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IL-20 MODULATES INNATE IMMUNE RESPONSES OF HUMAN CELLS TO *STAPHYLOCOCCUS AUREUS*

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PORTIA GOUGH

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TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT

As a leading cause of infection worldwide, *Staphylococcus aureus* is a versatile commensal microbe that can coexist with its host harmoniously and undetected, but is capable of causing invasive and life threatening infections. Due to its unusually extensive arsenal of factors that serve invasion of host tissues and evasion of the immune system, effective treatment and prevention measures for *S. aureus* have remained largely elusive for researchers. Efforts to understand the immune response to *S. aureus* have yielded an amalgam of findings, but a common thread throughout is the importance of neutrophils: both for their robust bactericidal functions and their ability to damage host tissue.

Neutrophils possess multiple antimicrobial mechanisms that are critical for protection of the host against infection with *S. aureus*, as patients with defects in number or function of these cells are rendered vulnerable to staphylococcal infections. Recruitment and activation of neutrophils at sites of infection is driven by cytokine and chemokine signals that directly target neutrophils via specific cell surface receptors. The IL-20 subfamily of cytokines has been reported to act at epithelial sites and contribute to psoriasis, wound healing, and anti-inflammatory effects during cutaneous *S. aureus* infection. Adding to previous work that demonstrated the effects of IL-20 on human keratinocytes during infection with *S. aureus*, our results show that IL-20 is produced by human bronchial epithelial cells in response to *S. aureus*. Here, IL-20 modulated production of a key antimicrobial peptide and affected the pro-inflammatory signaling generated for recruitment of neutrophils. IL-20 and its related cytokines have been

studied almost exclusively for their effects on epithelial cell function; however, the ability of these cytokines to directly affect neutrophil function remains incompletely understood.

Here, we show that human neutrophils altered their expression of IL-20 receptor chains upon migration and activation *in vivo* and *in vitro*. Such activation of neutrophils under conditions mimicking infection with *S. aureus* conferred responsiveness to IL-20 that manifested as modification of actin polymerization and inhibition of a broad range of actin-dependent functions, including phagocytosis, granule exocytosis, and migration. Consistent with the previously described homeostatic and anti-inflammatory properties of IL-20 on epithelial cells, the current studies provide evidence that IL-20 directly targets and inhibits key inflammatory functions of neutrophils during infection with *S. aureus*.

INTRODUCTION

Staphylococcus aureus is a commensal species of human skin and anterior nares that is also capable of causing invasive infection of multiple sites in the body including the blood, in the form of sepsis, and most peripheral tissues, particularly the skin and lung (Daum and Spellberg, 2012; Lowy, 1998; Miller and Diep, 2008; Miller and Cho, 2011). In the United States, approximately 30% of people are colonized with *S. aureus*, and on an annual basis ~80,000 people are diagnosed and treated for invasive infections, making it one of the most prevalent infections (Brown et al., 2014; Dantes et al., 2013). Though these infections are common, options for treatment are diminishing, as antibiotic resistance is on the rise and no preventative vaccine is available (Daum, 2007; Fowler and Proctor, 2014; Spellberg and Daum, 2012). In the effort to expand the narrowing field of treatment and prevention options for this prevalent pathogen, understanding how the host resolves infection with *S. aureus* via a protective immune response is a critical component of addressing this increasingly urgent issue.

Neutrophils are essential for resolving infection with *S. aureus*, particularly infections at epithelial sites (Miller and Cho, 2011; Rigby and DeLeo, 2012). The indispensible nature of these cells can be noted in the significantly increased incidence, recurrence and severity of *S. aureus* infections, particularly of the skin and lungs, in patients that have disorders of neutrophil number or function, such as chronic granulomatous disease or neutropenia (Winkelstein et al., 2000). Considerable focus has been placed on the bactericidal functions of neutrophils, as they are able to rapidly

migrate to a site of infection and phagocytose a foreign particle in seconds. In their granules, they are equipped with pre-formed proteins, including subunits of NADPH oxidase, proteases and anti-microbial peptides, that aid in their robust bactericidal activity (Amulic et al., 2012; Nathan, 2006; Nauseef and Borregaard, 2014). More recently, other aspects of neutrophil function have come into focus, as they are able to integrate multiple signals at the site of infection and adjust their function accordingly. They are also able to signal to other populations of leukocytes, playing a key role in shaping the immune response (Mantovani et al., 2011; Scapini and Cassatella, 2014; van Rees et al., 2016). Although neutrophils are central to controlling and resolving infections with *S. aureus*, the nuances of their function, particularly how they adjust their function in response to host signaling at the site of infection, remain incompletely understood.

Epithelial Infection with *S. aureus*: Cell Signaling in Pursuit of Protection

Skin and soft tissue infections are the most frequent manifestation of disease caused by *S. aureus*, and several host factors comprise the protective immune response at this site (Figure 1) (Miller and Cho, 2011; Parker and Prince, 2012). When staphylococci breach the epithelial barrier of the corneal layer of the skin and reach the epidermis, they are detected via binding of the cell wall component lipoteichoic acid (LTA) by TLR2 on keratinocytes or resident dendritic cells and macrophages in the dermis (Bardoel and Strijp, 2011; Fournier, 2012; Peres and Madrenas, 2013). Subsequent signaling by TLR2 via MyD88 initiates the pro-inflammatory response by activating the release and production of IL-1α from keratinocytes and IL-1β by the

resident leukocytes (Cho et al., 2012; Miller et al., 2006). IL-1α and IL-1β then induce expression of antimicrobial peptides (AMPs) from keratinocytes, including human β-defensin 3 (hBD-3), which is known to have direct bactericidal activity towards *S. aureus* (Ryu et al., 2014; Schauber and Gallo, 2008). The IL-1 cytokines also induce production of downstream pro-inflammatory factors, including TNFα and IL-6, and chemokines CXCL1, CXCL2 and CXCL8 (IL-8), which recruit neutrophils and other leukocytes to the site of infection (Abtin et al., 2014; Miller et al., 2007). Additionally, IL-17 is produced mostly by γδ T cells in the dermis, and has a similar effect as IL-1β (Cho et al., 2010; Maher et al., 2013). Thus, early signaling in the skin in response to infection with *S. aureus* is geared towards signaling that generates factors that can directly inhibit bacterial growth, in the form of AMPs, and signals that recruit neutrophils to kill *S. aureus*.

Cytokine and chemokine signaling in the lung in response to *S. aureus* infection is similar to that in the skin. *S. aureus* LTA is detected by TLR2, which again initiates production of IL-1α and IL-1β to drive production of AMPs, including hBD-3 and RegIIIγ, and other cytokines, and chemokines that recruit neutrophils from circulation to the site of infection to kill the invading staphylococci (Parker and Prince, 2012). Importantly, IL-1β induces the production of IL-17A in the lungs as well, and again this signaling is critical to generating a protective immune response that recruits neutrophils in sufficient quantity to control and clear *S. aureus* (Hoogerwerf et al., 2008; von Aulock et al., 2003).

IL-17 has a clear role in the protective immune response to *S. aureus* infection of the skin and lung (Cho et al., 2010; Kao et al., 2004). Hyper-IgE syndrome (HIES)

patients have a deficiency in IL-17 production, due to a lack of functioning T_H17 cells, and suffer from recurrent *S.aureus* infections of the skin and lung (Freeman and Holland, 2008). Using an *in vitro* model of infection it was shown that among the human epithelial cell types, keratinocytes and bronchial epithelial uniquely require IL-17, along with IL-1α and IL-1β, to elicit a pro-inflammatory response (Minegishi et al., 2009). Mice deficient in IL-17 are also more susceptible to cutaneous infection with *S. aureus*, but not to bacteremia caused by this pathogen (Ishigame et al., 2009). Protective immune responses of the skin and lung share major similarities, where a particular set of signals are required to act in concert to generate the appropriate signals and AMPs to protect the host and resolve infection.

IL-20: From Sterile Inflammation to Infection

IL-20 is a recent addition to the host signals that play a role in the inflammatory response during infection with *S. aureus* (Figure 1) (Myles et al., 2013). A member of the IL-20 subfamily of cytokines, which includes IL-19, IL-20, IL-22, IL-24 and IL-26, are a subclass of the IL-10 family of cytokines (Ouyang et al., 2011; Sabat, 2010). IL-10 is a potent anti-inflammatory cytokine, which is produced by leukocytes and signals to these cells via the IL-10 receptor to facilitate the resolution of inflammation (Fiorentino et al., 1989; Moore et al., 2001). IL-19, IL-20 and IL-24 are closely related to IL-10: these cytokines share a chromosomal locus and appear to be the result of a recent gene duplication, sharing amino acid sequence and structural homology with IL-10 (Blumberg et al., 2001; Logsdon et al., 2012). The other members of the subfamily, IL-22 and IL-26, are located elsewhere in the genome (Commins et al., 2008). Shared receptor

complexes further link IL-19, IL-20 and IL-24 together, as they signal through heterodimeric Type I or Type II IL-20 receptors, comprised of IL-20RA or IL-22RA, respectively, paired with IL-20RB (Blumberg et al., 2001; Logsdon et al., 2012).

IL-20 receptor expression has been primarily characterized in epithelial cells of the skin and lung (Parrish-Novak et al., 2002). IL-20 and its receptors are more highly expressed in psoriatic skin compared to normal skin, although its precise role (pro- vs anti-inflammatory) in this condition has not been fully characterized (Otkjaer et al., 2005). Additionally, this cytokine and its receptor are highly expressed in the synovial membranes and fluids of rheumatoid arthritis patients compared to healthy individuals (Hsu and Chang, 2015; Hsu et al., 2006). The cellular sources of IL-20 in psoriasis and rheumatoid arthritis are keratinocytes and synovial fibroblasts, respectively, and it is thought to engage an autocrine loop that activates production of pro-inflammatory factors such as IL-8 to recruit neutrophils and other leukocytes to the area (Hsu et al., 2006; Otkjaer et al., 2005). From this evidence, IL-20 was thought to have a pro-inflammatory role in these autoimmune disorders involving sterile inflammation. However, these immune modulatory effects could be a result the known wound healing and epithelial proliferation functions of IL-20. Again, the exact mechanism of how IL-20 contributes to these sterile inflammatory environments remains unknown.

A solely pro-inflammatory role for IL-20 is unexpected, given the close relationship of IL-20 to the canonical anti-inflammatory cytokine, IL-10. As described previously, infection of the skin and lung with *S. aureus* induces a robust and complex inflammatory response involving the production of multiple cytokines and chemokines to orchestrate a protective immune response that attracts the appropriate leukocyte

populations and modulates their function to allow removal of the invasive staphylococci. It was recently discovered, using knockout mice for IL-20RB, that IL-20 receptor signaling has an anti-inflammatory role during cutaneous infection with *S. aureus*. In the absence of IL-20 receptors, there was enhanced production of IL-1β and IL-17A, which increased the neutrophils recruited to the site of infection and resulted in significantly smaller lesion size and reduced CFUs (Myles et al., 2013). IL-20 subfamily cytokines were detected in both the cutaneous mouse model of infection and human keratinocytes infected *in vitro.* Mice preferentially expressed IL-19, while IL-20 was the most highly produced by human keratinocytes. Addition of recombinant IL-20 to human keratinocytes *in vitro* significantly inhibited the production of IL-1β as observed in the murine model (Myles et al., 2013). The inhibition of IL-1β by IL-20 receptor signaling indicated that IL-20 subfamily cytokines act as anti-inflammatory factors during infection with *S. aureus*, in a manner that contributes to the pathogenesis of infection by reducing the number of neutrophils recruited to infected lesions.

The function of IL-20 and its receptors have primarily been studied in the context of the aforementioned autoimmune disorders. And, the majority of studies evaluating IL-20 receptor expression have been limited to epithelial cells (Blumberg et al., 2001; Parrish-Novak et al., 2002). The expression of IL-20 receptors by leukocytes has mostly been evaluated on resting cells, namely B cells, T cells, monocytes and NK cells, and shown that there is some low constitutive expression of IL-20RB (Wolk et al., 2002). However, there has been investigation of expression of IL-20 receptors by cells obtained from the synovial fluid of rheumatoid arthritis patients. These cells are mostly neutrophils that have migrated in response to inflammation, and they have elevated

expression of IL-20 receptor chains (Hsu et al., 2006; Kragstrup et al., 2015). This indicates that the expression of these receptor chains on neutrophils may change in response to activation. And, given the expression of IL-20 by keratinocytes during infection with *S. aureus* and its demonstrated effects on the outcome of infection in the cutaneous model of infection in mice, it appeared that IL-20 may directly affect neutrophils.

Neutrophil Recruitment during Infection: Heeding the Call

Pro-inflammatory signaling serves to recruit neutrophils from circulation and activate their activities. During infection with *S. aureus,* this process is mediated by several cytokines, including IL-1β, IL-17, IL-6 and TNFα, which activate the production of chemokines, such as IL-8, that guide neutrophils to the site of infection (Figure 1) (Bardoel and Strijp, 2011; Fournier and Philpott, 2005). This signaling enhances the expression of adhesion molecules on the nearby endothelium, P- and E-selectin and intercellular adhesion molecule-1 (ICAM-1), allowing neutrophils passing by in circulation to roll along and adhere to the endothelium (de Oliveira et al., 2016; Sadik et al., 2011). The neutrophils are then able to extravasate from the endothelium into the peripheral tissue, and are directed to the site of infection by a chemotactic gradient, traveling along an increasing concentration of chemokines and bacterial products (Ley et al., 2007).

Neutrophils migrate rapidly, and this is facilitated by an intricate choreography of changes to the cytoskeleton, including changes to the quantity and location of polymerized actin to create a leading edge that forms a pseudopod to pull the cell along

a gradient (Weiner et al., 1999). Once it has arrived at the site of infection, the neutrophil must arrest migration, a process that requires the integration of multiple signals that indicate that the target site has been reached (Heit et al., 2002; Liu et al., 2012). In the process of migrating to the site of infection, neutrophils go from their resting state, as they are in circulation, to a primed state in which, once fully activated, they have enhanced antimicrobial functions (Daniels et al., 1992; Sullivan et al., 1989). Importantly, the primed neutrophil also has upregulated expression of receptors for various cytokines and other signals that are at the site of infection, allowing it to respond to the complex environmental cues generated by the host (Figure 1) (Berger et al., 1984; Detmers et al., 1990).

The changes that neutrophils undergo during migration and encountering pro-inflammatory factors *in vivo* can be mimicked *in vitro* (Figure 2). This requires the use of a binary signal, in the form engagement of integrin receptors and pro-inflammatory cytokine, such as TNFα (Dapino et al., 1993; Nathan and Sanchez, 1990). Neutrophils used in this work were subjected to this form of priming stimulation to replicate conditions where they encounter *S. aureus in vivo*. Additionally, *in vitro* migration, induced by bacterial products, host signaling or a combination of the two, has an activating effect on neutrophils that induces detectable changes to their receptor expression. This strategy was also employed in this work, again to observe neutrophil responses in conditions relevant to infection (Figure 2). Ultimately, whether *in vivo* or *in vitro*, the processes of migrating from circulation and encountering pro-inflammatory factors results in neutrophils that have undergone changes that allow them to fulfill their

functions at the site of infection: bacterial killing, and responding and signaling to other host cells.

Neutrophil Bactericidal Activity: Protection through Destruction

Once they encounter *S. aureus*, neutrophils have multiple mechanisms, both intracellular and extracellular, at their disposal to eliminate the pathogen. Neutrophils are professional phagocytes that can rapidly take up particles (in less than 20 seconds) opsonized by host antibodies or complement (Nordenfelt and Tapper, 2011; Segal, 2005; Segal et al., 1980). They can also recognize microbes directly via their array of pattern recognition receptors (PRRs) and internalize without opsonization (Ofek et al., 1995). Newly formed phagosomes in neutrophils undergo a maturation process distinct from the traditional endosomal pathway. Rather, their preformed granules fuse with the nascent compartment to release antimicrobial factors (defensins and proteases) and NADPH oxidase subunits are incorporated into the phagosomal membrane (Figure 3) (Johansson et al., 1995; Karlsson and Dahlgren, 2002; Lee et al., 2003). The preformed proteins in neutrophil granules allow them to have rapid responses to microbial challenges, and granule fusion is central to killing staphylococci in the phagosome, as it allows multiple NADPH oxidase to reach the phagosomal membrane (Karlsson and Dahlgren, 2002; Segal, 2005). The multiple NADPH oxidase generate a higher concentration of ROS compared to other phagocytes, and this robust ROS generation is a major factor in neutrophil bactericidal activities and makes them comparatively well-suited to kill *S. aureus*, a microbe that is resistant to ROS (Jann et al., 2011; Liu et al., 2005; McGovern et al., 2011).

Granules not only fuse with the phagosome, they are also released into the extracellular space (Figure 3) (Sengelov et al., 1995; Sengelov et al., 1993). There are three types of granules, distinguished by their generation during neutrophil maturation and their corresponding protein contents (Borregaard et al., 2007; Le Cabec et al., 1996). Primary (azurophilic) and secondary (specific) granules are peroxidase positive, as they harbor NADPH oxidase components; additionally, primary granules contain the most potent antimicrobial factors such as lytic enzymes and α-defensins, and secondary granules have additional antimicrobial factors, some of which protect the host via nutrient sequestration, such as lactoferrin (Bainton and Farquhar, 1968). Tertiary, or gelatinase, are peroxidase negative granules that contain proteins that degrade collagen to facilitate neutrophil extravasation from circulation, as well as membrane proteins that aid in migration (Kjeldsen et al., 1993; Kjeldsen et al., 1992). These granule populations differ not only in their contents, but also the timing and targeting of their release (Sheshachalam et al., 2014).

Because granules contain high concentrations of proteins that are potentially damaging to host tissues, their release must be tightly controlled. Degranulation occurs in a hierarchical manner, as each granule population is released to first facilitate migration, with tertiary granule exocytosis, and then bacterial killing, with secondary and then primary granule exocytosis or phagosomal fusion (Lacy and Eitzen, 2008). This stepwise release process is controlled in part through the differential associations of each granule population with actin, and the actin cytoskeleton serves to regulate exocytosis by limiting granule access to the plasma membrane (Jog et al., 2007). Tertiary granules are the most readily released, and are the most highly associated with

actin compared to primary and secondary granules, which are more resistant to release upon neutrophil activation and have little to no association with actin (Brumell et al., 1995; Jog et al., 2007). Little is known about the mechanisms that dictate the hierarchical release of neutrophil granules, but the differential association with actin is thought to play a role (Jog et al., 2007). Primary and secondary granules appear to be more reliant on microtubule dynamics for recruitment to their target membrane to allow subsequent release (Lacy and Eitzen, 2008). To prevent aberrant release of granules, exocytosis is activated by external cues, releasing only as the neutrophil becomes adherent during recruitment and encounters a pathogen or host pro-inflammatory factors.

The potent antimicrobial proteins, NADPH oxidase components and proteases that neutrophils carry in their granules allow for rapid deployment of killing mechanisms when they encounter *S. aureus*. To further enhance the effectiveness of the bactericidal factors in their granules, neutrophils are capable of undergoing a specialized form of cell death, NETosis, to form neutrophil extracellular traps (NETs), which have demonstrated ability to kill *S. aureus* (Brinkmann et al., 2004). This process involves the translocation of neutrophil elastase from primary granules to the nucleus, where it degrades specific histones. The resulting chromatin is then released from the cell along with the neutrophil elastase and MPO in a process distinct from lysis, that leaves the plasma membrane intact, and forms an extracellular trap that restricts the dissemination of bacteria from the site of infection and creates an area of concentrated antimicrobial factors from neutrophil granules (Brinkmann and Zychlinsky, 2007, 2012). In a mouse model of skin

infection with *S. aureus* using *in vivo* imaging, this process has been shown to occur along with phagocytosis to effectively limit its dissemination (Yipp et al., 2012).

There is an interplay between the multiple bactericidal mechanisms that neutrophils can employ during their response to infection with *S. aureus*. Migration to the site of infection, ROS production upon phagocytic uptake, and NETosis are all linked to granule exocytosis, as these processes rely on proteins contained within the granules. Detection of both host signals and staphylococcal components determine the timing and magnitude of the engagement of granule exocytosis and the associated bactericidal mechanisms, allowing the neutrophil to perform its destructive functions while limiting damage to host tissue at the site of infection.

Neutrophil Crosstalk: No Cell is an Island

Neutrophil activation extends beyond the neutrophil itself and its killing of *S. aureus*, as their bactericidal functions, cytokine release and production and their death all serve as signals to other leukocytes. They are the most numerous population at the site of infection, making them a major source of cytokines that signal to orchestrate the immune response. Macrophages, dendritic cells (DCs), NK cells, B cells and T cells are all targets of signaling from neutrophil derived cytokines, mostly stored in secretory vesicles, during infection (Figure 3) (Mocsai, 2013; Nathan, 2006; Scapini and Cassatella, 2014).

Neutrophil derived chemokines recruit monocytes and DCs to the site of infection, and affect the survival and maturation of these cells. For example, IL-18 from neutrophils activates NK cells to produce IFNγ and engage their cytotoxic activity

against intracellular pathogens (Sporri et al., 2008). The importance of neutrophil signaling for adaptive immune responses has been observed in human patients with defects in neutrophil function or development, who have normal B cells but strongly reduced levels of marginal zone B cells and an associated reduction in antibodies against T cell independent antigens. From observations of these patients and in other studies, it was found that neutrophils facilitate marginal zone B cell maturation during infection through their migration to the spleen and their production of BAFF (Puga et al., 2011). Therefore, neutrophils not only respond to signals from other cells, they are also a source of signaling that organizes both innate and adaptive immune responses.

The bactericidal activities and apoptosis of neutrophils also serve to modulate responses of other cells at the site of infection. NETs and MPO from neutrophils have an inhibitory effect on DCs and cause them to promote Th2 type T cell responses. ROS and granule products released by neutrophils regulate the activities of NK cells and γδ T cells (Costantini and Cassatella, 2011; Mantovani et al., 2011). Neutrophil apoptosis is a major anti-inflammatory signal at the site of infection. As they undergo this non-lytic form of cell death, neutrophils express phosphatidyl serine on their surface, signaling to macrophages to efferocytose them (El Kebir and Filep, 2013; Jones et al., 2016). Efferocytosis of apoptotic neutrophils initiates an anti-inflammatory response that includes the production of pro-resolving lipid mediators, resolvins and protectins, which inhibit further neutrophil recruitment, promote neutrophil apoptosis and reduce vascular permeability (Buckley et al., 2014; Ferracini et al., 2013). The effects of neutrophil activities extend far beyond the killing of *S. aureus* and other microbes, as the products of their bactericidal activities and their release and production of cytokines and

chemokines communicate to and shape the responses of other leukocytes during infection.

Multiple factors determine the outcome of epithelial infections with *S. aureus*: whether the microbe is cleared from its invasive position, or the infection progresses to an expanded area or other tissues. The first of these is the microbe itself, *S. aureus* is armed with a multitude of virulence factors including toxins and effectors that allow it to evade detection and destruction during the host immune response (Rigby and DeLeo, 2012). An additional factor is the infected epithelium itself, here the pathogen is detected and the resident cells produce factors to directly protect the tissue, such as anti-microbial peptides, and cytokines and chemokines to recruit and direct the actions of immune cells (Menzies and Kenoyer, 2005; Miller and Cho, 2011). Finally, the leukocytes themselves that are recruited to kill the pathogen and/or protect host tissue from invasion. In this process, neutrophils are the most abundant of these immune cells, and they must integrate multiple signals to preserve host tissues while simultaneously performing their destructive function of microbe killing. This work focuses on how signaling from the host epithelium in response to *S. aureus*, in the form of IL-20 acting in concert with other host factors, affects the epithelium itself and the multiple functions of neutrophils during staphylococcal infection.

CHAPTER I

IL-20 MEDIATES RESPONSES OF HUMAN BRONCHIAL EPITHELIAL CELLS DURING *IN VITRO* INFECTION WITH *STAPHYLOCOCCUS AUREUS*

Introduction

Staphylococcus aureus can persist in the respiratory tract in asymptomatic colonization or it can cause a range of infections, the most severe being necrotizing pneumonia (Parker and Prince, 2012). The most virulent strains of *S. aureus*, such as USA300, cause a relatively more exuberant pro-inflammatory response compared to other strains, contributing to the high mortality associated with them (DeLeo et al., 2010; Montgomery et al., 2008). Although neutrophils are required for clearance of *S. aureus* from peripheral tissues, excessive amounts of neutrophils and their bactericidal factors present during such heightened inflammatory responses can cause damage to host tissues (Diep et al., 2010); thus, the intensity of the immune response to *S. aureus* contributes heavily to the outcome of infection.

Once *S. aureus* invades the airways, initial detection of staphylococcal lipoteichoic acid occurs via TLR2 expressed on bronchial cells of the airway epithelium (Hoogerwerf et al., 2008). Activation of signaling through TLR2 induces production of IL-1β and other factors that then activate dendritic cells and macrophages, initiating a cascade that leads to production of GM-CSF, TNFα and IL-8 to recruit neutrophils (Parker and Prince, 2012; Sorrentino et al., 2008). IL-17 production is also a critical

aspect of a protective immune response that contributes to neutrophil recruitment during infection with *S. aureus,* and its importance has been demonstrated in multiple contexts.

During co-infections with influenza and *S. aureus*, there is increased pathology and mortality due to *S. aureus* caused by an influenza-mediated impaired IL-17 response (Kudva et al., 2011; Lee et al., 2010; Small et al., 2010). Additionally, Hyper IgE Syndrome (HIES) patients have increased incidence of and susceptibility to infection of the skin and lungs with *S. aureus* because they lack Th17 cells, a major source of IL-17 during staphylococcal infection (Freeman and Holland, 2008; Ma et al., 2008). This requirement for IL-17 is a shared aspect of protective immune responses to *S. aureus* infection in the skin and lung, as signaling by IL-17 facilitates downstream signaling from resident cells in these tissues to allow sufficient neutrophil recruitment for clearance of staphylococci (Minegishi et al., 2009).

The pro-inflammatory response mediated by cytokine and chemokine signaling from host epithelial cells plays a central role in activating an appropriate immune response to *S. aureus*. A recent addition to this increasingly complex signaling milieu is IL-20, a member of the IL-20 subfamily of cytokines that also includes IL-19 and IL-24, all of which signal through Type I or Type II IL-20 receptors (Blumberg et al., 2001). It was previously found that, during *in vitro* challenge with *S. aureus* USA300, primary human keratinocytes produced IL-20 and addition of exogenous IL-20 inhibited the production of IL-1β by these cells (Myles et al., 2013). This corresponded to observed affects of signaling through IL-20 receptors on IL-1β production in a cutaneous mouse model of *S. aureus* infection; however, the dominant cytokines signaling through IL-20 receptors during infection in mice were IL-19 and IL-24 (Myles et al., 2013). Inhibition of

IL-1β production by signaling through IL-20 receptors led to a reduction in IL-17 in lesions in infected mice, and contributed to pathology of infection with increased CFUs and lesion sizes in wild-type mice, compared to IL-20 receptor knockout mice (Myles et al., 2013). Because IL-20 receptor signaling decreased the production of these key pro-inflammatory cytokines by infected tissue or keratinocytes in pure culture, signaling through this receptor was concluded to have an anti-inflammatory role during *S. aureus* infection of the skin (Kaplan, 2013; Myles et al., 2013).

The IL-20 receptors and IL-20 itself are also expressed in human lung tissue (Hsing et al., 2006; Parrish-Novak et al., 2002). In addition to its anti-inflammatory role in the skin during infection, IL-20 also plays a role in antimicrobial peptide (AMP) production (Sabat, 2010; Wegenka, 2010). AMPs provide important protection to the lung epithelium during *S. aureus* infection. In particular, RegIIIγ has direct bacteriostatic and bactericidal activity against *S. aureus*, demonstrated both *in vivo* in a murine pneumonia model and *in vitro* using purified RegIIIγ (Choi et al., 2013; Lehotzky et al., 2010). Antibody blockade of RegIIIγ in mice intranasally infected with *S. aureus* USA300 resulted in significantly increased CFUs recovered from the lungs, and administration of recombinant RegIIIγ to mice deficient in its production rescued their defect in clearance of *S. aureus* from the lung (Choi et al., 2013). Production of RegIIIγ in the lung is initiated via activation of STAT3, a known pathway activated in epithelial cells by the IL-20 receptors (Dumoutier et al., 2001; Xu et al., 2007). Given the parallels between cytokine-mediated immune responses of the skin and the lung, and the expression of IL-20 receptors in lung epithelial cells where STAT3 mediated RegIIIγ production in response to *S. aureus* infection is protective, we investigated whether IL-20 modulates

cytokine or AMP expression in human bronchial epithelial cells during *in vitro* challenge with *S. aureus*.

Results

BEAS2b cells express an IL-20 receptor and produce IL-20 in response to *S. aureus*

The human bronchial epithelial cell line BEAS2b was selected to evaluate how IL-20 mediates human airway epithelial cell responses to *S. aureus*, as it is a cell line derived from immortalized healthy bronchial cells that maintain most morphological features of freshly obtained primary bronchial epithelial cells (Ke et al., 1988; Kinnula et al., 1994). BEAS2b cells were evaluated for both Type I and Type II IL-20 receptor expression by immunofluorescence microscopy. IL-20 receptors are heterodimers of IL-20RB paired with either IL-20RA or IL-22RA for Type I or Type II receptors, respectively (Blumberg et al., 2001; Wegenka, 2010). Based on microscopy of unstimulated BEAS2b cells for all three receptor chains, these cells express IL-22RA and IL-20RB, indicating that they express the Type II, but not Type I, IL-20 receptor (Figure 4A). IL-20 has been reported to signal through STAT3 in epithelial cells via both Type I and Type II receptors (Blumberg et al., 2001; Dumoutier et al., 2001). IL-20 mediated activation of this pathway via the IL-20 receptor expressed by these cells was confirmed by western blot of lysates from BEAS2b cells that were treated with recombinant human IL-20 (rhIL-20) in the presence or absence of antibody blockade of IL-20RB (Figure 4B). Dose-dependent STAT3 activation was observed in cells incubated with rhIL-20, and this signaling was blocked by addition of an anti-IL-20RB antibody (Figure 4B). Taken

together, these data show that BEAS2b cells express an IL-20 receptor that is activated by IL-20 and signals via STAT3.

