$^+$ γδ T cells upon reinfection. Groups of mice were exposed to *S. aureus* (5×10^8 CFU) via i.p. injections on days 0, 7, and 14. Mice were allowed to recover for 21 d before being rechallenged with *S. aureus* (5×10^8 CFU) on day 35, as were a group of naive controls. At 1 and 3 h postinfection, PEC were cultured with brefeldin A, but not PMA and ionomycin, stained for surface CD3, γδTCR, and intracellular IL-17, and analyzed by flow cytometry (**A**, **B**). IL-17 expression by individual Vγ subsets in the peritoneum was also assessed at 3 h postchallenge (**C**). The proportions of total γδ T cells expressing IL-17$^+$ and CD44 (**D**) or CD27 (**E**) were examined. Results expressed as mean ± SEM of n = 9–12 mice/group, with representative FACS plots. Data represent two to three independent experiments. $^*p < 0.05$, $^{***}p < 0.001$.

Discussion

Our study demonstrates for the first time, to our knowledge, that exposure to *S. aureus* can prime a subset of IL-17–producing γδ T cells that are capable of protecting against a subsequent staphylococcal infection. Previous studies have established that IL-17 plays an important role in immune protection against *S. aureus* infection both in humans (23, 24) and animal models (15, 32), with γδ T cells identified as the primary source of innate IL-17 in models of acute *S. aureus*–induced pneumonia, cutaneous infection, and surgical site infection (14, 15, 17). In this study, we have identified a population of *S. aureus*–primed γδ T cells that exhibit a more rapid expansion and clearance of infection than in the primary response and, in a manner similar to the memory response of conventional αβ T cells, are capable of conferring protection against S. *aureus* upon transfer to naive mice. Furthermore, we demonstrate the redundancy of IL-1 signaling in the IL-17–mediated secondary response of memory γδ T cells to S. *aureus* infection, compared with its critical requirement by γδ T cells in

