Staphylococcus aureus SaeR/S-Regulated Factors Decrease Monocyte-Derived Tumor Necrosis Factor- α to Reduce Neutrophil Bactericidal Activity

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Background. The ability of *Staphylococcus aureus* to evade killing by human neutrophils significantly contributes to disease progression. In this study, we characterize an influential role for the *S. aureus* SaeR/S 2-component gene regulatory system in suppressing monocyte production of tumor necrosis factor alpha (TNF- α) to subsequently influence human neutrophil priming.

Methods. Using flow cytometry and TNF- α specific enzyme-linked immunosorbent assays we identify the primary cellular source of TNF- α in human blood and in purified peripheral blood mononuclear cells (PBMCs) during interaction with USA300 and an isogenic saeR/S deletion mutant (USA300 Δ saeR/S). Assays with conditioned media from USA300 and USA300 Δ saeR/S exposed PBMCs were used to investigate priming on neutrophil bactericidal activity.

Results. TNF- α production from monocytes was significantly reduced following challenge with USA300 compared to USA300 Δ saeR/S. We observed that priming of neutrophils using conditioned medium from peripheral blood mononuclear cells stimulated with USA300 Δ saeR/S significantly increased neutrophil bactericidal activity against USA300 relative to unprimed neutrophils and neutrophils primed with USA300 conditioned medium. The increased neutrophil bactericidal activity was associated with enhanced reactive oxygen species production that was significantly influenced by elevated TNF- α concentrations.

Conclusions. Our findings identify an immune evasion strategy used by *S. aureus* to impede neutrophil priming and subsequent bactericidal activity.

Keywords. *Staphylococcus aureus*; neutrophil; SaeR/S; monocyte; TNF- α .

Staphylococcus aureus is a ubiquitous Gram-positive bacterium estimated to permanently colonize 1 of 3 individuals [1]. *Staphylococcus aureus* typically causes treatable cutaneous abscesses, but infections can progress into dermonecrotic lesions and life-threatening infections, including septicemia and necrotizing pneumonia [2, 3]. Methicillin-resistant *S. aureus* (MRSA) further represents a major public health concern as it is one of the most common antimicrobial-resistant pathogens to cause infections in both hospital and community settings [4]. This pervasiveness is in part attributed to the ability of *S. aureus* to circumvent protective immune responses, including evasion of human neutrophil killing (reviewed in [5]). We and others have demonstrated that factors under the regulation of the *S. aureus* SaeR/S 2-component gene regulatory system (SaeR/S TCS) contribute

significantly to this ability [6–9]. However, the molecular mechanisms dependent on SaeR/S that promote *S. aureus* survival following neutrophil interaction are incompletely defined.

The SaeR/S TCS regulates genes that control cytolytic and immunomodulatory factors that have been shown to significantly contribute to exacerbated infections in murine models of infection and ex vivo human models of infection [6, 10–16]. The contribution of immunomodulation to these negative outcomes has been exemplified through the ability of SaeR/S-regulated factors to induce specific inflammatory signals that promote a detrimental immune response [7, 11, 17, 18]. For example, SaeR/S-regulated staphylococcal binding immunoglobulin protein (Sbi) was shown to induce robust production of interleukin 6 (IL-6) and modulate levels of CXCL-1 during invasive infection [19]. This alteration in cytokine production during the early stages of infection subsequently influenced neutrophil recruitment and contributed to an overall deleterious inflammatory response. Similarly, we have demonstrated the ability of SaeR/S-regulated virulence factors to induce robust production of inflammatory cytokines, including interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), IL-6, and interleukin 2 (IL-2), that are associated with increased bacterial survival and host mortality [11, 18]. In line with these immunomodulatory roles, we further demonstrated that SaeR/S-regulated factors can suppress phosphorylation of nuclear factor- κ B (NF- κ B) and reduce production of interleukin 8 (IL-8) in

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human neutrophils to promote cell death and enhance bacterial survival [7]. Collectively, these findings highlight immune evasion strategies by which SaeR/S-regulated factors influence inflammatory signaling to exacerbate infection. However, the specific immune cells that are targeted for immunomodulation by SaeR/S-regulated factors and the subsequent influence on downstream immune responses remain incompletely defined.

