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Innate immunity to *S. aureus*: evolving paradigms in soft tissue and invasive infections

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Abstract

Staphylococcus aureus causes a wide range of diseases that together embody a significant public health burden. Aided by metabolic flexibility and a large virulence repertoire, *S. aureus* has the remarkable ability to hematogenously disseminate and infect various tissues, including skin, lung, heart, and bone, among others. The hallmark lesions of invasive staphylococcal infections, abscesses, simultaneously denote the powerful innate immune responses to tissue invasion, as well as the ability of staphylococci to persist within these lesions. In this manuscript, we review the innate immune responses to *S. aureus* during infection of skin and bone, which serve as paradigms for soft tissue and bone disease, respectively.

Introduction

Staphylococcus aureus is a Gram-positive bacterium that colonizes approximately 30% of the population (1). Despite this relatively innocuous lifestyle, *S. aureus* is capable of breaching tissue barriers, circulating through the bloodstream, and infecting nearly every organ system in the body. *S. aureus* is the most common cause of bacterial skin and soft

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tissue infections in the United States (2, 3). Other infection sites include but are not limited to bone, lung, kidney, and heart. A critical tenant in the battle against staphylococcal infections is to understand host risk factors, including those that parse out individuals capable of local control of infection, versus those that progress to invasive disease. A better understanding of the innate immune responses to *S. aureus* will also aid the development of new adjunctive therapies to ameliorate the morbidity of staphylococcal disease.

Historical perspectives on anti-staphylococcal immunity

In the early 1880's, Dr. Alexander Ogston examined purulent material from patients with soft tissue infection, noting microscopic "masses or clusters, like the roe of a fish, to which I gave the name 'staphylococcus'" (4, 5). Following Ogston's landmark discovery, it is clear that *S. aureus* is the preeminent bacterial pathogen causing purulent infections. Although much is known regarding the architecture of staphylococcal abscesses and the cellular contributors to pyogenic immune responses (6), many questions remain unanswered. In the sections that follow, we review the key events underlying effective recognition and microbiologic control of *S. aureus* skin and bone infection.

Immune responses to *S. aureus* in the skin

Skin is a complex organ that performs vital functions including immune responses, hormone and vitamin production, and formation of a protective mechanical and chemical barrier (7). Skin is composed of an outer epidermis overlying an inner dermis, separated by a basement membrane. The physical and biochemical barriers are derived from the association of keratinocytes (KCs) with the products of sweat, lipid and antimicrobial peptides (AMP) (7). The epidermis is formed by KCs in different maturation stages, Langerhans cells (LC) and T cells. The dermis contains extracellular matrix (ECM) components such as connective tissues, collagen, and elastin fibers (8). The fibers provide a structural framework to host blood vessels, adipocytes, fibroblasts, skin-resident macrophages, dermal dendritic cells, mast cells, T and B lymphocytes, plasma cells and NK cells (8). As such, resident immune cells are abundant in skin, and these cells are all involved in the control of *S. aureus* infection by influencing different arms of the immune response (9, 10).

Key players involved in bacterial recognition and inflammatory response

KCs, together with the other resident immune cells in the skin, participate in the recognition and response to invading pathogens (10, 11). KCs are typically the first cells that encounter pathogens, and recognize pathogen associated molecular patterns (PAMPs) via different pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-1 and −2, and the scavenger receptors CD36 and MARCO (10–12). Signaling through these receptors induces activation of transcription factors such as NFκB, AP1, and CREB to generate cytokines (IL-1α, IL-1β, IFN-γ, TNFα and IL-17A, IL-17F and IL-22), chemokines (CXCL1, CXCL2, CXCL9, CXCL10, CXCL11, CCL27, and CCL20) and antimicrobial effectors, such as antimicrobial peptides (AMPs) and inducible nitric oxide synthase (7, 12, 13) (Figure 1A; **left)**.

TLR1, −2, and- 6 recognizes the *S. aureus* cell wall components, specifically lipopeptides and peptidoglycan. These TLRs utilize the signaling adapter MyD88 to induce robust and efficient transcriptional programs that lead to inflammatory responses. TLR1, −2, and −6 are involved in many stages of *S. aureus* infection. Initially, TLR2 on KCs recognize bacteria to produce neutrophil chemoattractants and antimicrobial peptides, such as the cathelicidin LL-37 and defensins, which form pores in bacterial membranes (13). TLR2 is highly expressed on resident macrophages and recruited neutrophils and monocytes, which promptly respond to *S. aureus* and further stimulate cytokine production and phagocytosis. Therefore, it is expected that TLR2 is critical for both systemic and localized *S. aureus* infection. Mice deficient in TLR1, −2, −6 and MyD88 are highly susceptible to *S. aureus* infection in intranasal and intravenous infection as evidenced by increased bacterial load, poor inflammatory response and enhanced mortality or morbidity in various models of disease (14–18). In skin, the role of TLR2 is controversial. This may be due to differences in virulence of bacterial strains, infectious dose, and measured endpoints. While Miller et al., have demonstrated that TLR2 is dispensable to control *S. aureus* infection (17), Hoebe et al., have shown that TLR2-/- mice are more susceptible to infection (19). The strain used in the Hoebe et al. manuscript (ALC2906) shows higher lesion sizes and dermonecrosis, while the Xen 8.1 parental strain 8325-4 is less virulent *in vivo*. Distinct bacterial strains express unique virulence factors and toxins that could underlie different TLR2 requirements. Furthermore, the infection inoculum varies between these studies (2.5×10^6 vs. 10^5 CFU). If TLR2 is required for fine-tuning the immune response, higher amounts of bacteria (as used by Miller et al.) could override the TLR2 requirement to induce an efficient response, while lower doses of the bacterial could require TLR2 to mount a robust immune response.

