Video Article

# Quantifying the Cytotoxicity of *Staphyloccus aureus* Against Human Polymorphonuclear Leukocytes

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## Abstract

*Staphylococcus aureus* is capable of secreting a wide range of leukocidins that target and disrupt the membrane integrity of polymorphonuclear leukocytes (PMNs or neutrophils). This protocol describes both the purification of human PMNs and the quantification of *S. aureus* cytotoxicity against PMNs in three different sections. Section 1 details the isolation of PMNs and serum from human blood using density centrifugation. Section 2 tests the cytotoxicity of extracellular proteins produced by *S. aureus* against these purified human PMNs. Section 3 measures the cytotoxicity against human PMNs following the phagocytosis of live *S. aureus*. These procedures measure disruption of PMN plasma membrane integrity by *S. aureus* leukocidins using flow cytometry analysis of PMNs treated with propidium iodide, a DNA binding fluorophore that is cell membrane impermeable. Collectively, these methods have the advantage of rapidly testing *S. aureus* cytotoxicity against primary human PMNs and can be easily adapted to study other aspects of host-pathogen interactions.

## Video Link

The video component of this article can be found at https://www.jove.com/video/60681/

## Introduction

*Staphylococcus aureus* is a Gram-positive bacterium that causes a wide spectrum of diseases in humans. This prominent pathogen produces numerous virulence factors that contribute to different aspects of infection. These include surface molecules that allow *S. aureus* to adhere to different types of host tissue[1], extracellular proteins that interfere with the host immune response[2], and an array of secreted toxins that target different types of host cells[3]. In this report, we describe a method that quantifies the cytotoxicity of extracellular proteins produced by *S. aureus* against human polymorphonuclear leukocytes (PMNs or neutrophils), primary effector cells of the host innate immune response.

PMNs are the most abundant leukocytes in mammals. These circulating immune cells are rapidly recruited to the site of host tissue insult in response to danger signals produced by resident cells or by compounds unique to invading microbes. The extracellular input from these molecules and from direct contacts with activated resident host cells during extravasation increase the activation state of PMNs in a process known as priming[4,5]. Primed PMNs that have reached distressed tissue then execute important innate immune responses designed to prevent the establishment of infection. These include the binding and internalization, or phagocytosis, of invading microorganisms that triggers a cascade of intracellular events cumulating in microbe destruction by a battery of potent antimicrobial compounds[5].

PMNs play an essential role protecting humans from invading pathogens and are particularly important for preventing *S. aureus* infection[4]. However, this bacterium produces a wide range of virulence genes that impede different PMN functions. These include extracellular proteins that block recognition of signaling molecules, prevent adhesion to host tissue, inhibit production of antimicrobial compounds, and compromise plasma membrane integrity[4]. *S. aureus* orchestrates the temporal expression of these virulence genes through the collective input from multiple two-component sensory systems that recognize specific environmental cues. The SaeR/S two-component system is a major up-regulator of *S. aureus* virulence gene transcription during infection[6,7,8,9,10,11]. In particular, this two-component system has been shown to be critical for the production of bi-component leukocidins that specifically target human PMNs[12].

This protocol is broken into three different sections. The first section describes the purification of PMNs from human blood using density gradient centrifugation using a protocol that has been adapted from methods established by Bøyum[13] and Nauseef[14]. The second and third sections detail two different techniques to examine *S. aureus* cytotoxicity; one intoxicates PMNs with extracellular proteins produced by *S. aureus* while the other examines the ability of living bacteria to damage PMNs following phagocytosis. These procedures use propidium iodide to measure the loss of PMN plasma membrane integrity caused by *S. aureus* pore-forming toxins. Propidium iodide is a DNA-binding fluorophore that is normally cell membrane impermeable but can cross plasma membranes that have been disrupted by *S. aureus* toxins. Flow cytometry analysis allows the rapid quantification of propidium iodide-positive PMNs to measure the relative cytotoxicity of *S. aureus* strains. Methicillin-resistant *S. aureus*

(MRSA) identified as pulsed-field gel electrophoresis type USA300 and an isogenic deletion mutant of *saeR/S* in this strain (USA300Δ*saeR/S*) have been used as models to demonstrate how these procedures can quantify the cytotoxicity of *S. aureus* against human PMNs.

## Protocol

Heparinized venous blood from healthy donors was collected in accordance with protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in this study.

