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## A TSLP-complement axis mediates neutrophil killing of methicillin-resistant *Staphylococcus aureus*

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### Abstract

Community-acquired *Staphylococcus aureus* infections often present as serious skin infections in otherwise healthy individuals, and have become a world-wide epidemic problem fueled by the emergence of strains with antibiotic resistance, such as methicillin-resistant *S. aureus* (MRSA). The cytokine, thymic stromal lymphopoietin (TSLP), is highly expressed in the skin and other barrier surfaces and plays a deleterious role by promoting T helper type 2 (T<sub>H</sub>2) responses during allergic diseases; however, its role in host-defense against bacterial infections has not been well-elucidated. Here, we describe a previously unrecognized non-T<sub>H</sub>2 role for TSLP in enhancing neutrophil killing of MRSA during an *in vivo* skin infection. Specifically, we demonstrate that TSLP acts directly on both mouse and human neutrophils to augment control of MRSA. Additionally, we show that TSLP also enhances killing of *Streptococcus pyogenes*, another clinically important cause of human skin infections. Unexpectedly, mechanistically TSLP mediates its anti-bacterial effect by directly engaging the complement C5 system to modulate production of reactive oxygen species by neutrophils. Thus, TSLP increases MRSA killing in a neutrophil- and complement-dependent manner, revealing a key connection between TSLP and the innate complement system, with potentially important therapeutic implications for control of MRSA infection.

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## Introduction

The gram positive bacterium, *Staphylococcus aureus*, is the most common cause of bacterial skin infections, causing millions of outpatient and emergency room visits per year (1). Whereas *S. aureus* infections are endemic in hospitals world-wide and were once mainly considered to be hospital-acquired, the emergence of new and more virulent antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), has resulted in an increase in community-acquired infections in otherwise healthy people, which are not limited to the hospital setting. USA300 is the most prevalent community-acquired MRSA strain in the United States and presents as a skin infection in approximately 90% of cases (2). Despite typically beginning as skin infections, MRSA infections can lead to life threatening and invasive infections, including pneumonia, sepsis, and meningitis. Overall, MRSA infections cause more deaths in the United States than HIV, viral hepatitis, and tuberculosis combined, with 94,360 severe invasive infections and 18,650 deaths in 2005 (2–4). To combat this increasing epidemic, particularly in an era of decreased antibiotic efficacy, an understanding of the factors governing the protective immune response is required to help develop immunotherapies to combat these infections.

Neutrophils are the first line of defense against bacterial infections and play a vital role in host-defense against *S. aureus* by employing multiple mechanisms to kill bacteria, including phagocytosis, production of reactive oxygen species (ROS), anti-microbial peptides, and proteinases and acid hydrolases that degrade bacterial components (4). The importance of neutrophils in combating *S. aureus* infection is underscored by the recurrent *S. aureus* infections in patients with chronic granulomatous disease, which is characterized by defects in neutrophil respiratory burst and NADPH oxidase (5). Additionally, neutropenic cancer patients have an increased incidence of *S. aureus* infections, resulting in increased mortality and morbidity (6). In the mouse, neutrophil abscess formation is required for bacterial clearance in the skin, and mice depleted of neutrophils have a non-healing skin infection with increased bacteremia (7). Thus, neutrophils play a critical role in host-defense against *S. aureus* infections.

TSLP was originally described as a stromal factor with actions on B and T cells (8) but subsequently was shown to be produced by a broad range of non-hematopoietic cells, with additional actions on dendritic cells, basophils, eosinophils, macrophages, smooth muscle cells, macrophages, and mast cells (9, 10). TSLP has been most extensively studied in the context of allergic diseases, including asthma and atopic dermatitis, where it promotes disease in atopic individuals in a T<sub>H</sub>2 dependent manner(10–14). A recent study reported that TSLP inhibits production of IL-22 by type 3 innate lymphoid cells (ILC3 cells) in the gut during *Citrobacter rodentium* infection, thereby reducing the host's ability to control this bacterial infection (15); however, little is known of the role of TSLP in host defense to other bacterial infections, including those in the skin. Better understanding the role of TSLP in host defense is crucial, as responses and the role(s) of specific cytokines can differ greatly based on the site of disease/infection, with for example, contrasting clinical outcomes to IL-17 blockade in patients with psoriasis versus Crohn's disease (16, 17). Since TSLP is highly expressed in the skin (18) and there is an increasing prevalence of MRSA skin infections (19), we investigated whether TSLP can contribute to host-defense against skin

MRSA infection and now demonstrate that TSLP acts directly on both human and mouse neutrophils to enhance MRSA clearance in a complement- and ROS-dependent manner.

## Results

### TSLP enhances MRSA killing in a whole blood assay

We initially assessed whether TSLP promotes MRSA killing in an *in vitro* whole blood assay. Incubating TSLP together with MRSA in mouse blood significantly increased bacterial killing at both 2 and 3 hours, as compared to that observed with the addition of PBS and MRSA (assayed by colony forming units, CFU) (Fig. S1, A and B, and Fig. 1, A). We excluded the possibility that the increased killing of MRSA by TSLP resulted from a direct action of TSLP on the bacteria (Fig. S1C), and we thus sought to define the cell type that mediated TSLP-induced killing of the bacteria. Neutrophils are critical for host-defense against *S. aureus* (20), and we found that mouse bone marrow neutrophils not only express the TSLP binding protein (receptor), TSLPR, but that TSLPR expression was further increased upon *in vitro* stimulation with heat-killed *S. aureus* (HKSA) in these cells (Fig. 1B). These data suggested that mouse neutrophils might exhibit enhanced responsiveness to TSLP during MRSA infection. To determine if neutrophils were required for the action of TSLP, we depleted mice of neutrophils by using anti-Ly6G (Fig. 1C). When neutrophil-depleted blood was used in the whole blood killing assay, TSLP no longer augmented MRSA killing (Fig. 1D), demonstrating that the increased killing of MRSA induced by TSLP was neutrophil-dependent. Importantly, neutrophils are potent killers of bacteria and while depletion of neutrophils in the blood resulted in reduced control of bacteria in general, in line with the important role neutrophils play in bacterial clearance, it did not result in complete loss of bacterial control (Fig. S1D), consistent with the contributions of other cell types, such as macrophages, to MRSA clearance. Taken together, these data demonstrate that TSLP-enhanced killing of MRSA is neutrophil-dependent.

### TSLP acts directly on both mouse and human neutrophils to increase killing of MRSA

To determine whether TSLP could act directly on neutrophils, we next purified thioglycollate-elicited mouse peritoneal neutrophils, as less mature bone marrow neutrophils are incapable of killing MRSA *in vitro* (Fig. S2A), and first demonstrated that they expressed TSLPR (Fig. 2A). Moreover, when these neutrophils were incubated with MRSA and TSLP for 2 h, they exhibited increased killing as compared to cells incubated with MRSA and PBS (Fig. 2B), demonstrating that TSLP can act directly on mouse neutrophils *in vitro* to enhance MRSA killing. This direct effect of TSLP on neutrophils was TSLPR-dependent, as TSLP did not increase the killing of MRSA by *Tslpr*<sup>-/-</sup> neutrophils (Fig. S2B).

We next investigated whether TSLP exerts similar effects on human neutrophils. Indeed, TSLP treatment resulted in increased killing of MRSA in a whole blood killing assay (a representative donor is shown in Fig. 2C, with all donors shown in Fig. S2C). Although two previous studies reported that a synthetic short form of human TSLP could have direct antimicrobial activities on some pathogens, they observed little or no killing with *S. aureus* (21, 22). Consistent with this, we found that the increased killing of MRSA by

TSLP did not result from a direct action of TSLP on the bacteria, as MRSA and TSLP incubated together with serum alone (i.e., in the absence of cells) resulted in a similar bacterial titer to that observed when control PBS was used in place of TSLP (Fig. S2D). To determine whether TSLP-induced killing of MRSA in human whole blood was mediated by neutrophils, analogous to what we found for the mouse, we purified neutrophils from whole blood from healthy donors. These human neutrophils expressed mRNA for *CRLF2* (encoding TSLPR), and its expression was significantly enhanced by stimulation with heat killed *S. aureus* (HKSA), ranging from 5–76 fold enhancement depending on the donor (one donor shown in Fig. 2D). This increase in *CRLF2* expression by HKSA was likely due to TLR2 activation, as we found that stimulation of neutrophils with peptidoglycan, a TLR2 agonist present on gram positive bacteria including *S. aureus*, also increased *CRLF2* expression (Fig. S2E). Consistent with these mRNA expression data, the purified human neutrophils also expressed TSLPR protein, with higher expression upon HKSA stimulation (Fig. 2E), indicating that human neutrophils might also be able to respond to TSLP. Indeed, when freshly isolated human neutrophils were incubated with PBS or TSLP and MRSA for 3 h, TSLP markedly lowered the CFU (a representative donor is shown in Fig. 2F and all donors tested are depicted in Fig. S2F). Since priming of neutrophils can enhance their function (23, 24), we primed freshly isolated human neutrophils with HKSA and either PBS or TSLP and found that TSLP increased the ability of primed neutrophils to kill MRSA *in vitro* (Fig. S2G), analogous to unprimed neutrophils. Consistent with our experiments in mice, these data demonstrate that TSLP acts directly on both unprimed and primed human neutrophils to increase their killing of MRSA.

