

## *Staphylococcus aureus* Inhibits Neutrophil-derived IL-8 to Promote Cell Death

Olivia W. Zurek, Kyler B. Pallister, and Jovanka M. Voyich

Department of Microbiology and Immunology, Montana State University, Bozeman

**While *Staphylococcus aureus* accelerates human neutrophil cell death, the underlying host- and pathogen-derived mechanisms remain incompletely defined. Previous studies demonstrated that the *S. aureus* SaeR/S sensory system is essential for pathogen survival following neutrophil phagocytosis. Herein, we demonstrate that the SaeR/S system promoted accelerated cell death, suppressed phosphorylation of nuclear factor- $\kappa$ B, and reduced interleukin-8 (IL-8) production in human neutrophils. Treatment of neutrophils with recombinant IL-8 significantly reduced bacterial burden and apoptosis. Our findings demonstrate a mechanism by which *S. aureus* suppresses the early neutrophil-derived IL-8 response to disrupt cell fate and promote disease.**

**Keywords.** cell fate; IL-8; neutrophils; NF- $\kappa$ B; pathogenesis; SaeR/S; *Staphylococcus aureus*.

*Staphylococcus aureus* is a common pathogen that can infect immunocompromised as well as healthy individuals. Human polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against *S. aureus*. It follows that the ability of *S. aureus* to initiate infection includes survival after PMN phagocytosis and alteration of PMN cell fate [1–3]. Although it has been shown that *S. aureus* alters neutrophil lifespan by inducing rapid neutrophil death (referred to as programmed necrosis or necroptosis), the host and pathogen mechanisms associated with this phenotype have not been fully elucidated [2, 3]. To advance our understanding of how *S. aureus* influences PMN cell fate to promote disease, we investigated the role of the SaeR/S 2-component system (TCS) in PMN cell death.

The SaeR/S TCS regulates production of virulence factors that promote evasion and destruction of neutrophils [1, 4, 5]. Moreover, neutrophil components, including  $\alpha$ -defensin, trigger activation of the SaeR/S TCS and induce a transcriptional response tailored for its environment [6]. In this study, we demonstrate that the SaeR/S TCS contributes to accelerated neutrophil cell death via decreased nuclear factor- $\kappa$ B (NF- $\kappa$ B) phosphorylation and reduced interleukin-8 (IL-8) production by human neutrophils. Furthermore, we identified IL-8 to be an essential neutrophil-derived cytokine that can prolong PMN survival and promote bacterial clearance during staphylococcal disease.

### MATERIALS AND METHODS

#### Bacteria Cultures

USA300, USA400, and isogenic USA300 $\Delta$ saeR/S ( $\Delta$ saeR/S) and USA400 $\Delta$ saeR/S mutant strains were previously generated [4, 7] and prepared for use in human PMN assays as described in [7].

#### Human PMN Isolation

Human neutrophils were isolated from heparinized venous blood obtained from healthy donors [7]. Purity (less than 1% peripheral blood mononuclear cell [PBMC] contamination) and viability were assessed via flow cytometry (fluorescence-activated cell sorter [FACS], FACSCalibur, BD Biosciences). PMNs ( $1 \times 10^6$ ) were plated onto serum-coated 96- or 24-well plates and exposed to  $2 \times 10^6$  (low multiplicity of infection [MOI], 2:1) or  $1 \times 10^7$  (high MOI, 10:1) colony forming units (CFUs) of USA300 or  $\Delta$ saeR/S and phagocytosis was synchronized [7].

#### Cell Death Assays

At designated time points, PMNs exposed to USA300 or  $\Delta$ saeR/S were subjected to Annexin V (Trevigen) and propidium iodide (PI) or nuclear condensation or terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assays as described [8]. PMN lysis was quantified using lactate dehydrogenase (LDH) assays and calculated as percent lysis compared to maximum LDH release (USA300 + PMNs at 10:1 ratio) (Promega, [1]). Casp-GLOW assays (eBioscience) were used to analyze PMN caspase activity via FACS per manufacturer's suggestion.

#### NF- $\kappa$ B Inactivation and IL-8 Assays

Neutrophils ( $1 \times 10^6$ ) were plated in duplicate and incubated with 0.2% dimethyl sulfoxide (DMSO) or 20  $\mu$ M parthenolide (Sigma) in DMSO for 30 minutes at 37°C and 5% CO<sub>2</sub>. USA300 or  $\Delta$ saeR/S were added (low MOI) and phagocytosis was synchronized [7]. At designated time points, samples were either

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Correspondence: Jovanka M. Voyich, PhD, Department of Microbiology and Immunology, Montana State University, Cooley Laboratory, Bozeman, MT 59718 (jovanka@montana.edu).