## 1. Purification of human polymorphonuclear leukocytes and isolation of human serum

NOTE: All reagents should be routinely checked for the presence of endotoxin using a commercially available endotoxin detection kit and should contain <25.0 pg/mL endotoxin to prevent unwanted priming of PMNs.

1. Bring 50 mL of 3% dextran-0.9% NaCl (w/v), 35 mL of 0.9% NaCl (w/v), 20 mL of 1.8% NaCl (w/v), 12 mL of 1.077 g/mL density gradient solution, and 20 mL of injection- or irrigation-grade water to room temperature.
2. To isolate human serum, incubate 4 mL of freshly drawn human blood without anti-coagulant at 37 °C in a 15 mL glass tube for 30 min. After incubation, centrifuge sample at 2,000–3,000 × *g* for 10 min at room temperature. Transfer the upper serum layer into a fresh 15 mL conical centrifuge tube and place on ice.
3. Combine 25 mL of freshly drawn heparinized (1000 units/mL) whole human blood with 25 mL of room temperature 3% dextran-0.9% NaCl (1:1 ratio) in two replicate 50 mL conical centrifuge tubes (50 mL total volume per tube). Mix by gently rocking each 50 mL conical tube and then let stand at room temperature for 30 min.
4. After incubation at room temperature, two separate layers will appear. Transfer the top layer of each dextran-blood mixture into new 50 mL conical tubes and centrifuge at 450 x *g* for 10 min at room temperature with low or no brakes.
5. Carefully aspirate both supernatants and discard without disturbing the cell pellets. Gently resuspend each cell pellet in 2 mL of room temperature 0.9% NaCl, combine the resuspended pellets in a single 50 mL conical tube, then add the remaining 0.9% NaCl (final volume of 35 mL).
6. Carefully underlay 10 mL of room temperature of 1.077 g/mL density gradient solution beneath the cell suspension using a hand pipette. Spin at 450 x *g* for 30 min at room temperature with low or no brakes. Gently aspirate the supernatant without disturbing the cell pellet. Supernatant will contain peripheral blood mononuclear cells that can be collected as previously described[14].
7. Lyse the red blood cells by resuspending the cell pellet in 20 mL of room temperature water. Mix gently by rocking the tube for 30 s. The lysis of red blood cells will be accompanied by a distinct decrease in turbidity.
8. Immediately add 20 mL of 1.8% NaCl (at room temperature [RT]) and centrifuge sample at 450 × *g* for 10 min at room temperature.
   NOTE: It is important to minimize the time that PMNs are left in water alone following red blood cell lysis to maximize PMN yield and prevent PMN lysis and/or activation.
9. Carefully aspirate the supernatant without disturbing the cell pellet. Gently resuspend the cell pellet in 2 mL of RT RPMI 1640 medium and place on ice.
10. Count cells using a hemocytometer. Resuspend purified PMNs at a concentration of 1 x $10^7$ cells/mL with ice-cold RPMI and keep on ice.
11. Combine 100 µL of purified PMNs (1 x $10^6$ cells) with 300 µL of ice-cold Dulbecco's phosphate-buffered saline (DPBS) containing 1 µL of propidium iodide stain in two replicate flow cytometry tubes. For a positive control for plasma membrane damage, add 40 µL of 0.5% Triton X-100 solution into one of the flow cytometry tubes and mix thoroughly.
12. Use flow cytometry to measure the forward scatter, side scatter, and propidium iodide staining (excitation/emission maxima at 535/617 nm) of purified cells (**Figure 1**).
    NOTE: Forward and side scatter analysis will identify unwanted populations of lymphocytes and monocytes. Propidium iodide will only stain cells with a compromised plasma membrane and purified PMNs that have pronounced populations of propidium iodide positive cells should not be used. For these studies, purified PMNs were only used if they comprised >98% of purified cells and <5% stained positive for propidium iodide.
13. Prepare a 96-well plate for PMN cytotoxicity assays by coating individual wells that will be used in this assay with 100 µL of 20% isolated human serum that has been diluted with DPBS.
    NOTE: Plating PMNs directly on plastic or glass will cause activation of the cells. Be sure to include at least one negative control well that will only receive media and at least one positive control well that will receive 0.05% Triton X-100.
14. Incubate the plate at 37 °C for 30 min. Following incubation, wash the coated wells twice with ice-cold DPBS to remove any excess serum. Gently tap the plate upside down to remove any residual DPBS and place on ice.
15. Gently add 100 µL of purified human PMNs at 1 x $10^7$ cells/mL to each coated well (1 x $10^6$ PMNs/well). Allow PMNs to settle in wells by incubating the plate on ice for at least 5 min. Keep the plate level to allow even distribution of cells in each well and leave on ice to avoid unwanted activation of PMNs.