### ***Tslpr*-deficient mice have increased MRSA titers during an *in vivo* skin infection**

We next investigated whether the TSLP-neutrophil axis also enhanced MRSA killing *in vivo* by using a skin infection model in which MRSA was injected intradermally (i.d.) into the mouse ear. Interestingly, TSLP protein was potently increased in the ears at days 1 and 2 post-infection (p.i.) with MRSA, as compared to naïve PBS-injected controls (Fig. 3A). Additionally, TSLPR was expressed by ear neutrophils (Fig. 3B). To elucidate the role of TSLP in skin MRSA infection, we infected mice with MRSA i.d. and found that *Tslpr*-deficient (*Tslpr*<sup>-/-</sup>) mice had a significantly higher bacterial burden than did wild-type (WT) mice (Fig. 3C), indicating that TSLP helps to control MRSA *in vivo*. The increased bacterial burden in *Tslpr*<sup>-/-</sup> mice was not due to reduced recruitment of neutrophils to the ear, as *Tslpr*<sup>-/-</sup> and WT mice had similar percentages (Fig. 3, D and E) and numbers (Fig. 3F) of neutrophils in their infected ears. To eliminate the possibility that the *in vivo* results we had observed resulted from compensatory mechanisms in *Tslpr*<sup>-/-</sup> mice, we treated WT mice with either a human IgG1 Fc isotype control or TSLPR-Fc fusion protein i.d. at the time of MRSA infection and found that the mice with *in vivo* TSLP blockade (TSLPR-Fc treated) had significantly increased MRSA titers in the ear compared to isotype control treated mice, confirming that TSLP enhances bacterial control during *in vivo* MRSA skin infection (Fig 3G).

### **TSLP treatment enhances MRSA killing *in vivo* in normal WT hosts**

We next sought to determine whether increased TSLP signaling could augment MRSA killing in the skin of normal hosts and therefore injected PBS or TSLP plus MRSA i.d.

into the ears of WT mice. TSLP treatment significantly reduced the bacterial burden in the ears at day 2 p.i. (Fig. 4A), and this effect was sustained as one injection of TSLP at the time of infection resulted in significantly reduced titers even at days 3 and 6 p.i. (Fig. S3A). Because bacterial titers can only be assessed at one time-point per mouse we also evaluated if TSLP has a more cumulative effect by assessing pathology in these mice and found that TSLP also decreased pathological changes, with significantly decreased inflammation in the skin after MRSA infection compared to that observed in PBS-treated animals (Fig. 4, B and C). Moreover, the effect of TSLP was mediated by its functional receptor rather than an off-target effect, as *Tslpr*<sup>-/-</sup> mice treated with TSLP had similar MRSA titers to those treated with PBS (Fig. S3B). To determine whether TSLP's ability to increase *in vivo* killing of bacteria was limited to MRSA, we tested whether TSLP could also enhance the killing of both a non-MRSA strain of *S. aureus* (MW2) and *Streptococcus pyogenes*, another bacterial strain that causes clinically significant human skin infections (25). Indeed, WT mice treated with TSLP had significantly lower *S. aureus* MW2 and *S. pyogenes* titers compared to PBS-treated control mice (Figs. S3C and 4D). Thus, treatment with TSLP not only can decrease MRSA burden *in vivo* but also can kill a non-MRSA strain of *S. aureus* and another pathogenic bacterial strain (*S. pyogenes*) in the skin as well.

Additionally, *Tslpr*<sup>-/-</sup> infected mice had a similar bacterial burden to that observed in neutrophil-depleted WT mice (Fig. 4E), suggesting that TSLP-enhanced MRSA killing *in vivo* might be dependent upon neutrophils. Importantly, in contrast to its ability to enhance MRSA control in mice treated with an isotype control antibody, TSLP treatment did not increase MRSA control in neutrophil-depleted (anti-Ly6G treated) WT mice, thus demonstrating that TSLP-enhanced MRSA killing *in vivo* was dependent on neutrophils (Fig. 4F).

### TSLP acts directly on neutrophils *in vivo* to decrease MRSA burden

Having shown above that TSLP acts directly on both mouse and human neutrophils to enhance MRSA killing *in vitro* and that TSLP effects *in vivo* were neutrophil-dependent, we next investigated whether TSLP acts directly on neutrophils *in vivo*. Unfortunately, a neutrophil-specific Cre is not available, and LysM-Cre affects monocytes/macrophages as well as neutrophils (26). We thus used a cell transfer approach in which we co-transferred equal numbers of purified WT and *Tslpr*<sup>-/-</sup> bone marrow neutrophils into naïve mice, which could be distinguished by their expression of different isoforms of the congenic marker CD45. After infection i.d. with MRSA in the ear, transferred *Tslpr*<sup>-/-</sup> neutrophils were recruited to the infection site and accumulated there equally well as WT neutrophils (Fig. 5A). We next adoptively transferred an equal number of CMDFA-labeled WT or *Tslpr*<sup>-/-</sup> purified bone marrow neutrophils into *Tslpr*<sup>-/-</sup> mice and then injected these mice with MRSA and TSLP i.d. in the ear, as outlined in Fig. 5B. In these experiments, only the transferred WT neutrophils can respond to TSLP. On day 1 p.i., the *Tslpr*<sup>-/-</sup> mice that received WT neutrophils exhibited significantly greater MRSA killing (i.e., lower CFU) than mice receiving *Tslpr*<sup>-/-</sup> neutrophils (Fig. 5C). Importantly, this difference in MRSA titer was not due to less efficient recruitment of *Tslpr*<sup>-/-</sup> neutrophils than of WT neutrophils, as the percent of transferred *Tslpr*<sup>-/-</sup> neutrophils was even slightly higher than for WT neutrophils (Fig. 5, D and E), and the overall numbers of *Tslpr*<sup>-/-</sup> and WT transferred neutrophils in

the ear were similar (Fig. 5F). Given that TSLP does not directly act on MRSA (Figs. S1C and S2C) and requires TSLPR signals to act both *in vitro* (Fig. S2B) and *in vivo* (Fig. S3B), these data together demonstrate that TSLP acts directly on neutrophils *in vivo* to enhance MRSA clearance.

### A non-transcriptional mechanism for TSLP-mediated MRSA killing by neutrophils

We next sought to elucidate the mechanism underlying TSLP-mediated killing of MRSA. We initially performed RNA sequencing (RNA-Seq) on purified human neutrophils treated with PBS or TSLP with or without HKSA for 4 and 24 h. We found that TSLP did not significantly alter the transcriptional profile of human neutrophils at either 4 or 24 h, whereas HKSA greatly increased the number of differential expressed genes (1394 genes common to both donors at 4 h and 1252 at 24 h). As compared to HKSA alone, the addition of TSLP plus HKSA resulted in the common induction in both donors of only a single gene (CCL22) at 24 h (Table S1). These data suggest that TSLP-mediated neutrophil killing of MRSA is not due to transcriptional activation of new gene expression during the time of the killing assays and that proximal signaling events instead might be involved. Indeed, studies using inhibitors of mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) kinase or phosphatidylinositol 3-kinase (PI3K) showed that both of these pathways are necessary for TSLP-mediated killing of MRSA by human neutrophils, as pre-treatment with these inhibitors blocked TSLP-increased MRSA killing (Fig. S4, A and B for MAPK/ERK inhibition and Fig. S4, A and C for PI3K inhibition) but did not eliminate the basal ability of human neutrophils to kill MRSA (Fig. S4D). Given the rapid TSLP-induced neutrophil-mediated killing of MRSA (2–3 h for the *in vitro* assay) and the fact that the MAPK/ERK and PI3K pathways can mediate non-transcriptional effects in neutrophils (27–29), our results indicate that TSLP-mediated MRSA killing by neutrophils is a rapid response that does not require *de novo* gene induction.

### TSLP-enhanced killing of MRSA in both mouse and human is ROS-dependent

As phagocytosis of microbes is an important rapid response of neutrophils, we hypothesized that TSLP might increase neutrophil phagocytosis. Pathogen uptake is likely necessary for TSLP-enhanced killing of MRSA as treatment of neutrophils with cytochalasin D, an inhibitor of phagocytosis, eliminated TSLP-enhanced killing of MRSA *in vitro* (Fig S5A). To our surprise, however, TSLP treatment did not affect expression of CD11b (a component of the phagocytic CR3 receptor) on human neutrophils *in vitro* (Fig. S5B) or on mouse neutrophils *in vivo* (Fig. S5C). Moreover, TSLP did not augment the phagocytic uptake of *S. aureus* by either human (Fig. S5D) or mouse (Fig. S5, E and F) neutrophils.

A major mechanism used by human and mouse neutrophils to eliminate bacteria is the production of reactive oxygen species (ROS) (20), and we therefore evaluated the role of ROS in TSLP-driven MRSA killing *in vivo* utilizing the mouse skin infection model. Strikingly, neutrophils from infected *Tslpr*<sup>-/-</sup> mice had lower ROS levels (Fig. 6, A and B) compared to neutrophils from infected WT mice, indicating that ROS might contribute to TSLP-enhanced neutrophil killing of MRSA. Consistent with this notion, TSLP treatment did not enhance MRSA killing when a ROS scavenger, N-acetyl-L-cysteine, (NAC) (30) was administered i.d. (Fig. 6C), demonstrating that ROS is essential for TSLP-induced

neutrophil-mediated killing of MRSA *in vivo*. To eliminate the possibility that these data resulted from non-specific actions of NAC, we used *Gp91<sup>phox</sup>-/-* (*Nos2<sup>-/-</sup>*) mice, which are deficient in an integral component of the NADPH oxidase complex that generates ROS. TSLP treatment did not increase the killing of MRSA in *Gp91<sup>phox</sup>-/-* mice infected i.d. *in vivo* with MRSA, unlike its effect in WT controls (Fig. 6D), demonstrating that ROS is essential for TSLP-induced neutrophil-mediated killing of MRSA *in vivo*. Consistent with an essential role for ROS in TSLP-enhanced MRSA killing in the mouse skin infection model, pre-treatment of purified human neutrophils with Diphenyliodonium (DPI), an NADPH-oxidase inhibitor (36), eliminated the ability of TSLP to enhance their killing of MRSA (Fig. 6E), demonstrating that ROS is also essential for TSLP-augmented control of MRSA by human neutrophils.

