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The rise and rise of *Staphylococcus aureus*: laughing in the face of granulocytes

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Summary

Recent developments in the study of host–pathogen interactions have fundamentally altered our understanding of the nature of *Staphylococcus aureus* infection, and previously held tenets regarding the role of the granulocyte are being cast aside. Novel mechanisms of pathogenesis are becoming evident, revealing the extent to which *S. aureus* can evade neutrophil responses successfully by resisting microbicides, surviving intracellularly and subverting cell death pathways. Developing a detailed understanding of these complex strategies is especially relevant in light of increasing staphylococcal virulence and antibiotic resistance, and the knowledge that dysfunctional neutrophil responses contribute materially to poor host outcomes. Unravelling the biology of these interactions is a challenging task, but one which may yield new strategies to address this, as yet, defiant organism.

Keywords: apoptosis, inflammation, necrosis, neutrophil, *S. aureus*

Introduction

Staphylococcus aureus is a formidable and resilient human pathogen, as evidenced by its inexorable rise over recent years. Multiple mechanisms of virulence, together with the evolution of strategies to resist antibiotics, have contributed to its disquieting success. Duly, it is the subject of major political as well as scientific attention. Classically, neutrophils represent the major host defence cells against this organism, yet recent work suggests that staphylococcal actions render granulocytes ineffectual. Restoring their potency may offer the key to reversing failures of innate immunity.

Clinical relevance

Approximately 30% of the population is colonized with *S. aureus* either chronically or intermittently [1], although this is of no pathological consequence *per se*. Colonization is, however, linked intimately to disease as it is a major risk factor for invasive infection, and it is partly these carriage rates which help *S. aureus* to thrive as an opportunist [2,3]. Typically, *S. aureus* exploits vulnerable populations such as the elderly, immunosuppressed or debilitated. Major risk factors include breaches of the skin barrier, often by trauma, intravenous drug use or medical instrumentation and impaired mucosal immunity, for example, due to cystic fibrosis, artificial ventilation or post-influenza infection. These deficiencies provide bacterial access to local tissue and to the bloodstream, facilitating dissemination of infection. Local infections may be highly destructive *in situ*, while haematogenous spread results in deep-seated invasive disease including septic arthritis, osteomyelitis, pneumonia and endocarditis. In UK hospital patients the impact is manifest: *S. aureus* is the major cause of surgical site infections and the second most common cause of hospital-acquired bacteraemia [4] and, in this latter setting, mortality rates approach 30% [5]. Resistance to conventional antibiotics exacerbates virulence, exemplified by the ongoing nosocomial methicillin-resistant *S. aureus* (MRSA) epidemic. The emergence of exceptionally virulent community MRSA strains infecting even the young and immunocompetent underlines further the potency of this robust and versatile pathogen [6,7].

The importance of neutrophils in *S. aureus* infection

There are two key controversies surrounding the interactions between neutrophils and *S. aureus*. The first questions neutrophil bactericidal function in relation to *S. aureus*, postulating bacterial survival or even replication within the

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Table 1. *Staphylococcus aureus* mechanisms to evade neutrophil responses.

Virulence factor	Effect on neutrophil immunity	Reference
CHIPS	Inhibits neutrophil chemotaxis, phagocytosis and oxidative burst	[103–105]
SCIN	Inhibits neutrophil chemotaxis, phagocytosis and oxidative burst	[103,106]
ClfA	Inhibits neutrophil phagocytosis	[107]
Eap	Binds ICAM-1 and inhibits neutrophil recruitment	[108]
Staphylokinase	Inhibits alpha-defensins	[109]

CHIPS, chemotaxis inhibitory protein of staphylococci; SCIN, Staphylococcal complement inhibitor; ClfA, clumping factor A; Eap, extracellular adherence protein; ICAM-1, intercellular adhesion molecule-1.

phagolysosome of this usually inhospitable cell. The second questions the effects of *S. aureus* on neutrophil cell death. Because both the timing and mode of neutrophil death is linked intimately to their microbicidal function and to the resolution of inflammation, understanding these events will provide insights into pathogenesis.