The intracellular PRRs NOD1 and NOD2 also detect bacterial peptidoglycan to induce inflammation, antimicrobial peptide production, and phagocytic effector functions. NOD2 recognizes muramyl-dipeptide derived from *S. aureus* peptidoglycan. NOD2-deficient mice are highly susceptible to *S. aureus* skin and systemic infections when compared to WT counterparts (20–24). Finally, scavenger receptors CD36, SRBII and MARCO are required for optimal *S. aureus* skin host defense (25–27). Consequently, CD36-/- mice show increased bacterial loads and develop severe alpha-toxin-induced dermonecrosis (25).

Skin resident macrophages assist in the initial clearance of *S. aureus* and in conjunction with, e.g., perivascular macrophages, they regulate the recruitment of neutrophils and monocytes to the site of infection (28, 29). Dermal macrophages can phagocytose and kill *S. aureus* efficiently by producing reactive oxygen and nitrogen species, AMPs, and chelating proteins that starve bacteria of essential nutrients (9, 30). Furthermore, dermal macrophages secrete different chemoattractants that provide signals for neutrophil recruitment in a manner dependent on IL-1R and MyD88 (17). These cells are also involved in the clearance of dead cells at the site of infection, which is essential for resolution of disease (9, 30).

Once neutrophils arrive to the site of infection, they ingest *S. aureus* and attempt to control microbial growth by producing different antimicrobial effectors (see below) (9, 31, 32). Neutrophils are short-lived cells that readily undergo apoptosis and need to be cleared from the site of infection. However, *S. aureus* produces several toxins, such as alpha-toxin, γ-hemolysin, Panton–Valentine leukocidin (PVL), and phenol soluble modulins (PSMs), that

can hasten neutrophil cell death by inducing necrosis and leading to release of the danger associated molecular patterns (DAMPs) (6, 9, 31, 33–36). DAMPs released during *S. aureus* infection include IL-33, IL-1α, HGMB1, calprotectin and ATP (37–39). How these different modes of cell death lead to differential outcomes during infection is an active area of study.

Skin LCs and dermal DCs "sample" their surroundings, capturing antigens before traveling to skin-draining lymph nodes (28, 40). We and others have shown that during *S. aureus* infection of skin, LCs ingest the bacteria, are activated by PAMPs, and then migrate to draining lymph nodes, where LCs elicit *S. aureus*-specific adaptive responses (41, 42). Although there are several distinct DC subsets in the skin, their roles in *S. aureus* skin infection are not well understood (28).

Effector mechanisms of bacterial control

S. aureus can be ingested using receptors that recognize both opsonized and non-opsonized bacteria (9, 33, 43). When coated with opsonins (e.g. C3b and IgG), *S. aureus* elicits various antimicrobial effector functions (44). Reactive oxygen species (ROS) (such as O_2^-, H_2O_2 and HOCl) are produced following phagocytosis through the actions of NADPH oxidase and myeloperoxidase, and can directly kill bacteria or facilitate further killing by other mechanisms (45, 46). Nitric oxide (NO) is a major reactive nitrogen species (RNS) that is produced from nitric oxide synthase and has antimicrobial and immunomodulatory activity (47). Both genetic deletion and pharmacologic inhibition of NO formation render mice highly susceptible to *S. aureus* infection (48, 49). However, high concentrations of NO can exert anti-inflammatory effects. High NO levels may therefore predispose to infection by inhibiting cell proliferation, inducing host cell death, and preventing phagocyte-induced TNFα production and antigen presentation. Furthermore, *S. aureus* utilizes NO to proliferate and precludes induction of the stress regulon via lactic acid fermentation (50, 51).

Neutrophils kill pathogens by degranulation of toxic components (52). Degranulation induces the secretion of specific granules containing AMPs, including LL37 (human) and cathelicidin-related antimicrobial peptide (CRAMP, mouse homolog), and defensins. Degranulation also releases azurocidin, cathepsins, lactoferrin, lysozymes, proteinase-3, and elastase (53, 54).