### TSLP-enhanced killing of MRSA is complement-dependent

The complement system is a highly conserved innate defense system poised to rapidly respond to invading pathogens (31, 32), and binding of the complement activation fragment C5a to the C5a receptor 1 (CD88, C5aR1) expressed on neutrophils drives ROS production in these cells (33, 34). In our whole blood assays above where TSLP promotes the killing of MRSA, we had collected blood with sodium citrate; however, we observed that treatment of mouse blood with EDTA, which prevents complement activation and C5a generation (35), eliminated TSLP-mediated MRSA killing in neutrophils (Fig. S6A). We therefore investigated whether a complement-dependent mechanism was involved in this process. Importantly, local injection of WT mice with a C5-blocking antibody (anti-C5) during i.d. MRSA ear infection decreased ROS production by neutrophils as compared to ROS production by neutrophils from isotype control treated animals (Fig. 7A), showing that C5 can drive neutrophil ROS production in this model. To elucidate the potential role of C5 in TSLP-mediated MRSA killing *in vivo*, we treated WT mice with an isotype control or anti-C5 antibody along with TSLP or PBS during i.d. MRSA infection. Strikingly, whereas TSLP enhanced MRSA killing in isotype control antibody-treated animals, it had no effect in animals treated with anti-C5 (Fig. 7B), demonstrating that C5 is necessary for TSLP-induced neutrophil killing of MRSA *in vivo*. Although the C5a fragment of C5 is an anaphylotoxin that can act as a chemotactic factor for neutrophils (23, 33, 34), local blockade of C5 did not affect neutrophil recruitment to the site of infection, as animals treated i.d. with either anti-C5 or control antibodies had similar numbers of neutrophils in the ear after MRSA infection (Fig. S6, B and C). Additionally, TSLP treatment of WT mice increased C5aR1 expression on neutrophils during MRSA skin infection (Fig. 7, C and D). Expression of C5aR1 indeed appeared critical, as TSLP treatment did not increase MRSA killing in *C5ar1<sup>-/-</sup>* mice (Fig. 7E). Thus, TSLP induces *in vivo* killing of MRSA by neutrophils in a C5- and C5aR1-dependent fashion, via induction of anti-bacterial ROS generation in neutrophils.

We next investigated whether complement C5 was also necessary for the TSLP-enhanced neutrophil killing of MRSA by human neutrophils. Importantly, the C5-axis was also required for TSLP-enhanced killing by human neutrophils, as incubating purified human neutrophils with PMX-53, a peptide antagonist of C5aR1 that has been used in clinical trials (37, 38), prevented the TSLP-induced MRSA killing (Fig. 7F). Thus, TSLP enhanced

MRSA killing by human neutrophils is both ROS- and complement C5-dependent, in agreement with our *in vivo* mouse data. Of note, neither the ROS inhibitor (DPI) nor C5aR1 antagonist (PMX-53) abolished the overall killing of MRSA by human neutrophils (Fig. S6, D and E) but only eliminated the ability of TSLP to enhance neutrophil-killing of MRSA (Figs. 6E and 7F), indicating the critical engagement of this “C5-ROS axis” by TSLP. Moreover, incubation of purified human neutrophils with TSLP increased their secretion of C5a (Fig. 7G), indicating that TSLP may increase conversion of C5 to C5a or cycling of C5a, thereby creating more ligand for C5aR1. These data demonstrate that TSLP engages the C5 system for MRSA killing in both mouse neutrophils in an *in vivo* skin infection and in human neutrophils *in vitro*.

## Discussion

TSLP has been well-characterized as a cytokine that promotes T<sub>H</sub>2-type responses, with detrimental effects in asthma, atopic dermatitis, and other allergic diseases (10–14). Analogously, TSLP modulates intestinal inflammation by enhancing T<sub>H</sub>2-type responses and limiting proinflammatory T<sub>H</sub>1-type responses (39). In the context of infection, however, the role of TSLP is less well known. Respiratory syncytial virus can stimulate TSLP production by lung epithelial cells, promoting an immunopathogenic T<sub>H</sub>2-type response that may be further enhanced in asthmatics, leading to disease exacerbation (40). Consistent with this, TSLP is important for immunity to the nematode *Trichuris muris* due to its promoting a T<sub>H</sub>2-type response and worm expulsion (39), and a recent study demonstrated that TSLP lowers the ability to control *Citrobacter rodentium* infection in the gut (15). Thus, previous studies have generally focused on TSLP's enhancement of T<sub>H</sub>2-type responses. In contrast, our experiments reveal a key unexpected non-T<sub>H</sub>2 function for TSLP that can be protective *in vivo*, with TSLP acting directly on neutrophils to enhance control of both methicillin resistant and sensitive strains of *S. aureus* and *S. pyogenes* in the skin. Conceivably, these results may extend to the control of other bacterial species in the skin, an area for future investigation.

We hypothesize that multiple factors together likely determine whether TSLP plays a beneficial or detrimental role in infection, potentially including the specific infectious agent, the context (e.g., if there is a chronic ongoing T<sub>H</sub>2 response, such as in allergy/asthma), and the site of infection. This notion aligns with the growing understanding that immune responses and their regulation can be drastically distinct in different organs. For example, IL-17 inhibitors, anti-IL-17 and anti-IL17R, are therapeutic for psoriatic skin lesions (16), whereas a neutralizing antibody to IL-17 did not have therapeutic benefit for Crohn's disease and even exacerbated disease in some patients(17). Thus, our identification of a host protective role for TSLP by its enhancing killing of MRSA, *S. aureus* MW2 and *S. pyogenes* in the skin, in contrast to the detrimental effect of TSLP observed in the gut observed with *Citrobacter rodentium* (15), underscores the importance of carefully assessing the impact of TSLP in different organs and in the context of different infections. This idea is further supported by two recent studies that highlight the controversial role of TSLP in sepsis (41, 42). While one study demonstrated that TSLP may play a detrimental role by increasing morbidity and mortality during cecal ligation and puncture (CLP)-induced sepsis in mice (42), a recent study showed the opposite, indicating that TSLP reduces mortality



and morbidity by decreasing inflammation (41). However, both studies show that blockade of TSLP (or use of *Tslpr*<sup>-/-</sup> mice) decreases bacterial titers during CLP-sepsis, implying that TSLP is detrimental for septic bacterial clearance and again demonstrating that the role of TSLP in bacterial infections may depend greatly on either, or both, the location of infection and the infectious agent.

Finally, we identify an unanticipated critical crosstalk between TSLP and the complement system, with therapeutic implications for MRSA skin infections. Given that anti-TSLP (43) and anti-C5 (eculizumab or Soliris) (44) are currently being used therapeutically, our study indicates that one should be cognizant of possible diminished host defense in these settings.

## Material and Methods

### Study Design

The aim of this study was to elucidate and characterize the role of TSLP in neutrophil function, including for MRSA skin infections. The experimental design involved *in vivo* (mouse) and *in vitro* (mouse and human) experiments, including flow cytometric, histological, RNA-seq, and RT-PCR analysis along with bacterial colony enumeration. The animal experiments were not randomized. The investigators were not blinded to allocation during experiments and analyses unless otherwise indicated. Experimental replication is indicated in the figure legends.

### Mice

In experiments where only WT mice were used, 6–9 week old WT BALB/c mice or C57/BL6 mice were obtained from The Jackson Laboratory. *Tslpr*<sup>-/-</sup> (14) and *C5aRI*<sup>-/-</sup> (Jackson Laboratory) mice were bred in our facility. *Gp91<sup>phox</sup>*<sup>-/-</sup> mice were purchased from the Jackson Laboratory. For experiments, using both knockout mice and WT mice, littermate control WT mice were used. 6–9 week old strain-, age- and sex- matched mice were used for experiments. All experiments were performed under protocols approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee and followed National Institutes of Health guidelines for the use of animals in intramural research.

### Bacteria

The USA 300 clinical isolate (FPR3757) of MRSA was used in these studies, except where indicated. For whole blood killing assays, MRSA was plated overnight on a blood agar plate, 1 colony was picked and grown overnight at 37° C shaking in 2 ml of Tryptic Soy Broth (TSB) (Fisher Scientific) and then washed 2 times with PBS. The non-MRSA *S. aureus* strain, MW2, and *S. pyogenes* strain, NZ131, (both from ATCC) were used in the same way as MRSA, except NZ131 was grown in Todd Hewitt Broth under static culture conditions. For intradermal (i.d.) ear infections, bacteria in logarithmic growth were used.

### Whole blood killing assays

Whole blood killing assays were adapted from Kaplan et al. (45) In brief, whole mouse blood was collected into 4% sodium citrate, and whole human blood from healthy donors was collected into sodium citrate tubes. 75 µl of whole blood, 5 µl of 4% sodium citrate,

10  $\mu$ l of PBS or mouse or human TSLP (100 ng/ml final concentration; both from R&D Systems) and 25  $\mu$ l of MRSA (at a 1:1800 dilution of OD<sub>600</sub>= 0.25) were sequentially added to capped 2 ml skirted tubes and the tubes were slowly rotated in a 37° C incubator for 3 h. Serial 10-fold dilutions were then made, and the blood was spread on blood agar plates and incubated overnight. Colonies were counted the following day to determine the colony forming units (CFU)/tube. Experiments were performed with triplicate samples.

### Mouse neutrophil isolation

For eliciting peritoneal neutrophils, mice were injected i.p. with 1 ml of 3% thioglycollate and 4 h later their peritoneums were lavaged with 10 ml cold PBS and cells were collected. For bone marrow neutrophils, femurs from mice were excised under sterile conditions and the cells were flushed out using 2% FBS in PBS + 1 mM EDTA. Both peritoneal and bone marrow neutrophils were purified using either a Miltenyi Biotech negative selection Neutrophil Isolation Kit, or by a 55%/65%/ 75% percoll gradient.

