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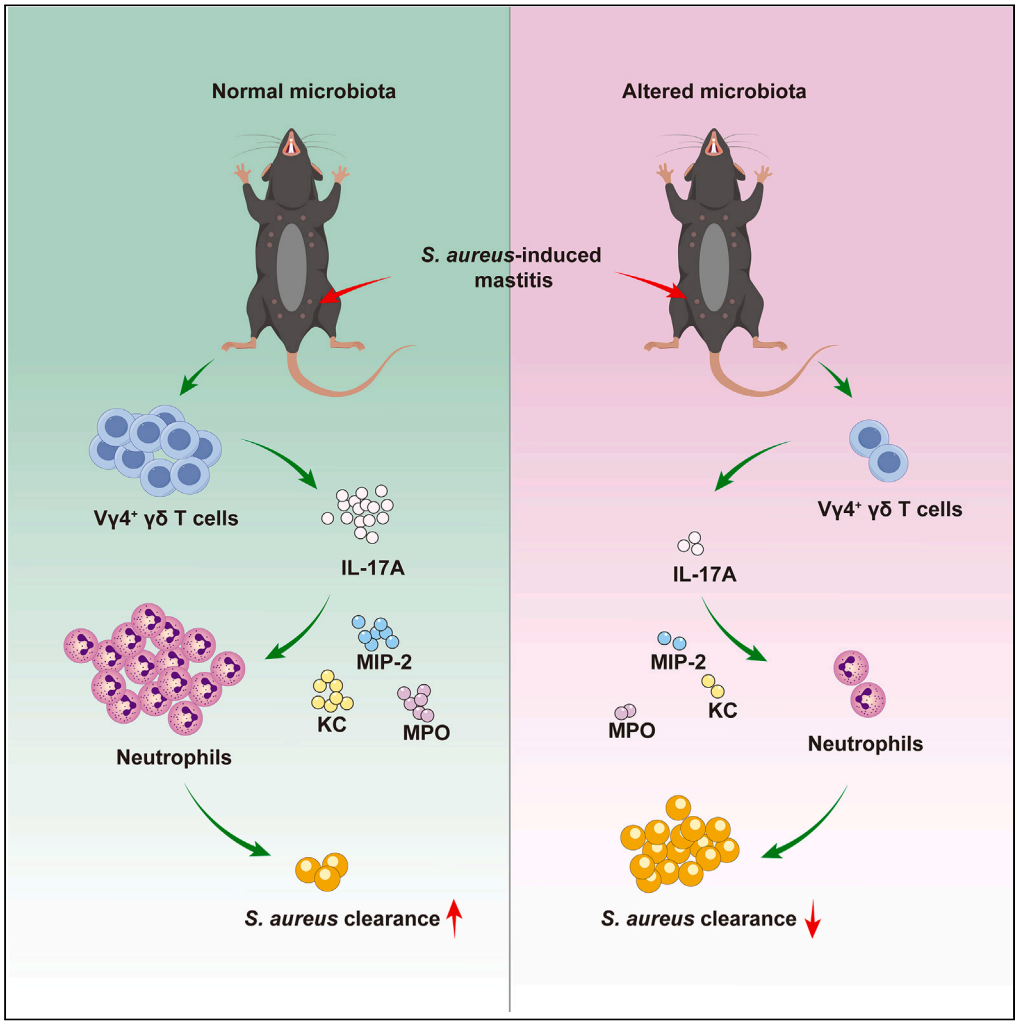

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Highlights

IL-17A/neutrophil axis promotes host defense against *S. aureus*-induced mastitis

The rapid activation of γδ T cells triggered the IL-17A-mediated immune response

The potent source of IL-17A was from a population of clonotypic Vγ4$^+$ γδ T cells

The protection mediated by γδT17 cells dependents commensal microbiota

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Mammary γδ T cells promote IL-17A-mediated immunity against *Staphylococcus aureus*-induced mastitis in a microbiota-dependent manner

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SUMMARY

Mastitis, a common disease for female during lactation period that could cause a health risk for human or huge economic losses for animals, is mainly caused by *S. aureus* invasion. Here, we found that neutrophil recruitment via IL-17A-mediated signaling was required for host defense against *S. aureus*-induced mastitis in a mouse model. The rapid accumulation and activation of Vγ4$^+$ γδ T cells in the early stage of infection triggered the IL-17A-mediated immune response. Interestingly, the accumulation and influence of γδT17 cells in host defense against *S. aureus*-induced mastitis in a commensal microbiota-dependent manner. Overall, this study, focusing on γδT17 cells, clarified innate immune response mechanisms against *S. aureus*-induced mastitis, and provided a specific response to target for future immunotherapies. Meanwhile, a link between commensal microbiota community and host defense to *S. aureus* mammary gland infection may unveil potential therapeutic strategies to combat these intractable infections.

INTRODUCTION

Bovine mastitis is one of the most prevalent infectious diseases of cattle that results in a huge economic loss in the dairy industry.[1] While mastitis is caused by several etiologic agents, *Staphylococcus aureus* is the most frequently isolated major pathogen from intramammary infections.[2] With a dearth of prevention strategies, *S. aureus*-induced mastitis is often treated with antibiotics.[3] Although antibiotic treatment is an option for individual animals, it is unfavorable because of costs and the potential risk of the development of antibiotic resistance, and is unsuitable for addressing the problem of long-term persistence of pathogenic *S. aureus* in udder tissue.[1,4] In addition, better understanding of protective immunity against mammary gland infection by *S. aureus* is further contributing to the urgent need to address bovine mastitis in immune-based therapies.

Innate immune responses play a key role in host defenses against *S. aureus* infection.[5,6] Specifically, effective immune response against *S. aureus* depends on recruitment of neutrophils and macrophages, and on abscess formation necessary for bacterial clearance.[7,8] In a mouse model, neutrophil depletion resulted in delayed bacterial clearance and reduced survival of infected mice.[9] In humans, neutropenic cancer patients showed high susceptibility to *S. aureus* infections, which increased morbidity and mortality.[10] Recruitment of neutrophils via expression of two CXC chemokines (macrophage inflammatory protein 2, MIP-2 and keratinocyte-derived cytokine, KC) is essential for control of *S. aureus* during mucosal and cutaneous infections.[11,12] Macrophages, in addition to their role in neutrophil recruitment, are recruited to infection sites and contribute to *S. aureus* clearance.[13–15] In summary, innate immunity is essential for defense against *S. aureus* infections.

Besides well-known innate immune cells (neutrophils, macrophages), T cells that bear γδ T cell receptor (TCR) (termed γδ T cells) are abundant in barriers sites, such as the skin, gut, and lungs, and play a key role in mucosal host defenses against bacterial infection.[16,17] Studies of mouse models have clearly demonstrated the importance of γδ T cells in immune responses to bacterial infection. Following lung challenge by bacteria, γδ T cells helped regulate initial immune response by altering recruitment of neutrophils, dendritic cells, or macrophages.[16,18,19] IL-17A-producing γδ T cells play a specialized protective role in innate immunity against *S. aureus* cutaneous infection.[20,21] γδ-T-cell-deficient mouse strains had impaired defenses against infection by *Klebsiella pneumonia*, *Mycobacterium tuberculosis*, or *Streptococcus pneumoniae*.[22–24] In the case of *S. aureus*-induced mastitis, however, functions of γδ T cells have received little research attention. A wide variety of bacteria inhabit the mammary gland ecosystem during a health lactation period in rodents and humans, and disturbance of balance state and overused of antibiotic are associated with increased risk of mastitis.[25–27] However, the existence of intramammary microbiota and its crosstalk with immune system are matter of controversy in dairy ruminants.[28] The role of commensal bacteria in regulating the local immune

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homeostasis and host resistance to pathogen infection by crosstalk between the microbiota and γδ T cells has been investigated in multiple organs.[29,30] However, the mechanism of interaction between microbiota and γδ T cells to regulate the resistance to mammary gland infection remains unclear.

Despite recent advances, the identity of the immune cells that are critical for mammary gland defense and the cytokines that shape the inflammatory response are poorly defined. In particular, information on the role of IL-17A in *S. aureus*-induced mastitis and their cellular sources is still lacking. In the present study, we have developed a mouse model of *S. aureus* mastitis to explore the interactions of *S. aureus* with the mammary gland and to characterize the innate immune defenses that come into play during mastitis. We noticed an important role for IL-17A/neutrophils axis in pathogen clearance during *S. aureus*-induced mastitis, and Vγ4$^+$ γδ T cells are main source of IL-17A. The immune surveillance of γδT17 cells in mammary gland upon *S. aureus* infection relies on commensal bacteria. Collectively, we revealed mechanism of γδT17 cells-mediated immunity in *S. aureus*-induced mastitis host defense, and paved the way for microbiota-based therapies for diseases involving these cells.

