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

Alison G. Murphy,* Kate M. O’Keeffe,* Stephen J. Lalor,* Belinda M. Maher,* Kingston H. G. Mills,† and Rachel M. McLoughlin*

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 antistaphylococcal 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 Vγ4⁺ subset and accumulate at the site of infection subsequent to an initial Vγ1L1⁺ and Vγ2⁺ T cell response. Moreover, these Vγ4⁺ 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: 000–000.

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

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

\γ\text{T} cells have recently been identified as a potent source of innate IL-17 and implicated in host protection in murine models of \emph{S. aureus} infection. In a cutaneous infection model, \γ\text{T} cell–deficient mice had reduced neutrophil recruitment to the infection site and impaired bacterial clearance (15). \γ\text{T} cells were also shown to have a protective role in \emph{S. aureus}–induced pneumonia through their capacity to produce IL-17 (14). We have recently reported that \γ\text{T} cells are the dominant source of IL-17 in a surgical site infection model in which IL-17R was validated from the Trinity College Dublin Bioresources Ethics Committee.

To European Union regulations, and experiments were performed under the Trinity College Dublin Bioresources facility. All mice were maintained according to specific pathogen-free conditions at the Trinity College Dublin’s facility. All mice were maintained according to 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 \emph{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 \γ\text{T} cells in a model of Listeria monocytogenes enteric infection (29).

In this study, we demonstrate for the first time, to our knowledge, that \γ\text{T} cells are the predominant source of IL-17 during \emph{S. aureus}–induced peritonitis. Interestingly, we have identified two waves of \γ\text{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\text{T} cells were expanded for a prolonged period and responded more vigorously through IL-17 production during subsequent \emph{S. aureus} infection, which was associated with enhanced protection. Induction of this IL-17 response by memory \γ\text{T} cells was not dependent on IL-1 signaling, in contrast to its critical requirement for IL-17 production by \γ\text{T} cells in naive mice. Furthermore, transfer of \emph{S. aureus}–primed \γ\text{T} cells conferred protection against \emph{S. aureus} infection in naive mice. Our findings demonstrate that a \γ\text{T} cell memory response can be induced upon exposure to \emph{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 \γ\text{T} cells may be an important property of a protective vaccine against \emph{S. aureus}.

Materials and Methods

Mice

Age- and sex-matched wild-type (WT) C57BL/6 and IL-1RI \text{−/−} (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

\emph{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\text{8} 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.

\emph{S. aureus}–induced peritonitis

Mice were exposed to \emph{S. aureus} by i.p. injection of 100 μl bacterial suspension (5 × 10\text{9} CFU). The recurrent peritonitis model involved repeated exposure to \emph{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 \emph{S. aureus} (5 × 10\text{8} CFU), in addition to a group of naive mice that had not previously been exposed to \emph{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 (10% FCS; Biorender; 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 complete RPMI. Kidneys, lungs, liver, spleen, and peritoneal lavages were homogenized in 3 ml sterile PBS. The 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.

\textit{In vitro coculture of purified γδ T cells with \emph{S. aureus}–infected macrophages}

PEC from naive mice were isolated as described above, transferred to a 96-well flat-bottom plate (2 × 10\text{5} cells/well), and macrophages allowed to adhere for 1.5 h at 37°C and 5% CO\text{2} in cRPMI. The media was then aspirated and replaced with RPMI lacking antibiotics. Macrophages were infected with 2 × 10\text{5} CFU/well \emph{S. aureus} (multiplicity of infection [MOI] 1:10) for 3 h at 37°C and 5% CO\text{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\text{5} purified \γ\text{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\text{2}. Purified \γ\text{T} cells were obtained by negative selection of CD3\text{+} T cells from the peritoneal cavity and the MLN of both naive and \emph{S. aureus}–exposed mice using the murine Pan T Cell Isolation Kit II (Milltenyi Biotec), followed by FACS sorting of \γ\text{T} cells using Abs specific to the γδTCR (Beckman Coulter [Dako-Cytomation] MoFlo Cell Sorter). Some experiments were carried out in the presence or absence of anti–IL-1R1 mAb (R&D Systems; 4 or 1 μg/ml) or anti–IL-23R mAb (R&D Systems; 1 μg/ml).