As stated previously, primary human keratinocytes were shown to produce IL-20 in response to *S. aureus* USA300, and addition of rhIL-20 with *S. aureus* had an anti-inflammatory effect by inhibiting IL-1β production (Myles et al., 2013). Given the similarities between cytokine responses to *S. aureus* in the skin and lung (Minegishi et al., 2009), BEAS2b cells were evaluated for their production of select pro-inflammatory and IL-20 subfamily cytokines; and, a murine pneumonia model was used to evaluate IL-20 subfamily cytokine expression *in vivo* in the lungs of mice (Figure 5). *S. aureus* USA300 was added at a MOI of 1 to BEAS2b cells cultured on collagen coated flasks, and cytokine expression at the transcript and protein levels were examined at two and six hours, respectively. Comparing uninfected BEAS2b cells to those incubated with *S. aureus* USA300, there was the expected induction of the pro-inflammatory factors IL-1β and IL-8 by *S. aureus* at the transcript level (Figure 5A), and high levels of IL-8 were detected in the supernatants of infected cells (Figure 5B). Infection with *S. aureus* also strongly induced the production of IL-20 transcripts (Figure 5A), and IL-20 was also detected in the supernatants of BEAS2b cells that were infected with *S. aureus* (Figure 5B). IL-19 was also detectable, but at approximately ten fold lower levels than IL-20 at both the transcript and protein levels (Figure 5A and 5B). Also, BEAS2b cells do not appear to express a Type I IL-20 receptor, which is required for binding IL-19, making it unlikely that IL-19 acts on these airway epithelial cells (Dumoutier et al., 2001; Logsdon et al., 2012; Parrish-Novak et al., 2002).

In the murine pneumonia model, mice expressed IL-19 and IL-24 by 6 h p.i. but no IL-20 was detected in murine lung tissue at either 6 or 48 h p.i. (Figure 5C). This difference in IL-20 subfamily cytokine expression in response to *S. aureus* infection is similar to previously reported findings in the skin, where mice also expressed IL-19 and IL-24, but human keratinocytes expressed mainly IL-20 (Myles et al., 2013). Evaluation of cytokine production by BEAS2b cells infected with *S. aureus* showed that these cells have the expected pro-inflammatory cytokine response of epithelial cells and that, like primary human keratinocytes, they produce IL-20 in response to infection with *S. aureus* USA300.

IL-20 receptor signaling modulates IL-8 and RegIIIγ production by BEAS2b cells during infection with *S. aureus*

To determine the effects of IL-20 on their pro-inflammatory signaling during infection with *S. aureus*, BEAS2b cells were pre-incubated with an anti-IL-20RB antibody to block signaling by IL-20. When signaling through the IL-20 receptor was blocked, there was a significant increase in IL-8 detected in BEAS2b cell supernatant at 4 hours post-infection with *S. aureus* USA300 at MOI 1 (Figure 6A). Because IL-8 is a major signal that stimulates neutrophil recruitment to a site of infection, neutrophil migration in the presence or absence of IL-20 receptor blockade was evaluated using a transwell migration assay in which human neutrophils were added to transwell inserts in 24 well plates containing BEAS2b cells at 2 hours post-infection (p.i.). Neutrophils were recovered from the lower chamber containing the infected epithelial cells after being allowed to migrate from the transwell insert for three hours and enumerated by counting

in a hemacytometer. Similar to the previously reported effects of IL-20 receptor signaling seen in cutaneous infection in mice (Myles et al., 2013), blockade of IL-20 receptor signaling *in vitro* enhanced neutrophil migration to infected BEAS2b cells (Figure 6B).

IL-20 has been shown to modulate the production of AMPs in human keratinocytes and reconstituted epidermis, where it enhances the production of β-defensins (Sa et al., 2007). Because of its dependence on STAT3 activation for its production and its demonstrated anti-staphylococcal activity (Choi et al., 2013), RegIIIγ was evaluated in the supernatants of BEAS2b cells that were infected with *S. aureus* as described previously. RegIIIγ was produced by BEAS2b cells in response to *S. aureus*, and addition of rhIL-20 with *S. aureus* significantly increased concentration of RegIIIγ in supernatants (Figure 7A). Additionally, treatment of BEAS2b cells with an anti-IL-20RB antibody significantly decreased the amount of RegIIIγ detected in supernatants of infected cells (Figure 7B). Taken together, these data demonstrate that signaling through the IL-20 receptor by IL-20 directly modulates innate immune responses of human bronchial epithelial cells to *S. aureus* infection *in vitro* by inhibiting the production of IL-8 and enhancing the production of RegIIIγ.

Modulation of innate immune response from BEAS2b cells by IL-20 does not provide protection during *in vitro* infection with *S. aureus*

Although the anti-inflammatory response mediated via IL-20 receptor signaling was previously shown to contribute to the pathology of cutaneous infection with *S. aureus* in mice (Myles et al., 2013), its reduction of neutrophil recruitment *in vivo* in mice

and *in vitro* in a transwell migration assay, along with its induction of RegIIIγ in BEAS2b cells, indicate that its signaling may be geared to protect host tissues during infection. To evaluate this potential role for IL-20 receptor signaling, lysis of BEAS2b cells during infection with *S. aureus* was evaluated using a lactate dehydrogenase (LDH) release assay. Compared to uninfected cells, LDH levels were significantly increased in BEAS2b cell supernatants at 4 hours p.i. with *S. aureus* (Figure 8A). Blockade of IL-20 receptor signaling led to a slight decrease in cell lysis, but this was not a significant change in cell survival (Figure 8A).

Staphylococci in the wells of infected BEAS2b cells were also quantified to determine if the inhibition of RegIIIγ production observed with antibody blockade of the IL-20 receptor resulted in a detectable change in bacterial growth over the course of infection. The significant reduction in RegIIIγ observed with antibody blockade of IL-20 receptor signaling did not lead to a concomitant significant increase in staphylococcal growth in culture with infected BEAS2b cells (Figure 8B). There was slightly less *S. aureus* growth in the culture media of cells treated with the anti-IL-20RB antibody, though this was not a significant difference from the isotype control or media alone (Figure 8B). Although a protective mechanism for IL-20 receptor signaling in infected BEAS2b cells could not be identified using the above measurements, cell lysis and bacterial growth, this does not exclude the possibility that anti-inflammatory signaling by the IL-20 receptor may provide some protection during *in vivo* infections.

Discussion

The immune response to *S. aureus* in the airways is initiated by cytokines and chemokines released from epithelial cells, which also produce AMPs to provide

protection via their direct antimicrobial activity (Choi et al., 2013; Parker and Prince, 2012). BEAS2b cells, derived from healthy primary bronchial epithelial cells, express IL-20 and an IL-20 receptor. Transcript levels of IL-20 are increased by approximately 70-fold by these cells in response to *S. aureus*, indicating a role for this cytokine, signaling through the IL-20 receptor, in the response of airway epithelial cells to staphylococcal infection. The origin of the signaling that initiates expression of IL-20 in response to *S. aureus* is not clear. In the skin, IL-20 is known to be induced by IL-1β in keratinocytes; however, a link between IL-1β and IL-20 production in lung epithelial cells was not established, as IL-1β protein was not detected in the supernatant of BEAS2b cells. In the previously reported murine cutaneous model of infection, IL-20 receptor signaling promoted an anti-inflammatory response driven by inhibition of IL-1β that led to downstream inhibition of IL-17A and reduced neutrophil recruitment to the site of infection (Myles et al., 2013). Here, in bronchial epithelial cells, signaling through the IL-20 receptor did not affect transcript levels of IL-1β, rather it directly affected production of the chemokine IL-8, a potent attractor of neutrophils (Baggiolini et al., 1989; Colditz et al., 1989). IL-20 receptor-mediated inhibition of IL-8 was shown to affect neutrophil recruitment in a transwell migration assay, as the blockade of IL-20 receptor signaling resulted in significantly more neutrophils migrating to the infected BEAS2b cells. This signaling could potentially have a similar consequence of reducing neutrophil recruitment *in vivo* in a manner that contributes to pathology associated with *S. aureus* pneumonia; however, lung tissue has a more fragile structure compared to skin, so it is possible that the reduced neutrophil recruitment mediated by IL-20 would aid in preserving host tissue.

The production of AMPs by epithelial cells in response to infection with *S. aureus* or other bacterial pathogens serves as an innate immune mechanism that can be directly protective for these cells (Bartlett et al., 2008). The present study showed that IL-20 induces production of RegIIIγ and inhibition of IL-20 receptor signaling significantly reduces the concentration of this AMP in the supernatants of BEAS2b cells infected with *S. aureus*. RegIIIγ is produced by lung epithelial cells in a STAT3 dependent manner and has direct antimicrobial and bacteriostatic activity against *S. aureus,* and is protective *in vivo* in a mouse model of pneumonia (Choi et al., 2013). IL-20 activated STAT3 in BEAS2b cells via the IL-20 receptor, so its induction of STAT3-regulated RegIIIγ is in line with previous findings regarding regulation of this AMP. Although no protective mechanism could be identified for the IL-20 mediated responses of lung epithelial cells infected with *S. aureus,* the regulation of the innate immune response in lung epithelial cells by this cytokine is a novel aspect of innate immunity in this tissue where balancing inflammation is critical to the survival of the host.

CHAPTER II

HUMAN NEUTROPHILS ALTER THEIR IL-20 RECEPTOR EXPRESSION AND IL-20 INHIBITS NEUTROPHIL MIGRATION IN VITRO

The work provided for this chapter is a portion of a published manuscript in Journal of Immunology entitled: IL-20 signaling in activated human neutrophils inhibits neutrophil migration and function. The authors of that manuscript were Portia Gough, Sundar Ganesan and Sandip K Datta.

Introduction

Neutrophil recruitment and bactericidal activity are required to resolve and control infection of skin and other epithelial sites with *Staphylococcus aureus* (Cho et al., 2012; Miller and Cho, 2011; Minegishi et al., 2009; Robertson et al., 2008). The critical nature of neutrophils to the host response to staphylococcal infection of the skin and lungs can be appreciated in the enhanced susceptibility of patient populations with defects in neutrophil number or function to such infections (Pozzi et al., 2015; Winkelstein et al., 2000). A hallmark of the response to infection at a localized site in the body is the initiation of inflammation: after detecting a pathogen, epithelial cells and resident leukocytes release pro-inflammatory factors to attract neutrophils from circulation (Janeway and Medzhitov, 2002; Medzhitov, 2007; O'Shea and Murray, 2008).
This essential component of the host response is driven by cytokine and chemokine signals that directly target neutrophils via specific cell surface receptors through which

these host signals modulate multiple neutrophil functions (El-Benna et al., 2008; Kielland et al., 2009; Rigby and DeLeo, 2012; Tufano et al., 1991).

IL-19, IL-20, and IL-24, members of the IL-20 subfamily of cytokines, were recently identified as part of the epithelial cytokine signaling response to *S. aureus* skin infection, contributing to the pathogenesis of infection by inhibiting the production of pro-inflammatory factors, such as IL-1β and IL-17, and thereby reducing neutrophil recruitment (Myles et al., 2013). In these studies, a species-specific pattern of induction for these cytokines was noted: human keratinocytes produced IL-20 at high levels when challenged with *S. aureus in vitro,* whereas IL-19 and IL-24 expression was more prevalent in mice during cutaneous infection (Myles et al., 2013). A similar pattern of expression of these cytokines was found in models of lung infection with *S. aureus*. Human bronchial epithelial cells expressed mostly IL-20 when infected with *S. aureus in vitro,* and mice expressed IL-19 and IL-24, but not IL-20, in a *S. aureus* pneumonia model (Figure 5). Both humans and mice express IL-20 subfamily cytokines in response to infection with *S. aureus*, but the prevalence of individual cytokines differs between these species, as human epithelial cells preferentially produce IL-20 instead of IL-19 and IL-24.

The IL-20 subfamily cytokines signal through two distinct receptor complexes that consist of the common IL-20RB chain dimerized with either IL-20RA (Type I) or IL-22RA (Type II), and all three receptor chains have been identified in skin and lung tissues of humans (Blumberg et al., 2001; Parrish-Novak et al., 2002; Wegenka, 2010). Expression of IL-20RB has not been reported in neutrophils, but expression of IL-20RA and IL-22RA has been described in synovial fluid cells, which consist mostly of

neutrophils that have migrated to an inflammatory joint (Hsu et al., 2006; Kragstrup et al., 2008). However, the functional consequences of expression of these receptors in neutrophils have not been well characterized.

To facilitate appropriate responses to signaling cues at infected sites, neutrophils undergo changes in their receptor expression as they transition from their resting state in circulation, with low levels of receptor expression for a limited repertoire of cytokines, to their activated state, with an expanded variety of receptors (Borregaard et al., 2007). This transition from limited to high responsiveness originates from secretory vesicles within the neutrophil, which serve as the primary source of surface receptors as they fuse with the plasma membrane during migration from circulation into peripheral tissues (Borregaard et al., 1987; Sengelov et al., 1995; Sengelov et al., 1993). The origin of cytokine receptors from preformed compartments, rather than *de novo* synthesis, allows neutrophils to rapidly alter their functionality in response to pathogenic threats and is a fundamental aspect of their biology.

Receptors for IL-8 and TNFα, notably potent activators of neutrophils that are produced in response to *S. aureus*, are expressed on resting neutrophils, and their effects on neutrophil function have been well characterized (Fournier, 2012; Nathan, 2006). IL-8 is a chemotactic factor that induces directional migration of neutrophils, and TNFα enhances phagocytosis, ROS production and degranulation in adherent neutrophils (Ferrante et al., 1993; Hammond et al., 1995). The effects of these and other cytokines require dynamic changes in the actin cytoskeleton of neutrophils to modulate multiple functions including migration, ROS production, phagocytosis and

granule exocytosis (Castellano et al., 2001; El-Benna et al., 2008; Jog et al., 2007; Weiner et al., 1999).

Chemotaxis stimulated by IL-8 and other chemokines requires the polarization of polymerized actin within the neutrophil, creating a leading edge that pulls the cell along increasing concentration of chemotactic factor in a gradient toward its originating source (Weiner et al., 1999; Zhelev et al., 2004). While IL-8 acts as a trail of chemical breadcrumbs of increasing concentration that leads the neutrophil to the site of infection, TNFα is encountered once the neutrophil is at the target site and aids in arresting migration here (Lokuta and Huttenlocher, 2005). Rather than stimulating the formation of a leading edge that is consistent with migration, TNFα causes neutrophils to form focal adhesions and adopt a 'spread' morphology (Kutsuna et al., 2004; Lokuta and Huttenlocher, 2005). Thus, the different functions mediated by these two pro-inflammatory factors can be observed in the different ways that they affect neutrophil morphology through corresponding rearrangements of the actin cytoskeleton that support these functions.

Like IL-8 and TNFα, IL-20 is also produced by epithelial cells and resident or recruited leukocytes in response to *S. aureus*, but receptor expression for this cytokine and its potential effects on neutrophils have not been characterized. In addition to its effects on pro-inflammatory signaling in epithelial cells during infection with *S. aureus*, IL-20 has been shown to affect actin polymerization and actin mediated processes such as migration in endothelial and dendritic cells (Bech et al., 2016; Hammer et al., 2009; Tritsaris et al., 2007). Given the importance of neutrophils in the innate immune response to *S. aureus* infection in the skin and lung, and the documented production of

IL-20 in response to such infections in the skin and lung, we sought to better characterize the determinants and functional consequences of IL-20 receptor expression directly on neutrophils. Here, we report that human neutrophils alter their IL-20 receptor chain expression upon exposure to conditions that mimic those encountered during infection.

Results

Human neutrophils express IL-20RB when activated

To assess IL-20 receptor expression on human neutrophils, we first examined neutrophils isolated from peripheral whole blood of healthy volunteers. Detection of each receptor chain (IL-20RA, IL-22RB, and IL-20RB) by flow cytometry revealed expression of IL-22RA on a proportion of neutrophils that varied between donors (Figure 9A). In contrast, there was little to no IL-20RA and IL-20RB detected on peripheral blood neutrophils (Figure 9A).

Since neutrophils harbor receptors in their secretory vesicles and granules and alter cell surface expression of receptors upon migration to a site of inflammation or infection (Borregaard et al., 2007; Kolaczkowska and Kubes, 2013), we extended our evaluation to include neutrophils that had extravasated from circulation *in vivo* in response to an inflammatory stimulus. To do this, we harvested neutrophils recruited into suction blister chambers on healthy volunteers that contained saline or lethally irradiated strains of *S. aureus*. These extravasated neutrophils displayed altered expression of IL-20 receptor chains compared to those in peripheral blood. Compared to each donor's own peripheral blood neutrophils, a lower frequency of their blister

neutrophils displayed IL-22RA and a higher frequency displayed IL-20RB (Figure 9B). Extravasation into the saline-containing blister chamber accounted for most of the alteration in receptor expression, but exposure to *S. aureus* seemed to further alter expression in some donors (Figure 9B).

To determine whether migration of neutrophils toward infected epithelial cells *in vitro* could mimic the IL-20R expression pattern seen in the *in vivo* blister studies, we infected human bronchial epithelial (BEAS2b) cells with *S. aureus*. Neutrophils were then allowed to migrate for three hours across a semi-permeable transwell membrane into the well containing the infected epithelial cells. The migrated neutrophils had significantly upregulated IL-20RB and downregulated IL-22RA compared to peripheral blood neutrophils (Figure 10A). Low levels of migration to uninfected cells occurred, but did not significantly alter receptor expression (Figure 10A). Migration toward infected cells appeared to be a key trigger for this pattern of receptor expression, as incubating adherent neutrophils with sterile filtered supernatant from infected cells similarly reduced IL-22RA expression but did not alter IL-20RB expression (Figure 10A).

Neutrophils encounter and respond to cytokines released at sites of inflammation. IL-8 is an important neutrophil chemoattractant, and incubation with TNFα is known to 'prime' neutrophils and enhance their subsequent functionality (Ferrante et al., 1993; Hammond et al., 1995; Tufano et al., 1991). To mimic migration induced by IL-8 and determine whether this induced changes in IL-20 receptor chain expression, neutrophils were allowed to migrate across a semi-permeable transwell insert into wells containing either recombinant human (rh) IL-8 alone or rhIL-8 with *S. aureus*. Migration to rhIL-8 caused a significant reduction in IL-22RA expression, but no significant change

in IL-20RB. Migration across the transwell membrane toward *S. aureus* induced both a significant increase in IL-20RB expression and a decrease in IL-22RA, and this was not further altered by concurrent administration of rhIL-8 (Figure 10B). Priming of neutrophils by direct incubation with TNFα similarly reduced the frequency of IL-22RA+ cells and increased the frequency of IL-20RB+ cells (Figure 10C). Direct incubation with *S. aureus* did not alter IL-20RB surface expression in unprimed neutrophils, but further augmented it in those primed with TNFα (Figure 10C). IL-20RA expression was examined separately since detection antibodies for IL-20RA and IL-22RA were both generated in rabbits and could not be used together. Collectively, these results indicate that human neutrophils modulate their expression of the IL-20 receptor chains IL-22RA and IL-20RB. Downregulation of IL-22RA seems to be a common response to migration, inflammatory cytokines, and bacteria. In contrast, induction of IL-20RB appears to be part of a more stringently controlled neutrophil response to infection, as it was stimulated only by *S. aureus*-induced migration or infection of TNFα-primed neutrophils.

IL-20 modulates actin polymerization in neutrophils

Changes to the actin cytoskeleton drive key neutrophil processes during infection including phagocytosis, granule exocytosis, and migration (Castellano et al., 2001; Jog et al., 2007; Kredel et al., 2014; Weiner et al., 1999). To directly assess the effect of IL-20 on the actin cytoskeleton in neutrophils, polymerized actin (F-actin) was visualized by phalloidin staining and confocal microscopy of infected neutrophils treated with IL-8, TNFα, or IL-20 for 1 minute. These conditions were selected based on modulation of IL-

20 receptor expression, particularly upregulation of IL-20RB. Morphological changes with increased peripheral polarization of F-actin attributable to IL-20 was observed in neutrophils treated with either IL-8 or TNFα and was especially prominent in those treated with TNFα (Figure 11A). For each condition, an automated tiling method was used to collect 25 unbiased fields of view that were then used for fluorescence quantification that was normalized to the number of cells per field to compare levels of F-actin. IL-8- and TNFα-primed neutrophils infected with *S. aureus* had significantly increased F-actin content when treated with IL-20, while those infected with *S. aureus* alone responded to IL-20 with significantly reduced levels of F-actin (Figure 11B). These results identify that IL-20 modifies F-actin content and organization in *S. aureus*-infected human neutrophils.

IL-20 signals via ERK1/2 in activated neutrophils

IL-20 has been reported to activate STAT3, p38 MAPK, or ERK1/2 in different cell types (Bech et al., 2016; Blumberg et al., 2001; Hammer et al., 2009; Parrish-Novak et al., 2002). After exposure of neutrophils to IL-20, we found STAT3 phosphorylation to be minimal and inconsistent (not shown). Western blot of lysates from neutrophils that were incubated with *S. aureus* (MOI 1) and 100 ng/mL IL-8 or 10 ng/mL TNFα for 10 minutes showed that ERK1/2 was phosphorylated in the presence of 10 ng/mL IL-20 (Figures 12A and 12B). In agreement with published findings regarding signaling by TNFα in human neutrophils, phosphorylation of p38 MAPK was detected (Figure 12C); and, IL-20 did not alter p38 MAPK activation in TNFα-primed neutrophils (Figure 12C). Treatment of neutrophils with small molecule inhibitors SB 202190 or PD 98059,

inhibited signaling by p38 MAPK or ERK1/2, respectively (Figure 12A-12C). The inhibitor SB 202190 does not inhibit p38 MAPK phosphorylation directly, but inhibits activation of its downstream targets, including ATF-2 (Wu et al., 2006); accordingly, this inhibition was confirmed by Western blot (Figure 12C). Based on western blots of neutrophil lysates, IL-20 activates ERK1/2 in these cells and this signaling can be inhibited by treatment with PD 98059.

IL-20 inhibits neutrophil migration *in vitro*

Neutrophils have a hierarchical response to multiple signals that allows them to reach a site of infection and arrest movement at the point of threat, be it wound or infection (Heit et al., 2002; Liu et al., 2012). Actin dynamics that establish polarization and pseudopod extension and other morphological changes are a critical component of neutrophil migration (de Oliveira et al., 2016; Zhelev et al., 2004). To determine whether IL-20 affects this actin-mediated process, neutrophils were incubated with *S. aureus* and IL-8 and random migration was observed using live imaging via time lapse microscopy. Neutrophils were seeded in coverglass slides in separate chambers and infected with *S. aureus* in media alone or media supplemented with IL-8 or IL-8+IL-20. Neutrophils in the presence of IL-20 began to arrest movement at approximately 15 minutes, while the neutrophils in the other chambers continued their movement (Figure 13: Supplemental Videos 1-3), indicating that IL-20 inhibits neutrophil migration induced by IL-8 and *S. aureus*.

The aforementioned BEAS2b cells produce IL-20 and IL-8 in response to infection with *S. aureus* (Figures 5A and 5B). Supernatants from these cells were

obtained 4 hours post-infection and sterile filtered, and depleted of IL-20 by immunoprecipitation (Figure 14A). Untreated and IL-20-depleted ('αIL-20') supernatants were added to cell culture plates and transwell inserts were placed in wells to which $1.5x10^5$ neutrophils added. Neutrophils were allowed to migrate from the insert to the supernatants for 3 hours before being collected from the lower chamber and enumerated by manual count in a hemacytometer. Significantly more neutrophils were recovered from the wells containing IL-20-depleted supernatant was relative to untreated or isotype control-treated supernatant, and addition of recombinant human IL-20 to the depleted supernatant returned migration to the lower levels seen with untreated supernatant (Figure 14B).

In this system, inhibition of ERK1/2, but not p38 MAPK, increased migration to levels similar to that seen with IL-20 depletion and this was not further affected by IL-20 depletion or repletion (Figure 15A). Addition of IL-20 also inhibited migration of neutrophils from transwell inserts toward recombinant IL-8 and *S. aureus* (Figure 15B). Inhibition of ERK1/2 did not affect migration induced by IL-8 and *S. aureus*, but abrogated the inhibition of migration by IL-20 (Figure 15B). The transwell migration assays and time lapse microscopy show that IL-20 inhibits neutrophil migration, and this inhibition is dependent on activation of ERK1/2.

Discussion

Neutrophil function is modulated by the cytokines and chemokines encountered during infection. Prior work by our lab in a model of *S. aureus* skin infection identified anti-inflammatory effects of IL-20 receptor signaling that included diminished

recruitment of neutrophils to infected lesions in mice (Myles et al., 2013). IL-20 has been reported to similarly inhibit neutrophil recruitment in a mouse model of corneal wound healing (Zhang et al., 2017). These studies focused on the impact of IL-20 on neutrophil recruitment by epithelial cells, recognized targets of this cytokine in models of psoriasis and wound healing (Otkjaer et al., 2007; Sa et al., 2007). However, analyses of synovial fluid neutrophils from patients with rheumatoid arthritis have suggested neutrophils may directly respond to this cytokine (Hsu et al., 2006; Kragstrup et al., 2008). Our current study identifies the ability of inflammatory and infectious stimuli to induce receptor expression on human neutrophils in a manner that allows IL-20 to directly target these cells and influence their anti-bacterial and inflammatory properties.

Two IL-20 receptors have been reported, comprised of a common IL-20RB chain that forms a heterodimer with either IL-20RA (Type I) or IL-22RA (Type II) (Blumberg et al., 2001; Wegenka, 2010). Both receptors have been found on epithelial cells, but their expression on leukocytes has been less well characterized (Blumberg et al., 2001; Parrish-Novak et al., 2002). RT-PCR data has shown expression of IL-20RB alone on peripheral blood mononuclear cells, B cells, T cells, and NK cells (Wolk et al., 2002). IL-20RB expression has not been reported on neutrophils, but IL-20RA and IL-22RA receptor chains have been detected by flow cytometry on synovial membranes and synovial fluid cells, including neutrophils, from patients with rheumatoid arthritis (Hsu et al., 2006; Kragstrup et al., 2008), suggesting that the expression of these receptors may be regulated by environmental cues.

In the present study, freshly isolated peripheral blood neutrophils expressed detectable IL-22RA on their surface but did not express IL-20RA or IL-20RB. We found

that expression of IL-20RB, common to both Type I and Type II IL-20 receptors, was upregulated in response to migration from circulation *in vivo* or stimulation with *S. aureus* and pro-inflammatory host factors *in vitro*. These same conditions caused a decrease in IL-22RA expression, indicating a possible balance between IL-20 receptor and IL-22 receptor signaling since IL-22RA is used by both. Because detection of both IL-20RA and IL-22RA depended on rabbit antibody reagents, we were limited in our ability to determine co-expression of IL-20RA but found that its expression on neutrophils was low and was further decreased on infected neutrophils after priming with TNFα. Expression of IL-20RB correlated with responsiveness of neutrophils to IL-20, suggesting that IL-20RB imparted its function by pairing with low levels of IL-22RA or IL-20RA. However, we cannot rule out the possibility that IL-20 signals through a non-canonical receptor in neutrophils.

Consistent with utilization of JAK-STAT signaling pathways by the Class II cytokine receptor family (Bazan, 1990; Kotenko and Pestka, 2000; Ouyang et al., 2011), IL-20 receptor signaling has been described to involve STAT3 in epithelial cells (Blumberg et al., 2001; Parrish-Novak et al., 2002). Activation of p38 MAPK and ERK1/2 by the cytokine IL-20 has also been reported in dendritic cells and endothelial cells, respectively (Bech et al., 2016; Hammer et al., 2009). Furthermore, IL-20 has been shown to activate ERK1/2 in rheumatoid arthritis synovial fibroblasts, which are comprised of mostly neutrophils (Hsu et al., 2006). We detected inconsistent and minimal phosphorylation of STAT3 in neutrophils in response to IL-20, and show that activation of ERK1/2 occurs in neutrophils and is required for the functional effects of IL-20 observed during infection *in vitro*.

Our observed inhibitory effects on neutrophil migration are consistent with decreased neutrophil accumulation reported in *in vivo* models (Myles et al., 2013; Zhang et al., 2017), but contrast with the reported ability of IL-20 to directly induce neutrophil chemotaxis *in vitro* (Hsu et al., 2006). These discrepancies may reflect differential neutrophil activation states that are determined by environmental cues. We suggest that the inhibition of neutrophil function seen in our *in vitro* models mimicking infection with *S. aureus* is linked to the altered polymerization of actin seen by confocal microscopy of neutrophils after exposure to IL-20, since migration requires dynamic actin rearrangement (Weiner et al., 1999). This work establishes IL-20 as a cytokine that can directly target neutrophils at a site of infection with *S. aureus* and shows that its effects are mediated via activation of ERK1/2. Because IL-20 affects actin dynamics in neutrophils, and these cells rely on changes to the cytoskeleton to perform multiple functions, it is worthwhile to consider other potential functions that IL-20 may affect: namely, phagocytosis and granule exocytosis.

CHAPTER III

IL-20 INHIBITS RESPONSES OF TNFα-PRIMED NEUTROPHILS TO INFECTION WITH STAPHYLOCOCCUS AUREUS

The work provided for this chapter is a portion of a published manuscript in Journal of Immunology entitled: IL-20 signaling in activated human neutrophils inhibits neutrophil migration and function. The authors of that manuscript were Portia Gough, Sundar Ganesan and Sandip K Datta.

Introduction

Neutrophils are the most abundant leukocyte in humans and exhibit multiple antimicrobial mechanisms that are critical for protection of the host against infection with extracellular bacterial and fungal pathogens (Miller and Cho, 2011; Rigby and DeLeo, 2012; Spaan et al., 2013). For example, their central role in controlling and resolving infections with the bacterial pathogen *Staphylococcus aureus* is illustrated by the increased severity and frequency of these infections in patients with disorders of neutrophil number or function (Winkelstein et al., 2000). *S. aureus* is a leading cause of infectious morbidity and mortality even in immunocompetent hosts. It most frequently displays its propensity to transition from a colonizer to a pathogen at epithelial barrier sites, such as the skin and lung, but it can also disseminate from these sites and cause severe invasive disease (Foster, 2005; Lowy, 1998).