## 2. Cytotoxicity assay of *S. aureus* extracellular proteins against human polymorphonuclear leukocytes

1. Culture *S. aureus* overnight in tryptic soy broth (TSB) using a shaking incubator set at 37 °C. For these studies, 20 mL of TSB in separate 150 mL Erlenmeyer flasks were inoculated with frozen cultures of *S. aureus* strains USA300 or USA300Δ*saeR/S* and grown for approximately 14 h with shaking at 250 rpm.
2. Subculture *S. aureus* by performing a 1:100 dilution of overnight bacterial culture with fresh media. Incubate at 37 °C with shaking until the bacteria reach early stationary growth phase.
   NOTE: For these experiments, 20 mL of tryptic soy broth in 150 mL Erlenmeyer flasks were inoculated with 200 µL of overnight cultured USA300 or USA300Δ*saeR/S* and incubated at 37 °C with shaking at 250 rpm for 5 h.

3. When bacteria have reached early stationary growth phase, transfer 1 mL of subcultured *S. aureus* into a 1.5 mL microcentrifuge tube and centrifuge at 5,000 × *g* for 5 min at room temperature.
4. Following centrifugation, transfer supernatant into a 3 mL syringe. Pass supernatants through a 0.22 µm filter and into a new 1.5 mL microcentrifuge tube on ice.
5. Perform serial dilutions of supernatants with ice-cold media used to culture *S. aureus*.
   NOTE: For the experiments shown, supernatants from USA300 and USA300Δ*saeR/S* underwent four consecutive 1/2 log dilutions with ice-cold TSB.
6. Gently add supernatant samples or media alone (for negative and positive controls) to individual wells of 96-well plate containing PMNs on ice from step 1.15. For these experiments, 10 µL of USA300 or USA300Δ*saeR/S* supernatant samples were added to each well. Gently rock plate to distribute supernatants in wells and incubate at 37 °C.
7. At desired times, remove the plate from incubator and place on ice. Add 40 µL of 0.5% Triton X-100 to the positive control well.
8. Gently pipette the samples up and down in each well to completely pull off all PMNs adhered to plate, then transfer the samples to flow cytometry tubes on ice that contain 300 µL of ice-cold DPBS with 1 µL of propidium iodide.
9. Measure the proportion of propidium iodide-positive PMNs using flow cytometry (**Figure 2A**). When bound to DNA, propidium iodide has excitation/emission at 535/617 nm.

## 3. *S. aureus* cytotoxicity assay against human polymorphonuclear leukocytes following phagocytosis

NOTE: Growth curves defined by the optical density at 600 nm ($OD_{600}$) and concentration of bacteria must be determined empirically for the *S. aureus* strains to be tested before beginning this assay. Success of these experiments requires the consistent harvest of equal concentrations of each *S. aureus* strain tested at mid-exponential growth phase using the $OD_{600}$ of sub-cultured bacteria.