As an additional effector mechanism to control *S. aureus* infection, neutrophils secrete DNA rich structures, termed neutrophil extracellular traps (NETs). NETs are produced in a MyD88- and TLR2-dependent mechanism and are necessary for containing *S. aureus* in the skin to prevent bacteremia (55) (Figure 1A, **middle)**. NETs limit the spread of pathogens, since they are rich in antimicrobial molecules such as AMPs, cathepsins, elastase, histones, and proteases (56). However, *S. aureus* can destroy NETs, and the degradation product 2'-deoxy-adenosine induces apoptosis in macrophages which increases bacterial survival in the abscess (57).

Immune mechanisms of abscess formation

Abscesses are the hallmark inflammatory lesions during *S. aureus* infection, and function to restrain and eliminate the pathogen (6, 9, 58). The abscess core contains fibrin, viable and

necrotic neutrophils, tissue debris, and live bacteria. Abscess maturation is accompanied by formation of a fibrous capsule at the periphery; however, if the abscess is not tightly organized, systemic spread of infection may occur via the bloodstream (6, 9, 58). Interestingly, macrophages are localized to the periphery of the abscess in areas near the fibrous capsule, which may suggest a role in neutrophil chemotaxis toward and egress from the abscess (6, 9).

The immune mechanisms involved in abscess formation are beginning to be uncovered. Cho et al. have shown that neutrophil-derived IL-1β is required for *S. aureus*-induced abscess formation (59). Recently, Feuerstein et al. suggested that resident macrophages expressing MyD88 contribute to abscess maturation (14). Our unpublished data show that the lipid mediator leukotriene B_4 (LTB_4) is essential for neutrophil direction to the infectious focus, microbial killing, and fibrous capsule formation (manuscript under review). Furthermore, an ointment containing LTB_4 increases *S. aureus* clearance and decreases lesion size. These findings correlate with neutrophil recruitment, abscess formation, ROS production, and IL-1β generation. Although there is much more to learn regarding the host-derived products that contribute to formation of abscess, a considerable amount of research has focused on the staphylococcal factors that promote survival within abscesses.

Among the *S. aureus* virulence factors involved in abscess formation, staphylocoagulase (Coa), von Willebrand factor binding protein (vWbp) and clumping factor A (ClfA) are all required for abscess formation. These proteins promote coagulation leading to fibrin generation and the formation of a pseudocapsule surrounding “staphylococcal abscess communities” within individual abscess lesions (6, 60).

Taken together, understanding the immune responses to *S. aureus* in skin, as well as host and bacterial mechanisms of abscess formation and survival, will aid in understanding the dynamics of staphylococcal pathogenesis and could lead to effective therapeutic strategies to prevent deeper infection (Figure 1A, **right)**.

Immune responses to *S. aureus* during skeletal infection

Osteomyelitis as a paradigm for invasive staphylococcal infection

Beyond skin infections, *S. aureus* has a remarkable ability to invade and proliferate within nearly every organ system. Of the many tissues that *S. aureus* is capable of colonizing, bone is one of the most frequently infected, and unfortunately, one of the most debilitating manifestations of disease.

S. aureus is by far the most common cause of osteomyelitis (61, 62). Treatment regimens include prolonged antimicrobial therapy in conjunction with surgery to remove infected or devitalized bone. These surgical procedures are necessary given that *S. aureus* triggers profound bone destruction, which is accompanied by a loss of vascular architecture, and thus decrease delivery of antimicrobials to the site of infection. *S. aureus* is also the most common cause of septic arthritis, which can trigger subchondral bone destruction or even frank osteomyelitis if contiguous spread occurs (63, 64). Osteomyelitis is therefore paradigmatic for invasive staphylococcal infections that are recalcitrant to treatment and

carry considerable morbidity. In the following sections, we detail advances in our understanding of the innate immune responses to *S. aureus* infection of bone.

Bone as a target tissue for *S. aureus* infection

Bone is a complex tissue consisting of a mineralized organic matrix that is constantly remodeled by the coordinated actions of osteoblasts, bone-forming cells, and osteoclasts, bone-resorbing cells. While osteoblasts differentiate from mesenchymal stem cells, osteoclasts develop from monocytic progenitors, providing an inherent link between innate immunity and bone remodeling. *S. aureus* is capable of colonizing skeletal tissues following hematogenous dissemination, via direct inoculation following trauma, or by spread of a contiguous infection. Upon colonization of bone, *S. aureus* is capable of establishing chronic infection, often surviving within traditional abscess lesions in the bone marrow, or invading directly into damaged bone through the network of osteocytic canaliculi (65). In addition to invading into healthy bone tissue, *S. aureus* can also invade and adhere to pieces of devitalized bone known as 'sequestra', creating a niche for chronic infection (Figure 2) (65). The mechanisms utilized by staphylococci to persist within bone are an area of ongoing investigation and are outside the scope of this review (66–70). However, the events leading to detection of invading staphylococci by the immune system in bone are poorly understood in comparison to studies in skin. Moreover, innate immune responses to bacterial pathogens in bone lead to profound effects on bone remodeling, which in turn influence the outcome of infection (66, 71–75).