As professional phagocytes that produce large amounts of reactive oxygen species (ROS), both in the phagosome and extracellular space, neutrophils are particularly well suited to killing *S. aureus*, compared to other phagocytes (Rigby and DeLeo, 2012). When they encounter staphylococci or other bacteria, they are able to phagocytose the invading agent in as little as 20 seconds (Segal et al., 1980). Once the phagosome has formed, neutrophil granules fuse with it to provide proteases, antimicrobial peptides and NADPH oxidase components that create a hostile environment for invading microbes (Karlsson and Dahlgren, 2002). Additionally, these antimicrobial granule contents are released into the extracellular space (Figure 3) (Borregaard et al., 2007). The chromosomal content of neutrophils also serves to restrict bacterial growth as neutrophils undergo a specific form of cell death called NETosis, releasing their chromatin into the extracellular space to ensnare bacteria and create a concentrated area of antimicrobial peptides and proteases (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012; Fuchs et al., 2007). Phagocytosis, granule fusion and exocytosis, ROS production and NETosis all combine to make neutrophils particularly well-suited to killing *S. aureus*, a bacterial pathogen with multiple mechanisms to evade destruction.

During epithelial infections with *S. aureus*, cytokines are produced at the site of infection to attract the appropriate leukocytes and modulate their function, both initiating and resolving the inflammatory response (Hutchins et al., 2013; Medzhitov, 2007). TNFα is an important pro-inflammatory cytokine produced in response to *S. aureus* infection, as it arrests migration at the site of infection and enhances neutrophil antimicrobial functions (Ferrante et al., 1993; Lokuta and Huttenlocher, 2005; Lowy,

1998). In order to fully respond to TNFα with enhanced antimicrobial functions, neutrophils require a second signal, via engagement of their integrin receptors by ligands such as ICAM-1 (Nathan, 1987). Adherent neutrophils that are treated with TNFα *in vitro* are 'primed', a state between resting and activated that is defined by increased production of ROS, degranulation and rate of phagocytic uptake upon encountering a microbial threat (Chakrabarti et al., 2006; El-Benna et al., 2008; Wewers et al., 1990). Changes in receptor expression can also result from activation of TNFα-primed neutrophils, as shown in the previous chapter where IL-20 receptor expression was altered under these conditions.

In the previous chapters, it was shown that IL-20 is produced by human epithelial cells during *in vitro* infection with *S. aureus*, that neutrophil IL-20 receptor expression changes upon priming with TNFα and that IL-20 can alter actin polymerization in these cells. Actin polymerization changes rapidly in neutrophils to facilitate multiple functions, including migration, phagocytosis and granule exocytosis (Nordenfelt and Tapper, 2011; Weiner et al., 1999). Migration requires a polarized morphology that allows the cell to pull itself along a surface, phagocytosis is associated with localized polymerization and depolymerization underneath the particle bound to the cell surface, and granule exocytosis is aided by depolymerization of actin along the plasma membrane of the cell (Downey et al., 1990; Nathan, 2006). TNFα has been shown to induce morphological changes in neutrophils that cause them to become adherent by altering the actin cytoskeleton to induce a spread morphology, rather than polarized, and create focal adhesions (Lokuta and Huttenlocher, 2005). These cytoskeletal changes are thought to arrest migration and allow increased granule exocytosis observed in TNFα-primed

neutrophils (Chakrabarti et al., 2006; Kutsuna et al., 2004; Lokuta and Huttenlocher, 2005). Changes in the actin cytoskeleton are accompanied by, and can be linked to, detectable changes in neutrophil functions.

Neutrophil granules are specialized compartments that contain preformed proteins that are relevant to the multiple functions of neutrophils. Produced during maturation, the contents of both the granule lumen and membrane allow these cells to respond to their environment rapidly, without requiring transcription and translation (Amulic et al., 2012; Borregaard et al., 2007). There are three main granule populations: primary, secondary and tertiary, which differ in the timing of their release, a process called graded exocytosis (Lew et al., 1986; Sengelov et al., 1995). The differential release of granule contents corresponds to the different functions that neutrophils perform during their response to infection.

Early in the response to infection neutrophils migrate from circulation and into peripheral tissues, a process aided by the release of matrix metalloproteinases (MMPs), collagenases that degrade the extracellular matrix, contained in tertiary granules (Borregaard et al., 1987; Delclaux et al., 1996). Once neutrophils are at the site of infection, primary and secondary granules containing antimicrobial peptides, proteases and NADPH oxidase components are released to the extracellular space and phagosome (Lacy and Eitzen, 2008). Little is known of the exact mechanics of graded exocytosis, but it is thought to be controlled in part by different associations with cytoskeletal components. Tertiary granules are associated with the most actin compared to primary and secondary granules; thus, their release is most affected by changes to the actin cytoskeleton (Jog et al., 2007).

The actin cytoskeleton has a central role neutrophil bactericidal functions, and IL-20 affects actin polymerization in multiple cell types, including neutrophils (Bech et al., 2016; Hammer et al., 2009). Here, IL-20 is shown to inhibit the actin-dependent processes of phagocytosis and tertiary granule exocytosis in TNFα-primed neutrophils during *in vitro* infection with *S. aureus.*

Results

IL-20 inhibits killing of *S. aureus* by TNFα-primed neutrophils *in vitro*

Since priming of neutrophils with TNFα modulated their IL-20 receptor expression, we evaluated whether TNFα-primed neutrophils had altered responsiveness to IL-20. Epithelial cells produce IL-20 in response to infection with *S. aureus*, and incubation with *S. aureus* enhances neutrophil expression of IL-20RB; so, IL-20 effects on neutrophil bactericidal mechanisms were evaluated using an *in vitro* killing assay. To model the epithelium and fully induce the priming response in neutrophils, the assay was carried out with cells that were primed while adherent to cell culture plate wells that were coated with ICAM-1 and autologous donor serum. IL-20 was then added along with the inoculum of *S. aureus*, which was at MOI 1 to closely mimic the amount of staphylococci in infected lesions (Leyden et al., 1974; Travers et al., 2010).

Priming with TNFα is known to enhance multiple bactericidal mechanisms in neutrophils (Ferrante et al., 1993; Tufano et al., 1991). Consistent with this, fewer colony forming units (CFU) of *S. aureus* were recovered in the presence of TNFα-primed neutrophils (Figure 16B) compared to unprimed neutrophils (Figure 16A). In the presence of IL-20, unprimed neutrophils had a slight, but significant, increase in CFUs

of *S. aureus* at 4 hours post-infection (Figure 16A). Addition of IL-20 significantly increased CFU recovered from TNFα-primed neutrophils at 2 and 4 hours post-infection (Figure 16B).

A potential cause of the increased in CFUs mediated by IL-20 could be a difference in cell death. If IL-20 induced apoptosis or allowed neutrophils to become more susceptible to the leukotoxins produced by *S. aureus*, this would hinder their ability to kill the staphylococci. To rule out differences in cell death as the cause of altered bactericidal activity, neutrophils were stained with propidium iodide (PI) and an antibody for Annexin V, to label dead and apoptotic cells, respectively, at 4 hours post-infection (Figure 17A). Quantification of the populations of Annexin V+ and PI+ cells showed no significant difference, in the presence or absence of IL-20, in the percentage of dead cells or those undergoing apoptosis (Figure 17B). Lysis of neutrophils was assessed at multiple timepoints by LDH release assay, and this showed that neutrophils infected with *S. aureus* undergo lysis over the course of the killing assay, but IL-20 did not significantly alter the percent of lysed cells (Figure 17C). In the absence of a difference in cell death, the increased CFUs in the presence of IL-20 indicates that it affects one or more bactericidal mechanisms of neutrophils.

IL-20 does not affect ROS production or NET formation

Production of reactive oxygen species (ROS) is a major mechanism utilized by neutrophils to kill staphylococci (Rigby and DeLeo, 2012; Segal, 2005). Total ROS production was evaluated by addition of luminol, which generates luminescence when reacting with ROS, to wells containing neutrophils that were either uninfected or infected

with *S. aureus* as before. Measurements were taken every four minutes for four hours and showed that, as expected, priming uninfected neutrophils with TNFα caused an initial spike in ROS production (Figure 18A) (Dewas et al., 2003; Nathan, 1987). Infection with *S. aureus* caused ROS production to increase by over two orders of magnitude, and addition of IL-20 did not significantly change this response (Figure 18B).

NETS are another means by which neutrophils kill and control the growth of staphylococci, releasing their nuclear contents into the extracellular space to trap microbes in concentrated regions of antimicrobial factors (Brinkmann et al., 2004). Because this mechanism relies on the release of dsDNA, the formation of NETs can be measured by quantifying dsDNA or inhibited by addition of DNase to neutrophils infected with *S. aureus*. Measurement of dsDNA in supernatants of neutrophils showed that infection with *S. aureus* induces the release of neutrophil nuclear contents, and that IL-20 does not affect the quantity of dsDNA released (Figure 19A). Addition of DNase to infected neutrophils resulted in reduced bactericidal activity, but did not abrogate the IL-20 mediated inhibition of bacterial killing, confirming that IL-20 does not modulate NET formation (Figure 19B). Although IL-20 inhibits killing of *S. aureus* by neutrophils, it does not affect the bactericidal mechanisms of ROS production or NETs.

IL-20 inhibits tertiary granule exocytosis

Granule exocytosis contributes to different aspects of neutrophil functions in response to bacteria or other activating stimuli and requires translocation to the plasma membrane via cytoskeletal remodeling (Jog et al., 2007; Mitchell et al., 2008). Primary, secondary, and tertiary granules each contain different compositions of proteases,

antimicrobial peptides, and other proteins that contribute to their distinct functional roles in the response to infection (Borregaard et al., 2007; Cowland and Borregaard, 2016). Exocytosis of each granule population occurs in a hierarchical manner dependent on differential association with actin (Jog et al., 2007).

To evaluate whether IL-20 affects granule exocytosis, we measured the release of protein markers for each granule population in supernatants of neutrophils that were infected with *S. aureus in vitro*. IL-20 did not affect the concentration of neutrophil elastase and lactoferrin, markers of primary and secondary granules, respectively (Figure 20A-B). Granule exocytosis can also be detected based on expression of certain membrane proteins that are known to originate from primary or secondary granules. CD63 serves as a marker for primary granules, while secondary granule exocytosis results in CD66b expression on the neutrophil surface (Bainton et al., 1987; Borregaard et al., 2007). Staining neutrophils that were either uninfected or infected with *S. aureus* for these markers and evaluating them by flow cytometry revealed that, as expected, priming with TNFα alone induces secondary granule release (CD66b expression) (Figures 20D and 20F); however, release of primary granules requires both TNFα-priming and infection with *S. aureus* (Figures 20C and 20E). Again, IL-20 did not affect the membrane expression of either CD63 or CD66b, confirming that it does not modulate the release of primary or secondary granules.

Matrix metalloproteinases (MMPs), also called gelatinases, comprise the predominant protein population in tertiary granules (Cowland and Borregaard, 2016; Kjeldsen et al., 1992). The activity of gelatinases in neutrophil supernatants was evaluated by gelatin zymography, where the clearing of gelatin in polyacrylamide gels is

visualized by Coomassie stain. IL-20 reduced gelatinase activity in the supernatants of TNFα-primed neutrophils that were infected with *S. aureus* (Figure 21A). The concentration of the major constituent of tertiary granules, MMP9, was also significantly reduced after exposure of infected neutrophils to IL-20 (Figure 21B). The reduced levels of gelatinase activity and MMP9 in supernatants of neutrophils that were treated with IL-20 suggests that the release of tertiary granules is inhibited by IL-20. Since tertiary granules are more highly associated with actin than primary and secondary granule populations, the inhibition of their release implies modulation by IL-20 of actin dynamics in neutrophils during *in vitro* infection with *S. aureus.*

IL-20 inhibits phagocytic uptake of *S. aureus* by TNFα-primed neutrophils

To investigate the potential involvement of another pathway of bactericidal activity by IL-20, we next assessed the effect of IL-20 on phagocytosis of *S. aureus* by neutrophils, an actin-dependent process essential for intracellular killing (Castellano et al., 2001; Rigby and DeLeo, 2012). Inhibition of actin polymerization by pre-treatment of neutrophils with cytochalasin D resulted in a total abrogation of bactericidal activity, as there was no difference in the quantity of CFUs of *S. aureus* in the presence or absence of neutrophils at 4 hours post-infection (Figure 22A). This inability of cytochalasin D-treated neutrophils to control the growth of *S. aureus* indicated that an actin-mediated process was required for killing *S. aureus* in the *in vitro* assay.

Phagocytosis relies on intricate actin dynamics of polymerization to engulf a particle, and depolymerization to take up the phagosome (Lee et al., 2003). A gentamicin protection assay was used to evaluate this whether this process was

affected by IL-20. In this assay, extracellular bacteria were killed by gentamicin treatment, following this the cells were lysed and the detectable CFUs reflect phagocytosed intracellular bacteria. By one hour after infection, IL-20 exposure lowered the percentage of intracellular *S. aureus* recovered from TNFα-primed neutrophils (Figure 22B), suggesting impairment of phagocytosis by IL-20. Inhibition of this function that neutrophils require for killing staphylococci, along with the demonstrated effects of IL-20 on actin polymerization in neutrophils, provides a mechanism by which IL-20 inhibits neutrophil bactericidal activity *in vitro*.

Inhibition of phagocytosis and tertiary granule exocytosis by IL-20 is mediated by ERK1/2

In the previous chapter, it was shown that IL-20 activates ERK1/2 in TNFα-primed neutrophils during infection with *S. aureus*, and does not affect p38 MAPK activation. Additionally, activation of p38 MAPK or ERK1/2 could be inhibited by treatment with SB 202190 or PD 98059, respectively. To determine whether ERK1/2 activation by IL-20 was required for the observed inhibitory effects of this cytokine, bacterial killing and phagocytosis were evaluated as before using neutrophils treated with SB 202190 or PD 98059. Inhibition of ERK1/2 by PD 98059 abrogated the IL-20-mediated differences in bacterial killing and phagocytic uptake by TNFα-primed neutrophils (Figure 23A and 23B). Additionally, when ERK1/2 was inhibited, IL-20 treated neutrophils displayed similar levels of tertiary granule release, as indicated by gelatin zymography and measurement of MMP9 in supernatants of TNFα-primed neutrophils that were infected with *S. aureus in vitro* (Figure 23C and 23D). Taken together, the enhanced

phosphorylation of ERK1/2 observed in the lysates of IL-20 treated neutrophils and restoration of phagocytosis and tertiary granule release in PD 98059-treated neutrophils indicates that the inhibitory effects of IL-20 are dependent on ERK1/2 activation.

Discussion

During infection with *S. aureus* at epithelial sites, neutrophils are primed and their properties and responses are altered as they migrate from circulation and arrive at the site of infection. Over the course of this process, neutrophils are responding to pro-inflammatory signaling in a manner that enhances their capacity to respond to their environment, with expanded receptor expression, and kill microbial threats, with increased ROS production and degranulation (Amulic et al., 2012; El-Benna et al., 2008). In the previous chapter, it was shown that treating neutrophils with TNFα *in vitro*, which mimics the priming process that neutrophils undergo *in vivo*, alters IL-20 receptor expression. This results in downregulation of IL-22RA, expressed by resting neutrophils in circulation, and upregulation of IL-20RB. The data shown in the present chapter showed that neutrophils with enhanced IL-20RB expression, via priming with TNFα and *in vitro* infection with *S. aureus*, became responsive to IL-20 in a manner that inhibited the actin-mediated functions of phagocytosis and tertiary granule exocytosis.

Neutrophils that have been primed with TNFα have significantly increased killing of *S. aureus in vitro* (Ferrante et al., 1993); however, bactericidal activity was inhibited when these cells were treated with IL-20, resulting in significantly higher CFUs recovered at 2 and 4 hours when IL-20 was present. In this assay, neutrophils required changes in actin polymerization to destroy staphylococci, as treatment with cytochalasin

D abrogated killing activity. Evaluation of multiple killing mechanisms revealed that ROS production and release of neutrophil elastase and dsDNA, major components of NETs, were unaffected by IL-20. However, phagocytosis, a necessary function for bactericidal activity that relies on specific changes to the actin cytoskeleton (Castellano et al., 2001; Lee et al., 2003), did appear to be affected by IL-20. When TNFα-primed neutrophils were treated with IL-20, there was a lower percentage of staphylococci recovered from neutrophils that were treated with gentamicin before lysis, indicating an IL-20 mediated inhibition of this process.

Mechanisms of granule exocytosis vary in their dependence on different cytoskeletal components; and, compared to primary and secondary granules, tertiary granule exocytosis is linked most closely with actin dynamics (Jog et al., 2007). IL-20 inhibited only tertiary granule exocytosis, based on reduced gelatinase activity and MMP9 concentration in neutrophil supernatants, and not primary or secondary exocytosis. This specificity of affected granule population provides further evidence that IL-20 modulates the actin cytoskeleton in a manner that affects specific neutrophil functions that rely heavily on rapid and specific changes to this cellular component.

In the previous chapter, phalloidin staining with qualitative and quantitative analysis of confocal microscopy images showed that IL-20 causes changes to morphology and significantly increased actin polymerization in TNFα-primed neutrophils. Phagocytic uptake of target particles requires actin polymerization, followed quickly by depolymerization, while tertiary granule exocytosis is thought to require dissolving of the cortical actin ring at the periphery of the neutrophil (Muallem et al.,

1995; Saiepour et al., 2006). The finding that IL-20 modulates actin polymerization in neutrophils is commensurate with downstream effects on these processes.

Overall, the observed inhibitory effects of IL-20 on the neutrophil responses to infection are consistent with the anti-inflammatory properties of this cytokine on epithelial cells during cutaneous staphylococcal infection (Myles et al., 2013). Further verification in appropriate infectious and inflammatory models will be needed to confirm the inhibitory nature of IL-20 activity on neutrophil function during infection *in vivo*. This may be complicated because of differences between mouse and human neutrophils (Eisenhauer and Lehrer, 1992; Haley, 2003; Yaffe et al., 1999), and the preferential expression of IL-20 by human cells compared to the tendency of murine cells to secrete IL-19 and IL-24 instead (Myles et al., 2013). The identification of IL-20 as a regulator of neutrophil function during infection with *S. aureus in vitro* adds to the repertoire of host signaling factors that can affect the outcome of these frequent, incompletely understood and potentially deadly infections.

CHAPTER IV

CONCLUSION

The immune response to a pathogen consists of a multitude of factors from both host and the invasive agent. Infections with *S. aureus* comprise an especially complex interplay of these factors, as this pathogen has a particularly large arsenal of virulence factors and immune evasion mechanisms that can affect the host response (Foster, 2005). Additionally, *S. aureus* can infect multiple sites of the body, and different sites vary in their response, such that a protective response to skin and soft tissue infection differs from that of bacteremia (Cohen, 2002; McNicholas et al., 2014; Miller and Cho, 2011). Though the sum of factors contributed by both host and pathogen create a complex biological puzzle, it is essential to understand how each piece contributes to the ultimate outcome: resolution of infection or morbidity and mortality. As tools for treatment wane with the rise of antibiotic resistance and there remains no preventative vaccine, understanding the pieces of this puzzle to develop effective treatment and prevention options becomes an increasingly urgent task.

An essential component of understanding the immune response is the signaling, in the form of cytokines and chemokines, generated by the host in response to *S. aureus*. Upon detection of a pathogen, signaling is engaged by the cells at the infected site to initiate an inflammatory response, which recruits the appropriate leukocytes and activates them according to the microbial threat present. Cytokines are central to organizing the immune response as they dictate which immune cells respond and how

these cells behave once they are active in that response (Iwasaki and Medzhitov, 2004, 2010). A few key cytokines have been identified as pieces of a protective immune response to skin and soft tissue infection with *S. aureus*, namely IL-1β and IL-17, for their pro-inflammatory role in neutrophil recruitment (Cho et al., 2012; Cho et al., 2010). IL-20 was then shown to have an anti-inflammatory effect, inhibiting the production of these cytokines during such infections, and contributing to pathogenesis of infection by reducing neutrophil recruitment (Myles et al., 2013). The study of these factors focused on their role in mobilizing this leukocyte population to the site of infection, but less is known about how host signaling affects the function of neutrophils. There is some appreciation of the enhancing effects of pro-inflammatory cytokines on neutrophil functions at the site of infection, but relatively little is known about how anti-inflammatory signaling affects the functions of these immune cells. Neutrophils are the best equipped immune cells to kill *S. aureus* and are well appreciated as an essential part of a protective immune response. The addition of IL-20 as a host factor that affects their function adds a valuable piece to the puzzle of the immune response to *S. aureus*.

Establishing an *in vitro* Model: Obtaining Meaningful *in vitro* Pieces of an *in vivo* Puzzle

When one examines an immune response to epithelial infection with *S. aureus*, two major features of pathogen control and elimination can be observed: those that directly serve to eliminate the pathogen through bactericidal activity, and those that indirectly serve this purpose by signaling to or otherwise modifying the function of host cells. These direct and indirect features are interdependent, and the latter comprises a

multitude of signaling molecules, both bacterial and host derived. The major advantage of *in vivo* experiments is that they allow examination of a response to infection that includes all of these components. However, these components differ considerably between humans and, the most frequently used model organism for infection with *S. aureus*, mice (Mestas and Hughes, 2004; Seok et al., 2013).

A model for human infection should closely mimic the responses and functions of human cells. *S. aureus* is a complex human pathogen that expresses multiple factors that respond to the immune system of humans, some of which are considerably less toxic or ineffective in mice (Salgado-Pabon and Schlievert, 2014; White et al., 2014). Though mice are frequently used to model *S. aureus* infection in humans, there are key differences in their immune systems that must be considered when studying the immune response to this pathogen, particularly the differences between human and murine phagocytes. As stated previously, the neutrophil is central to controlling and resolving *S. aureus* infections, particularly those of the skin and lung. In humans, neutrophils are the most abundant leukocyte, comprising 50-70% of circulating white blood cells, while lymphocytes are 20-40% of the population. Whereas the circulating leukocytes in mice are only 15-20% neutrophils and 50-70% lymphocytes (Mestas and Hughes, 2004; Mizgerd and Skerrett, 2008). This is a fundamental difference in the composition of the immune systems between humans and mice.

There are also key functional differences, as mouse neutrophils lack α-defensins, antimicrobial peptides that make up 30-50% of proteins in human neutrophils (Eisenhauer and Lehrer, 1992). Humans that lack α-defensins, a rare disorder called specific granule deficiency (SGD), suffer from recurrent infections of the skin, lung and

other sites, with multiple bacterial pathogens including *S. aureus* (Glenthørj et al., 2014; Sakura et al., 1993; Wada et al., 2005). However, though they lack α-defensins that are critical to human immune defense, mice are relatively resistant to infection with *S. aureus* (Salgado-Pabon and Schlievert, 2014). There are other major differences in granule contents, including significantly lower levels of other antimicrobial proteins such as lysozyme and alkaline phosphatase in murine neutrophils (Rausch and Moore, 1975). Additionally, the neutrophils of mice contain 10-20% less myeloperoxidase, an enzyme that contributes to killing of microbes by human neutrophils (Noguchi et al., 2000; Rausch and Moore, 1975). Thus, for studies that seek to investigate and better understand human neutrophil function, the mouse is not an ideal model.

Given their importance in the host response to infection with *S. aureus*, the functions of human neutrophils during infections with *S. aureus* merit investigation. This requires the development of a suitable *in vitro* system that provides neutrophils with an environment that resembles an *in vivo* site of infection. The *in vitro* models used in the studies presented here were designed to provide such an environment via using a sufficient quantity of neutrophils (Li et al., 2002, 2004), a MOI that reflects *in vivo* infections in human tissue (Leyden et al., 1974; Travers et al., 2010), exposure to host factors at physiologically relevant concentrations, and the use of adherent cells (rather than stirred suspension) for a tissue-like environment. Although mouse models have contributed much to our knowledge of staphylococcal pathogenesis and immune responses (Kim et al., 2014), there is also value in robust *in vitro* modeling with human cells to expand our knowledge of functions and responses that are unique to our species.

IL-20 effects on epithelial cell responses during *S. aureus* infection

Since its discovery in 2001, IL-20 has been almost exclusively studied for its role in disorders that are mediated by sterile inflammation: psoriasis and rheumatoid arthritis (Hsu and Chang, 2015; Leng et al., 2011; Stenderup et al., 2007). In these inflammatory disorders, IL-20 is upregulated and has therefore been considered a pro-inflammatory cytokine (Ouyang et al., 2011). Response to a cytokine requires receptor expression, and heterodimeric IL-20 receptors, consisting of IL-20RB dimerized with either IL-20RA or IL-22RA, are mostly expressed in skin and lung tissue (Dumoutier et al., 2001; Parrish-Novak et al., 2002). In spite of high expression of these receptors in the skin and lungs and known expression of these cytokines during inflammatory responses, there has been limited exploration of the role of IL-20, and the other members of its subfamily, IL-19 and IL-24, in the response to infection. One common finding for each of these IL-20 subfamily members is activation of antimicrobial peptide (AMP) production, indicating that this group of cytokines comprises a component of the innate immune response in the skin and lungs (Sa et al., 2007).

In one of the few studies carried out to examine the role of IL-20 receptor signaling in epithelial cells during infection, it was shown to modulate the immune response to cutaneous infection with *S. aureus* in an anti-inflammatory manner, inhibiting production of IL-1β, an effect observed in both mice and primary human keratinocytes (Myles et al., 2013). IL-19 and IL-24 were expressed in the murine model of cutaneous infection, while human keratinocytes expressed mostly IL-20 (Myles et al., 2013). In the present work, primary human epithelial cell line BEAS2b was found to

express IL-20 receptor chains for the Type II receptor, comprised of IL-22RA and IL-20RB. Examination of BEAS2b that were infected with *S. aureus* cells showed that IL-20, and to a lesser extent, IL-19, are produced in response to staphylococci. Conversely, IL-20 was not expressed in the lungs of mice that were intranasally inoculated with *S. aureus* in a model of pneumonia, rather transcripts for IL-19 and IL-24 were detected (Figure 5). The significance of this preferential expression of IL-20 by human cells and of IL-19 and IL-24 by murine tissue that was observed in both studies of responses to *S. aureus* is not known. Though IL-19, IL-20 and IL-24 are all studied in the context of inflammation, they appear to have unique functions (Ouyang et al., 2011; Rutz et al., 2014). The initial study of IL-20 receptor signaling in mice did not differentiate between the different cytokines, as the mice used were IL-20RB knock-outs and that receptor chain is required for signaling by all three. Administration of recombinant murine IL-19 or IL-20 to mice had similar effects in the cutaneous model (recombinant IL-24 was unavailable). However, human keratinocytes responded only to IL-20 and not IL-19 during *in vitro* challenge with *S. aureus* (Myles et al., 2013). Taken together with the higher expression and production levels of IL-20 from human keratinocytes and bronchial epithelial cells during infection, this indicates that IL-20 dominates the IL-20 receptor-based innate immune response to *S. aureus*.

In epithelial cells, IL-20 receptor signaling acts via STAT3 activation (Dumoutier et al., 2001), which was detected in BEAS2b cells that were treated with IL-20. This response was inhibited with antibody blockade of IL-20RB, further verifying that STAT3 was activated by ligation of IL-20 to its receptor. With receptor activation established, the effects of IL-20 on the responses of BEAS2b cells to *S. aureus* were evaluated by

examining both signaling and AMP production. Blockade of IL-20RB inhibited production of IL-8, a direct chemotactic factor for neutrophils. Additionally, production of the AMP RegIIIγ was activated in response to *S. aureus* and was mediated by IL-20, as addition of recombinant IL-20 significantly increased RegIIIγ concentration, while blockade of IL-20RB inhibited its production. While IL-20 subfamily cytokines are known to control AMP production, the effects of IL-20 on RegIIIγ, an AMP with known anti-staphylococcal activity (Sa et al., 2007), has not been previously reported. The enhancement of AMP production, along with inhibiting IL-8 production in lung epithelial cells, indicates that IL-20 receptor signaling has an anti-inflammatory role in the lungs during infection with *S. aureus*, similar to observations made in the murine model of cutaneous infection and human keratinocytes. Although protective effects mediated by AMP production and inhibition of IL-8 could not be observed in the *in vitro* modeling used here, the possibility of this purpose for IL-20 during infection should not be ruled out. Its inhibition of neutrophil migration, which was detrimental in the cutaneous model, and upregulation of a protein that can directly kill *S. aureus* may be critical for protecting the comparatively more fragile tissue of the lung, where damage from exuberant inflammatory responses can have fatal consequences.

IL-20 receptor expression and signaling in human neutrophils

While expression of heterodimeric IL-20 receptors is readily detectable in skin and lung tissue (Blumberg et al., 2001; Parrish-Novak et al., 2002), their expression in leukocytes has been elusive (Kunz et al., 2006; Wolk et al., 2002). In the first decade after the discovery and characterization of IL-20 receptors, there were two reports of

investigations that sought to characterize whether immune cells could be targets of the cytokines that signal through these receptors, namely IL-19, IL-20 and IL-24. In the first, Wolk et al. examined expression in resting human monocytes, NK cells, B cells and T cells by RT-PCR and found transcripts for IL-20RB, but not IL-20RA or IL-22RA; this study did not include granulocytes (Wolk et al., 2002). Later, a study by Kunz et al. evaluated IL-20 receptors in the same human leukocyte populations, again excluding granulocytes, but the cells were stimulated with various known activating agents; again, only IL-20RB expression was detected by RT-PCR (Kunz et al., 2006). The latter study also sought to detect IL-20 receptors on peripheral blood monocytes (PBMCs) by immunohistochemistry, and again found only IL-20RB expression. Additionally, the PBMCs did not respond to stimulation by IL-20 receptor cytokines IL-19, IL-20 or IL-24 with the expected STAT3 phosphorylation (Kunz et al., 2006). From these studies, which found no heterodimeric receptor expression or associated downstream signaling, it was concluded that immune cells are not targets of signaling by IL-20 or its related cytokines. However, the expression of these receptors on granulocytes was not evaluated and the significance of the expression of IL-20RB was not explored.