How well do neutrophils kill *S. aureus*?

Neutrophils are highly efficient at killing phagocytosed pathogens. They engage a complex cascade of cellular events to eradicate pathogens via oxidative and non-oxidative mechanisms. Following bacterial phagocytosis, the nicotinamide adenosine dinucleotide phosphate (NADPH) enzyme complex and nitric oxide (NO) synthase immediately generate reactive oxygen and nitrogen intermediates (ROI, RNI) within the phagosomal compartment, molecules which are implicated directly in microbicidal activity [8]. Lysosomes laden with proteases, cathepsins, defensins and other antimicrobial proteins fuse rapidly with the phagosome, discharging their potent contents into the phagolysosome. The generation of superoxide by the NADPH complex permits activation of granule proteases within the acidified phagolysosome, thus linking oxidative and non-oxidative bactericidal mechanisms [9]. Neutrophils are also capable of killing non-phagocytosed pathogens through the formation of neutrophil extracellular traps (NETs), comprising tangles of chromatin and granule proteins which are released by rupture of the neutrophil cell membrane. These structures ensnare bacteria and kill them by exposure to high local concentrations of anti-microbial molecules [10,11].

The consequences of neutrophil deficiencies in number or function substantiate their critical bactericidal role, as affected patients succumb to repeated bacterial infections. Evidence provided by patients with genetic defects implicates neutrophils in opposing *S. aureus* specifically [12,13]. Patients susceptible to recurrent *S. aureus* infection include those with chronic neutropenia such as severe congenital neutropenia (SCN), impaired neutrophil migration such as leucocyte adhesion deficiency 1 [13,14] and those with disorders of intracellular killing. This latter group includes patients with chronic granulomatous disease (CGD), who exhibit profoundly impaired oxidative killing due to defective assembly of the NADPH oxidase complex [15,16] and Chediak Higashi patients, in whom degranulation is impaired due to failure of phagolysosome maturation [13]. Furthermore, experimental work using murine models has attributed roles for specific neutrophil microbicidal proteases to particular pathogens; for example, selectively knocking out neutrophil cathepsin G, but not neutrophil elastase, predisposes to *S. aureus* infection [17]. There are also numerous *in vitro* studies that support the neutrophil as the key innate effector cell in controlling *S. aureus* infection [18–21].

Consistent with the notion that neutrophils are a major resource in the conflict against invading *S. aureus*, the bacterium invests in a panoply of virulence determinants to avoid recognition and phagocytosis by neutrophils (see Table 1). Several secreted and cell-bound proteins act in concert to effectively thwart neutrophil responses at multiple stages including chemotaxis, opsonization, activation and phagocytosis. These sophisticated mechanisms equip the bacterium with major advantages over neutrophils and are reviewed in more detail by Foster and Rooijakkers [22,23]. Additionally, the acquired immune response is considered weak in the face of this pathogen because the presence of anti-staphylococcal antibodies does not confer protection against further infection [24,25].

Despite this, there is a growing body of evidence which suggests that neutrophil defences are of only limited efficacy against staphylococcal insult. Abscess formation is a typical pathology during *S. aureus* infection which comprises bacteria and recruited neutrophils, many of which are merely corpses, walled off by a fibrin mesh. This is clearly a neutrophil-rich site and yet it is often a focus of persistent infection, allowing speculation that an abscess represents a frustrated immune response: it can contain infection but is unable to resolve it. Also, patients rendered neutropaenic acutely through the administration of chemotherapy are susceptible to a broad range of pathogens and *S. aureus*, although important, does not predominate [26]. Although the epidemiology and microbiology depend upon numerous factors, including the presence of intravascular devices and the selective pressure of antibiotics, it is intriguing to speculate how such a ubiquitous, colonizing opportunist is kept in check under these circumstances.