Peripheral blood mononuclear cells (PBMCs) have been shown to regulate inflammatory signals that promote neutrophil recruitment and enhance their antimicrobial mechanisms in response to *S. aureus* infection [20–22]. Many PBMC products such as monocyte-derived interleukin 1 (IL-1) and TNF- α also act as neutrophil priming agents [21, 23]. Neutrophil priming is a mechanism that regulates the level of neutrophil activation to provide an appropriate response to a secondary stimulus [23]. Despite the importance of inflammatory mediators produced by PBMCs that are involved in neutrophil priming, there is a lack of studies examining the impact of *S. aureus* on this process. Previous studies have investigated the effects of conditioned media (CM) containing inflammatory signals produced by human PBMCs following exposure to heat-killed *S. aureus* on neutrophil functions [24, 25]. These studies observed that neutrophil staphylocidal activity was enhanced following priming with CM and that this effect was dependent on PBMC production of TNF- α [25]. While these effects were observed using CM from heat-killed *S. aureus*, it remains to be elucidated if CM from live bacteria elicit a similar response on neutrophil priming.

In the current study, we developed a co-culture model using human PBMCs and neutrophils, as well as CM assays, to demonstrate the importance of TNF- α during monocyte–neutrophil interactions following challenge with live *S. aureus*. We observed that SaeR/S-regulated factors significantly decreased monocyte production of TNF- α . Diminished TNF- α concentrations significantly influenced neutrophil staphylocidal activity by limiting neutrophil priming and subsequent production of reactive oxygen species (ROS). Collectively, this study demonstrates that the response of PBMCs to *S. aureus* significantly impacts the outcome of neutrophil–*S. aureus* interactions.

METHODS

Bacterial Strains and Cultures

Community-associated MRSA pulsed-field gel electrophoresis type USA300 (strain LAC), USA400 (strain MW2), and isogenic *saeR/S* and *hla* deletion mutants (USA300 Δ *saeR/S*, USA400 Δ *saeR/S*, and USA300 Δ *hla*), generated in previous studies [6, 10, 15], were grown in tryptic soy broth containing 0.5% glucose and harvested at midexponential growth, as described previously [6, 10, 12, 15].

Assessment of TNF- α Production From Human Cells

Heparinized venous blood from healthy donors was collected in accordance with the protocol approved by the Institutional

Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. For whole-blood experiments, 20 μ L of Roswell Park Memorial Institute medium (RPMI) only or RPMI with 2×10^6 colony-forming units (CFU) of bacteria was added to 980 μ L of freshly drawn human whole blood in 1.5-mL Eppendorf tubes. Samples were placed on an end-over-end mixer (20 RPM, 37°C, 5% carbon dioxide) and, at designated time points, samples were stained with fluorescein isothiocyanate (FITC) mouse antihuman CD14, phycoerythrin mouse anti-human CD3, and allophycocyanin mouse antihuman TNF or immunoglobulin G1 (IgG1) isotype control (BD Biosciences). Intracellular staining was performed using a BD Pharmingen intracellular stain kit following the manufacturer's protocol, and all samples were analyzed using a FACSCalibur Flow Cytometer (BD Bioscience). Alternatively, serum was collected by centrifugation (524g for 10 minutes), filtered using a 0.2- μ m filter (PALL Life Science), and stored at –80°C until quantitation of TNF- α was performed using a TNF- α -specific enzyme-linked immunosorbent assay (ELISA, BD Biosciences).

Human neutrophils (polymorphonuclear leukocytes [PMNs]) and peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (<25 pg/mL) as previously described [6, 12]. Purity (<1% PBMC contamination) and viability (<2% propidium iodide positive) of neutrophil preparations were assessed by flow cytometry (FACSCalibur). For co-culture assays, 7×10^5 neutrophils were combined with 3×10^5 PBMCs at a physiological ratio and exposed to 2×10^6 CFUs of *S. aureus* opsonized with 50% normal human serum (NHS) in a 96-well NHS-coated plate. After bacteria were added, phagocytosis was synchronized by centrifugation (524g for 7 minutes at 10°C) and then incubated at 37°C [12]. At designated time points, samples were stained as above and analyzed using flow cytometry.

For PBMC CM experiments, 5×10^6 PBMCs were challenged with 1×10^7 opsonized bacteria in a 24-well serum-coated plate that was then synchronized and incubated at 37°C [12]. At designated time points, samples were filtered using a 0.2- μ m filter (PALL Life Science), plated to ensure they did not contain live bacteria, and stored at 4°C for further use as CM or at –80°C for assessment of intracellular TNF- α production using ELISA as described above.