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subjected to nuclear condensation or TUNEL assays described above or used to determine PMN bactericidal activity (CFUs enumerated as described in [7]). To determine the role of IL-8, neutrophils were treated with recombinant IL-8 (25 ng/mL, R&D) or with anti-IL-8 (1 µg/mL, R&D) and isotype (1 µg/mL, R&D) 30 minutes prior to bacterial treatment. At 6 hours postinfection, nuclear condensation of PMNs was assessed along with bacterial survival.

### Intracellular Detection of Phosphorylated NF-κB p65 Subunit and IL-8

Heparinized whole blood was treated with Roswell Park Memorial Institute (RPMI) medium or RPMI medium containing  $1 \times 10^7$  CFUs USA300 or  $\Delta$ saeR/S and incubated with end-over-end mixing (20 rpm, 37°C with 5% CO<sub>2</sub>) for 5, 60, and 120 minutes. Using BD Biosciences whole-blood intracellular stain kit, samples were stained with anti-CD11b (BD Biosciences) or isotype control for 15 minutes at room temperature (RT). The optimal intracellular stain with anti-NF-κB-p65(P) (eBioscience) or corresponding isotype control was achieved after a 30 minute-incubation at RT. PMNs were determined by forward scatter/side scatter profile and high CD11b expression. For IL-8 detection, at 3 and 6 hours postexposure, infected PMNs or PMNs in media were stained with anti-CD11b or isotype control, fixed, and permeabilized per manufacture's recommendation (eBioscience). Additionally, cells were incubated with anti-IL-8 or corresponding isotype control (BD biosciences) for 30 minutes and analyzed by FACS.

### Cytokine Assays

Supernatants from PMNs ( $1 \times 10^6$ ) or PBMCs ( $1 \times 10^4$ ) subjected to USA300 or  $\Delta$ saeR/S (low MOI) were collected and sterile filtered at 6 and 18 hours. Procarta assays (Affymetrix) were used to detect interferon-γ (IFN-γ), IL-6, macrophage inflammatory protein-1α (MIP-1α), IL-1α, IL-1β, IL-8, vascular endothelial growth factor (VEGF), and tumor necrosis factor-α (TNF-α). The samples were read in duplicate using a Luminex cytometer (Bio-Rad). Additionally, supernatants from PMNs ( $1 \times 10^6$ ) or PBMCs ( $1 \times 10^4$ ) subjected to USA300 or  $\Delta$ saeR/S (low MOI) were collected and sterile filtered at 6 and 18 hours and assayed in duplicate using R&D Quantikine enzyme-linked immunosorbent assay (ELISA) for IL-8 and VEGF.

### Study Approval

Healthy blood donors were informed and gave written consent prior to participation. All studies were conducted in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University.

## RESULTS AND DISCUSSION

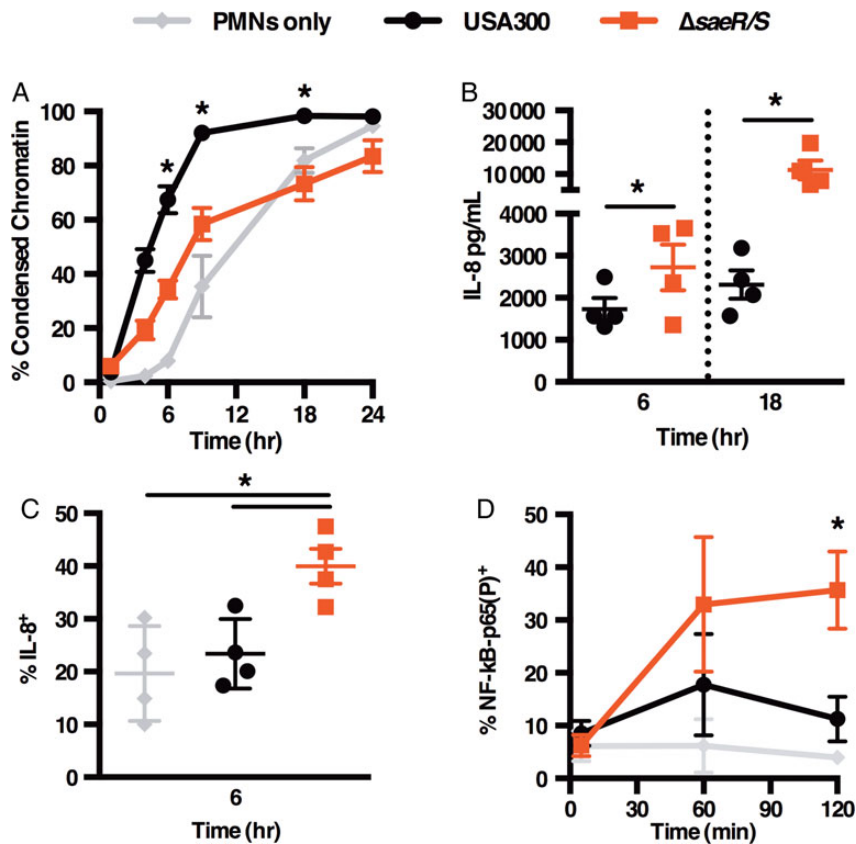
To test the hypothesis that the SaeR/S TCS influences PMN cell fate, we subjected human PMNs to USA300 or USA300 $\Delta$ saeR/S