### Human neutrophil isolation and *in vitro* MRSA killing assays

Whole blood from healthy donors was collected in EDTA tubes, and neutrophils were isolated directly from the blood by negative selection using a kit (Stem Cell). For neutrophil killing assays, 3–4  $\times 10^5$  neutrophils (either purified human blood neutrophils or thioglycollate-elicited mouse peritoneal neutrophils) were added to a capped 2 ml skirted tube in RPMI medium. PBS or TSLP (100 ng/ml final), and/or PMX-53 (5  $\mu$ M; Tocris Bioscience) were added and incubated for 5 min. 50  $\mu$ l of coated MRSA or *S. pyogenes* (bacteria at a 1:50 dilution of OD<sub>600</sub>= 0.25) pre-incubated in 10% autologous human or mouse serum) was added/tube, for a final total volume of 200  $\mu$ l. In some experiments, neutrophils were primed with HKSA (Invivogen) plus either PBS or TSLP for 2 h before addition of MRSA. The tubes were slowly rotated in a 37° C incubator for 2–3 h as indicated in the figure legends. For DPI treatment, neutrophils were incubated with 2  $\mu$ M DPI for 30 min, washed, counted, and then used in the killing assay as described above. Each treatment was done in triplicate. Whole blood from healthy human NIH blood bank volunteer donors was obtained without donor identification and met the criteria for exemption from informed consent and institutional review board review as defined in The Code of Federal Regulations Title 45 (Public Welfare), Department of Health and Human Services, Part 46 (Protection of Human Subjects), and their distribution was in accord with National Institutes of Health guidelines for the research of human subjects.

### Neutrophil depletion

Neutrophil-depleted blood was obtained by injecting mice i.p. with 0.5 mg of anti-Ly6G antibody (1A8, Bioxcel) two days before blood was collected. For infection studies of neutrophil-depleted mice, mice were injected i.p. with 0.5 mg of anti-Ly6G antibody two days before and again on the day of infection. Neutrophil depletion was ~93–98% efficient as assessed by flow cytometric staining with Gr-1 and Ly6C antibodies (Biolegend).

### Intradermal ear infection

6–9 week old WT, *Tslpr*<sup>-/-</sup>, or *C5ar1*<sup>-/-</sup> BALB/c mice or neutrophil-depleted WT BALB/c mice were injected intradermally (i.d.) using a 29 ½ -gauge 3/10 ml insulin syringe (BD Biosciences) with MRSA or *S. pyogenes* mixed with either TSLP (2 µg) or PBS (final OD<sub>600</sub>=0.125 in a total volume of 10 µl). In some experiments, 10 µg of anti-mouse C5 blocking antibody (BB5.1, Hycult Biotech) or mouse IgG1 isotype control (MOPC-21, Bioxcel) was additionally added, but the total volume injected was still 10 µl. For *in vivo* ROS inhibition, 1.3 µg of NAC (N-acetyl-L-cysteine, Sigma-Aldrich) was co-injected with PBS or TSLP and MRSA i.d. into the ears. Each experiment included 6–12 ears per group. Some samples were excluded at the time of infection due to a poor injection.

### Neutrophil *in vivo* transfer experiments

Equal numbers of purified WT and *Tslpr*<sup>-/-</sup> bone marrow neutrophils were either co-transferred (~3 ×10<sup>6</sup> of each) into WT mice or labeled with 5 µM CMDFA, as previously described (46), and transferred separately (~15 ×10<sup>6</sup>) into *Tslpr*<sup>-/-</sup> mice i.v. 30 min prior to infection with MRSA i.d. in the ear.

### Ex-vivo detection of ROS

Mouse ear samples were processed as described above, and cells were incubated in medium with 5 µM of Cell Rox<sup>®</sup> Deep Red reagent (Life Technologies) for 30 min at 37° C, washed 3 times with PBS, and fixed with 4% paraformaldehyde before staining for CD11b<sup>+</sup> Ly6G<sup>+</sup> (Ly6C<sup>low</sup>).

### CRLF2 RT-PCR

Human neutrophils were isolated and stimulated with medium or 10<sup>9</sup> HKSA/ml (Heat killed *S. aureus* or 10 µg/ml peptidoglycan (InvivoGen) for 4 h. Probes for *CRLF2* (Hs00845692\_m1) and *RPL7* (Hs02596927\_g1) were from Life technologies.

### Statistics

Statistical significance was calculated as indicated in the figure legends, using GraphPad Prism 6 software. For all statistical analyses, data were considered significant when P < 0.05 (\*), P < 0.01 (\*\*), P < 0.001 (\*\*\*) or P < 0.0001 (\*\*\*\*). Variances were similar between groups in all experiments, as determined by the F test using GraphPad Prism 6 software.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

We thank Melanie Faivre-Charmoy for instruction on how to perform intradermal ear injections and ear cell processing and Phillip A. Swanson II for critical comments.

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## References and Notes

1. McCaig LF, McDonald LC, Mandai S, Jernigan DB. Staphylococcus aureus-associated skin and soft tissue infections in ambulatory care. *Emerging infectious diseases*. 12 :1715. Nov. 2006 [PubMed: 17283622]
2. DeLeo FR, Chambers HF. Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era. *The Journal of clinical investigation*. 119 :2464. Sep. 2009 [PubMed: 19729844]
3. Klevens RM, et al. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *Jama*. 298 :1763. Oct 17. 2007 [PubMed: 17940231]
4. Miller LS, Cho JS. Immunity against Staphylococcus aureus cutaneous infections. *Nature reviews Immunology*. 11 :505. Aug. 2011
5. Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Current opinion in immunology*. 15 :578. Oct. 2003 [PubMed: 14499268]
6. Gonzalez-Barca E, Carratala J, Mykietiak A, Fernandez-Sevilla A, Gudiol F. Predisposing factors and outcome of Staphylococcus aureus bacteremia in neutropenic patients with cancer. *European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology*. 20 :117. Feb. 2001
7. Moine L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. *Infection and immunity*. 68 :6162. Nov. 2000 [PubMed: 11035720]
8. Friend SL, et al. A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Experimental hematology*. 22 :321. Mar. 1994 [PubMed: 8112430]
9. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. *Nature immunology*. 11 :289. Apr. 2010 [PubMed: 20300138]
10. West EE, Kashyap M, Leonard WJ. TSLP: A Key Regulator of Asthma Pathogenesis. *Drug discovery today Disease mechanisms*. 9 Dec 1. 2012
11. Divekar R, Kita H. Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation. *Current opinion in allergy and clinical immunology*. 15 :98. Feb. 2015 [PubMed: 25479313]
12. Yoo J, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *The Journal of experimental medicine*. 202 :541. Aug 15. 2005 [PubMed: 16103410]
13. Zhou B, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nature immunology*. 6 :1047. Oct. 2005 [PubMed: 16142237]
14. Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *The Journal of experimental medicine*. 202 :829. Sep 19. 2005 [PubMed: 16172260]
15. Giacomini PR, et al. Epithelial-intrinsic IKK $\alpha$  expression regulates group 3 innate lymphoid cell responses and antibacterial immunity. *The Journal of experimental medicine*. 212 :1513. Sep 21. 2015 [PubMed: 26371187]
16. Garber K. Anti-IL-17 mAbs herald new options in psoriasis. *Nature biotechnology*. 30 :475. Jun. 2012
17. Hueber W, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*. 61 :1693. Dec. 2012 [PubMed: 22595313]
18. Soumelis V, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nature immunology*. 3 :673. Jul. 2002 [PubMed: 12055625]
19. David MZ, Daum RS. Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. *Clinical microbiology reviews*. 23 :616. Jul. 2010 [PubMed: 20610826]
20. Rigby KM, DeLeo FR. Neutrophils in innate host defense against Staphylococcus aureus infections. *Seminars in immunopathology*. 34 :237. Mar. 2012 [PubMed: 22080185]