Studies on rheumatoid arthritis synovial fibroblasts (RASF) and membranes from human subjects, an assessment of tissue and cells that can include activated neutrophils engaged in an inflammatory response, revealed expression of IL-20 receptors as well as IL-20 itself (Hsu et al., 2006; Kragstrup et al., 2008). Although the specific cell population from which signaling originated was not identified, cells from these sources were shown to respond to IL-20 with ERK1/2 phosphorylation (Hsu et al., 2006). It appeared from these studies of rheumatoid arthritis that neutrophils may

express an IL-20 receptor during an inflammatory response that activates ERK1/2, instead of STAT3, in response to binding IL-20.

In the work presented here, IL-20 receptor expression on neutrophils was assessed, revealing expression patterns that were particular to the activation state of the neutrophil. Circulating, or resting, a highly variable percentage of neutrophils expressed IL-22RA, as little as 0.24% up to 95% with an average of 32% (Figure 9A). The other IL-20 receptor chains, IL-20RA and the common chain of the two types of heterodimeric receptor, IL-20RB, were rarely detected on resting neutrophils, an average of 3% and 0.7%, respectively (Figure 9A). Neutrophils are known to alter their cytokine and chemokine receptor expression as they migrate from circulation and go from their resting state to fully activated during a response to injury or infection (Borregaard et al., 2007; Nathan, 2006). Additionally, data from the aforementioned studies indicated that neutrophils may express a receptor for and respond to IL-20.

Compared to resting neutrophils isolated from peripheral blood of healthy volunteers, neutrophils that were activated by *in vivo* migration into blister chambers containing saline or different strains of γ-irradiated *S. aureus*, had reduced IL-22RA expression and a higher percentage of cells that expressed IL-20RB. The extravasated neutrophils appeared to express either one chain or the other, co-expression of IL-22RA and IL-20RB on the same cell was rarely detected. This receptor expression pattern could be mimicked with appropriate activating stimuli *in vitro*, namely, migration to infected BEAS2b cells or *S. aureus* alone in the presence or absence of IL-8, or adherent neutrophils that were primed with TNFα and infected with *S. aureus* (Figure 10).

In functional *in vitro* studies with neutrophils, these conditions that enhanced IL-20RB expression rendered neutrophils responsive to IL-20, such that activation of ERK1/2 was detected and required for IL-20-mediated effects on multiple functions relevant to infection. The requirement for IL-20RB was further supported by the low levels of expression of this receptor by unprimed neutrophils, and their relative lack of response to IL-20. However, the receptor expression profile induced by these stimuli, where neutrophils expressed IL-20RB instead of IL-22RA, does not appear to be that of the canonical heterodimer described from studies of IL-20 receptors on epithelial cells. Rather, it adds to the evidence from the previous studies of IL-20 receptors on different populations of leukocytes that found only IL-20RB expression (Kunz et al., 2006; Wolk et al., 2002). It is possible that leukocyte populations express a different type of IL-20 receptor, and ERK1/2 activation observed in the present study and in RASFs (Hsu et al., 2006), instead of the STAT3 activation observed in epithelial cells, provides further evidence of an alternate receptor that likely relies on IL-20RB expression. While the work presented here establishes that neutrophils that respond to IL-20 also express IL-20RB, additional experiments would be required to directly establish that IL-20RB, either alone or in concert with some other receptor chain, is necessary for cellular responses to IL-20.

Neutrophil migration is inhibited by IL-20

Multiple neutrophil functions require dynamic changes to the actin cytoskeleton, and cytokines and chemokines affect actin polymerization and distribution in defined ways that can be linked to downstream functional changes (Kutsuna et al., 2004; Lokuta

and Huttenlocher, 2005). TNFα is known to enhance neutrophil adhesion, granule exocytosis and arrest migration *in vitro*. When F-actin is visualized by fluorescence microscopy of phalloidin-stained neutrophils TNFα-primed neutrophils have a spread morphology with focal adhesions, and their cortical ring of polymerized actin is dissolved (Lokuta and Huttenlocher, 2005; Nathan, 2006). Similar examination of F-actin in neutrophils incubated with IL-8, the potent chemotactic factor, reveals a polarized morphology as the cells form a defined leading edge of F-actin to facilitate their migration (Lokuta and Huttenlocher, 2005; Weiner et al., 1999). Observation of neutrophil morphology and quantification of actin polymerization in the present study showed that treatment of infected neutrophils with IL-20, in conditions that enhanced IL-20RB expression, induced a slightly elongated morphology and significant changes to the amount of F-actin (Figure 11). As with TNFα and IL-8-mediated changes to the actin cytoskeleton, this effect of IL-20 indicated that this cytokine could affect multiple neutrophil functions.

Neutrophil migration requires specific coordinated cytoskeletal changes to facilitate forward motion and the integration of multiple signals to accurately guide the cell to its target site (Insall and Machesky, 2009; Weiner et al., 1999). ERK1/2 and p38 MAPK are known to act downstream of chemotactic factors and cytokines that activate them to affect migration in defined ways (Cara et al., 2001; Huang et al., 2004). A study by Liu et al., showed that ERK1/2 acted as a ‘stop’ signal for neutrophils in chemotaxis along a fMLF gradient, as it arrested migration, while p38 MAPK activity stimulated migration (Liu et al., 2012). The arrest of migration is an important aspect of neutrophil

recruitment, as it facilitates the switch from forward motion to engagement of bactericidal mechanisms.

The observed inhibitory effects of IL-20 on migration in time-lapse microscopy videos, transwell migration assays with IL-8 and *S. aureus* and its activation of ERK1/2 in neutrophils indicate that it directly inhibits migration by activating the pathways that act as a 'stop' signal for neutrophils (Figures 13-15). IL-20 also has demonstrated indirect effects on neutrophil migration, as it inhibited IL-8 production from infected BEAS2b cells, and IL-1β production by human keratinocytes and in the cutaneous model of infection in mice (Figure 5) (Myles et al., 2013). Taken together, these provide evidence that IL-20 functions to inhibit the recruitment of neutrophils from circulation by affecting the production of other host signals. It is possible that IL-20 serves both functions, directly arresting the migration of neutrophils that have reached the target site where it is being produced, and inhibiting recruitment by reduction of pro-inflammatory IL-1β or IL-8. IL-20 was previously shown to activate ERK1/2 in other cell types, namely, endothelial cells and RASFs (Hsu et al., 2006; Tritsaris et al., 2007); additionally, it has also been shown to inhibit the migration of dendritic cells *in vitro* (Bech et al., 2016). Additional studies would be required to define whether IL-20 directly modulates migration *in vivo.*

IL-20 regulates multiple neutrophil effector functions

Once neutrophils have arrested migration after being recruited to a site of *S. aureus* infection, they engage their bactericidal functions. A neutrophil's greatest capacity to destroy staphylococci lies in its ability to rapidly phagocytose the invading

organism and subject it to high amounts of ROS and other antimicrobial factors within the phagosome (Nauseef and Borregaard, 2014; Segal, 2005). In other words, though they can release NETs and other antimicrobial factors to the extracellular space, neutrophils rely largely on phagocytic uptake and intracellular killing mechanisms to destroy bacterial pathogens (Ferrante et al., 1993; Jann et al., 2011; Lee et al., 2003; Nordenfelt and Tapper, 2011). This appeared to be true in the *in vitro* killing assay used in the present study, as actin polymerization was required for the killing activity of TNFα-primed neutrophils (Figure 22). Significantly increased CFUs recovered from TNFα-primed neutrophils treated with IL-20 were linked to IL-20-mediated inhibition of uptake of *S. aureus*, as a lower percentage of bacteria were recovered from gentamicin protection assays (Figures 18 and 22). Other known killing mechanisms of neutrophils, including primary and secondary granule exocytosis, ROS production and NET release appeared to be unaffected by IL-20. However, phagocytosis relies on precisely controlled dynamic changes to the actin cytoskeleton, and the demonstrated IL-20-mediated changes to this fundamental aspect of neutrophil biology provides additional evidence that it affects this process. Of particular consideration is the significant increase in polymerized actin detected around the periphery of TNFα-primed neutrophils in the presence of IL-20, as phagocytosis requires depolymerization of actin in the final stage of particle uptake (Lee et al., 2003; Nordenfelt and Tapper, 2011).

Exocytosis of tertiary granules, rather than primary or secondary, also requires rearrangement of the actin cytoskeleton to dissolve the cortical ring of polymerized actin (Jog et al., 2007; Lacy and Eitzen, 2008; Mitchell et al., 2008). Indeed, IL-20 was found to inhibit only exocytosis of tertiary granules, as markers of primary and secondary

degranulation were unaltered in IL-20 treated neutrophils. This specificity, altering tertiary granule release alone, is likely due to the aforementioned affects of IL-20 on F-actin content and distribution in neutrophils. The significant reduction in gelatinase activity and MMP9 concentration (Figure 21), markers of tertiary granule exocytosis, in IL-20-treated neutrophils has potential downstream functional consequences in line with other observations of the effects of IL-20.

MMP9 has been directly linked to neutrophil migration through its collagenase activity, which is thought to play a role in allowing extravasating neutrophils to move through the extracellular matrix to reach the site of infection (Delclaux et al., 1996). Additionally, pro-IL-1β and IL-8 are targets of MMP9 proteolysis. Cleavage by MMP9 activates pro-IL-1β to its active form, and truncates IL-8 in a manner that increases its chemotactic and activating effects on neutrophils (Koymans et al., 2016; Van den Steen et al., 2000). The importance of this function during *S. aureus* infection has been demonstrated in studies of staphylococcal superantigen-like proteins 1 and 5 (SSL1 and SSL5), which bind to MMP9 to inhibit its function, thereby reducing cleavage of IL-8 and downstream neutrophil recruitment and activation (Itoh et al., 2010; Koymans et al., 2016). Other studies have directly linked MMP9 to neutrophil killing activity, as it enhanced killing of *S. aureus* by transendothelial neutrophils *in vitro* (Liu et al., 2016); and, neutrophils from MMP9 deficient mice were significantly inhibited in phagocytic uptake and killing of *Streptococcus pneumoniae in vitro* (Hong et al., 2011). Thus, MMP9 is a neutrophil protein with multiple functions during infection, which have both protective and deleterious consequences.

Its ability to activate pro-IL-1β and IL-8 indicates a pro-inflammatory role for MMP9, as this activity increases neutrophil recruitment and activity. In conjunction with its cleavage of extracellular matrix components, MMP9 has potential to aid host defense but also damage host tissue through its activities. Its damaging effects in the lungs have been documented in conditions like emphysema and chronic obstructive pulmonary disease (COPD), where it's significantly elevated due to increased neutrophil infiltration (Betsuyaku et al., 1999; Paone et al., 2011). Modulation of MMP9 release by IL-20 during infection with *S. aureus* is a major pathway through which the responses of neutrophils can be controlled, potentially providing a way to reduce neutrophil recruitment to limit damage to host tissue while having the detrimental effect of inhibiting neutrophil bactericidal functions.

Host signaling and the outcome of infection

This work has demonstrated how IL-20, a cytokine that is produced during skin and lung infection with *S. aureus*, directly affects the functions of neutrophils, the cells that are at the front line of host defense at these sites. When considering its significant inhibition of key pro-inflammatory signaling in multiple infection models, both *in vivo* and *in vitro*, and its dampening of key neutrophil functions, the evidence indicates that IL-20 acts as an anti-inflammatory mediator during infection. Infection induces an inflammatory response to protect the host, but there must be control of its damaging effects and an eventual return to homeostasis. Just as signaling is required to initiate inflammation, so is it required to resolve inflammation. To date, the most potent and well-known mediator of the anti-inflammatory response is IL-10, to which IL-20 is closely

related in both a shared chromosomal locus, sequence homology and secondary structure (Kotenko and Pestka, 2000; Logsdon et al., 2012; Ouyang et al., 2011). Although there are limited studies on the effects of IL-10 on neutrophils directly, the available evidence is similar to those observed with IL-20. Beyond its known anti-inflammatory effects on neutrophil cytokine production (Bazzoni et al., 2010), IL-10 has also been shown to inhibit phagocytosis of multiple pathogens, including *S. aureus,* by neutrophils (Roilides et al., 2000). Since its discovery, IL-20 has been studied almost exclusively in the context of its overexpression in rheumatoid arthritis and psoriasis, which established it as a pro-inflammatory mediator. However, its more recently established anti-inflammatory functions during infection align with its high level of homology with IL-10.

When combined with the previous study of IL-20 receptor signaling during infection with *S. aureus*, IL-20 can be incorporated into a model for the innate immune response to staphylococci. Once *S. aureus* is detected by epithelial cells and resident leukocytes, pro-inflammatory factors, including TNFα, IL-1β and IL-17, are produced to induce expression of chemokines to attract neutrophils. In the process of migrating from circulation into the infected tissue, neutrophils become primed so that their antimicrobial responses are enhanced and their cytokine receptor repertoire is expanded. This altered cytokine receptor expression includes upregulated expression of IL-20RB, likely allowing the neutrophils to respond to IL-20, which is also produced at the site of infection. IL-20 can signal to epithelial cells through Type I and/or Type II heterodimeric IL-20 receptors in epithelial cells to enhance production of antimicrobial peptides, to directly control growth of staphylococci, and inhibit production of pro-inflammatory

factors, to reduce neutrophil recruitment. For neutrophils that reach the site of infection, IL-20 can affect the dynamics of actin polymerization to dampen key effector functions, reducing the release of tertiary granule protein MMP9, arresting migration and inhibiting phagocytosis.

The balance between controlling infection and preserving host tissue is delicate, as the anti-inflammatory responses necessary for returning to homeostasis can contribute to the pathogenesis of infection by interfering with bactericidal mechanisms. In the attempt to preserve host tissue, IL-20 receptor signaling has demonstrated detrimental consequences in the cutaneous mouse model of infection with *S. aureus* (Kaplan, 2013; Myles et al., 2013). However, it is possible that IL-20 may succeed in providing protection to host tissue during lung infection with *S. aureus*. Bronchial epithelial cells produce this cytokine, and the lung is more vulnerable than the skin to the damaging effects of neutrophil proteases (Parker and Prince, 2012). The novel findings on the effects of IL-20 on neutrophils presented here add to the body of knowledge of the immune response to *S. aureus* and open up potential new avenues of investigation to better understand how cytokines affect the ultimate outcome of infection.

CHAPTER V

EXPERIMENTAL METHODS

Ethics statement

All human sample collection and processing were performed with approval of the National Institute of Allergy and Infectious Disease IRB, which approved the associated clinical trial (NCT02262819). All subjects gave full consent to sample collection, and all participants provided their written consent to the research protocol. All animal experiments were conducted in compliance with guidelines approved by the NIAID institutional animal care and use committee.

Cell culture, analysis and infection of BEAS2b cells

Culture of BEAS2b cells. Human bronchoepithelial cell line BEAS2b was obtained from the American Type Culture Collection (ATCC CRL-9609). BEAS2b cells were maintained at no greater than 75% confluence in collagen coated flasks with complete bronchial epithelial growth medium (BEGM, Lonza) at 37°C and 5% CO_2. For *in vitro* infection procedures, cells were seeded at low density in collagen coated 24 well plates and allowed to grow to 75% confluence before use.

Immunofluorescence microscopy of BEAS2b cells. Immunofluorescence microscopy was performed on BEAS2b cells that were cultured as described above on collagen coated glass coverslips. Cells were blocked with 10% goat serum and 5% BSA in PBS for at least one hour. IL-20 receptors were labeled using rabbit-derived αIL-20RA or αIL-22RB (Abcam), for Type I or Type II receptor, respectively, with rat-derived αIL-

20RB (eBioscience). Primary antibodies were incubated in blocking buffer at room temperature for one hour, followed by washing with PBS containing 0.1% Tween (Sigma). Immunoglobulin class matched isotype controls from same species and manufacturer were used for labeling controls. Secondary antibodies were anti-rabbit Alexa fluor 594 (red) or anti-rat Alexa fluor 488 (green), incubated in the same manner as primary antibodies. Cells were counterstained with 4'-6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) and mounted with ProLong Gold (ThermoFisher) according to manufacturer instructions.

Western blot for STAT3 signaling. For detection of STAT3 signaling by western blot, cells were grown in 24 well plates as described above and BEGM was replaced with either fresh BEGM alone or with indicated amounts of recombinant human IL-20 (rhIL-20) (R & D Systems). For select conditions, IL-20RB was blocked by addition of 1 μg of αIL-20RB or isotype control with fresh BEGM and allowed to incubate for 1 hour prior to addition of rhIL-20. Cells were lysed with RIPA buffer, 1 hour after the addition of rhIL-20, supplemented with protease and phosphatase inhibitor cocktails (Roche). Samples were stored at -80°C until use in western blot procedure. For SDS-PAGE, lysates were diluted in 6X sample buffer (0.5 M Tris, 10% SDS, 30% glycerol, 0.6M DTT, 0.012% bromophenol blue) and boiled for 10 minutes. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked for one hour (Odyssey blocking buffer, Licor). Membranes were probed with primary antibodies for rabbit-derived αPhospho-STAT3-Tyr705 or mouse-derived total STAT3 (Cell Signaling Technology), followed by washing and secondary antibodies anti-rabbit IRDye 680LT and anti-mouse IRDye 800CW (Licor). Blot images were acquired using a Licor scanner.

In vitro *infection of BEAS2b cells.* For measurement of their responses to *S. aureus,* or for assays where neutrophils migrated to BEAS2b cells or their supernatants, the epithelial cells were grown in 24 well plates as described above and BEGM was removed the night before the experiment and replaced with minimal media (BEBM, Lonza). The following day, BEBM was replaced with fresh BEBM alone or BEBM with *S. aureus* USA300 (MOI 1). In select procedures, IL-20RB was blocked as described previously for 1 hour before addition of *S. aureus*. Lysates for RNA isolation were obtained using RLT lysis buffer according to manufacturer's instructions (Qiagen) at 2 hours post-infection (p.i.). Supernatants were collected from wells and centrifuged at 8000 rpm for 5 minutes to obtain cell-free preparations at 6 hours p.i.

Measurement of BEAS2b cell responses to S. aureus. To measure gene expression, RNA was isolated from lysates collected as described above using RNeasy kit (Qiagen) according to manufacturer's instructions. RT-PCR was performed using Taqman gene expression assay primer-probe pairs for indicated targets in an Applied Biosystems 7500 Fast RT-PCR thermal cycler (ThermoFisher). Concentration of indicated cytokines was determined using BioPlex assay (Bio-Rad). Briefly, beads conjugated with antibodies for each target were mixed and added to 96 well plates, followed by addition of standards and samples to wells. After incubating and washing beads according to manufacturer's instructions, biotin-conjugated detection antibodies for each target were added and incubated and washed as before. Streptavidin-PE was then added and beads were washed following incubation. Signal from beads was acquired using a Luminex, and protein quantity was determined based on signal from standards.

RegIIIγ concentration was determined using ELISA with capture and HRP-conjugated detection antibody pairs (R & D Systems). After coating plate with capture antibody diluted in PBS overnight, plates were washed (0.05% Tween in PBS) and blocked (5% BSA in PBS) prior to addition of supernatant samples and protein standard. After 2 hours incubation, plates were washed four times and detection antibody was added and incubated for 1 hour at room temperature. Colorimetric substrate TMB (eBioscience) was added and 2N sulfuric acid was used to stop the reaction, and the plate was read at A_{405} (Beckman Coulter).

LDH assay was performed according to manufacturer's instructions (Promega), with colorimetric substrate added to cell supernatants in equal volume and incubated for 30 minutes. Total lysis sample was obtained by lysing all cells in select wells using provided lysis buffer. Equal volume of acetic acid was added to stop the reaction and the plate was read at A_{485}. Percent lysis was calculated by comparing the absorbance value for each sample to the value for the total lysis sample.

Staphyococcal growth in cell culture was measured by collection of cell supernatants at indicated times, serial dilution in sterile PBS and plating on BHi agar (Fisher Scientific). CFUs were enumerated on the following day after overnight incubation of agar plates at 37°C.

Intranasal infection of mice

To measure cytokine signaling in mouse lungs during infection with *S. aureus*, 10-12 week old C57Bl/6 mice were intranasally inoculated as follows. *S. aureus* USA300 was grown from refreshed overnight culture to OD_{600} of 1.0, washed two times with an equal

volume of sterile PBS and suspended to a final concentration of 1.4 x 10^9 CFU/mL. For inoculation, mice were anaesthetized with Isofluorane and 50 μL of sterile PBS or *S. aureus* suspension (7 x 10^7 CFU/mouse) was slowly administered into the nose. Mice were held upright for approximately 5 seconds and returned to their cage. After 6 or 48 hours, lungs were collected and placed in RNALater (Qiagen) and stored according to manufacturer's instructions. After homogenizing tissue in a TissueLyser I using sterile stainless steel beads (Qiagen), RNA was isolated using an RNeasy kit according to manufacturer's instructions, and subjected to RT-PCR as described previously.

Neutrophil Isolation and IL-20 receptor expression

Isolation from peripheral blood. Neutrophils were isolated from fresh heparinized venous blood obtained from healthy volunteers. All steps were performed at room temperature. Dextran was added to blood at a final concentration of 3% and erythrocytes were allowed to settle for 30 minutes. The leukocyte-rich supernatant was overlaid on Ficoll PLUS (GE Healthcare) and spun at 400xg for 40 minutes. Remaining erythrocytes were removed with hypotonic lysis, and neutrophils were counted using a hemacytometer and suspended in neutrophil media (RPMI+1% human serum albumin and 20 mM HEPES) at a final concentration of 1x10^7 cells/mL. Purity of the final cell suspension was assessed by flow cytometry and ≥97% were CD66b+ CD45+ neutrophils and contained ≤0.1% CD14+ cells (monocytes). Cells were >99% viable by Trypan blue exclusion and were used immediately following isolation. For relevant experiments, neutrophils were treated with vehicle (DMSO diluted 1:2000) or inhibitors: SB 202190 (10 μM) or PD 98059 (30 μM) at 37°C for 30 minutes.

Exudate neutrophils. Neutrophils that extravasated from circulation were obtained from healthy volunteers after induction of suction blisters as previously described (25). Briefly, blisters were induced on the forearm by suction and the epidermal roofs of the resulting blisters were removed. Chambers were placed around the dermal blister bases and filled with saline alone, or saline containing γ-irradiated strains of *S. aureus* from a healthy volunteer (SA1) or a patient with atopic dermatitis (SA2). Fluid from the chamber was removed after 24 hours, and cells were pelleted and resuspended in HBSS.

Flow cytometry for IL-20 receptor expression. All steps were performed with cold reagents and cells were kept on ice at all times. This procedure was performed on peripheral blood neutrophils, exudate neutrophils, neutrophils that migrated *in vitro* or TNFα-primed neutrophils. For neutrophils that migrated *in vitro*, labeling for IL-20 receptor chains was performed on cells that passed through transwell inserts to either infected BEAS2b cells (described above) or *S. aureus* in neutrophil media alone or supplemented with recombinant human IL-8 (R & D Systems). Cells were suspended in FACS buffer (Cell Staining Buffer, BioLegend) blocked with Human Fc block for 10 minutes prior to addition of antibodies for IL-20RA, IL-22RA (Abcam), IL-20RB (eBioscience), or species-appropriate isotype control antibodies at 10 μg/mL. Secondary and conjugate antibodies were added at concentrations according to manufacturer instructions: Brilliant Violent 421-anti-Rabbit, PE-anti-Rat, Alexa Fluor 647-anti-CD66b, FITC-anti-CD45 (BioLegend). Cell viability was determined by Zombie NIR Fixable Viability Kit (BioLegend) according to manufacturer instructions. Labeled cells were kept on ice and acquired on the flow cytometer (LSRFortessa, BD

Biosciences) immediately. Analysis was performed with FlowJo software, cells were gated on singlets, and dead cells were excluded before gating on target populations.

Measurement of neutrophil responses to IL-20.

Infection of human neutrophils with S. aureus*.* The *S. aureus* USA300 LAC strain was kindly provided by M. Otto (NIAID, Bethesda, MD). *S. aureus* was grown to mid-exponential phase in BHI (BD Biosciences) from a refreshed overnight culture and washed with HBSS. Neutrophils were added to 48 well plates that were coated with Coating Buffer, at $1x10^6$ cells per well in media. Neutrophils were either left unprimed or primed with TNFα at 10 ng/mL. For relevant experiments, cytochalasin D (10 μg/mL) or 10 U/mL DNase (Sigma) was added. Neutrophils were then incubated for 30 minutes at 37°C. *S. aureus* was then added at MOI 1, diluted in either media alone or media containing 50 ng/mL rhIL-20, and plates were centrifuged at 1200 rpm for 8 minutes to synchronize phagocytosis.

Samples were taken in quadruplicate at multiple time points between 0.5-4 hours for CFU enumeration. Neutrophils were lysed by adding pH 11 dH_2O to ensure lysis of phagosomes and total dispersal of staphylococci from cell membrane ghosts (Decleva et al., 2006). Lysate was serially diluted in sterile PBS and plated on BHI agar plates that were then incubated at 37°C, and CFUs were enumerated the following day. To enumerate intracellular staphylococci, 100 μg gentamicin was added at indicated time points and plates were incubated on ice for 20 minutes. Cells were washed two times with ice cold PBS to remove excess antibiotic, and then lysed, serially diluted and CFU were enumerated as described. Samples were taken in duplicate.

Immunofluorescence and confocal microscopy. For immunofluorescence by confocal microscopy, neutrophils were seeded on acid washed coverslips that were treated with Coating Buffer and placed in 24 well plates as described (26). Neutrophils were infected with *S. aureus* as described above and incubated at 37°C for 1 minute, supernatant was removed and cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Cells were permeabilized with 0.1% Triton-X 100 for 10 minutes at room temperature and cells were stained with Alexa Fluor 488-phalloidin (Invitrogen) according to manufacturer instructions. After counterstaining with DAPI, coverslips were mounted with Prolong Gold (Invitrogen).

Confocal images were collected using a Leica DMI8000 confocal microscope (Leica Microsystems) enabled with 63x oil immersion objective NA 1.4. Images were acquired using constant laser intensity for Argon Laser and 488 nm wavelength for Alexa fluor 488. Photons were collected using constant photomultiplier electronic gain between the samples to quantify the differences in absolute fluorescence intensity levels from different conditions. Twenty-five fields of view from each condition were collected in an unbiased manner using an automated tiling method. Acquired images were further analyzed using Imaris image processing software (Bitplane USA) to quantitate the absolute total fluorescence intensity per cell. Cumulative intensity calculated from 25 fields of view were plotted as average mean intensity normalized to total number of cells.

Western blotting. Neutrophil lysates were obtained from neutrophils infected with *S. aureus* as described above, incubated for 10 minutes at 37°C. Supernatant was aspirated from wells and lysis buffer (50 μL/well) was added to wells while the plate was

on ice. Samples were stored at -80°C until use. SDS-PAGE, transfer to nitrocellulose membrane and blocking were performed as described above. Membranes were probed with antibodies for phospho-ERK1/2 (9101), total ERK1/2 (9102), phospho-p38 MAPK (9211) or phospho-ATF-2 (9221) from Cell Signaling Technology or Actin (ab8227) from Abcam. Corresponding secondary antibodies (anti-rabbit 680LT or anti-mouse 800CW; Licor) were added. Membranes were stripped and reprobed as needed to assess multiple targets. Immunoblots were imaged using a Licor scanner.

Live imaging. For live imaging of neutrophils, Nunc Lab-Teck II Chambered Coverglass (8 well, ThermoFisher) was coated with Coating Buffer as described previously, and neutrophils were seeded at $5x10^5$ cells/well. The slide was placed inside a heated chamber (37°C) on a Leica AF 6000 LX microscope, stimulations were added (GFP-expressing *S. aureus* with 100 ng/mL IL-8 +/- 10 ng/mL IL-20). Images were taken in three locations in each chamber every minute for at least 30 minutes.

Transwell migration assays. For assays where neutrophils migrated to BEAS2b cells or supernatants, the epithelial cells were grown in 24 well plates as described above and BEGM was removed the night before the experiment and replaced with minimal media (BEBM, Lonza). The following day, BEBM was replaced with fresh BEBM alone or BEBM with *S. aureus* USA300 (MOI 2). Infected BEAS2b cells were incubated for 2 hours before addition of transwell inserts (3.0 μm pore, Corning) in each well, to which $1.5x10^6$ neutrophils were added. Neutrophils were allowed to migrate for three hours, at which point transwell inserts were removed and neutrophils were recovered from the bottom chamber by gentle pipetting. These cells were stained for IL-20 receptor and neutrophil markers as described above.

Alternatively, BEAS2b cells were incubated with *S. aureus* as described for four hours and supernatants were removed, sterile filtered (0.2 μm filter) and stored at -20°C until use. To remove IL-20 from supernatant, Protein G DynaBeads (ThermoFisher) were used according to manufacturer instructions with anti-IL-20 antibody (R & D Systems). Removal of IL-20 was confirmed by BioPlex assay (Bio-Rad).

To quantify neutrophil migration in response to BEAS2b cell supernatants or recombinant human (rh) IL-8 and/or rhIL-20 (R & D Systems), 24 well plates were coated with Coating Buffer: 20% autologous donor serum and 12 ng/mL ICAM-1 (R & D Systems) in HBSS with divalent cations (Cellgro) at 37°C for one hour and washed two times with HBSS. BEAS2b cell supernatant or neutrophil media containing 100 ng/mL rhIL-8 and/or 10 ng/mL rhIL-20 was added to the wells, with supernatant from uninfected cells or neutrophil media alone, respectively, used as negative controls. Neutrophils were allowed to migrate to BEAS2b cell supernatant for 3 hours or neutrophil media with recombinant human proteins for 1 hour. Neutrophils were then recovered from the lower chamber by gentle pipetting and counted manually using a hemacytometer. Each condition was performed in duplicate.

