**Staphylococcus aureus** infection of mice expands a population of memory γδ T cells that are protective against subsequent infection

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**Abstract**

The development of vaccines against *S. 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 anti-staphylococcal 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 re-infection with the bacterium. These cells are comprised largely of the Vγ4+ subset and accumulate at the site of infection subsequent to an initial Vγ1.1+ 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 signalling 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*.

**Keywords**

*S. aureus*; γδ T cells; T cell memory; IL-17
Introduction

*Staphylococcus aureus* is a Gram-positive coccoid 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, bacteraemia, 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 vancomycin (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 pre-clinical 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 (ClfA), we have recently demonstrated vaccine-induced protection against acute systemic *S. aureus* infection in mice in the complete absence of an antibody 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), whilst 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 anti-bacterial immunity, through its role in neutrophil recruitment (18-22). Patients with hyper IgE syndrome (HIES), 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 colonisation by *S. aureus* (25), due in part to decreased IL-17 responses (26). These and other studies have highlighted the key role for IL-17 in anti-staphylococcal 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, where 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 more than 50% of peripheral T cells within days of infection onset (27). Using a humanised chimeric severe-combined immunodeficiency
(SCID) model, 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 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, characterised by distinct subsets. Initially, a rapid influx of Vγ1.1 and Vγ2 cells (Garman nomenclature), which 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 signalling, in contrast to its critical requirement for IL-17 production by γδ T-cells in naïve mice. Furthermore, transfer of S. aureus-primed γδ T cells conferred protection against S. aureus infection in naïve 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 C57BL/6 and IL-1RI−/− (6-8 weeks old) were housed under specific pathogen-free conditions at the TCD Bioresources facility. All mice were maintained according to EU regulations and experiments were performed under licence from the Irish Department of Health and Children and with approval from the TCD 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 5×10⁹ CFU/ml by measuring the optical density of solutions at 600 nm. CFUs were verified by plating serial dilutions of each inoculum onto Tryptic Soy Agar (TSA).

S. aureus-induced peritonitis

Mice were exposed to S. aureus by i.p. injection of 100 μl bacterial suspension (5×10⁸ CFU). The recurrent peritonitis model involved repeated exposure to S. aureus via i.p. injection on d 0, 7 and 14 before mice were allowed to recover for 21 d. On d 35 the previously exposed, infection-free mice received an i.p. challenge of S. aureus (5×10⁸ CFU), in addition to a group of naïve mice that had not previously been exposed to S. aureus. At specific time points post challenge 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 PECs re-suspended in cRPMI (RPMI: Biosera, 10% fetal calf serum: Biosera, 100mM L-glutamine: Gibco, 100μg/ml penicillin/streptomycin: Gibco). 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, cells washed and re-suspended in...
cRPMI. Kidneys, liver and spleen were homogenized in 3 ml of sterile PBS. Total tissue bacterial burden was established by plating serial dilutions of peritoneal lavage or tissue homogenate on TSA plates for 24 h at 37°C. Results are expressed as CFU per ml.

**In vitro co-culture of purified γδ T cells with S. aureus infected macrophages**

PECs from naive mice were isolated as described above, transferred to a 96-well flat-bottomed plate (2×10^5 cells/well) and macrophages allowed to adhere for 1.5 h at 37°C and 5% CO₂ in cRPMI. The media was then aspirated and replaced with RPMI lacking antibiotics. Macrophages were infected with 2×10^6 CFU/well of S. aureus (MOI 1:10) for 3 h at 37°C and 5% CO₂. After 3 h the supernatant was aspirated and replaced with RPMI supplemented with gentomycin (100 μg/ml). Macrophages were then co-cultured with 1×10^5 purified γδ T cells, isolated from naïve or previously exposed mice (21 d post final exposure i.e. d 35), for 24 h at 37°C and 5% CO₂. Purified γδ T cells were obtained by negative selection of CD3⁺ T cells from the peritoneal cavity and the MLN of both naïve 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 antibodies specific to the γδ TCR (Beckmann Coulter (Dako) MoFlo Cell Sorter). Some experiments were carried out in the presence or absence of anti-IL-1RI monoclonal antibody (R&D, 4 μg/ml or 1 μg/ml) or anti-IL-23R monoclonal antibody (R&D, 1 μg/ml).

