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How methicillin-resistant *Staphylococcus aureus* evade neutrophil killing

Mallary Greenlee-Wacker[a,b,c], **Frank R. DeLeo**[d], and **William M. Nauseef**[a,b,c]

[a]Inflammation Program, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa

[b]Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa

[c]Veterans Administration Medical Center, Iowa City, Iowa

[d]Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

Abstract

Purpose of review—Methicillin-resistant strains of the important human pathogen *Staphylococcus aureus* pose a significant public health threat in the community, as they are easily transmitted, especially prone to cause invasive disease, and infect otherwise healthy individuals. The mechanistic basis for the ability of these organisms to evade the innate immune responses remains incompletely defined.

Recent findings—The success of pathogens such as *S. aureus* rests, in part, on their capacity to overcome neutrophil-mediated host defense to establish infection and cause human disease. *S. aureus* has the potential to thwart effective neutrophil chemotaxis, and phagocytosis, and succeeds in evading killing by neutrophils. Furthermore, *S. aureus* surviving within neutrophils promotes neutrophil cytolysis, with release of host-derived molecules that promote local inflammation. Here, we provide a brief overview of our understanding of the mechanisms by which *S. aureus* – including methicillin-resistant *S. aureus* – avoids neutrophil-mediated host defense and causes disease.

Summary—Understanding the molecular mechanisms by which *S. aureus* avoids neutrophil-mediated responses and initiates signaling cascades that culminate in neutrophil lysis will provide insights prerequisite to the development of novel targets for treating staphylococcal infections.

Keywords

cytolysis; inflammation; necroptosis; staphylococcal infection

Correspondence to: William M. Nauseef, MD, Inflammation Program and Department of Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, D160 MTF, 2501 Crosspark Road, Coralville, IA 52241, USA. Tel: +1 319 335 4278; fax: +1 319 335 4194; william-nauseef@uiowa.edu.

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INTRODUCTION

Infection from methicillin-resistant *Staphylococcus aureus* (MRSA) causes a wide range of diseases, including skin and soft-tissue infections, pneumonia, osteomyelitis, endocarditis, toxic shock syndrome and sepsis [1,2], and imposes a major burden on the healthcare system. Furthermore, staphylococcal infections are common and often deadly; in 2011, approximately 11 000 of an estimated 80 000 invasive MRSA infections in the United States were fatal [3▪▪]. Until recently, strains recovered in the United States classified as pulsed-field type USA100 and USA200 were primarily isolated from patients with hospital-associated infections, whereas USA300 and USA400 were isolated almost exclusively from community-associated infections (and were thus community-associated-MRSA) [4]. However, USA300 has now emerged as the predominant MRSA isolate recovered from both nosocomial and community settings [5▪▪]. Although progress has been made in elucidating mechanisms underlying the shift in epidemiology, the basis for the success of USA300 to establish itself widely in the environment remains incompletely determined.

Successful pathogens overcome mechanical barriers, withstand attack by soluble antimicrobial factors in the circulation, and avoid destruction by phagocytes in order to survive and disseminate. The capacity of an organism to subvert, evade, or endure immune responses contributes to its survival and ability to promote disease, namely to its virulence. In addition, the local consequences of infection can be magnified by derailing or delaying the resolution phase of the inflammatory response. With respect to all of these features, *S. aureus* represents a virulent pathogen par excellence. Long recognized for its ability to resist killing by neutrophils [6], *S. aureus* causes infections characterized by exuberant inflammation, local tissue necrosis, and a propensity for distant spread. Survival within neutrophils represents the essential initial step in the cascade culminating in staphylococcal disease.

