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IFN-γ Regulated Chemokine Production Determines the Outcome of *Staphylococcus aureus* Infection[1]

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Immunomodulatory therapy represents an attractive approach in treating multidrug-resistant infections. Developing this therapy necessitates a lucid understanding of host defense mechanisms. Neutrophils represent the first line of systemic defense during *Staphylococcus aureus* infections. However, recent research suggests that survival of *S. aureus* inside neutrophils may actually contribute to pathogenesis, indicating that neutrophil trafficking to the infection site must be tightly regulated to ensure efficient microbial clearance. We demonstrate that neutrophil-regulating T cells are activated during *S. aureus* infection and produce cytokines that control the local neutrophil response. *S. aureus* capsular polysaccharide activates T cell production of IFN-γ in a novel MHC class II-dependent mechanism. During *S. aureus* surgical wound infection, the presence of IFN-γ at the infection site depends upon $\alpha\beta$TCR$^+$ cells and functions to regulate CXC chemokine production and neutrophil recruitment in vivo. We note that the reduced neutrophil response seen in IFN-$\gamma^{-/-}$ mice during *S. aureus* infection is associated with reduced tissue bacterial burden. CXC chemokine administration to the infection site resulted in an increased survival of viable *S. aureus* inside neutrophils isolated from the wound. These data demonstrate that T cell-derived IFN-γ generates a neutrophil-rich environment that can potentiate *S. aureus* pathogenesis by facilitating bacterial survival within the neutrophil. These findings suggest avenues for novel immunomodulatory approaches to control *S. aureus* infections. *The Journal of Immunology,* 2008, 181: 1323–1332.

In the past decade, the emergence of methicillin-resistant *Staphylococcus aureus* strains has increased dramatically. A recent study estimated that 94,360 invasive methicillin-resistant *S. aureus* infections occurred in the United States in 2005, with 18,650 of these cases associated with mortality (1). As a consequence of this rapidly growing epidemic, coupled with the decreasing effectiveness of currently available antimicrobial agents, an alternative novel approach to anti-infective therapy is to modulate the host's immune response (2–5). An in-depth understanding of host-pathogen interaction is therefore vital to delineate effective mechanisms of host response that combat infection.

The polymorphonuclear leukocyte (PMN)[3] response is the body's first line of systemic defense against invasion by *S. aureus* and a critical determinant in the outcome of infection (6–8). Patients who are neutropenic or who have congenital or acquired defects in PMN function are more susceptible to infection with this extracellular pathogen (9), and various animal model studies have identified a critical role for PMNs in the clearance of *S. aureus* infections (8, 10). However, contrasting studies suggest that PMNs may also contribute to pathogenesis by harboring viable *S. aureus* intracellularly (11). Our own studies have also demonstrated that heightened PMN recruitment is associated with increased disease pathogenesis in surgical wound infections (12). Given the potential contrasting roles played by neutrophils during *S. aureus* infection, it is clear that any dysregulation in PMN trafficking could facilitate pathogenesis. Thus, it is imperative that we understand precisely the local cytokine signals controlling neutrophil trafficking during *S. aureus* infection.

To date, the role played by T cells during *S. aureus* infection has been underappreciated. Outside the array of toxin-mediated diseases (toxic shock syndrome, scalded skin syndrome), very little is known about how T cells respond to *S. aureus*. However, recent developments have identified a significant role for T cells in regulating *S. aureus* pathogenesis in three distinct animal models of infection. This effect could not be attributed to the actions of superantigens (12). In these studies, animals deficient in $\alpha\beta$TCR signaling or CD4$^+$ T cells developed significantly less severe infections than wild-type (WT) animals. Decreased bacterial burden in these mice was associated with a dysregulated PMN response, suggesting that *S. aureus*-activated T cells can regulate CXC chemokine production and associated PMN recruitment to the infection site (12). Until now, the mechanism by which the T cell regulates CXC chemokine-driven neutrophil recruitment during *S. aureus* infection has not been described.

PMN recruitment has traditionally been regarded as a T cell-independent process. However, recent studies have challenged this dogma and highlighted the existence of a subpopulation of T cells with the ability to produce distinct cytokines and chemokines, which impact (directly or indirectly) PMN proliferation, maturation, chemotaxis, and survival (13–15). IFN-γ is one such cytokine that can modulate neutrophil trafficking in inflammatory diseases (16–20). Depending on the nature of the infection and the stage of the disease, IFN-γ plays conflicting roles during *S. aureus* pathogenesis. This suggests a distinction between the local and the systemic activities of this cytokine (21, 22). IFN-γ was shown to be protective against septicemia while on the other hand promoting the development of septic arthritis in a mouse model (22). Similarly, it has been shown that IFN-γ is required for an acquired

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; WT, wild type; GalU, polygalacturonic acid; CP, capsular polysaccharide; MPO, myeloperoxidase; MHCII, MHC class; SEA, staphylococcal enterotoxin A; TT, tetanus toxoid; PNAG, poly-*N*-acetylglucosamine.

resistance against *S. aureus* infection but is not involved in the secondary Ab response (23). To date, the role played by IFN-γ in regulating the host's neutrophilic response during *S. aureus* infection has not been described.

Capsular polysaccharides (CPs) are well-characterized virulence factors expressed by many extracellular bacteria. Their main function is to enhance microbial virulence by rendering the bacterium resistant to phagocytosis. Most CPs are uncharged or carry a net negative charge (24). However, CPs produced by a few bacterial species have a zwitterionic charge, i.e., the CP-repeating units include both positively and negatively charged sugar residues (24). Seminal advances in CP biology have identified a novel mechanism by which zwitterionic CPs can activate T cells in a MHC class II (MHCII) pathway-dependent manner, a pathway previously considered to be exclusive to protein Ags (25, 26). The majority of *S. aureus* strains express CPs of serotype 5 (CP5) or 8 (CP8) (27), which are structurally very similar (28, 29). Previous studies have hypothesized that *S. aureus* may produce multiple cell-associated polymers that under certain conditions possess a zwitterionic charge motif, conferring upon them the ability to activate T cells (28). In this study, we investigated a superantigen-independent mechanism of T cell activation by *S. aureus* and report CP8 activation of $CD4^+$ T cells as a mechanism by which *S. aureus* can activate T cell production of IFN-γ at sites of infection.

Materials and Methods

Mouse model of S. aureus surgical wound infection

Male WT C57BL/6 (stock no. 000664) and congenic gene-deficient mice (IFN-γ-deficient: B6.129S7Ifng^{tm1}Ts/J (stock no. 002287), αβTCR-deficient: B6.129-Tcrb^{tm1mom}/J (stock no. 002118)) were obtained at 6–8 wk of age from The Jackson Laboratory. Animal experiments were performed in accordance with the guidelines of the Harvard Medical School Standing Committee on Animals. Surgical wound infection was established in mice as previously described (12, 30) using *S. aureus* strain PS80, a streptomycin-resistant capsule serotype 8 strain (12, 28). At specific time points after induction of infection, wound tissue was excised and analyzed as follows.

For histological analysis, excised tissue was fixed in formalin, embedded in paraffin, and stained with H&E for microscopic examination of leukocyte infiltration. Individual tissue sections were examined and scored for relative neutrophil infiltration by two individuals blinded to the identity of the sample using a Zeiss Axioskop 2 plus microscope. Tissue sections were ranked in severity and assigned a score of between 1 and 4. Neutrophil accumulation at the wound site was quantified by measuring tissue myeloperoxidase (MPO) levels as previously described (31). MPO activity was calculated per gram of tissue weight. For cytokine and chemokine analysis, wound tissue was homogenized in a lysis buffer and CXCL1 (KC), CXCL2 (MIP-2), CXCL5 (LIX), and IFN-γ levels were measured by ELISA (R&D Systems).

