Phagocytosis of *Staphylococcus aureus* by Human Neutrophils Prevents Macrophage Efferocytosis and Induces Programmed Necrosis


*J Immunol* published online 11 April 2014
http://www.jimmunol.org/content/early/2014/04/11/jimmunol.1302692
Phagocytosis of *Staphylococcus aureus* by Human Neutrophils Prevents Macrophage Efferocytosis and Induces Programmed Necrosis

Mallary C. Greenlee-Wacker,*,† Kevin M. Rigby,‡ Scott D. Kobayashi,§ Adeline R. Porter,§ Frank R. DeLeo,§ and William M. Nauseef*‡

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) pose a significant threat to human health. Polymorphonuclear leukocytes (PMN) are the first responders during staphylococcal infection, but 15–50% of the initial ingested inoculum survives within the PMN phagosome and likely contributes directly or indirectly to disease pathogenesis. We hypothesize that surviving intracellular CA-MRSA undermine effective phagocyte-mediated defense by causing a decrease in macrophage uptake of PMN containing viable *S. aureus* and by promoting PMN lysis. In support of this hypothesis, PMN harboring viable CA-MRSA strain USA300 (PMN-SA) upregulated the “don’t eat me” signal CD47, remained bound to the surface, and were inefficiently ingested by macrophages. In addition, coculture with PMN-SA altered the macrophage phenotype. Compared to macrophages fed USA300 alone, macrophages challenged with PMN-SA produced more IL-8 and less IL-1 receptor antagonist, TNF-α, activated caspase-1, and IL-1β. Although they exhibited some features of apoptosis within 3 h following ingestion of *S. aureus*, including phosphatidylserine exposure and mitochondrial membrane depolarization, PMN-SA had sustained levels of proliferating cell nuclear Ag expression, absence of caspase activation, and underwent lysis within 6 h following phagocytosis. PMN lysis was dependent on receptor-interacting protein 1, suggesting that PMN-SA underwent programmed necrosis or necroptosis. These data are the first demonstration, to our knowledge, that bacteria can promote sustained expression of proliferating cell nuclear Ag expression, absence of caspase activation, and underwent lysis within 6 h following phagocytosis. Together, these findings demonstrate that *S. aureus* surviving within PMN undermine the innate immune response and may provide insight into the pathogenesis of *S. aureus* disease. The Journal of Immunology, 2014, 192: 000–000.
S. AUREUS PROMOTES RIP-1-DEPENDENT CELL DEATH OF NEUTROPHILS

protein 1 (RIP-1) kinase inhibitor necrostatin-1 (Nec-1). Taken together, our findings demonstrate the complex manner in which viable S. aureus undermine early phagocyte-mediated defenses and promote persistent infection and inflammation.

Materials and Methods

Reagents, Abs, and cells

All reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise indicated. Clinical grade dextrans T500 (Pharmacia, Uppsala, Sweden), Ficol-Hypaque PLUS (GE Healthcare, Piscataway, NJ), sterile endotoxin-free water, and 0.9% sterile endotoxin-free sodium chloride (Baxter, Deerfield, IL) were used in neutrophil preparations. HEPES, HBSS (with [HBSS**] and without divalent cations), and Dulbecco’s PBS were purchased from Mediatech (Manassas, VA). A HEMA-3 staining kit was obtained from Fisher Scientific, and 25% human serum albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 was purchased from Lonza (Hampden, MD), and RPMI medium was purchased from Cambrex Scientific (VWR, Rockville, MD). PMN (hereafter referred to as Aged PMN) were generated by culturing PMN in RPMI 1640 containing 10% FBS in DMEM for 18–24 h. Aged and freshly isolated PMN were labeled with CFDA SE (Invitrogen, Carlsbad, CA) and used for phagocytosis studies. RPMI 1640 medium containing 10% FBS and opsonized with pooled human serum was added to each well. After 6 or 12 h, cell-culture plates were washed and the supernatants were collected, centrifuged at 20,000 × g, and frozen. Human cytokine 10-plex, including GM-CSF, IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TNF-α, was purchased from Invitrogen. Supernatants were minimally diluted 1:10, and the assay was performed according to the manufacturer’s protocol and analyzed by flow cytometry.