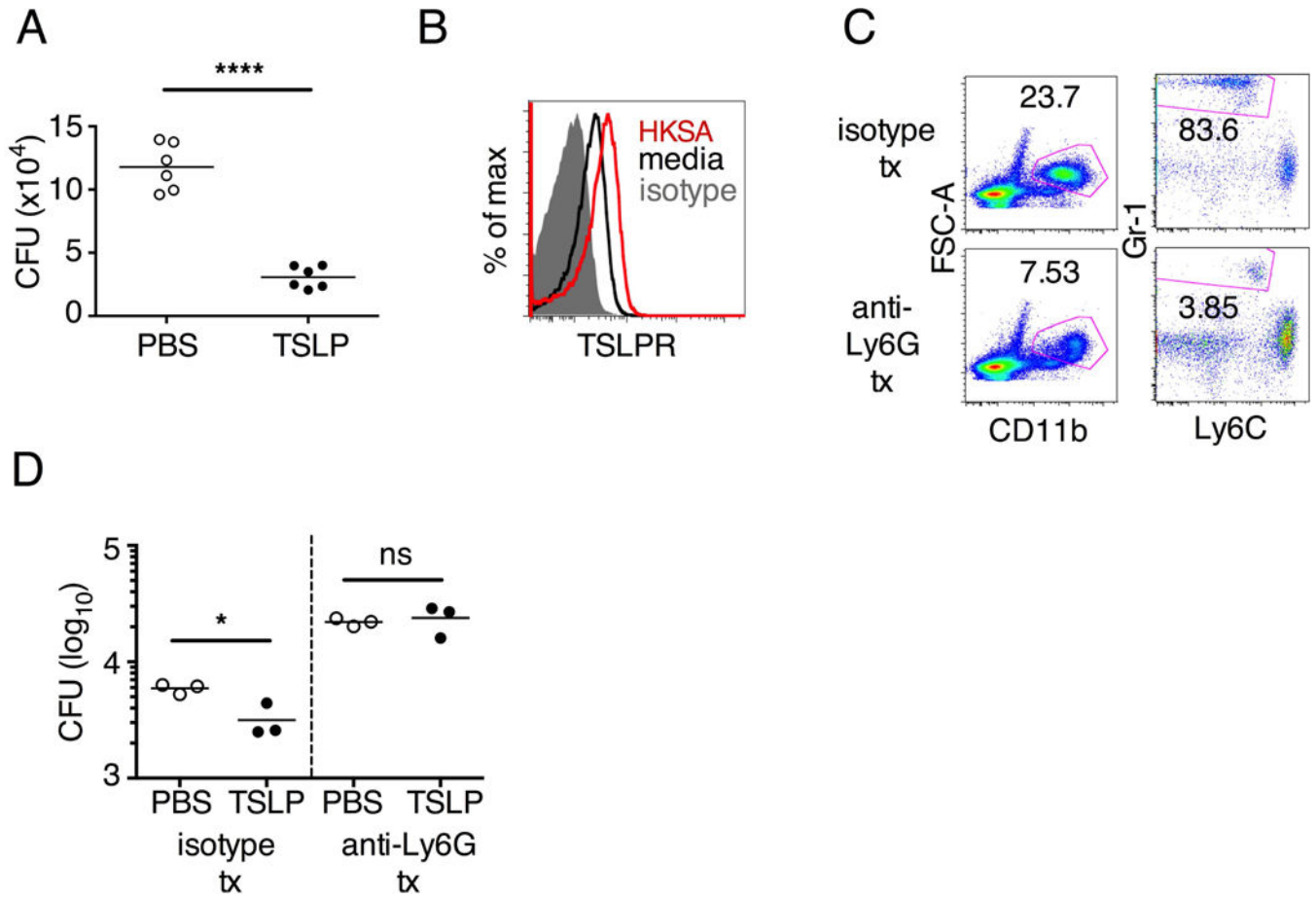
21. Bjerkan L, et al. The short form of TSLP is constitutively translated in human keratinocytes and has characteristics of an antimicrobial peptide. *Mucosal immunology*. 8 :49. Jan. 2015 [PubMed: 24850429]
22. Sonesson A, et al. Thymic stromal lymphopoietin exerts antimicrobial activities. *Experimental dermatology*. 20 :1004. Dec. 2011 [PubMed: 22092577]
23. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nature reviews Immunology*. 13 :159. Mar. 2013
24. McPhail LC, Clayton CC, Snyderman R. The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. *The Journal of biological chemistry*. 259 :5768. May 10. 1984 [PubMed: 6425297]
25. Cunningham MW. Pathogenesis of group A streptococcal infections. *Clinical microbiology reviews*. 13 :470. Jul. 2000 [PubMed: 10885988]
26. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic research*. 8 :265. Aug. 1999 [PubMed: 10621974]
27. Hawkins PT, Stephens LR, Suire S, Wilson M. PI3K signaling in neutrophils. *Current topics in microbiology and immunology*. 346 :183. 2010; [PubMed: 20473789]
28. Zhong B, et al. Human neutrophils utilize a Rac/Cdc42-dependent MAPK pathway to direct intracellular granule mobilization toward ingested microbial pathogens. *Blood*. 101 :3240. Apr 15. 2003 [PubMed: 12511425]
29. Hommes DW, Peppelenbosch MP, van Deventer SJ. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut*. 52 :144. Jan. 2003 [PubMed: 12477778]
30. Allen IC, et al. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity*. 30 :556. Apr 17. 2009 [PubMed: 19362020]
31. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nature immunology*. 11 :785. Sep. 2010 [PubMed: 20720586]
32. Kolev M, Le Fric G, Kemper C. Complement-tapping into new sites and effector systems. *Nature reviews Immunology*. 14 :811. Dec. 2014
33. Sarma JV, Ward PA. New developments in C5a receptor signaling. *Cell health and cytoskeleton*. 4 :73. Jul 1. 2012 [PubMed: 23576881]
34. Klos A, et al. The role of the anaphylatoxins in health and disease. *Molecular immunology*. 46 :2753. Sep. 2009 [PubMed: 19477527]
35. Muller-Eberhard HJ. Complement. *Annual review of biochemistry*. 38 :389. 1969;
36. Bauernfeind F, et al. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *Journal of immunology*. 187 :613. Jul 15. 2011
37. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Molecular immunology*. 48 :1631. Aug. 2011 [PubMed: 21549429]
38. Tamamis P, et al. Insights into the mechanism of C5aR inhibition by PMX53 via implicit solvent molecular dynamics simulations and docking. *BMC biophysics*. 7 :5. 2014; [PubMed: 25170421]
39. Taylor BC, et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *The Journal of experimental medicine*. 206 :655. Mar 16. 2009 [PubMed: 19273626]
40. Lee HC, et al. Thymic stromal lymphopoietin is induced by respiratory syncytial virus-infected airway epithelial cells and promotes a type 2 response to infection. *The Journal of allergy and clinical immunology*. 130 :1187. Nov. 2012 [PubMed: 22981788]
41. Piliponsky AM, et al. Thymic Stromal Lymphopoietin Improves Survival and Reduces Inflammation in Sepsis. *American journal of respiratory cell and molecular biology*. Mar 2. 2016
42. Kuethe JW, et al. Thymic stromal lymphopoietin mediates the host response and increases mortality during sepsis. *The Journal of surgical research*. 191 :19. Sep. 2014 [PubMed: 24990542]
43. Gauvreau GM, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *The New England journal of medicine*. 370 :2102. May 29. 2014 [PubMed: 24846652]

44. Morgan BP, Harris CL. Complement, a target for therapy in inflammatory and degenerative diseases. *Nature reviews Drug discovery*. Oct 23. 2015
45. Kaplan A, et al. Failure to induce IFN-beta production during *Staphylococcus aureus* infection contributes to pathogenicity. *Journal of immunology*. 189 :4537. Nov 1. 2012
46. Swamydas M, Lionakis MS. Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. *Journal of visualized experiments: JoVE*. :e50586. 2013 [PubMed: 23892876]
47. Ribeiro-Gomes FL, et al. Site-dependent recruitment of inflammatory cells determines the effective dose of *Leishmania major*. *Infection and immunity*. 82 :2713. Jul. 2014 [PubMed: 24733090]

### Editor's summary

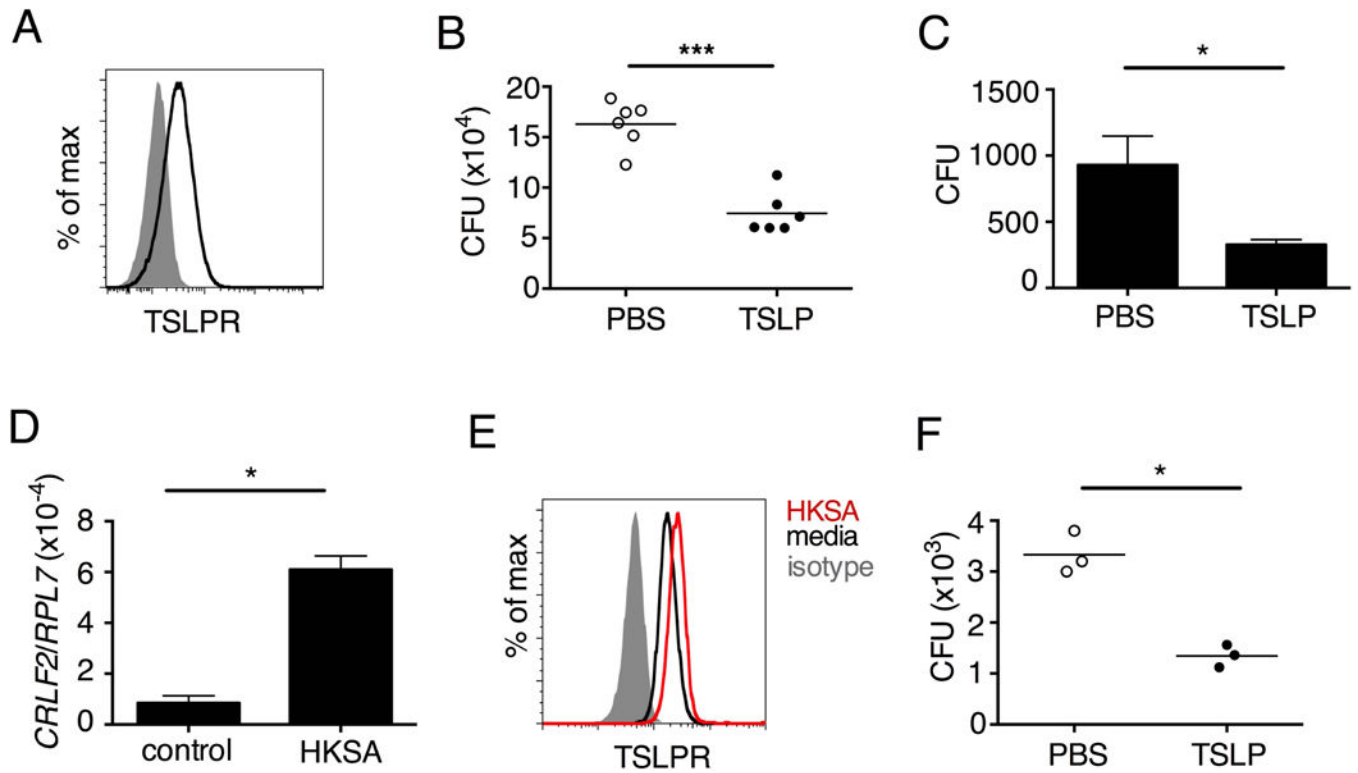
#### 'Complement'ary MRSA Fight

Thymic stromal lymphopoeitin (TSLP) is a cytokine thought to promote allergic responses; however, its role in fighting infectious diseases is less clear. Now West *et al.* report that TSLP in the skin can enhance killing of methicillin-resistant *S. aureus* (MRSA). TSLP enhances killing in both mouse and human neutrophils, in part through interactions with the complement system that induce reactive oxygen species in neutrophils. This enhanced killing is not limited to MRSA, as TSLP also boosts killing of *Streptococcus pyogenes*. These data suggest that TSLP may augment innate immune cells and complement to fight bacterial infection.