Total tissue bacterial burden was established by homogenizing tissue in tryptic soy broth and plating on tryptic soy agar plates supplemented with 500 μg/ml streptomycin (Sigma-Aldrich). Results are expressed as CFU/g of tissue weight.

Human mononuclear cell-$CD4^+$ T cell coculture assay

Total mononuclear cell and $CD4^+$ T cell cocultures were established as previously described (32). Following Ag stimulation for 72 h, cell-free supernatants were harvested and IFN-γ levels were quantified by ELISA using a R&D Systems Duoset. To inhibit endosomal processing, baflomycin A (20 nM) was added to the coculture system for the duration of the incubation. To inhibit MHCII-dependent presentation of Ags to the $CD4^+$ T cells, total mononuclear cells were incubated for 30 min with blocking Abs to the MHCII molecules HLA-DR (Biolegend), HLA-DP, HLA-DQ (Neomarkers), and the MHC class I molecules HLA-A, HLA-B, and HLA-C (Biolegend) as well as appropriate isotype controls before the addition of $CD4^+$ T cells and Ags. Blocking Abs remained in the coculture for the duration of the experiment.

Human B lymphoma line-$CD4^+$ T cell coculture assay

The human Burkitt lymphoma cell line (Raji) expressing MHCII and MHC class I molecules and its MHCII transcriptional mutant cell line (RJ2.2.5) have been previously described (32). Cocultures were established using either $MHCII^{+/+}$ or $MHCII^{-/-}$ B cells and $CD4^+$ T cells as outlined above. Cocultures were stimulated with Ags and IFN-γ production was quantified by ELISA at specific time points.

Purification of CP8

CP8 was purified and characterized as previously described (28) from cultures of *S. aureus* strain PS80 or an isogenic mutant of PS80 (MA20) that had the enterotoxin gene cluster (33) deleted by allelic replacement mutagenesis (34). CP8 purified from either strain demonstrated a similar level of activity in our assay system (data not shown), confirming the lack of superantigen contamination in our polysaccharide preparations. Polysaccharide purity was assessed by UV (260 and 280 nm) and nuclear magnetic resonance spectroscopy. Techoic acid contamination, assessed using a standard microphosphorus assay (35), was <1%. LPS contamination was established using an Endochrome Endpoint Chromogenic *Limulus* Amebocyte Lysate assay (Charles River Laboratories) and found to be <1 endotoxin unit/1 mg CP8.

CP8 labeling

CP8 was radiolabeled using a carbodiimide reaction. A 1-mg/ml solution of CP8 was prepared in H_2O and the pH adjusted to 4.75 before addition of an appropriate amount of carbodiimide reagent (*n*-cyclohexyl-*N*′-[2,4-morpholinyl-ethyl]carbodiimide-methyltolulenesulfonate) to modify ~20% of the carboxylic acids. The treated CP8 was neutralized with NaOH and reacted with [^{3}H]$NaBH_4$ followed by unlabeled $NaBH_4$ to neutralize any unreacted centers. The ^{3}H-labeled CP8 was then dialyzed against sterile H_2O before use.

APC processing of CP8

Raji B cells (1×10^8) were cultured in the presence of 0.5 mg of ^{3}H-labeled CP8 for 12–18 h at 37°C and microsomal depolymerization of CP8 was assessed as previously described (25). Microsomal CP8 was analyzed by molecular sieve chromatography on a Superose 12 column using fast protein liquid chromatography (Biologic). Fractions were assayed for radioactivity to determine the elution profile. The elution profile of microsomal CP8 was compared with that of intact CP8 recovered from the culture medium.

Quantification of intracellular bacteria

Surgical wound infection was established in IFN-$\gamma^{-/-}$ mice using 30 CFU of *S. aureus*. At 4 and 24 h, mice were injected directly into the wound tissue with recombinant CXCL2 or CCL3 (400 ng; R&D Systems). At the indicated time points, wound tissue was excised and the tissue was pooled from two mice before digestion with collagenase IV (1.5 mg/ml) for 1 h at 37°C. Neutrophils were sorted from the cell suspension using Abs to the neutrophil surface marker Ly6G (BD Pharmingen) and FACSAria cell-sorting system (BD Biosciences). Purified neutrophils were treated with lysostaphin (10 μg/ml) for 20 min (34) to kill extracellular organisms. The cells were washed in PBS, lysed in sterile water, and then plated on tryptic soy agar. The number of CFU was expressed per 10^6 neutrophils for each individual mouse pool.

Statistical analyses

Data are expressed as means ± SEM and statistical analysis was performed using an unpaired Student's *t* test or as medians and analyzed using a Mann-Whitney *U* test (Prism 4 GraphPad software). In both cases, a $p < 0.05$ was considered statistically significant.

Results

IFN-γ is produced locally at sites of S. aureus infection by $\alpha\beta TCR^+$ T cells

To investigate the local production of IFN-γ during *S. aureus* infections, we used a previously established murine model of *S. aureus* surgical wound infection. Using this model, infection can be established in the mouse with inocula as low as 10^1 CFU (12, 30). To establish infection, we used a serotype 8 strain of *S. aureus* (PS80). We inoculated WT mice with 10^2 CFU of *S. aureus* strain PS80. IFN-γ was measured in the wound tissue and was increased at 6 h after induction of infection, with the levels decreasing to that of the PBS-treated control wounds over time (Fig. 1*A*). To identify the cellular source of IFN-γ at the infection site, we compared IFN-γ production in the wound tissue of WT or $\alpha\beta TCR^{-/-}$ mice

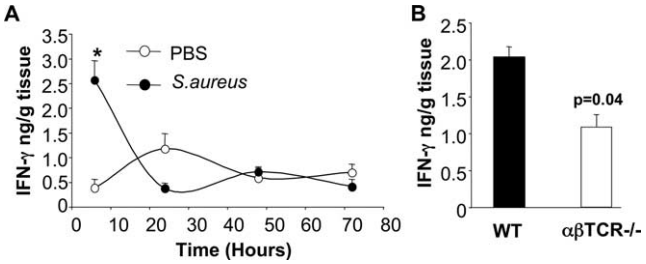


FIGURE 1. IFN-γ is rapidly produced at the site of *S. aureus* surgical wound infection. *A,* Surgical wounds were established in WT mice and challenged with *S. aureus* PS80 (10^2 CFU) or PBS. Wound tissue was excised at defined time points and IFN-γ tissue levels were measured by ELISA. *B,* Surgical wounds were established in WT and $\alpha\beta$TCR$^{-/-}$ mice challenged with *S. aureus* (10^2 CFU). IFN-γ levels were measured in the tissue at 6 h post induction of infection and were shown to be reduced significantly in the absence of $\alpha\beta$TCR signaling (*, $p < 0.05$, $n = 5$–10 mice/group).

during *S. aureus* infection. A significant reduction in wound tissue IFN-γ levels was observed in the $\alpha\beta$TCR$^{-/-}$ mice compared with the WT mice at 6 h (Fig. 1*B*). These results identify $\alpha\beta$TCR$^+$ T cells as a major source of IFN-γ during *S. aureus* infection and suggest that the bacteria possess some mechanism to induce IFN-γ production by this cell type.