Macrophage phagocytosis assay

Phagocytosis assays were performed (as described in Ref. 13) with the following modifications. Macrophages were obtained (as described in Ref. 14). After 6–8 d of culture in Teflon jars with RPMI 1640 containing 20% autologous serum, cells were plated overnight in RPMI 1640 containing 10% pooled human serum. Macrophages adhered to plastic, and nonadherent cells were washed away prior to the start of the assay. Apoptotic PMN (hereafter referred to as Aged PMN) were generated by culturing freshly isolated PMN in 10% FBS in DMEM for 18–24 h. Aged and freshly isolated PMN were labeled with CFDA SE at a 1:1000 dilution for 20 min at 37°C, washed with HBSS++ containing 0.1% HSA, and resuspended at 1 × 107 cells/ml. Following CFDA SE labeling, freshly isolated PMN were fed USA300-expressing sGFP for 10 min, spun at 500 × g to remove extracellular bacteria, and incubated for 60 min at 37°C while monitoring the bacteria with a Nikon inverted microscope. Freshly isolated PMN, Aged PMN, and PMN-SA were then stained with 10% pooled human serum, centrifuged at 500 × g, and resuspended in phagocytosis buffer (RPMI 1640 containing 5 mM CaCl2). Macrophages were cocultured with PMN, Aged PMN, or PMN-SA at a ratio of 1:20 (macrophage/target) in four-well Lab Tek chamber slides. Phagocytosis was synchronized by centrifugation at 500 × g for 5 min at 4°C, and cells were incubated for indicated time points at 37°C in 5% CO2. Nonadherent cells were washed away with PBS, and the remaining cells were harvested by trypsinization. Cells were washed three times with FACS buffer and analyzed using an Accuri flow cytometer (BD Biosciences). Each data set was first gated on CD14+ macrophages. Percent internalized was calculated (CTFR/CD15 cells/total CD14* gated cells × 100), and percent bound was calculated as ((CTFR/CD15* cells/total CD14* gated cells × 100). Three populations of CD14+ macrophages fed PMN-SA were assessed for the presence of GFP* USA300: CTFR−PMN-SA (no PMN associated), those with bound PMN, and those with internalized PMN. USA300 association was calculated as (GFP*CD14*total CD14* macrophages) for each population of cells. For CD47 blocking experiments, the anti-CD47 B6H12.2 isotype control was obtained from Thermo Scientific. Cells were washed twice with FACS buffer and incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L) (Invitrogen). Data were acquired with an Accuri flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

Macrophage phagocytosis assay

Phagocytosis assays were performed (as described in Ref. 13) with the following modifications. Macrophages were obtained (as described in Ref. 14). After 6–8 d of culture in Teflon jars with RPMI 1640 containing 20% autologous serum, cells were plated overnight in RPMI 1640 containing 10% pooled human serum. Macrophages adhered to plastic, and nonadherent cells were washed away prior to the start of the assay. Apoptotic PMN (hereafter referred to as Aged PMN) were generated by culturing freshly isolated PMN in 10% FBS in DMEM for 18–24 h. Aged and freshly isolated PMN were labeled with CFDA SE at a 1:1000 dilution for 20 min at 37°C, washed with HBSS++ containing 0.1% HSA, and resuspended at 1 × 107 cells/ml. Following CFDA SE labeling, freshly isolated PMN were fed USA300-expressing sGFP for 10 min, spun at 500 × g to remove extracellular bacteria, and incubated for 60 min at 37°C while monitoring the bacteria with a Nikon inverted microscope. Freshly isolated PMN, Aged PMN, and PMN-SA were then stained with 10% pooled human serum, centrifuged at 500 × g, and resuspended in phagocytosis buffer (RPMI 1640 containing 5 mM CaCl2). Macrophages were cocultured with PMN, Aged PMN, or PMN-SA at a ratio of 1:20 (macrophage/target) in four-well Lab Tek chamber slides. Phagocytosis was synchronized by centrifugation at 500 × g for 5 min at 4°C, and cells were incubated for indicated time points at 37°C in 5% CO2. Nonadherent cells were washed away with PBS, and the remaining cells were harvested by trypsinization. Cells were washed three times with FACS buffer and analyzed using an Accuri flow cytometer (BD Biosciences). Each data set was first gated on CD14+ macrophages. Percent internalized was calculated (CTFR/CD15 cells/total CD14* gated cells × 100), and percent bound was calculated as ((CTFR/CD15* cells/total CD14* gated cells × 100). Three populations of CD14+ macrophages fed PMN-SA were assessed for the presence of GFP* USA300: CTFR−PMN-SA (no PMN associated), those with bound PMN, and those with internalized PMN. USA300 association was calculated as (GFP*CD14*total CD14* macrophages) for each population of cells. For CD47 blocking experiments, the anti-CD47 B6H12.2 isotype control was obtained from Thermo Scientific. Cells were washed twice with FACS buffer and incubated with Abs for 30 min on ice with gentle shaking every 10 min.