More persuasive, and counter-intuitive, is direct evidence of intracellular survival of *S. aureus* within neutrophils.

Although considered classically an extracellular pathogen, *S. aureus* is known to possess many virulence determinants which protect it from neutrophil microbicides. For example, physical and electrochemical cell wall properties resist the effects of neutrophil defensins and lysozyme, while neutralizing enzymes and carotenoid pigment confer resistance to ROI [22,27,28]. Both *in vitro* and *in vivo* work has supported this premise with the caveat that many of these studies relate to the highly virulent community-acquired MRSA strains. Palazzolo-Balance *et al.* have demonstrated that *S. aureus* up-regulates a plethora of virulence factors, including haemolysins, leucotoxins, iron scavengers and stress response genes, when exposed to purified neutrophil-derived anti-microbial factors. Moreover, these potent microbicides, including hydrogen peroxide, hypochlorous acid and azurophilic granule proteins, merely exerted bacteriostatic rather than bactericidal effects [29]. Importantly, Gresham *et al.*, established that intracellular bacteria remain viable and virulent. They described the recovery of viable *S. aureus* from neutrophils isolated from a murine peritonitis model and that infected neutrophils were sufficient to establish infection in a naive mouse [30]. Electron microscopy revealed that *S. aureus* strains better able to survive within neutrophils were localized within large vacuoles termed 'spacious phagosomes' and phagosomal membranes sometimes appeared partially degraded, suggestive of an early stage of bacterial escape into the cytoplasm. Notably, neutrophil depletion resulted in improved outcome of infection in this study and others [31,32], suggesting that an excess of neutrophils may perversely facilitate infection and the persistence of inflammation. Voyich *et al.* have also demonstrated *S. aureus* survival within the neutrophil phagolysosome and, crucially, have also shown spontaneous microbial escape via host cell lysis. Community-acquired strains of MRSA appeared to be more resistant to neutrophil killing and caused more cell lysis compared with hospital strains, correlating bacterial survival directly with aberrant neutrophil death [33]. In accordance with this, Kubica *et al.* describe the intracellular survival of *S. aureus* within macrophages whereby the bacteria exist 'silently' inside phagolysosomes for several days and subsequently escape by inducing spontaneous cell lysis. This process is dependent upon multiple virulence factors, in particular α-haemolysin [34]. By also exploiting macrophages in this way, *S. aureus* readily outmanoeuvres two invaluable professional phagocytes that, together, form the first line of cellular innate immune defence.

How does *S. aureus* influence neutrophil cell death pathways?

Neutrophil cell death pathways. Neutrophils are short-lived cells and in the absence of an activation signal undergo constitutive apoptosis. Apoptosis is a favourable mode of death, as it leads to functional down-regulation of superfluous cells while preserving membrane integrity and promoting clearance by macrophages. Apoptosis is a highly complex and tightly regulated series of intracellular events that culminate in neatly packaged cell corpses which are phagocytosed by tissue macrophages. Molecules central to the apoptotic process include members of the Bcl-2 family, which comprises pro- (Bax, Bak, Bim, Bad) and anti-apoptotic (Bcl-2, Bcl-xl, A1, Mcl-1) proteins, which typically regulate mitochondrial membrane stability. The cysteinyl protease family of caspases, which are held in the cell as inactive proforms, are also fundamental in the death programme [35].

By contrast, necrosis is an unfavourable mechanism of cell death, whereby the potent anti-microbial molecules bound previously within the cell spill into the extracellular space and cause local tissue damage. Unlike apoptosis, necrosis is thought typically to be a passive mode of cell death, occurring as a result of injurious processes including infarction, cancer and inflammation [36]. Because necrotic cells lack the 'eat me' signals expressed by apoptotic corpses, clearance of necrotic debris is less efficient, resulting in inappropriate persistence of tissue inflammation [37] as well as impaired bacterial clearance. In light of this, many pathogens, including *S. aureus*, manipulate neutrophil cell death in order to promote their own survival within the host.