NEUTROPHILS IN HOST DEFENSE

Recruited early to the site of infection, neutrophils ingest invading microbes and sequester them within the phagosome, a membrane-bound vacuole in which antimicrobial agents are delivered and generated by the combined actions of degranulation and activation of the NADPH oxidase, respectively [7▪]. The collaborative activities of NADPH-oxidase derived reactive oxygen species, such as H_2O_2 and HOCl, antimicrobial peptides and proteolytic enzymes originating from cytoplasmic granules (reviewed in [8,9]) kill and degrade a wide range of microbial targets. Following eradication of ingested microbes, neutrophils undergo apoptotic cell death. Engulfment of apoptotic cells, or efferocytosis, by macrophages initiates the resolution phase of inflammation and promotes a return to normal tissue homeostasis [10,11].

Although neutrophils eliminate the vast majority of invading bacteria, some microbes circumvent killing by these leukocytes. Indeed, *S. aureus* sabotages neutrophil-mediated host defense by compromising neutrophil viability and disrupting normal chemotaxis, phagocytosis, antimicrobial action, and efferocytosis [6,9,12▪▪, 13▪,14,15▪].

CAVEATS REGARDING STUDIES OF NEUTROPHILS VERSUS MICROBES

Much of our understanding of neutrophil function in host defense derives from studies performed *in vitro* using neutrophils isolated from blood. Experimental variables, with respect to phagocytes and bacteria, greatly influence the fate of both microbe and host cell. The expression of virulence factors varies with growth phase, and staphylococci in early exponential or mid-log phase succumb more readily to neutrophil attack than do organisms in stationary growth phase [14]. Functional properties of adherent and suspended human neutrophils differ markedly [16], and adherent neutrophils more readily kill ingested *S. aureus* [16]. Furthermore, the efficiency of killing a given inoculum of bacteria improves as the magnitude of the microbial challenge decreases; a lower multiplicity of infection results in more complete antimicrobial action [17].

In addition to variations in experimental design, in-vitro studies with isolated neutrophils lack the contributions that circulating factors and bystander cells, including tissue macrophages, platelets, and lymphocytes, may provide to host defense. For example, the acute phase plasma protein Group IIA phospholipase A2 (gpIIA-PLA2) exhibits potent antimicrobial action against Gram-positive bacteria, including *S. aureus* [18,19]. At nanomolar concentrations, gpIIA-PLA2 acts synergistically with neutrophils in an NADPH oxidase-dependent fashion to kill and degrade ingested staphylococci [20]. The significant contributions of gpIIA-PLA2 to neutrophil efficacy would be lacking from in-vitro studies in the absence of plasma.

Given the limitations inherent to in-vitro experimental systems, one might be tempted to employ animal models to probe neutrophil function against *S. aureus*. However, neutrophils from rodents, rabbits, and other mammals, including nonhuman primates, differ from human neutrophils in many ways. For example, in contrast to human neutrophils, murine neutrophils contain less myeloperoxidase and lack defensins, two important antimicrobial agents (reviewed in [21▪,22]). Furthermore, responses of nonhuman neutrophils to *S. aureus* and its products often diverge from those of human neutrophils, and such differences often bring into question whether animal infection models are appropriate for mimicking human staphylococcal disease [23,24▪].

Although both reductionist in-vitro studies and animal model in-vivo systems have intrinsic shortcomings, they each yield observations that provide insight into the processes underlying neutrophil–*S. aureus* interactions. With the limitations of the experimental systems in mind, we will highlight several ways in which *S. aureus* overcomes neutrophil-mediated immunity to cause disease.

INHIBITION OF NEUTROPHIL RECRUITMENT AND PHAGOCYTOSIS

As first responders to microbial invasion, neutrophils migrate to sites of infection by a chemical gradient of chemokines, anaphylatoxins, and other chemoattractants, including molecules produced or shed by microbes. To succeed as a human commensal, *S. aureus* has adapted to coexist with humans and to that end acquired capabilities to inhibit fundamental neutrophil functions, such as chemotaxis and phagocytosis. Toxins and membrane-associated proteins that can block chemokine signaling and extravasation include