Osteoimmunology: Reciprocal interactions between the skeleton and the immune system

The intricate cellular interactions that lead to bone remodeling took many decades to delineate and are still an active area of research. In the late 1980's, osteoblasts were linked to the regulation of osteoclastogenesis, even before the primary signals for osteoclastogenesis had been identified (76–78). M-CSF was identified as a critical factor supporting osteoclastogenesis, which was in keeping with the observation that osteoclasts arise from myeloid cells during co-culture experiments (79, 80). These early discoveries paved the way for the identification of a TNF-family cytokine, receptor activator of NFκB-ligand (RANKL), as the canonical osteoclast differentiation factor (81, 82), as well as the discovery of a related inhibitory molecule known as osteoprotegrin (OPG) (83, 84). Osteoblast-lineage cells produce both RANKL and OPG to maintain the balance between bone formation and resorption (81, 82, 85).

The field of "osteoimmunology" emerged from decades of work dating back to the 1970s, in which the effects of various immune cell-derived factors and cytokines on bone homeostasis were examined (86, 87). TNFα, IL-1, and IL-6 favor bone resorption by promoting osteoclast differentiation and function. Indeed, IL-1 was initially described as "osteoclast activating factor" due to its effects on bone (88, 89). IL-1, IL-6, and TNFα trigger osteoblast lineage cells to upregulate RANKL (90), while IL-1 and TNFα can also act on osteoclasts to promote differentiation, survival, and bone resorbing activity (91–93). However, both TNFα and IL-1 can only affect osteoclast precursors that have first been primed with RANKL (94,

95). Interestingly, bone remodeling mediated by TNFα is in part driven by its ability to alter osteoblastic expression of IL-1 and the IL-1R (96). In addition to these cytokines, T_H17 cells contribute to bone loss during arthritis, as IL-17 triggers RANKL production and osteoclastogenesis (97, 98). In contrast to IL-1, IL-6, TNFα, and IL-17, anti-inflammatory and T_H2 cytokines are largely anti-osteoclastogenic. IL-10 can signal directly onto pre-osteoclasts to suppress RANKL-induced transcription factors (99, 100). Similarly, IL-4 and IL-13 inhibit osteoblast proliferation, favor production of OPG, and decrease RANK expression on osteoclasts (101–104). Therefore, pro-inflammatory and anti-inflammatory cytokines have major impacts on osteoclastogenesis, with the major common mechanism being modulation of osteoblast-lineage RANKL production.

Bone cells as innate sensors of bacterial pathogens

S.aureus has an extraordinary virulence repertoire that facilitates binding to host tissues, subsequent tissue invasion, host cell death, and bacterial dissemination (105–108). Yet these virulence factors also serve as potent stimuli for activation of innate immune responses.

Staphylococcal adhesins allow binding to ECM components found in bone, including fibronectin and collagen (109). Select adhesins also promote endocytic uptake into non-professional phagocytic cells such as osteoblasts (109, 110). Once internalized, *S. aureus* can escape into the cytoplasm by lysing the endosome (111–114). This close association with bone cells triggers immune responses, as osteoblasts, osteoclasts, and their precursor cells express a repertoire of PRRs (115–120).

Depending on the cell type, PRR ligation has variable outcomes. PRR stimulation prevents myeloid precursor cells from subsequently becoming osteoclasts, but enhances RANKL-primed, pre-osteoclast differentiation (115). Additionally, osteoblast PRR activation leads to production of pro-osteoclastogenic cytokines, such as TNFα and RANKL, as well as other cytokines and AMPs (115, 121, 122). RANKL signaling on myeloid cells induces signaling cascades through TRAF6, NIK, IKK, p38, ERK, and JNK, activating non-canonical and canonical NFκB, AP-1, MITF, and NFATc1 transcription factors (123). These differentiation pathways overlap with immune-mediated signaling and provide potential for crosstalk downstream of immune activation. IL-1 cytokines also signal through TRAF6 to activate p38 MAPK, leading to enhanced osteoclastogenesis (96, 124). Taken together, the effect of TLR/IL-1R ligation on osteoclast differentiation is complex, but once cells are primed with RANKL, these stimuli appear to enhance osteoclastogenesis.