**ELISA**

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

**Flow Cytometry**

PEC and MLN cells were incubated in the presence of Brefeldin A (Sigma) for 4 h at 37°C and 5% CO₂ to block cytokine secretion. Cells were then incubated with Fcγ block (1 μg/ml) on ice before surface staining with fluorochrome-conjugated antibodies against CD3 (BD, clone 500A2), γδ TCR (Biolegend, clone GL3), IL-1R (Biolegend, clone JAMA147), Vγ1.1 (Biolegend, clone 2.11), Vγ2 (BD, clone UC3-10A6) and Vγ3 (BD, clone 536). Garman nomenclature is used throughout this study (31). Cells were fixed and permeabilised using the Dako cytomation Intrastain Kit, before intracellular staining with a fluorochrome-conjugated antibodies against IL-17A (eBioscience, clone 1B7) and IFNγ (eBioscience, clone XMG1.2). Flow cytometric data was acquired with a BD FACSCanto II and analysed using FlowJo software (Tree Star, Inc). Gates are set on respective Fluorescence Minus One (FMOs).

**Amplification 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 sorting of Vγ1.1⁺, Vγ2⁺ and Vγ3⁺ γδ T cells using antibodies specific for each cell subset (Beckmann Coulter (Dako) 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, as 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, Fw 5' TTCTGCTGCTCTGGGTTTTT-3' and Rev 5'-TCCCTCCTAAGGGTCGTTGAT-3'; Vγ2, Fw 5'-TGGTACCGGCAAAAAACAAATCA-3' and Rev 5'-CAATACACCCTATGACATCG-3'; Vγ3, Fw 5'-TTGCAAGCTCTCTGGGTTC-3' and Rev 5'-TCCCTCCTAAGGGTCGTTGAT-3' and
Adoptive transfer of S. aureus primed \( \gamma \delta \) T-cells

Purified \( \gamma \delta \) 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 days after the final exposure to \( S. \) aureus) using the murine Pan T cell isolation Kit II (Miltenyi Biotec), followed by FACS sorting of \( \gamma \delta \) T cells using antibodies specific to the \( \gamma \delta \) TCR (Beckmann Coulter (Dako) MoFlo Cell Sorter). CD3\(^+\)\( \gamma \delta \)\(^+\) or CD3\(^+\)\( \gamma \delta \)\(^-\) T cells were injected i.p. to naive mice (\( 1 \times 10^5 \) cells/ mouse). At 3 h post-transfer, mice received an i.p. injection of \( S. \) aureus (\( 5 \times 10^8 \) CFU). At 72 h post-infection, 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 statistical analysis software. Differences between groups were analysed by unpaired Students \( t \) test or a one way ANOVA with Tukeys post-test comparison where indicated. \( P < 0.05 \) was considered significant.