S. aureus CP8 activates CD4$^+$ T cells to produce IFN-γ in a MHCII-dependent mechanism

To identify a mechanism by which *S. aureus* activates T cell production of IFN-γ, we adopted an in vitro coculture, using purified human CD4$^+$ T cells as responder cells and unfractionated mononuclear cells as APCs. Following 72 h (a previously identified optimal time point; data not shown) of stimulation, both heat-killed *S. aureus* PS80 and purified *S. aureus* CP8 induced significant IFN-γ production by the CD4$^+$ T cells in the cocultures at levels comparable to that produced by the superantigen staphylococcal enterotoxin A (SEA) and the conventional protein Ag tetanus toxoid (TT) (Fig. 2*A*). Stimulation of CD4$^+$ T cells alone or total mononuclear cells alone did not result in IFN-γ production, indicating that the APC-CD4 cell interaction is required to mediate CP8-induced T cell activation. Stimulation of the cocultures with the negatively charged polysaccharide control, polygalacturonic acid (GalU), did not induce cytokine production. Similar experiments were conducted using purified *S. aureus* CP5 and poly-*N*-acetylglucosamine (PNAG) (36, 37). These polymers also resulted in CD4$^+$ T cell-induced IFN-γ production, although at a somewhat lower level than that seen with CP8 (data not shown). These data indicate that CP8 can induce T cell cytokine production and that the APC is required in this process, which suggests that CP8 must first be presented by the APC for optimal T cell activation.

To explore this concept, we investigated the role of the MHCII pathway in CP8 activation of CD4$^+$ T cell-dependent IFN-γ production. The MHCII pathway involves uptake of the Ag into the APC and subsequent depolymerization or processing of the Ag. Using our previously described cell-based system to describe MHCII-dependent Ag processing of zwitterionic polysaccharides (25), we investigated whether *S. aureus* CP8 is depolymerized in a similar manner. [^{3}H]CP8 was incubated with an EBV-transformed human B cell line (Raji), which served as the APC in this system. Following 18 h of incubation, endosomes were extracted from lysed cells. Using a superose 12 chromatography system, we detected the presence of a smaller molecular mass species of CP8 (~15–20 kDa) in the endosomes compared with the size of the intact CP8 (~100 kDa) in the tissue culture medium (Fig. 2*B*). The endosomal presence of degraded CP8 implies that it is processed by the MHCII pathway.

We next confirmed that CP8 requires processing by the MHCII pathway for optimal CD4$^+$ T cell production of IFN-γ in our coculture system. We incubated the APCs with baflomycin A1, a proton pump inhibitor that prevents acidification of the MHCII vesicle, a crucial step in the MHCII processing pathway (25, 38, 39). In our coculture system, baflomycin A1 treatment significantly reduced IFN-γ production by the CD4$^+$ T cells in response to both heat-killed *S. aureus* and purified CP8, confirming that Ag processing of this carbohydrate is required to facilitate robust T cell cytokine production in response to the polysaccharide alone or the intact organism (Fig. 2*A*). Baflomycin treatment did not have any effect on the activation of T cells by SEA, because superantigens do not require intracellular processing for T cell activation. This result also demonstrates the lack of toxic effects of this drug on our coculture system. In contrast, baflomycin treatment completely abolished IFN-γ production induced by the conventional protein Ag TT, which relies on intracellular processing and presentation on the surface of the APC to activate T cells (Fig. 2*A*).

The involvement of MHCII in CD4$^+$ T cell-derived IFN-γ production in response to CP8 was confirmed using an isogenic MHCII-deficient Raji B cell line as APCs. Raji cells (MHCII positive) or the corresponding MHCII transcriptional-deficient cell line (RJ2.2.5) were cocultured with purified CD4$^+$ T cells and stimulated with CP8. Only the MHCII-expressing Raji cells supported IFN-γ production by the CD4$^+$ T cells in response to CP8 (Fig. 2*C*).

To establish further a role for MHCII in mediating the actions of CP8, we pretreated total mononuclear cells with blocking Abs specific for MHCII molecules HLA-DR, HLA-DP, HLA-DQ, the MHC class I molecules HLA-A, HLA-B, and HLA-C, or matched isotype control Abs, before adding the CD4$^+$ T cells and heat-killed *S. aureus* or CP8 as stimuli. Fig. 2*D* demonstrates that blockade of the MHCII molecule HLA-DR inhibited CP8-induced IFN-γ production by the CD4$^+$ T cells. IFN-γ production was significantly reduced (to ~25% of the no-Ab control) in the presence of Abs to HLA-DR but not by Abs specific for HLA-DP, HLA-DQ, or HLA-A, HLA-B, and HLA-C or the appropriate isotype control Abs. Similarly, IFN-γ production in response to heat-killed *S. aureus* PS80 was significantly reduced (to 50% of the no-Ab control) in the presence of blocking Abs to the HLA-DR molecule, confirming that a substantial proportion of *S. aureus*-induced T cell cytokine production can be attributed to the presence of bacterial Ags presented in the context of the HLA-DR MHCII molecule (Fig. 2*E*).

CP8 induces CXC chemokine-driven PMN recruitment in vivo and these effects are dependent upon activation of $\alpha\beta$TCR$^+$ T cells

We next determined whether purified CP8 could induce a robust inflammatory response in vivo in our surgical wound infection model and whether this effect was dependent upon signaling through the $\alpha\beta$TCR. WT mice challenged with CP8 (50 μg) produced significant CXCL1 and CXCL2 locally at the wound site (Fig. 3*A*). CXCL1 levels peaked at 6 h, whereas CXCL2 levels increased to a maximum at 48 h. The consequence of this disparate profile in CXC chemokine production is not understood but is consistent with what we observed in wounds infected with live *S. aureus* (12) and with findings in other surgical injury models (40). In mice lacking the $\alpha\beta$TCR, CXC chemokine production was significantly lowered in response to CP8 compared with the levels observed in WT mice (Fig. 3*A*). To investigate whether the effects