TNF- α Inhibition Assays

Calbiochem TNF- α inhibitor $C_{32}H_{32}F_3N_3O_2 \cdot 2 HCl \cdot 1/2 H_2O$ (CAS 1049741-03-8; Millipore Sigma) was resuspended according to manufacturer protocol and used at the final concentration of 4.6 μ M. TNF- α inhibitor was added to PMNs (10^6 cells) only, or PMNs (7×10^5 cells) plus PBMCs (3×10^5 cells) for 10 minutes on ice. After treatment, cells were exposed to 2×10^6 opsonized bacteria, and phagocytosis was synchronized using centrifugation (524g for 7 minutes at 10°C), then incubated at 37°C with 5% carbon dioxide [12].

Bactericidal Assays

PMNs or PMNs supplemented with PBMCs (co-cultures) were exposed to opsonized bacteria at a 2:1 multiplicity of infection (MOI, bacteria:cell ratio) in a 96-well serum-coated plate and phagocytosis was synchronized as described above [12]. At designated time points, 100 μ L of sample was placed in 900 μ L reverse osmosis water, vortexed, and placed on ice for 15 minutes. Serial dilutions were performed and CFUs were enumerated by plating on tryptic soy agar as previously described [6, 10, 12]. For PBMC bactericidal assays, 5×10^6 PBMCs in a 24-well serum-coated plate were challenged with 1×10^7 opsonized bacteria, synchronized using centrifugation (524g for 7 minutes at 10°C) and incubated at 37°C [12]. At designated time points, bacterial survival was assessed using serial dilutions in water and CFUs were enumerated on tryptic soy agar.

Neutrophil Priming With TNF- α or PBMC Conditioned Media

For ROS assays, neutrophils were loaded at room temperature with 25 μ M 2',7'-dihydrochlorofluorescein diacetate (DCF; Molecular Probes) for 30 minutes in RPMI/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [12]. DCF-containing PMNs (10^6) were then added to a 96-well serum-coated plate containing 50 μ L of PBMC CM, RPMI only, or PBMC CM containing TNF- α inhibitor, for 30 minutes at 37°C. TNF- α inhibitor was added to CM for a final concentration of 4.5 ng/mL for 10–20 minutes at room temperature prior to addition to PMNs. After treatment, PMNs were exposed to opsonized bacteria at a ratio of 1:2 (PMN:bacteria), and phagocytosis was synchronized using centrifugation (524g for 7 minutes at 10°C) [12]. Measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California) or by flow cytometry using 488/530 nm emission/excitation wavelengths.

RESULTS

SaeR/S-Regulated Factors Decrease TNF- α Production in Human

Whole Blood

To determine if SaeR/S-regulated factors influenced TNF- α production, viable wild-type *S. aureus* (USA300) or isogenic *saeR/S*-deletion mutant (USA300 Δ *saeR/S*) was exposed to human whole blood and TNF- α secretion was quantitated at 1, 3, and 5 hours using ELISA (Figure 1). Production of TNF- α occurred as early as 1 hour postexposure to *S. aureus* and, as expected, challenge with USA300 had significantly increased production compared with USA300 Δ *saeR/S* (Figure 1A). However, by 3 hours, whole blood exposed to USA300 Δ *saeR/S* resulted in significant increase in TNF- α concentrations compared with USA300, and this trend continued at 5 hours (Figure 1A). We then used flow cytometry to identify the source of TNF- α in blood following exposure to *S. aureus*. CD14⁺ monocytes were identified as the primary source of TNF- α , and challenge with USA300 Δ *saeR/S* resulted in a significant increase in TNF- α production from CD14⁺ cells at 3 hours postchallenge compared to challenge with

USA300 (Figure 1B and 1C). Experiments using an isotype control antibody verified specificity of intracellular TNF- α production (data not shown). Additionally, the proportions of CD14⁺ cells between challenge with USA300 and USA300 Δ *saeR/S* were similar (5%–10% of total cells, Supplementary Figure 1), indicating that these results did not arise from differences in total monocyte populations. The USA300 Δ *saeR/S* strain also demonstrated increased bacterial survival compared with USA300 at 1 hour, but by 3 hours there were no significant differences between the strains (Figure 1D). These results reflect previous observations showing that the USA300 Δ *saeR/S* strain does not have a growth defect in blood at early time points [15].