( $\Delta$ saeR/S) strains at low MOI and measured cellular features associated with programmed cell death (Figure 1A, [Supplementary Figure 1A–D](#)). Consistent with published observations [3], USA300 promoted significant exposure of phosphatidylserine and plasma membrane damage within 6 hours of interaction ([Supplementary Figure 1A and B](#)). By 9 hours, the majority of USA300-treated cells were undergoing late apoptosis and/or necrosis, depicted by condensed nuclei (Figure 1A), fragmented DNA ([Supplementary Figure 1C](#)), and nearly complete PMN lysis ([Supplementary Figure 1D](#)). The aforementioned apoptotic features were significantly reduced in neutrophils infected with the  $\Delta$ saeR/S mutant (Figure 1A and [Supplementary Figure 1A–D](#)), demonstrating that SaeR/S contributes to accelerated neutrophil death following *S. aureus* phagocytosis.

To determine if SaeR/S interferes with caspase activation, reported to be inhibited by USA300 [2], we measured active caspases in PMNs following interaction with USA300 and  $\Delta$ saeR/S at high MOI ([Supplementary Figure 1E](#)). The substantial decrease in caspase activity in PMNs exposed to USA300 as opposed to  $\Delta$ saeR/S demonstrated that caspase inhibition is dependent on SaeR/S-regulated factors. At low MOI, however, caspases were neither activated by USA300 nor  $\Delta$ saeR/S mutant ([Supplementary Figure 1F](#)). Given these observations, we aimed to identify additional host-derived factors that contribute to SaeR/S-mediated cell death.

Because cytokines play an essential role in modulating neutrophil lifespan [9] and because the SaeR/S system has been shown to influence cytokine levels in whole blood and murine models of infection [5], we examined the influence of SaeR/S on secreted cytokine levels in PMNs during infection with USA300 and  $\Delta$ saeR/S at 6 and 18 hours. We identified cytokines predominantly made by neutrophils by comparing cytokine profiles in purified PMNs to PBMCs representative of the contamination in the cell preparation ( $1 \times 10^4$  PBMCs was equivalent to <1.0% contamination in PMN preparations). Based on the cytokine survey, IFN-γ, IL-6, MIP-1α, IL-1α, IL-1β, and TNF-α were mainly produced by PBMCs (data not shown). However, IL-8 and VEGF were neutrophil-derived and, therefore, examined in subsequent experiments. At 6 and 18 hours, USA300-treated PMNs produced significantly less IL-8 than the  $\Delta$ saeR/S group (Figure 1B). VEGF levels were higher in USA300-infected PMNs than the  $\Delta$ saeR/S group at 6 hours and similar between the 2 groups at 18 hours ([Supplementary Figure 2A](#)); thus, reduced IL-8 during USA300 infection was not a result of a general protein synthesis decline.

To further demonstrate that PMNs were the source of IL-8, we measured intracellular IL-8 in PMNs by FACS. Infection with the  $\Delta$ saeR/S mutant promoted a significant increase of IL-8 expressing PMNs in contrast to PMNs only at 3 hours ([Supplementary Figure 2B](#)) and when compared to both PMNs only and USA300-treated PMNs at 6 hours postexposure (Figure 1C). This trend was also observed during USA400 and USA400 $\Delta$ saeR/S infection