\textit{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\text{2} to block cytokine secretion. Cells were then incubated with Fc\γ receptor 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 (BioScience; clone 17B7) and IFN-γ (eBioscience; clone XMG1.2). Flow cytometric data were acquired with a BD FACSCan等到 II (BD Biosciences) and analyzed using FlowJo software (Tree Star). Gates are set on respective fluorescence-minus-ones.

\textit{Amplification of the Vγ4 gene in purified Vγ1.1+ Vγ2+ cells}

Purified subsets of γδ T cells were obtained by negative selection of CD3\text{+} 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 (Milltenyi 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 to 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′-TTCTGC-CTGGCCTCGGTTT3′ and reverse 5′-TCCCTCTAAGGGTGTTG-TGAT-3′; Vγ2, forward 5′-TGTTGACCCGAAAAAACAACATC-3′ and
The Journal of Immunology 3

rebase 5'-CAATACACCTTTATGACATCGT-3'; Vγ3, forward 5'-TTCGA-
-
-GTCTCTGGGT-TG-3' and reverse 5'-GGCAGCTAGTCTGCGC-
-CTT-3'; Vγ4, forward 5'-GGGAAGCAGTCTAGTCGCCC-3' and reverse 5'-
-GTCCATGCTCTGGGTACTA-3'; Vγ5, forward 5'-GATCCACTCTG-
-TCAGTCTACACA-3' and reverse 5'-AAGGAGAAGAAGTTGAC-
-CAGC-3', and; 18S, forward 5'-CCTGCGGTTAATTGTACT-3' and re-
verse 5'-AACATGAGCCGCGAC-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 [DakoCyomation] MoFlo Cell Sorter),
CD3γδ6 or CD3γδ6 T cells were injected i.p. to naive mice (1 × 107 cells/
mouse). At 3 h posttransfer, mice received an i.p. injection of S. aureus (5 ×
106 CFU). At 72 h postinfection, the peritoneum was lavaged and the kid-
nels, 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 sig-
ificant.

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
process of generating vaccines that will induce protective cellular immu-
nity. 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 × 106 CFU) or
sterile PBS as a control. IL-17A and IL-1γ 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 stimu-
lation, revealed that IL-17 was exclusively produced by CD3+ T cells. Moreover, γδ 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 cy-
tokine 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
升高 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 ap-
peared in the peritoneal cavity by 72 h postinfection (Fig. 1D, Supple-
mmental Fig. 1B), which had subsided by 5 d postinfection (data not shown). This second phase of IL-17+ γδ T cell accumu-
lation 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-
1R−/− mice were infected with S. aureus (5 × 106 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-1R−/− mice (Supplemental
Fig. 1C). However, IL-17 production by these γδ T cells was
abrogated in the IL-1R−/− 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 cyto-
kine 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-1R−/− 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 × 106 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 Vγ1.1+ T cells and Vγ2+ T cells (Fig. 2A, 2B). By 3 h
postinfection, a population of Vγ1.1+Vγ2+ cells had also ac-
cumulated in the peritoneal cavity. Vγ3+ cells were almost
detectable in the peritoneal cavity by flow cytometry (0.6 ± 0.2
Vγ3Vγδ6). Based on previous studies, therefore, we hypothesized
that peritoneal Vγ1.1+Vγ2+ 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 Vγ1.1+Vγ2+
PEC and MLN cells. Gel electrophoresis of the amplimers indicates
that the Vγ1.1+Vγ2+ cells were indeed Vγ4+ cells (Supplemental
Fig. 1E). Furthermore, the translated sequence of the amplified DNA,
excised from the gel, aligned with the Vγ4 protein sequence con-
fiming that the Vγ1.1+Vγ2+ cells were Vγ4+ 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 Vγ4+ cells was signif-
iacantly increased, whereas the frequency of Vγ2+ cells had sig-
nificantly decreased in the peritoneal cavity (Fig. 2B). The
frequency of Vγ1.1+ 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 Vγ1.1+ and Vγ4+
T cells at 72 h postinfection (Fig. 2B). Vγ3+ 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
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).
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 × 10⁸ 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 γδ+ 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 γδ+ 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 × 10⁸ 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.
because in mice recurrently infected and subsequently rechallenged with an alternative *S. aureus* strain, SH1000 (36, 37), an increase in IL-17 production by \( \gamma \delta \) T cells in the peritoneal cavity was also observed (36.1 \( \pm \) 3.1 versus 31.7 \( \pm \) 4.7\% IL-17 + \( \gamma \delta \) cells respectively; \( n = 5 \) per group). Analysis of the individual \( \gamma \delta \) 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 \( V_\gamma^4 \) population (Fig. 3C, Supplemental Fig. 2C). A similar increase in IL-17 expression by \( \gamma \delta \) T cells from naive or previously exposed mice was compared (E). Results expressed as mean \( \pm \) SEM of \( n = 8 \) mice/group. Data are representative of two independent experiments. *\( p < 0.05 \), **\( p < 0.005 \), ***\( p < 0.001 \). Exp, exposure.