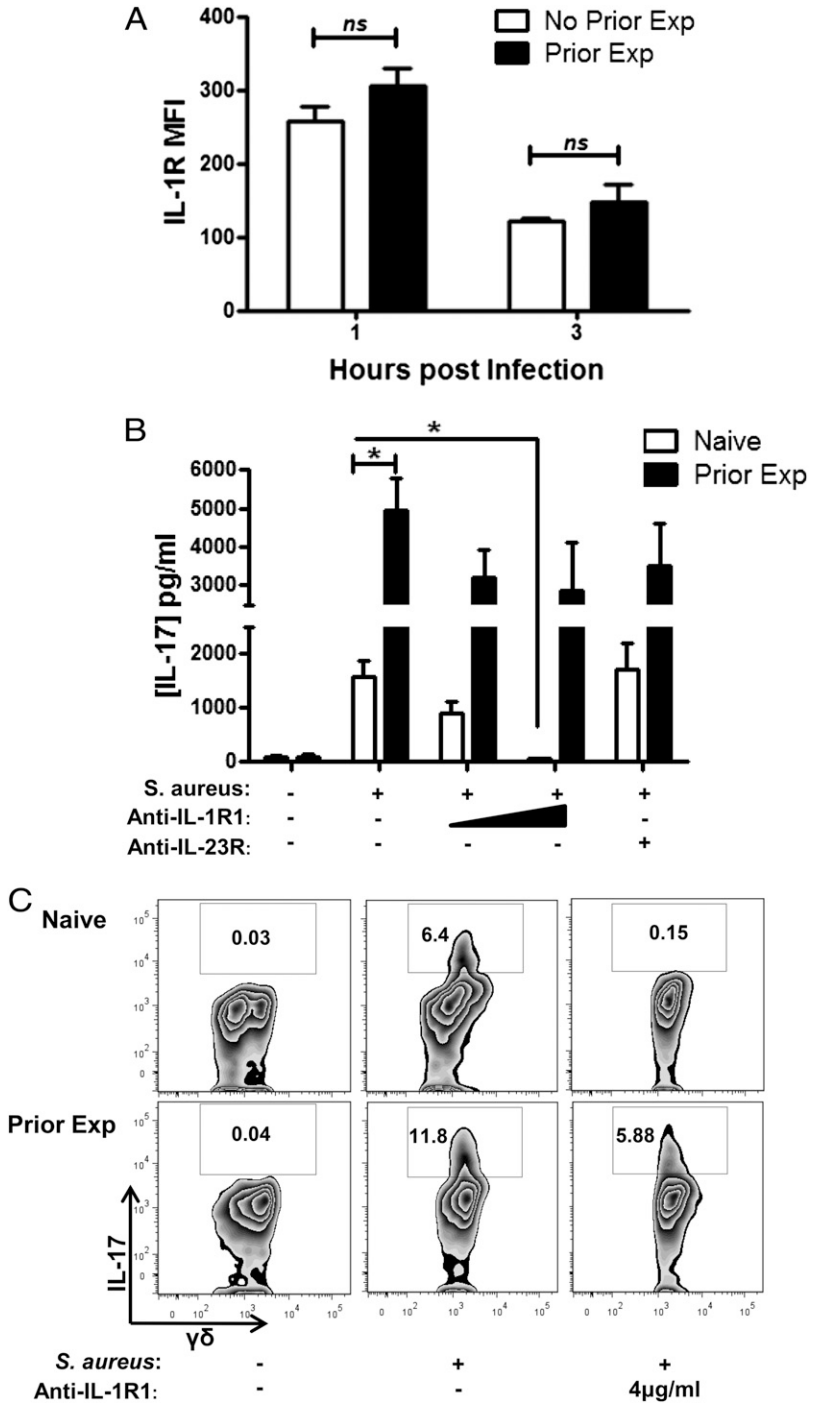

FIGURE 6. IL-1R or IL-23R signaling is dispensable for the secondary IL-17 response of primed γδ T cells in vitro. Groups of mice were exposed to *S. aureus* (5×10^8 CFU) via i.p. injections on days 0, 7, and 14. Mice were allowed to recover for 21 d before being rechallenged with *S. aureus* (5×10^8 CFU) on day 35, as were a control group of naive mice. PEC were cultured with brefeldin A, but not PMA and ionomycin, stained for surface CD3, γδTCR, and IL-1RI expression and intracellular IL-17, and analyzed by flow cytometry (**A**). Results expressed as mean ± SEM for $n = 9$ mice/group. Data are representative of two independent experiments. γδ T cells purified (>98% pure) from previously exposed or naive mice were cocultured overnight with *S. aureus*–infected macrophages (MOI 1:10), in the presence or absence of mAbs to IL-1RI (4 or 1 μg/ml) or IL-23R (1 μg/ml). Uninfected macrophages were used as a control. IL-17 concentration in supernatants was quantified by ELISA (**B**). Results expressed as mean ± SEM for $n = 4$ to 5 replicates/group. Cells were incubated with brefeldin A, but not PMA and ionomycin, stained for surface CD3 and γδTCR and intracellular IL-17, and analyzed by flow cytometry (**C**). Data are representative of four to five independent experiments. $^*p < 0.05$. MFI, mean fluorescence intensity.

naive mice. These findings reveal that γδ T cells are an important source of IL-17 in adaptive immunity to *S. aureus* and indicate that targeting the induction of nontraditional lymphocytes such as specific subsets of γδ T cells that secrete IL-17, one of the few known correlates of antistaphyloccoal immunity, could significantly benefit future anti–*S. aureus* vaccine design.

Employing a model of *S. aureus*–induced peritonitis, we demonstrate the rapid recruitment of IL-17–producing γδ T cells were to the peritoneal cavity, accumulating in significant numbers as early as 1 h postinfection. IL-17–producing γδ T cells were also detected in the MLN, the primary draining lymph node for the peritoneal cavity (43, 44). The rapid response to *S. aureus* infection is characteristic of the innate-like function of γδ T cells. However, we also observed the γδ T cell response adapt to *S. aureus* infection over time. At 1 h postinfection, $V\gamma1.1^+$ and $V\gamma2^+$ cells comprised the majority of γδ T cells recruited to the site of infection; however by 72 h, a large population of $V\gamma4^+$ cells had accumulated in the peritoneal cavity. Moreover, in a novel model of recurrent peritonitis, we found that this composition of γδ T cells was maintained up to 3 wk after final exposure to the bacterium. Significantly, these $V\gamma4^+$ T cells were maintained in the peritoneal cavity and the MLN in the absence of bacteria.