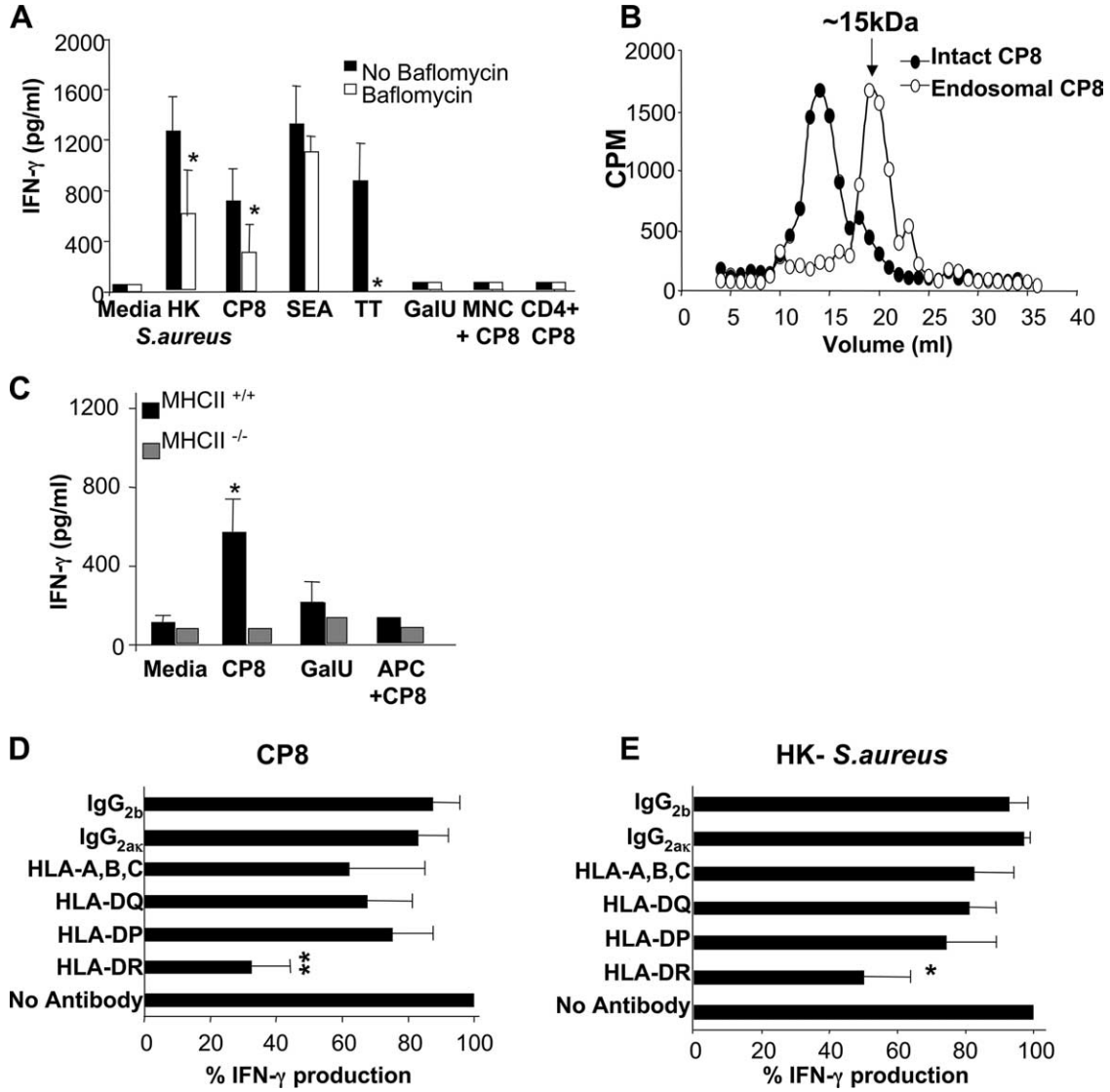


FIGURE 2. *S. aureus* CP8 activates T cells to produce IFN-γ in a MHCII-dependent mechanism. *A,* Total mononuclear cell (1×10^5)-$CD4^+$ T cell (1×10^5) cocultures were stimulated with the following Ags for 72 h: heat-killed (HK) *S. aureus* (10^2 CFU), *S. aureus* CP8 (50 μg), the non-zwitterionic polysaccharide polygalacturonic acid (GalU; 50 μg), the superantigen SEA (10 nM), or the conventional protein Ag TT (100 μg). Levels of IFN-γ present in the supernatant were measured by ELISA. For some experiments, baflomycin A1 (20 nM) was included in the coculture system for the duration of the experiment (results expressed as mean ± SEM for $n = 7$ individual donors; ∗, $p < 0.05$). *B,* Raji B cells were incubated in the presence of ^{3}H-labeled CP8 for 18 h. Endosomes were extracted from these cells, analyzed on a Superose 12 column, and the radioactivity levels of individual fractions were assayed. The presence of small-molecular-mass species CP8 (~15 kDa) was detected in the endosomes. The culture media were also removed from the cells and run on the same column to establish the size of the intact CP8 (~100 kDa) present in the culture media (representative of $n = 4$ experiments). *C,* Cocultures of $CD4^+$ T cells (1×10^5) with either MHCII-expressing B cells (Raji) or MHCII-deficient B cells (R2.2.5; 0.5×10^5) or monocultures of either B cell line alone were stimulated with CP8 (50 μg) or GalU (50 μg) for 72 h, and IFN-γ levels in the supernatant were quantified by ELISA (results expressed as mean ± SEM for $n = 3$ experiments; ∗, $p < 0.05$). Total mononuclear cells were incubated with blocking Abs (1 μg) to the MHC II molecules HLA-DR, HLA-DP, HLA-DQ, or the MHCI molecules HLA-A, HLA-B, and HLA-C and appropriate isotype control Abs for 30 min prior to the addition of $CD4^+$ T cells. Cocultures were then stimulated with either CP8 (50 μg, *D*) or HK *S. aureus* (10^2 CFU, *E*). IFN-γ levels in the supernatant were quantified by ELISA (results expressed as mean fold reduction vs no Ab control ± SEM for $n = 4$ individual donors; ∗, $p < 0.05$, ∗∗, $p < 0.01$).

of CP8 on local CXC chemokine production translated into an effect on PMN recruitment, we examined H&E-stained wound tissue sections from mice challenged with CP8 (50 μg), GalU (50 μg), or PBS. WT mice responded to the CP8 challenge with an inflammatory infiltrate that was more robust that that seen in the mice receiving PBS or GalU. Furthermore, the $\alpha\beta TCR^{-/-}$ mice challenged with CP8 demonstrated impaired leukocyte infiltration to the wound site compared with WT mice (Fig. 3*B*). We quantitated the differences in PMN infiltration among groups using a MPO assay (41) (42). WT mice challenged with CP8 demonstrated significantly higher MPO levels compared with mice receiving PBS or GalU. Consistent with the histology findings, the $\alpha\beta TCR^{-/-}$ mice receiving CP8 showed significantly reduced tissue MPO levels (Fig. 3*C*).

CP8 activation of CXC chemokine-driven neutrophil recruitment is IFN-γ dependent

We hypothesized that the in vivo effects of CP8 on CXC chemokine-driven neutrophil recruitment were mediated indirectly by IFN-γ produced by $\alpha\beta TCR^+$ T cells. Furthermore, our in vitro analyses have identified a mechanism by which purified *S. aureus* CP8 can activate $CD4^+$ T cells to produce IFN-γ, but these same