**Enhanced IL-17 expression by \( \gamma \delta \) 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 \( \gamma \delta \) 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-1a or IL-18 secretion was detected (Supplemental Fig. 4). IL-1alpha and IL-18, which can also stimulate IL-17 production by \( \gamma \delta \) 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 × 10^3 CFU) i.p. injections on days 0, 7, and 14. Mice were then rechallenged with S. aureus (5 × 10^3 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 γδ TCR 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.

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 re-exposure 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 × 10^3 γδ T cell–depleted CD3^+ T cells (i.e., CD3^+δ^− 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 × 10^3 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.
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...
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 antistaphylococcal 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, Vγ1.1+ and Vγ2+ cells comprised the majority of γδ T cells recruited to the site of infection; however by 72 h, a large population of Vγ4+ 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 Vγ4+ T cells were maintained in the peritoneal cavity and the MLN in the absence of bacteria.

### 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 × 10⁶ 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 × 10⁶ 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, γδ TCR, 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 γδ TCR 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.
Adoptively transferred S. aureus–primed 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 × 10⁵ cells transferred i.p. to naive syngeneic hosts. A total of 1 × 10⁵ 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 infected with S. aureus i.p. (5 × 10⁸ 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.
Consistent with previous studies (15, 17), we found that IL-1β was dispensable for IL-17 production by γδ T cells during S. aureus–induced peritonitis in 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. McLaughlin, 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 CD3 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+γδ 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 pliteptotic 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 diptheria (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 Vγ9Vδ2+ 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.


Corrections


In Supplemental Fig. 1F, sequencing analysis of our PCR-amplified cDNA from FACS-sorted Vγ1.1−Vγ2− 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 AVSRHLWGHSRGGKEIRFSSNKQVFRSPHMTYTGTKRSQASVSKECCVVQKKTL 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 Vγ1.1−Vγ2− cells we have identified are Vγ4+ 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 Vγ4+ γδ T cells and does not in any way impact the conclusions or interpretation of findings reported in the original publication.

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Supp Fig 1
Supp Figure 1: γδ T cell accumulation in the peritoneal cavity during S. aureus infection
Mice were infected with S. aureus (5x10⁸ CFU) via i.p. injection. At the indicated time points post-infection, PECs were cultured with Brefeldin A, but not PMA and ionomycin, stained for surface CD3 and γδ TCR, and intracellular IL-17 and IFNγ, and analysed by flow cytometry. (A) Representative FACS plot at 3 h post-infection. (B) The absolute numbers of IL-17-producing γδ T cells in the peritoneal cavity are expressed as mean ± SEM of n=5 mice/group. Data are representative of 2 independent experiments. WT and IL-1Rγc-/- mice were infected with S. aureus (5x10⁸ CFU) via i.p. injection and γδ T cell recruitment (C) and IL-17 production (D) assessed by flow cytometry at 3 h post-infection. Results are expressed as mean ± SEM of n=10 mice/group. Data are representative of 2 independent experiments. Mice were infected with S. aureus (5x10⁸ CFU) via i.p. injection. PECs were harvested from mice at 3 h post-infection and Vγ1.1-Vγ2+ cells purified by FACS. RNA was extracted from the purified Vγ1.1-Vγ2+ cells, reverse transcribed into cDNA and each Vγ gene amplified by PCR (E). Amplified DNA was excised from the gel and sequenced. This was translated to the amino acid sequence and aligned with that of the Vγ4 reference sequence (NCBI reference gene (NG_007033.1)) (F).
Supp Figure 2: Prior exposure to *S. aureus* leads to expansion of IL-17+ Vγ4 T cells and protects against dissemination of bacteria during subsequent infection.