Differential expression of Vγ and/or Vδ chains is used to categorize γδ T cell subsets, which often display tissue-specific homing and distinct cytokine profiles (35). $V\gamma1.1^+$ cells have been reported to produce IL-17 in *Aspergillus*-infected mice with chronic granulomatous disease (45), but are traditionally thought to be more anti-inflammatory, with depletion studies indicating increased

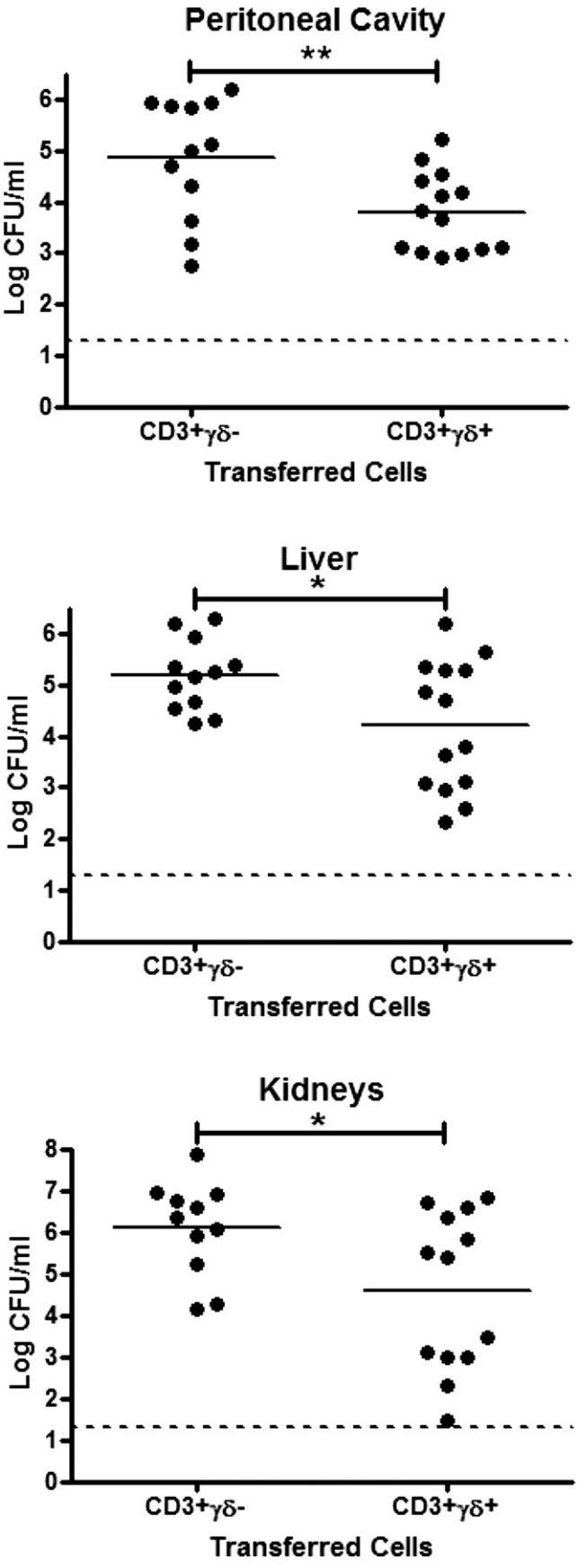

FIGURE 7. Adoptively transferred *S. aureus*–primed γδ T cells protect naive mice against subsequent infection. γδ T cells were purified (>98% pure) from the peritoneum of S. *aureus*–infected mice on day 35 (i.e., 21 d after the final exposure to *S. aureus*) and 1×10^5 cells transferred i.p. to naive syngeneic hosts. A total of 1×10^5 γδ T cell–depleted $CD3^+$ T cells (i.e., $CD3^+\gamma\delta^-$ cells) isolated from the same mice previously exposed to *S. aureus* were transferred to a separate group of naive mice as a control. Three hours posttransfer, mice were infected with *S. aureus* i.p. (5×10^8 CFU). At 72 h postinfection, bacterial burden was assessed in the peritoneal cavity, kidneys, and liver. Results are expressed as log CFU/ml of n = 12–14 mice/group. Data are representative of three independent experiments. $^*p < 0.05$, $^{**}p < 0.005$.

inflammatory responses to infection with *Listeria monocytogenes* (46) and coxsackievirus B3 infection (47). Conversely, $V\gamma2^+$ cell depletion led to reduced inflammatory responses in both of these models (46, 47). Moreover, these same proinflammatory $V\gamma2^+$ cells appear to be pathogenic in autoimmune models and were identified as the predominant γδ T cell subset infiltrating the CNS of mice with experimental autoimmune encephalomyelitis and the joints of mice with collagen-induced arthritis (34, 48). $V\gamma4^+$ cells contribute to the clearance of bacterial infections such as *L. monocytogenes* (49), *Bacillus subtilis* (50), and *Escherichia coli*, in which IL-17–producing $V\gamma4^+V\delta1^+$ T cells control the early recruitment of neutrophils (20, 51, 52).