Luminex assays and ELISA

For measuring cytokines by Luminex or ELISA the following modifications were made to the protocol. PMN were not stained, and macrophages were fed PMN-SA at a 1:5 and 1:15 ratio or USA300 at an MOI of 1:1 in a 24-well cell-culture plate. After 1 h of phagocytosis, unengested cells or bacteria were washed away, and RPMI 1640 was added to each well. After 6 or 12 h, supernatants were collected, centrifuged at 20,000 × g, and frozen. Human cytokine 10-plex, including GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, INF-γ, and TNF-α, was purchased from Invitrogen. Supernatants were minimally diluted 1:10, and the assay was performed according to the manufacturer’s protocol and analyzed by flow cytometry.
using Bio-Rad Bioplex (Bio-Rad). GM-CSF, IL-2, -4, -5, -10, and IFN-γ were below the level of detection at the 1:10 dilution and therefore were not measured in the assay. Abs for IL-1β ELISA were purchased from BD Biosciences, the IL-1 receptor antagonist (IL-1RA) DuoSet ELISA was purchased from R&D Systems, and IL-8, TNF-α, and IL-6 ELISA were purchased from E Bioscience.

**Immuno blot analyses**

For caspase-1 detection, macrophages were lysed (50 mM Tris-HCl [pH 8], 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100 containing protease inhibitor mixture of 0.5 mM benzamidine, 0.5 mM PMSF, 0.1 mg/ml aprotinin, 0.1 mg/ml phosphoramidon, 0.1 mg/ml tosyllysine chloromethylketone hydrochloride, 0.1 mg/ml tosylphenylalanyl chloromethyl ketone, 0.1 mg/ml 4-aminophenylmethanesulfonyl fluoride, 0.1 mg/ml E-64, 0.05 mg/ml leupeptin, and 0.01 mg/ml pepstatin) at indicated time points, proteins were resolved in a gradient SDS-PAGE gel, and transferred to polyvinylidene difluoride membrane for immunoblotting. For caspase-3, PCNA, and β-actin detection, PMN were lysed as previously described (16). For immunoblotting, Fas stimulation was used as a positive control for apoptosis using 500 ng/ml anti-Fas Ab. The following dilutions were used for immunoblot: 1:1,000 anti–caspase-1, 1:200 anti–caspase-3, 1:200 anti–PCNA, and 1:20,000 anti–β-actin. The pixel intensity of bands in immunoblots was measured using a phosphoimager (Typhoon 9410 Variable Mode Imager; GE Healthcare).

**Caspase activity assays**

To measure PMN caspase activity following phagocytosis of serum opsonized *S. aureus*, synchronized phagocytosis assays were performed as described previously using 96-well culture plates (7). The bacteria-to-PMN ratio was ~10:1 for these experiments, and assay plates were incubated at 37°C with 5% CO₂ in a humidified incubator for up to 6 h. At the desired time point, assay plates were centrifuged to pellet cells, culture supernatant was removed, and cells were stored at ~80°C. PMN caspase activity was determined using ApoAlert Caspase Assay Plates (Clontech, Mountain View, CA) according to the manufacturer’s instructions.

**Results**

PMN fed *S. aureus* differentially regulate “eat me” and “don’t eat me” signals

Despite efficient uptake by PMN, 15–50% of the ingested inoculum of USA300 or RN6390 remained viable 180 min after phagocytosis, as judged by two independent measures of bacterial viability (12) (Supplemental Fig. 1). Furthermore, PMN acquire many of the morphologic signs of apoptosis early on, including exposure of PS and nuclear condensation, when challenged with USA300 at an MOI of 10:1 (7). PMN fed USA300 or RN6390 at an MOI of 1:1 exhibited greater Annexin V staining than did control cells, and this Annexin V staining increased over time (Fig. 1A). Importantly, the majority of Annexin V<sup>+</sup> cells remained negative for PI during this time (Fig. 1A), indicating that the SA-laden PMN remained viable. Increasing the MOI to 5:1 resulted in a similar percentage of Annexin V<sup>+</sup> cells, but a greater percentage of cells positive for PS and nuclear condensation.

**Cytotoxicity assays**

For experiments using a caspase inhibitor, PMN were pretreated with or without 40 μM Nec-1 and/or 10 μM Q-VD-OPh for 20 min prior to challenge with USA300 at an MOI of 1:1. Alternatively, PMN were pretreated with 0, 10, 50, 100, 200, or 500 μM Nec-1, and USA300 was added to assays at an MOI of 10:1 as indicated. Lactate dehydrogenase (LDH) activity was measured from supernatants in duplicate wells 6 h following phagocytosis as reported previously (7).