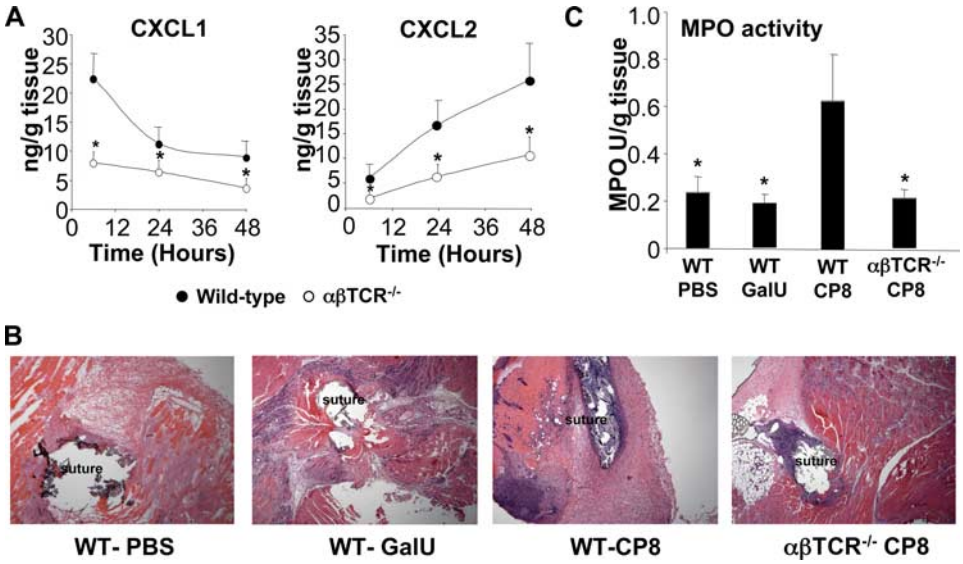


FIGURE 3. CP8-induced CXC chemokine production and associated neutrophil recruitment in vivo is $\alpha\beta$TCR dependent. *A,* Surgical wounds were established in WT and $\alpha\beta TCR^{-/-}$ mice and challenged with purified *S. aureus* CP8 (50 μg). At specific time points, the wound tissue was excised and homogenized and CXC chemokine levels were measured in the tissue by ELISA (results expressed as mean ± SEM, $n = 6$ mice; *, $p < 0.05$). *B,* H&E-stained paraffin-embedded tissue sections were prepared from excised surgical wound tissue on day 3 following challenge with either PBS, GalU (50 μg), or CP8 (50 μg). CP8 induced a robust inflammatory infiltrate in WT mice compared with either PBS or GalU mice. This infiltration was reduced in the $\alpha\beta TCR^{-/-}$ mice (representative section of $n = 3$ mice). *C,* Tissue MPO levels were measured in excised wound tissue on day 3 following challenge with PBS, GalU (50 μg), or CP8 (50 μg) in WT mice and CP8 (50 μg) in $\alpha\beta TCR^{-/-}$ mice. MPO levels were significantly elevated in WT mice following challenge with CP8 compared with $\alpha\beta TCR^{-/-}$ mice or WT mice challenged with either PBS or GalU (results expressed as mean ± SEM, $n = 6$ mice; *, $p < 0.05$).

cells did not produce significant levels of CXCL8 in response to CP8 or heat-killed *S. aureus* stimulation (data not shown), suggesting that T cells are not directly chemotactic for neutrophils. Surgical wounds were established in groups of WT and IFN-$\gamma^{-/-}$ mice, and the mice were challenged with purified CP8 (50 μg). CXCL1 and CXCL2 production was impaired in the IFN-$\gamma^{-/-}$ mice at 6 and 48 h, respectively, compared with the WT mice (Fig. 4*A*). This correlated with a reduction in PMN infiltration to the wound site at 72 h in the IFN-$\gamma^{-/-}$ mice (Fig. 4*B*) and the reduced histological score assigned to these tissues (Fig. 4*C*). To quantify further the CP8-driven PMN response in these animals, we measured MPO activity in the wound tissue at 72 h postchallenge. CP8

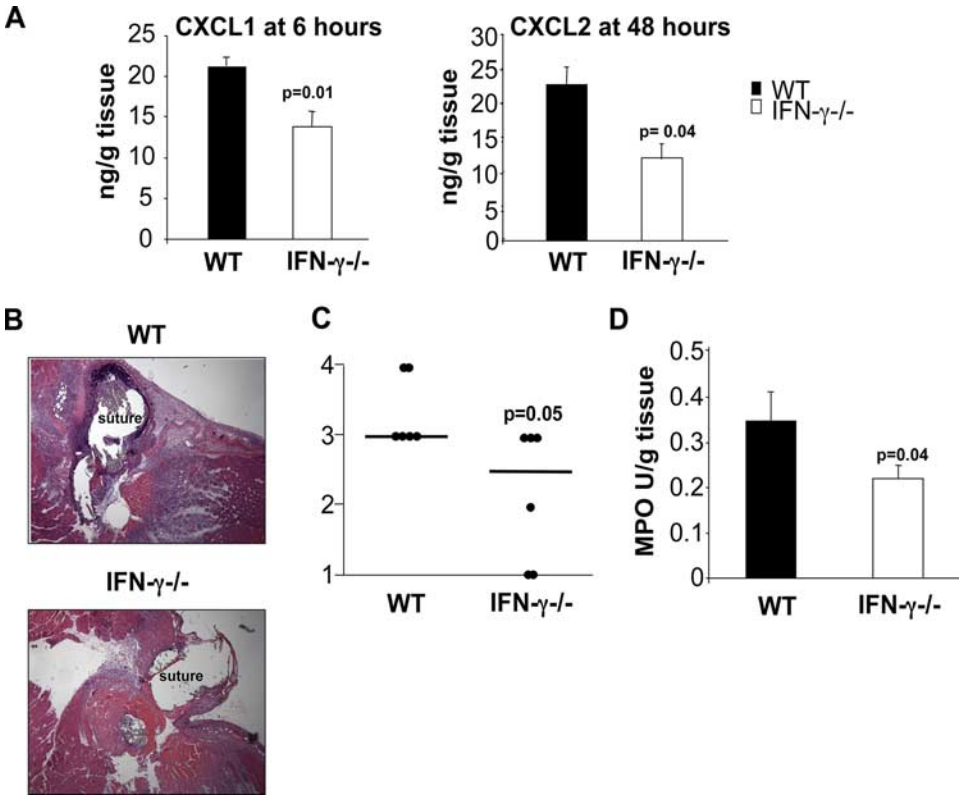
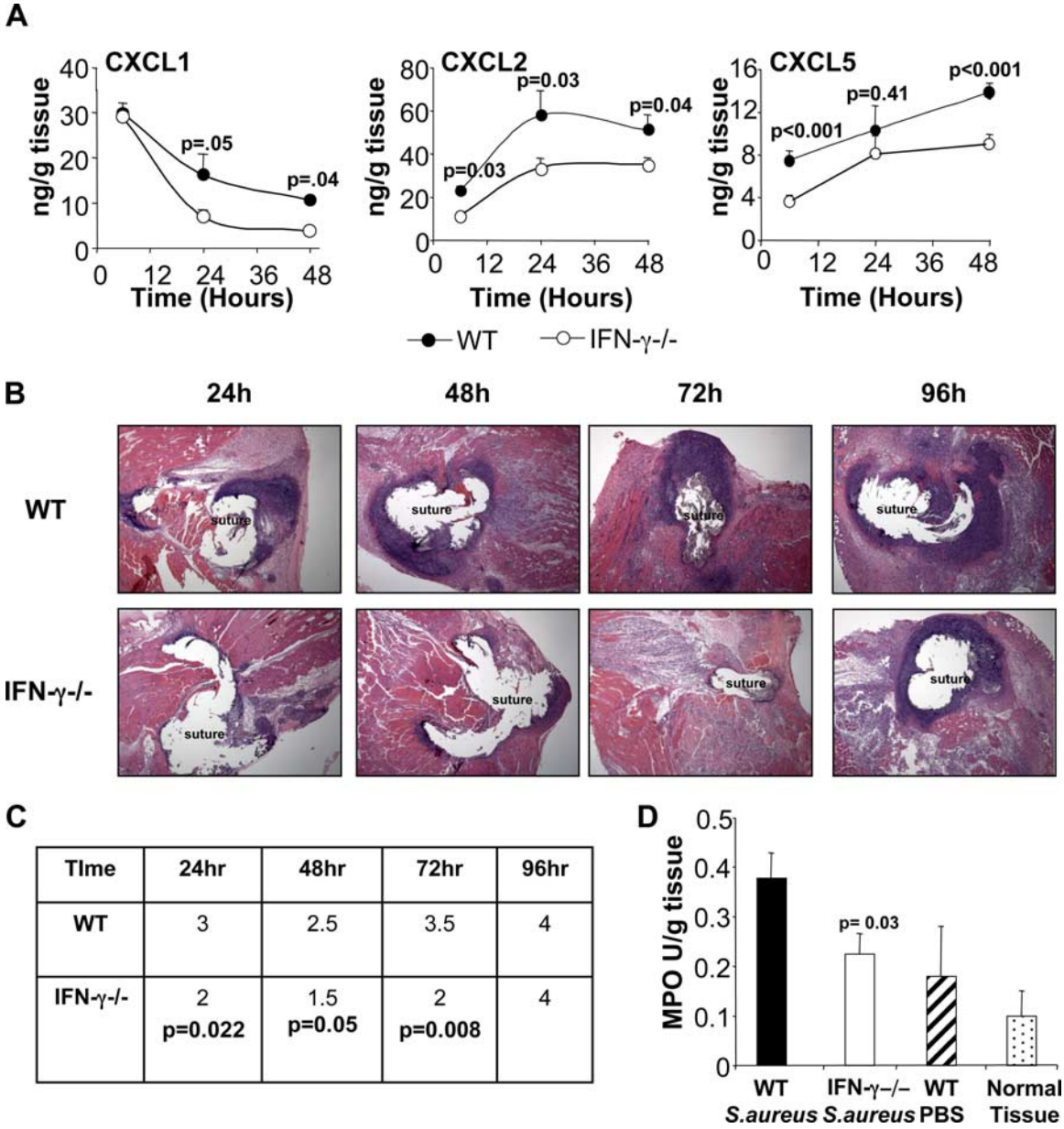