Assessment of neutrophil apoptosis and cell death. Annexin V-FITC antibody and propidium iodide (Sigma) were added to neutrophil samples, according to manufacturer's instructions, taken from indicated conditions at 4 hours p.i. Cells were washed, acquired on flow cytometer and analyzed using FlowJo software. LDH release assay (Promega) was performed on samples from indicated timepoints as described above.

Measurement of ROS production. Neutrophils were added to 96 well plates ($5x10^5$ cells/well) and primed and infected as described above. Each condition was set up in triplicate. Luminol (1 mM) was added to wells immediately following addition of *S. aureus*. The plate was incubated in a luminometer (Beckman Coulter DTX 880 Multimode Detector) set to 37°C and measurements were taken every four minutes for four hours.

Measurement of dsDNA release and granule exocytosis. Supernatants were taken from neutrophils infected with *S. aureus* as described above and spun down at 8000 rpm for 5 minutes to ensure removal of cells. Concentration of dsDNA was assessed as described by Fuchs et al.: 500 mU/mL of MNase (Sigma) was added to supernatants prior to centrifugation and activity was stopped with 5 mM EDTA (Fuchs et al., 2007). A Picogreen dsDNA kit was used to quantify concentration in samples and standard (Invitrogen).

Select targets for granule exocytosis were stained on neutrophils in indicated conditions at 1 hour p.i. using FITC-conjugated αCD63 (primary granules) and PerCP Cy5.5-conjugated αCD66b. Cells were acquired on a BD LSRFortessa flow cytometer and analyzed as described previously.

Primary (azurophilic) granule exocytosis was also measured by neutrophil elastase activity assay. Supernatants were incubated at 37°C overnight with equal volume of the colorimetric substrate N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (1 mM), and the reaction was read at A_{405}. Concentration of neutrophil elastase in samples was calculated based on a standard curve generated from recombinant neutrophil elastase. Sandwich ELISAs for lactoferrin and MMP9 were used to measure secondary (specific)

and tertiary (gelatinase) granule release, respectively. Antibody pairs for lactoferrin (capture: 265-1K1 and detection: HRP conjugate) and MMP9 (capture: IA5 and detection: biotinylated IIA5) were obtained from Thermo Fisher, and corresponding recombinant proteins were used as standards.

Zymograms were performed according to manufacturer (Invitrogen) instructions by separating samples on Novex 10% Zymogram Gelatin Protein gels, using renaturing buffer to renature proteins in the gel and developing buffer allowing digestion of gelatin to proceed overnight at 37°C. Areas of digested gelatin were visualized by Coomassie staining.

Data Analysis

All data are shown as means ± SEM and are representative or compiled from at least three separate experiments using blood from different donors. Statistical differences were analyzed by t-test or Two-way ANOVA using GraphPad Prism 7 software.

APPENDIX A

FIGURES

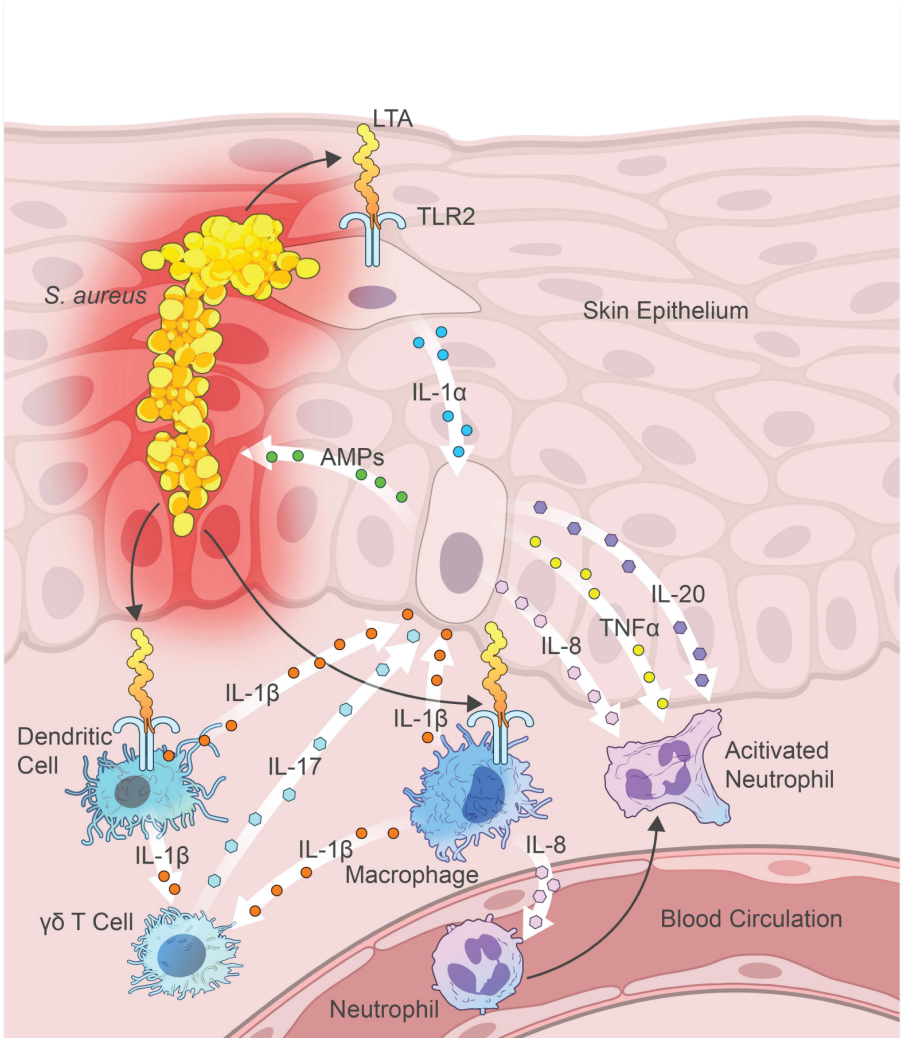


Figure 1. Immune signaling in skin during infection with *S. aureus*. Staphylococci breach the epithelial barrier and are detected by pattern recognition receptors, such as TLR2 binding lipoteichoic acid from the *S. aureus* cell wall, on epithelial cells (keratinocytes) or resident macrophages. Engagement of TLR2 results in release of IL-1α from keratinocytes and IL-1β from macrophages and other resident leukocytes. IL-1α activates production of antimicrobial peptides (AMPs) and chemokines from keratinocytes and IL-1β induces downstream inflammatory signaling, including IL-17 production by γδ T cells. Other pro-inflammatory factors, such as TNFα, are also produced. Together, the pro-inflammatory factors and *S. aureus* itself activate chemokine signaling, such as IL-8, to recruit neutrophils from circulation. *S. aureus* also induces IL-20 production from keratinocytes. Migrating from circulation alters neutrophil function and morphology, priming them for enhanced anti-microbial responses and altering their receptor expression to render them more responsive to the rich signaling environment encountered in the infected epithelium. Here, the neutrophils will encounter multiple signals simultaneously, including, but not limited to: IL-8, TNFα and IL-20. [Illustration courtesy of Ryan Kissinger, Department of Visual and Medical Arts, Research Technologies Branch, National Institute of Allergy and Infectious Disease.]

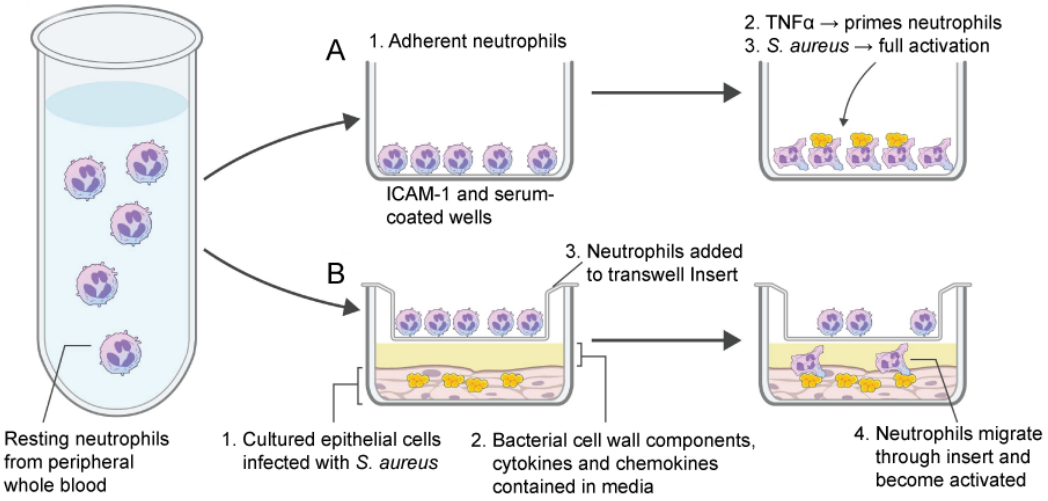


Figure 2. Priming and activation of human neutrophils *in vitro*. Neutrophils isolated from peripheral whole blood have a round morphology and are in a 'resting' state. To activate neutrophils *in vitro*, they can be either (A) adherent to ICAM-1 and serum coated wells and primed with TNFα and infected with *S. aureus*, or (B) allowed to migrate through transwell inserts to *S. aureus*-infected epithelial cells. [Illustration courtesy of Ryan Kissinger, Department of Visual and Medical Arts, Research Technologies Branch, National Institute of Allergy and Infectious Disease.]

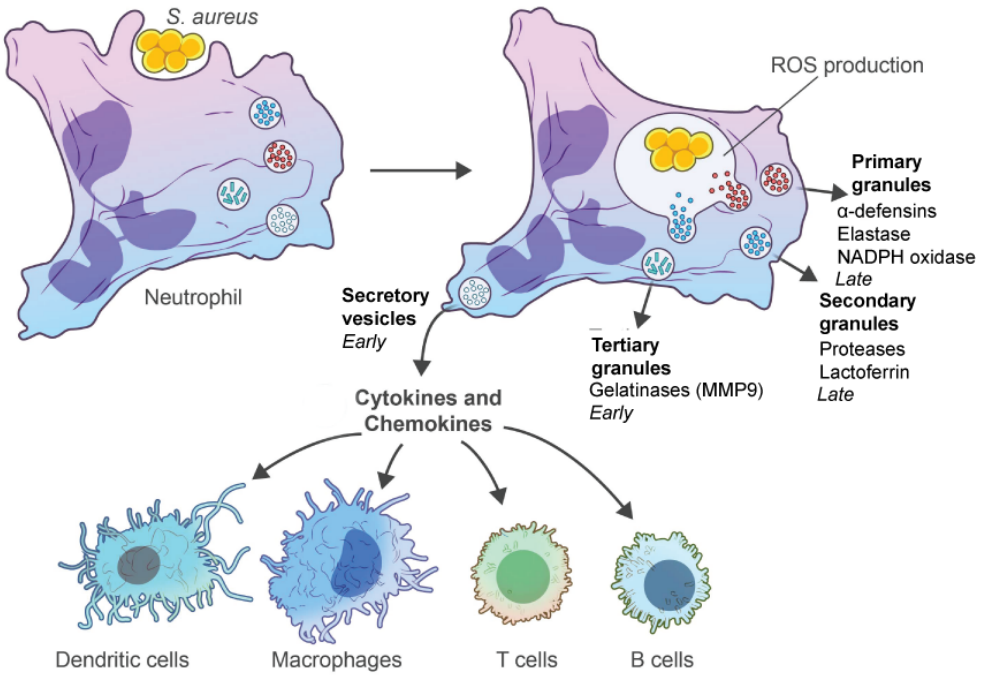


Figure 3. Overview of granule contents and degranulation. When a neutrophil migrates to infection and becomes activated by encountering host signaling factors and/or *S. aureus* it mobilizes its granules in a hierarchical manner. Early in the process secretory vesicles and tertiary granules are released. Secretory vesicles contain plasma proteins and various cytokines and chemokines that can signal to other cells. Tertiary granules contain enzymes that can degrade extracellular matrix components, and may aid in neutrophil migration. Later, once the neutrophil encountered *S. aureus* and phagocytosed staphylococci, primary and secondary granules fuse with the phagosome and are also released into the extracellular space. Primary and secondary granules contain antimicrobial factors such as α-defensins, NADPH oxidase components and proteases. [Illustration courtesy of Ryan Kissinger, Department of Visual and Medical Arts, Research Technologies Branch, National Institute of Allergy and Infectious Disease.]

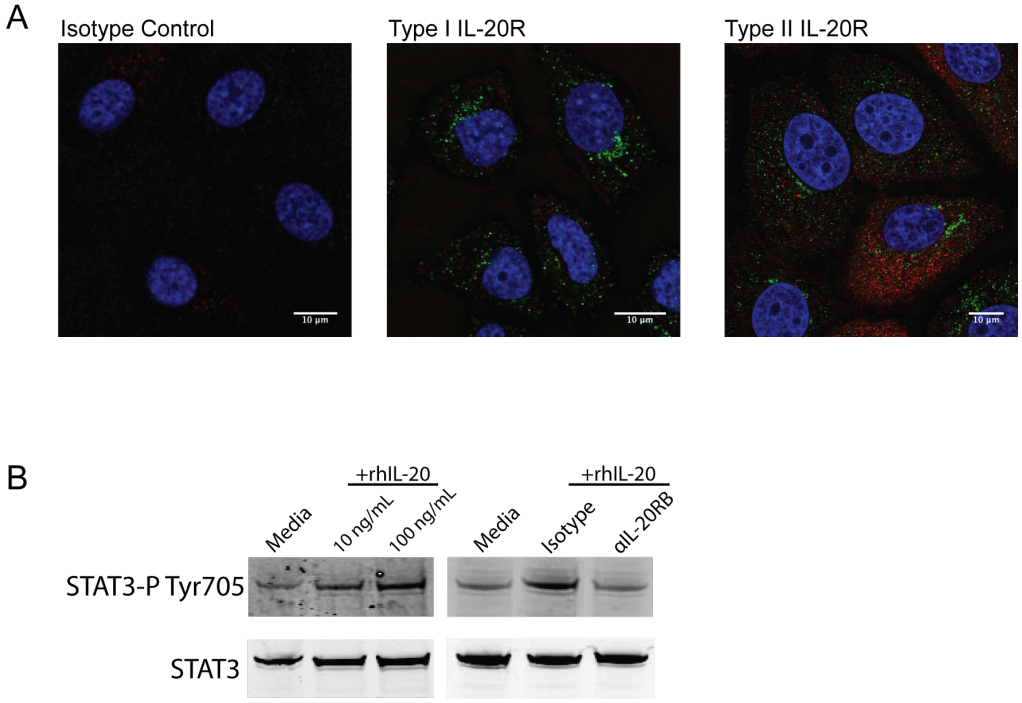


Figure 4. BEAS2b cells express a Type II IL-20 receptor that signals via STAT3 activation. (A) Immunofluorescence staining for Type I or Type II IL-20 receptor was performed on BEAS2b cells cultured on collagen-coated glass coverslips. For both Type I and Type II receptors, rat-derived anti-IL-20RB labeled with anti-rat Alexa fluor 488 (green). For Type I receptor, rabbit-derived anti-IL-20RA labeled with Alexa fluor 594 (red) and for Type II receptor, rabbit-derived anti-IL-22RA labeled with Alexa fluor 594 (red). Isotype control is Rat IgG2a, labeled with Alexa fluor 488 (green) and rabbit IgG labeled with Alexa fluor 594 (red). In all panels, DAPI is blue. (B) Western blot of BEAS2b cell lysates for STAT3 activation after 1 hour incubation with rhIL-20 at indicated doses or after 20 minute pre-incubation with 1 μg/mL isotype control or anti-IL-20RB antibody and subsequent 1 hour incubation with 10 ng/mL rhIL-20. Data shown is representative of three independent experiments.

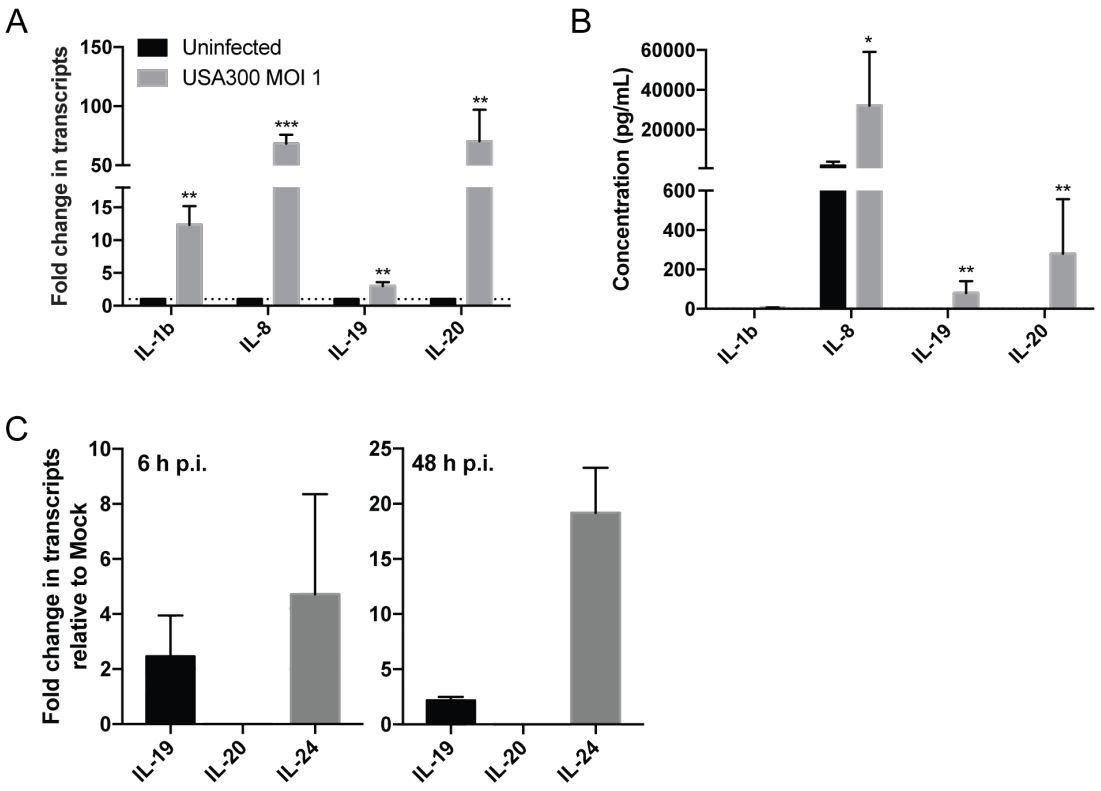


Figure 5. IL-20 is expressed by BEAS2b cells during *in vitro* infection with *S. aureus*. BEAS2b cells were incubated with *S. aureus* USA300 at a MOI=1 and lysates and supernatants were subjected to assays for select cytokines. (A) Fold expression in cells at 2 hours p.i. by RT-PCR of indicated cytokines in infected cells relative to uninfected, normalized to GAPDH. (B) Concentration of indicated cytokines measured by BioPlex assay of supernatants from uninfected and infected cells at 6 hours p.i. (C) C57/Bl6 were inoculated intranasally with $8x10^7$ CFU *S. aureus* USA300 in a sublethal pneumonia model. RNA was isolated from lungs that were removed at indicated timepoints and subject to RT-PCR . Gene expression was normalized to GAPDH and is shown as fold increase in transcripts relative to mock infected mice that were inoculated with sterile PBS. Data for all graphs are combined from three independent experiments, and statistical significance was determined by t-test.

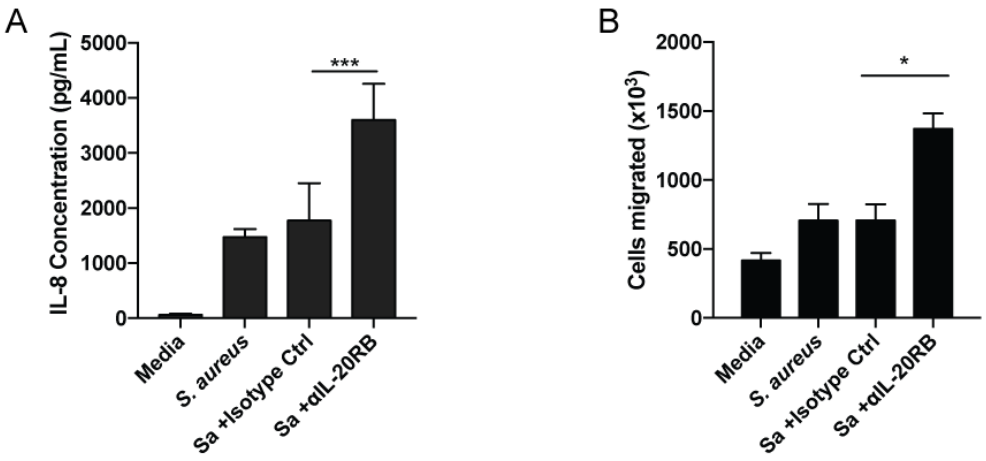


Figure 6. Signaling through IL-20 receptor inhibits production of IL-8 and reduces neutrophil migration. BEAS2b cells were pretreated with 1 μg/mL isotype control or anti-IL-20RB antibody for one hour and infected with *S. aureus* USA300 at a MOI=1. (A) Concentration of IL-8 in BEAS2b cell supernatants from indicated conditions at 4 hours p.i., measured by BioPlex assay. (B) Quantification of neutrophils that migrated during transwell migration assay from transwell inserts with 3 μm pore into chamber containing infected BEAS2b. Transwell inserts were added to wells containing BEAS2b cells at 2 hours p.i. and neutrophils were allowed to migrate for 3 hours. Each condition was performed in duplicate. Inserts were then removed and neutrophils were collected in supernatant from the lower chamber and counted manually in a hemacytometer. Data is representative of three independent experiments, and statistical significance was determined by t-test.

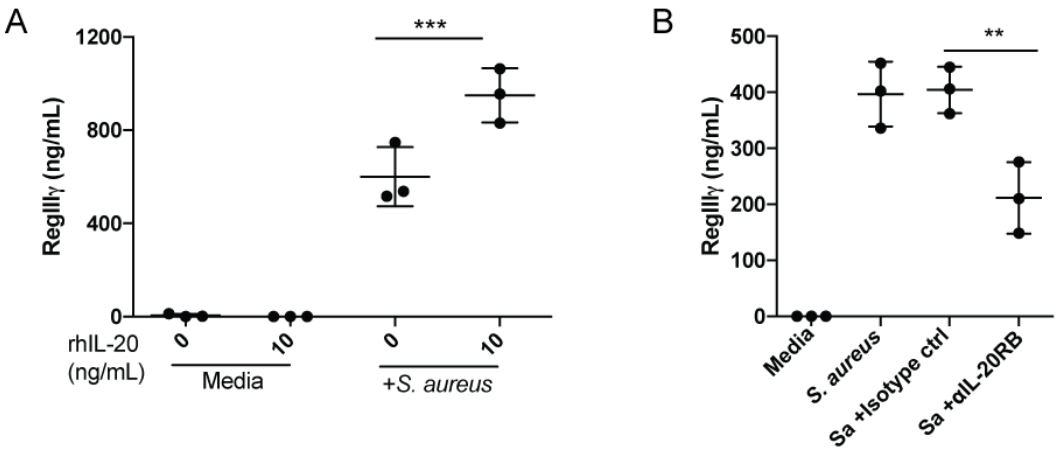


Figure 7. IL-20 and IL-20 receptor signaling enhances RegIIIγ production. Detection of RegIIIγ in supernatants of BEAS2b cells at 4 hours p.i. that were infected with *S. aureus* USA300 at a MOI=1 and (A) 0 or 10 ng/mL rhIL-20 or (B) pretreated for one hour with 1 μg/mL isotype control or anti-IL-20RB antibody. Data is combined from three independent experiments, and statistical significance was determined by t-test.

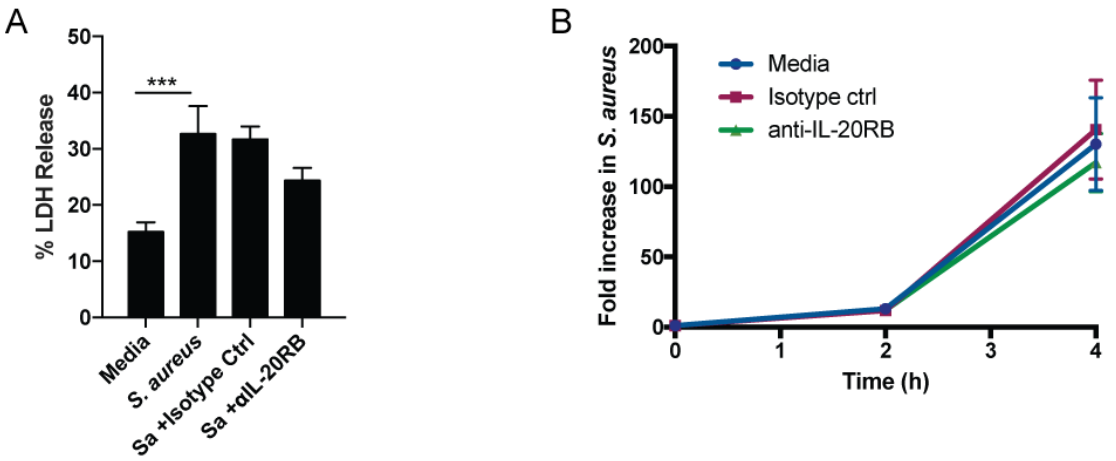


Figure 8. IL-20 receptor signaling does not provide protection for BEAS2b cells during *in vitro* infection with *S. aureus*. BEAS2b cells were pretreated with 1 μg/mL isotype control or anti-IL-20RB antibody for one hour and infected with *S. aureus* USA300 at a MOI=1. (A) Percent cell lysis was measured by LDH release assay of supernatants at 4 h p.i., percent shown is relative to lysis of all cells. (B) *S. aureus* growth in culture media of wells containing BEAS2b cells in the indicated conditions expressed as fold increase of CFUs relative to *S. aureus* incubated in wells containing cell culture media alone. Data is combined from three independent experiments, and statistical significance was determined by t-test.

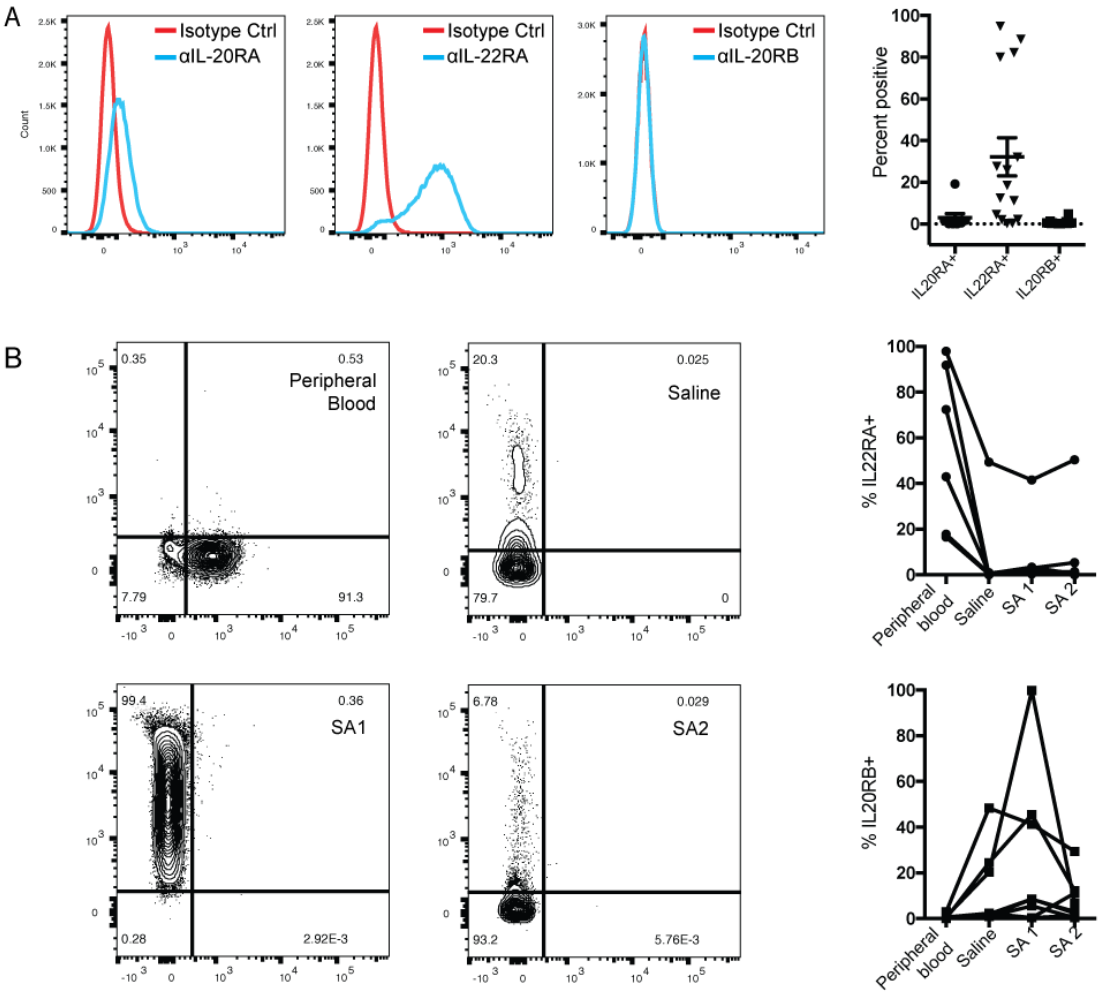


Figure 9. Human exudate neutrophils that migrated *in vivo* have altered IL-20 receptor expression. (A) Detection of indicated IL-20 receptor chains on live neutrophils isolated from peripheral whole blood. Flow cytometry histograms from a representative donor (left) and summary data from multiple donors of percentage of neutrophils staining positive for each receptor chain (right) are shown. (B) Detection of IL-20R receptor chains on live neutrophils that extravasated from circulation into suction blisters induced on healthy volunteers. The suction blisters contained saline or killed *S. aureus* (SA1 or SA2, two distinct clinical isolates). Flow cytometry dot plots from a representative blister subject (left) and summary data from multiple subjects (right, each line connects data from a unique subject, n=6) are shown.