Responses of neutrophils to staphylococci and impact on cell survival. As critical innate immune cells, neutrophils express many of the Toll-like receptor (TLR) family of pattern recognition receptors (PRRs) [38,39] and it is via these receptors, in part, that neutrophils first detect *S. aureus*. Toll-like receptor (TLR)-2 is considered to be the primary PRR for Gram-positive bacteria, as lipoteichoic acids (LTA) signal through this receptor [40]. TLR-2 is important for both limiting *S. aureus* carriage [41] and infection [42], as TLR-$2^{-/-}$ mice are highly susceptible to *S. aureus* challenge [43]. This also holds true in human disease for deficiencies in downstream TLR signalling molecules, including myeloid differentiation primary response gene (MyD88) and interleukin (IL)-1 receptor-associated kinase (IRAK-4) [44,45], although these molecules are also involved in IL-1 signalling. Staphylococcal-produced phenol-soluble modulins have also been shown to signal via TLR-2 [46], supporting further the role of these receptors in initiating host responses to *S. aureus*. The other major cell wall component of *S. aureus* is peptidoglycan. Once thought to be a TLR-2 agonist, peptidoglycan (PGN) is now considered to signal dominantly via intracellular PRRs of the nucleotide-binding oligomerization domain (NOD) family [47] which have been shown recently to play a crucial role in defence against *S. aureus in vivo* [48].

Both TLRs and nucleotide-binding oligomerization domain (NOD)-2 couple into classic proinflammatory pathways, involving mitogen-activated protein kinase (MAP) kinase and nuclear factor kappa (NF-κB) activation [49] that

delay neutrophil apoptosis. There is some variation in the described magnitude of neutrophil survival to *S. aureus*-derived molecules, but in particular a pro-survival effect of LTA via TLR-2 ligation has been demonstrated [50,51]. Kobayashi *et al.* examined changes in neutrophil gene expression following exposure to bacteria including *S. aureus*, and found that many genes associated with the cell death programme, such as members of the Bcl-2 family and TLR-2 signalling components, were up-regulated [52]. The initial arrest of spontaneous neutrophil apoptosis potentially enhances the functional longevity of the cell, facilitating its response against the pathogen [39]. This host advantage is circumvented quickly by *S. aureus* and, given that the micro-organism may be able to persist inside neutrophils, delayed apoptosis may even favour the microbe.

Conversely, exposure to *S. aureus* may instigate apoptosis through the mechanism of phagocytosis-induced cell death (PICD) because this itself is a potent stimulus of apoptosis via ligation of Fcγ receptors [53]. PICD may represent a physiological mechanism evolved to link bacterial killing to safe neutrophil disposal, rather than a pathological consequence of infection, as the phenomenon is also seen with heat-killed *S. aureus* [54] and inert particles [53].

Regulation of neutrophil apoptosis by *S. aureus*

Although neutrophils detect the presence of staphylococci and may initiate a response resulting in activation and survival, the bacterium itself also exerts powerful effects on neutrophil survival. There are conflicting reports regarding the influence of *S. aureus* as a pro- or anti-apoptotic stimulus to neutrophils. The outcome can depend upon the experimental conditions; in particular, MOI, duration of challenge, the presence of contaminating cells and bacterial strain. Findings are therefore context-dependent, which emphasizes the complexity of neutrophil lifespan regulation by multiple influences during bacterial infection. Ocana *et al.* conclude that MOI is crucial: low bacteria to neutrophil ratios led typically to an inhibition of apoptosis and high bacteria to neutrophil ratios induced apoptosis [55]. The delay of apoptosis was consistent with a mechanism involving bacterial recognition by cell surface receptors such as TLRs. This pro-survival effect can also be achieved indirectly by the actions of cytokines [56], and has been linked recently to autocrine/paracrine IL-6 production by neutrophils themselves in response to *S. aureus* challenge [55]. In keeping with these observations, in murine models of *S. aureus* infection mice deficient in the proinflammatory cytokine IL-1β developed larger lesions and fared worse than wild-type counterparts due to impaired neutrophil recruitment [18]. Because IL-1β exerts many anti-apoptotic effects on leucocyte populations, it is conceivable that the lack of cell survival in these deficient mice also contributes to worsening of infection. However, neutrophils respond only poorly or not at all to IL-1β directly, suggesting that the actions of IL-1β may be mediated indirectly by downstream action of tissue cells or other leucocytes [57,58].

