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Proinflammatory mechanisms of streptococcal pyrogenic exotoxins

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Abstract

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By Anders F. Johnson

Group A Streptococcus (GAS) is an obligate human pathogen responsible for the severe infections scarlet fever and streptococcal toxic shock syndrome (STSS). The toxins behind scarlet fever and STSS are streptococcal pyrogenic exotoxins, most of which are superantigens that interact with the immune system to cause a cytokine storm. The exception to this toxin family is the cysteine protease SpeB, which degrades and activates many host and pathogenic factors. Prior work has shown that SpeB acts as a bacterial caspase and activates proinflammatory host factors IL-1β and GSDMA. We present data showing that SpeB activates IL-18 secreted by keratinocytes independent of infection. This activation is exclusive to strains of GAS producing SpeB and is not found in commensal microbes of the skin. This active cytokine goes on to be recognized by the immune system to generate an inflammatory response to bacterial infection. These data suggest keratinocyte-secreted IL-18 is acting as a sensor and sounds an early alarm to skin pathogens. Streptococcal superantigens have also been shown as targets of SpeB. Prior literature has shown the superantigen SpeA is resistant to degradation, while SmeZ is susceptible to degradation. However, the interaction of SpeB with the other streptococcal superantigens had not been characterized. Here we present data showing the variety of degradation tendencies of SpeB on streptococcal superantigens. We show that there is a range of susceptibility to SpeB, that is independent of superantigen grouping and species of origin. We also present data showing that SpeB synergizes with superantigens to enhance their proinflammatory capabilities, instead of strictly antagonizing and degrading them. Altogether, SpeB is shown to be a tool of GAS to activate the host proinflammatory immune response, which when overactivated, is a major cause of morbidity and mortality during infection.

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Chapter 1

Introduction

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Introduction

Streptococcus pyogenes (group A *Streptococcus;* GAS) is a ubiquitous human pathogen responsible for over half a million deaths per year worldwide[1]. No vaccine exists, and current treatment depends on conventional antibiotics and symptom management. While the β-lactam penicillin remains the antibiotic of choice for mild to moderate infections, severe or prolonged infections require additional measures for effective clearance. The standard recommendation is to utilize the lincosamide clindamycin in combination with penicillin[2]. Any resistance is a serious issue because of the reliance on these antibiotics, so surveillance is important. GAS has no resistance to penicillin, but treatment failure remains a major concern. Clindamycin has been very effective, but global rates of resistance continue to rise and make the implementation of universal guidelines a challenge.

GAS produces many virulence factors that modulate and shape the host immune response[3]. SpeB is a cysteine protease that degrades immune antimicrobials, tissue matrix, and other host factors to promote dissemination. It can also activate some cytokines resulting in a highly proinflammatory immune response[4,5]. Superantigens are another virulence factor that non-specifically activate the host immune system, generating a cytokine storm[6]. This inflammation directly contributes to the morbidity and mortality during infections and is responsible for more severe presentations of disease like scarlet fever and toxic shock syndrome[7,8]. It is not yet clear why inducing inflammation through SpeB and superantigens is beneficial for GAS, but conservation of these mechanisms suggests an essential role in routine infection and transmission.

GAS Infections

GAS colonizes the nasopharynx, where it can cause disease, disseminate to other sites in the body, and transmit to other humans. GAS is isolated from this site in 12-24% of healthy children and 37% of those with a sore throat[9]. Pharyngitis, or strep throat, is the most common disease caused by GAS and is estimated to occur more than 600 million times per year[1]. Common symptoms of pharyngitis are a sore throat, fever, enlarged tonsils, and coughing with throat pain, induced by pro-inflammatory exotoxins secreted by GAS[10,11]. Some individuals are susceptible to recurring pharyngitis[10] which may be prevented with tonsillectomy, though 33% of children lacking tonsils are still colonized by GAS[12]. GAS exotoxins also promote colonization of the skin and more serious invasive infections, and are major drivers of pathogenesis[13].

Beyond pharyngitis, GAS is responsible for both mild and severe disease manifestations on the skin. Pyoderma, also known as impetigo, results in the formation of pustules on the skin that rupture to form scabs[14]. Spread by direct contact, it is most common in children and those living in crowded conditions with limited medical care[14]. There is an estimated disease burden of 111 million cases every year[1]. Severe complications manifest when GAS successfully beaches the epithelial layer. One common complication is cellulitis, the inflammation of the lower dermis and subcutaneous tissue[15]. Cellulitis is estimated to be present in 20% of invasive infections[16]. If an infection is untreated and GAS reaches the bloodstream, this results in bacteremia. Necrotizing fasciitis may also occur, with reported fatality rates of 29% in the United States[17] and 32% in Europe[18].

Rapid treatment is necessary as severe complications may occur from untreated infections. Famously, untreated pharyngitis can lead to scarlet fever, an inflammatory disease with resurging outbreaks[19–21] and fatality rates up to 30%[22]. Scarlet fever is mediated by the streptococcal

pyrogenic exotoxin superantigens which induce an inflammatory cytokine storm[23]. In the bloodstream, superantigens are responsible for streptococcal toxic shock syndrome (STSS), a multi-organ disease with a fatality rate up to 44%[18,24]. STSS often co-occurs with necrotizing fasciitis, an invasive infection of the skin[25] where surgery within 24 hours is often necessary for survival due to tissue damage and bacteremia[26]. Untreated GAS infections further have the risk of immune sequelae such as rheumatic fever (RF), where the immune system mistakenly recognizes host tissue as foreign antigens[27,28]. When targeted toward the heart this results in rheumatic heart disease (RHD), a chronic condition that is a major source of GAS morbidity and mortality[16]. The risk of any of these complications is thus limited when GAS infections are rapidly treated.