**Statistical analyses**

One-way ANOVA and Dunnett or Tukey posttests were used to calculate statistical significance. Unless indicated otherwise, *p* values were obtained from the posttests used to correct for multiple comparisons (*p* < 0.05).
both Annexin V and PI (data not shown), suggesting that the PMN plasma membrane was more readily compromised at an increased bacterial MOI.

Accompanying the increased expression of PS on the surface of cells, PMN fed USA300 or RN6390 exhibited greater loss of mitochondrial membrane potential compared with that in unstimulated PMN, as measured by the fluorescent membrane potential sensor JC-1. After 10 min of treatment with the mitochondrial membrane depolarizer carbonyl cyanide 3-chlorophenylhydrazone, 97–99% of cells lost red/green fluorescence associated with JC-1 accumulation in intact, polarized mitochondria (data not shown). In like fashion, PMN mitochondrial membrane depolarization occurred 60 min following phagocytosis of USA300 or RN6390, with the greatest loss occurring in PMN fed USA300 (Fig. 1B). Increased mitochondrial membrane depolarization in PMN fed USA300 was confirmed using DIOC6 to assess mitochondrial integrity (data not shown). Taken together, these data demonstrate that intact PMN containing a population of viable USA300 exhibited some features typical of apoptosis, results consistent with previous studies (7).

In general, increased expression of PS by apoptotic cells is accompanied by a loss of “don’t eat me” signals such as CD47 (17, 18). To determine if ingestion of USA300 altered expression of “don’t eat me” signals on PMN-SA, we compared the expression of CD47 on PMN that were freshly isolated, aged, or fed opsonized USA300. Surprisingly, although PMN-SA exhibited enhanced PS exposure, PMN-SA challenged at an MOI of 1:1 expressed 1.3–7.2- and 1.3–1.7-fold higher levels of CD47 compared with Aged PMN and PMN left alone in buffer, respectively (Fig. 1C, 1D). There was ∼31% less CD47 on the surface of apoptotic PMN compared with that on fresh PMN, but the decrease was not statistically significant (Fig. 1D). These data suggest that PMN-SA exhibited an altered phenotype that was distinct from that typical of apoptotic cells. Furthermore, the increased expression of CD47 on PMN-SA may influence their subsequent interactions with macrophages, as loss of CD47 from the surface of apoptotic cells inhibits SIRPα signaling and promotes efferocytosis (17, 18).

**Macrophages readily bind but inefficiently ingest S. aureus–laden PMN.**

Typically, macrophages rapidly bind and ingest apoptotic cells. However, we reasoned that the atypical phenotype of PMN-SA might prompt an interaction with macrophages that differed from that normally observed between macrophages and apoptotic PMN. To explore the fate of PMN-SA with respect to efferocytosis, we developed a flow cytometry–based assay to assess both binding and internalization of PMN by macrophages. As expected, PMN that underwent spontaneous apoptosis after being cultured for 24 h (Aged PMN) transiently bound to and were readily engulfed by macrophages in a time-dependent fashion (Fig. 2A, 2B, Supplemental Fig. 2A). Freshly isolated PMN bound to macrophages to a similar degree but were not internalized; internalization of Aged PMN was 4.8-fold greater than that of freshly isolated PMN after 30 and 60 min of phagocytosis (*p < 0.05; Fig. 2B). The interactions between PMN-SA and macrophages were strikingly different from those of macrophages with either fresh or Aged PMN, whereas there was increased binding of PMN-SA to macrophages, internalization was significantly decreased in comparison with uptake of Aged PMN (*p < 0.05).

To determine if S. aureus used PMN as a vehicle to gain access to macrophages, we examined the distribution of viable sGFP–USA300 associated with macrophages fed PMN-SA. In general, USA300 could associate with macrophages in one of three different ways: as PMN-SA bound to the surface of the macrophage, as PMN-SA ingested by macrophages, or as free S. aureus released from lysed PMN and ingested by macrophages. Overall, the majority of CD14+ macrophages (50.2 ± 17.9% at 60 min; n = 4) associated with viable S. aureus that appeared to be distributed in one of these three different populations (Fig. 2C). Most sGFP–USA300 were found within PMN bound extracellularly to macrophages, whereas 6–9% were in PMN that had been ingested by macrophages, and only 2 to 3% were free within macrophages. These data suggest that macrophages did not effectively ingest PMN-SA and that USA300 did not efficiently exploit PMN as a Trojan horse to gain entry into macrophages. The flow cytometry results indicating that most PMN-SA were bound to the surface of macrophages was confirmed by confocal microscopy (Supplemental Fig. 2B).