SaeR/S Decreases TNF- α Production in Human Monocytes

As CD14⁺ monocytes were the predominant source of TNF- α production in human blood exposed to *S. aureus*, we next investigated TNF- α production in purified PBMCs. To reduce the potential influence of bacterial cytolytic activity between USA300 and USA300 Δ *saeR/S*, we used a low MOI of 2:1 (bacteria:PBMC). Similar to observations using whole blood, CD14⁺ monocytes were identified as the primary producers of TNF- α , and significant increases in intracellular monocyte TNF- α expression were observed at 3 hours following challenge with USA300 Δ *saeR/S* compared with USA300 (Figure 2A). To demonstrate that the observed effects of the SaeR/S TCS on TNF- α production were not specific to strain USA300, we also challenged PBMCs with USA400 (strain MW2) and an isogenic *saeR/S*-deletion mutant (USA400 Δ *saeR/S*). In congruence with our findings, PBMCs challenged with USA400 showed a significant decrease in TNF- α production from CD14⁺ monocytes compared to challenge with USA400 Δ *saeR/S* (Supplementary Figure 2A).

We have previously demonstrated that at high MOIs (10 bacteria:1 monocyte), the SaeR/S-regulated α -toxin (Hla) significantly increases monocyte programmed cell death and plasma membrane permeability [15]. To determine if observed increases in TNF- α at a lower MOI were due to the impact of SaeR/S-regulated factors on monocyte viability, we investigated TNF- α production and viability of PBMCs following exposure to USA300, USA300 Δ *saeR/S*, and USA300 Δ *hla*. ELISA results demonstrated that PBMCs exposed to USA300 Δ *hla* elicited a similar TNF- α response to PBMCs challenged with USA300, but a significantly lower TNF- α response than PBMCs challenged with USA300 Δ *saeR/S* at 3 and 5 hours postexposure (Figure 2B). Additionally, PBMCs challenged with USA300 caused significant increases in monocyte cell membrane permeability compared with USA300 Δ *saeR/S* and USA300 Δ *hla*. However, there were no significant differences in membrane permeability of CD14⁺ cells following exposure to USA300 Δ *saeR/S* and USA300 Δ *hla* (Figure 2C). These results confirm previous findings demonstrating that Hla significantly contributes

Figure 1. SaeR/S suppresses tumor necrosis factor alpha (TNF- α) production in human blood from CD14⁺ monocytes. Human whole blood was exposed to USA300, USA300 Δ saeR/S, lipopolysaccharide, or RPMI medium for 1, 3, and 5 hours and TNF- α production was measured. *A*, Serum TNF- α cytokine concentrations were measured at designated time points using enzyme-linked immunosorbent assay. *B*, Representative flow cytometry dot plots of human blood stained for monocyte (CD14⁺), T-cell (CD3⁺), and production of TNF- α , at 3 hours postchallenge with USA300 and USA300 Δ saeR/S. *C*, Compiled flow cytometry results of TNF- α and CD14⁺ positive monocytes of total CD14⁺ monocyte population. *D*, Bacterial survival of USA300 and USA300 Δ saeR/S in human whole blood. Data shown are the mean $\pm$ standard error of the mean of 10 experiments, and statistics were determined by 1-way analysis of variance followed by Tukey posttest. * P < .05, ** P < .01. Abbreviations: CFU, colony-forming units; LPS, lipopolysaccharide; ns, not significant; TNF- α , tumor necrosis factor alpha.

to monocyte cell membrane damage, but indicate that there are other SaeR/S-regulated factors and mechanisms responsible for reducing monocyte production of TNF- α [15]. Last, we also assessed bacterial viability following challenge with PBMCs (Figure 2D). PBMCs were unable to control challenge with USA300 and USA300 Δ hla, but bacterial survival of USA300 Δ saeR/S was significantly reduced at 3 and 5 hours (Figure 2D). Collectively, these results demonstrate that the SaeR/S TCS is required for bacterial survival following exposure to PBMCs and suggest that reduced monocyte production of TNF- α by SaeR/S-regulated factors is independent of cell lysis.