Specific PRRs on bone cells that sense *S. aureus* include TLR2 recognition of peptidoglycan and lipoteichoic acid (118, 125, 126), TLR9 endosomal recognition of bacterial DNA, and NOD-mediated recognition of cytoplasmic bacteria following escape from the endosome. Similar to the interactions with resident skin cells, *S. aureus* activates TLR2 on osteoblasts *in vitro*, leading to release of AMPs and cell death (121, 127). Once internalized, *S. aureus* in osteoblasts can be killed in the endosome through TLR9-mediated induction of oxidative stress, though not as robustly as professional phagocytes (128, 129). *S. aureus* also triggers expression of NOD2 by osteoblasts (130, 131), and cooperation between TLR2 and NOD2 induces RANKL production (116, 117, 132). Finally, the NLRP3 inflammasome can be

activated by *S. aureus* peptidoglycan and bone particles in myeloid cells (133, 134). Consequently, recognition of *S. aureus* by multiple PRRs on bone cells induces a robust inflammatory response and alters bone remodeling (Figure 1B). *S. aureus* recognition by PRRs such as TLR2 and NOD2 allows for shared innate mechanisms between resident skin and bone cells, emphasizing the importance of response to general bacterial motifs.

Deconvolution of the innate immune responses to *S. aureus* osteomyelitis using animal models

Animal models of osteomyelitis can be used to define critical immune responses leading to inflammation and alterations in bone remodeling (66, 68, 70, 71, 135–139). In a murine model of post-traumatic *S. aureus* osteomyelitis, Yoshii et al. found high levels of IL-1, IL-6, and TNFα in bone early after infection, with TNFα remaining elevated for the 28-day course of infection (140). The chemokines CCL3, CXCL2, and CCL2 have also been detected at high levels during osteomyelitis, and importantly, CCL3 and CXCL2 can trigger osteoclastogenesis and enhance bone loss (141, 142).

Downstream of PRRs, signaling through MyD88 is critical for osteoclastogenesis enhanced by PAMPs and IL-1 (115, 143). Just as MyD88/IL-1R are important in neutrophil recruitment and *S. aureus* clearance in skin infection models (17), these signaling pathways are also crucial for bacterial control on implants in a post-arthroplasty model of infection (144). Furthermore, IL-1R–deficient mice were found to have a higher frequency and severity of septic arthritis in a systemic *S. aureus* model (145). The role of TLR2 in *S. aureus* infection is largely dependent on the model system employed and the target tissue examined (see above). TLR2 enhances bone resorption in response to injection of heat-killed *S. aureus*, but not a lipoprotein-deficient strain (146). This supports a mechanism whereby TLR2 senses systemic bacterial components and can mediate changes in bone homeostasis. These studies corroborate that MyD88-dependent PRRs and cytokines are critical for bone remodeling and control of *S. aureus* infection.

S. aureus secreted virulence factors induce bone cell death and contribute to the pathogenesis of osteomyelitis

S. aureus pathogenesis is partially dependent on secreted virulence factors, including cytolytic toxins and proteins that modify immune functions. In experimental models of osteomyelitis, several *S. aureus* proteins impact bone architecture and contribute to comorbidities such as sepsis. Abscess formation in the bone marrow and around devitalized bone leads to a hypoxic environment, which subsequently alters quorum sensing and toxin production (67). PSMs mediate approximately 30% of the cortical bone loss observed in a murine model of osteomyelitis, with direct cytolytic effects on osteoblasts (66, 67). Bone destruction can also be triggered by the superantigen TSST-1 and staphylococcal protein A (Spa), which both activate osteoclast signaling to enhance bone resorption (75, 147, 148). PVL enhances early bacterial survival in bone and promotes bacterial spread to nearby muscles and joints in a rabbit model of osteomyelitis (149). Furthermore, alpha-hemolysin (Hla) contributes to severe sepsis-related mortality following osteomyelitis in rabbits (150).

In addition to their role in osteomyelitis, staphylococcal toxins significantly contribute to the pathogenesis of infection in other organ systems. For example, PSMs are small, amphipathic pore-forming toxins that are relatively promiscuous in their ability to induce toxicity among several cell types and species (67, 151). In the skin, PSMs stimulate keratinocytes to release proinflammatory cytokines (152). PVL contributes to staphylococcal skin disease by facilitating spread to neighboring muscle during skin infection (153). Taken together, these findings highlight the essential role of staphylococcal secreted virulence factors in disease pathogenesis, and highlight the broad tissue tropism of cytolytic toxins.

Limited but compelling evidence implicates the *S. aureus* toxin repertoire in disease severity during human infection. *S. aureus* strains expressing PVL are associated with more severe local disease and a greater systemic inflammatory response in children with osteomyelitis (154). Additionally, PVL has been shown to be mediate lysis of human myeloid cells, including osteoclasts, after binding the C5a receptor (147). Yet, the contribution of staphylococcal toxins regarding disease severity and pathogenesis varies based on the infection site and the repertoire of virulence factors expressed by the infecting *S. aureus* strains, which may not be fully assessed in experimental models.