Soluble factors secreted by *S. aureus* also modulate neutrophil apoptosis. Lundqvist-Gustafsson *et al.* describe the pro-apoptotic effect of a heat-labile, secreted staphylococcal product mediated in part by activation of p38 MAP kinase [59]. In other studies, the pore-forming toxin Panton–Valentine leucocidin (PVL) is a powerful inducer of neutrophil cell death [60]. While PVL is predominantly a necrosis-inducing toxin, at low concentrations it induces neutrophil apoptosis [60].

Finally, because bacterial uptake and killing are linked to the initiation of neutrophil apoptosis, intracellular survival of *S. aureus* within neutrophils and subsequent escape via lysis implies again that the bacterium has evolved strategies to suppress apoptosis in particular circumstances.

S. aureus exotoxins modulate neutrophil function and induce necrosis

Neutrophil necrosis not only depletes a pool of valuable immune cells but also generates a proinflammatory environment by releasing toxic intracellular contents. Furthermore, the vicious circle of self-perpetuating inflammation, necrotic chaos and abscess formation may enhance bacterial survival both by providing a 'niche' where microorganisms are relatively hidden from the immune system and by weakening the bacterial defences on infiltrating neutrophils, for example by cleavage of chemokine receptors [61]. It is well recognized that *S. aureus* secretes a number of exotoxins, many of which are activatory or leucolytic *in vitro,* although their clinical relevance remains highly controversial. This group includes the haemolysins (α, β, γ and δ), leucocidins and phenol-soluble modulins.

Haemolysins facilitate the scavenging of iron, although many of them also have leucolytic properties. Alpha-haemolysin is a pore-forming toxin (PFT) and a prominent virulence factor secreted by almost all clinical strains of *S. aureus* [62–64]. There is good evidence to support its pathological significance in a number of animal models of disease including pneumonia, peritonitis and septic arthritis [65–68]. Cellular susceptibility to α-haemolysin varies widely and human neutrophils demonstrate a remarkable degree of resistance to this lytic agent [69]. None the less, the neutrophil-α-haemolysin interaction is not inert, instead modulating neutrophil adherence to endothelial cells and leukotriene generation [70–72]. This potential sequence of events may result in endothelial dysfunction, which has been implicated in animal models of sepsis [73].

Beta-haemolysin is a magnesium-dependent sphingomyelinase C with more potent haemolytic than leucolytic activity. Variable effects have been reported on nucleated cells, in some studies with human lymphocytes and neutrophils appearing susceptible *in vitro* [74,75]. More recent work has revealed that β-haemolysin is a potent inhibitor of

IL-8 production from endothelial cells and thus impedes neutrophil transmigration [76]. The pathological relevance of this exotoxin in human disease is by no means clear, however, with few experimental mammalian models to date supporting its role [77].

Delta-haemolysin, another PFT, exerts significant proinflammatory effects by activating neutrophils *in vitro.* It can act both as a priming agent in the presence of lipopolysaccharide (LPS) or tumour necrosis factor (TNF)-α [78], and also as a direct activator of neutrophils, generating ROI and platelet-activating factor, provoking the release of granule enzymes and modulating leukotriene generation [79,80].