TNF- α Production by PBMCs Increases PMN Bactericidal Activity

TNF- α has been shown to influence neutrophil bactericidal activity and ROS production [26–28]. To determine if the influence of SaeR/S-regulated factors on monocyte-derived TNF- α could directly influence neutrophil staphylocidal activity, we developed a co-culture model that combined isolated human neutrophils and PBMCs at a ratio of 7:3 (PMN:PBMC). The neutrophil bactericidal activity following challenge with USA300 Δ saeR/S was not significantly increased by the addition of PBMCs (Figure 3A). However, we did demonstrate the importance of TNF- α in these co-cultures by treating them with a TNF- α -specific inhibitor, which led to

Figure 2. SaeR/S decreases tumor necrosis factor alpha (TNF- α) production in isolated human peripheral blood mononuclear cells (PBMCs). Isolated human PBMCs were exposed to RPMI medium, USA300, USA300 Δ saeR/S, or USA300 Δ hla at a multiplicity of infection of 2:1 (bacteria:PBMC) for 1, 3, and 5 hours. **A**, Representative flow cytometry dot plots of CD14⁺ and CD3⁺ cells producing TNF- α at 3 hours. **B**, At designated time points, TNF- α from PBMCs exposed to bacteria was quantified using enzyme-linked immunosorbent assay. **C**, CD14⁺ PBMC plasma membrane permeability was assessed by propidium iodide uptake and measured using flow cytometry. **D**, Bacterial survival following exposure to PBMCs was assessed at designated time points. Data shown are the mean $\pm$ standard error of the mean for 10 experiments. Statistics were determined by 1-way analysis of variance followed by Tukey posttest. * P < .05. Abbreviations: CFU, colony-forming units; ns, not significant; PI, propidium iodide; TNF- α , tumor necrosis factor alpha.

significant increases in USA300 Δ saeR/S survival (Figure 3A). Inhibition of TNF- α had no significant influence on neutrophil bactericidal activity in USA300-challenged co-cultures. These data suggest that the levels of TNF- α following challenge with USA300 are significantly reduced by SaeR/S-regulated factors and are insufficient to augment neutrophil bactericidal activity. ELISA results further demonstrated that co-cultures challenged with USA300 Δ saeR/S resulted in significantly increased TNF- α cytokine production at 3 hours postchallenge compared with USA300, and also confirmed the specificity of the TNF- α inhibitor (Figure 3B). Taken together, these results demonstrate that SaeR/S-regulated factors influence early monocyte

production of TNF- α , which significantly influences neutrophil bactericidal activity.

Conditioned Media From PBMCs Challenged With USA300 Δ saeR/S Primes Neutrophils

To address if PBMCs exposed to *S. aureus* produce secreted factors that could prime neutrophils, neutrophils were treated with filtered (bacteria-free) CM from PBMCs challenged with USA300 or USA300 Δ saeR/S for 3 hours. CM-primed neutrophils were then challenged with a secondary stimulus of USA300 and subsequent ROS production was measured. Approximately 60 minutes after exposure, USA300-challenged neutrophils

Figure 3. Tumor necrosis factor alpha (TNF- α) production from peripheral blood mononuclear cells (PBMCs) increases polymorphonuclear leukocyte (PMN) bactericidal activity. PMNs supplemented with PBMCs only, or pretreated with 4.6 μ M TNF- α inhibitor (iTNF), were challenged with USA300 or USA300 Δ saeR/S at a multiplicity of infection of 2:1 (bacteria to PMN). **A**, Bactericidal activity of untreated or iTNF-treated PMNs supplemented with PBMCs was compared to bactericidal activity of PMNs alone at 0, 1, and 3 hours. **B**, TNF- α cytokine concentrations were quantitated using enzyme-linked immunosorbent assay. Data shown are the mean $\pm$ standard error of the mean of 10 experiments and statistics were determined by 1-way analysis of variance followed by Tukey posttest. * P < .05, ** P < .01, *** P < .001. Abbreviations: CFU, colony-forming units; iTNF, tumor necrosis factor alpha inhibitor; ns, not significant; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leukocyte; TNF- α , tumor necrosis factor alpha.

that were primed with USA300 Δ saeR/S CM had significantly increased ROS production compared with infected neutrophils primed with USA300 CM or unprimed neutrophils (Figure 4A and 4B). Furthermore, treatment of USA300 Δ saeR/S CM with the TNF- α inhibitor, prior to neutrophil priming, significantly attenuated the enhanced ROS response (Figure 4A and 4B). In congruence with these findings, neutrophils primed with USA400 Δ saeR/S CM also demonstrated significant increases in ROS production approximately 60 minutes after exposure compared to neutrophils primed with USA400 CM. Additionally, the increased ROS response in neutrophils primed with USA400 Δ saeR/S CM was significantly attenuated by addition of the TNF- α inhibitor (Supplementary Figure 2B and 2C). These results indicate that the disruption of neutrophil priming by the SaeR/S-modulation of TNF- α is conserved between different strains of *S. aureus*.