RESULTS

IL-17A dependent recruitment of neutrophils is required for host defense during *S. aureus* mammary gland infection

Previous studies have discussed intramammary immune responses and cytokine profiles during *S. aureus*-induced mastitis.[31] To assess comparative immune responses in early-stage host defense against *S. aureus* mammary gland infection, we measured expression of the cytokines IL-17A, IL-17F, IL-23, IL-22, IFN-γ, and IL-4, which are produced by many different important T cells subsets. IL-17A, IL-23, and IFN-γ levels in mammary gland showed significant increases at 3 and 6 h post-infection (p.i.), whereas those of IL-17F, IL-22, and IL-4 showed no notable change (Figure 1A). These findings indicate that IFN-γ and IL-17A are the major mediator cytokines in early stage of *S. aureus* mammary gland infection.

We next focus on the role of IL-17A because it has been implicated in a variety of mouse models of *S. aureus* infection as being critical to host defense.[18,20,21,32,33] The role of IL-17A was examined by intramammary infusion of 10^7 *S. aureus* colony-forming units (CFUs) infecting wild-type (WT) and IL-17A knockout (KO) mice. Mammary gland bacterial counts at 6 h p.i. were significantly higher in IL-17A KO than in WT mice (Figure 1B). Similar results were obtained from Gram staining analysis; i.e., counts of Gram-positive bacteria in mammary gland sections were higher for IL-17A KO than for WT mice (Figure 1C). Mammary gland sections were also stained with hematoxylin/eosin (H&E), and histological examination revealed greater neutrophil infiltration in WT than in IL-17A KO mice (Figure 1C). IL-17A-dependent neutrophils recruitment has been shown to be crucial for baseline protection against *S. aureus* infections.[34] Neutrophil levels in mammary gland were examined by flow cytometry at 6 h p.i. based on co-expression of CD11b and Ly-6G, and found to be significantly lower in IL-17A KO than in WT mice (Figure 1D). IL-17 regulates production of CXC chemokines such as MIP-2/CXCL2 and KC/CXCL1, which is necessary for neutrophil recruitment.[35] To further elucidate the mechanism for impairment of neutrophil infiltration, we compared mammary gland expression of neutrophil-inducing cytokines and chemokines in WT and IL-17A KO mice at 6 h p.i. KC and MIP-2 production were lower in IL-17A KO than in WT mice (Figure 1E), and the same was true for myeloperoxidase (MPO) production, an important marker of neutrophil function (Figure 1F). These findings clearly demonstrate that IL-17A deletion impairs neutrophil recruitment and bacterial clearance in mammary gland following *S. aureus* infection.

γδ T cells are accumulated and activated rapidly following *S. aureus* mammary gland infection

IL-17A is produced by many different T cell subsets. To assess comparative expansion and activation dynamics of major T cell subsets in early-stage host defense against *S. aureus* mammary gland infections, we performed immunophenotyping analysis of CD4$^+$, CD8$^+$, NKT, and γδ T cells. Lactating C57BL/6 mice were infected with 5×10^7 CFU *S. aureus* by intramammary infusion, and frequency and activation kinetics of major T cell subsets in mammary gland and spleen were evaluated by flow cytometry. γδ T cells frequency in mammary gland showed significant increase at 3 or 6 h p.i., whereas CD4$^+$, CD8$^+$, and NKT cells showed no notable change (Figure 2A). γδ T, CD4$^+$ T and NKT cells frequency in spleen showed no significant change at 3 or 6 h p.i. in addition to CD8$^+$ T cells (Figure S1). γδ T cells activation was assessed by staining with the established markers CD44 and CD69.[36] These markers were highly expressed in γδ T cells at 3 or 6 h p.i. (Figure 2B). The contrasting timelines of frequency and activation changes observed for γδ T cells clearly indicate an active role of these cells in early-stage *S. aureus*-induced mastitis. IL-17A-producing ability of γδ T cells from infected mammary gland tissue was assessed by intracellular staining. γδT17 cells increased rapidly at 3 and 6 h p.i. (Figure 2C). These results indicated that γδ T cells were rapidly activated and γδT17 cells were rapidly expanded in early-stage *S. aureus*-induced mastitis.

γδ T cells-deficient mice develop impaired IL-17A/neutrophil axis response and increased susceptibility to *S. aureus*-induced mastitis

Mammary gland abscesses in WT and TCRδ KO mice at 6 h p.i. were examined in order to evaluate the role of γδ T cells during *S. aureus* infection. Abscesses in TCRδ KO mice were notably larger than those in WT (Figure 3A). Mammary gland of TCRδ KO and WT mice were collected, and bacterial counts were performed. *S. aureus* counts were much higher in TCRδ KO than in WT mice (Figure 3B). Severity of these lesions was much greater in TCRδ KO mice; they showed larger hemorrhage area and a badly organized cellular arrangement reminiscent of mammary acini (Figure 3C). In WT mice, *S. aureus* was barely detectable by Gram staining, presumably because of phagocytosis and clearance of bacteria by neutrophils within the abscess. In contrast, blue-stained areas of Gram-positive bacteria were widespread throughout the

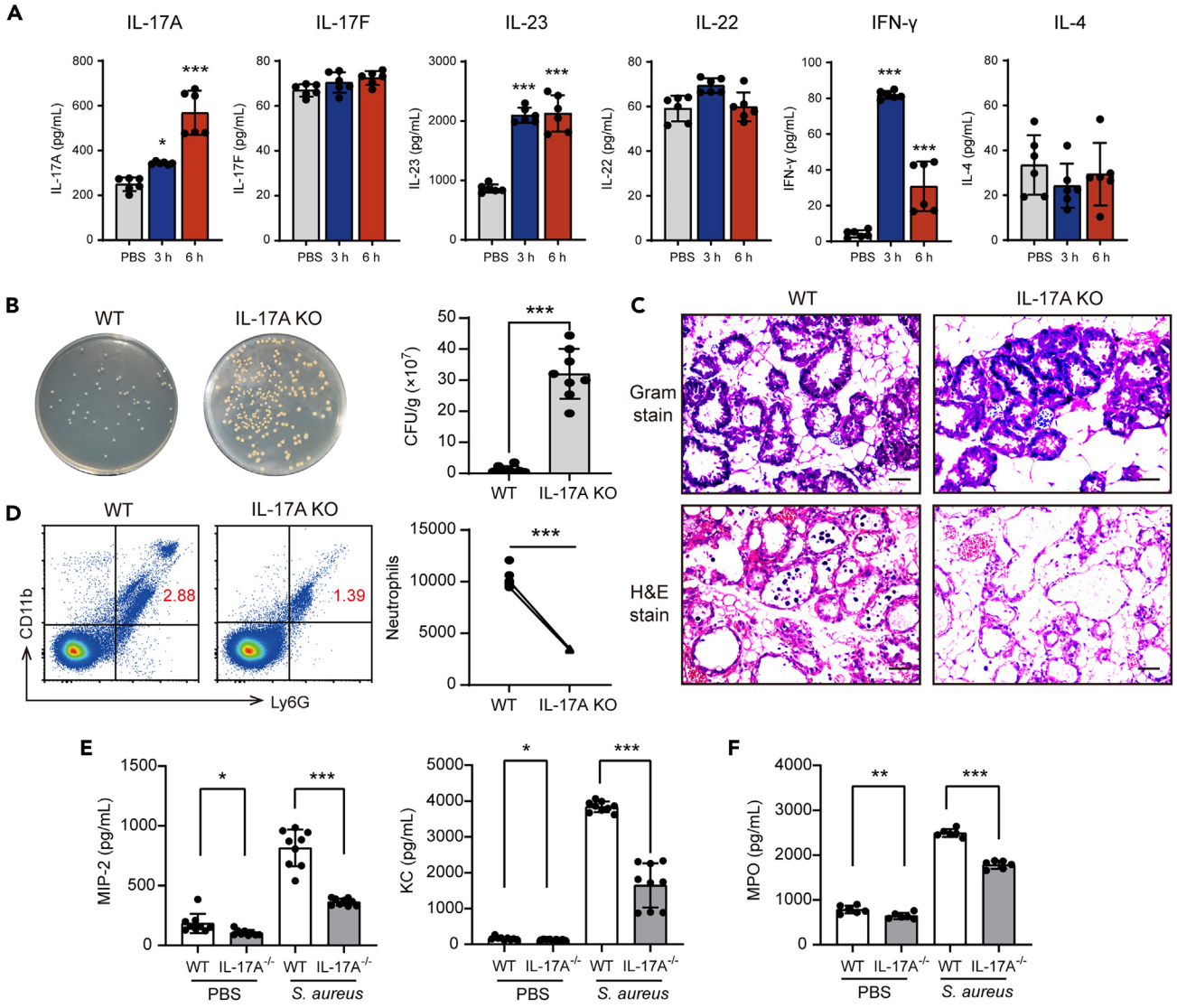

Figure 1. IL-17A dependent recruitment of neutrophils is required for host defense during *S. aureus* mammary gland infection

(A) Seven days post-parturition, lactating C57BL/6 mice were intramammary (i.m.m.) injected with *S. aureus* ATCC 27543 and sacrificed at various times p.i. Levels of IL-17A, IL-17F, IL-23, IL-22, IFN-γ, and IL-4 were determined in mammary gland tissues (n = 6 per group). Data shown were pooled from three independent experiments. WT and IL-17A KO mice were i.m.m. injected with *S. aureus* ATCC 27543.