Streptococcal pyrogenic exotoxin B

The GAS cysteine protease streptococcal pyrogenic exotoxin B (SpeB) is a virulence factor essential for invasive infections. It is encoded on the chromosome and is expressed by most GAS isolates[29]. Originally referred to as streptococcal cysteine protease, it was characterized in 1945 as degrading M protein[30]. SpeB was also first reported with SpeA as one of the causative agents of scarlet fever, known then as A and B toxin before being renamed[31,32]. The nucleotide sequence of *speB* was reported in 1990 and found to correlate with streptococcal cysteine protease, merging the two into one protein now referred to as SpeB[33].

SpeB expression is regulated by several transcription factors. RopB is an activator located upstream of *speB* and is essential for expression[34]. RopB binds near the primary promoter of *speB*, and mutations have shown that loss of RopB leads to decreased or eliminated *speB* expression[35]. RopB is a sensor of the quorum sensing peptide SIP, thus inducing SpeB when the bacteria reach

high density[36]. Another important component is the CovR/S two-component system that regulates GAS virulence[37]. CovR binds upstream of *speB* to repress expression, however, it is unclear if it directly represses the *speB* transcript or acts upon the essential activator RopB[38,39]. Strains with mutations that inactivate CovS have lower expression of SpeB, and mutants of CovR have higher expression of SpeB[39]. Another known regulator of SpeB is CcpA, a response regulator that binds at catabolite response element sites on DNA. CcpA binds near *speB* promoter regions, resulting in additional upregulation under conditions where it is not repressed[40].

SpeB acts as a cysteine protease by cleaving the covalent bonds between amino acids in a protein chain. Function is based on the Cys192 and His340 residues for catalytic activity, and the Trp357 residue for binding, as shown by site-specific mutagenesis[41,42]. Activity is lost with Cys192Ser or His340Ala mutants, and greatly reduced with Trp357Ala mutant[42,43]. SpeB is translated with a signal sequence for secretion, but remains an inactive zymogen upon signal sequence removal[44]. Generation of the catalytically active form of SpeB results after additional autocatalytic cleavages[45]. To prevent activity within GAS, the inhibitor Spi is co-transcribed with SpeB to prevent its activation and activity in the cytosol. Spi is lost upon secretion, allowing for extracellular activation of SpeB[46].

SpeB can target numerous proteins for proteolysis because of its broad substrate specificity. Substrate cleavage by SpeB occurs after an Ala, Asn, Asp, Glu, Gly, Ser, or Thr amino acid that has a preceding hydrophobic residue[47,48]. These residues must have their sidechains solvent-accessible for recognition by SpeB[49]. Regions that are highly flexible or with a strong negative charge are also more likely to be targeted[47]. Activity can be prevented with thiol-reactive drugs or other inhibitors of cysteine-family proteases, such as E-64[50].

Targets of SpeB

SpeB degrades many host and bacterial proteins. Host proteins degraded fall into two broad categories, structural proteins in the extracellular matrix, and factors involved in the immune response. Structural proteins, such as plasminogen[51], fibrinogen[52], desmogleins[53], and junction proteins[54] are degraded. This tissue destruction promotes colonization and dissemination of GAS. Immune factors cleaved are anti-microbial cytokines[4], chemokines[55], small peptides such as LL-37[56], and complement factors[57]. SpeB is also capable of cleaving immunoglobulins, however research has proposed this is unique to laboratory settings, and is not physiological[58]. The host proteins targeted by SpeB are essential to antimicrobial functions of the immune response, making GAS highly resistant to many critical immune effectors.

While SpeB degrades many immune factors, it has been found to also activate proteins that require protease cleavage for activity. In the human host, SpeB has been shown to activate proteins canonically activated by caspase-1 as part of the inflammasome. One example is IL-1β, a proinflammatory cytokine that restricts bacterial growth. Canonically, IL-1β is produced in an inactive pro form that is then cleaved and activated by caspase-1[59]. SpeB cleaves near the caspase-1 cleavage site, also activating IL-1β[4]. Activated IL-1β acts as a sensor which is then used to restrict the growth of GAS. This sensor can be bypassed though, as patients taking inhibitors of IL-1 or those infected with GAS with reduced SpeB infection tend to have more invasive infections with severe symptomology. Altogether, this suggests that IL-1β is used as a sensor of bacterial infection, and the presence of SpeB triggers a clearing response to limit pathogenesis.

Another protein activated by SpeB is Gasdermin A (GSDMA). GSDMs are pore forming proteins responsible for pyroptosis that are part of the inflammasome[60]. GSDMA is produced in skin cells such as keratinocytes[61]. It induces cell death by forming pores in the outer membrane,

preventing the replication of intracellular pathogens, and releasing cytokines to drive the proinflammatory response to pathogens[62]. While caspase-1 has been shown to cleave other GSDMs, GSDMA had no known activators. Our lab has shown recently that SpeB cleaves and activates GSDMA, leading to cell death and the release of cytokines[5,63]. This shows that GSDMs can act as sensors of proteases, and act to prevent pathogens infecting the skin.