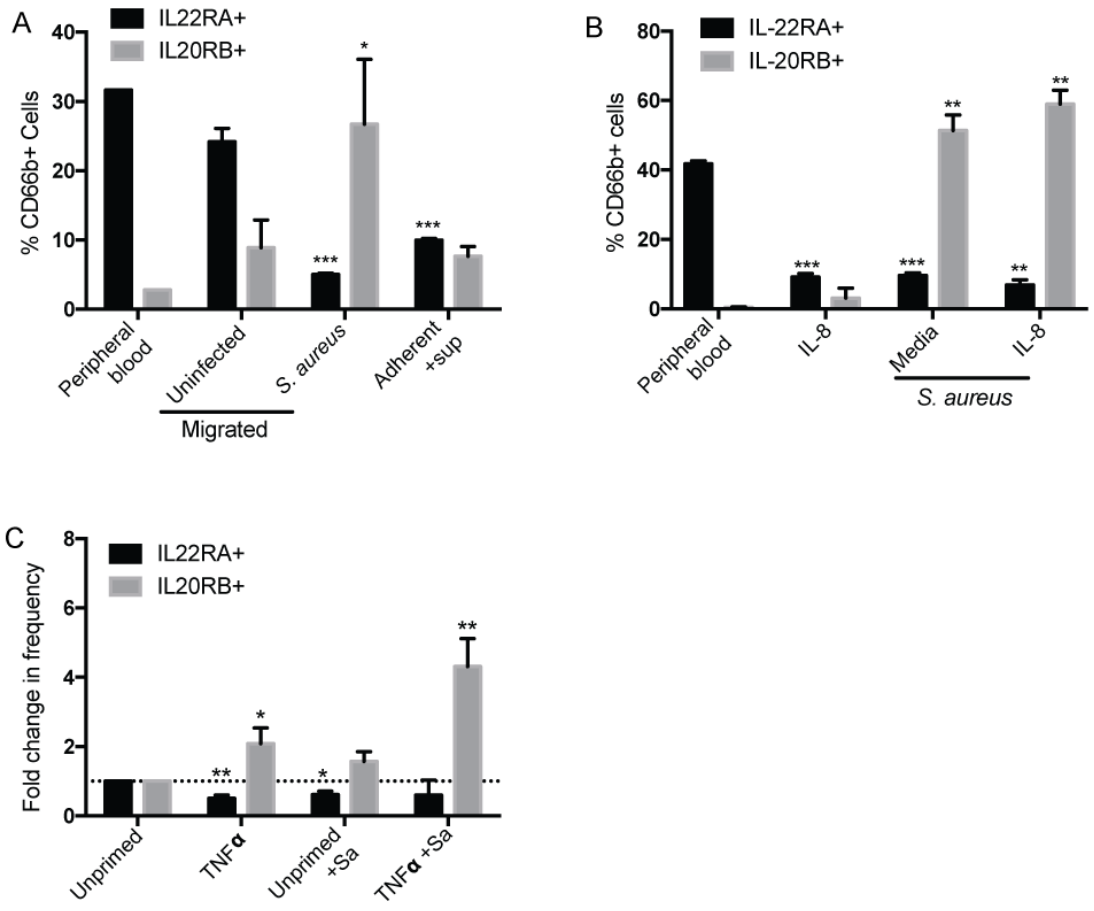


Figure 10. Neutrophil IL-20 receptor expression is altered via migration or activation *in vitro*. Healthy donor-derived peripheral blood neutrophils were assessed for IL-20R chain expression after exposure to indicated conditions *in vitro*. In (A), neutrophils were stained directly after isolation (peripheral blood), after migration across transwell inserts to uninfected or S. aureus-infected BEAS2b cells, or after adherence and exposure to supernatant from S. aureus-infected BEAS2b cells (adherent+sup). In (B), neutrophils were stained directly after isolation (peripheral blood) or after migration across transwell inserts into wells containing IL-8 (100 ng/ml), S. aureus ($2x10^6$ CFU/well), or both. In (C), neutrophils were incubated in media (unprimed) or primed with TNFα (10 ng/ml, 30 minutes) and then incubated for 1 hour in the absence or presence of *S. aureus* (Sa, $1x10^6$ CFU/well=MOI 1). Data show mean frequency of live CD66b+ cells that express indicated receptor chains from three healthy volunteers (A-B), or fold-change in frequency of receptor-positive cells relative to unprimed neutrophils in 6 healthy volunteers (C). Error bars reflect SEM. *p< 0.05, **p<0.01, ***p<0.001 compared to peripheral blood (A-B) or unprimed (C) neutrophils.

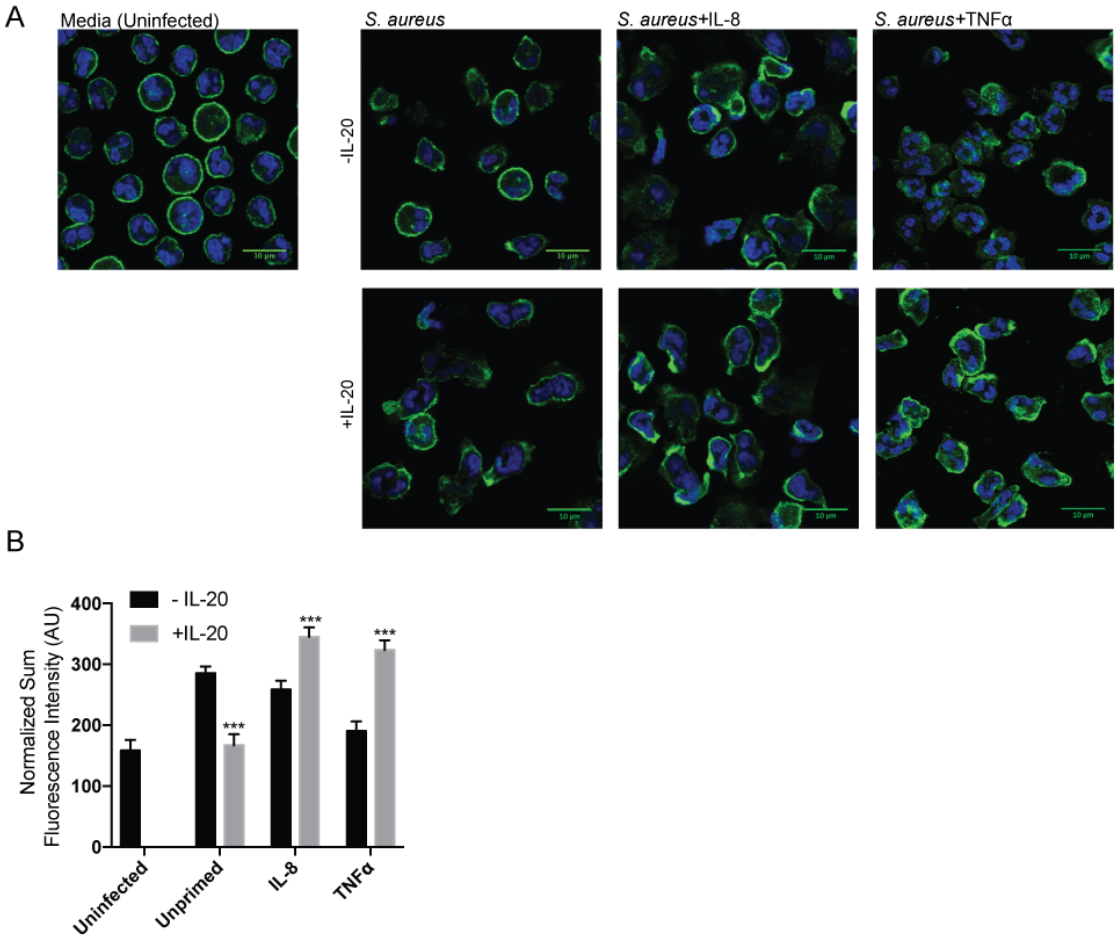


Figure 11. IL-20 modulates actin polymerization in neutrophils. (A) Representative confocal microscopy images of neutrophils incubated with indicated stimulation for 1 minute (Sa, *S. aureus* 2x10^6 CFU/well; IL-8, 100 ng/mL; TNFa, 10 ng/ml). Green, Alexa Fluor 488-phalloidin; blue, DAPI. (B) Quantification of Alexa Fluor 488-phalloidin fluorescence intensity per cell from analysis of 25 fields of view per condition that were generated by an automated tiling method and normalized to the total number of cells across all images with standard deviation. Data is representative of results from three different donors. ***p<0.001 by t test.

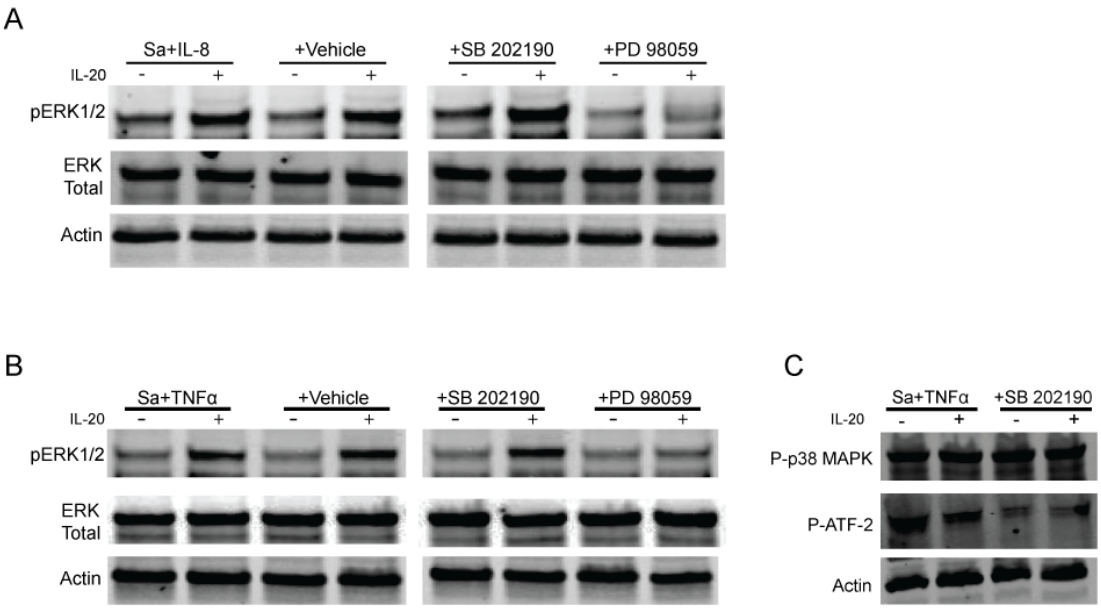


Figure 12. IL-20 signals via ERK1/2 in activated neutrophils. Western blots of lysates from neutrophils that were untreated or treated with indicated inhibitors for 30 minutes, then infected with *S. aureus* (MOI 1) and (A) stimulated with IL-8 alone or with IL-20 for 10 minutes or (B and C) TNF alone or with IL-20 for 10 minutes. Results shown are representative of neutrophils from 3 healthy donors.

Figure 13. Live imaging videos in supplemental file. IL-20 inhibits random neutrophil migration. Live imaging of neutrophils in chambered coverglass ($5x10^5$ cells/chamber) with media alone (Video 1), GFP-expressing *S. aureus* (MOI 1) + IL-8 (100 ng/mL) (Video 2), or GFP-expressing *S. aureus* (MOI 1) + IL-8 and IL-20 (10 ng/mL) (Video 3). An image was acquired from three different positions per condition every 1 minute for 30 minutes. Data shown is representative of images obtained from three different healthy donors.

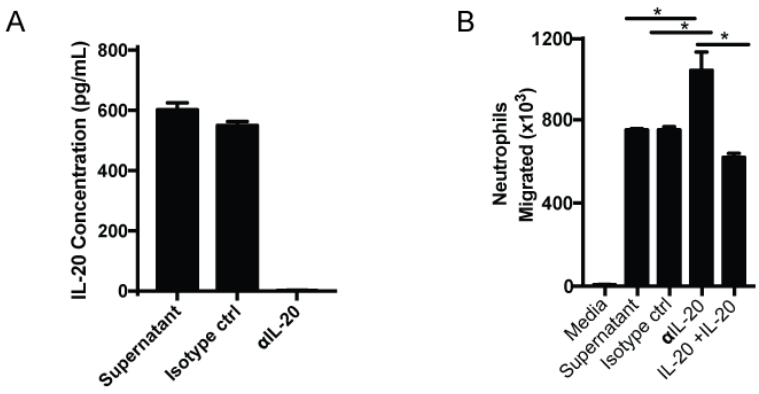


Figure 14. Depletion of IL-20 from supernatant of infected BEAS2b cells enhances neutrophil migration. (A) Sterile filtered supernatant from BEAS2b cells that were infected with *S. aureus* (MOI 2) for 4 hours and assayed via BioPlex for IL-20 concentration. Supernatant was was subjected to immunoprecipitation with an anti-IL-20 antibody or isotype control. (B) $1.5x10^6$ peripheral blood neutrophils were placed in the upper chamber of transwell plates. After 3 hours, the number of cells that migrated across the transwell insert to lower chambers was counted using a hemacytometer. The lower chambers contained media or supernatants from human bronchial epithelial (BEAS2b) cells that had been infected with *S. aureus* (MOI 2) for 4 hours, sterile-filtered, and then subjected to immunoprecipitation with isotype control or IL-20 antibody. Under some conditions (αIL-20+IL-20), recombinant IL-20 (10 ng/mL) was added back to supernatant after IL-20 immunoprecipitation. Data shown reflect mean +/- SEM of results from three donors, and *$p<0.05$, **$p<0.01$ by t test.

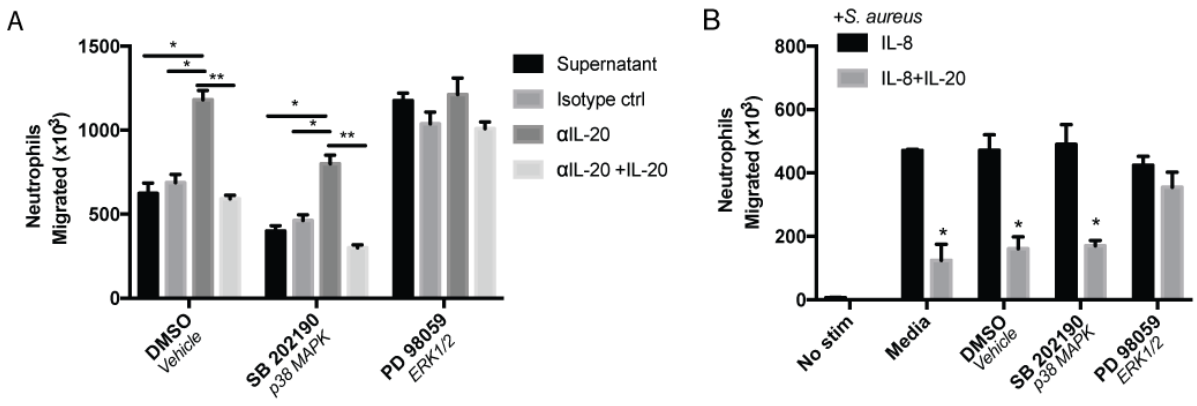


Figure 15. Inhibition of ERK1/2 abrogates IL-20 effects on neutrophil migration. (A) Neutrophils were treated with indicated inhibitors and stimulated to migrate across a transwell chamber into a lower chamber containing supernatants from BEAS2b cells that had been infected with *S. aureus* (MOI 2) for 4 hours, sterile-filtered, and then subjected to immunoprecipitation with isotype control or IL-20 antibody. Under some conditions (αIL-20+IL-20), recombinant IL-20 (10 ng/mL) was added back to supernatant after IL-20 immunoprecipitation. (B) Neutrophils untreated or treated with indicated inhibitors and stimulated to migrate across transwell inserts for 1 hour to wells containing *S. aureus* ($2x10^6$ CFU/well) + IL-8 (100 ng/mL) alone or with IL-20 (10 ng/mL). Data shown reflect mean +/- SEM of results from three donors, and *$p<0.05$, **$p<0.01$ by t test.

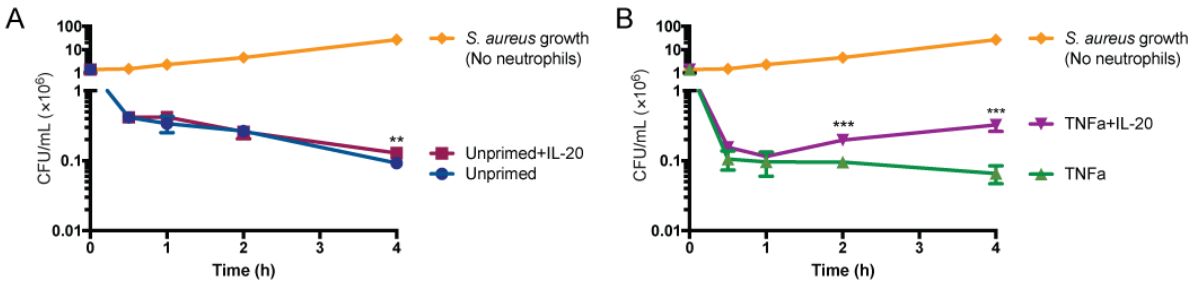


Figure 16. IL-20 inhibits killing of *S. aureus* by TNFα-primed neutrophils *in vitro*. Bacterial CFU recovered from adherent peripheral blood neutrophils that were unprimed (A) or primed with TNFα (10 ng/mL, 30 min) (B) and then incubated with *S. aureus* (MOI 1) in the presence or absence of IL-20 (50 ng/mL) for the indicated time. Data shown are the mean of results from seven donors with each donor assayed in quadruplicate.

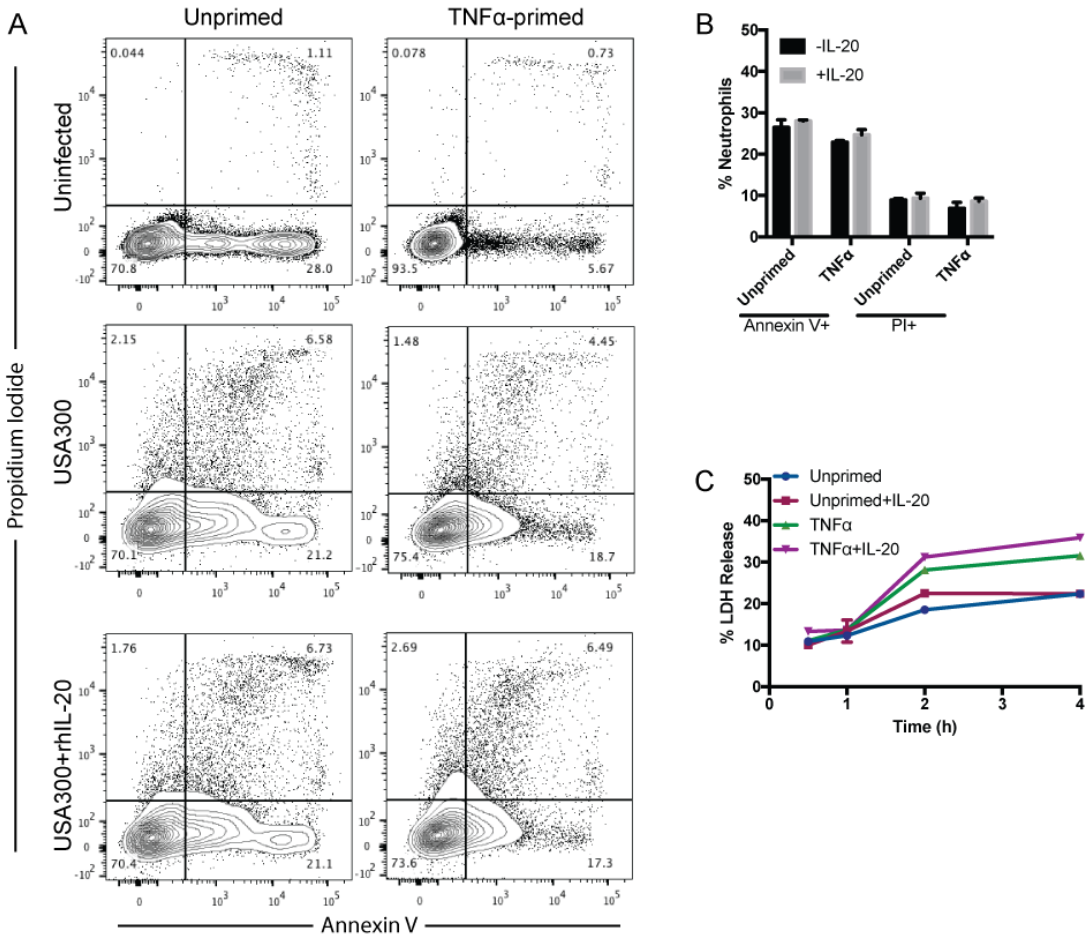


Figure 17. Neutrophil apoptosis and lysis are not altered by IL-20. Apoptosis and cell death of neutrophils in the *in vitro* killing assay was evaluated by staining cells with anti-Annexin V antibody and propidium iodide (PI) at 4 hours post-infection. (A) Dot plots of Annexin V and PI stained cells acquired via flow cytometry. (B) Quantification of flow cytometry dot plots indicates total percentage of Annexin V+ (apoptotic) and PI+ (dead) cells. (C) Percent cell lysis, based on comparison to sample of fully lysed cells, by LDH assay of cell free neutrophil supernatants in same conditions at indicated timepoints. Data shown reflect mean +/- SEM of results from four donors and no significant differences were found by t test.

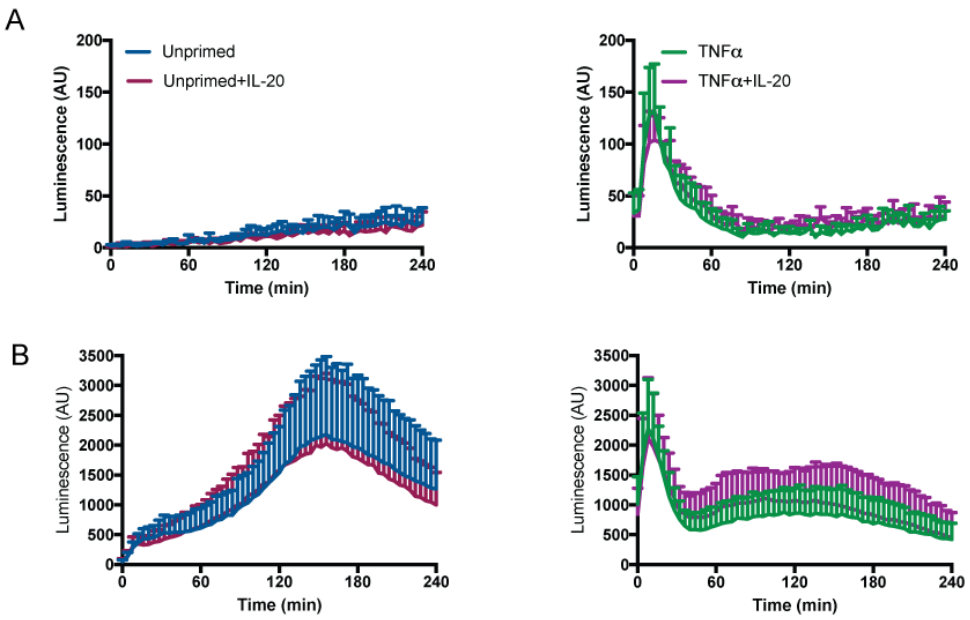


Figure 18. IL-20 does not affect ROS production by neutrophils. ROS detected by luminescence of luminol (1 mM) added to wells with unprimed or TNFα-primed neutrophils that were (A) uninfected or (B) infected with *S. aureus* (MOI 1) in the presence or absence of IL-20 (50 ng/mL) as described for the *in vitro* killing assay. Conditions were performed in triplicate and measurements were taken every 4 minutes; data shown as mean +/- SEM of results from four donors.

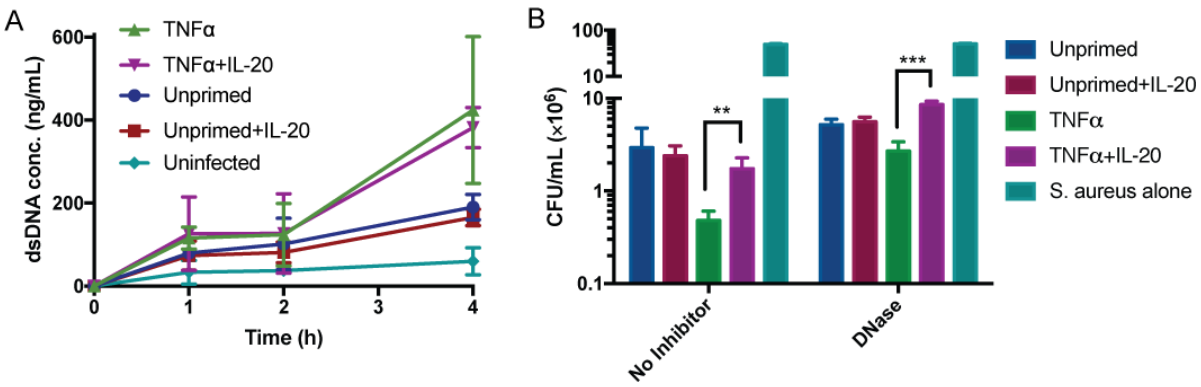


Figure 19. IL-20 does not alter release of dsDNA from neutrophils during infection with *S. aureus*. (A) Concentration of dsDNA in DNase treated cell-free supernatants from indicated timepoints of neutrophils that were primed with TNFα (10 ng/mL) or unprimed and infected with *S. aureus* in the presence or absence of IL-20 (50 ng/mL). Values for unprimed uninfected neutrophils are also included. (B) CFUs recovered at 4 hours post-infection when neutrophils were incubated as previously described with *S. aureus* in indicated conditions without inhibitor or with 10 U/mL DNase to inhibit NET formation. Data shown are mean +/- SEM of results from three donors **p<0.01, ***p<0.001 by t test.

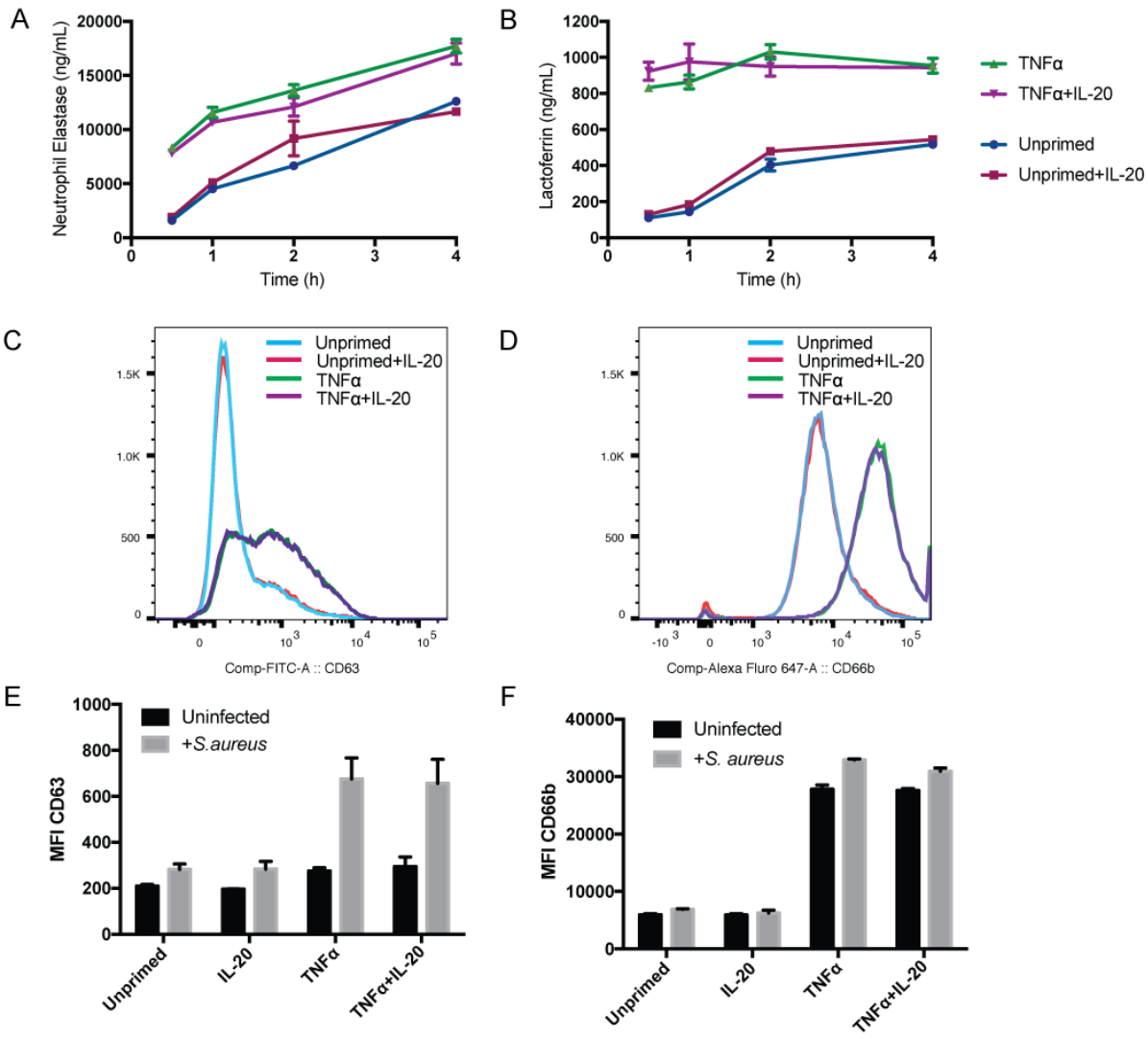


Figure 20. Primary and secondary granule exocytosis are not affected by IL-20. (A) Concentration of neutrophil elastase (primary/azurophilic granule marker) in supernatants from human neutrophils, incubated with *S. aureus* MOI 1 for indicated times, measured by reaction with colorimetric substrate, calculated using standard of known concentration. (B) Lactoferrin concentration, marker for secondary (specific) granules, measured by ELISA of neutrophil supernatants. Data shown in (A) and (B) as mean +/- SEM from three healthy donors. (C and D) Representative histograms of (C) CD63 and (D) CD66b expression on neutrophils that were incubated with *S. aureus* for 1 hour, membrane markers of primary or secondary granule exocytosis, respectively. (E and F) Graphs showing quantification of (E) CD63 or (F) CD66b expression by mean fluorescence intensity (MFI) obtained from histograms for uninfected or infected neutrophils in the indicated conditions. Data shown in C-F is representative of four different donors.