(B) *S. aureus* CFUs in L4 mammary gland homogenates at 6 h p.i. (n = 8 per group).

(C) Representative photomicrographs of Gram-stained and H&E-stained sections of mammary gland at 6 h p.i. (bar: 200 μm).

(D) Representative flow cytometry plots and total number of mammary gland neutrophils at 6 h p.i. (n = 4 per group).

(E) Mean protein levels of MIP-2 and KC in mammary gland homogenates at 6 h p.i. (n = 9 per group).

(F) Mean MPO activity in mammary gland homogenates at 6 h p.i. (n = 6 per group). Data are represented as mean ± SEM.

section (Figure 3C). These results indicated that γδ T cells deletion reduced bacteria clearance and increased susceptibility to *S. aureus*-induced mastitis.

Mammary gland production of IL-17A was much lower in TCRδ KO mice (Figure 3D). Neutrophil recruitment was examined by flow cytometry in mammary gland of WT and TCRδ KO mice at 6 h p.i., and found to be significantly lower in TCRδ KO than in WT mice (Figure 3E). MPO activity following *S. aureus* infection was significantly higher in WT than in TCRδ KO mice (Figure 3F). We used enzyme-linked immunosorbent assay (ELISA) to measure levels of soluble factors known to have direct neutrophil chemotactic activity (i.e., neutrophil chemokines KC and MIP-2) at 6 h p.i. Mammary gland production of all three chemokines was much lower in TCRδ KO than in WT mice (Figure 3G). These findings demonstrate that γδ T cells deletion impaired IL-17A/neutrophil axis response to mammary gland following *S. aureus* infection.

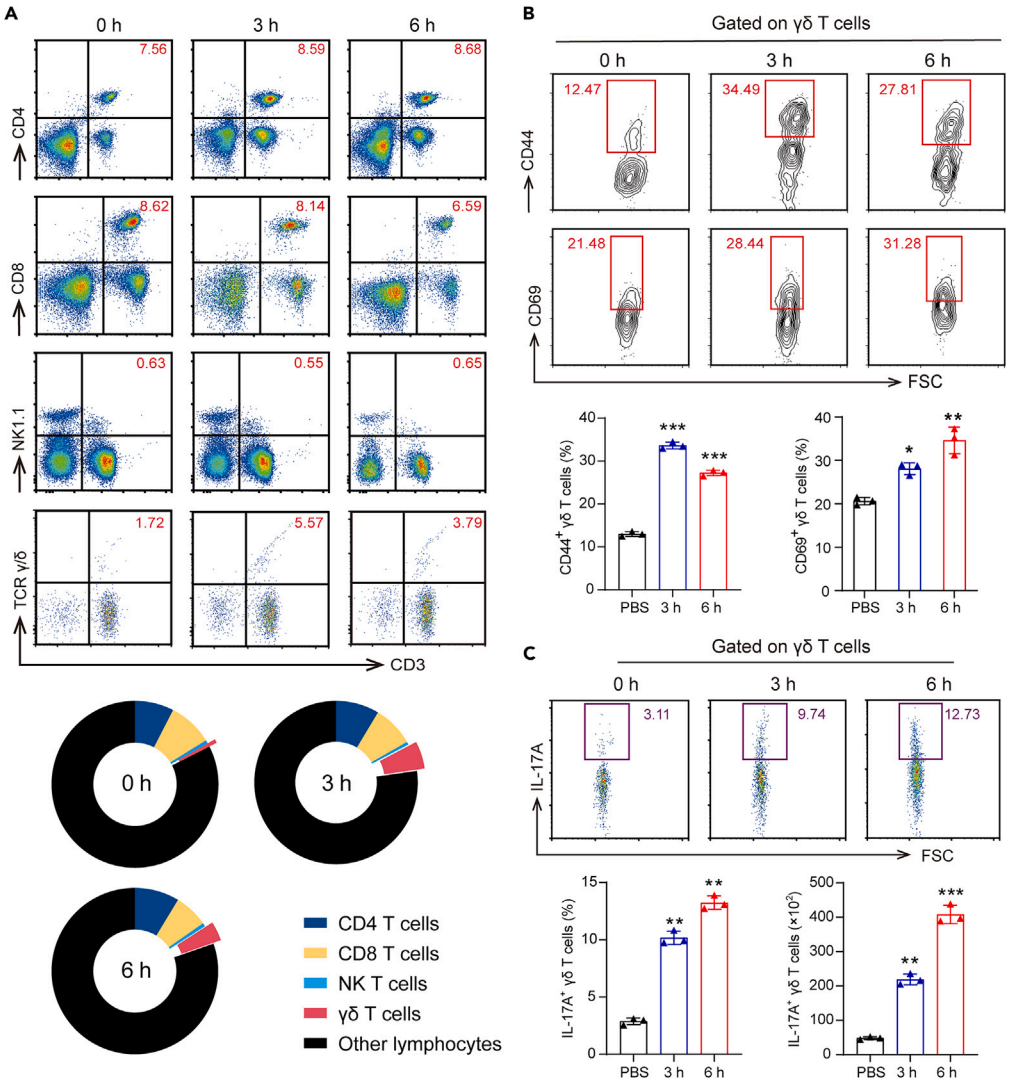

Figure 2. γδ T cells are accumulated and activated rapidly following *S. aureus* mammary gland infection

WT mice were i.m.m. injected with *S. aureus* ATCC 27543.

(A) Lymphocytes from mammary glands were prepared at the indicated times, and CD4, CD8, NKT, and γδ T cells were stained for flow cytometric analysis. FACS plot and pie chart showing frequencies of CD4, CD8, NKT, and γδ T cells in mammary gland of 0, 3, and 6 h p.i. (n = 6).

(B) Flow cytometric plots of CD44 and CD69 expression on mammary gland γδ T cells at 3 and 6 h p.i. Frequency of activated ($CD44^+$ or $CD69^+$) γδ T cells (n = 3 per group).

(C) Percentage and absolute numbers of $IL\text{-}17A^+$ γδ T cells in mammary gland (n = 3 per group). Data are represented as mean ± SEM.

$V\gamma4^+$ γδ T cells are a potent source of innate IL-17A during *S. aureus*-induced mastitis

γδ T cells comprise several heterogeneous subsets, and different subsets have different cytokine expression characteristics.[37] $V\gamma1^+$ and $V\gamma4^+$ are two important subtypes of γδ T cells, and play an important role in maintaining local homeostasis and immune surveillance.[37] We examined dynamic changes of these two types of γδ T cells in mammary gland during *S. aureus* infection. Absolute count of $V\gamma1^+$ γδ T cells showed steady increase and peaked at 6 h p.i., while that of $V\gamma4^+$ γδ T cells showed steady increase and peaked at 3 h p.i. (Figure 4A). To determine the association between γδ T cells subsets and IL-17A production, flow cytometric analysis of γδ T cells from mammary gland tissue was performed. A high proportion of $V\gamma4^+$ γδ T cells produced IL-17A at 3 and 6 h p.i. (Figure 4C), whereas the proportion of $IL\text{-}17A^+$ $V\gamma1^+$ T cells was small in uninfected mice and did not increase notably following *S. aureus* mammary gland infection (Figure 4B). WT mice were *in vivo* administration of anti-TCR Vγ1 or Vγ4 mAb, and $V\gamma1^+$ or $V\gamma4^+$ γδ T cells in mammary gland were significantly decreased on day 5 after mAb administration (Figure S2B and S2C). Mammary gland production of IL-17A at 6 h p.i. was much lower in $V\gamma4^+$ T-cell-depleted mice than in controls (Figure 4D). Percentage of $V\gamma4^+$ $IL\text{-}17A^+$ T cells in mammary gland was also much lower in $V\gamma4^+$ T cell-depleted mice than in controls (Figure 4E). These findings indicate that the $V\gamma4^+$ subset of γδ T cells is a potent source of innate IL-17A during *S. aureus* infection.