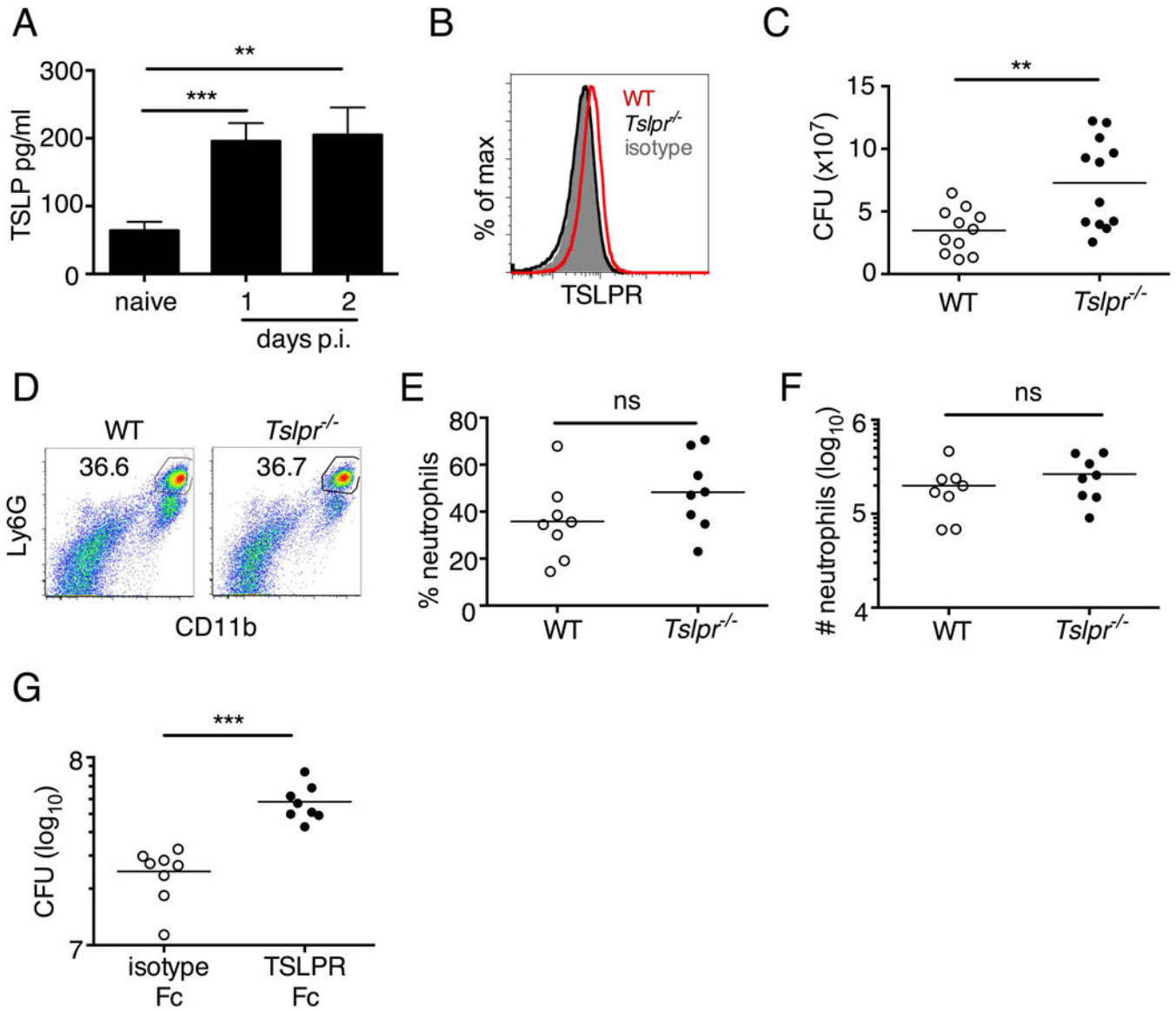
**Fig. 1. TSLP increases MRSA killing in mouse blood in a neutrophil-dependent manner** (A, C, D) Mouse blood was incubated with PBS or TSLP and MRSA for 3 h. (A) CFU analysis (shown is a representative experiment from blood from 2 mice performed in triplicate). (B) TSLPR expression on mouse neutrophils incubated with medium or HKSA. (C) Flow-cytometric staining of blood neutrophils in mice treated with a control antibody or depleted of neutrophils using anti-Ly6G antibody. (D) A representative experiment showing CFU of MRSA after an *in vitro* whole blood killing assay performed with blood from mice treated with an isotype control or anti-Ly6G antibodies (blood from 2–3 mice were combined for each treatment condition and assayed in triplicate). \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; ns = not significant, two-tailed Student's *t*-test. Data are representative of 6 independent experiments for (A), 2 experiments for (B) and 3 experiments for (C) and (D).