Putting it all together: Staphylococcal immune response in humans

Individuals with diseases that impact innate immunity are at enhanced risk of staphylococcal infection. Genetic diseases that predispose individuals to *S. aureus* infections include chronic granulomatous disease (CGD) (155), deficiencies in MyD88 (156), IRAK-4 (157), TIRAP (158), and RAC2 (159), Wiskott-Aldrich Syndrome (159), leukocyte adhesion deficiency (160), severe congenital neutropenia (160), and allelic variants of cytokines IL-1α, IL-4, and IL-6 (161), among others. Increased risk of *S. aureus* infection has also been associated with co-morbidities such as diabetes (162, 163), malnutrition (164), bone marrow transplantation (165), and HIV infection (166). In general, these conditions are associated with extreme dysregulation of the immune response. While people with malnutrition (164, 167), newborns (168, 169) and bone-marrow transplant recipients (170) are functionally immunocompromised, subjects with uncontrolled diabetes (171–173), obesity (174, 175) and advancing age (176, 177) exhibit chronic low-grade inflammation and are also susceptible to infection. However, the common ground that favors *S. aureus* infection remains to be determined.

Remaining questions and future research

The innate immune response to *S. aureus* mediates infection outcomes and is dependent on host genetics and comorbidities, the tissue environment, and mechanisms of immune evasion by bacterial pathogens. Skin and bone cells participate in the induction of innate immunity and subsequent tissue remodeling events. Future research should therefore investigate how tissue resident cells instigate immune responses through the elaboration of cytokines, the recruitment of phagocytes, and the production of antimicrobial compounds. At the same time, these studies must address the consequences of immune activation on tissue homeostasis and remodeling, factors which play a large role in the morbidity of infectious diseases and the eventual recovery of a functional organ system. Specific questions remain

about the contribution of individual cell lineages to immunity in both skin and bone. Targeted inactivation of innate pathways in tissue resident cells using genetic tools such as CRISPR-Cas or Cre-lox technology will be necessary to study their contribution to anti-staphylococcal immunity *in vivo*. Additional areas of future research include the redundancy or compensation between PRRs, crosstalk downstream of common PRR and tissue-specific signaling pathways, and mechanisms of adaptive immunity that limit morbidity from primary innate immunodeficiency. Furthermore, the cellular and species tropism of secreted *S. aureus* virulence factors is worthy of ongoing investigation (178). The contribution of individual toxins to disease pathogenesis is controversial when considering data from different animal models. For example, PVL activity is restricted to the human and rabbit C5a receptor, thus the effects of this toxin cannot be elucidated using murine models (179). Similarly, other staphylococcal bi-component toxins have species-specific interactions with receptors, therefore not all animal models are appropriate to measure toxin effects (178). While innate immune responses are the first line of defense to prevent dissemination of *S. aureus*, these early events influence subsequent adaptive responses. A thorough understanding of immune protection from staphylococcal disease will therefore only result from study of both arms of the immune system.

Conclusions

In conclusion, innate immunity to *S. aureus* infection is multi-faceted and tissue specific. Decades of research on staphylococcal pathogenesis have elucidated important roles for key PRRs such as TLR2 and NOD2, as well as for specific cytokine signaling pathways such as IL-1. The roles of tissue resident cells in these signaling processes are beginning to be explored, and will be facilitated by new mammalian genetic tools. Understanding how innate immune responses impact tissue homeostasis is a critical future direction, given that tissue pathology is a significant driver of morbidity, mortality, and treatment failure. New therapies aimed at boosting innate immunity or blocking immunoevasive factors produced by *S. aureus* hold considerable progress as adjunctive therapies for the treatment of invasive infection (180–182).