1. Start overnight cultures of *S. aureus* strains and subculture bacteria as described in steps 2.1.1 and 2.1.2.
2. Harvest subcultured *S. aureus* when it has reached mid-exponential growth by transferring 1 mL of cultured bacteria to a 1.5 mL microcentrifuge tube and centrifuging at 5,000 × *g* for 5 min at room temperature.
   NOTE: Under our growth conditions, USA300 and USA300Δ*saeR/S* reached mid-exponential growth phase after approximately 135 min of incubation[6].
3. Wash *S. aureus* following centrifugation by aspirating the supernatant, resuspending the pelleted bacteria in 1 mL of DPBS, vortexing the sample for 30 s, and centrifuging at 5,000 × *g* for 5 min at room temperature.
4. Opsonize *S. aureus* by resuspending the bacterial pellet in 1 mL of 20% human serum diluted with DPBS and incubating at 37 °C with agitation for 15 min.
5. Centrifuge opsonized bacteria at 5,000 x g for 5 minutes at room temperature. Wash *S. aureus* following centrifugation by aspirating the supernatant, resuspending the pelleted bacteria in 1 mL DPBS, then vortex the sample until the bacterial pellet is completely broken apart plus an additional 30 seconds. Centrifuge bacteria at 5,000 × *g* for 5 min at room temperature.
6. Resuspend opsonized *S. aureus* strains in 1 mL RPMI, vortex the sample until bacterial pellet is completely broken apart, and then for an additional 30 s. Place bacteria on ice.
7. Dilute opsonized *S. aureus* strains to the desired concentration with ice-cold RPMI. Vortex for 30 s and place on ice.
8. Confirm the concentration of opsonized *S. aureus* by plating 1:10 serial dilutions of bacteria on tryptic soy agar.
   NOTE: Because differences in the concentration of bacteria used in this assay can have a major impact on subsequent PMN plasma membrane permeability (**Figure 3A**), it is very important that the concentration of each strain tested is determined for every experiment and is equivalent between strains.
9. Gently add 100 µL/well of each *S. aureus* strain or RPMI (for positive and negative controls) to PMNs in the 96-well plate on ice from step 1.14. Gently rock plate to distribute *S. aureus* in wells.
10. Synchronize phagocytosis by centrifuging the plate at 500 × *g* for 8 min at 4 °C[15]. Incubate plate at 37 °C immediately following centrifugation (T = 0).
11. At desired times, remove plate from incubator and place on ice. Add 40 µL of 0.5% Triton X-100 to the positive control well.
12. Gently pipette the samples up and down in each well to completely pull off all PMNs adhered to plate, then transfer the samples to flow cytometry tubes on ice containing 200 µL of ice-cold DPBS with 1 µL of propidium iodide.
13. Analyze samples for propidium iodide staining using flow cytometry as described in step 2.9.

## Representative Results

We have demonstrated how the procedures described above can be used to relatively quantify the cytotoxicity of *S. aureus* against human PMNs using MRSA PFGE-type USA300 and an isogenic deletion mutant of *saeR/S* in this strain (USA300Δ*saeR/S*) generated in previous studies[6]. PMNs isolated using the procedures described in section 1 of this protocol were stained with propidium iodide and examined using flow cytometry. Forward and side scatter plots were used to illustrate contamination of purified PMNs by monocytes or lymphocytes (**Figure 1A,B**) and PMN integrity was determined using propidium iodide staining (**Figure 1C**). The described method of human PMN purification can consistently yield 0.5 x $10^7$ to 1 x $10^8$ PMNs that are >98% pure and are >95% propidium iodide negative.

The cytotoxicity of extracellular proteins produced by USA300 and USA300Δ*saeR/S* were tested against purified PMNs (**Figure 2**) following the procedures described in section 2 of this protocol. These experiments demonstrate a concentration dependent increase in the propidium iodide staining of purified PMNs following 30 min of intoxication with extracellular proteins produced by USA300 (**Figure 2B**). Previous studies have demonstrated that the SaeR/S two-component system is important for expression of numerous bi-component leukocidins that target human PMNs[6,10,11,16]. Congruent with these previous findings, very few propidium iodide-positive PMNs were detected following exposure to extracellular proteins produced by USA300Δ*saeR/S* (**Figure 2B**). Further experiments demonstrated a steady increase in the proportion of lysed PMNs following intoxication by USA300 extracellular proteins that plateaued after approximately 30 min (**Figure 2C**). Minimal lysis of human

PMNs was noted at all timepoints following exposure to extracellular proteins produced by USA300Δ*saeR/S*. These results illustrate the utility of this assay for the relative quantification of cytotoxicity by extracellular *S. aureus* proteins against human PMNs.

We tested USA300 and USA300Δ*saeR/S* using the *S. aureus* cytotoxicity assay against human PMNs following phagocytosis that is described in section 3 of this protocol (**Figure 3**). A concentration dependent increase in the proportion of propidium iodide positive PMNs was observed 90 min after the phagocytosis of USA300 (**Figure 3A**). A significant decrease was observed in the proportion of PMNs that were propidium iodide positive following the phagocytosis of USA300Δ*saeR/S* (**Figure 3A**), supporting other results that indicate the SaeR/S two-component system is important for the cytotoxicity of *S. aureus* against human PMNs (**Figure 2**)[7,11]. As previously mentioned and demonstrated in **Figure 3A**, differences in *S. aureus* concentration have a pronounced impact on PMN lysis following phagocytosis. Enumeration of the USA300 and USA300Δ*saeR/S* inoculum used in each of these experiments demonstrated that the contrast in cytotoxicity between these strains was not due to differences in the concentration of bacteria used (**Figure 3B**). These findings show how the *S. aureus* cytotoxicity assay against human PMNs following phagocytosis can be used to assess the ability of different *S. aureus* strains to compromise human PMN plasma membrane integrity.