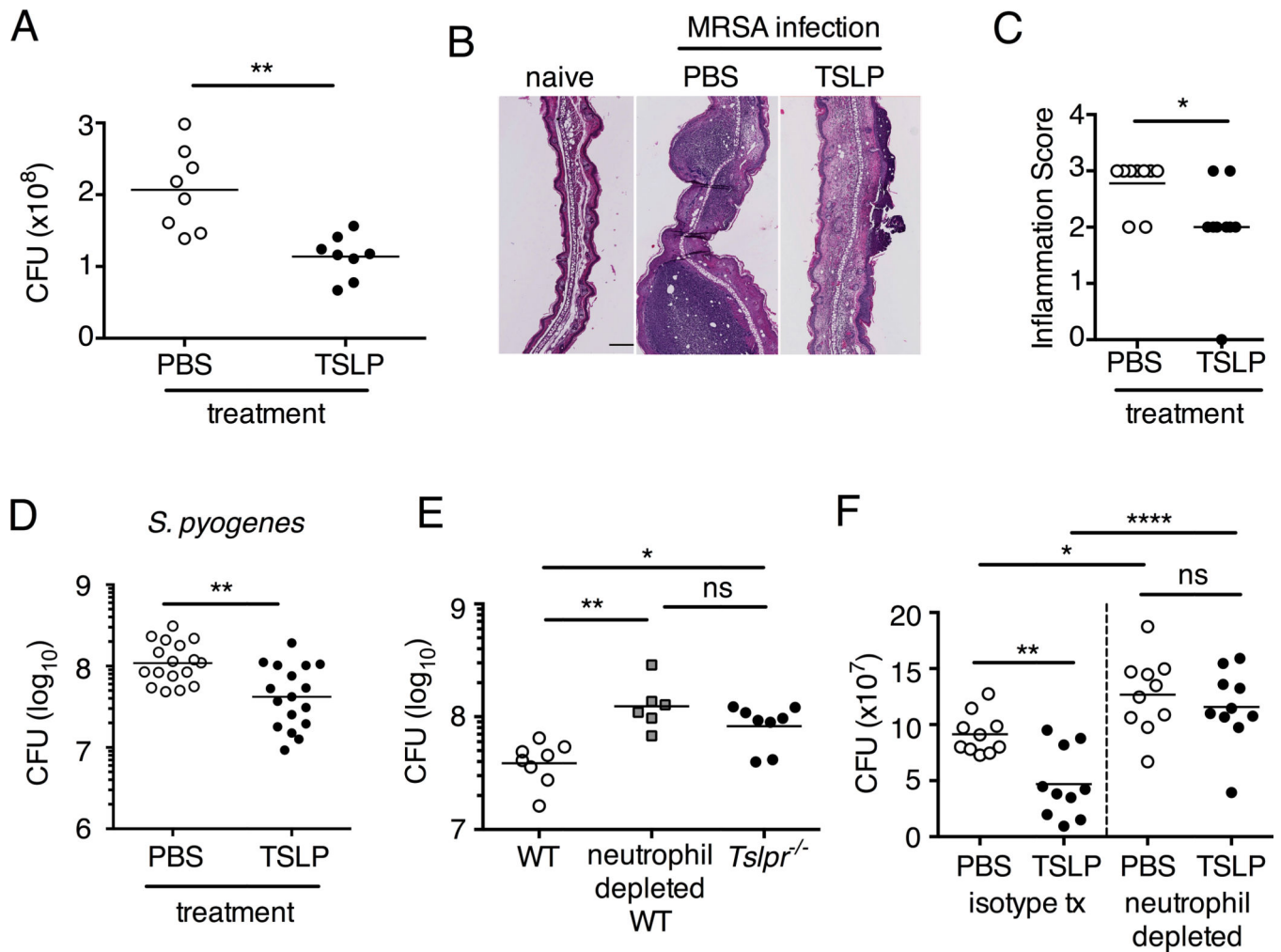


**Fig. 2. TSLP acts directly on both mouse and human neutrophils to increase MRSA killing *in vitro***

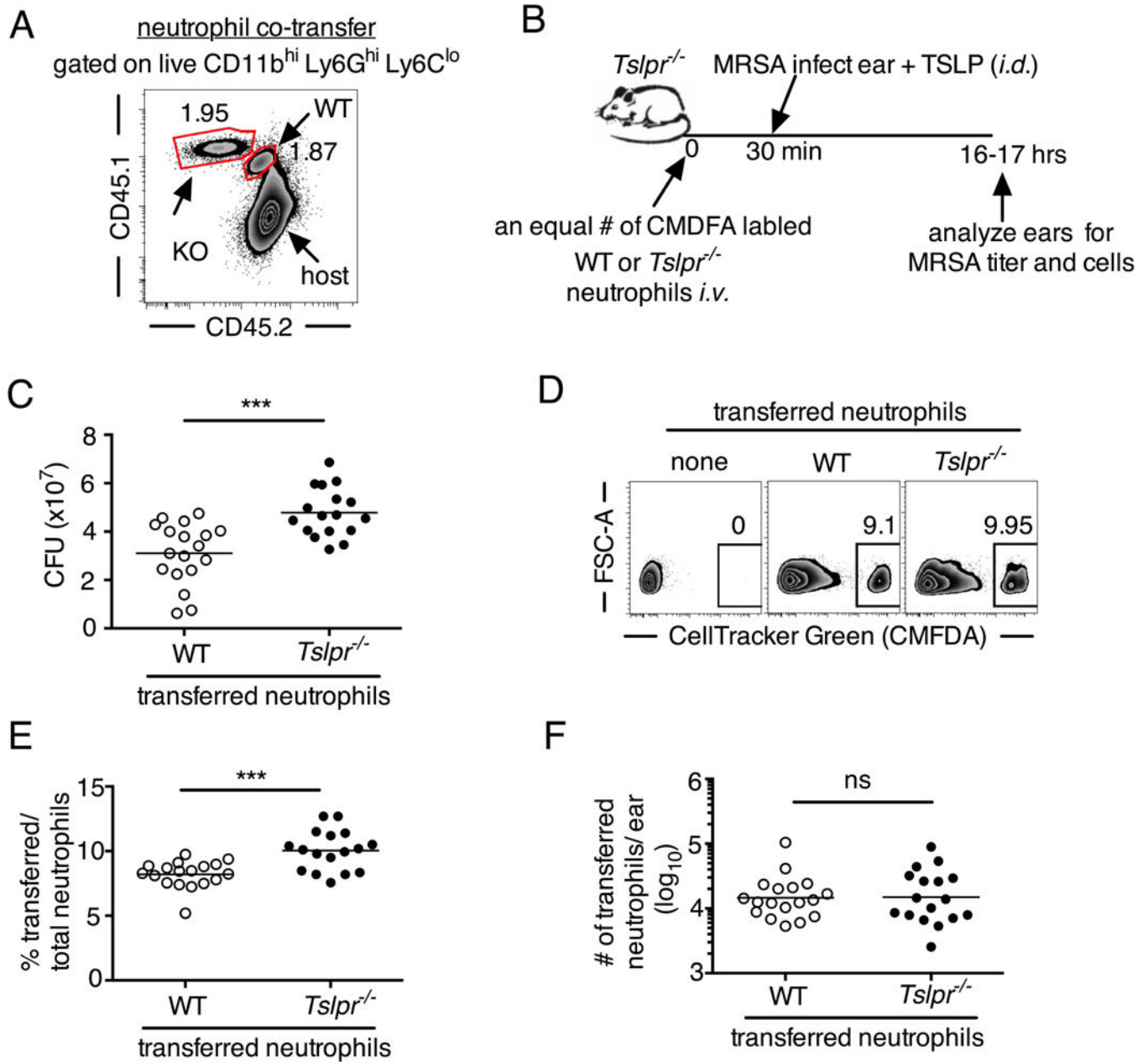
(A,B) Thioglycollate-elicited mouse neutrophils were used. (A) Representative flow-cytometric staining of TSLPR expression on neutrophils. (B) CFU after purified neutrophils were incubated with PBS or TSLP and MRSA for 2 h. (C) Whole human blood was incubated with MRSA and PBS or TSLP for 3 h and CFU determined (representative graph of 1 donor shown in triplicate, statistics shown are of two-tailed paired *t*-test from 6 donors). (D–F) Purified human blood neutrophils were used. (D) *CRLF2* expression by human blood neutrophils determined by RT-PCR after 4 h treatment with medium (control) or HKSA (representative donor shown) and normalized to expression of *RPL7*. (E) Representative flow-cytometric staining of TSLPR on human blood neutrophils. (F) CFU after neutrophils were incubated with MRSA and PBS or TSLP for 3 h (shown is a representative graph of 1 donor done in triplicate; statistics shown are of two-tailed paired *t*-test from 7 donors). \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; ns = not significant, using two-tailed Student's *t*-test for unless indicated. Data are representative of at least 3 independent experiments.



**Fig. 3. *Tslpr*-deficient mice have increased MRSA burden during *in vivo* skin infection**  
 (A–G) Mice were infected with MRSA i.d. in the ear. (A) TSLP protein expression in the ear after i.d. MRSA infection ( $n=4$ ), naïve controls were mock-infected with PBS only. (B–G) Ears were analyzed on day 1 p.i. (B) Representative TSLPR expression on neutrophils. (C) Analysis of CFU in the ear of WT ( $n=11$ ) and *Tslpr*<sup>-/-</sup> mice ( $n=12$  ears). (D–F) Analysis of neutrophils in the ear. Shown are representative FACS plots (D) and percent (E) and total number (F) of neutrophils from WT and *Tslpr*<sup>-/-</sup> mice ( $n=8$  ears). (G) CFU of MRSA in the ear on day 2 post-i.d. ear infection of WT mice treated with human IgG1 Fc isotype control or TSLPR Fc (two-tailed Mann-Whitney test) ( $n=8$  ears). \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ; ns = not significant. a–f, two-tailed Student's *t*-test. Data are representative of 3 (or 2 for (G)) independent experiments.



**Fig. 4. TSLP treatment enhances *in vivo* MRSA and *S. pyogenes* killing during a skin infection** Mice were infected with MRSA i.d. in the ear. **(A)** CFU of MRSA at day 2 p.i. in the ears of WT mice treated with PBS or TSLP (two-tailed Mann-Whitney test) ( $n=10$  ears). **(B)** Representative images of hematoxylin and eosin (H&E)-stained ear sections on day 2 p.i. (5X magnification: bar indicates 200 microns). **(C)** Inflammation score according to blinded histological analysis ( $n=9$  ears). **(D)** CFU of *S. pyogenes* in the ears of WT mice treated with PBS or TSLP on day 1 post-i.d infection (two-tailed Mann-Whitney test) ( $n=17$  ears, shown are results of two combined independent experiments). **(E)** CFU in the ear of WT, neutrophil-depleted WT, and *Tslpr*<sup>-/-</sup> mice on day 1 p.i. (two-tailed Mann-Whitney test) ( $n=8$  ears for WT and *Tslpr*<sup>-/-</sup>,  $n=6$  for neutrophil depleted WT) **(F)** CFU of MRSA in the ears of WT mice or neutrophil-depleted WT mice treated with PBS or TSLP on day 2 p.i. (two-tailed Mann-Whitney test) ( $n=10$  ears). \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ; ns = not significant. Data are representative of 2 (**B, C, E, F**) or 4 (**A**) independent experiments.



**Fig. 5. TSLP acts directly on neutrophils *in vivo* to enhance killing of MRSA during a skin infection**

(A) Equal numbers of purified bone marrow neutrophils from WT (CD45.1<sup>+/2+</sup>) and *Tslpr*<sup>-/-</sup> (KO; CD45.1<sup>+/1+</sup>) mice were co-transferred i.v. into WT C57BL/6 host mice (CD45.2<sup>+/2+</sup>) and were then infected with MRSA i.d. in the ear. Shown is a representative flow cytometric plot of the neutrophil populations in the ear on day 1 p.i. (n=10, gated on neutrophils = live CD11b<sup>hi</sup>Ly6G<sup>hi</sup>Ly6C<sup>lo</sup> cells). (B) Experimental design for (C–F), where an equal number of purified CellTracker Green (CMDFA) labeled WT or *Tslpr*<sup>-/-</sup> bone marrow neutrophils were transferred i.v. into *Tslpr*<sup>-/-</sup> host mice, which were subsequently injected with MRSA + TSLP i.d. in the ear. (C) CFU of MRSA in the ears 16–18 h p.i. (n=17–18). (D) Representative flow-cytometric plot showing the percent of transferred