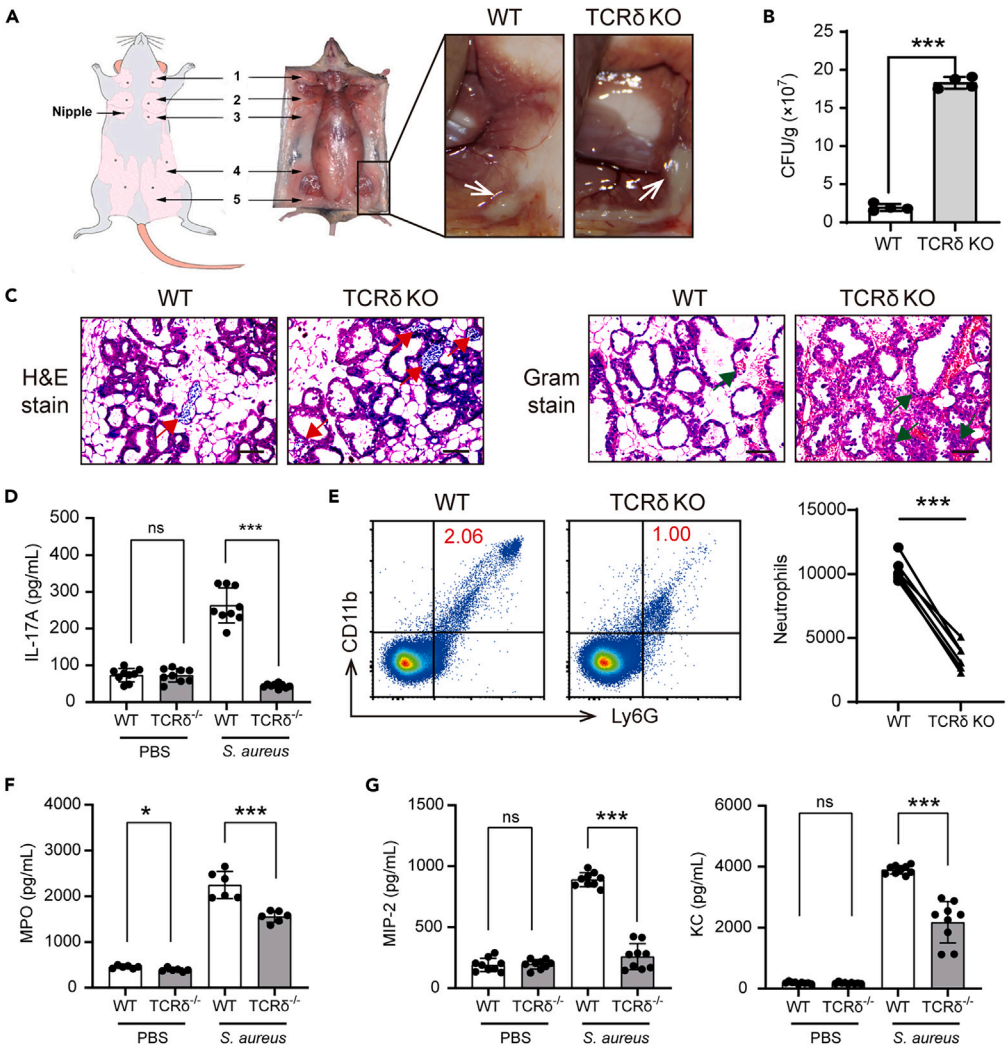

Figure 3. γδ T cells-deficient mice develop impaired IL-17A/neutrophil axis response and increased susceptibility to *S. aureus*-induced mastitis

WT and TCRδ KO mice were i.m.m. injected with *S. aureus* strain ATCC 27543.

(A) The schematic of the relative positions of the cervical (#1), thoracic (#'s 2 and 3), abdominal (#4) and inguinal (#5) glands.[58] Clinical manifestations of R4 mammary gland at 6 h p.i. after infection.

(B) *S. aureus* CFUs in L4 mammary gland at 6 h p.i. (n = 4 per group).

(C) Representative photomicrographs of Gram-stained and H&E-stained sections of mammary gland at 6 h p.i. (bar: 200 μm).

(D) Mean protein levels of IL-17A in mammary gland homogenates at 6 h p.i. (n = 9 per group).

(E) Representative flow cytometry total number of mammary gland neutrophils at 6 h p.i. (n = 6 per group).

(F) Mean MPO activity in mammary gland homogenates at 6 h p.i. (n = 6 per group).

(G) Mean protein levels of MIP-2 and KC in mammary gland homogenates at 6 h p.i. (n = 9 per group). Data are represented as mean ± SEM.

The protective effects mediated by γδ T cells and IL-17A during *S. aureus*-induced mastitis depend on commensal bacteria

Bidirectional γδ T cell-microbiota interactions have been discussed in several studies, including the microbiota differential shaping of γδ T cell populations and the roles of γδ T cell-microbiota crosstalk in local homeostasis and immune surveillance.[29] To evaluate the role of commensals in host defense against mammary gland infection, we exposed pregnant mouse to a cocktail of antibiotic (ABX) for 3 weeks (Figure 5A). The bacteria in feces and mammary glands of ABX and No ABX mice were cultured under anaerobic and aerobic conditions, which preliminarily showed that long-term antibiotic exposure resulted in changes in the gut and intramammary gland microbiota (Figure S3A). We further evaluated the species richness and change of composition in intestinal and mammary gland after antibiotic treatment by 16S sequencing, and found that microbiota in mammary gland and intestine showed various degrees of overlapped after antibiotic treatment and also had unique microbiota composition (Figure S3B). Besides, the relative abundance of Firmicutes was significantly reduced while Protebacteria, Bacteroidota, and Campylobacterota were increased (Figure 5B), and Lactobacillaceae in Firmicutes were reduced in both intestinal and mammary gland (Figure S3C). After treatment with antibiotics, the percentages of $CD4^+$ T cells in the mammary gland of mice decreased, and γδ T cells

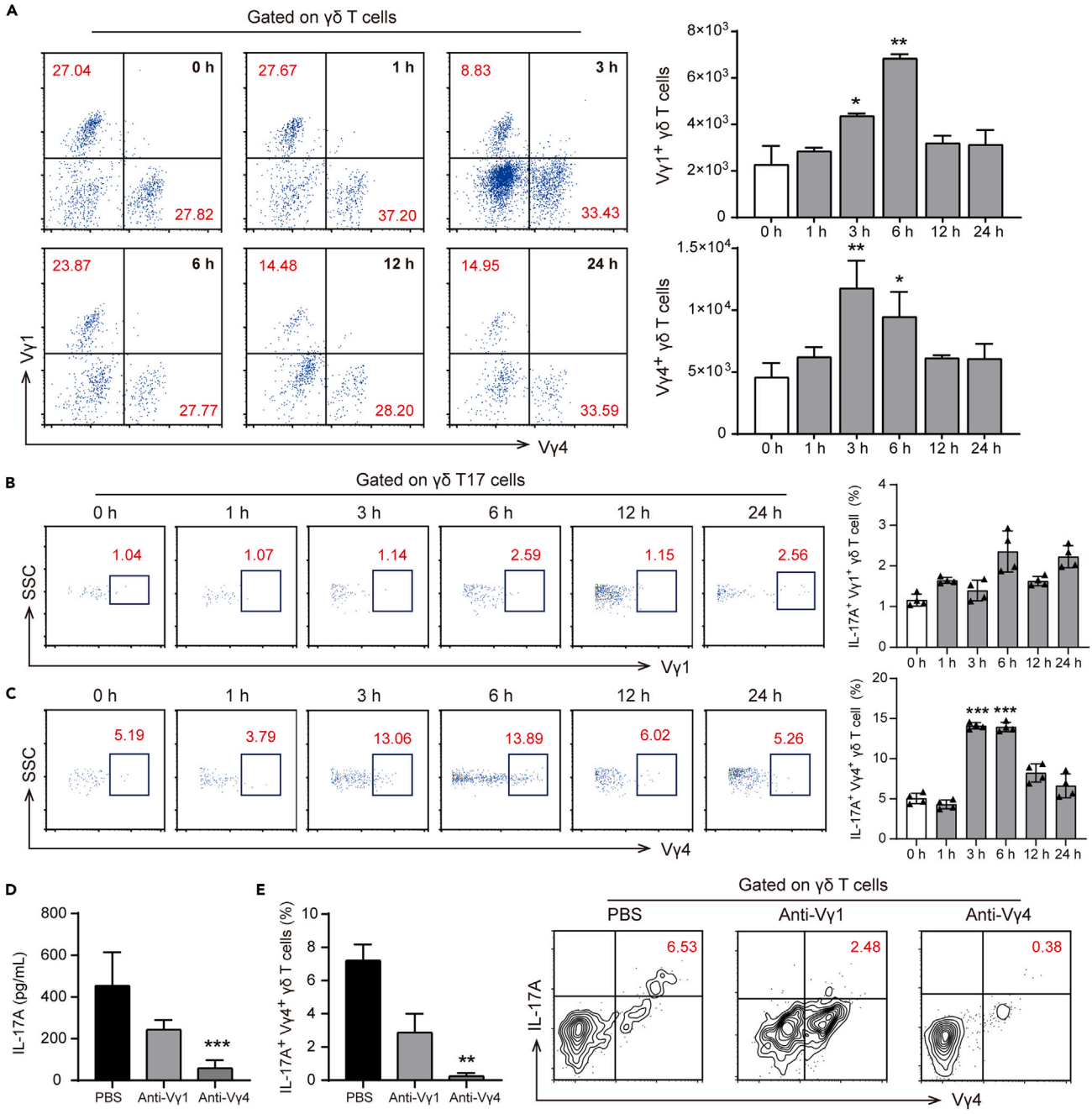

Figure 4. Vγ4$^+$ γδ T cells are a potent source of innate IL-17A during *S. aureus*-induced mastitis

Lymphocytes from mammary gland of WT mice infected with *S. aureus* were pooled and nylon-wool purified.