superantigen-like 5 (Ssl5), Ssl3, Ssl10, staphopain A, chemotaxis inhibitor protein, FPR-like 1 inhibitor proteins (FLIPr and FLIPr-like), and extracellular adherence protein. In addition, staphylococcal factors, including aureolysin, staphylococcal complement inhibitor, extracellular fibrinogen-binding molecule, Ssl7, staphylococcal protein A, Ssl10, staphylococcal IgG-binding molecules (Sbi-III and Sbi-IV), and staphylokinase inhibit complement activation and opsonization. Consequently, the enhanced virulence phenotype of community-associated-MRSA strains, such as USA300, might be attributed, in part, to their ability to subvert these key neutrophil functions. However, the extent to which such devices serve as virulence factors to promote human disease remains unclear, as purulence and abscess formation, both clinical manifestations of successful neutrophil recruitment, and phagocytosis are robust in human *S. aureus* infections. As detailed descriptions of these *S. aureus* molecules are beyond the scope of this article, we refer the reader to recent review articles on the topic [9,12▪▪,13▪].

MODERATION OF PHAGOCYTE REACTIVE OXYGEN SPECIES

The combined actions of reactive oxygen species, including H_2O_2, HOCl, and their derivatives, and granule proteins create in the neutrophil phagosome an environment often lethal to many species of bacteria and fungi. However, a significant fraction of the ingested inoculum of *S. aureus* remains viable, albeit not replicating, within neutrophils, presumably because it has evolved redundant means to resist oxidative damage and survive within phagosomes. Indeed, *S. aureus* can survive in the presence of millimolar concentrations of hydrogen peroxide [25]. For example, *S. aureus* produces super-oxide dismutase, which converts superoxide anion, the proximal product of the NADPH oxidase, to H_2O_2, and catalase, which consumes H_2O_2 to yield O_2 and H_2O, thereby eliminating oxidants generated by stimulated neutrophils. Furthermore, *S. aureus* strains express the pigment staphyloxanthin, which consumes oxidants and renders cells resistant to oxidant-dependent killing and surface factor promoting resistance to oxidative killing, which protects bacteria from singlet oxygen via an undefined mechanism [26–30]. Ingested *S. aureus* not only undermine oxidant attack but also repair oxidative damage incurred within neutrophils. Methionine sulfoxide reductases (Msr), highly conserved enzymes that support oxidative repair in a wide range of organisms [31,32], contribute to survival of bacteria within neutrophils [33,34▪]. Deletion of *msrA1* and *msrB*, the predominant Msr isoforms in staphylococci, from USA300 impairs the ability of ingested organisms to survive in the presence of H_2O_2, HOCl, or within neutrophils [34▪]. Of note, *S. aureus* upregulates *Msr* expression when fed to neutrophils that lack NADPH oxidase activity, due to either pharmacologic inhibition or genetic absence, or to purified granule proteins, demonstrating that not all oxidant stress in ingested neutrophils derives from the phagocyte NADPH oxidase. Together, the combined effects of limiting and repairing oxidant damage promote survival of *S. aureus* within neutrophils.

Ingested staphylococci sense and react to the toxic onslaught within neutrophils. Studies of the *S. aureus* transcriptome following neutrophil phagocytosis indicate that genes involved in stress response, virulence, capsule synthesis, metabolism, and gene regulation are upregulated, whereas those involved in protein synthesis, cell division, and replication are downregulated. These findings suggest that phagocytosed *S. aureus* devote significant

energy and effort to self-preservation rather than to growth and replication [14]. Similar changes in gene expression occur when *S. aureus* encounters in-vitro sublethal amounts of H_2O_2, HOCl, or neutrophil azurophilic granule proteins [25]. These studies demonstrate the dynamic nature of events within neutrophil phagosomes, with both captor and prey actively exchanging responses.