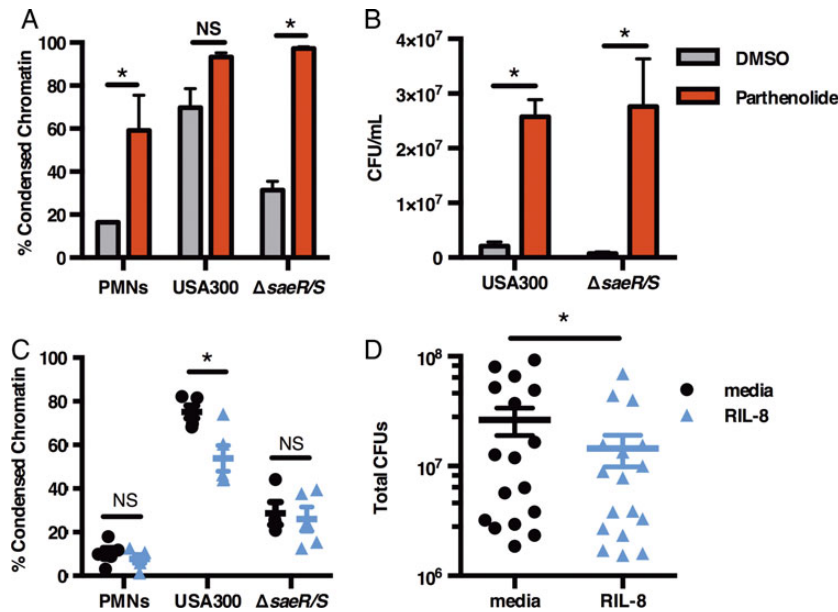
**Figure 1.** SaeR/S alters cell fate and suppresses IL-8 production in neutrophils. PMNs were subjected to USA300 or  $\Delta$ saeR/S at low MOI. Percent PMNs with condensed chromatin assessed via FACS at designated times (A). IL-8 release from PMNs assessed by ELISA (B) and intracellular IL-8 measured in PMNs at 6 hours postinfection via FACS (C). Whole blood was treated with USA300 or  $\Delta$ saeR/S and percent NF- $\kappa$ B-p65(P)<sup>+</sup> cells from CD11b<sup>Hi</sup> population was quantified by FACS at designated time points (D). Data shown are the means  $\pm$  SEM of 4–10 experiments (A) or 3–4 experiments (B–D). \**P* < .05 determined by 1-way ANOVA with Tukey’s posttest (A, C, and D) comparing USA300 to  $\Delta$ saeR/S groups or paired *t* test (B). Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IL-8, interleukin-8; MOI, multiplicity of infection; nuclear factor- $\kappa$ B, NF- $\kappa$ B; NF- $\kappa$ B-p65(P), phosphorylated NF- $\kappa$ B p65 subunit; PMNs, polymorphonuclear leukocytes; SEM, standard error of the mean.

(Supplementary Figure 2C), confirming the SaeR/S-dependent inhibition of PMN-derived IL-8. Last, we assessed SaeR/S system’s global impact on IL-8 release in whole blood and observed a significant decrease in IL-8 released in serum samples from the USA300-treated group when compared to the  $\Delta$ saeR/S-treated blood at 3 hours postinfection (Supplementary Figure 2D). VEGF levels were similar between both groups, further highlighting the specificity of SaeR/S-dependent IL-8 suppression (Supplementary Figure 2E).

IL-8 is an essential chemokine that promotes neutrophil recruitment, antimicrobial functions, and prolonged survival in vitro [9] and during infection with pathogenic microorganisms, including *Anaplasma phagocytophilum*, *Chlamydomphila pneumoniae*, and *Paracoccidioides brasiliensis* [10, 11]. Given that USA300 does not promote de novo IL-8 transcription [3], we wanted to determine if IL-8 reduction during USA300 infection is associated with SaeR/S-dependent modulation of NF- $\kappa$ B activity. To test this, we employed FACS to examine phosphorylation

of the phosphorylated NF- $\kappa$ B p65 subunit (NF $\kappa$ B-p65[P]) in PMNs from human blood infected with USA300 or  $\Delta$ saeR/S. At 2 hours, the percentage of PMNs with NF- $\kappa$ B-p65(P) was significantly reduced following interaction with USA300 when compared to  $\Delta$ saeR/S-infected cells (Figure 1D).

Our observation that *S. aureus* suppresses NF- $\kappa$ B is supported by published data linking NF- $\kappa$ B inhibition to accelerated cell death following infection with *Escherichia coli*, *Yersinia pestis*, and *Salmonella typhimurium* [12]. Given NF- $\kappa$ B’s regulation of antiapoptotic factors such as myeloid cell leukemia-1 (Mcl-1) and caspase inhibitors that can extend cell lifespan [11], we wanted to determine whether functional NF- $\kappa$ B is necessary for neutrophil survival during infection with *S. aureus*. To test this, we measured nuclear condensation and DNA fragmentation in PMNs pretreated with the NF- $\kappa$ B inhibitor parthenolide. PMN lifespan was shortened significantly in  $\Delta$ saeR/S-infected PMNs following parthenolide pretreatment compared to untreated neutrophils infected with  $\Delta$ saeR/S (Figure 2A, confirmed