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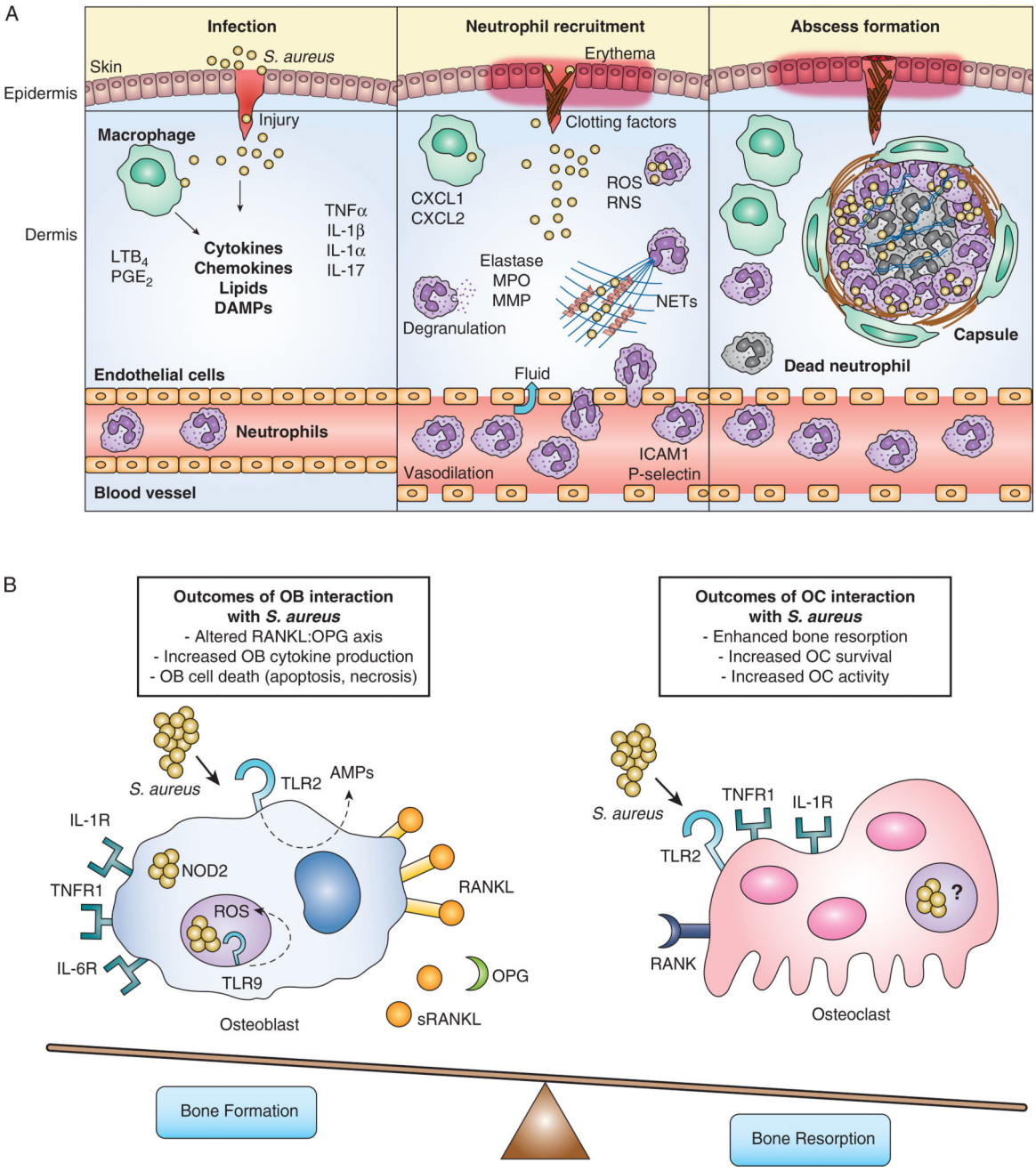

Figure 1. Innate immune responses to *S. aureus* during skin and bone infection

(A) Left panel: *S. aureus* infects skin after breaching the epithelial layers. Keratinocytes and skin-resident macrophages produce inflammatory mediators that promote neutrophil responses. Middle panel: Neutrophils are recruited to the skin where they phagocytose bacteria, undergo degranulation, and produce extracellular traps that aid in bacterial killing. Right panel: *S. aureus* infection is contained by abscess formation. Live and dead neutrophils and bacteria are found within the abscess. The abscess becomes encapsulated with fibrous material and surrounded by macrophages. **(B)** Bone remodeling activities of osteoblasts and osteoclasts are altered following interactions between innate immune

receptors and *S. aureus*. In osteoblasts, TLR2 recognition of extracellular *S. aureus* leads to production of AMPs, TLR9 detection of bacterial CpG DNA in the endosome induces an antibacterial ROS response, and NOD2 sensing of cytoplasmic *S. aureus* occurs following escape from the endosome. The culmination of osteoblastic innate recognition results in production of pro-inflammatory cytokines, such as TNFα, IL-1, and IL-6. These cytokines allow osteoblasts to favor increased production of RANKL and decreased release of the RANKL inhibitory cytokine OPG. The increased RANKL:OPG ratio and pro-inflammatory cytokine production have a net effect to enhance osteoclast differentiation. However, OB activation and the effects of staphylococcal toxins may also result in osteoblast cell death through apoptosis and necrosis. RANKL production allows for enhanced differentiation of osteoclast precursors. Pro-inflammatory cytokines such as TNFα and IL-1 can signal directly onto osteoclast precursors to increase osteoclast survival and bone resorption activity. Osteoclast expression and ligation of TLR2 has been shown to allow for the further differentiation down the osteoclast lineage, however this occurs only in cells that have first been stimulated with RANKL. Whether or not *S. aureus* can invade osteoclasts or activate endosomal or cytoplasmic PRRs remains to be determined.