(A) Representative flow cytometry lots of Vγ1$^+$ and Vγ4$^+$ T cells at 0, 1, 3, 6, 12, and 24 h p.i., and total number of Vγ1$^+$ and Vγ4$^+$ T cells after *S. aureus* infection (n = 4 per group).

(B) Representative flow cytometry plots and frequency of IL-17A$^+$ Vγ1$^+$ γδ T cells from mammary gland of WT mice at 0, 1, 3, 6, 12, and 24 h p.i (n = 4 per group).

(C) Representative flow cytometry plots and frequency of IL-17A$^+$ Vγ4$^+$ γδ T cells from mammary gland of WT mice at 0, 1, 3, 6, 12, and 24 h p.i (n = 4 per group).

(D) Mean IL-17A protein levels in mammary gland homogenates of mice treated with PBS, anti-Vγ1$^+$ (clone 2.11), and anti-Vγ4$^+$ (clone UC3-10A6) antibodies at 6 h p.i. (n = 4 per group).

(E) Representative flow cytometry plots and frequency of IL-17A$^+$ Vγ4$^+$ γδ T cells from mammary gland of mice treated as in (D), at 6 h p.i. (n = 4 per group). Data are represented as mean ± SEM.

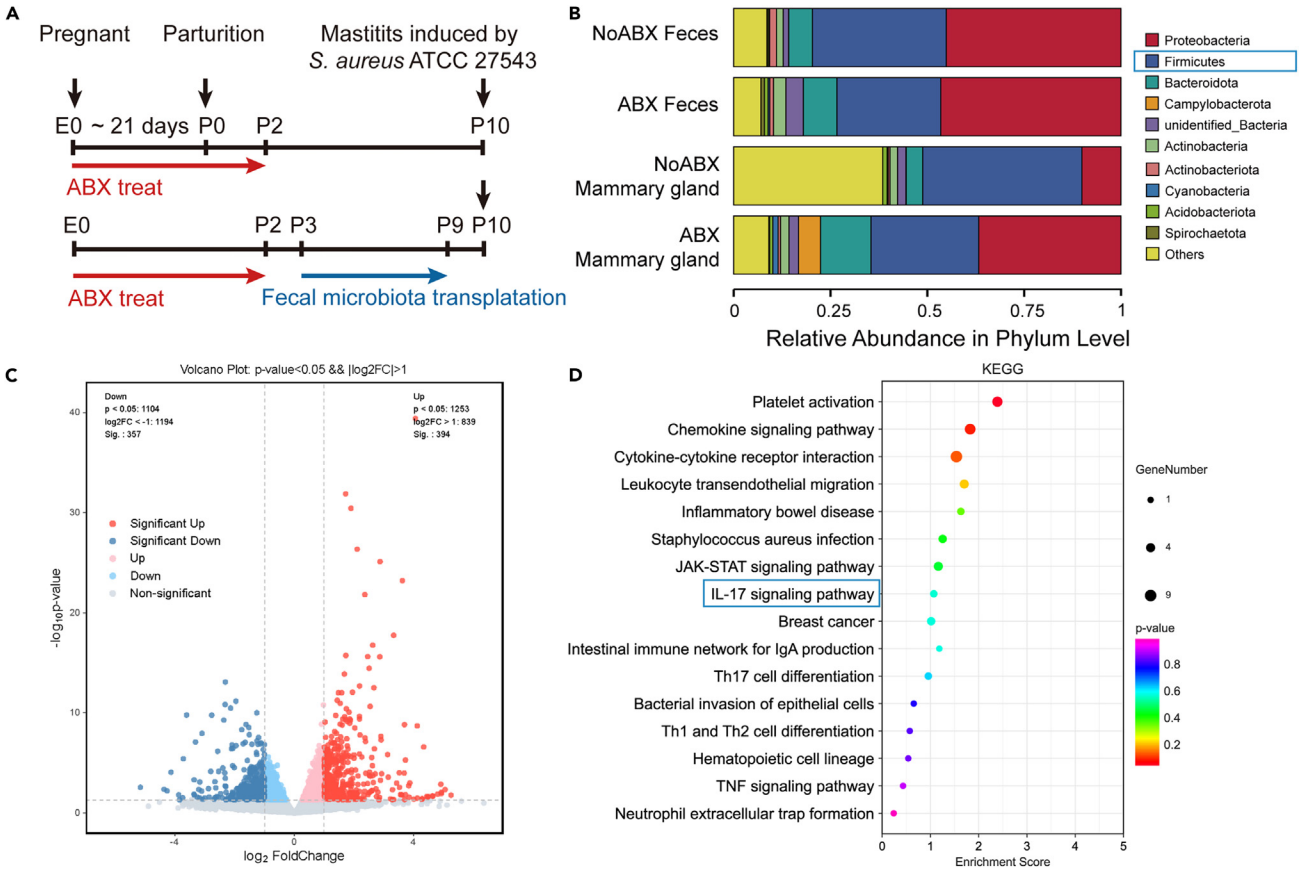

Figure 5. Commensal microbiota may regulate IL-17A signaling pathway during *S. aureus*-induced mastitis

(A) Schematic representation. The pregnant mice were treated with a cocktail of antibiotics for 3 weeks, and infected with *S. aureus* on postnatal day 7 (P7). ABX-exposed mice were reconstituted intestinal commensal microbiota by FMT on day P3–P9.

(B) Relative abundance of phylum-level commensals obtained from 16S rRNA sequencing of the feces and mammary gland tissue of ABX- or No ABX-exposed mice (n = 3 per group).

(C) Volcano map showing the up and down genes in mammary gland tissues of ABX vs. No ABX mice (n = 3 per group).

(D) Kyoto encyclopedia of genes and genomes (biological function) showing the difference expression genes in mammary gland tissues of ABX vs. No ABX mice (n = 3 per group). Data are represented as mean ± SEM.

and NKT cells increased (Figure S3D). The effect of commensal microbiota to γδ T cells/IL-17A axis during mastitis was investigated by RNA sequencing. The disturbance of microbiota caused significant differences in gene expression (Figure 5C), including downregulation of genes related to IL-17A signaling pathway (Figure 5D), and revealed that microbes may have influenced the host susceptibility to *S. aureus* infection by γδ T cells/IL-17A axis.

During *S. aureus* mastitis, an increased bacteria load in mammary gland was observed as well as more severe abscesses and lesions in ABX-exposed mice as compared to No ABX mice, and feces microbiota transplantation (FMT) restored defense against *S. aureus*-induced mastitis in ABX mice, whereas it was not significantly affected in TCRδ KO and IL-17A KO mice (Figures 6A and 6B).To further probe the cellular mechanisms of microbiota-mediated protection against *S. aureus*-induced mastitis, we analyzed the impact of commensals on the percentage of γδT17 cells and neutrophils during *S. aureus* infection. Our data showed a significant decrease in γδT17 cells and neutrophiles abundance infection in ABX mice during *S. aureus*-induced mastitis, while reconstitution of microbiota abrogated these declines (Figures 6C–6F). Furthermore, Vγ4^{+} γδ T cell was the main subtype of microbiota-dependent expansion γδ T cells during infection, while Vγ1^{+} γδ T cell showed reverse trend (Figures 6G–6J). These finding indicated that commensals promote host defense to *S. aureus* mammary gland infection in mouse model that is likely dependent on γδ T cells and IL-17A, and microbiota are required for the expansion of Vγ4^{+} γδ T cell associated with defense to *S. aureus*-induced mastitis.