To determine if soluble cues from PMN-SA inhibited the capacity of macrophages to mediate efferocytosis, we compared ingestion of Aged PMN by macrophages in the presence or absence...
of supernatant conditioned by PMN-SA. Conditioned supernatants failed to alter uptake or binding of Aged PMN (data not shown). Therefore, soluble factors did not significantly contribute to enhanced binding or decreased engulfment of PMN-SA. The ability of PMN-SA to resist uptake by macrophages could also likely reflect augmented expression of CD47 and possibly other “don’t eat me” signals by PMN harboring viable S. aureus. Our results indicate that blocking CD47 with F(ab’)_2 prepared from B6H12.2 (an Ab known to bind CD47) enhanced the engulfment of viable cells, as previously reported by Gardai et al. (17). When compared with ingestion of Aged PMN by macrophages, macrophages ingested 26% more viable PMN opsonized with anti-CD47 F(ab’)_2 than viable PMN opsonized with the isotype control (Supplemental Fig. 2C). Using the same blocking Ab fragments, we were unable to demonstrate that blocking CD47 significantly enhanced internalization of PMN-SA (data not shown), suggesting that CD47 modulation alone cannot account for altered efferocytosis of PMN-SA.

PMN-SA alter macrophage proinflammatory cytokine production

Macrophages secrete cytokines when they ingest microorganisms or apoptotic PMN (19, 20). To determine if PMN-SA were likewise stimulatory, we compared the cytokine profiles of macrophages fed PMN-SA or USA300 alone (Fig. 3). We compared cytokine production by macrophages after 6 h of exposure to PMN-SA with that of macrophages cultured in the absence of agonists or fed USA300 alone. Macrophages stimulated with USA300 produced more IL-1RA, IL-6, and TNF-α than unstimulated macrophages. There was a trend for enhanced IL-8 production from macrophages fed USA300 versus unstimulated macrophages, but this difference was not statistically significant. Macrophages fed PMN-SA at a ratio of five PMN-SA per macrophage produced significantly less IL-1RA than those fed USA300 at a 1:1 ratio, and the level of IL-1RA did not exceed basal levels produced by unstimulated macrophages. Although IL-6, IL-8, and TNF-α were detected above basal levels, production was relatively depressed (versus USA300 alone); however, these decreases were not statistically significant. Increasing the ratio of PMN-SA per macrophage to 15:1 resulted in levels of IL-6 and TNF-α equivalent to those elicited by USA300 alone, triggered significantly more IL-8 compared with that from macrophages fed USA300, but caused no change in IL-1RA release. Similar relative profiles were observed after 12 h of stimulation (data not shown). Thus, the cytokine profile of macrophages fed PMN-SA differed significantly from that of macrophages fed USA300 alone.

Under identical experimental conditions, macrophage IL-10 was measured 12 h after these phagocytes were fed USA300 alone, PMN-SA, or Aged PMN (Supplemental Fig. 3). Macrophages fed USA300 alone or PMN-SA at a ratio of five PMN per macrophage produced more IL-10 than unstimulated macrophages. Increasing the ratio of PMN-SA per macrophage to 15:1 resulted in significantly more IL-10 than unstimulated macrophages. Increasing the level of IL-10 produced from macrophages fed Aged PMN did not exceed basal levels (Supplemental Fig. 3). Importantly, macrophages were capable of producing elevated levels of IL-10 in response to Aged PMN when the macrophages were cocultured with Aged PMN in the presence of serum as previously reported (19 and data not shown). Overall, macrophages fed PMN-SA had enhanced IL-10 production to compared with those exposed to USA300 alone.

Inflammasome activation by PMN-SA versus S. aureus alone

Given that inflammasomes contribute to innate host response to various microbial agents (21), including NLRP3 inflammasome activation by S. aureus in murine macrophages (22), we compared IL-1β production and caspase-1 activation by macrophages stimulated with USA300 or PMN-SA. Macrophages fed USA300 alone produced significantly more IL-1β than did unstimulated macrophages (Fig. 4A, 4B). Macrophages fed PMN-SA produced 2–2.6-fold less IL-1β than did those fed USA300 alone when assayed by Luminex (Fig. 4A). Cytokine analysis by ELISA resulted in a similar trend, as macrophages that were fed PMN-SA compared with those fed USA300 alone produced 6.6-fold less IL-1β. As a positive control for NLRP3 inflammasome activation of macrophages, we measured IL-1β production in response to LPS and