Some of the most important leucolytic toxins secreted by *S. aureus* are the bicomponent leucotoxins, including γ-haemolysin and leucocidins such as PVL. Gamma haemolysin is a prominent haemolysin, secreted by 99% of *S. aureus* strains. Its leucolytic and activatory actions on neutrophils are also well established [81–83], and a number of studies have validated its role as a relevant virulence determinant in mammalian models of disease [67,84,85]. By contrast, PVL demonstrates a remarkable and exclusive affinity for leucocytes alone, with little haemolytic effect in humans [86]. Pore formation is clearly demonstrable in neutrophil membranes, forming non-specific cation channels which leads to degranulation and the release of proinflammatory mediators prior to cytolysis [82,86–89]. Recent work suggests that at high concentrations leucotoxins may also inhibit neutrophil oxidative burst, potentially undermining their bactericidal capacity [90].

While PVL's potent cytolytic effects on human neutrophils were first characterized in the 1960s [91], it has been implicated more recently as a key virulence factor associated with necrotizing pneumopathies caused by highly virulent community-acquired MRSA. These infections typically affect immunocompetent individuals, and histological findings are dominated by severe tissue necrosis and neutrophil lysis [92]. A number of factors incriminated PVL as a probable aetiological candidate, including the preferential targeting of leucocytes with a high degree of specificity for neutrophils [83]. Furthermore, epidemiological associations exist between community-acquired MRSA, necrotizing pneumonia and PVL [92–95]. Although some mammalian models of pneumonia have supported this view [96] other groups dispute this, citing a limited role for PVL and implicating alternative virulence factors [65,97–99].

Finally, novel and highly effective leucolytic agents are emerging in association with CA-MRSA. Phenol-soluble modulins are cytolytic peptides first described in *S. epidermidis* infections, which are generating considerable interest as important virulence determinants of CA-MRSA. These pore-forming toxins attract, activate and lyse neutrophils [100,101]. Not only is leucocidal activity demonstrable *in vitro*, but there is also supportive evidence of *in vivo* virulence in murine skin abscess and bacteraemia models [101].

Conclusions

Reconciling the effects of so many influences on neutrophil lifespan is problematic. With respect to the regulation of programmed cell death, it is likely that competing pro- and anti-apoptotic stimuli converge in the neutrophil and their summative effect, to some extent, determines outcome. In most circumstances neutrophil apoptosis is a desirable consequence, aiding neutrophil clearance and the resolution of infection, but manipulation of neutrophil death by acceleration, suppression or bypass of apoptosis may commandeer the process to pathogen advantage [52].

With respect to the induction of neutrophil lysis, the repertoire of secreted leucolytic agents and the clinical manifestations of necrotizing infection reflect how successful *S. aureus* can be as an extracellular pathogen. However, in light of mounting evidence that *S. aureus* can survive intracellularly after phagocytic uptake, these toxins may in fact play a key role in facilitating escape from the phagosome [102]. Subsequent neutrophil lysis could enable *S. aureus* to escape infection foci and allow pathogen dissemination. In either case, cell death proceeds via the unsafe mechanism of cellular necrosis and the adverse consequences of failure of neutrophil apoptosis are evident. A schematic representation of the potential ways that *S. aureus* may subvert the neutrophil response is shown in Fig. 1.

Overall, we have a limited and inadequate understanding of the complex interactions between *S. aureus* and the innate immune response. This is especially evident when contrasting its peaceful existence as a colonizer compared with its demonstrable aggression as a pathogen. *S. aureus* has proved itself to be a sophisticated pathogen. It is remarkably capable of disabling the neutrophil, resisting potent bactericidal mechanisms and inducing aberrant neutrophil death. The emergence of highly pathogenic strains, which are adept at targeting the neutrophil, underlines not only their own virulence but also the key importance of the neutrophil. Gaining insight into the molecular basis of staphylococcal success may enable future therapies targeted at enhancing neutrophilic defences against *S. aureus.* Restoring neutrophil potency may rest not only on addressing bactericidal mechanisms but also on re-engaging subverted mechanisms of cell death.

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Disclosure

The authors have no conflicts of interest to declare.