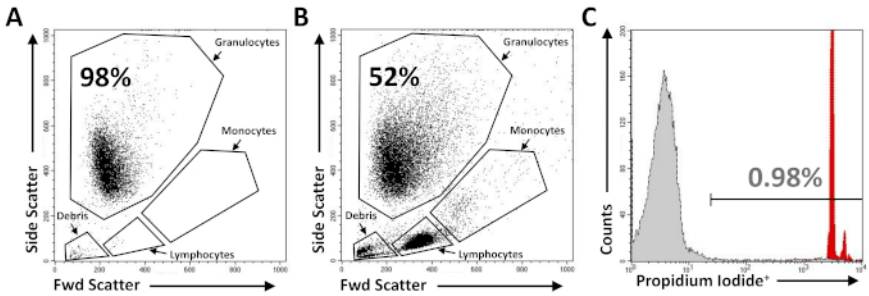


**Figure 1: Flow cytometry analysis of purified PMNs.** Representative flow cytometry dot plots of (**A**) purified human PMNs and (**B**) PMNs that have been purposely contaminated with peripheral blood mononuclear cells. (**C**) Representative flow cytometry histogram demonstrating minimal propidium iodide staining (<1%) of purified PMNs (shaded grey) as compared to PMNs treated with 0.05% Triton X-100 (shaded red). Please click here to view a larger version of this figure.

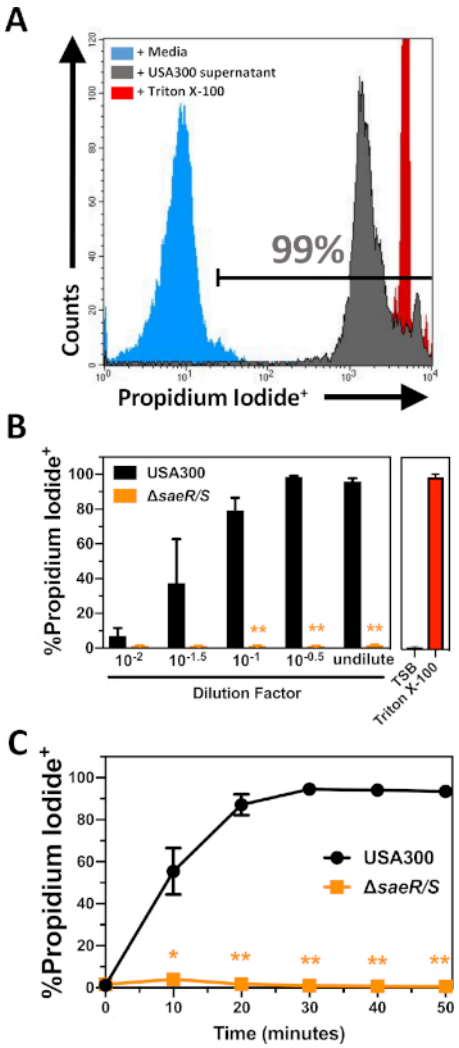


**Figure 2: Flow cytometry analysis of PMNs intoxicated with extracellular proteins produced by *S. aureus*.** (**A**) Representative flow cytometry histogram of PMNs stained with propidium iodide after 30 min of incubation with media control (shaded blue), filtered USA300 supernatant at a final concentration of 1:110 (shaded grey), or 0.05% Triton X-100 (shaded red). (**B**) The proportion of propidium iodide positive PMNs after 30 min of incubation with different concentrations of USA300 or USA300Δ*saeR/S* supernatants. (**C**) The proportion of propidium iodide positive PMNs over time following incubation with USA300 or USA300Δ*saeR/S* supernatant at a final concentration of 1:110. Data are presented as mean ± SEM of at least 3 separate experiments with * p ≤ 0.05 and ** p ≤ 0.005 as determined by two-tailed t-test. Please click here to view a larger version of this figure.