Human γδ T cells, like αβ T cells, are capable of Ag recall and can recognize a bacterial Ag upon re-exposure to the organism, allowing for a more rapid and efficient immune response (53–56). In nonhuman primate models, phosphoantigen-specific $V\gamma2^+V\delta2^+$ γδ T cells have been shown to exhibit Ag-specific responses during mycobacterial infection (54). $V\gamma2^+V\delta2^+$ cells, the predominant subset present in human blood, recognize metabolites from isoprenoid synthesis. One such phosphoantigen, hydroxymethyl-but-2-enyl-pyrophosphate (HMBPP) is a potent stimulator of $V\gamma2^+V\delta2^+$ cells (27) and is produced during infections with *E. coli*, *L. monocytogenes*, and *Mycobacterium tuberculosis* (57, 58). Although HMBPP is not produced by *S. aureus*, transfer of HMBPP-activated γδ T cells was protective against *S. aureus* infection in SCID mice (28).

Murine γδ T cells resembling adaptive αβ T cells and bearing the characteristics of pathogen-specific human γδ T cells have recently been identified in a model of *L. monocytogenes* infection (29). In that study, a population of $CD27^-CD44^+$ memory γδ T cells were induced in response to a secondary infection via the oral mucosal route, but not the systemic route, suggesting that mucosal priming plays a key role in the expansion of memory γδ T cells in that model. Critically, this was associated with enhanced protection against secondary infection with listeria but not *Salmonella typhimurium*. The authors further demonstrated that the memory γδ T cell population comprised predominantly of $V\gamma4^+V\delta1^+$ cells. These cells were retained long term and produced elevated levels of IL-17 and IFN-γ upon secondary oral infection, largely in an MHC class II–dependent manner. Similarly, Roark et al. (59) demonstrated high levels of CD44 expression on a population of effector memory $V\gamma4^+V\delta1^+$ cells. These studies suggest that pathogen-specific murine γδ T cells are capable of generating protective memory.

In the current study, *S. aureus*–primed $CD27^-V\gamma4^+$ γδ T cells were also $CD44^{hi}$ and displayed enhanced cytokine production upon re-exposure to *S. aureus* both in vivo and in vitro, reflecting an effector memory phenotype. In our S. *aureus*–induced peritonitis model, however, $CD27^-V\gamma4$ T cells secreted only IL-17, and not IFN-γ as reported by Sheridan et al. (29). This polarized cytokine expression profile was evident in both the primary infection model and upon rechallenge and is consistent with the reported thymic imprinting of γδ T cells, which was found to be stable even during infection with *Plasmodium berghei* (38).

Consistent with previous studies (15, 17), we found that IL-1β was indispensable for IL-17 production by γδ T cells during *S. aureus*–induced peritonitis in naive mice. Similarly, IL-1R blockade with an mAb abrogated IL-17 production by γδ T cells from naive mice, in coculture studies with *S. aureus*–infected macrophages in vitro. In contrast, *S. aureus*–primed γδ T cell secretion of IL-17, which was significantly elevated compared with that produced by γδ T cells from naive mice, was independent of IL-1R signaling. Moreover, IL-1R expression on peritoneal γδ T cells did not differ from that seen on γδ T cells encountering S. *aureus* for the first time. As IL-23R signaling was not critical for IL-17 expression by γδ T cells in naive or prior-exposed mice, and IL-1α and IL-18 were undetectable in the peritoneal cavity of both groups of mice, we hypothesize that these primed γδ T cells are in fact memory cells capable of rapid recall of staphylococcal Ags upon re-exposure. Interestingly, preliminary experiments by our group indicate that inhibition of MHC class II signaling interferes with the ability of memory γδ T cells to respond to Ag stimulation (A.G. Murphy and R.M. McLoughlin, unpublished observations). This is consistent with the recent data published by Sheridan et al. (29), and further investigation into these mechanisms is warranted.