**FIGURE 3.** Macrophage cytokine production is skewed by PMN-SA. Human monocyte-derived macrophages were left alone (Mac), fed USA300 (SA) at an MOI of 1:1, or fed PMN-SA at a ratio of five PMN-SA per macrophage or 15 PMN-SA per macrophage. Following a 6 h incubation, supernatants were analyzed by Luminex or ELISA. Bars represent the mean of three individual experiments ± SEM. Statistical analyses were performed using one-way ANOVA and Tukey posttest. *p < 0.05 versus macrophages, †p < 0.05 versus macrophages + SA.
silica (Fig. 4B). The decreased IL-1β production reflected depressed NLRP3 engagement, as caspase-1 activation was less than when macrophages were fed PMN-SA versus USA300 alone (Fig. 4C). Taken together, these data indicate that PMN-SA elicited altered cytokine profiles in macrophages and failed to activate the NLRP3 inflammasome.

**PMN-SA exhibit limited features of accelerated apoptosis**

Because macrophages did not scavenge PMN-SA, we reasoned that PMN lysis would be the predominant fate for PMN following ingestion of CA-MRSA. In fact, we and others (7, 9) had demonstrated that PMN undergo lysis >180 min after phagocytosis of USA300, even at MOI as low as 1:1. Lysis requires viable USA300 and is partially dependent on staphylococcal toxin production (7, 9, 23). Furthermore, PMN lysis late after ingestion of USA300 is inhibited by pretreatment of PMN with inhibitors of transcription or protein synthesis (7 and M.C. Greenlee-Wacker and W.M. Nauseef, unpublished observations), demonstrating that PMN actively engage endogenous responses that culminate in cell disruption.

As demonstrated earlier (Fig. 1), PMN-SA initially exhibited increased surface expression of PS and mitochondrial membrane

**FIGURE 4.** Inflammasome activation is dampened by PMN-SA. Human monocyte-derived macrophages (Mac) were treated with buffer or primed with LPS for 2 h prior to treatment buffer alone, silica, USA300, or PMN-SA. Supernatants were analyzed for IL-1β production by Luminex for following a 6 or 12 h incubation (A) or by ELISA following a 6 h incubation (B). Caspase-1 cleavage was analyzed by immunoblotting following a 6 h incubation (C). Bars represent the mean of five experiments ± SEM (B) or three experiments (C). For (A), p values were determined using one-way ANOVA and Tukey posttest. *p < 0.05 versus macrophages, ^p < 0.05 versus macrophages + SA. For (B), an outlier experiment was identified using Prism software (GraphPad) and excluded from the analysis. p values were then determined using repeated-measures one-way ANOVA. *p < 0.05 versus macrophages + LPS, ^p < 0.05 versus macrophages + LPS + Silica, p < 0.05 versus macrophages + SA.

**FIGURE 5.** Analysis of PCNA and caspase-3 in human PMN following phagocytosis. Lysates from PMN that were in buffer alone, challenged with USA300 at an MOI of 1:1 (+SA), or treated with anti-Fas Ab (+αFas) as indicated were analyzed by immunoblot for PCNA, caspase-3, and actin. Shown is a representative of three experiments (A). To quantify band intensity, immunoblots were scanned and analyzed using a phosphor-imager. Data were plotted as the signal above background versus time 0 for the indicated bands ± SEM (B), n = 4.
depolarization, features characteristic of the initial stages of apoptosis. In contrast to apoptotic PMN, however, PMN-SA upregulated surface expression of CD47 and were not engulfed by macrophages (Figs. 1, 2). These data suggest that PMN-SA may derail the apoptotic cell death pathway. PCNA, a cell-cycle protein expressed in PMN cytoplasm, promotes PMN survival by scavenging procaspases and thereby preventing their activation (24). As decreased levels of cytoplasmic PCNA typically accompany PMN apoptosis, we speculated that PMN-SA may increase PCNA over time, sequester procaspases, and thereby contribute to prolonged survival. PCNA decreased and caspase-3 increased in cultured PMN in a time-dependent fashion, results consistent with the induction of apoptosis in normal PMN over time in culture (Fig. 5A). In contrast, PMN-SA exhibited a sustained level of PCNA compared with that of PMN in culture and cytoplasmic PCNA was observed as late as 24 h following phagocytosis (Fig. 5B). Furthermore, whereas anti-Fas Ab promoted caspase-3 activation in PMN, as previously demonstrated (25), PMN-SA failed to activate caspase-3 with similar kinetics (Fig. 5). To determine if a higher MOI would induce caspase activation in PMN, we challenged PMN with USA300 at an MOI of 10:1 and assessed caspase activation (Fig. 6). Even at the higher MOI, PMN fed USA300 failed to activate caspase-3 (Fig. 6A). In addition, PMN-SA failed to activate caspase-8, -9, and -2, whereas opsonized beads or anti-Fas Ab prompted time-dependent increase in all caspases tested (Fig. 6B–D). Taken together, the sustained levels of cytoplasmic PCNA and the failure to activate caspase-3 demonstrate a divergence of the phenotype of PMN-SA from that typical for the apoptotic death pathway.