DISCUSSION

Mastitis is one of the most severe diseases for humans and animals, especially in the dairy industry.[38] T cells and their cytokine responses have been repeatedly implicated in host defense against *S. aureus* infections, but it is unclear whether a predominant T cells subset

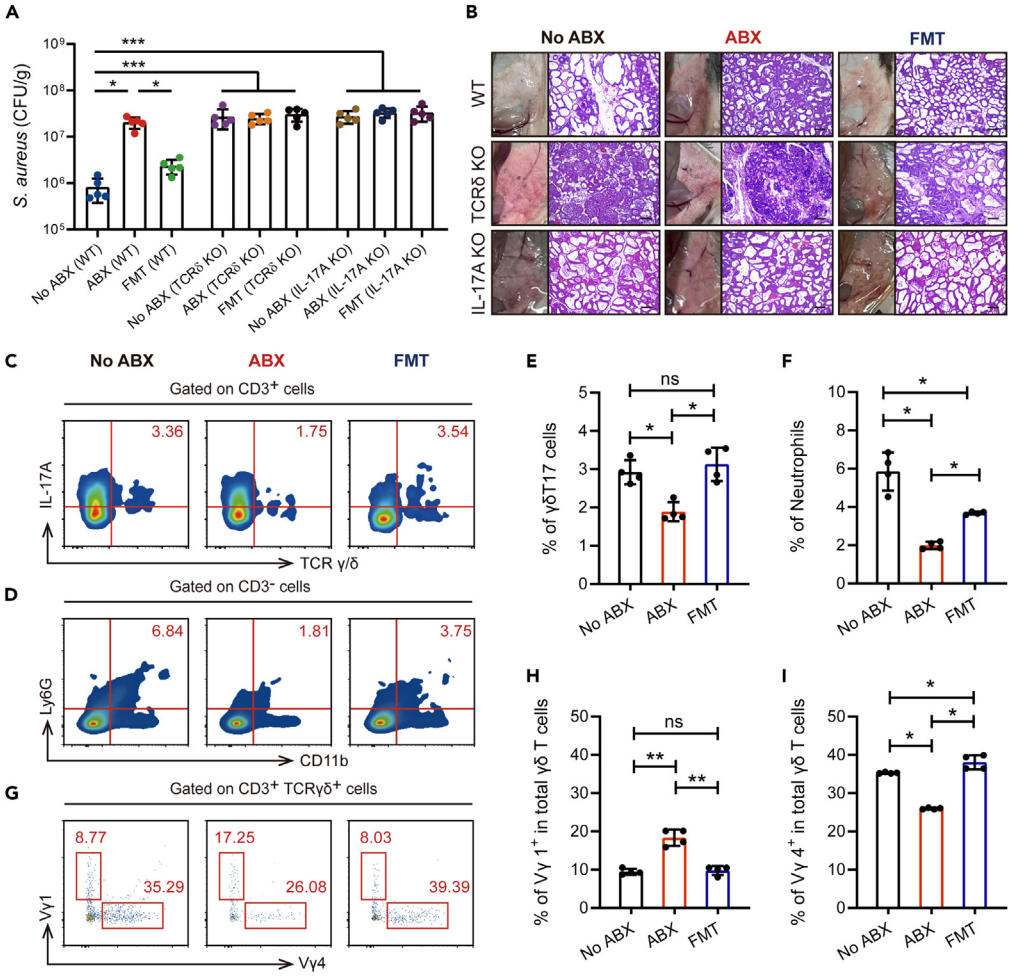

Figure 6. The protective effects mediated by γδ T cells and IL-17A during *S. aureus*-induced mastitis depend on commensal bacteria

(A) *S. aureus* CFUs in L4 mammary gland of WT, TCRδ KO, or IL-17A KO mice exposed to ABX, No ABX, and FMT mice (n = 5 per group).

(B) Clinical manifestations of R4 mammary gland at 6 h p.i., and representative photomicrographs of H&E-stained section at 6 h p.i. (bar: 100 μm).

(C and D) Representative FACS plots of γδT17 cells and neutrophils in mammary gland at 6 h p.i.

(E and F) Frequency of γδ T cells and neutrophils (n = 4 per group).

(G) Representative FACS plots of $V\gamma1^+$ and $V\gamma4^+$ γδ T cells in mammary gland at 6 h p.i.

(H and I) Frequency of $V\gamma1^+$ and $V\gamma4^+$ in total γδ T cells (n = 4 per group). Data are represented as mean ± SEM.

mediates such protection. In the present study, we employed a mouse model of *S. aureus*-induced mastitis and found that expansion of γδ T cells during early-stage infection was critical in mediating IL-17A immune responses, including induction of neutrophil recruitment, production of proinflammatory cytokines. Moreover, the potent T cell source of IL-17A was a population of clonotypic $V\gamma4^+$ γδ T cells. The accumulation and influences of γδ T cells in host defense against *S. aureus* mammary gland infections depends on commensal microbiota.

First, using an IL-17A KO mice, we determined that signaling through the IL-17A is required to prevent *S. aureus*-induced mastitis. Expansion of IL-17A and IL-17F genes during *E. coli* mammary gland infection raise the point of the contribution to the host defense against bacterial infection.[39,40] IL-17A-mediated immunity plays an important, well-documented role in defense against *S. aureus* infections in humans and animal models.[20,41] IL-17A influences the course of infection via regulating the influx of neutrophils to the infection site. IL-17A-deficient mice showed impairment to neutrophil recruitment and host defense against *S. aureus* cutaneous challenge.[20] We observed, similarly, that IL-17A deletion impaired bacterial clearance (Figures 1B and 1C), which proved that IL-17A is involved in the response of the mammary gland infection by *S. aureus*, and that increase in IL-17A production in local at the onset of infection improved the containment of infection. The observation also corroborates the important role of neutrophils early in the course of infection (Figure 1D), as reported for *E. coli* mastitis in the cow[42] and the mouse mastitis model.[43] Neutrophils were shown to play an essential role in mammary gland defense, in relation to the production of chemokines KC/CXCL1 and MIP-2/CXCL2.[43] We also noticed that the early production of IL-17A was paralleled by an early increase in MIP-2 KC and MPO (Figures 1E and 1F).

Second, we found that γδ T cells are accumulated and activated rapidly in the early stage of *S. aureus* mammary gland infection, and are the main source of IL-17A during *S. aureus*-induced mastitis. γδ T cells are the first T cells that appear in the thymus, and the major tissue-resident T cell component of mucosal barrier tissues such as gut, skin, and lung.[37] γδ T cells, as innate T cells, respond rapidly to infections and play important roles in defenses against a variety of diseases.[44] In particular, they have been shown in numerous studies to be involved in responses to *S. aureus* infections, including peritonitis, cutaneous infection, surgical wound infection, and pneumonia.[16,20,45,46] We observed substantial accumulation of γδ T cells in mammary gland as early as 3 and 6 h p.i. γδ T cells displayed increased CD69 and CD44 expression following *S. aureus* mammary gland infection (Figures 2A and 2B), suggesting that they were preferentially activated by the bacteria. In this study, γδ T cells were the main source of IL-17A in *S. aureus*-induced mastitis, and γδ T cell depletion resulted in reduced IL-17A and neutrophils in early-stage infection (Figures 3D and 3E). Thus, accumulation of γδ T cells in mammary gland appears to promote host protective responses during early-stage *S. aureus*-induced mastitis.

γδ T cells comprise several heterogeneous subsets, and different subsets display unique functions during bacterial infection. In a model of *S. pneumoniae* pulmonary infection, numbers of Vγ1, Vγ4, and Vγ6 γδ T cells in lung were significantly elevated.[24] Vγ4 γδ T cells play an essential role in neutrophil-mediated host defense against *S. pneumoniae* infection by promoting synthesis of TNF-α, and possibly of MIP-2, in lungs.[47] During *Bordetella pertussis* infection, Vγ4 γδ T cells in lungs produced IL-17 as early as 2 h p.i., thereby providing protection against primary infection through induction of antimicrobial peptides Different subsets of γδ T cells have different cytokine expression characteristics, and their crucial role in innate immunity against various infections is increasing clear.[45,48–50] Especially, IL-17A-producing γδ T cell was found to be important in neutrophil recruitment and host defense against *S. aureus* infections.[20,21,45,51] Seven γδ T cell subsets are known; however, our RT-PCR analyses revealed the presence of only two subsets, Vγ1 and Vγ4, in mammary gland (data not shown). Similarly, we found that $V\gamma4^+$ γδ T cells, rather than $V\gamma1^+$ γδ T cells, are the producers of IL-17A and mediating clearance of pathogens via recruitment neutrophils during early-stage *S. aureus* mastitis in a mouse model (Figures 4D and 4E).