FIGURE 4. CP8 effects on neutrophil recruitment are dependent upon local IFN-γ production. *A,* Surgical wounds were established in WT and IFN-$\gamma^{-/-}$ mice and challenged with CP8 (50 μg). CXCL1 and CXCL2 levels were measured in the tissue at 6 and 48 h, respectively, after induction of infection and were reduced in the IFN-$\gamma^{-/-}$ mice (results expressed as mean ± SEM, $n = 10$). *B,* Paraffin-embedded tissue sections were prepared from WT and IFN-$\gamma^{-/-}$ mice on day 3 following CP8 challenge. H&E staining demonstrated impaired neutrophil infiltration in the IFN-$\gamma^{-/-}$ mice compared with WT animals (representative section of $n = 5$ mice). *C,* Tissue sections were scored by two individuals blinded to the identity of the sample and results were plotted for individual mice; the bar indicates median score ($n = 5$). *D,* To quantify neutrophil infiltration further, tissue MPO levels were assessed in wound tissue on day 3 following challenge with CP8 (50 μg). IFN-$\gamma^{-/-}$ mice had significantly reduced levels of MPO at the wound site compared with WT mice (results expressed as mean ± SEM, $n = 5$).



TIme	24hr	48hr	72hr	96hr
WT	3	2.5	3.5	4
IFN-γ-/-	2 p=0.022	1.5 p=0.05	2 p=0.008	4

FIGURE 5. The neutrophil response during *S. aureus* surgical wound infection was altered in IFN-$\gamma^{-/-}$ mice. *A,* Surgical wound infection was established in WT and IFN-$\gamma^{-/-}$ mice and tissue levels of CXCL2, CXCL5, and CXCL1 were measured at the indicated time points (results expressed as mean ± SEM, $n = 10–15$). *B,* Wound tissue was also excised at 24, 48, 72, and 96 h after induction of infection, formalin fixed, and embedded in paraffin. Tissue sections were H&E-stained to visualize inflammatory infiltration around the wound suture site (representative section of $n = 3–6$ individual mice). *C,* Tissue sections were scored and results are presented as median values ($n = 3–6$). *D,* Surgical wound infection was established in WT and IFN-$\gamma^{-/-}$ mice and, at 72 h, wound tissue was excised and homogenized, and tissue MPO levels were quantified (results expressed as mean ± SEM, $n = 5$).

induced a robust MPO response in WT mice that was significantly reduced in the IFN-$\gamma^{-/-}$ mice (Fig. 4*D*). These data suggest that CP8 mediates its effects on PMN trafficking indirectly through its ability to induce IFN-γ production by $CD4^+$ T cells.

CXC chemokine-driven PMN recruitment during S. aureus infection is dependent upon the presence of IFN-γ

To determine the involvement of IFN-γ in coordinating PMN recruitment during live *S. aureus* infection, surgical wound infection was induced in groups of WT and IFN-$\gamma^{-/-}$ mice using an inoculum of 10^2 CFU of *S. aureus* PS80. CXC chemokine levels were measured in the wound tissue at 6, 24, and 48 h after bacterial challenge. CXCL2 levels were significantly reduced in the wound tissue of IFN-$\gamma^{-/-}$ mice compared with that of WT mice at all time points; similarly, CXCL5 levels were significantly reduced at 6 and 24 h. CXCL1 levels, however, were significantly reduced in the IFN-$\gamma^{-/-}$ mice only at 24 and 48 h (Fig. 5*A*). CXCL1 levels are not different between the WT and IFN-$\gamma^{-/-}$ mice at the 6-h time point, suggesting that CXCL2 and CXCL5 rather than CXCL1 are more significant targets for the actions of IFN-γ. All three CXC chemokines were produced with distinct profiles, suggesting that their production is regulated by distinct pathways and also that they may play specific roles in regulating the neutrophil response in vivo (40, 43). This may be due to the fact that IFN-γ production at the wound site peaks at 6 h, the same time as CXCL1; therefore, IFN-γ does

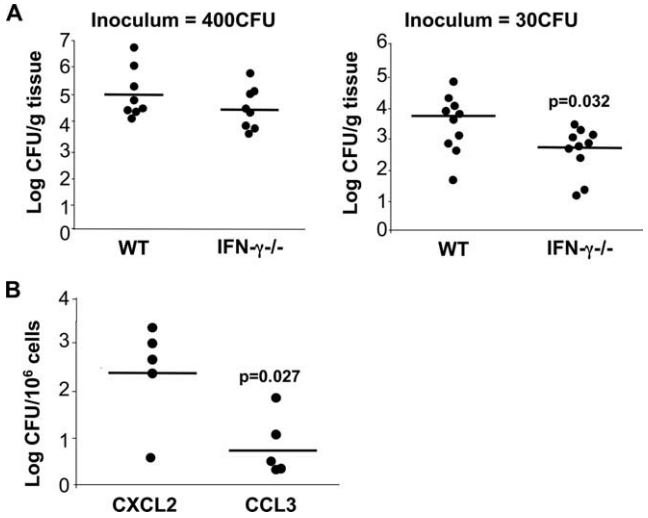


FIGURE 6. IFN-$\gamma^{-/-}$ mice exhibit decreased tissue bacterial burden compared with WT mice as a consequence of the reduced CXC chemokine production seen in these mice. *A,* Surgical wound infection was established in WT and IFN-$\gamma^{-/-}$ mice with either 30 or 400 CFU. Wound tissue was excised on day 3 and total tissue bacterial burden was quantitated by plate counts. Limit of detection of the assay was ~1.6 log. Results are expressed as log CFU per g of tissue, means indicated by bar ($n = 10$). *B,* IFN-$\gamma^{-/-}$ mice were challenged with *S. aureus* (10 CFU) and treated with either CXCL2 or CCL3 (400 ng). Neutrophils were isolated from the wound tissue by collagenase digestion on day 3 of infection. The cell suspension was sorted on the Ly6G$^+$ neutrophils, and the levels of viable organisms present were intracellular quantified. CXCL2 treatment increased the numbers of viable organisms internalized per 10^6 neutrophils compared with CCL3 treatment (*, $p < 0.05$). The limit of detection of this assay was ~0.38 log.

not have a chance to modulate the early production of this cytokine.