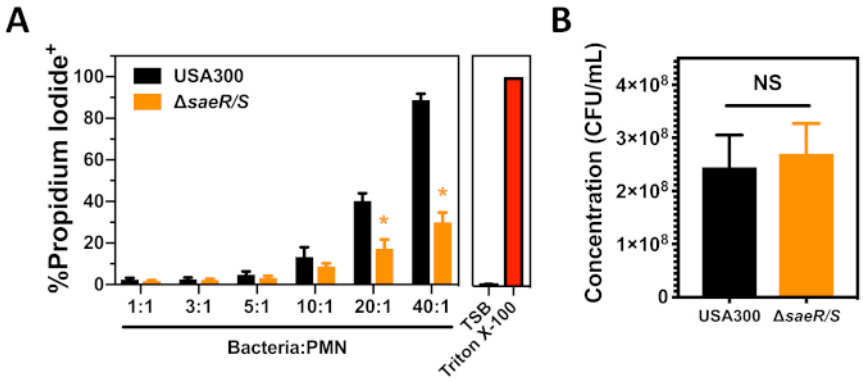


**Figure 3: Flow cytometry analysis of PMNs following phagocytosis of *S. aureus*.** (**A**) The proportion of propidium iodide positive PMNs 90 min after the phagocytosis of different concentrations of USA300 or USA300Δ*saeR/S*. (**B**) Concentration of opsonized *S. aureus* strains used for the experiments shown in panel A. Data are presented as mean ± SEM of 4 separate experiments with * p ≤ 0.01 as determined by two-tailed t-test. Please click here to view a larger version of this figure.

## Discussion

This protocol describes the purification of PMNs from human blood and two distinct assays that use propidium iodide for quantifying the cytotoxicity of *S. aureus* against these important innate immune cells. The success of these procedures will depend upon the quality of purified PMNs and the appropriate preparation of *S. aureus* and extracellular proteins produced by this pathogen. For the isolation of PMNs, it is important to minimize PMN activation during and after purification by using reagents free of endotoxin contamination, treating cell preparations gently, and keeping cells at the appropriate temperature. Signs that indicate activation of PMNs include clumping of cells during purification and when more than 5% of isolated cells stain positive for propidium iodide. Because of the relatively short life span of PMNs, these cells must be isolated from human blood and tested in the same day. PMNs will begin to exhibit signs of spontaneous apoptosis if left on ice for more than 3 h after purification. As mentioned earlier, it is very important that every PMN preparation is carefully evaluated using flow cytometry analysis of forward and side scatter as well as propidium iodide staining to ensure the purity and integrity of isolated cells.

The expression of bi-component leukocidins by *S. aureus* is responsible for the majority of compromised PMN plasma membrane integrity that is observed using the assays described in this protocol. Variation in the expression of these toxins and other pore-forming peptides, such as phenol-soluble modulins, between strains of *S. aureus* will produce differences in cytotoxicity against human PMNs. Significant deviations during in vitro growth between *S. aureus* strains will also influence expression of pore-forming toxins and subsequent cytotoxicity. In addition, the ratio of *S. aureus* to PMNs in phagocytosis assays has a major impact on subsequent PMN plasma membrane permeability (**Figure 3A**) and these experiments require the consistent harvest of equal concentrations of each *S. aureus* strain tested at mid-exponential growth phase using the $OD_{600}$ of subcultured bacteria. Given these considerations, it is very important to define growth curves for all strains that will be examined before beginning cytotoxicity assays. We do not recommend these methods for analyzing *S. aureus* cytotoxicity with strains that exhibit significant growth differences in vitro.

USA300 is a virulent MRSA isolate that is known to be highly cytotoxic against human PMNs[15] and the loss of SaeR/S in this strain dramatically reduces transcription of numerous bi-component leukocidins that target human PMNs[6,12], making these strains ideal models for comparing cytotoxicity using the assays described. However, there is extensive genetic variation between different *S. aureus* isolates and the parameters detailed in these protocols may not result in substantial changes in cytotoxicity against human PMNs when testing other *S. aureus* strains. Tailoring the growth conditions, volumes of supernatants added, or ratio of bacteria to PMNs may be required for success with these methods using other strains of *S. aureus*.

## Disclosures

The authors have nothing to disclose.

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