Finally, the role of microbiota in the development and function of γδ T cells has been investigated in several researches. Commensal bacteria are a key factor in the maintenance of $IL\text{-}1R1^+$ γδ T cells which is a potential source of IL-17A that can be activated by IL-23 and IL-1 in both infectious and noninfectious settings *in vitro* and *in vivo*.[52] The expansion of IL-17-producing $V\gamma6^+$ γδ T cells in testicular depends on commensal microbiota and promotes immune surveillance against *Listeria monocytogenes*.[53] Oral probiotics for the treatment of breast infections have been evaluated in human and rodents.[26,54,55] In this work, we proved that commensals promote defense against *S. aureus* mammary gland infection and drive the expansion of γδT17 cells and neutrophils during infection in a mouse model (Figures 6A–6F). We found that $V\gamma4^+$ γδ T cell was the main subtype of microbiota-dependent expansion γδ T cells during infection (Figures 6G and 6J). However, the expansion of $V\gamma1^+$ γδ T cell showed an opposite tendency to $V\gamma4^+$ γδ T cell during *S. aureus*-induced mastitis, and the mechanism remains unclear (Figures 6G and 6H). In addition, the mechanism by which the microbiota activates γδ T cells to promote local IL-17 and IFN-γ production during *S. aureus* mammary gland infection remains to be elucidated. Nevertheless, this concept of microbiota treating or preventing mastitis is incompatible with current knowledge concerning intramammary microbiota and local immune system of dairy ruminants.[28] It is likely that the mammary gland of ruminants is not part of the common mucosal immune system as originally defined in monogastric species.[56,57] Therefore, a bovine mastitis model is indispensable to further verify the regulatory role of intramammary microbiota and γδ T cells in local defense.

Collectively, this research elucidated the expansion of γδ T cells, specifically $V\gamma4^+$ γδ T cells, were critical in IL-17A-mediated immune responses, including induction of proinflammatory cytokines production, neutrophil recruitment, and bacterial clearance. The crucial role that γδT17 cells play in promoting host defense may depended on commensal microbiota. Our findings help to clarify local immune responses mechanisms and provide an insight into therapeutic strategies against *S. aureus*-induced mastitis.

Limitations of the study

The current study provided insights into γδT17 cells-mediated innate protection in against *S. aureus*-induced mastitis, while there are some limitations to consider. First, the study focuses on IL-17A, while IFN-γ, which was also increased in the early stage of infection, has not been explored. It has been reported that γδ T cells may also be the potential sources of IFN-γ, and the role of IFN-γ during *S. aureus*-induced infection remains to be further explored. Second, to verify the anti-infection of the commensal microbiota, antibiotics were used to reduce the bacterial counts and disrupt homeostasis, rather than using strictly sterile Germ-free mice; further investigation and experimental validation are warranted to dissect the precise functions and mechanisms involved.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108453.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.W., L.X., and N.P.; methodology, L.X., N.P., Y.X., Y.L., H.Z., X.B., B.L., Y.F., and H.G.; formal analysis, J.W., H.L., C.M., S.S., and T.W.; writing – original draft, N.P. and L.X.; writing – review & editing, N.P.; funding acquisition, X.W.; supervision, X.W., L.X., and N.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research. We avoided "helicopter science" practices by including the participating local contributors from the region where we conducted the research as authors on the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC-labeled anti-mouse CD3e (145-2C11)	eBioscience	Cat# 17-0031-82; RRID: AB_469315
PE/Cy7-labeled anti-mouse TCR γ/δ (GL3)	eBioscience	Cat# 25-5711-82; RRID: AB_2573464
FITC-labeled anti-mouse CD4 (RM4-5)	eBioscience	Cat# 11-0042-82; RRID: AB_464896
FITC-labeled anti-mouse CD8 (53–6.7)	eBioscience	Cat# 11-0081-82; RRID: AB_464915
FITC-labeled anti-mouse NK1.1 (PK136)	eBioscience	Cat# 11-5941-82; RRID: AB_465318
PE-labeled anti-mouse F4/80 (BM8)	eBioscience	Cat# 12-4801-82; RRID: AB_465923
FITC-labeled anti-mouse CD11b (M1/70)	eBioscience	Cat# 11-0112-82; RRID: AB_464935
PE-labeled anti-mouse Ly-6G (1A8-Ly6g)	eBioscience	Cat# 11-9668-82; RRID: AB_2572532
FITC-labeled anti-mouse CD44 (IM7)	eBioscience	Cat# 11-0441-82; RRID: AB_465045
FITC-labeled anti-mouse CD69 (H1.2F3)	eBioscience	Cat# 12-0691-82; RRID: AB_465732
PE-labeled anti-mouse IL-17A (eBio17B7)	eBioscience	Cat# 12-7177-81; RRID: AB_763582
PE-labeled anti-mouse IFN-γ (XMG1.2)	eBioscience	Cat# 12-7311-82; RRID: AB_466193
PE-labeled anti-mouse TCR Vγ1.1 (2.11)	BioLegend	Cat# 141104; RRID: AB_10697031
FITC-labeled anti-mouse TCR Vγ2 (UC3-10A6)	BioLegend	Cat# 137704; RRID: AB_10569353
InVivoMAb anti-mouse TCR Vγ1.1/Cr4 (2.11)	Bio X Cell	Cat# BE0257; RRID: AB_2687736
InVivoMAb anti-mouse Vγ2 TCR (UC3-10A6)	Bio X Cell	Cat# BE0168; RRID: AB_10950109
Bacterial and virus strains		
Staphylococcus aureus ATCC27543	ATCC	ATCC: NCDO-1499
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Coolaber	Cat# CA2031
Neomycin sulfate	Coolaber	Cat# CN7741
Metronidazole	Coolaber	Cat# CM7361
Vancomycin hydrochloride	Coolaber	Cat# CV11721
PBS 1×	Gibco	Cat#14040-133
FBS	BI	Cat# 04-007-1A
Collagenase D	Sigma	Cat# COLLD-RO
RPMI 1640 Medium	Gibco	Cat# 11875093
BSA	Solarbio	Cat# A8010
Red blood cell lysis buffer	Solarbio	Cat# R1010
PMA/Phorbol 12-myristate 13-acetate	Sigma	Cat# P8139-1MG
Ionomycin	Sigma	Cat# I3909-1ML
Brefeldin A	eBioscience	Cat# 00-4506-51
4% Paraformaldehyde	Solarbio	Cat# P1110
TRIzol reagent	Invitrogen	Cat# 15596026
Critical commercial assays		
BD Cytofix/Cytoperm Fixation/Permeabilization Kit	BD	Cat# 554714; RRID: AB_2869008
Mouse IL-4 DuoSet ELISA	R&D	Cat# DY404-05
Mouse IL-17 DuoSet ELISA	R&D	Cat# DY421-05
Mouse IL-17F DuoSet ELISA	R&D	Cat# DY2057

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse IL-22 DuoSet ELISA	R&D	Cat# DY582-05
Mouse IL-23 DuoSet ELISA	R&D	Cat# DY1887-05
Mouse IFN-γ DuoSet ELISA	R&D	Cat# DY485-05
Mouse CXCL1/KC DuoSet ELISA	R&D	Cat# DY453-05
Mouse CXCL2/MIP-2 DuoSet ELISA	R&D	Cat# DY452-05
Qiagen gel extraction kit	Qiagen	Cat# 28704
TruSeq DNA PCR-free sample preparation kit	Illumina	Cat# FC-121-3001
TruSeq stranded mRNA LT sample preparation kit	Illumina	Cat# RS-122-2103
Deposited data		
16S amplicon sequencing data	NCBI	http://ncbi.nlm.nih.gov/sra; NCBI SRA: PRJNA954128
Transcriptome sequencing data	NCBI	http://ncbi.nlm.nih.gov/sra; NCBI SRA: PRJNA1031084
Experimental models: Organisms/strains		
Mouse: C57BL/6 (7-12-week-old)	Spfbiotech	N/A
Mouse: IL-17A$^{-/-}$	Cyagen Biosciences	Cat# S-KO-02627
Mouse: TCRδ$^{-/-}$	Zhinan Yin Lab	N/A
Oligonucleotides		
Primer: IL-17A$^{-/-}$ confirmation; IL17AF1: 5′-GCAGCTTCAGATATGTCCATACAC-3′	This paper	N/A
Primer: IL-17A$^{-/-}$ confirmation; IL17AR1: 5′-GTTACGCTTCAAAGCTATCTTG-3′	This paper	N/A
Primer: IL-17A$^{-/-}$ confirmation; IL17AR2: 5′-CTGTGATCTGGGAAGCTCAGTG-3′	This paper	N/A
Primer: TCRδ$^{-/-}$ confirmation; TCRδF1: 5′-CTTGGGTGGAGAGGCTATTC-3′	This paper	N/A
Primer: TCRδ$^{-/-}$ confirmation; TCRδF2: 5′-CAAATGTTGCTTGTCTGGTG-3′	This paper	N/A
Primer: TCRδ$^{-/-}$ confirmation; TCRδR1: 5′-AGGTGAGATGACAGGAGATC-3′	This paper	N/A
Primer: TCRδ$^{-/-}$ confirmation; TCRδR2: 5′-GTCAGTCGAGTGCACAGTTT-3′	This paper	N/A
Primer: 16S rRNA sequencing 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′	This paper	N/A
Primer: 16S rRNA sequencing 806R 5′-GGACTACHVGGGTTCTAAT-3′	This paper	N/A
Software and algorithms		
NovoExpress	Agilent	https://www.agilent.com.cn/; RRID:SCR_024676
GraphPad Prism	GraphPad Software	https://www.graphpad.com/; RRID:SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiao Wang (wangxiao@imu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The demultiplexed reads for 16S amplicon sequencing and transcriptome sequencing data have been deposited at the NCBI Sequence Read Archive [http://ncbi.nlm.nih.gov/sra] and are publicly available as of the date of publication. Accession number are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