Results

\( \gamma \delta \) 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, where mice received a single i.p. injection of \( S. \) aureus (\( 5 \times 10^8 \) CFU) or sterile PBS as a control. IL-17A and IL-1\( \beta \) 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-1\( \beta \) were maximal at 3 h and had decreased to the levels observed in mice administered PBS by 24 h post infection. IL-1\( \alpha \), 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 peritoneal exudate cells (PECs) at 3 h post-infection 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 mediastinal lymph nodes (MLN) during \( S. \) aureus infection. Again, \( \gamma \delta \) 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 \( \gamma \delta \) 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\( \gamma \) production by \( \gamma \delta \) T cells isolated from \( S. \) aureus-infected mice (Supp 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 post-infection, compared to naïve mice (time 0), and continued to increase up to 3 h post-infection (Fig 1D). By 24 h post-infection, 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 post-infection (Fig 1D; Supp Fig 1B), which had subsided by 5 d post-infection (data not shown). This second phase of IL-17+ γδ T cell accumulation was not detected in the MLN where the frequency of these cells was comparable to the PBS-treated controls by 12 h post-infection (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 cell subsets is IL-1β-dependent (17, 33). To confirm a role for IL-1 signalling in regulating IL-17 production by γδ T cells in the peritoneal cavity, WT and IL-1RI−/− mice were infected with S. aureus (5×10⁸ CFU) via a single i.p. injection. At 3 h post-infection, PECs 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-1RI−/− mice (Supp Fig 1C). However, IL-17 production by these γδ T cells was abrogated in the IL-1RI−/− mice (Fig 1F, Supp Fig 1D), demonstrating a critical role for IL-1 signalling 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 during S. aureus-induced peritonitis, mice were infected with S. aureus (CFU) via i.p. injection and PECs and MLN cells isolated at various time points post-infection. Flow cytometric analysis of PECs 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 & B). By 3 h post-infection a population of Vγ1.1−Vγ2− cells had also accumulated in the peritoneal cavity. Vγ3+ cells were almost undetectable in the peritoneal cavity by flow cytometry (0.6±0.2 Vγ3+γδ T). Based on previous studies therefore, we hypothesised that peritoneal Vγ1.1−Vγ2− cells were in fact Vγ4 cells, for which no fluorochrome-conjugated antibody is commercially available. To confirm this we PCR-amplified cDNA from FACS-sorted Vγ1.1−Vγ2− PECs and MLN cells. Gel electrophoresis of the amplimers indicates that the Vγ1.1−Vγ2− cells were indeed Vγ4+ cells (Supp Fig 1E). Furthermore, the translated sequence of the amplified DNA, excised from the gel, aligned with the Vγ4 protein sequence confirming that the Vγ1.1−Vγ2− cells were Vγ4+ cells (Supp Fig 1F). These cells are therefore referred to as Vγ4 cells in the figure and throughout the remainder of this report.

At 72 h post-infection, the frequency of Vγ4+ cells was significantly increased while the frequency of Vγ2+ cells had significantly 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 post infection (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 post-infection (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 (5×10^8 CFU i.p. on d 0, 7 and 14) and allowed to clear the infection (<1 log CFU/ml in peritoneal cavity, 21 d post the 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 amongst total CD3+ T cells, in the peritoneal cavity of previously exposed mice, compared with naïve mice (Fig 3A). A small but significant increase in both the frequency and absolute number of γδ+ T cells was also observed in MLN (Fig 3B). The total number of CD3+ cells did not differ significantly between naïve and prior exposed mice at this point (PEC: 0.21±0.04 vs 0.34±0.05 ×10^6, MLN: 1.55±0.06 vs 1.6±0.05 ×10^6 for naïve vs. previously exposed mice, respectively). Further analysis showed that the composition of the γδ T cell population observed 72 h post-infection 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 (Fig 3D). Crucially, in the absence of PMA and ionomycin stimulation, γδ T cells from previously exposed mice were undetectable in the peritoneal lavage fluid (data not shown).

Upon recovery (21 d post the final exposure) mice were re-challenged by i.p. injection of S. aureus (5×10^8 CFU), as were a group of naïve mice as a control. At specific time points post-challenge, bacterial burden was assessed and PECs and MLN cells isolated to characterise 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 naïve mice (Fig 4A and Supp Fig 2A). Enhanced bacterial clearance was associated with increased IL-17 secretion in the peritoneal cavity of previously exposed mice 1 and 3 h post challenge (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, Supp 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 naïve mice (Fig 5A & B). This effect was not strain specific because in mice recurrently infected and subsequently re-challenged 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% vs. 31.7±4.7% IL-17+γδ T cells respectively, n=5 per 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 post-infection, particularly IL-17 production by the Vγ4+ population (Fig 5C, Supp Fig 2C). A similar increase in IL-17 expression by γδ T cells was observed in the MLN of previously exposed mice (Supp Fig 3A & B), including a significant increase in the number of IL-17+Vγ4+ T cells, 3 h post-infection (Supp 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 hours post infection (Supp Fig 3D).