To establish the impact of the altered CXC chemokine profiles on the recruitment of neutrophils to the wound site, we assessed H&E-stained wound tissue sections from WT and IFN-$\gamma^{-/-}$ mice challenged with *S. aureus*. Compared with WT mice, neutrophil recruitment in IFN-$\gamma^{-/-}$ mice was significantly reduced up to 72 h (Fig. 5*B*). These differences in neutrophil infiltration were confirmed at 72 h by measurement of tissue MPO levels (Fig. 5*D*). At 96 h after induction of infection, PMN infiltration at the wound site appeared similar in WT and IFN-$\gamma^{-/-}$ mice, suggesting that IFN-γ is only required for early CXC chemokine-driven neutrophil recruitment. At later time points, other factors may compensate for the diminished levels of this cytokine in the IFN-$\gamma^{-/-}$ mice.

T cell production of IFN-γ can influence S. aureus surgical wound infection

We next investigated how the impaired PMN response seen in IFN-$\gamma^{-/-}$ mice would impact infection outcomes. *S. aureus* surgical wound infection was established in groups of WT and IFN-$\gamma^{-/-}$ mice using an inoculum of either 30 or 400 CFU, and the total tissue bacterial burden was evaluated 3 days postchallenge. Previous studies have demonstrated that maximal bacterial levels at the wound site were achieved by day 3 (12). At an inoculum of 400 CFU/wound, we observed a 0.5 log reduction in the tissue bacterial burden in IFN-$\gamma^{-/-}$ mice compared with WT mice (mean log CFU/g of tissue of 5.1 and 4.55 in WT and IFN-$\gamma^{-/-}$ mice, respectively), whereas an inoculum of 30 CFU resulted in a significant 1 log reduction in infection levels in the absence of IFN-γ (mean log CFU/g of tissue of 3.8 and 2.7 in WT and IFN-$\gamma^{-/-}$ mice, respectively) (Fig. 6*A*). CXC chemokine production and PMN infiltration to the wound site were similar in WT mice challenged with either 30 or 400 CFU *S. aureus* PS80 (data not shown). These data suggest that fewer PMN recruited to the wound site in IFN-$\gamma^{-/-}$ mice resulted in more efficient clearance of the infecting bacteria.

CXC chemokines increase the number of intracellular bacteria associated with neutrophils isolated from the S. aureus wound infection site

To explain the observation that decreased neutrophil infiltration to the wound site in IFN-$\gamma^{-/-}$ mice was associated with a reduction in tissue bacterial burden, we hypothesized that the bacteria may survive within the neutrophil. Therefore, a neutrophil-rich environment may actually exacerbate infection. To explore this concept, *S. aureus* surgical wound infection was induced in IFN-$\gamma^{-/-}$ mice using a low-dose inoculum (30 CFU) and recombinant CXC (CXCL2) or CC (CCL3) chemokines administered directly into the wound site to reconstitute the reduced chemokine levels seen in these mice during *S. aureus* infections (Fig. 4*A* and Ref. 12). At 72 h, neutrophils were isolated from the excised wound tissue by collagenase digestion, followed by cell sorting to enrich for Ly6G$^+$ cells. Following lysostaphin treatment to kill extracellular organisms, the cells were lysed and the levels of viable intracellular bacteria were quantified by plate count analysis. Reconstitution of CXC chemokine activity in the IFN-$\gamma^{-/-}$ mice resulted in a significant increase in neutrophil-associated intracellular *S. aureus* survival compared with administration of the CC chemokine CCL3 that has no effects on neutrophil trafficking or activation (Fig. 6*B*). CCL3 administration had no effect on tissue bacterial burden above the effects of PBS (data not shown).

Discussion

In this study, we identify the T cell cytokine IFN-γ as a critical regulator of neutrophil recruitment during staphylococcal wound infection. Neutrophils represent the major cell type involved in eradication of *S. aureus* infection. However, given the controversial findings that neutrophils may act as reservoirs for viable *S. aureus* during infection (11), it is clear that any dysregulation in neutrophil trafficking could facilitate bacterial pathogenesis. Therefore, it is vital that appropriate control mechanisms are in place to coordinate the timely recruitment to, and subsequent clearance of, neutrophils from the infection site.

During *S. aureus* surgical wound infection, $\alpha\beta$TCR$^+$ T cells were identified as a significant source of IFN-γ. The absence of $\alpha\beta$TCR$^+$ T cells did not result in a complete abolition of IFN-γ production at the surgical wound site, suggesting that other cell types contribute to *S. aureus*-induced IFN-γ production. However, previous studies have demonstrated that CD8$^+$ T cells do not play a significant role in determining the outcome of *S. aureus* surgical wound infection (12). A clear role for NK cells during *S. aureus* infections remains to be established, although most studies have attributed NK cell activation to the *S. aureus* superantigens. In our model of surgical wound infection, we are using a low enterotoxin-producing strain of *S. aureus*, suggesting that in this setting NK cells are not a major player in modulating the host's response.

In light of recent studies delineating the mechanisms by which zwitterionic CPs activate T cells (25, 44) coupled with the previously documented ability of CP8 to modulate intra-abdominal abscess formation in mice and activate human T cell proliferation in vitro (28), we identified this molecule as a candidate Ag expressed by *S. aureus* for activating T cell cytokine production. We demonstrate using an in vitro coculture system that *S. aureus* CP8 can activate CD4$^+$ T cells to produce the cytokine IFN-γ in a MHCII

pathway-dependent manner. Activation of $\alpha\beta TCR^+$ T cells by foreign Ags typically requires their uptake into APCs and then processing of the Ag through the MHCII endocytic pathway. Most pathogens are endocytosed by the APCs and are subsequently degraded through the cells' oxidative burst mechanisms. Endosomes then carry released peptides or carbohydrate molecules through the vesicular traffic for eventual loading onto the MHCII molecule and shuttling to the cell surface for recognition by $\alpha\beta TCR^+$ T cells. We provide evidence that CP8 can be taken up by APCs and processed or depolymerized to a low-molecular-mass species in the endosomes of these cells ready for loading on the MHCII molecule and presentation on the cell surface. Blocking studies using HLA-DR-specific mAbs categorically prove that an interaction between CP8 and the MHCII molecule HLA-DR is required for robust T cell cytokine production. We confirm that CP8 is not simply acting as a superantigen since baflomycin treatment significantly reduced CP8-induced T cell production of IFN-γ but did not inhibit the actions of the true superantigen SEA, which can bind to the MHCII molecule and activate T cells independent of intracellular processing.

Analysis of leukocytes isolated from the surgical wound infection site demonstrated the presence of $F480^+$ monocytes at the wound site as early as 1 h postinfection, with infiltration of these cells reaching a maximum at 3 h (data not shown). These monocytes can process and present CP8 to the $\alpha\beta TCR^+$ T cells in a rapid manner. Previous studies have demonstrated that presentation of polysaccharide Ags can occur in as little as 3 h (25), confirming this as a mechanism by which CP8 expressed by invading *S. aureus* can activate T cell production of cytokines early in the inflammatory response. Zwitterionic polysaccharides can activate T cells with a broad Vβ usage and cross-reactivity has been demonstrated between zwitterionic CPs from different bacterial species, indicating that these polysaccharides act in a nonbiased manner to promote robust cytokine production by T cells (45).