**S. aureus causes rapid destruction of PMN in an RIP-1–dependent manner**

Because previous studies and our current observations indicate that PMN-SA did not undergo apoptotic cell death but rather eventually lysed (7), we tested the hypothesis that necroptosis was the cell death program that dictated the fate of PMN-SA. Necroptosis is defined as RIP-1–dependent cell death that can be blocked by Nec-1 (26, 27). At an MOI of 10:1, 50 μM of Nec-1 inhibited lysis of PMN-SA by 21.1%, and there was a 53.7% reduction in PMN lysis with 500 μM of Nec-1 (PMN lysis was 62.2 ± 4.0% in the absence of Nec-1 [0 μM] versus 28.8 ± 3.2% for assays containing 500 μM Nec-1) (Fig. 7). At an MOI of 1:1, caspase inhibition in combination with 40 μM of Nec-1 was sufficient to completely inhibit lysis of PMN-SA (Supplemental Fig. 4). These data suggest that the higher inoculum of SA was sufficient to induce necroptosis of PMN-SA, whereas a lower bacterial burden required additional caspase inhibition to favor the necroptotic pathway. Importantly, concentrations of Nec-1 used in these assays did not affect S. aureus survival, and Nec-1 alone did not interfere with macrophage lysis (data not shown). In addition, Nec-1 did not affect neutrophil function, including phagocytosis, reactive oxygen species production in response to PMA, or CD11b up-regulation in response to USA300 or fMLF (data not shown). Combined, these data clearly demonstrate that USA300 induced RIP-1–dependent cell death.

**Discussion**

Staphylococcal infections are generally characterized by resistance to antimicrobial therapy, local persistence, distant metastases, and tissue necrosis (28). Despite their important role in the innate immune host response to invading microbes, PMN fail to kill and degrade all ingested S. aureus that are trapped in phagosomes. Furthermore, PMN-SA can transmit infection to naive mice or rabbits in experimental models (29, 30), thereby demonstrating their potential to contribute to persistence or progression of infection during human disease. Based on these clinical observations and experimental findings, we reasoned that the fate of PMN-SA

**FIGURE 6. Caspase activity in human PMN following phagocytosis.** (A–D) Human neutrophils were cultured with IgG and serum complement-coated latex beads (Beads; filled squares, 10 beads/PMN), USA300 (SA; red circles, MOI of 10:1), or anti-Fas Ab (α-Fas; filled triangles) as indicated. At each time point, samples were clarified by centrifugation, and caspase activity in culture supernatants and stimulated PMNs was determined with an ApoAlert Caspase Assay Plate kit (Clontech) according to the manufacturer’s instructions. Data for PMNs are the mean ± SEM of two to three experiments for all assays except the 2 h time point, in which case there is a single data point. The level of caspase activity for FAS-stimulated PMNs at 6 h is set as 100% (positive control), and all other data are expressed relative to the α-FAS data points at 6 h. Statistical analyses were performed using one-way ANOVA and Tukey posttest. *p < 0.05 for α-FAS versus SA, p < 0.05 for Beads versus SA, p < 0.05 for α-FAS versus Beads.

**FIGURE 7. Nec-1–dependent inhibition of PMN lysis.** Human PMN were cultured with USA300 at an MOI of 10:1 in the presence of Nec-1 or vehicle (0 μM Nec-1) or without bacteria (DMSO), and cell lysis was determined by release of LDH. Data for PMNs are the mean ± SEM of four to eight experiments. Statistical analyses were performed using one-way ANOVA and Dunnett posttest. *p < 0.05 for PMNs + USA300 treated with Nec-1 versus vehicle control (0 μM).
influences the initial innate immune response to staphylococci as well as the downstream effects that are manifested as the clinical features of staphylococcal disease.

Soon after ingestion of USA300, PMN-SA increased surface exposure of PS and depolarized mitochondrial membranes, changes typical for the early stages in the apoptotic pathway. However, unlike PMN proceeding toward an apoptotic cell death, PMN-SA increased expression of CD47, sustained cytoplasmic levels of PCNA, and failed to activate caspase-3, -8, -2, and -9 events that each likely influenced the subsequent fate of PMN-SA.