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Expression of the TNF receptor 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 antigen-experienced (CD27⁺) γδ T cells from antigen-naïve 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 present study, IL-17 producing γδ T cells present in the peritoneal cavity of S. aureus-infected naïve and previously exposed mice were also found to express a CD27⁻CD44hi phenotype (Fig 5C & D). 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 signalling

During S. aureus infection in naïve mice, IL-17 production by peritoneal γδ T cells was critically dependent upon IL-1 signalling (Fig 1A & F). 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 re-infection of previously exposed mice, compared with infection of naïve mice (Fig 4B), no difference in IL-1β or IL-23 secretion was detected (Supp Fig 4). IL-1α and IL-18, which can also stimulate IL-17 production by γδ T cells (41), were undetectable in the peritoneal cavities of either naïve or prior exposed mice following S. aureus infection (data not shown). Microbial colonisation 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 signalling (42). However, we did not detect any difference in the mean fluorescence intensity (MFI) of IL-1RI expression on peritoneal IL-17⁺ γδ T cells between previously exposed and naïve mice at 1 and 3 h post-challenge (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 to 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 naïve 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 naïve mice, γδ T cells isolated from mice previously exposed to S. aureus produced significantly more IL-17 when co-cultured with S. aureus infected macrophages (Fig 6B & C). 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 antibodies to both IL-1RI and the IL-23R were added to the co-cultures. Blockade of IL-1RI at the higher concentration of antibody (4 μg/ml) resulted in complete abrogation of IL-17 secretion by γδ T cells from naïve mice (Fig 6B & C). Conversely, blockade of IL-1RI, using either concentration of antibody, only partially reduced IL-17 production by previously exposed γδ T cells, and this reduction was not significant (Fig 6B & C). IL-23R blockade had no effect on IL-17 production by either naïve or prior-exposed γδ T cells (Fig 6B). Taken together, our in vitro and in vivo data demonstrate differential requirements for innate IL-1 signalling in the IL-17-mediated response to S. aureus infection by primed γδ T cells and those from naïve 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 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 & Supp 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. d 35), to naïve syngeneic hosts. Prior to transfer, purified S. aureus-exposed γδ T cells primarily consisted of the Vγ4 subset (> 90% Vγ4+, data not shown). 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 naïve mice as a control. 3 h post-transfer, mice were challenged by i.p. injection of S. aureus (5×10^8 CFU). At 72 h post-infection, 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 to 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 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 naïve 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 to its critical requirement by γδ T-cells in naïve 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 non-traditional lymphocytes such as specific subsets of γδ T cells that secrete IL-17 - one of the few known correlates of anti-staphylococcal 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 post-infection. 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 post-infection 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.

Differential expression of Vγ and/or Vδ chains are used to categorise γδ T cell subsets, which often display tissue-specific homing and distinct cytokine profiles (35). Vγ1.1+ 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 inflammatory responses to infection with L. monocytogenes (46) and coxsackievirus B3 infection (47). Conversely, Vγ2+ cell depletion led to reduced inflammatory responses in both of these models (46, 47). Moreover, these same pro-inflammatory Vγ2+ 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). Vγ4+ cells contribute to the clearance of bacterial infections such as L. monocytogenes (49), B. subtilis (50) and E. coli, where IL-17-producing Vγ4+Vδ1+ T cells control the early recruitment of neutrophils (20, 51, 52).