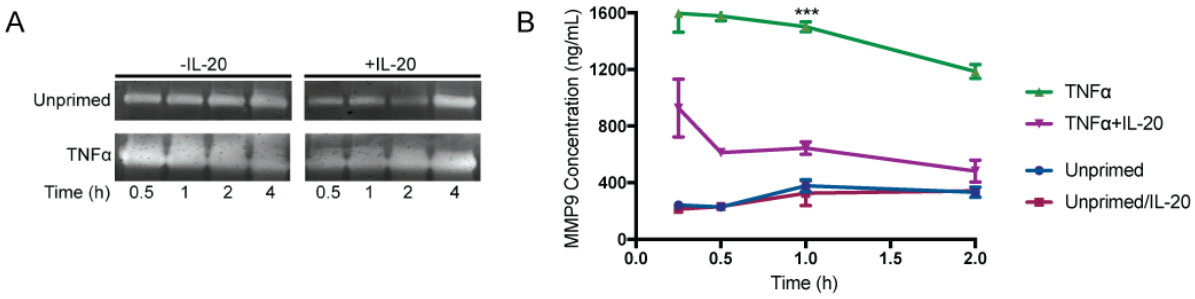


Figure 21. Exocytosis of tertiary neutrophil granules is inhibited by IL-20. (A) Gelatin zymography of supernatants from neutrophils infected for indicated times with *S. aureus* (MOI 1). Results shown are representative of neutrophils from four independent healthy donors. (B) Concentration of MMP9, marker for tertiary (gelatinase) granule release, in supernatants of neutrophils infected for indicated times with *S. aureus* (MOI 1) +/- IL-20. Data shown as mean +/- SEM from four healthy donors. ***p<0.001 for TNFα compared to TNFα+IL-20-treated neutrophils by Two-way ANOVA.

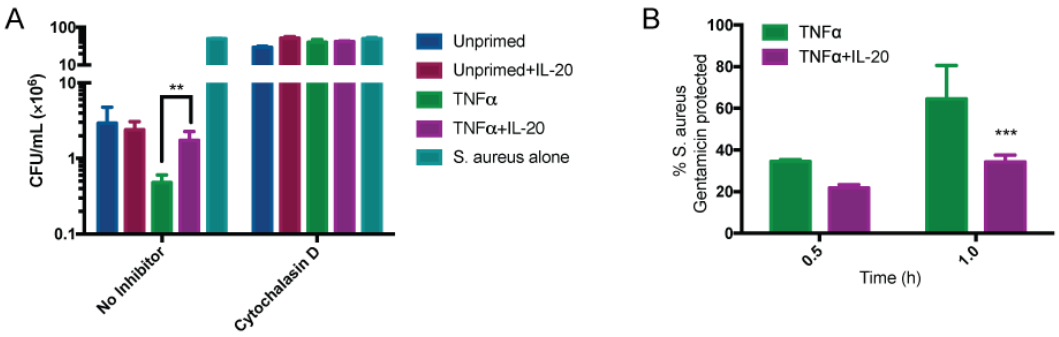


Figure 22. IL-20 inhibits phagocytic uptake of *S. aureus* by TNFα-primed neutrophils. (A) Neutrophils primed with TNFα and then incubated with *S. aureus* (MOI 1) +/- IL-20 as previously described, in the absence or presence of cytochalasin D (10 μg/mL). CFU were determined in quadruplicate for each sample at 4 hours post-infection. Data shown are mean +/- SEM of results from three donors. (B) Gentamicin was added at the indicated time points after infection to kill extracellular bacteria and CFU were enumerated following neutrophil lysis. Data shown as percent of *S. aureus* remaining compared to average of total *S. aureus* present in duplicate gentamicin-untreated wells and reflect mean +/- SEM of results from four donors. For (A and B): **p<0.01, ***p<0.001 by t test of TNFα compared to TNFα+IL-20-treated neutrophils.

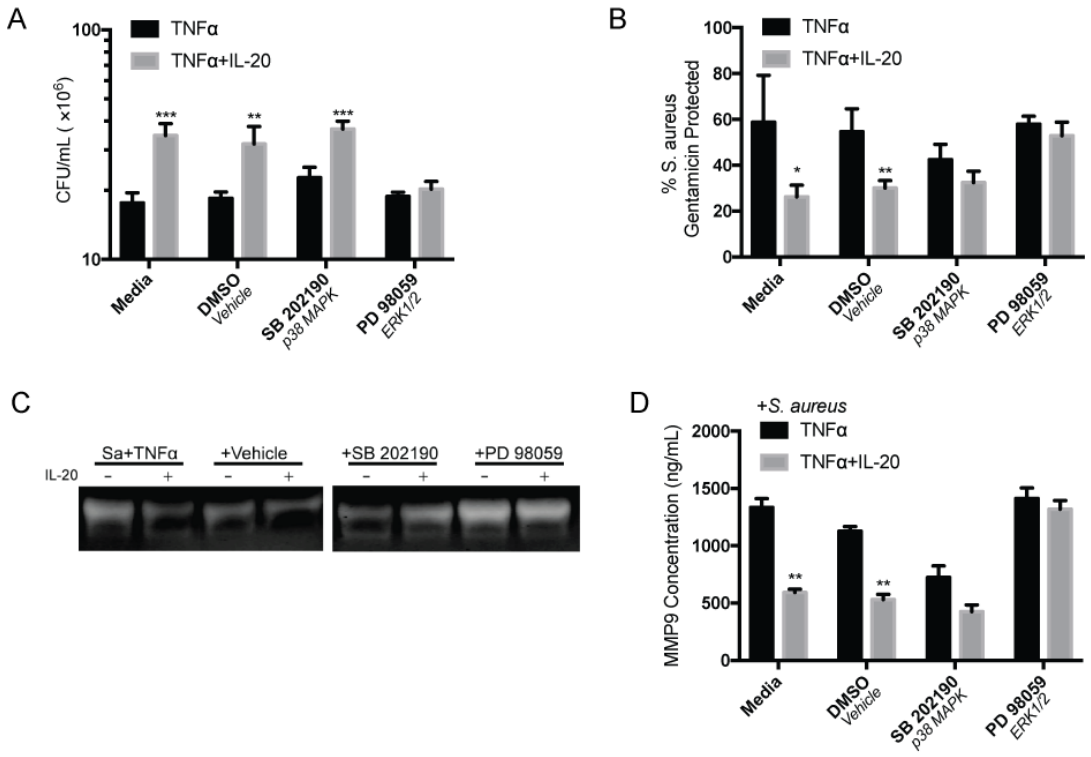
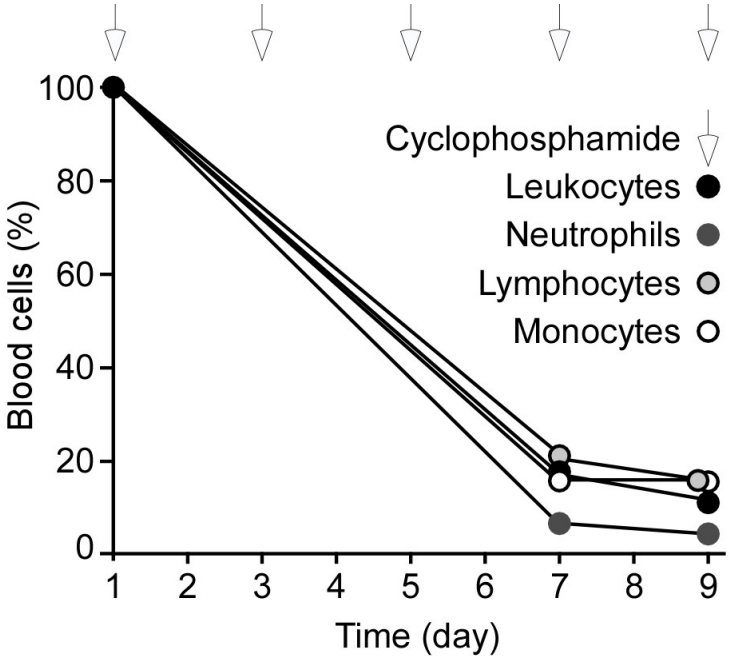


Figure 23. Inhibition of phagocytosis and tertiary granule exocytosis by IL-20 is mediated by ERK1/2. (A) *S. aureus* CFU recovered from adherent peripheral blood neutrophils that were treated with the indicated inhibitors and then incubated with *S. aureus* (MOI 1) and TNFα (10 ng/mL) in the presence or absence of IL-20 (50 ng/mL) for four hours. (B) Neutrophils were treated with indicated inhibitors and incubated with *S. aureus* and TNFα +/- IL-20 as previously described. Gentamicin was added at the indicated time points after infection to kill extracellular bacteria and CFU were enumerated after lysing neutrophils. Data shown as percent of *S. aureus* remaining compared to average of total *S. aureus* present in duplicate gentamicin-untreated wells. (C) Gelatin zymography of cell free supernatants from neutrophils incubated with for one hour with *S. aureus* (MOI 1) and TNFα +/- IL-20 after treatment with indicated inhibitors. Results shown are representative of neutrophils from three healthy donors. (D) Concentration of MMP9 in supernatants of neutrophils infected for one hour with *S. aureus* (MOI 1) +/- IL-20 after priming under indicated conditions. For (A), (B), and (D), data shown reflect mean +/- SEM of results from three donors, and *p<0.05, **p<0.01, ***p<0.001 by t test.

A



B

Cell Counts ($10^3/\mu L$)

Day	Leukocytes	Neutrophils	Lymphocytes	Monocytes
1	6.35±0.27	1.86±0.31	4.20±0.31	0.19±0.03
7	1.06±0.02	0.12±0.07	0.85±0.09	0.03±0.01
9	0.79±0.38	0.08±0.05	0.66±0.29	0.03±0.02

Figure B.1. Induction of leukopenia in CD1 mice by cyclophosphamide treatment. Six-week old CD-1 mice were treated every 48 hours with 150 mg/kg cyclophosphamide via intraperitoneal injection. On day 1, 7 and 9 mice were euthanized, blood sampled via cardiac puncture and anticoagulated through the addition of EDTA. The numbers of white blood cells (WBC) in each sample were enumerated by hemocytometry and plotted as a function of time (A). White arrows above the plot indicate days when animals received cyclophosphamide treatment. The average cell count (and the standard error of the means) of leukocytes, neutrophils, lymphocytes or monocytes were determined on days 1, 7 and 9 (B). On day 7, cyclophosphamide treatment had depleted 83% of leukocytes, 94% or neutrophils, 80% of lymphocytes and 84% of monocytes as compared to mock treated mice.

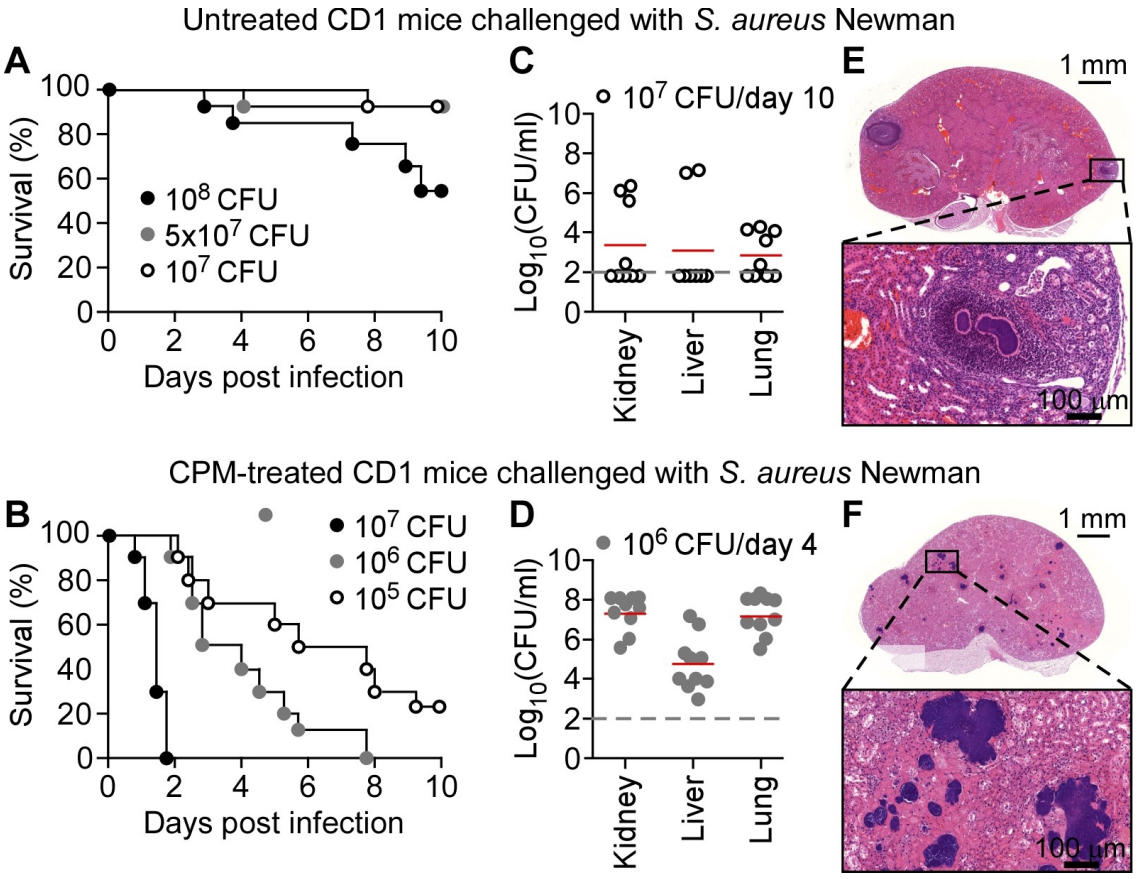


Figure B.2. *Staphylococcus aureus* bloodstream infection in leukopenic mice. (A-B) Cohorts of 7-week-old female CD-1 mice (n=10) mock treated (A) or treated with cyclophosphamide in 48 hour intervals (B) were infected by injection of *S. aureus* Newman (1×10^5, 1×10^6 , 1×10^7, 5×10^7 or 1×10^8 CFU) into the periorbital venous plexus and animal survival recorded over 10 days. (C-D) At day 10 (C) or 4 (D) post infection, kidney, liver and lung tissues of mock-treated (C) or cyclophosphamide-treated (D) CD-1 mice infected with *S. aureus* Newman bloodstream infection (1×10^6 or 1×10^7 CFU) were removed during necropsy; homogenized tissues were analyzed for staphylococcal load by plating serially diluted samples on agar plates and enumerating CFU. (E-F) Hematoxylin-eosin stained thin-sections of kidney tissues from mock-treated (E) or cyclophosphamide-treated (F) mice that had been euthanized on day 10 following bloodstream infection with 1×10^7 and 1×10^6 CFU *S. aureus* Newman, respectively, were viewed by light microscopy and images acquired. Bars indicate length measurements.

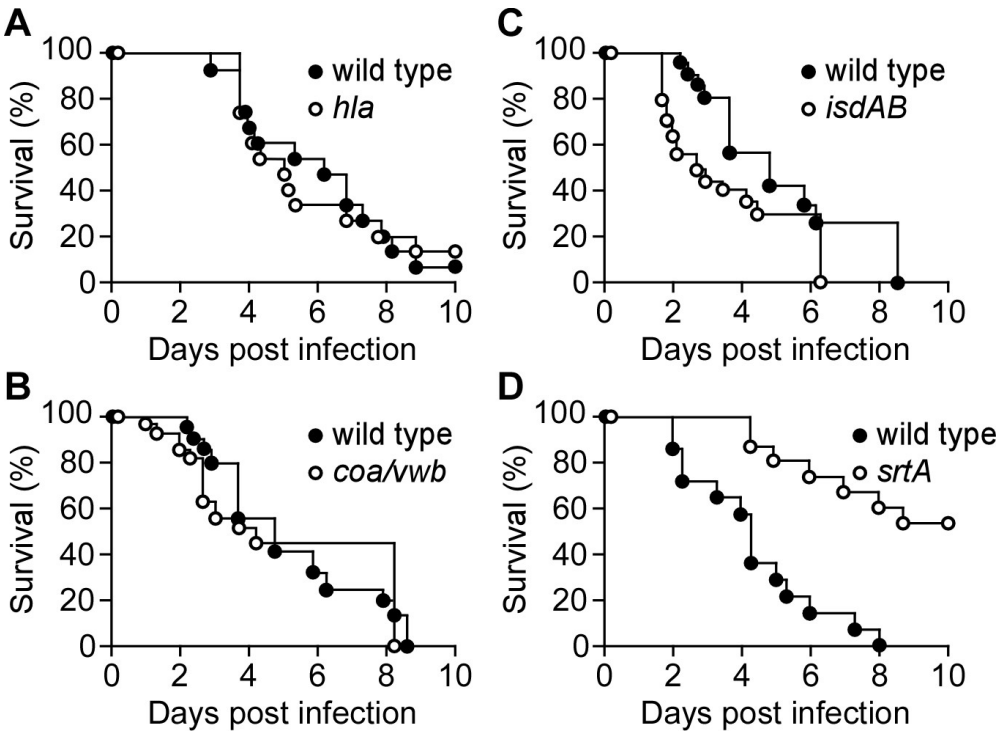


Figure B.3. Virulence factor contribution to *Staphylococcus aureus* bloodstream infection in leukopenic mice. Cohorts of 7-week-old female leukopenic (cyclophosphamide treated) CD-1 mice (n=10-15) were infected by intravenous injection with 2×10^6 CFU of *S. aureus* Newman wild-type or its isogenic variants with deletions or insertions in *hla* (A), *isdAB* (B), *coa/vwb* (C) or *srtA* (D). Animal survival was recorded over 10 days. Statistical significance in animal survival between two cohorts was analyzed with the two-tailed log-rank test; $P \leq 0.05$ was used to as criteria for significant difference.

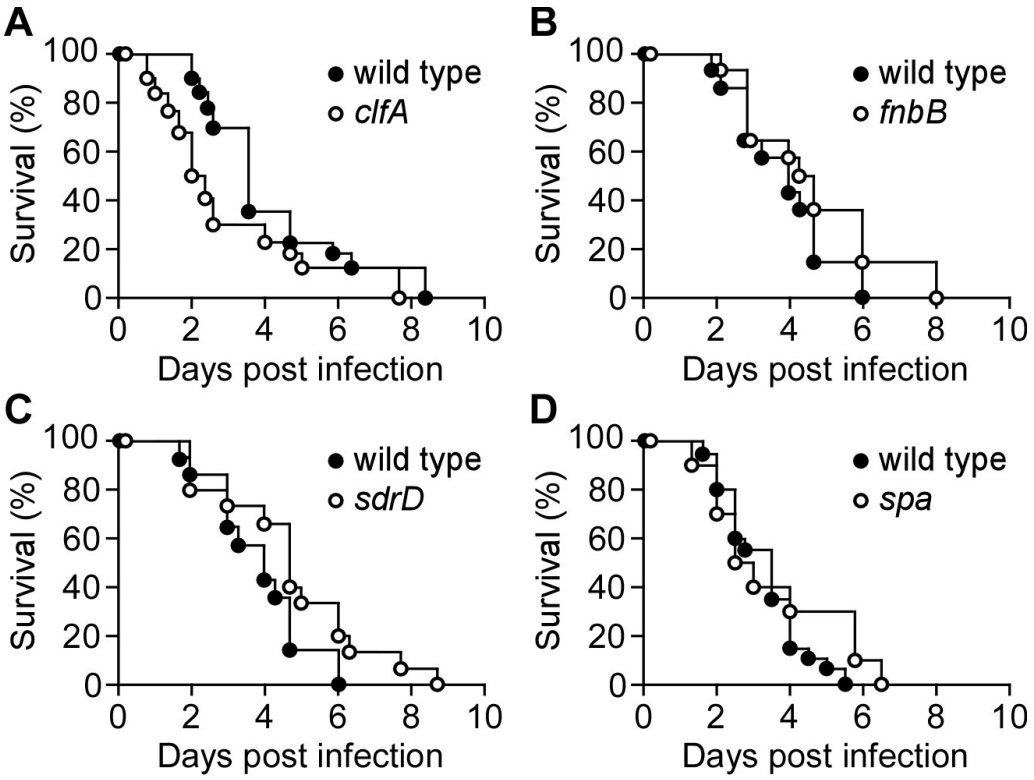


Figure B.4. Contributions of individual surface proteins to *Staphylococcus aureus* bloodstream infection in leukopenic mice. Cohorts of 7-week-old female leukopenic (cyclophosphamide treated) CD-1 mice (n=10-20) were infected by intravenous injection with 2×10^6 CFU of *S. aureus* Newman wild-type or its isogenic variants with deletions or insertions in *clfA* (A), *fnbB* (B), *sdrD* (C) or *spa* (D). Animal survival was recorded over 10 days. Statistical significance in animal survival between two cohorts was analyzed with the two-tailed log-rank test; $P \leq 0.05$ was used to as criteria for significant difference.

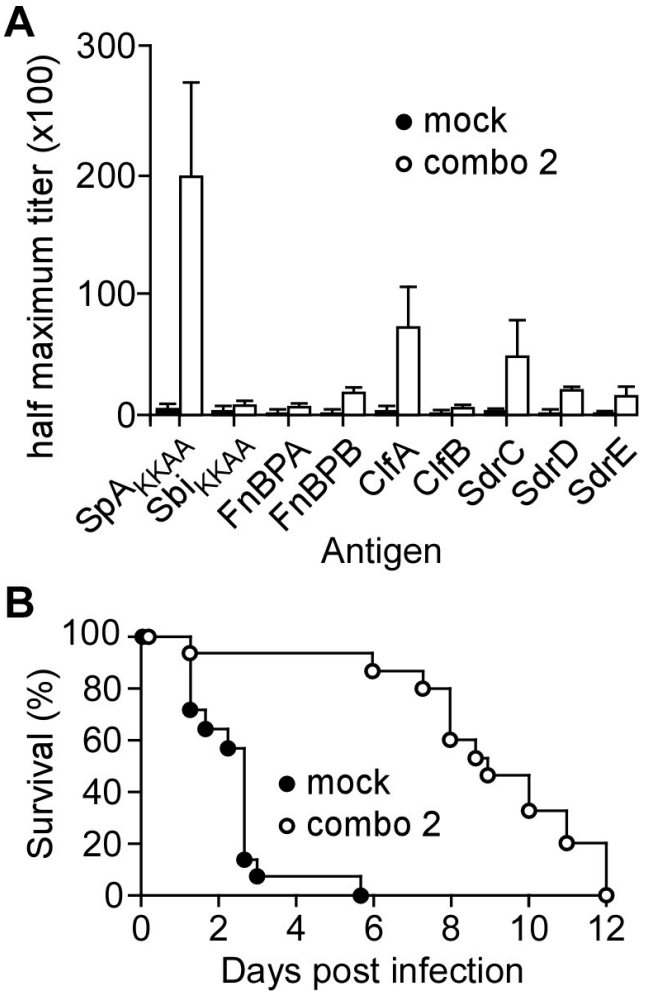


Figure B.5. Surface protein vaccine prolongs the time-to-death of leukopenic mice with *Staphylococcus aureus* bloodstream infection. Three-week-old CD-1 mice were immunized with adjuvant alone (mock) or with a cocktail of 25 µg each of four recombinant purified proteins emulsified in adjuvant: ClfA, FnBPB, SdrD and SpA_{KKAA}. Mock or booster immunizations occurred on day 12. Animals were treated with cyclophosphamide on day 21 and in 48 hour intervals thereafter for the duration of the experiment. Animals (n=5) were bled on day 26 and mouse serum half-maximal antibody titers against purified *S. aureus* surface proteins (SpA_{KKAA}, Sbi_{KKAA}, FnBPA, FnBPB, ClfA, ClfB, SdrC, SdrD, and SdrE) were determined by ELISA. Brackets denote standard error of the means. (B) Leukopenic (cyclophosphamide treated) CD-1 mice (n=10) that had been mock immunized or immunized with the surface protein vaccine (vaccinated) were challenged by intravenous injection with 2×10^6 CFU *S. aureus* Newman on day 27 following the first immunization. Animal survival was recorded over 10 days. Statistical significance in animal survival between two cohorts was analyzed with the two-tailed log-rank test; $P \leq 0.05$ was used as criteria for significant difference.

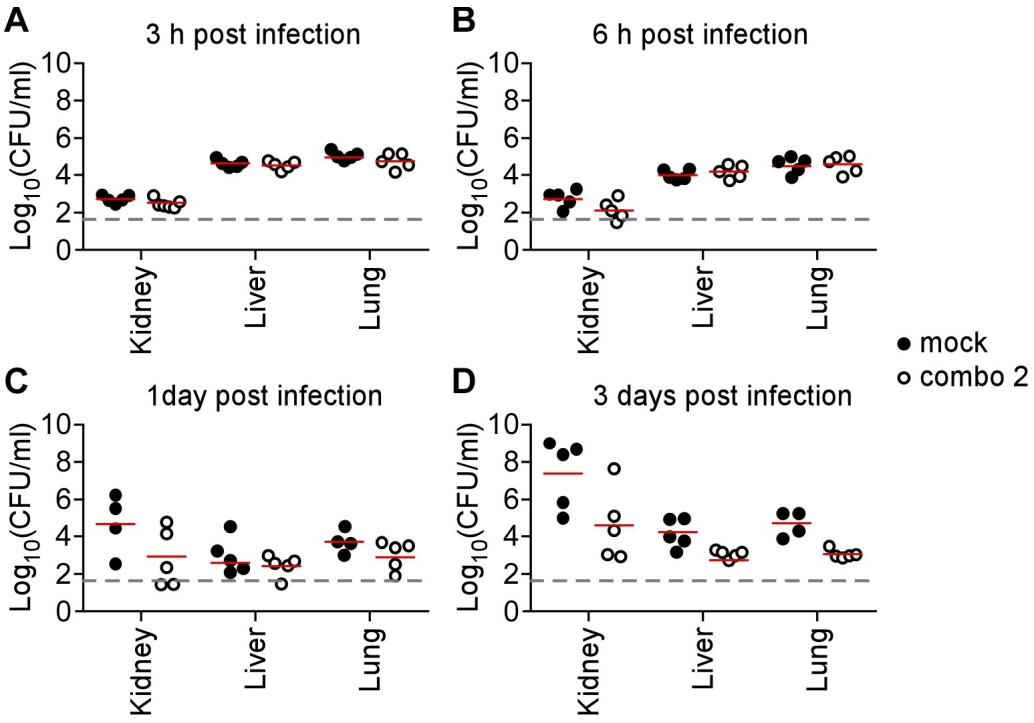


Figure B.6. Surface protein vaccine inhibits bacterial replication in leukopenic mice with *Staphylococcus aureus* bloodstream infection. Mock-immunized (closed circles) or vaccinated (ClfA, FnBPB, SdrD and SpA_{KKAA} - open circles) leukopenic (cyclophosphamide treated) CD-1 mice were infected with 2×10^6 CFU of *S. aureus* Newman as described in the legend to Fig. 5. Animals were euthanized 3 hours (A), 6 hours (B), 1 day(C) or 3 days (D) post challenge. Kidney, lung and liver tissues were removed during necropsy and analyzed for staphylococcal load by plating serially diluted samples on agar plates and enumerating CFU. Statistical significance of differences in bacterial load between mock treated and vaccinated leukopenic mice were analyzed with the Mann-Whitney test; $P \leq 0.05$ was used as criteria for significant difference.

APPENDIX B: VACCINE PROTECTION OF LEUKOPENIC MICE AGAINST STAPHYLOCOCCUS AUREUS BLOODSTREAM INFECTION.

Preface

During my time as a graduate student with the Department of Microbiology, I had the pleasure and valuable learning experience of working with my labmates in the Missiakas and Schneewind labs to contribute to the work in this manuscript, which was published in Infection and Immunity in November of 2014. Contributions to this work were also made by Sabine Rauch, Han Kim, Olaf Schneewind and Dominique Missiakas. The manuscript was prepared by Dominique Missiakas.

Abstract

The risk for *Staphylococcus aureus* blood stream infection (BSI) is increased in immunocompromised individuals, including patients with hematologic malignancy and/or chemotherapy. Due to the emergence of antibiotic-resistant strains, MRSA (methicillin resistant *S. aureus*), staphylococcal BSI in cancer patients is associated with high mortality, however neither a protective vaccine nor pathogen specific immunotherapy is currently available. Here we modeled staphylococcal BSI in leukopenic CD1 mice that had been treated with cyclophosphamide, a drug for leukemia and lymphoma patients. Cyclophosphamide treated mice were highly sensitive to *S. aureus* BSI and developed infectious lesions lacking immune cell infiltrates. Virulence factors of *S. aureus* that are key for disease establishment in immunocompetent hosts- α-hemolysin (Hla), iron-regulated surface determinants (IsdA and IsdB) and coagulases (Coa and vWbp)- are dispensable for the pathogenesis of BSI in leukopenic mice. In contrast, sortase A

mutants, which cannot assemble surface proteins, displayed delayed time-to-death and increased survival in this model. A vaccine with four surface antigens (ClfA, FnBPB, SdrD and SpA_{KKAA}), which was identified by genetic vaccinology using sortase A mutants, raised antigen-specific immune responses that protected leukopenic mice against staphylococcal BSI.