**Figure 2.** IL-8 promotes neutrophil survival and bacterial clearance. PMNs treated with 20  $\mu$ M parthenolide or DMSO control were infected with USA300 or  $\Delta$ saeR/S at low MOI for 6 hours followed by analysis of PMNs with condensed chromatin (A) and bacterial burden (B). In C–D, PMNs were exposed to RIL-8 (25 ng/mL) and infected with USA300 or  $\Delta$ saeR/S for 6 hours followed by FACS analysis of condensed chromatin (C) or assessment of USA300 survival. D, Data shown are the means  $\pm$  SEM of 3–5 separate experiments. \* $P$  < .05 determined by 1-way ANOVA with Tukey’s posttest (A–C) or by paired  $t$  test (D) comparing USA300 to  $\Delta$ saeR/S groups. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming unit; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorter; IL-8, interleukin-8; MOI, multiplicity of infection; NS, not significant; PMNs, polymorphonuclear leukocytes; RIL-8, recombinant IL-8; SEM, standard error of the mean.

by TUNEL shown in [Supplementary Figure 3A](#)). Notably, cell death of parthenolide-treated PMNs infected with USA300 did not increase significantly when compared to the USA300 group without treatment underpinning the intrinsic ability of USA300 to inhibit NF- $\kappa$ B in PMNs (Figure 2A). NF- $\kappa$ B involvement in spontaneous PMN apoptosis [13] was confirmed by PMNs that underwent accelerated death in the presence of parthenolide (Figure 2A). Using LDH assays, we showed that the observed effect of parthenolide was not due to cell poisoning ([Supplementary Figure 3B](#)). Parthenolide treatment also significantly ( $P$  < .05 determined by  $t$  test,  $n$  = 3) abolished IL-8 release by 95% in USA300-infected PMNs (DMSO 1426  $\pm$  94 pg/mL vs parthenolide 75  $\pm$  39 pg/mL), but did not alter VEGF production (DMSO 214  $\pm$  52 pg/mL vs parthenolide 215  $\pm$  21 pg/mL), further suggesting that NF- $\kappa$ B inhibition was specific.

Finally, bacterial survival of both USA300 and  $\Delta$ saeR/S was significantly increased in PMNs exposed to parthenolide (Figure 2B), while parthenolide alone did not promote bacterial growth (data not shown). The observation that inhibition of NF- $\kappa$ B in  $\Delta$ saeR/S-treated neutrophils resulted in accelerated cell death and bacterial survival similar to USA300-treated neutrophils emphasizes the importance of the PMN response to resolution of *S. aureus* infection. Herein, we demonstrate that the SaeR/S TCS reduced phosphorylation of NF- $\kappa$ B to promote

evasion of PMN killing. Our results are supported by skin infection models where lack of MyD88 protein, essential to NF- $\kappa$ B activation, led to larger abscess formation and *S. aureus* survival [14]. Also, patients with NF- $\kappa$ B signaling deficiencies are highly susceptible to staphylococcal infections [15], further emphasizing the importance of NF- $\kappa$ B-dependent inflammation during staphylococcal disease.

To determine the role of IL-8 during *S. aureus*–neutrophil interaction, PMNs were replenished with recombinant IL-8 (RIL-8). The percentage of USA300-treated PMNs undergoing cell death was significantly reduced when compared to PMNs exposed to USA300 without treatment (Figure 2C). IL-8 can inhibit PMN apoptosis via the PI3K/Akt pathway, leading to subsequent activation of NF- $\kappa$ B and antiapoptotic Mcl-1 production, needed for neutrophil survival [10]. Therefore, future studies are necessary to define the precise mechanism of how SaeR/S-derived IL-8 alters PMN cell fate. In correlation with improved neutrophil survival, RIL-8 significantly enhanced PMN bactericidal activity against USA300, demonstrated by a significant decrease in USA300 survival (Figure 2D). Finally, the importance of IL-8 in bacterial clearance was underscored by increased USA300 burden in PMNs treated with neutralizing IL-8 antibody ([Supplementary Figure 3C](#)).

Taken together, our findings identify a mechanism employed by *S. aureus* to evade human neutrophils by which the SaeR/S

TCS disrupts PMN cell fate via suppression of NF- $\kappa$ B and IL-8 production to promote bacterial survival. We propose that both timing and magnitude of inflammation in PMNs play major roles in dictating the outcome of staphylococcal disease and that alteration in the innate ability of PMNs to produce IL-8 may increase susceptibility to *S. aureus* infections.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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