To confirm if CM from USA300 Δ saeR/S-exposed PBMCs acted as a priming agent, we exposed DCF-labeled neutrophils to USA300 CM or USA300 Δ saeR/S CM and subsequently challenged with *N*-formyl-methionine-leucyl-phenylalanine (fMLF). fMLF is a bacterial-derived chemotactic peptide that induces a respiratory burst by binding to formyl peptide receptors that are present on neutrophils and upregulated following neutrophil priming [29]. In congruence with our previous findings, USA300 Δ saeR/S CM significantly enhanced the neutrophil ROS response compared to USA300 CM following fMLF stimulation and the influence of TNF- α was confirmed using the TNF- α inhibitor (Figure 4C). Additionally, no significant differences were observed in plasma membrane damage following priming

with CM from USA300 or USA300 Δ saeR/S (data not shown). Last, the influence of priming with USA300 Δ saeR/S CM on neutrophil bactericidal activity was investigated. Priming PMNs with USA300 Δ saeR/S CM significantly enhanced killing of *S. aureus* at 30 and 60 minutes and this effect was not observed following priming with USA300 CM (Figure 4D). The enhanced bactericidal activity of PMNs primed with USA300 Δ saeR/S CM was in part attributed to TNF- α , as treatment of USA300 Δ saeR/S CM with the TNF- α inhibitor significantly reduced the increased neutrophil staphylocidal activity (Figure 4D). These results demonstrate that factors regulated by the SaeR/S system reduce PBMC cytokine production of TNF- α to subsequently impact the neutrophil response to a secondary stimulus, including *S. aureus* and fMLF. Taken together, these data identify that disruption of neutrophil priming is a virulence mechanism used by *S. aureus* to impede an effective neutrophil response.

DISCUSSION

The success of *S. aureus* as a pathogen is largely attributed to evasion mechanisms that circumvent killing by neutrophils. These mechanisms can directly target specific neutrophil functions, including neutrophil recruitment, phagocytosis, and the generation of ROS [2, 3, 8, 30]. In addition to these strategies, we observed that SaeR/S-regulated virulence factors impeded neutrophil priming and ROS production by decreasing monocyte production of TNF- α .

Elevated TNF- α is historically considered to be a biomarker for predicting severe *S. aureus* bacteremia, with potentially fatal outcomes [31, 32]. In agreement, we have demonstrated

Figure 4. Conditioned media from USA300Δ*saeR/S*-exposed peripheral blood mononuclear cells (PBMCs) increases neutrophil reactive oxygen species (ROS) response to secondary challenge with USA300 and *N*-formyl-methionine-leucyl-phenylalanine (fMLF). Conditioned media (CM) from PBMCs exposed to USA300 or USA300Δ*saeR/S* (multiplicity of infection of 2:1) for 3 hours was used to prime neutrophils for 30 minutes prior to challenge with live USA300 or secondary stimulus, fMLF. *A*, 2',7'-dihydrochlorofluorescein diacetate (DCF)-labeled neutrophils unprimed or CM-primed were challenged with USA300 or RPMI medium only and ROS production was measured over 120 minutes via spectrometry. *B*, Data from (*A*) showing total relative fluorescent units calculated using area under the curve. *C*, Intracellular ROS production from primed DCF-labeled neutrophils following challenge with 1 μM fMLF was measured by flow cytometry at 40 minutes post-secondary challenge. *D*, Bacterial viability at 0, 30, and 60 minutes following interaction with neutrophils primed with USA300 CM, USA300Δ*saeR/S* CM, CM treated with tumor necrosis factor alpha inhibitor, or RPMI and challenged with USA300. Data shown are the mean ± standard error of the mean from 7 experiments for *A*, *B*, and *D*, and 9 experiments for *C*. Statistics were determined by 1-way analysis of variance followed by Tukey posttest. **P* < .05, ***P* < .01. Abbreviations: AUC, area under the curve; CFU, colony-forming units; CM, conditioned media; iTNF, tumor necrosis factor alpha inhibitor; ns, not significant; PMN, polymorphonuclear leukocyte; RFU, relative fluorescent unit; ROS, reactive oxygen species.

in invasive models that induction of TNF-α expression by SaeR/S-regulated factors was associated with increased bacterial burden and mortality [11]. In contrast, findings have also shown that local production of TNF-α by residential cells can correlate to a protective immune response [33, 34]. For example, TNF-α production by residential cells has been shown to significantly influence neutrophil recruitment from the microvasculature and that inhibition of TNF-α can reduce neutrophil recruitment by as much as 40%–70% [33, 34]. These findings highlight the pleiotropic influence of TNF-α during

different immune responses. Herein, our data indicate that elevated TNF-α concentrations correlate to enhanced neutrophil responses that we propose are important during the initial stages of infection.