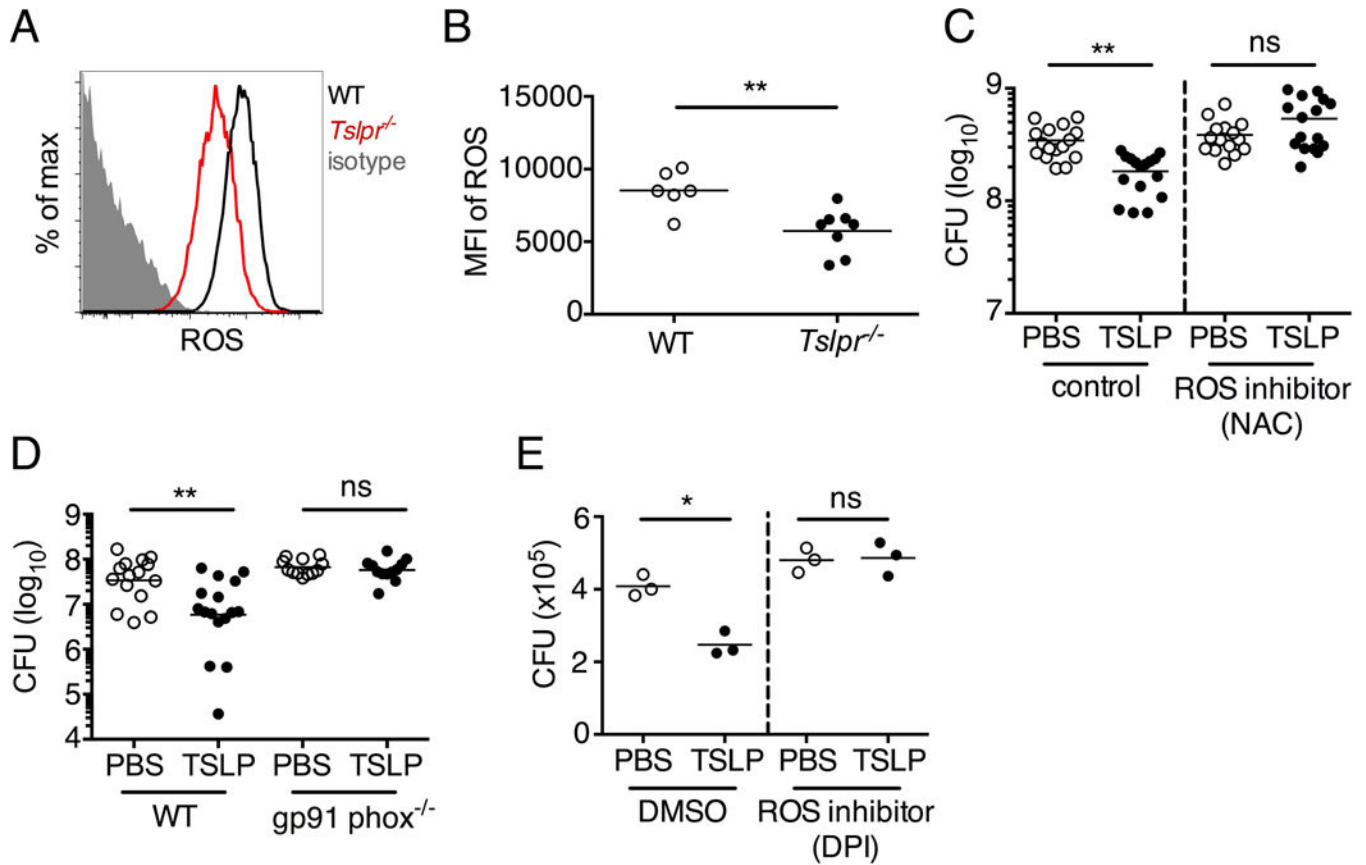
neutrophils (CMDFA<sup>+</sup>) out of total neutrophils in the ears of *Tslpr*<sup>-/-</sup> mice receiving no cells, WT neutrophils, or *Tslpr*<sup>-/-</sup> neutrophils (n=17–18 from two combined individual experiments, gated on total neutrophils). **(E)** Percent and **(F)** number of transferred neutrophils per ear (n=17–18). \*, p < .05; \*\*, p < .01; \*\*\*, p < .001; ns = not significant, using a two-tailed Mann-Whitney test.

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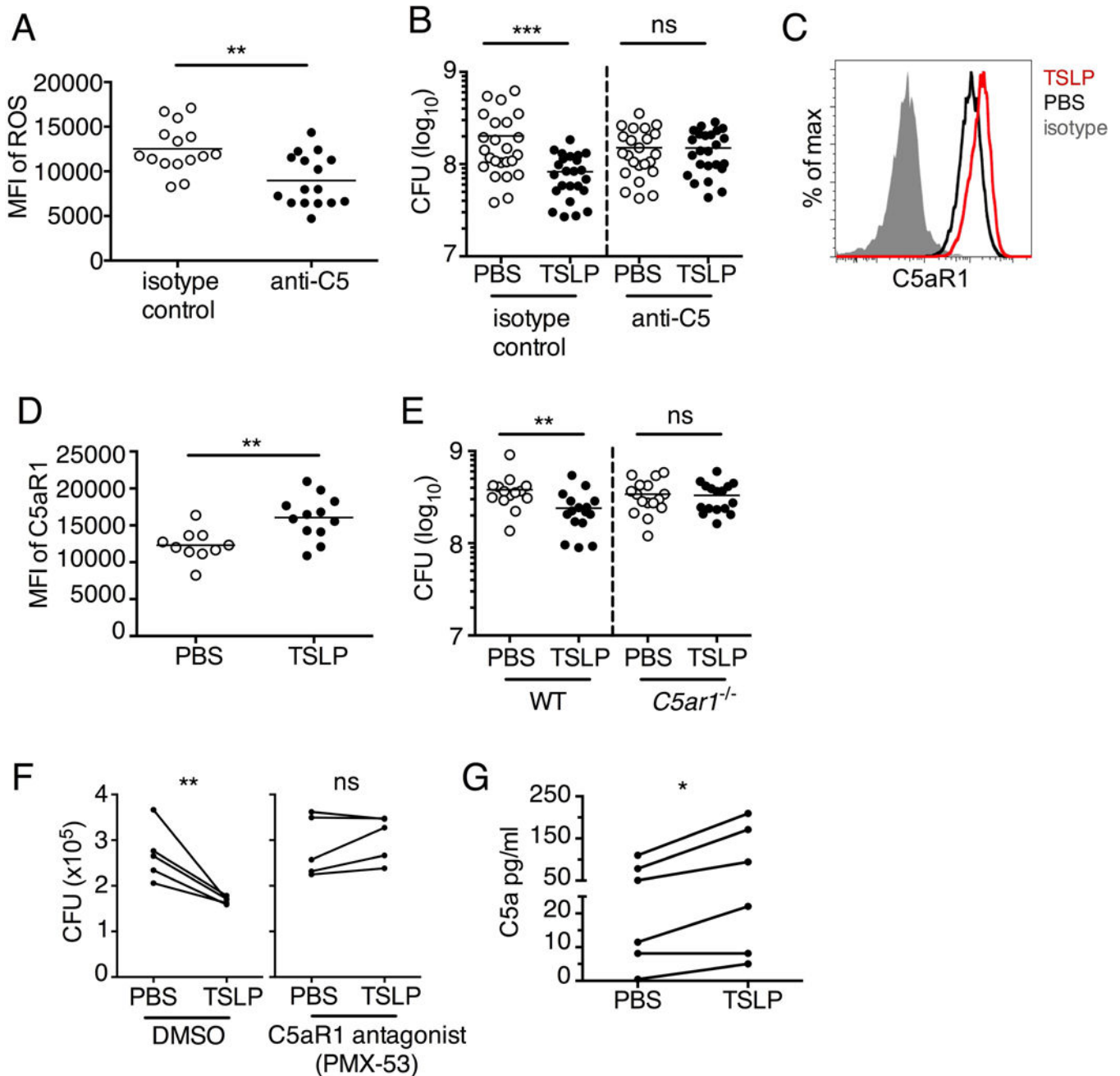
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**Fig. 6. TSLP induced killing of MRSA is mediated by reactive oxygen species**  
 (A–C) Day 1 p.i. of mice infected with MRSA i.d. in the ear. (A–B) ROS production of mouse neutrophils after staining with CellROX deep red. Shown are a representative FACS plot (A) and mean fluorescence intensity (MFI) (B) of WT ( $n=6$ ) and *Tslpr*<sup>-/-</sup> mice ( $n=8$  ears). (C) Mice were injected i.d. in the ear with MRSA and either PBS or TSLP along with either control (PBS) or a ROS inhibitor (NAC). CFU on day 1 p.i. (two-tailed Mann-Whitney test,  $n=16$  ears). (D) CFU in the ear on day 2 p.i. of WT and *Gp91 phox*<sup>-/-</sup> mice infected with MRSA and PBS or TSLP (two-tailed Mann-Whitney test,  $n=12$ –16 ears). (E) Purified human neutrophils were pretreated with DMSO or DPI, treated with PBS or TSLP, and incubated for 2 h with MRSA. CFU was then determined (representative donor shown in triplicate, statistics shown are using a two-tailed paired-*t* test of 3 donors, 3 independent experiments). \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ; ns = not significant. (b) Two-tailed Student's *t*-test. Data are representative of 3 independent experiments (A, B) or are combined data from two independent experiments (C&D).



**Fig. 7. TSLP induced killing of MRSA is mediated by reactive oxygen species and complement (A–G)** Day 1 p.i. of mice infected with MRSA i.d. in the ear. **(A)** MFI of ROS production of mouse neutrophils after day 1 p.i. with MRSA plus isotype control ( $n=15$ ) or anti-C5 antibodies injected i.d. ( $n=16$  ears). **(B)** Mice were infected with MRSA and PBS or TSLP with isotype control or anti-C5 antibodies given i.d. in the ear. CFU on day 1 p.i. (two-tailed Mann-Whitney test,  $n=15$  (PBS isotype) or  $n=16$  ears). **(C–D)** C5aR1 expression on mouse neutrophils as assessed by flow cytometry. Shown are a representative FACS plot **(C)** and MFI for multiple animals **(D)** ( $n=10$  (WT) or 12 (TSLP) ears). **(E)** CFU at day 1 p.i. of WT or *C5ar1*<sup>-/-</sup> mice infected with MRSA and PBS or TSLP ( $n=10$  (PBS WT) or 16 (all

other groups) ears. **(F)** Purified human neutrophils were treated with DMSO or PMX-53 and PBS or TSLP and incubated for 2 h with MRSA. CFU was then determined (n=5 donors, two-tailed paired-*t* test of 4 independent experiments). **(G)** Purified human neutrophils were incubated with PBS or TSLP for 30 min and supernatants were assessed for C5a protein (n=6 donors, ratio-paired two-tailed student's *t*-test, 3 independent experiments). \*, p < .05; \*\*, p < .01; \*\*\*, p < .001; ns = not significant. (A) Two-tailed Student's *t*-test. Data are representative of 3 independent experiments (A, C–D) or 3 combined independent experiments shown in (B) and 2 in (E).