In the in vitro coculture system, CP8 did result in some level of T cell-induced IFN-γ production even in the absence of the MHCII pathway. Similarly, IFN-γ production was seen in vivo even in the absence of $\alpha\beta$TCR signaling. It is possible that CP8 can activate TLR2 signaling, which can promote IFN-γ production by T cells in an IL-12-dependant manner (44). During surgical wound infection, *S. aureus* CP8 may activate TLR2 signaling, which could contribute to local T cell cytokine production. Although nothing is known about how CP8 activates TLR signaling, other *S. aureus* surface molecules such as lipoteichoic acid have been shown to activate cytokine production in a TLR2-dependent mechanism (46, 47). However, a recent study excluded the involvement of TLR2 signaling in regulating neutrophil recruitment during *S. aureus* skin infections and attributed these effects instead to the IL-1R/MyD88 pathway (48). Further research is required to delineate the mechanisms by which CP8 can activate these signaling pathways to modulate the host's immune response.

We have defined a mechanism by which *S. aureus* CP8 can activate T cell production of IFN-γ. CP8 is not unique, however, in its ability to activate T cells because other bacterial components such as PNAG, wall-teichoic acid, and lipoteichoic acid may also contribute to T cell cytokine production. In fact, preliminary studies indicate that both CP5 and PNAG can activate $CD4^+$ T cell production of IFN-γ in vitro with similar and slightly lower potency, respectively, to that seen with CP8 (data not shown). In addition, a previous study demonstrated the analogous ability of CP8, CP5, and wall-teichoic acid to modulate abscess formation in a T cell-dependent manner (28). To investigate the relative contribution of CP8 to IFN-γ production during *S. aureus* wound infection, we challenged WT mice with an isogenic acapsular mutant of strain PS80 (RMS-1). RMS-1 induced lower IFN-γ production at the wound site at 6 h compared with PS80 (762 ± 147 pg/ml vs 1047 ± 102 pg/ml, respectively), indicating a definite role for CP8 in activating IFN-γ production. However, it was not a significant reduction, suggesting that other components of the bacteria are capable of activating T cell cytokine production in the absence of CP8. It is naive to think that individual bacterial components can exclusively modulate aspects of the host immune response. *S. aureus* can express a disparate spectrum of virulence factors depending on the strain, phase of growth, and microenvironment. In an organism so complex, it is clear that there would be a redundancy of effects between the abilities of these components to modulate the immune response.

Previous studies have demonstrated that T cells present at sites of *S. aureus* infection can control the local neutrophil response (12). We now identify CP8-induced production of IFN-γ by the T cells as a mechanism by which this occurs. In vivo CP8 challenge resulted in increased production of CXC chemokines and associated neutrophil recruitment to the wound site. However, in the absence of either $\alpha\beta TCR^+$ T cells or IFN-γ, the effects of CP8 on neutrophil recruitment were significantly attenuated. Furthermore, we demonstrate that in the context of live *S. aureus* infection, IFN-γ is required for optimal CXC chemokine production and associated neutrophil recruitment. These in vivo observations are consistent with our in vitro observations that CP8 activation of $\alpha\beta TCR^+$ T cells results in the production of IFN-γ and the in vivo consequence of this IFN-γ production is to promote neutrophil recruitment.

During *S. aureus* surgical wound infection, it is likely that proinflammatory cytokines such as IL-1β and/or TNF-α are produced through activation of a MyD88 signaling pathway (49). These cytokines can induce chemokine production by cells present locally at the infection site such as infiltrating monocytes (50) and muscle tissue (51, 52). IFN-γ production by recruited T cells then serves to enhance this CXC chemokine response. In this respect, IFN-γ has previously been shown to act cooperatively with both IL-1β and TNF-α to control the expression of a variety of cytokines and chemokines (53) (19, 54). It is not surprising then that CXC chemokine production at the *S. aureus* wound site was not completely abolished in the $IFN\text{-}\gamma^{-/-}$ mice. Neutrophil recruitment to the infection site is impaired early in the $IFN\text{-}\gamma^{-/-}$ mice, but by 96 h has reached similar levels to those seen in the WT mice. The decrease in PMN infiltration confirms a role for IFN-γ early in the innate response to *S. aureus* infection and is consistent with previous studies that highlighted the ability of IFN-γ to influence the early effector arm of the neutrophil response (19). The impact of IFN-γ on later phases of the neutrophil response during staphylococcal infection, i.e., apoptotic clearance, remain to be established. Furthermore, we must consider a role for alternative T cell-derived cytokines e.g., IL-17 in regulating the neutrophil response. IL-17 has previously been shown to impact upon both recruitment and effector activities of neutrophils during the infectious process (55–57). Investigating a clear role for IL-17 in *S. aureus* infection will form the basis of future studies.

$IFN\text{-}\gamma^{-/-}$ mice had lower levels of bacteria recovered from the wound site compared with WT mice. The findings that heightened neutrophil levels are associated with increased levels of infection support the hypothesis that under some conditions a neutrophil-rich environment can actually exacerbate infection due to the ability of *S. aureus* to gain entry into and survive within the neutrophil (11, 12). Previously, studies have demonstrated that a higher mortality rate in mice challenged with *S. aureus* correlated with increased neutrophil numbers and an increased bacterial burden at the site of infection. The mechanism of intracellular survival in this

study was attributed to the uptake of *S. aureus* organisms into macropinosomes occurring as a consequence of membrane ruffling, which did not support efficient killing of the ingested organism. CXC chemokines were shown to enhance this process (58). Consistent with this finding, we demonstrated that local administration of the CXC chemokine CXCL2 but not the CC chemokine CCL3 directly into the *S. aureus* wound infection site in IFN-$\gamma^{-/-}$ mice (which demonstrate reduced neutrophil influx to the *S. aureus* infection site) increased the numbers of viable organisms present within the neutrophils isolated from the wound site.

S. aureus has evolved to develop a variety of virulence mechanisms that allow it to circumvent the host's immune response (59). Although not traditionally regarded as an intracellular pathogen, numerous studies have demonstrated the ability of *S. aureus* to survive for significant periods of time inside various cell types (60–63). The concept that neutrophils may act as reservoirs for viable *S. aureus* organisms and can therefore actually potentiate persistent infection has yet to be explored fully. In this study, we demonstrate that CXC chemokines can promote the intracellular survival of *S. aureus* within neutrophils, therefore exacerbating infection. In an alternative bacterial infection model, CXC chemokine effects on neutrophils have resulted in decreased killing of the invading microorganisms. Neutrophils harvested from periodontitis abscesses demonstrated reduced ability to kill *Porphyromonas gingivalis* as a consequence of decreased neutrophil H_2O_2 production due to excessive IL-8 priming (64). Further work outside the scope of this study is required to ascertain the precise mechanisms by which heightened CXC chemokine levels facilitate the survival of *S. aureus* at sites of surgical wound infection. If even a very small number of *S. aureus* cells can survive within the neutrophil and begin to replicate, this could exacerbate the local infection.

This study highlights the necessity for neutrophil trafficking to be tightly regulated during *S. aureus* infection and identifies a novel mechanism by which the T cell-derived cytokine IFN-γ facilitates a neutrophil-rich environment at the infection site by directing CXC chemokine-driven neutrophil recruitment. In the absence of appropriate regulatory signals, uncontrolled neutrophil trafficking has previously been shown to result in chronic inflammatory tissue damage (65). In the context of *S. aureus* infection, this situation could provide a reservoir for even a very small number of *S. aureus* organisms to exploit the host response by gaining entry and surviving inside the neutrophil. Studies such as ours, which expand our insights into the mechanisms of host response to infection, contribute to a more lucid understanding of host-pathogen interactions. This is a crucial step in developing specific drugs to treat the rapidly growing epidemic of resistant *S. aureus* infections.

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Disclosures

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