REGULATION OF VIRULENCE

Bacterial two-component gene regulatory systems (TCS) are known to be important for many processes, including growth, metabolism, virulence, and pathogenesis. In general, TCS are composed of a sensor kinase and a response regulator. The sensor kinase interacts with specific molecules present in the extracellular milieu – for example, reactive oxygen species – and then interacts with the response regulator protein, which in turn alters gene expression [35]. Although this review lacks sufficient space to review the 16 TCS encoded within the genome of *S. aureus* and links to pathogenic properties, we highlight a few of the discoveries on the role of SaeRS, a *S. aureus* TCS that has been the focus of recent studies.

Genes encoding SaeRS are upregulated following phagocytosis of *S. aureus* by neutrophils. Genetic deletion of *saeR* and *saeS* from the USA400 strain MW2 decreases pathogen survival in human blood and in the presence of human neutrophils [14,36]. Consistent with these findings, virulence of the mutant strain is reduced significantly in mouse models of *S. aureus* infection [14,36]. SaeRS induces expression of genes encoding molecules involved in virulence, such as *hla*, *LukAB*/*LukGH*, and *hlgA* [37▪], and those encoding Ssls protein and staphylococcal nuclease [38▪,39]. Collectively, these data support the idea that SaeRS is important for the ability of *S. aureus* to circumvent killing by neutrophils and thereby cause disease.

ROLE OF CYTOLYTIC TOXINS

Cytolytic toxins produced by *S. aureus* are recognized for their ability to cause lysis of host cells *in vitro*. Many of these toxins, including the two-component leukotoxins, alpha-hemolysin (Hla) and alpha-type phenol soluble modulins (PSMα), contribute to virulence in animal models of *S. aureus* infection. In addition, sublytic concentrations of Panton-Valentine leukocidin, one of the two-component leukotoxins, and PSMα can prime neutrophils for enhanced function, which could alter the outcome of host–pathogen interactions. Whether the findings with respect to cytolysis, neutrophil priming, or virulence in animal models of infection can be extrapolated to implicate toxins in human disease remains incompletely determined. There is evidence from the early 1900s that Hla contributed the human fatalities prior to the antibiotic era, but some of these reports are anecdotal. Inasmuch as a comprehensive understanding of *S. aureus* virulence and pathogenesis is important for development of new prophylactic and therapeutic approaches directed to treat or prevent severe *S. aureus* infections, the toxins have been the focus of intense investigation.

Recent studies [40,41▪,42,43▪,44▪] – including work from our collective laboratories – have provided evidence that leukotoxin GH (LukGH; also known as leukotoxin AB), Hla, and PSMα contribute to neutrophil lysis after phagocytosis. In other studies [41▪,43▪,44▪], these

toxins have been suggested to directly disrupt the *S. aureus* phagosome and promote bacterial escape to and replication in the cytoplasm. Although it is tempting to speculate that such phenomena explain the observed rapid *S. aureus*-mediated lysis of human neutrophils after phagocytosis, a few notable caveats should be considered. First, the toxins would need to accumulate in the phagosome at sufficient concentration to disrupt the phagosomal membrane. Although this is possible, reactive oxygen species, especially those generated by the myeloperoxidase–halide system, can inactive toxins such as the PSMs [45]. If the bacteria escape from the neutrophil phagosome – and we have found no evidence for such an event – the toxins must accumulate in the cytoplasm at sufficient concentration to disrupt the plasma membrane. For LukGH and Hla, the cytoplasmic face of the plasma membrane presents an incorrect topology (i.e., inside-out) for toxin interaction and formation of cytolytic pores. It is also interesting that neutrophil lysis after *S. aureus* phagocytosis occurs in the absence of PSMs, albeit at a reduced level [43▪]. Taken together, these data suggest that these toxins do not disrupt the phagosome membrane directly. Rather, the mechanism may be indirect and more work is needed to better understand these phenomena. As an alternative explanation for lysis of *S. aureus*-containing neutrophils, the toxins could induce changes in neutrophil gene expression, trigger signal transduction events, or both that culminate in lysis of neutrophils. We describe these events in greater detail in the next section.