IL-18 is another proinflammatory cytokine regulated by Caspase-1. First described as "IFN-γ inducing factor", it was later renamed and is more closely related to IL-1 family cytokines[64]. Canonically produced in monocytes as an inactive pro form, it is activated and secreted as part of the NLRP3 inflammasome, where it goes on to induce Th1 T-cells with IL-12, leading to the production of IFN-γ[65]. However, it is also constitutively secreted as inactive pro-IL-18 by keratinocytes and other epithelial cells[66,67]. We proposed that it is activated by SpeB, and present that work in Chapter 2 of this dissertation. IL-1β, GSDMA, and IL-18 all have SpeB cleavage sites in a location close to the canonical Caspase-1 cleavage site, thereby allowing for activation.

SpeB also cleaves and degrades many bacterial proteins, potentially negatively impacting GAS virulence when it destroys other essential virulence factors[50]. M protein, the major GAS surface protein is cleaved by SpeB to allow shedding from the surface[68]. The loose M protein goes on to impede the immune response by blocking neutrophils[69]. Other proteins degraded include C5a peptidase[68], streptokinase[70], and the superantigen SmeZ[71]. The degradation, or lack thereof, for other superantigens is presented in Chapter 3 of this dissertation. Cleavage of self-proteins is used to regulate infection, and progression of invasive disease is dependent on SpeB[72].

Superantigens

Superantigens are a class of proinflammatory toxins produced by GAS that non-specifically activate immune cells. Originally found as scarlet fever causing toxin[7], they are essential for scarlet fever and are the driving virulence factor of STSS. By bypassing the typical antigen processing pathway through binding of major histocompatibility complex class II (MHC-II) receptors and T cell receptors (TCR), superantigens overstimulate the immune system[8]. This activation leads to massive increases in proinflammatory cytokines such as IL-1β, IL-6, and IFNγ to create a cytokine storm[8,73]. This results in tissue damage, disseminated thrombosis, and organ failure[25].

There are many superantigens produced by GAS and *Staphylococcus aureus*. They can be broadly classified into 5 different groups based on sequence and structural homology[8]. The superantigens made by GAS are assigned to groups II, IV, and V, while those made by *S. aureus* are assigned to groups I, II, III, and V[74]. Of the 13 produced by GAS, most are named streptococcal pyrogenic exotoxin (Spe), with exception to streptococcal mitogenic exotoxin Z (SmeZ) and streptococcal superantigen (SSA)[3]. Those produced by *S. aureus* are staphylococcal enterotoxins (SE) and toxic shock syndrome toxin-1 (TSST-1)[74]. In GAS, genes *speG*, *speJ*, *speQ*, *speR*, and *smeZ* are on the chromosome, but not ubiquitous across strains[75]. The remaining superantigens are encoded on prophage and are transmissible across strains[76].

Broadly, superantigen binding is focused on Major Histocompatibility Class II (MHC-II) receptors and the T Cell Receptor (TCR). MHC-II receptors are exclusive to professional antigen presenting cells (APC) such as dendritic cells, B cells, and phagocytes[77]. Superantigens bind MHC-II through 4 mechanisms: bind the α-chain dependent or independent of the antigen peptide, bind the β-chain mediated by zinc, or a combination of all of these[78]. Superantigens produced by

GAS commonly bind to the HLA-DQ chain of the receptor[79], while those produced by *S. aureus* bind to the HLA-DR chain[80]. Binding of the TCR is done on the Vβ chain, where each superantigen will have unique interactions with the variable side chains[78]. CD28 on T cells, and CD80 or CD86 on antigen presenting cells act as co-factors and are necessary for proper binding[81].

Superantigens can activate up to 20% of host CD4 and CD8 T cells[6], triggering a proinflammatory cytokine storm within the host. This massive upregulation leads to increases in IL-2, IFN-γ, and IL-12[6]. The excessive IFN-γ present promotes intracellular replication within macrophages[82]. Upregulation of cytokines from antigen presenting cells including TNFα, IL-1β, and IL-6 from NFκB[83]. Cytokines lead to further T cell and APC recruitment, with additional activation, thus causing a cytokine storm[6,8]. One downstream consequence of CD4 T cell amplification is the promotion of the allergen response[84]. Superantigen induction of TNFα[85,86] helps aid in the recruitment of neutrophils[87], which are used by GAS to enhance infection[11].

While superantigens can activate T cells present throughout the human body, recent research has pointed to mucosal-associated invariant T (MAIT) cells as a major driver of the response to superantigens. The presence of MAIT cells in human peripheral blood is around 10% of the total T cell population. In patients with STSS, MAIT cells have been identified as 15% of TNF producing T cells, and from 41% to 60% of IFN-γ producing cells[88]. MAIT cells are also elevated in patients hospitalized with Acute Rheumatic Fever[89]. GAS lacks the riboflavin pathway that canonically activates MAIT cells[90]. Instead, MAIT cells are likely being activated in a cytokine dependent manner by IL-12 and IL-18[91], both known to be induced by superantigens, or in the case of IL-18, activated by GAS through SpeB. Depletion of MAIT cells from PBMCs prior to GAS infection resulted in significantly mitigated cytokine storm[92]. Altogether, MAIT cells are a major source of pathology from GAS infections.