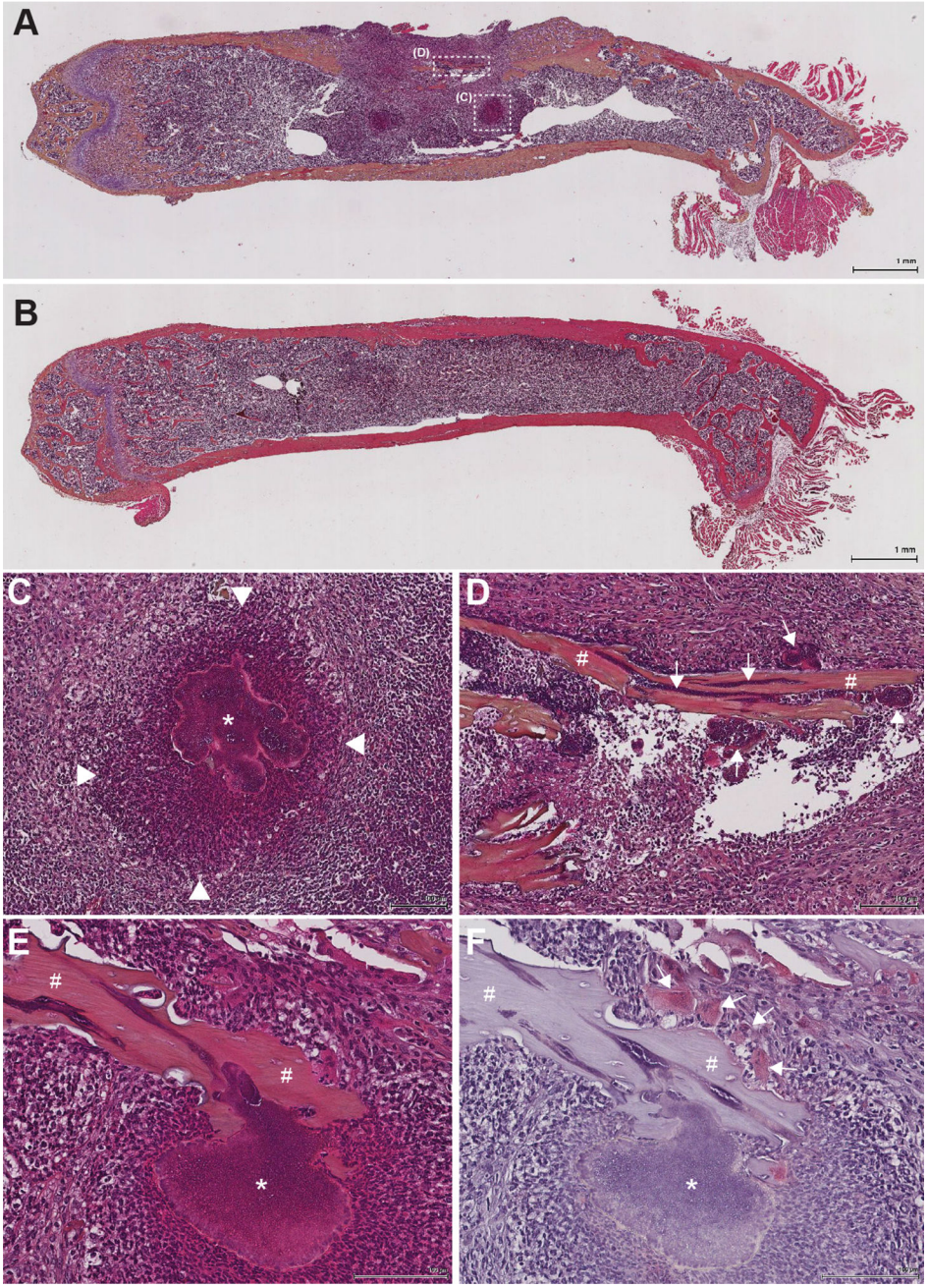

Figure 2. *S. aureus* forms traditional abscesses in bone marrow, but also grow directly on and invade into, living and dead bone fragments

Murine femurs were extracted, fixed in neutral buffered formalin, and dehydrated in 70% ethanol. Following decalcification in 20% EDTA pH 7.4, femurs were processed and embedded in paraffin. Femurs infected with *S. aureus* (**A**) or mock-infected with PBS (**B**) were sectioned and stained with a modified hematoxylin and eosin (H&E) stain prior to imaging at 1× magnification. Different abscess morphologies, including a traditional abscess (white box "C") in the bone marrow (**C**), and sequestra (white box "D") along cortical bone fragments (**D**) were observed in the *S. aureus* infected femurs upon imaging at 10×

magnification. Arrowheads in **C** denote the boundaries of the abscess' neutrophilic infiltrate. * denotes the staphylococcal abscess community surrounded by an eosinophilic pseudocapsule in the center of the abscess. # in **D** denotes a nonviable piece of cortical bone (sequestrum) with tightly adherent clusters of staphylococci (arrows) both on the surface of and within the sequestrum. **(E–F)** A second murine osteomyelitis sample was stained with both modified H&E (**E**) and tartrate-resistant acid phosphatase (mature osteoclast marker) (**F**) to demonstrate that *S. aureus* can also adhere to segments of living cortical bone (denoted by #), as osteoclasts (arrows) are visualized remodeling the same fragment of cortical bone. * denotes a large cluster of staphylococci directly adherent to the bone segment.