Experimental animal protocols were performed in accordance with guidelines of the Ethics Committee of Inner Mongolia University (IMU-mouse-2020-039). Specific-pathogen-free (SPF) C57BL/6 WT mice were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). IL-17A$^{+/-}$ mice (genetic background C57BL/6) were from Cyagen Biosciences (Suzhou, China). TCRδ$^{+/-}$ with C57BL/6 background were kindly donated by Dr. Zhinan Yin (Jinan University, Guangzhou, China). IL-17A KO (IL-17A$^{-/-}$) and TCRδ KO (TCRδ$^{-/-}$) mice were generated by crossing IL-17A$^{+/-}$ and TCRδ$^{+/-}$ mice. Mice were typed by PCR analysis of tail DNA. All experiments were performed using 7- to 12-week-old mice. These animals were housed at relatively constant humidity (40%–60%), temperature (20°C–22°C), and a 12-h light/dark cycle, and maintained on a normal chow diet with free access to water. One male and two females were co-housed in one micro-isolator cage. When pregnancy was confirmed, each female was housed individually, and used for further experiments when they were raising five of more pups.

Microbe strains

S. aureus strain ATCC27543 was purchased from American Type Culture Collection (Manassas, VA, USA). *S. aureus* strain ATCC27543 was streak onto tryptic soy agar (TSA) plates (tryptic soy broth [TSB] plus 1.5% agar [HuanKai Microbial, Guangdong, China]) and grown overnight at 37°C. Single colonies were cultured in TSB at 37°C in a shaking incubator (220 rpm) for 12 h, followed by a 2-h subculture of a 1:50 dilution of the overnight culture. The bacteria were collected by centrifugation (10,000×g, 10 min, 4°C), resuspended in sterile PBS, and washed 3 times. The absorbance (OD_{600}) was measured to estimate the CFU for inoculation, which was verified after overnight culture on TSA plates.

METHOD DETAILS

Mouse model of *S. aureus*-induced mastitis

To establish *S. aureus*-induced mouse mastitis model, lactating mice were anesthetized by intraperitoneal injection of ketamine/xylazine cocktail on day 7 post-parturition. The teats and surrounding areas were disinfected with 75% ethanol. The udder canals of the fourth and fifth pairs of mammary glands were held lightly with fine forceps and exposed. 1×10^7 CFU *S aureus* dissolved in 30 μL PBS was injected through the mammary gland ducts using blunt 31G needles. Mice were left to recover in their cage following the injection. Twenty-four hours later, the mice were sacrificed, and mammary glands were collected for analysis.

Bacterial counts

To access bacterial burden, the mammary gland tissues were homogenized in sterile PBS (0.9 mL/0.1 g tissue) using a tissue homogenizer (Analytikjena, Yena, Germany). Tissue homogenates were prepared in PBS, and serial dilutions were plated on tryptic soy agar for determination of bacterial counts to determine the bacterial burden. After 18 h incubation at 37°C, CFUs were measured by standard plate counting.

Histology analysis

Mammary gland tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and 4-μm sections were cut and subjected to H&E and Gram staining. The stained sections were imaged under a microscope.

Flow cytometry

Fluorescence-activated cell sorting (FACS), a type of flow cytometry was performed to measure expression of cell surface markers and intracellular cytokines. In brief, mammary glands were removed aseptically and cut into small pieces, digested at 37°C for 1 h with a concentration of 1 mg/mL collagenase D (Sigma), and then filtered through steel sieves (40 μm mesh). Total cell pellet was resuspended in 5 mL RPMI 1640 (Biological Industries, Beit HaEmek, Israel) and centrifuged (1000 rpm, 10 min, room temperature). Cells in precipitate were collected, washed with PBS, blocked with PBS plus 10% BSA on ice for 15 min, and stained for 30 min with the following specific Abs (all from eBioscience [San Diego, CA, USA] except the last two, which were from Tianjin Sungene Biotech Co. [Tianjin, China]): APC-*anti*-CD3 (clone 17A2), PE-cy7-*anti*-TCR γ/δ (clone GL3), FITC-*anti*-CD4 (clone RM4-5), FITC-*anti*-CD8 (clone 53–6.7), FITC-*anti*-NK1.1 (clone PK136), FITC-*anti*-CD11b (clone M1/70), FITC-*anti*-CD44 (clone IM7), FITC-*anti*-CD69 (clone H1.2F3), PE-*anti*-Ly-6G (clone 1A8-Ly6g), PE-*anti*-F4/80 (clone BM8), PE-*anti*-TCR Vγ1 (clone 2.11), and/or FITC-*anti*-TCR Vγ4 (clone UC3-10A6). For intracellular cytokine staining, cells were first stimulated in culture medium containing 50 ng/mL PMA (Sigma) and 1 μg/mL ionomycin (Sigma) for 4–6 h at 37°C, 5% CO_2, in the presence of 10 ng/mL Brefeldin A (eBioscience). After cell surface staining, cells were fixed and permeabilized with Perm/Wash solution (BD Bioscience; San Jose, CA, USA), and separately stained intracellularly with PE-*anti*-IFN-γ (XMG1.2) or PE-*anti*-IL-17A (TC11-18 H10) (eBioscience). FACS was performed based

on acquisition of ~300,000 events, and data were analyzed using software program NovoExpress. Cells were gated to identify mammary gland γδ T cells and γδ T17 cells as shown in Figure S2A.

Enzyme-linked immunosorbent assay (ELISA)

Mammary gland homogenates (from tissues collected at 6 h infection time) were obtained as above, and IL-4, IL-22, IL-17A, IL-17F, IL-23, IFN-γ, KC (CXCL-1), MIP-2 (CXCL-2), and MPO protein levels were measured by ELISA (R&D Systems; Minneapolis, MN, USA) as per manufacturer's protocol.

Depletion of Vγ1$^+$ and Vγ4$^+$ γδ T cells

Vγ1$^+$ (clone 2.11) and Vγ4$^+$ (clone UC3-10A6) γδ T cells were depleted using antibodies from Bio X Cell (Lebanon, NH, USA). Antibodies (dosage 200 μg per 100 μL PBS) were i.v. injected in tail vein three days before *S. aureus* mammary gland infection. Control mice were injected with sterile PBS.

Microbiota disruption and reconstitution

The process of microbiota disruption and feces microbiota transplantation was performed as previously described.[26,59] Mice were given broad-spectrum antibiotics (ampicillin 1 g/L, neomycin 1 g/L, metronidazole 1 g/L, and vancomycin 0.5 g/L) in drinking water for 3 weeks for microbiota disruption. Fresh feces were collected from untreated donor mice. Fecal pellets from different healthy mice were mixed with sterile PBS (0.1 g feces/1 mL PBS), then homogenized immediately. The homogenate was centrifuged (100×g, 2 min, 4°C), and the supernatant was collected for transplantation. A total of 200 μL of the supernatant was administrated to ABX treatment mice by oral gavage for 7 consecutive days for microbiota reconstitution. The mice of the No ABX group were administrated an equal volume of vehicle (200 μL PBS).

Microbiota assay

Fresh feces and mammary gland tissues were collected under sterile conditions and immediately frozen in liquid nitrogen and then stored at −80°C. Total bacteria genomic DNA samples were extracted using the CTAB/SDS method. 16S rRNA genes of distinct regions were amplified using specific primers (16S V4: 515F-806R) with barcodes. All PCR reactions were purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. Library quality was assessed on the Qubit @ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on a NovoSeq 6000 platform, and 250-bp paired-end reads were generated.

RNA sequencing

Mammary gland tissues were collected and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Libraries were constructed using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced on an Illumine HiSeq X Ten platform and 150 bp paired-end reads were generated. Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using software program GraphPad Prism V. 8 (San Diego, CA, USA). Values are expressed as mean ± SEM. Unpaired t-test or one-way analysis of variance (ANOVA) was used for comparison of two group or multiple groups. A non-parametric test analysis (Kruskal-Walis or Mann-Whitney) was used for comparison groups with few samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.