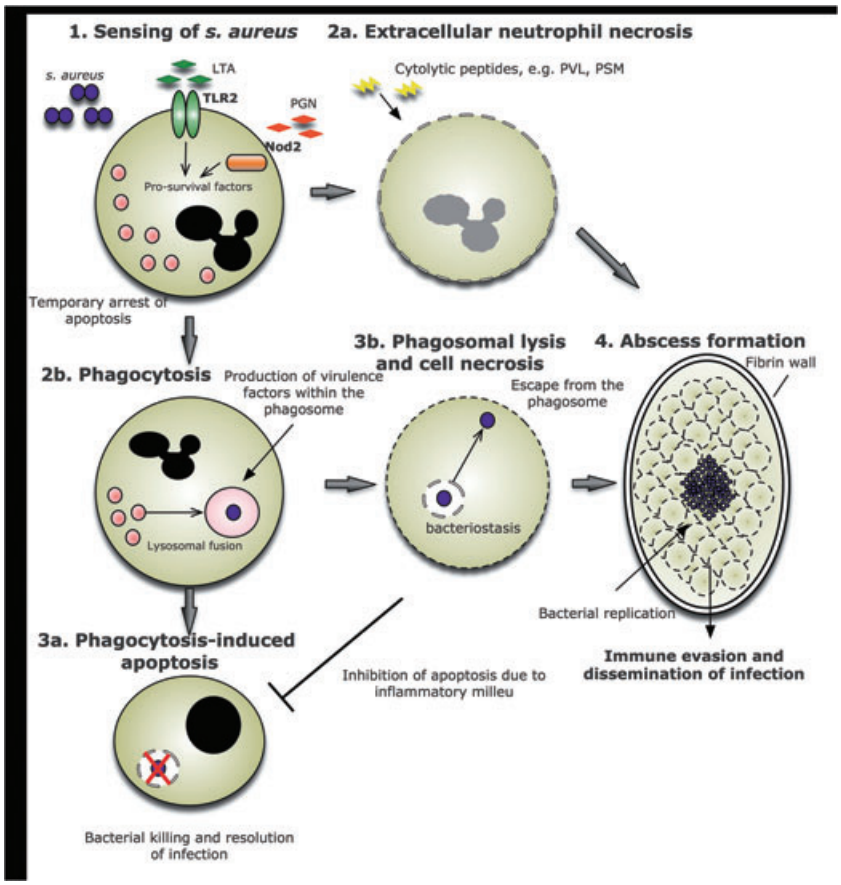

Fig. 1. Mechanisms by which *Staphylococcus aureus*–neutrophil interactions may lead to abscess formation. Initial sensing of *Staphylococcus aureus* is carried out mainly by the pattern recognition receptors (PRRs), Toll-like receptor (TLR)-2 and nucleotide-binding oligomerization domain (NOD)-2. The subsequent signalling leads to the generation of pro-survival molecules which serve to 'prime' the neutrophil and thus delay apoptosis temporarily (1). Extracellular bacterial factors, including Panton–Valentine leucocidin (PVL) and phenol-soluble modulins (PSM), have direct effects upon the neutrophil, leading to cell necrosis (2a). Intact neutrophils phagocytose *S. aureus,* which becomes confined within the phagosome (2b). The desirable outcome is for bacterial killing to take place in the phagosome as a result of activation of anti-microbial proteases and the generation of oxidative stress, following which the cell would undergo apoptosis and be cleared by tissue macrophages (3a). *S. aureus* may subvert this mechanism by synthesizing virulence factors including lytic proteins within the phagosome, which cause rupture of both the phagosomal and plasma membrane (3b). Bacteria then escape from the cell and potentially utilize the cell contents to fuel replication. Accumulation of cell corpses contributes to abscess formation and inflammation, providing a niche which is relatively hidden from infiltrating immune cells, allowing bacterial replication and ultimately systemic dissemination (4). LTA, lipoteichoic acid; PGN, peptidoglycan.

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