S. AUREUS ALTERS THE RESOLUTION OF INFLAMMATION

Daily production and turnover of neutrophils in healthy adults are extraordinary – in the order of 10^9 neutrophils per/kg body weight [7▪]. Inasmuch as neutrophils are the most abundant leukocyte in humans and contain or produce high levels of toxic molecules, such as reactive oxygen species and proteases, there is potential for host tissue damage should these cells be activated nonspecifically or lyse. Consequently, mechanisms closely regulate neutrophil activation and eliminate effete neutrophils. Aged or spent neutrophils typically undergo apoptosis and are subsequently removed by other phagocytic cells. Together with modulation of other components in the local milieu [46], the clearance of apoptotic neutrophils serves as a critical step in restitution of tissue homeostasis after inflammation.

Although most bacteria are killed by neutrophils, which in turn undergo apoptosis, *S. aureus* can survive within these host cells and ultimately cause cytolysis. Following phagocytosis of USA300, neutrophils have initial features typical of apoptosis, including surface expression of phosphatidylserine, mitochondrial membrane depolarization, nuclear condensation, and membrane blebbing [15▪,47]. *S. aureus*-laden neutrophils upregulate the prosurvival factor proliferating cellular nuclear antigen, fail to activate caspase 2, 3, 8, and 9, increase surface expression of CD47, a ‘don’t eat me’ signal, and resist engulfment by macrophages [15▪]. The recent recognition that the normal pathway toward apoptosis is derailed in neutrophils containing viable *S. aureus* has stimulated interest in elucidating the mechanisms that culminate in neutrophil lysis [14,47]. As indicated above, the contribution of *S. aureus* toxins to neutrophil lysis after phagocytosis may be indirect [40,41▪,42,43▪,44▪,47,48▪▪,49], as neutrophil lysis following phagocytosis of *S. aureus* depends on the presence of viable organisms and new protein synthesis by neutrophils [47]. As *S. aureus* activates the NLRP3 inflammasome in macrophages in a toxin-dependent manner [50–52], it is reasonable to

speculate that neutrophils die via a caspase-1-dependent cell death known as pyroptosis. However, neutrophils produce relatively low levels of IL-1β in response to stimuli, suggesting that caspase-1 activation is not robust in human neutrophils [53▪]. Consistent with this notion, the caspase 1 inhibitor YVAD has no effect on neutrophil lysis following phagocytosis of USA300 [47]. Data from our collective research groups indicate that neutrophils harboring viable *S. aureus* undergo programmed necrosis or necroptosis [15▪]. Necroptosis is a proinflammatory form of cell death dependent on receptor interacting protein-1 kinase, and it is associated with release of danger-associated molecular patterns (DAMPs) [54▪▪], which activate the immune system. Although this form of cell death releases DAMPs and thereby amplifies local inflammation, death by necroptosis delays or prevents the return to normal tissue homeostasis. Further studies are required to elucidate how persistence of viable *S. aureus* within neutrophils promotes necroptosis and whether the phenomenon contributes to pathogenesis of staphylococcal disease.

CONCLUSION

The success of *S. aureus* – including MRSA – as a pathogen depends on its ability to avoid killing by components of the innate immune system, especially neutrophils. Understanding the mechanisms by which *S. aureus* avoids destruction by the innate immune system is a prerequisite to the development of new prophylactic or therapeutic agents designed to prevent or treat infections.

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KEY POINTS

- Success of *S. aureus* as a pathogen rests on its ability to undermine initiation of an immune response, thwart the antimicrobial responses of the host, and inhibit the resolution of inflammation.
- Many community-associated-MRSA strains, especially the USA300 strain, survive within and ultimately cause lysis of neutrophils.
- *S. aureus* inhibits neutrophil-mediated resolution of inflammation by interfering with macrophage efferocytosis and promoting receptor interacting protein-1-dependent lysis of neutrophils.