There are several rationales proposed for why superantigens are produced during infection. The common thinking is that GAS utilizes them for colonization, as superantigens promote colonization in superantigen-susceptible mouse models[93–96]. Research has shown that bacteria can modulate the T cell response to superantigens through ligands from the bacterial cell wall[97]. Another idea proposed is that overstimulation by superantigens prevents an effective adaptive immune response. One mechanism is the development of T cell anergy due to overstimulation, slowing down the response to GAS[79]. In addition, patients with recurrent tonsillitis have shown a less effective antibody response to SpeA due to the death of B cells[10,98], preventing the formation of memory for subsequent infections. Altogether, superantigens modulate the host immune response in ways that benefits GAS during infection.

Increased expression of superantigens or acquisition of new superantigens has been linked to isolates responsible for major outbreaks of scarlet fever. There have been recent outbreaks of scarlet fever causing isolates in the United Kingdom by the $M1_{UK}$ clade of isolates, notable for increased expression of SpeA[20]. $M1_{UK}$ isolates found in Australia had a mutation in *ssrA*, resulting in readthrough into SpeA and enhancing expression[99]. The $M1_{UK}$ strain has been confirmed to also be present in Canada[100], the United States[101], and the Netherlands[102]. Another example was a 2011 outbreak of scarlet fever in Hong Kong, where a strain of GAS acquired SSA and SpeC expression from a phage[21,103]. The introduction of new superantigens, or increased expression of already present ones, is sufficient to cause outbreaks and increases the potential for global spread.

Antibiotic Treatment of GAS Infections

The β-lactam penicillin remains the gold standard for antibiotic treatment of many GAS infections[2]. β-lactams target penicillin-binding proteins (PBPs) to block peptidoglycan cross-linking in metabolically active bacteria, leading to bacterial death (**Fig 1**)[104]. Despite extensive use for decades, there has been minimal change in GAS susceptibility to penicillin[105]. Discovered in 1928 by Alexander Fleming, penicillin was brought to clinical trials in 1941. But it did not take long for resistance to be observed. Penicillinase-producing *E. coli* were observed in 1940, and strains of penicillin-resistant *S. aureus* were clinically found in 1942, with 80% resistant by the end of the 1960s. Semi-synthetic versions of penicillin such as methicillin were generated in response, however it would only take 20 years for methicillin resistance to become endemic[106].

In animal models and human infection, clindamycin is also effective against severe GAS infection[107,108]. Clindamycin is a semi-synthetic lincosamide antibiotic that targets the 50S subunit of the ribosome[109]. Inhibition of the ribosome occurs through blocking of the peptidyl transferase reaction, preventing protein synthesis in susceptible pathogens, commonly Gram-positive bacteria of *Streptococcus*, *Staphylococcus*, and *Clostridium* species[110]. Clindamycin is bacteriostatic and can limit the production of toxic proteins and virulence factors independent of its effects on growth (**Fig 1**)[111]. This is also true for GAS[112], where clindamycin inhibition of M protein synthesis promotes phagocytic killing[113] and inhibition of superantigens and other toxins [112,114] can mitigate septic shock[111]. Similar anti-toxin effects have been described for *Clostridium perfringens*[115] and *Clostridioides difficile*[116].

Because of their efficacy, both penicillin and clindamycin are recommended as of 2014 by Infectious Diseases Society of America guidelines for necrotizing GAS infections[2]. They should be used in combination with surgical interventions. Due to a mortality rate of 30% or higher from

severe symptoms, treatment should be rapid to minimize risk of death[117]. While penicillin and clindamycin are not antagonistic when prescribed together, there is no inherent bactericidal benefit to using both[118]. However, the added benefits of clindamycin may come from ribosome inhibition reducing the development of toxin-mediated symptoms like STSS[119]. Since penicillin treatment can lead to lysis and toxin release[107], protein synthesis inhibitors like clindamycin[120] that decrease toxin production can help mitigate excessive immune stimulation[107]. It remains to be determined whether adjunctive use of additional antibiotics improves treatment[121]. For clindamycin-resistant GAS, early experimental work suggests linezolid[122] as a suitable alternative, while gentamicin is also suggested as a potential candidate, albeit with potential toxicity[123].

Antibiotic Resistance

GAS develops resistance to clindamycin by two primary mechanisms: target site modification or efflux pumps. Methylation of clindamycin target sites on the 23S RNA by ErmA, ErmC, or enzymes are most common[124]. Isolates with methylated target sites can either have constitutive or inducible resistance to clindamycin[125]. Inducible resistance can result in treatment failure, as inducible clindamycin resistance is undetectable unless macrolides are also present[126]. Efflux pumps such as MsrA and MefA are a common resistance mechanism involved in macrolide resistance[127]. Despite the structural similarity of clindamycin and macrolides, these pumps have shown greater efficacy against macrolides[128]. *Staphylococcus* species may also enzymatically inactivate clindamycin through LinA[129]. Due to the abundance of antibiotic resistance genes on plasmids, there is concern of horizontal gene transfer generating new resistant strains[76].

Clindamycin resistance in the United States is on the rise, from an estimated at 0.5% in 2003[130] to currently as high as 15% in pediatric GAS infections[131]. Isolates from invasive infections are more commonly resistant, increasing from 2% to over 23% in this time[132]. Resistance rates are geographically variable; in China, resistance may approach 95.5%[133], where over similar period, northern Europe rates approximated 1%[134]. Despite the rapid change in resistance trends and the emergence of potentially hypervirulent, resistant strains, the recommendation remains continue the use of protein synthesis inhibitors such as clindamycin when necessary, but to be mindful and vigilant for resistant isolates[2].