TNF-α is a robust priming agent that can enhance the production of neutrophil ROS [23] and has been observed to increase staphylocidal activity in a dose-dependent manner [28, 35]. The importance of ROS in controlling *S. aureus* infections is illustrated by the observed increase in susceptibility to infections in individuals with mutations in components of NADPH

oxidase, as seen in patients suffering from chronic granulomatous disease [36]. *Staphylococcus aureus* uses many mechanisms to detoxify ROS, including superoxide dismutase and catalase [37, 38]. In previous studies, we demonstrated that SaeR/S-regulated factors also reduced neutrophil ROS products by decreasing hydrogen peroxide and hypochlorous acid production, likely inhibiting myeloperoxidase [8, 39]. In the current study, we observed that factors regulated by SaeR/S significantly impeded TNF- α production by PBMCs to diminish neutrophil ROS response to secondary challenge with either *S. aureus* or fMLE. Collectively, these observations underscore the importance of ROS production in controlling *S. aureus* and demonstrate that inhibition of ROS is a major mechanism used by this pathogen to survive neutrophil phagocytosis.

TNF- α also impacts neutrophil cell fate by activating NF- κ B [40], a transcription factor that regulates anti-apoptotic signals, including production of IL-8 and TNF- α [41, 42]. We have previously demonstrated that SaeR/S-regulated factors diminished both NF- κ B phosphorylation and IL-8 production, which accelerated neutrophil cell death and improved survival of *S. aureus* [7]. TNF- α has been shown to increase NF- κ B activation and IL-8 production in neutrophils [40]. Therefore, it is possible that reduced production of TNF- α in PBMCs by SaeR/S-regulated factors suppresses NF- κ B activation and IL-8 production in neutrophils. Alternatively, NF- κ B regulates TNF- α production, and suppression of NF- κ B activation by SaeR/S-regulated factors could impede proper TNF- α production and subsequent IL-8 production [42]. Future studies will investigate if these *S. aureus* immunomodulation mechanisms are linked and contribute to accelerated neutrophil death.

Although we identified that SaeR/S-regulated factors decreased TNF- α production from human monocytes, the mechanism responsible for this decrease remains undefined. As previous studies have shown that Hla is cytolytic toward human monocytes [43, 44], we used a USA300 Δ hla strain of *S. aureus* to determine if monocyte production of TNF- α was associated with cell viability. Our data demonstrate that Hla was not responsible for the observed decrease in TNF- α and suggest that cell lysis is not the predominant mechanism. The SaeR/S-regulated factor LukGH (LukAB) has been observed to target monocytes and contribute to mediating phagosomal escape from macrophages [10, 45, 46]. Although macrophages and monocytes are different, it is possible that LukGH is influencing monocyte phagosomal functions to induce variations in TNF- α cytokine production [47, 48]. Notably, LukGH and Pantone-Valentine leukocidin have also been observed to influence neutrophil priming [49, 50]. The SaeR/S-regulated immunomodulatory protein Sbi has been shown to increase TNF- α production in murine peritoneal macrophages in vivo during peritonitis [10, 19] and might also account for the differences in TNF- α production observed in this study. Focusing

on specific SaeR/S-regulated cytolytic and immunomodulatory factors responsible for reducing TNF- α will be the topic of future research.

In the current study, we developed a co-culture model of human neutrophils and PBMCs to demonstrate that SaeR/S-regulated factors significantly reduce early monocyte production of TNF- α and that this reduction influenced neutrophil responses to *S. aureus*, including ROS production and bactericidal activity. These data identify an immune evasion strategy used by *S. aureus* that targets neutrophil priming through SaeR/S-mediated reduction of monocyte-derived TNF- α to limit the initial inflammatory response and survive neutrophil phagocytosis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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