A memory response specifically by primed γδ T cells, and not CD4 or CD8 T cells (which did not produce IL-17 upon re-exposure to *S. aureus*), was confirmed by the demonstration that adoptive transfer of γδ T cells, but not $CD3^+\gamma\delta^-$ T cells, from the peritoneum of mice infected with S. *aureus* conferred protection to naive mice against challenge with *S. aureus*. The enhanced clearance of bacteria from the peritoneum also resulted in reduced dissemination to peripheral sites, including the kidneys and liver of γδ T cell recipient mice.

γδ T cells occupy a unique niche in the immune system due to their pleiotropic effector functions, their capacity to recognize distinct phosphoantigens and their preferential localization at mucosal sites, all of which support a prominent role for γδ T cells in anti-microbial immunity. Indeed, in a rare variant of SCID that results in decreased αβ T cells and increased numbers of γδ T cells, patients display normal Ab production and can respond effectively to vaccinations against diphtheria (60). This highlights the capacity of γδ T cell recall responses even in the absence of conventional αβ T cell help. Moreover, vaccines targeting γδ T cells have shown efficacy in West Nile virus infection, in which administration of α-glucans known to promote γδ T cell expansion resulted in attenuated viremia and mortality following lethal infection (61). γδ T cells are also currently being targeted in novel anticancer vaccines. In these studies, autologous $V\gamma9^+V\delta2^+$ cells were activated and expanded by phosphoantigen stimulation in vitro and then infused back into the patients, where they have been found to kill transformed cells (62).

This study significantly advances our understanding of the cellular immune response to *S. aureus*, identifying a subset of IL-17–producing γδ T cells that undergo rapid expansion following infection. Furthermore, these IL-17–secreting memory γδ T cells confer protective immunity following re-exposure to *S. aureus*. Future studies are now required to identify the specific staphylococcal Ags recognized by γδ T cells. *S. aureus* is of course a human pathogen, and there are differences between human and mouse γδ T cells. Therefore, studies in humans will be necessary to validate these findings from murine models. If IL-17–secreting memory γδ T cells are induced and found to have a role in anti-staphylococcal immunity in humans, these cells represent a potentially important and novel target for the rational design of future vaccines against *S. aureus*.

Acknowledgments

We thank Barry Moran for assistance with cell sorting.

Disclosures

K.H.G.M. is a cofounder of and shareholder in Opsona Therapeutics Ltd. and TriMod Therapeutics Ltd., university startup companies involved in the development of immunotherapeutics.

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Corrections

Murphy, A. G., K. M. O'Keeffe, S. J. Lalor, B. M. Maher, K. H. G. Mills, and R. M. McLoughlin. 2014. *Staphylococcus aureus* infection of mice expands a population of memory γδ T cells that are protective against subsequent infection. *J. Immunol.* 192: 3697–3708.

In Supplemental Fig. 1F, sequencing analysis of our PCR-amplified cDNA from FACS-sorted $V\gamma1.1^{-}V\gamma2^{-}$ cells had shown 100% homology to the National Center for Biotechnology Information's reference sequence of the Vγ4 gene (National Center for Biotechnology Information reference sequence NG_007033.1 [12827–12920, 13077–13388]) at nucleotides 13081–13310. We translated this nucleotide sequence to the amino acid sequence AVSRHLWGHMSSRGKEIRLFSNVKKQVFRSPMHTYTGTKRSQASVSKECCVVLQKKTL in a 5′–3′ reading frame.

In our error, we compared the alignment of a reading frame that covered a stop codon. An alternate 5′–3′ reading frame correctly predicts the protein sequence, identified as A0A075B5Y8 on the UniProt Web site (http://www.uniprot.org/), where Vγ4 is referred to as Vγ6 using Heilig and Tonegawa's nomenclature. Using CLUSTALW software, realignment of the translated amino acid sequence of our amplified cDNA obtained in this reading frame against the FASTA-formatted Vγ4 reference sequence (above) gives an alignment score of 100, and confirms that the $V\gamma1.1^{-}V\gamma2^{-}$ cells we have identified are $V\gamma4^{+}$ T cells. A corrected Supplemental Fig. 1F has already been published online. The supplemental material therefore differs from what was originally published.

Translation of the nucleotide sequence in this reading frame confirms the identification of these cells as $V\gamma4^{+}$ γδ T cells and does not in any way impact the conclusions or interpretation of findings reported in the original publication.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500594