β-lactams and macrolides are the drugs of choice for GAS, and therefore have the highest concern for the development of resistance. Along with rapid increases in erythromycin and clindamycin resistance, tetracycline resistance is widespread, and levofloxacin resistance has been observed[132] However, the challenges with GAS treatment are still typically antibiotic failure, not intrinsic drug resistance. No resistance to vancomycin or β-lactams has been observed.

β-lactam Resistance Concerns

The answer to why GAS has not developed resistance to β-lactams despite extensive use and widespread resistance in related species has remained elusive. A study in 1998 found no significant change in MIC over time[105], and this trend has continued[132]. While there have been clinical isolates with elevated penicillin MIC values reported in India, Japan, and Mexico[135–138], no mechanism has been provided. In other Streptococci, resistance is primarily conferred by mutations within PBPs. One proposal is that PBPs with low affinity for β-lactams are poorly tolerated by GAS[139]. Consistent with this, GAS engineered to express low-affinity PBPs had

growth defects, poor growth rates, and morphological abnormalities[140,141]. Additional work showed that decreases in M protein production could lead to resistance, at the cost of being avirulent[142]. Taken together, this suggests that penicillin sensitive PBPs are essential to GAS biology, and changes that would support resistance are either fatal or so detrimental that survival in a clinical setting is quite difficult. This has been partially backed up from recent work showing that three or fewer amino acid changes to PBP have occurred in 99% or more of the clinically relevant GAS strains[143].

A community outbreak of GAS in Seattle recently led to identification of two isolates with reduced susceptibility to β-lactams[144]. These isolates had a T553K substitution within *pbp2x* and a S79F substitution within *parC* of topoisomerase. These isolates had higher MIC values for ampicillin, amoxicillin, and cefotaxime than isogenic isolates, while the MIC for penicillin was unchanged. The two isolates have no confirmed epidemiologic link despite their genomes being nearly identical[144]. In the wake of these findings, there were concerns that these mutations were already worldwide. Subsequent studies have identified additional natural mutations in *pbp2x* responsible for reduced susceptibility[145]. Isogenic isolates with *pbp2x* mutations show no change in virulence in a mouse model, however they have a potential for increased fitness as more bacteria was recovered from tissue[146]. These mutations are concerning because of the similarities with antibiotic resistance in *Streptococcus pneumoniae*, another pathogen responsible for childhood disease[147]. Penicillin had been the antibiotic of choice for treatment, but resistance became widespread in the 1980s due to mutations in *pbp2x* and *pbp2b*[148]. One possible source of resistance was horizontal gene transfer into *S. pneumoniae* from other native oral streptococcal species such as *S. mitis*[149]. T550 in *S. pneumoniae pbp2x* corresponds to T553 in GAS *pbp2x*, suggesting future resistance could similarly arise[144].

Additional Considerations with Antibiotic Treatment

A penicillin allergy is one of the few reasons to consider another drug for most GAS infections. This allergy is estimated in 8% of patients, but an IgE mediated allergic response will only be visible in 1 in 20 people[150,151]. Allergy is often over reported or self-diagnosed, leading to other antibiotics being prescribed when unnecessary[152]. Vancomycin or linezolid are common alternatives for those with severe penicillin allergies[2]. Allergic reactions to clindamycin are rare, it has therefore become common as an alternative choice in instances of allergic reactions to other antibiotics[153]. Since infection is recurrent for many people, repeated use of penicillin may drive allergy development, select for resistance in other species of microbes present, and give rise to serious opportunistic infections by pathogens such as *Clostridioides difficile* from the use of antibiotics[154,155].

Mechanisms for Treatment Failure

Thus despite *in vitro* sensitivity to many antibiotics, including near universal sensitivity to penicillin, GAS remains a major public health burden. Treatment failure was first reported not long after the introduction of penicillin[156], and has remained a problem ever since in both common pharyngitis and more severe invasive infections[157–159]. Death due to treatment failure is not exclusively found in regions lacking access to antibiotics and prompt medical treatment, even in resource-rich countries invasive infections can have a high failure rate during treatment[160]. Since death is not always from overwhelming bacteremia, but rather pathological inflammation as sepsis, a bolus of antibiotic leading to massive bacterial lysis may transiently exacerbate disease or even

lead to death[161]. Individuals treated with only penicillin have also shown greater risk of recurrent tonsillitis, suggesting an inability to clear the infection fully[162].

Bacteria can survive at antibiotic concentrations beyond a minimal bactericidal concentration (MBC) by a process known as the Eagle Effect[163]. First observed in 1948[164], it is speculated to be related to penicillin having greater efficacy on bacteria in log phase growth, as they are actively rebuilding their peptidoglycan[156]. During infection, resource limitation and antimicrobial immune responses slowing bacterial growth may lead to decreased antibiotic efficacy (Fig 2). The Eagle Effect has manifested in treatment failure using the mouse model of GAS infection, where delaying penicillin treatment leads to significant reduction in survival[165].

Community-mediated resistance (Fig 2) is another mechanism that may contribute to failure, where β-lactamases secreted by the resident microbiota in the polymicrobial environment protect sensitive pathogens, including GAS[166,167]. One study showed β-lactamase producers were found in 40% of pediatric patients with orofacial or respiratory tract infections[168], with another suggesting rates as high as 74% in the tonsils[169]. One potential impact of clindamycin is therefore killing β-lactam resistant species that provided protection to GAS, allowing for later reinfection[162]. The deep tissue is commonly sterile, so community resistance is more likely to play a role during pharyngitis where these is an abundant polymicrobial community present.