Introduction

Staphylococcus aureus is a commensal of the human nares, skin and gastrointestinal tract that also causes invasive disease, including skin and soft tissue infections (SSTI), bacteremia, sepsis, endocarditis, pneumonia and osteomyelitis (Kuehnert et al., 2006; Lowy, 1998). *S. aureus* causes disease in healthy individuals, which most frequently manifests as purulent SSTIs (Klevens et al., 2007). Invasive disease is associated with bloodstream infection (BSI) that may develop into fulminant sepsis or endocarditis (DeLeo et al., 2010; Lessa et al., 2010). The treatment of *S. aureus* infections has been complicated by the emergence and spread of antibiotic-resistant strains, designated MRSA (methicillin-resistant *S. aureus*), that have evolved resistance traits against all known therapeutics (David and Daum, 2010; DeLeo et al., 2010).

Patients with indwelling catheters, endotracheal intubation, medical implantation of foreign bodies (prosthetic joints, implants and heart valves), trauma, surgical procedures, hemodialysis, peritoneal dialysis, immunosuppressive or cancer therapy, diabetes, as well as individuals with increased age and low birth weight are all at elevated risk of *S. aureus* infection (Kallen et al., 2010; Spellberg and Daum, 2012).

These patient populations can be broadly classified into three groups: individuals with a breach of barrier function, enabling staphylococci to cause invasive infections (trauma, surgery); individuals with implants that serve as a protective niche for staphylococci; and, individuals with diminished innate immune defenses, most importantly patients with reduced opsonophagocytic killing of bacteria, which is mediated by neutrophil granulocytes (Casanova et al., 2012; Puel et al., 2011). The latter can occur in individuals with primary immunodeficiency disorders, caused, for example in patients with chronic granulomatous disease, by mutations that affect the NADPH oxidase of neutrophil granulocytes to produce oxygen radicals for killing of staphylococci (Babior, 2004; Curnutte et al., 1974). Much more frequently, however, diminished opsonophagocytic killing of staphylococci occurs in individuals with hematologic malignancies and/or anti-cancer chemotherapy (Chemaly et al., 2010), a patient population where *S. aureus* BSIs cause significant morbidity and mortality (Gonzalez-Barca et al., 2001; Skov et al., 1995).

Cyclophosphamide is an alkylating agent that is used for the therapy of malignancies and autoimmune diseases and for bone marrow transplantation (Emadi et al., 2009). Cyclophosphamide therapy induces neutropenia and leukopenia and is associated with increased risk for *S. aureus* BSI (Bishop et al., 1981; Mahajan et al., 2012). Although cyclophosphamide induced leukopenia in mice has been used extensively as a preclinical model to assess therapeutic efficacy of antibiotics against bacterial infections, this model has heretofore not been used to analyze the virulence factors for *S. aureus* BSI in patients with diminished capacity for opsonophagocytic killing (Craig and Andes, 2008; Zuluaga et al., 2006).

Although there is a need for vaccines and immunotherapies that protect high risk patients against *S. aureus* infection, several clinical trials for staphylococcal vaccines failed to meet their study endpoints (Daum and Spellberg, 2012). These trials have focused on single staphylococcal antigens that, during preclinical testing, were demonstrated to act as virulence factors for the pathogenesis of specific disease and, when used as subunit vaccines, elicited immune responses that protect immunocompetent animals (Bubeck Wardenburg and Schneewind, 2008; Duthie and Lorenz, 1952; Fattom et al., 1990; Kuklin et al., 2006; Menzies and Kernodle, 1996). Clinical trials for *S. aureus* vaccines included capsular polysaccharides (CP types 5 and 8), α-hemolysin (Hla), coagulase (Coa), and the iron-regulated surface determinant B (IsdB) (Harrison, 1963; Kernodle, 2011; Shinefield et al., 2002).

Here, we use the leukopenic mouse model to analyze *S. aureus* variants lacking specific protective antigens to characterize targets for a staphylococcal vaccine in cancer patients. Using genetic vaccinology to derive vaccine targets, we report that a cocktail of four surface protein antigens (ClfA, FnBPB, SdrD and SpA_{KKAA}) provides protection against *S. aureus* BSI in leukopenic mice.

Materials and Methods

Animal care and regulatory compliance. All experiments involving the care and use of animals followed protocols that were reviewed, approved and performed under the regulatory supervision of The University of Chicago's Institutional Biosafety Committee and the Institutional Animal Care and Use Committee. Animal care was managed by The University of Chicago Animal Resource Center, accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health

and Human Services (A3523-01). Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Veterinary Care was under the direction of full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine. CD-1 mice (strain code 022) were purchased from Charles River.

Statistical analysis. Mouse survival was analyzed for significance using the two--tailed log-rank test. The bacterial load following *S. aureus* infection, represented as the $\log_{10}$ CFU/g of organ tissue, was analyzed with the Mann-Whitney test for statistical significance. Quantification of abscess formation was analyzed for statistical significance using the unpaired two-tailed Student's *t*-test. Statistical analyses were performed using GraphPad Prism 4 software. All animal experiments were examined for reproducibility using either two or three independent determinations.

Bacterial strains, media and growth conditions. Mutants harboring the *bursa aurealis* mariner transposon in defined genes were transduced with bacteriophage ϕ85 into *S. aureus* Newman (Baba et al., 2008). The *isdA*/*isdB* and *coa*/*vwb* double mutants have been described (Cheng et al., 2010; Mazmanian et al., 2003). *S. aureus* cultures were grown at 37°C in tryptic soy broth (TSB), and *bursa aurealis* mutant cultures were supplemented with 10 μg/ml of erythromycin. *Escherichia coli* strain BL21(DE3) was grown in Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin at 37°C when plasmid was present.

Protein purification. Recombinant clumping factor A (ClfA), fibrinogen binding protein A (FnBPA), FnBPB, serine-aspartate repeat C (SdrC), SdrD, SdrE, non-toxigenic staphylococcal protein A (SpA_{KKAA}) and staphylococcal immunoglobulin-binding protein (Sbi_{KKAA}) carrying hexa-histidine tags were produced from *E. coli* BL21(DE3) carrying plasmids described earlier (Kim et al., 2010b; Kim et al., 2011; Stranger-Jones et al., 2006). Briefly, overnight cultures of recombinant *E. coli* strains were diluted 1:100 into fresh medium and grown at 37°C to an absorbance of 0.5 at 600 nm (A_{600}), at which point cultures were induced with 1 mM isopropyl β-D-1--thiogalatopyranoside (IPTG) and grown for an additional 3 hours. Bacterial cells were sedimented by centrifugation (3,000 ×*g* for 5 min), washed and suspended in Buffer A (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 10 mM imidazole), and disrupted with a French pressure cell at 14,000 psi. Crude lysates were centrifuged (40,000 ×*g* for 40 min) and filtered supernatants subjected to affinity purification. His-tagged proteins were purified over nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity column by gravity flow and eluted with an imidazole gradient (10-500 mM). Protein purification was ascertained by separating sample aliquots on 12% SDS-PAGE and Coomassie staining. Fractions containing the proteins were pooled and subjected to 1% Triton X-114 extraction to remove endotoxin. Following detergent addition, samples were first kept on ice for 10 min and then incubated at 37°C for 10 min followed by centrifugation (16,000 ×*g* for 10 min). Triton X-114 extraction was repeated twice and the aqueous phase containing the proteins was subjected to gel filtration in endotoxin-free PBS (Cellgro) using Hi-Trap

desalting column (GE Healthcare). Protein concentration was assessed with the bicinchoninic acid assay (BCA).

Cyclophosphamide-induced leukopenia and Staphylococcus aureus challenge of animals. Six-week-old female CD-1 mice were treated every 48 hours via intraperitoneal injection with cyclophosphamide monohydrate (CPM; Sigma C0768) dissolved in 200 µl of sterile water at a dose of 150 mg/kg body weight or were mock (water) treated for 7 days prior to the infection and throughout the experiment. To confirm leukopenia, mouse blood from at least three mice per group was drawn via cardiac puncture into collection tubes lined with EDTA (Sarstedt, Microvette, 20.1278.100) and white blood cells were counted with a hemocytometer. For infection of animals, overnight cultures of *S. aureus* were inoculated 1:100 into fresh TSB and grown for 2 hours with shaking at 37°C. Staphylococci were sedimented by centrifugation, washed, suspended, and diluted in sterile, endotoxin-free phosphate buffered saline (PBS) to obtain a starting stock of 1×10^9 CFU/ml. Inocula were determined by CFU enumeration following serial dilution, plating on tryptic soy agar, followed by incubation of plates for 16 hours at 37°C. Seven-week-old cyclophosphamide or mock treated CD-1 mice were anesthetized by intraperitoneal injection with a cocktail of 65 mg ketamine and 2 mg of xylazine per kilogram of body weight, and infected with 100 µl of bacterial suspension (ranging from 10^5 to 10^8 CFU) via intravenous injection into the periorbital venous plexus. Infected animals were monitored for morbidity or recovery over a period of 10 days. At indicated times after infection, mice were killed by CO_2 inhalation, and organs were excised and homogenized in sterile phosphate buffered saline (PBS), 0.1% Triton

X-100 using a high shear lab homogenizer (Omni International). Homogenates were diluted, plated on agar and incubated 16 hours at 37°C for enumeration of colony forming units (CFU).

Histopathology. Mouse organs were excised during necropsy and fixed in 10% formalin for 24 hours at room temperature. Tissues were embedded in paraffin, thin--sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Immunization studies. For active immunization, 3-week-old CD-1 mice were injected intramuscularly with a mix of 25 µg of each recombinant protein (SpA_{KKAA}, SdrD, FnBPpB and ClfA) emulsified in complete Freund adjuvant (Difco), 100 µl volume each dose, followed by a boost on day 12 with 25 µg of each protein emulsified in incomplete Freund adjuvant (Difco). Cyclophosphamide and mock treatment were started on day 21 and continued throughout the experiment every 48 hours. Blood was collected on day 26 via peri-orbital vein puncture with heparinized micro-hematocrit capillary tubes (Fisher). Z-gel serum separation microtubes (Sarstedt) were used to collect serum, and specific antibody production was assessed via enzyme-linked immunosorbent assay (ELISA) as described previously (Kim et al., 2010a). On day 27, mice were challenged by intravenous injection with 2×10^6 CFU of *S. aureus* Newman. Antibody titers in mouse sera were analyzed via ELISA for the vaccine antigens SpA_{KKAA}, SdrD, FnBPpB and ClfA as well as for cross-reactivity toward Sbi_{KKAA}, FnBPpA, ClfB, SdrC and SdrE.

Results

Leukopenia following cyclophosphamide treatment of CD1 mice

Earlier work used outbred ICR mice and intraperitoneal injection of two doses (150 and 100 mg/kg body weight) of cyclophosphamide, administered 48 hours apart (days 1 and 3), to induce leukopenia (<10 neutrophils/mm^3 blood, 84% reduction in white blood cells, with 92% and 96% decline in lymphocytes and monocytes, respectively) for 3 days (Zuluaga et al., 2006). Others used Swiss mice and two injections of 150 mg/kg cyclophosphamide on day 1 and 4 to achieve neutropenia (Griffith et al., 2008). Using female outbread CD1 mice, we observed that three intraperitoneal injections of 150 mg/kg cyclophosphamide in 48 hour intervals (days 1, 3 and 5) were required to cause leukopenia (Figures B.1A and B.1B). At the day 7 interval, neutrophils were >90% depleted relative to the average cell count prior to cyclophosphamide treatment; other leukocyte populations were >80% depleted (Figures B.1A and B.1B). We also observed that leukopenia was not sustained for the ten day observation period of our experimental plan unless CD1 mice received continued treatment with cyclophosphamide in 48 hour intervals. Cyclophosphamide treatment did not affect the weight of animals as compared to mock treated animals (data not shown). On the basis of these observations, we developed a protocol whereby CD1 mice were either mock or cyclophosphamide (150 mg/kg) treated on days 1, 3 and 5 and challenged by intravenous inoculation with *S. aureus* on day 6. Animals received mock or cyclophosphamide treatment in 48-hour intervals (days 7, 9, 11, 13, 15) unless mice succumbed to the infectious challenge.

Staphylococcus aureus bloodstream infection in leukopenic mice

Cohorts of CD1 mice (n=10) were infected via intravenous injection with *S. aureus* Newman (Baba et al., 2008), a human clinical isolate that has been used for animal model development (Cheng et al., 2009; Duthie and Lorenz, 1952). Injection of 1×10^8 CFU of *S. aureus* Newman caused 50% mortality in immunocompetent CD-1 mice over the 10-day observation period, whereas lower inocula (5×10^7 or 1×10^7 CFU) resulted in reduced mortality (Figure B.2A). In contrast, all leukopenic mice that had been treated with cyclophosphamide succumbed to BSI when challenged with 1×10^6 or 1×10^7 CFU *S. aureus* Newman. Mice that received a higher dose (1×10^7) succumbed with a shortened mean time-to-death (<24 hours) as compared to animals challenged with 1×10^6 CFU (5 days) (Figure B.2B). Even a challenge dose of 1×10^5 CFU *S. aureus* Newman caused bloodstream infections with 80% mortality over the 10-day observation period (Figure B.2B).

Lethal outcome of *S. aureus* BSI was associated with staphylococcal replication in infected tissues (Figures B.2C-B.2F). Enumeration of *S. aureus* in organs of mock-treated animals that survived a bloodstream challenge of 1×10^7 CFU revealed small bacterial numbers in kidney [3.3 (±0.6) $\log_{10}$ CFU/ml], liver [3.1 (±0.7) $\log_{10}$ CFU/ml] and lung tissues [2.9 (±0.3) $\log_{10}$ CFU/ml] (Figure B.2C). In contrast, cyclophosphamide-treated animals harbored increased *S. aureus* load in infected kidney [7.8 (±0.2) $\log_{10}$ CFU/ml], liver [5.1 (±0.4) $\log_{10}$ CFU/ml] and lung tissues [6.9 (±0.6) $\log_{10}$ CFU/ml] at day 4 post infection (Figures B.2C and B.2D).

Following infection of immunocompetent mice, *S. aureus* BSI triggers the development of abscess lesions in many organ systems, which can be visualized in

hematoxylin-eosin stained thin-sectioned kidney tissues (Cheng et al., 2009). Renal tissues of mock--treated mice that had been euthanized ten days following BSI with *S. aureus* Newman harbored 1-4 lesions that presented with characteristic morphology (Cheng et al., 2009): a bacterial nidus (designated the *staphylococcal abscess community*), surrounded by an eosinophilic pseudocapsule composed of fibrin deposits and large infiltrates of immune cells (Cheng et al., 2010) (Figure B.2E). Hematoxylin-eosin stained renal tissues of cyclophosphamide-treated mice harbored >100 small lesions per kidney that were comprised of large numbers of staphylococcal cells and surrounded by eosinophilic deposits, likely comprised of fibrin; however, these lesions did not show immune cell infiltrates (Figure B.2D).

Impact of staphylococcal virulence factors on bloodstream infection in leukopenic mice

We wondered whether previously characterized *S. aureus* virulence factors might be required for the pathogenesis of bloodstream infections in leukopenic mice and examined three variants with knockout mutations in the *hla, coa/vwb* and *isdA/isdB* genes, respectively. Staphylococcal α-toxin (α-hemolysin) is encoded by the *hla* gene (Gray and Kehoe, 1984) and the primary translation product functions as a precursor that is cleaved by signal peptidase and secreted into the extracellular medium (Bhakdi et al., 1981). Association of the soluble, monomeric form of Hla with its ADAM10 host cell receptor triggers oligomerization and membrane pore formation (Wilke and Bubeck Wardenburg, 2010), causing injury to vascular

endothelial cells, epithelial cells as well as platelets and cells of the myeloid lineage (Becker et al., 2014; Inoshima et al., 2011). During bloodstream infection of immunocompetent mice, the *hla* mutant of *S. aureus* Newman displayed reduced mortality and increased time-to-death (Powers et al., 2012). In contrast, when tested in leukopenic CD1 mice, no differences in overall mortality or time-to-death were detected between animals that had been challenged with wild-type or *hla* mutant *S. aureus* Newman (Figure B.3A; P=0.8649).

Coagulase (Coa) and von-Willebrand factor binding protein (vWbp) are also secreted into the extracellular medium by *S. aureus* (Bjerketorp et al., 2002; Much, 1908). Both proteins associate with and non-proteolytically activate prothrombin to convert fibrinogen into fibrin clots (Friedrich et al., 2003), which promotes escape from phagocytic killing and establishment of abscess lesions in infected tissues via the formation of a fibrin capsule that restricts access of immune cells to staphylococcal abscess communities (Cheng et al., 2010). During bloodstream infection of immunocompetent mice, the *coa*/*vwb* mutant displayed delayed time-to-death and increased survival as compared to wild-type *S. aureus* Newman (McAdow et al., 2011). In contrast, during bloodstream infection of leukopenic mice, the *coa*/*vwb* mutant displayed similar mortality and time-to-death as wild-type *S. aureus* Newman (Figure B.3B; P=0.5149). Two surface proteins of *S. aureus*, IsdA and IsdB, contribute to heme-iron scavenging from host hemoglobin and to passage of the tetrapyrrol moiety across the bacterial envelope (Haley and Skaar, 2012; Mazmanian et al., 2003). *S. aureus isdAB* mutants display a small delay in time-to--death during bloodstream infections as well as reduced replication in infected tissues (Kim et al., 2010b; Torres et

al., 2006). During bloodstream infection in leukopenic mice, the *S. aureus isdAB* mutant caused similar mortality than the wild-type strain (Figure B.3C; P=0.1356).

Staphylococci require sortase during bloodstream infection in leukopenic mice

Sortase A is a transpeptidase that cleaves the LPXTG sorting signals of surface protein precursors and links their C-terminal carboxyl group to the peptidoglycan of *S. aureus* (Mazmanian et al., 1999; Ton-That et al., 1999). Sortase A mutants (*srtA*) cannot anchor any one of eighteen surface proteins of *S. aureus* Newman to the bacterial envelope (Mazmanian et al., 2000) and are unable to form abscess lesions or cause lethal BSI in immunocompetent mice (Cheng et al., 2009; McAdow et al., 2011). When analyzed during bloodstream infection of leukopenic mice, the *srtA* mutant displayed a delayed time-to--death and increased survival as compared to wild-type *S. aureus* Newman, indicating that the combined contributions of all surface proteins are required for staphylococcal disease processes in mice that lack most of their immune cells (Figure B.3D; P=0.0039).

Contribution of surface protein towards *Staphylococcus aureus* bloodstream infection in leukopenic mice

Earlier work had used genetic vaccinology to compare the immune response of immunocompetent mice infected with wild-type *S. aureus* and sortase A mutants to identify antigens that elicit protective immune responses, as infection with the *srtA* variant, but not-wild-type staphylococci, triggers protective immunity to recurrent infection (Kim et al., 2011). Using this technology, a vaccine was derived by combining

four antigens (ClfA, FnBPB, SdrD and SpA_{KKAA}) that elicited immune responses in immunocompetent mice and protected animals against challenge with wild-type *S. aureus* (Kim et al., 2011). Protein A (SpA) binds immunoglobulins via their Fcγ domain and the Fab heavy chains of V_H3 clan antibodies, attributes that interfere with opsonophagocytosis of bacteria and with the development of adaptive B cell responses (Falugi et al., 2013; Goodyear and Silverman, 2003). The SpA_{KKAA} variant has lost these functions and, unlike wild-type SpA, elicits neutralizing antibody responses that promote opsonophagocytic killing of staphylococci and development of B cells to establish adaptive immune responses (Kim et al., 2010a). ClfA binds to the C-terminal end of the fibrinogen γ-chain (Ganesh et al., 2008; McDevitt et al., 1994), which is also available for staphylococcal binding during fibrin fiber assembly (McAdow et al., 2011). Neutralizing antibodies can block ClfA binding to both fibrinogen and fibrin (McAdow et al., 2011; Patti, 2004). FnBPB binds to fibronectin, elastin and fibrinogen and these activities promote the pathogenesis of *S. aureus* BSI (Burke et al., 2011; Jonsson et al., 1991; Roche et al., 2004; Shinji et al., 2011). The molecular mechanisms whereby SdrD contributes to *S. aureus* pathogenesis are currently not known (Cheng et al., 2009). Here we asked whether any one of these four surface protein genes (*clfA*, *fnbB*, *sdrD* or *spa*) contributed to the pathogenesis of *S. aureus* BSI in leukopenic mice, studying in pairwise comparison the survival or time-to-death between wild-type and mutant strains. Of note, none of the four surface protein genes (*clfA*, *fnbB*, *sdrD* or *spa*) was necessary for *S. aureus* BSI associated mortality or was a determinant of time-to--death in leukopenic mice (Figure B.4; wild type vs. *clfA*, P=0.1479; wild type vs. *fnbB*, P=0.1726; wild type vs. *sdrD*, P=0.0736; wild type vs. *spa*, P=0.2330).

Surface protein vaccine protects leukopenic mice against *S. aureus* bloodstream infection

To address the question whether the surface protein vaccine derived via genetic vaccinology may protect leukopenic mice against *S. aureus* BSI, we first immunized immunocompetent CD1 mice with a cocktail of purified recombinant proteins emulsified in adjuvant, including ClfA, FnBPB, SdrD and SpA_{KKAA}. Animals received a booster immunization on day 12 and were bled to determine the concentration of serum IgG against each antigen. Compared to mock (adjuvant alone) immunized mice, animals that had received the surface protein vaccine, developed specific antibodies against SpA_{KKAA}, FnBPB, ClfA, SdrC, SdrD and SdrE but not against FnBPA, ClfB and Sbi_{KKAA} (Figure B.5A). These findings can be explained by the high degree of sequence identity (76%) between the A domain of the serine-aspartate repeat protein family (SdrCDE), which is lower between the A domains of FnBPA and FnBPB (60%) as well as ClfA and ClfB (50%). The immunoglobulin binding domains (IgBDs) of SpA display sequence homology to the N-terminal two IgBDs of Sbi (staphylococcal binder of immunoglobulin - 35% identity), which was also not sufficient to elicit a strong cross--reactive immune response. As expected, mock immunized animals did not generate antibodies against staphylococcal surface proteins (Figure B.5A).

Vaccinated mice were treated with cyclophosphamide starting on day 21 and, once animals were found to be leukopenic, they were challenged on day 27 with 2×10^6 CFU *S. aureus* Newman. Mock immunized animals succumbed to challenge within 1-5 days with a mean time-to-death of 2.5 days. Leukopenic animals that had received the

surface protein vaccine succumbed more slowly to BSI with a mean time-to-death of 8.5 days (mock vs. vaccine, P<0.0001) (Figure B.5B). To monitor the ability of the surface protein vaccine to reduce staphylococcal replication, cohorts of 5 animals (mock or vaccinated) were euthanized 3 hours, 6 hours, 1 day and 3 days post challenge and the bacterial load in kidney, liver and lung tissues was quantified via CFU enumeration. This experiment revealed that staphylococci disseminated within 3 hours into organ tissues (Figure B.6). While the load of *S. aureus* in liver and lung tissues increased slowly in mock immunized animals, mice that had received the surface protein vaccine did not increase their bacterial load in both organ tissues (Fig. 6D; P=0.0119 in liver and P=0.0195 in lung on day 3 post infection). *S. aureus* replicated very quickly in renal tissues and by day 3, 7.4 (±0.8) $\log_{10}$ CFU/ml was enumerated in mock immunized animals (Figure B.6D).

Leukopenic animals that had received the surface protein vaccine harbored a 2.8 $\log_{10}$ reduction in bacterial load [4.6 (±0.9) $\log_{10}$ CFU/ml] (Figure B.6D; mock vs. combo 2, P=0.0556). Thus, immunization with the surface protein vaccine not only extended the time-to-death of leukopenic animals with *S. aureus* BSI, the vaccine also limited staphylococcal replication in infected tissues.

Discussion

Chemotherapy of tumors, premature birth, human immunodeficiency virus (HIV) infection, leukemia, chronic granulomatous disease (CGD) and other inheritable diseases cause functional depletion or annihilation of phagocytic cells (Freeman and Holland, 2009). Unless diagnosed early and treated with antimicrobial prophylaxis,

such immunodeficiency is associated with significant morbidity and mortality because of bacterial infection (Freeman and Holland, 2009; Marin et al., 2014). *S. aureus* is a frequent cause of morbidity and mortality in individuals with immunodeficiency owing to its status as a commensal of humans and to the dissemination of antibiotic resistant MRSA strains (Chambers, 2005; DeLeo et al., 2010). For example, individuals infected with HIV experience a six-fold higher risk of *S. aureus* SSTI as compared to healthy individuals (Popovich et al., 2010).

Cyclophosphamide treatment of animals has been used extensively to induce leukopenia and examine the preclinical efficacy of antibiotics in immunocompromised hosts (Craig, 1998; Craig and Andes, 2008; Zuluaga et al., 2006). Cyclophosphamide induced leukopenia in mice has also been used for the study of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), antibiotic-resistant bacteria most frequently associated with nosocomial infection and therapy failure (Rice, 2008). While most of the work in leukopenic mice focused thus far on studying the efficacy of antibiotics (Guo et al., 2011; Thompson et al., 2014), progress was also documented for the development of vaccine and immunotherapeutics to prevent *P. aeruginosa* BSI (Cryz et al., 1983; Scarff and Goldberg, 2008; von Specht et al., 1995). Nevertheless, the development of vaccines that prevent *S. aureus* BSI in leukopenic mice was heretofore not investigated. Work on staphylococcal vaccines commenced more than a century ago (Meakins, 1910).

Preclinical work over several decades indicates that immunization of immunocompetent mice with purified Hla, Coa, ClfA or IsdB can elicit antigen-specific

immune responses that protect animals from *S. aureus* BSI associated mortality (Bubeck Wardenburg and Schneewind, 2008; Kuklin et al., 2006; Palmqvist et al., 2004). However, clinical trials with whole-cell killed (Rogers and Melly, 1965) or subunit vaccines formulated from secreted virulence factors, α-hemolysin (Hla) and coagulase, did not protect against recurrent SSTI (Harrison, 1963). Immunotherapy with antibodies neutralizing Hla (Kernodle, 2011; Parish and Cannon, 1960) or ClfA, the fibrinogen binding surface protein and agglutinin (McAdow et al., 2011; McDevitt et al., 1994), also did not protect against *S. aureus* infection (Projan et al., 2006; Weems et al., 2006). Conjugates of *S. aureus* type-5/8 capsular polysaccharide (CP5/CP8) with *Pseudomonas* exotoxin A raise opsonophagocytic antibodies (Fattom et al., 1990; Fattom et al., 2004), however the vaccine did not protect hemodialysis patients from *S. aureus* infection (Shinefield and Black, 2005). Finally, the clinical trial for V710, the recombinant IsdB vaccine (Kuklin et al., 2006; Moustafa et al., 2012), was recently terminated; multiorgan dysfunction and mortality following *S. aureus* infection occurred more frequently in individuals receiving V710 than in control cohorts and V710 immunization did not show clinical benefit (Fowler et al., 2013). A key concept explaining recent failures of human vaccine trials with single staphylococcal antigens is based on the observation that *S. aureus* requires many different secreted products to cause disease (Cheng et al., 2009; Stranger-Jones et al., 2006). If so, vaccine-induced neutralization of several different virulence factors may be required for the establishment of protective immunity (DeDent et al., 2012). Further, clinical trials that examine vaccine or immunotherapeutic efficacy focus on healthcare-related *S. aureus* infections in patients at high risk for staphylococcal BSI, which includes for example

end-stage renal disease with hemodialysis (Shinefield et al., 2002) or very-low-birth-weight neonates (Weisman, 2007), i.e. populations with at least partial defects in innate and adaptive immune responses (Shinefield and Black, 2005; Weisman et al., 2011).

Here we used cyclophosphamide-induced leukopenia in mice to ask whether specific virulence factors/protective antigens contribute to the pathogenesis of *S. aureus* BSI. Our results show that the structural genes for several protective antigens - *clfA*, *coa*/*vwb*, *fnbB*, *hla*, *isdAB*, *sdrD* and *spa*- are not required for the pathogenesis of staphylococcal BSI in leukopenic mice. If so, vaccines designed to raise neutralizing antibodies against any one of these secreted products would not be expected to provide protection against *S. aureus* BSI in leukopenic patients. Nevertheless, sortase A (*srtA*) mutant *S. aureus*, which cannot assemble any one of eighteen LPXTG surface proteins into the bacterial envelope (including ClfA, FnBPB, IsdAB, SdrD and SpA), displayed a significant decrease in virulence when tested in leukopenic mice.

Earlier work demonstrated that *S. aureus* infection in mice does not elicit protective immune responses that prevent subsequent infections with this pathogen (Burts et al., 2005). This phenotype requires *S. aureus* expression of SpA, the B cell superantigen (Falugi et al., 2013). Mice that have been infected with *spa* mutants lacking the immunoglobulin binding attributes of SpA elicit antibody responses against many different antigens and provide protection against subsequent disease (Falugi et al., 2013). A similar phenotype is observed in mice that have been infected with *srtA* mutants (Kim et al., 2011). Linear regression analysis was used to identify the antibody responses mediating protective immunity against *S. aureus* (Kim et al., 2011). This approach, designated genetic vaccinology, identified four surface protein antigens -

ClfA, FnBPB, SdrD and SpA - as protective antigens (Kim et al., 2011; Kim et al., 2012). Indeed, when used as a combination vaccine in immunocompetent mice, ClfA, FnBPB, SdrD and SpA_{KKAA} elicit protective immunity against *S. aureus* BSI (Kim et al., 2011). Here we show that the surface protein vaccine - ClfA, FnBPB, SdrD and SpA_{KKAA} - elicits immune responses that protect leukopenic mice against *S. aureus* BSI. Of note, vaccine protection is limited to a delay in time-to-death in these severely immunocompromised mice. Although increases in survival were not recorded, we believe the observed protection may have clinical relevance for the following two reasons. First, chemotherapy of hematologic malignancy rarely diminishes the blood concentration of neutrophils and macrophages to the same degree as was achieved here with continued cyclophosphamide treatment of mice. If so, the residual opsonophagocytic capacities of chemotherapy or leukemia patients may elevate vaccine-induced protection in this patient population. Second, even the delayed time--to-death protection affords clinical opportunities of initiating antibiotic therapies against *S. aureus*, which may enable recipients of the surface protein vaccine to survive BSI.

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