The higher levels of CD47 expressed by PMN-SA likely contributed to their ability to resist uptake by macrophages and to persist extracellularly (Fig. 1). Gresham et al. (29) reported that deficiency in CD47 results in less bacterial burden and increased survival of mice i.p. given a lethal dose of S. aureus. Although the authors attributed this phenotype to decreased PMN infiltration into the peritoneal cavity, the absence of CD47 may also increase the scavenging of dying PMN by macrophages and thereby promote the resolution of infection (29). We confirmed previous reports that blocking CD47 on freshly isolated PMN enhanced internalization by macrophages (data not shown), suggesting that the ability of PMN-SA to resist uptake by macrophages relies on signals in addition to CD47, including CD31, urokinase-type plasminogen activator receptor, and plasminogen activator inhibitor-1 (31–33), that may cooperate with CD47 in suppressing uptake of PMN-SA.

In addition to failing to undergo efficient effecrocotosis by macrophages, PMN-SA prompted cytokine production and inflammatory activation by macrophages at levels less than those produced by macrophages in response to USA300 alone (Figs. 3, 4). In contrast to the response to USA300 alone, macrophages challenged with PMN-SA produced more IL-8 but significantly less IL-1β and IL-1RA (Fig. 4). Although enhanced IL-8 would likely elicit greater PMN chemotaxis (34), decreases in the other cytokines might result overall in a dampened inflammatory response. As inflammasome-mediated production of IL-1β promotes PMN recruitment and host defense in mouse models of cutaneous infection due to S. aureus (35–37), the decreased IL-1β response to PMN-SA might contribute to exacerbated disease pathology during S. aureus infection.

Along with the modulation of macrophage phagocytosis of PMN-SA, CA-MRSA strains such as USA300 ultimately induce the lysis of PMNs. In contrast to spent PMN undergoing apoptosis, PMN-SA regulated prosurvival determinants, including PCNA and caspasases (Figs. 5, 6). Because PCNA can sequester pro-caspases and prevent their activation (24), it is possible that PCNA serves as the master switch that caused depressed caspase activity in PMN-SA.

PMN-SA not cleared by macrophages eventually undergo lysis, a process that requires viable S. aureus and is actively promoted by PMN, as inhibitors of eukaryotic transcription and translation block LDH release from PMN-SA (7 and data not shown). Despite the upregulation of PCNA, PMN-SA lysed in an RIP-1–dependent manner. To our knowledge, this is the first report that PMN are capable of programmed necrosis or necrototic cell death and that necroptosis can be induced by intraphagosomal bacteria (Fig. 7). During necroptosis, downregulation of inhibitor of apoptosis proteins favors the assembly of the ripoptosome, a multimeric platform comprised of RIP-1, RIP-3, caspase-8, and FADD (38, 39). Conditions that promote caspase-8 inhibition, such as treatment with a pan caspase inhibitor or situations in which the cellular level of cellular FLICE/caspase 8 inhibitory protein short isoforms exceed those of cellular FLICE/caspase 8 inhibitory protein long isoforms, direct cells to undergo programmed necrosis (40). Although additional studies are required to investigate the downstream signaling events in PMN-SA, it is noteworthy that necroptosis of PMN-SA at low MOI required the addition of caspase inhibitors, whereas no supplemental inhibition was required at the higher MOI (10:1).

From a clinical perspective, necroptosis of PMN-SA would be anticipated to exacerbate staphylococcal disease, both locally and systemically. Lysis of PMN-SA releases viable SA that could propagate infection to distant sites, perhaps with organisms pheno-typically more virulent by virtue of adaptations that allowed them to survive attack in PMN phagosomes. In addition, necroptosis of PMN-SA releases PMN constituents that would serve as danger-associated molecular pattern molecules that would amplify local tissue damage.

In summary, we demonstrate that CA-MRSA undermines effective innate immune response of human phagocytes in several ways. Diversion of PMN-SA from a typical apoptotic pathway compromised macrophage effecrocytosis and cytokine production, promoted programmed necrosis, and culminated in escape of viable bacteria. Collectively, these events may contribute to the persistent nature of staphylococcal infection as well as its propensity for causing local tissue necrosis. Elucidation of the staphylococcal signals that trigger the PMN responses that drive the downstream cellular changes dictating PMN-SA fate may provide important and novel insights with therapeutic potential.

Acknowledgments
We thank Sarah Bloomberg, Michael Carlson (Beckman Coulter), Matthew Long, Jamie Schwartz, and Dr. Mark White for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References