Biofilms are an aggregate of bacteria encased in an extracellular matrix and contribute to many bacterial species' ability to resist immune effectors and antibiotics. Aggregates of GAS consistent with biofilm formation have been observed in the nasopharyngitis[12] and skin[170,171]. The GAS biofilm requires cell surface anchored proteins such as pili and the serotype-specific M protein to contribute to a hydrophobic cell surface and aggregation of GAS chains on biotic and abiotic surfaces[172–175]. Host proteins recruited by cell surface-anchored virulence factors further

contribute to aggregation and shield GAS from antimicrobials[176–178]. This protection is also extended toward antibiotics (Fig 2), with biofilm formation associated with a reduced efficacy of antibiotics *in vitro* and *in vivo*[174,179,180], including a 2500-fold increase in penicillin tolerance in one study[181].

While dual role of biofilms in pathogenesis and antibiotic failure is well-recognized, and a target for future therapeutics, this connection is less explored with other virulence factors. GAS can invade macrophages[182,183], epithelial[184], and other host cells and resist autophagy and other mechanisms to promote their intracellular growth[185]. Intracellular GAS are shielded from penicillin (Fig 2), which cannot cross the cell envelope, and the ability to invade cells is correlated with eradication failure during treatment of pharyngitis[186]. Thus virulence factors required for cell invasion may promote penicillin failure, but not failure of cell-penetrating antibiotics such as clindamycin or erythromycin are more effective against intracellular GAS[184]. Penetration of antibiotic into tissue is also a hurdle that is worsened during severe infections[133,156,187,188]. Edema, thrombosis, and tissue necrosis are pervasive during necrotizing fasciitis and other invasive GAS infections and drastically limit antibiotic perfusion (Fig 2); for this reason surgical removal of infected tissue is often required, even for highly antibiotic-sensitive GAS[2]. This pathology is caused directly by streptolysin O and other GAS toxins[189].

Together, these observations suggest that the virulence factors GAS use to escape the immune system are tied to its ability escape antibiotics. Neutralizing antibodies and small drug inhibitors of GAS virulence factors thus have the potential to not only reduce pathogenesis and restore the effectiveness of the immune response, but to work synergistically with conventional antibiotics to break the resistance/tolerance mechanisms of GAS.

Antivirulence Treatment

Since inhibiting toxin production has therapeutic benefits, neutralizing their activity may also be therapeutically useful. Intravenous immunoglobulin (IVIG) is an experimental adjunctive treatment for severe GAS infections that targets toxicity and promotes effective immune responses[190]. IVIG is generated from the pooled serum from healthy human donors and thus contains a panel of antibodies against diverse, but undefined, bacterial targets[191]. These likely include major toxins and surface-anchored virulence factors[13]. Through their neutralization[192] and increased opsonization of the bacterium, IVIG antibodies can decrease bacterial burden and limit proinflammatory cytokine storms (Fig 1)[193]. The repertoire of virulence factors produced by GAS is variable, as is the repertoire of specific antibodies between donors used for IVIG[194], so the ability to neutralize toxins will vary between treatments and requires optimization[195,196]. Typical side effects include headaches or nausea[197], but there are risks for rare but severe complications[198]. Additional technical restrictions on using IVIG are the high cost of generation, storage requirements, and the risk of bloodborne pathogens found in any human blood.

In mice, IVIG has clear efficacy in models of STSS[199] and necrotizing fasciitis[200]. Because cases of severe GAS infections are rare, the opportunity to perform proper control trials are limited and many findings may be underpowered. In some hospitals, IVIG is routinely used in tandem with clindamycin, though in one study this did not provide statistically significant improvement compared to clindamycin alone[108,201]. One trial was cancelled due to limited enrollment, but the IVIG group had significant improvement compared to placebo[202], while another trial of 100 patients found no benefit over antibiotics alone[203].

Because of their role in severe infections, there have been efforts to synthesize therapeutics specifically targeting superantigens[204]. There have been efforts to utilize neutralizing antibodies

against superantigens from *S. aureus*[205]. While there has been some success in mouse models, administration must be done early as the antibodies will only prevent superantigen binding, not the resulting cytokine storm. Likewise, there have been efforts to develop small peptides to block superantigen binding, with mixed results[206,207]. However targeting CD28 with antibodies has shown success[208,209]. But like the antibodies, blocking receptor binding must be done before initial superantigen binding, and runs the risk of interfering with typical immune function.

Vaccines for GAS

There is currently no vaccine for GAS infection, as attempts in the past have been unsuccessful. From the 1920s trials occurred for 50 years, starting with whole killed GAS and the cell wall, eventually narrowing to the M protein[210]. In the 1960s a trial using purified M3 protein resulted in three participants developing rheumatic fever (RF)[211]. This was one of the main reasons in 1979 the FDA banned all vaccine trials involving GAS components in the United States for nearly 30 years[212].