Human γδ T cells, like αβ T cells, are capable of antigen recall and can recognise a bacterial antigen upon re-exposure to the organism, allowing for a more rapid and efficient immune response (53-56). In non-human primate models, phosphoantigen specific Vγ2+Vδ2+ γδ T cells have been shown to exhibit antigen specific responses during mycobacterial infection (54). Vγ2+Vδ2+ cells, the predominant subset present in human blood, recognise metabolites from isoprenoid synthesis. One such phosphoantigen, hydroxymethyl-but-2-enyl-pyrophosphate (HMBPP) is a potent stimulator of Vγδ T cells have been shown to exhibit antigen specific responses during mycobacterial infection (54). Vγ2+Vδ2+ cells, the predominant subset present in human blood, recognise metabolites from isoprenoid synthesis. One such phosphoantigen, hydroxymethyl-but-2-enyl-pyrophosphate (HMBPP) is a potent stimulator of Vγ2+Vδ2+ cells (27) and is produced during infections with E.coli, L. monocytogenes and M. 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 Listeria 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 Vγ4+Vδ1+ cells. These cells were retained long term and produced elevated levels of IL-17 and IFNγ upon secondary oral infection, largely in an MHC class II-dependent manner. Similarly, Roark et al. demonstrated high levels of CD44 expression on a population of effector memory Vγ4+Vδ1+ cells (59). These studies suggest that pathogen-specific murine γδ T cells are capable of generating protective memory.

In the current study, S. aureus-primed CD27−Vγ4+ γδ T cells were also CD44hi 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−Vγ4 T cells secreted only IL-17, and not IFNγ as reported by Sheridan et al. (29). This polarised cytokine expression profile was evident in both the primary infection model and upon re-challenge 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 naïve mice. Similarly, IL-1R-blockade with a monoclonal antibody abrogated IL-17 production by γδ T cells from
naïve mice, in co-culture 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 to that produced by γδ T cells from naïve mice, was independent of IL-1R signalling. 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 signalling was not critical for IL-17 expression by γδ T cells in naïve or prior-exposed mice, and IL-1α and IL-18 were undetectable in the peritoneal cavity of both groups of mice, we hypothesise that these primed γδ T cells are in fact memory cells capable of rapid recall of staphylococcal antigens upon re-exposure. Interestingly, preliminary experiments by our group indicate that inhibition of MHC II signalling interferes with the ability of memory γδ T cells to respond to antigen stimulation (Murphy and McLoughlin, unpublished data). 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⁺γδ⁻ T cells, from the peritoneum of mice infected with *S. aureus* conferred protection to naïve 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 recognise distinct phosphoantigens and their preferential localisation at mucosal sites, all of which support a prominent role for γδ T cells in antimicrobial 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 antibody 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, where 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 anti-cancer vaccines. In these studies, autologous Vγ9⁺Vδ2⁺ 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 antigens 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*.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**Abbreviations used in this paper**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PEC</td>
<td>Peritoneal Exudate Cells</td>
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<tr>
<td>MLN</td>
<td>Mediastinal Lymph nodes</td>
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**References**


FIGURE 1.