Groups of mice were exposed to *S. aureus* (5x10⁸ CFU) via i.p. injections on d 0, 7 and 14, and allowed to recover for 21 d. Previously exposed mice were then re-challenged with *S. aureus* (5x10⁸ CFU) on day 35, as were a control group of naive mice. At the indicated time points following challenge, bacterial burden was assessed in the kidneys and liver (A). Results expressed as log CFU/ml of n=12-15 mice/group. At 3 h post-infection, MLN cells were cultured with Brefeldin A, but not PMA and ionomycin, and stained for surface CD3, CD4, CD8 and γδTCR, and intracellular IL-17, and analysed by flow cytometry (B). At 1 h post infection isolated PECs were cultured with Brefeldin A, but not PMA and ionomycin, and IL-17 production by individual γδ T cell subsets analysed by flow cytometry (C). Results expressed as mean ± SEM of n=12 mice/group. *p<0.05, **p<0.005, ***p<0.001. Data represent 4 independent experiments.
Supp Fig 3

A

No Prior Exp  Prior Exp

1 hr p.i.

7.01  20.6

3 hrs p.i.

6.71  38.2

B

% IL-17+ γδ cells

0  5  10  15  20  25

1  3

Hours post Infection

*  **  ***

C

1 hr post infection

3 hrs post infection

IL-17+ γδ cells x10^4

0  0.2  0.4  0.6  0.8  1

Vγ1.1  Vγ2  Vγ4

*  ***  ***

D

% IL-17+ γδ+ cells

0  5  10  15  20  25

No Prior Exp  Prior Exp

*  **  ***

IL-17+ γδ cells x10^4

0  2  4  6  8

No Prior Exp  Prior Exp

*  **
**Supp Figure 3:** Prior exposure to *S. aureus* results in the expansion of IL-17-producing Vγ1.1+ and Vγ4+ T cells in the MLN and spleen upon re-challenge.

Groups of mice were exposed to *S. aureus* (5x10⁸ CFU) via i.p. injections on d 0, 7 and 14. Previously exposed mice were then re-challenged with *S. aureus* (5x10⁸ CFU) on d 35, as were a control group of naive mice. At 1 and 3 h post-challenge, 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 analysed by flow cytometry. Results expressed as mean ± SEM of n=9 mice/group, with representative FACS plots (A & B). IL-17 expression by individual Vγ subsets amongst total γδ T cells in the MLN was also assessed at 1 and 3 h post-challenge (C). Results expressed as mean ± SEM of n=9-12 mice/group. At 3 h post-challenge, spleen cells were cultured with Brefeldin A, but not PMA and ionomycin, stained for surface CD3, γδTCR and intracellular IL-17, and analysed by flow cytometry (D). Results expressed as mean ± SEM of n=5 mice/group. *p<0.05, **p<0.005, ***p<0.001. Data represent 2-3 independent experiments.
Supp Figure 4: Elevated IL-17 production by γδ T cells upon re-challenge is not associated with increased IL-1β or IL-23 secretion in the peritoneal cavity.

Groups of mice were exposed to *S. aureus* (5x10^8 CFU) via i.p. injections on d 0, 7 and 14. Previously exposed mice were then re-challenged with *S. aureus* (5x10^8 CFU) on d 35, as were a control group of naive mice. At 1 and 3 h post challenge secreted IL-1β and IL-23 in the peritoneal fluid was measured by ELISA.