While there are numerous issues related to global strain diversity and antigenic variation, the major concern is the development of autoimmune sequelae[213]. Historically most vaccines target the M protein, the most abundant protein on the surface of GAS, as well as the protein responsible for strain typing. However, antibodies for M protein can be cross reactive with myosin found in the heart, leading to autoimmune conditions such as RF and RHD[3]. RF and RHD are side effects of GAS infections where the host generates an immune response to itself, primarily in the heart and joints. There is an additional risk of post-streptococcal glomerulonephritis (PSGN) in the

kidneys[214]. Due to the risk of causing these side effects, it has made generating a vaccine based on M protein difficult.

Despite these risks, there has been substantial work in recent years to develop a vaccine[215]. To address the variety of circulating strains, most modern vaccines contain multiple N-terminal fragments of different M proteins. As of this writing, the furthest along trial is a 30-valent M protein vaccine[216]. By including 30 variations of M protein, it is estimated to reach almost 50% of relevant pathogenic strains worldwide[217]. In addition, reports have shown that cross-opsonization is occurring, which will allow for antibodies to react against variations of M protein not directly included within the vaccine[218].

There have also been attempts to create a vaccine that does not target the M protein. The logical choice would be to use the GAS hyaluronic acid capsule as a potential candidate; however, hyaluronic acid is also found in human tissue and used by GAS as a form of molecular mimicry[219]. Forming an immune response would trigger a response to self-proteins. Another surface target of interest is the group A carbohydrate, a component of the cell wall[220]. After removal of the N-acetylglucosamine side chains, it was found to protect mice from cutaneous, but not invasive, GAS infections[221]. GAS antigens have also been considered for multi-component vaccines[222]. Some candidates include a SLO, SpyCEP and SpyAD trivalent vaccine[223], SpeA[94], and C5a peptidase[224].

Closing Comments

Until a vaccine is developed for GAS, antibiotics will remain essential for treating infection. The gold standard, penicillin, has been effective at treating GAS for over 80 years with no resistance, but a low, consistent, rate of failure. Since other bacteria eventually gain resistance

to the antibiotics commonly used in their treatment, it can be expected that GAS may eventually become resistant, which will lead to massive increases in morbidity and mortality. If the mutations in *pbpx2* of GAS continue to follow the same progression as *S. pneumoniae*, this may not be in the distant future[144,148]. However, all mutations identified thus far are insufficient for non-susceptibility and carry a fitness cost, both of which will require additional compensatory mutations for GAS to overcome[225]. Therefore, dedicated surveillance is essential as the emergence of penicillin resistance by GAS would constitute a public health crisis.

Other methods of treatment beyond β-lactams are essential for handling severe GAS infections. While resistance is on the rise globally, clindamycin is one of the most effective treatments available alongside β-lactams to manage necrotizing fasciitis or STSS. With rapidly rising resistance we lose this tool and will require new therapeutic strategies. As with penicillin, surveillance is crucial to determine current resistance trends. Properties that would be desired in these drugs, to complement the shortcomings of penicillin, include the targeting of vegetative bacteria in biofilms and intracellular bacteria. IVIG is a promising method to improve survival during severe infections, but it may not be a replacement for clindamycin or another effective antibiotic. Understanding how resistance develops and the global profile of resistance will ensure that new drugs can be developed and deployed in the proper locations.

In this dissertation, novel interactions of SpeB with host cytokines and streptococcal superantigens are presented. Chapter 2 introduces IL-18 as another target of SpeB, reinforcing its role as a bacterial caspase. Using primary human keratinocytes, we show that secreted pro-IL-18 is matured by SpeB into an active form. Because of the proinflammatory nature of IL-18, it remains to be determined if the active IL-18 is used to respond to the pathogenic GAS, or if it is used by the bacteria to modulate the host immune system in a favorable manner. Chapter 3 further

characterizes the interaction of superantigens with SpeB. Using a panel of superantigens from GAS and *S. aureus*, we show that not all superantigens are cleaved and degraded by SpeB. It is then further shown that SpeB goes on to synergize and enhance the resulting cytokine storm generated by these toxins. Taken together, the findings presented here further the understanding of how GAS uses SpeB to modulate the host immune system during infection. The implications of these findings and future directions will be discussed in chapter 4.

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Figures

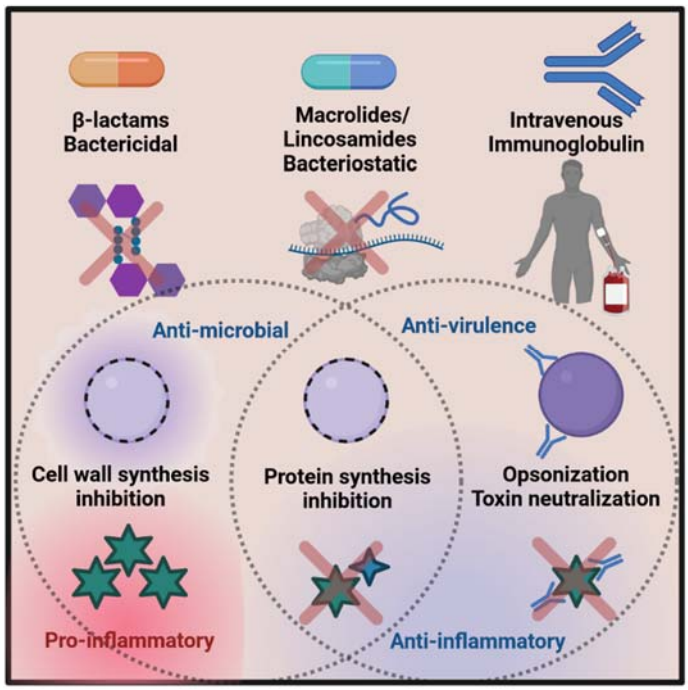

Figure 1. Summary of treatment methods discussed in this review.