γδ T cells are the primary source of IL-17 during S. aureus induced peritonitis. Mice were infected with S. aureus (5×10⁸ CFU) via i.p. injection. At the indicated times post–infection, the peritoneal cavity was lavaged and the MLN collected. Secreted IL-17A and IL-1β in the peritoneal fluid was measured by ELISA (A). 3 h post-infection (B, C), and at the indicated time-points (D & E), PECs (B & D) and MLN cells (C & E), cultured with Brefeldin A but not PMA and ionomycin, were stained for surface CD3, CD4, CD8 and γδ TCR, and intracellular IL-17, and analysed by flow cytometry. IL-1R1−/− and WT mice were infected with S. aureus (5×10⁸ CFU) via i.p. injection and at 3 h post-infection PECs, cultured with Brefeldin A but not PMA and ionomycin, were stained for surface CD3 and γδ TCR, and
intracellular IL-17, and analysed by flow cytometry (F). Results expressed as mean ± SEM of n=10 mice/ group, with representative FACS plots. *<p<0.05, **p<0.005, ***p<0.001. Data representative of 2 independent experiments.
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. PECs (A & B) and MLN cells (C) were harvested from mice at the indicated times post-infection, surface stained with fluorochrome-conjugated antibodies against CD3, γδ TCR, Vγ1.1, Vγ2 and Vγ3 and analysed by flow cytometry. (A) FACS plots are representative of PECs at 3 h post-infection. (B & C) Results expressed as mean frequency ± SEM of each subset within the γδ+ gate of n=9-12 mice/group. *p<0.05, **p<0.005, ***p<0.001. Data representative of 2-3 independent experiments.
FIGURE 3.
Elevated frequency of Vγ4+ cells in mice previously exposed to *S. aureus*
Groups of mice were exposed to S. aureus (5×10⁸ CFU) via i.p. injections on d 0, 7 and 14. Following recovery on d 35, isolated PECs and MLN cells were cultured with Brefeldin A, but not PMA and ionomycin, and stained for surface CD3, γδTCR, Vγ1.1, Vγ2 and Vγ3, and intracellular IL-17, and analysed by flow cytometry. γδ T cell frequencies amongst 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 the 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 per group. *p<0.05, **p<0.005, ***p<0.001. Data representative of 2 independent experiments.
FIGURE 4.
Prior exposure 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 d 0, 7 and 14. Mice were then re-challenged with *S. aureus* (5×10^8 CFU) on d 35, as were a group of naive controls. At the indicated times post-challenge, the bacterial burden in the peritoneum was assessed (A). Results expressed as log CFU/ml of lavage fluid collected from n=5 mice/group per time point. At 1 and 3 h post-challenge, secreted IL-17 in the peritoneal fluid was measured by ELISA (B). At 3 h post-infection, isolated PECs were cultured with Brefeldin...
A, but not PMA and ionomycin, stained for surface CD3, CD4, CD8 and \( \gamma\delta \) TCR, and intracellular IL-17, and analysed by flow cytometry (C). Results expressed as mean ± SEM of n=12 mice/group. Data representative of 3 independent experiments. *p<0.05, **p<0.005, ***p<0.001.
Prior exposure to *S. aureus* results in an enhanced IL-17 response by predominantly Vγ4+ γδ T cells upon re-infection.

Groups of mice were exposed to *S. aureus* (5×10^8 CFU) via i.p. injections on d 0, 7 and 14. Mice were allowed to recover for 21 d before being re-challenged with *S. aureus* (5×10^8 CFU) on day 35, as were a group of naïve controls. At 1 and 3 h post infection PECs were cultured with Brefeldin A, but not PMA and ionomycin, stained for surface CD3, γδTCR and intracellular IL-17, and analysed by flow cytometry (A & B). IL-17 expression by individual Vγ subsets in the peritoneum was also assessed at 3 h post-challenge (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. *p<0.05 **p<0.005, ***p<0.001. Data represent 2-3 independent experiments.
FIGURE 6.
IL-1R or IL-23R signalling 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 d 0, 7 and 14. Mice were allowed to recover for 21 d before being re-challenged with *S. aureus* (5×10^8 CFU) on day 35, as were a control group of naïve 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 analysed by flow cytometry (A). Results expressed as mean ± SEM for n=9 mice/group. Data are representative of 2 independent experiments. γδ T cells
purified (> 98% pure) from previously exposed or naive mice were co-cultured overnight with *S. aureus* infected macrophages (MOI 1:10), in the presence or absence of monoclonal antibodies to IL-1RI (4μg/ml or 1μg/ml) or IL-23R (1μg/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=4-5 replicates/group. *p<0.05. Cells were incubated with Brefeldin A, but not PMA and ionomycin, stained for surface CD3 and γδTCR, and intracellular IL-17, and analysed by flow cytometry (C). Data representative of 4-5 independent experiments.
FIGURE 7.
Adoptively transferred *S. aureus*-primed γδ T cells protect naïve mice against subsequent infection
γδ T cells were purified (> 98% pure) from the peritoneum of *S. aureus*-infected mice on d 35 (i.e. 21 days after the final exposure to *S. aureus*), and 1×10^5 cells transferred i.p. to naïve syngeneic hosts. 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 naïve mice as a control. 3 h post-transfer, mice were infected with *S. aureus* i.p. (5×10^8^ CFU). 72 h post-infection, bacterial burden was assessed in the peritoneal cavity, kidneys
and liver. Results are expressed as log CFU/ml of n=12-14 mice/group. *p<0.05, **p<0.005, ***p<0.001. Data representative of 3 independent experiments.