Bactericidal β-lactams such as penicillin target the peptidoglycan of the cell wall, leading to cell lysis. This can lead to an efflux of virulence factors and other cellular proteins, resulting in inflammation. Macrolides and lincosamides are bacteriostatic, blocking protein synthesis by targeting the bacterial ribosome. Preventing toxin synthesis works to reduce inflammation. Intravenous immunoglobulin (IVIG) is an infusion of pooled antibodies from human donors, which works to induce opsonization and neutralize toxins, reducing inflammation. Originally created for Johnson and LaRock 2021[226] with Biorender.com.

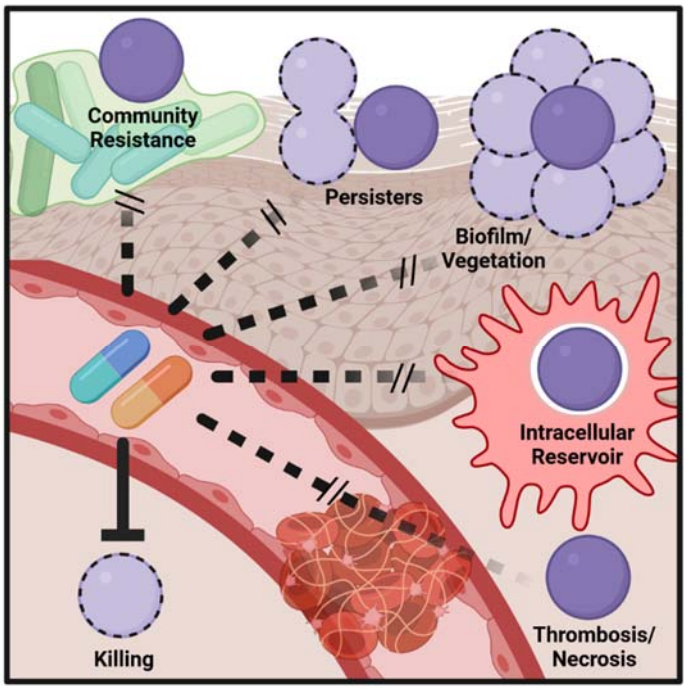

Figure 2. Model of mechanisms contributing to antibiotic failure during GAS infections.

Community-mediated resistance mediated by protection by endogenous microbiota is likely most prevalent during pharyngitis and not invasive infections, where GAS most often exists as a monoculture. Persisters, resistant through altered growth rates or other epigenetics states, can contribute to treatment failure of any infection. Formation of biofilms, invasion of epithelial cells, and survival within phagocytes can similarly occur during any infection and serve to shield single bacteria from antibiotic action. During invasive infections in particular, inflammation- and toxin-mediated necrosis of tissue and thrombosis of dermal vasculature can limit antibiotic perfusion, necessitating surgical removal of infected tissue. Originally created for Johnson and LaRock 2021[226] with Biorender.com.

Chapter 2

Constitutive secretion of pro-IL-18 allows keratinocytes to initiate inflammation during bacterial infection

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Abstract

Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is an obligate human pathogen that commonly infects the skin. Keratinocytes are one of the first cells to contact GAS, and by inducing inflammation, they can initiate the earliest immune responses to pathogen invasion. Here, we characterized the proinflammatory cytokine repertoire produced by primary human keratinocytes and surrogate cell lines commonly used *in vitro*. Infection induces several cytokines and chemokines, but keratinocytes constitutively secrete IL-18 in a form that is inert (pro-IL-18) and lacks proinflammatory activity. Canonically, IL-18 activation and secretion are coupled through a single proteolytic event that is regulated intracellularly by the inflammasome protease caspase-1 in myeloid cells. The pool of extracellular pro-IL-18 generated by keratinocytes is poised to sense extracellular proteases. It is directly processed into a mature active form by SpeB, a secreted GAS protease that is a critical virulent factor during skin infection. This mechanism contributes to the proinflammatory response against GAS, resulting in T cell activation and the secretion of IFN-γ. Under these conditions, isolates of several other major bacterial pathogens and microbiota of the skin were found to not have significant IL-18-maturing ability. These results suggest keratinocyte-secreted IL-18 is a sentinel that sounds an early alarm that is highly sensitive to GAS, yet tolerant to non-invasive members of the microbiota.

Author Summary

Timely and appropriate inflammation is critical for an effective immune response against an invading pathogen. Some immune effectors are stored in an inert form that is enzymatically activated. This can allow for responses much more rapidly than transcriptionally regulation, on the order of minutes. An important regulator of these rapid responses is the inflammasome, which

among its activities, processes the proinflammatory cytokines IL-1β and IL-18 into truncated forms that gain the ability to bind their cognate receptors. These cytokines are typically stored intracellularly until activation, but human keratinocytes constitutively secrete IL-18 in its unprocessed form. We show that this pool of IL-18 initiates an immune response to the human pathogen group A *Streptococcus*, but not non-pathogens. Mechanistically, this occurs from IL-18 cleavage by SpeB, a major virulence factor that is required for tissue invasion. Keratinocytes live in close association with diverse microbes and face a challenge in selectively responding to pathogens while tolerating normal microbiota. Our findings outline a strategy for discriminating species with high virulence potential through their use of proteolytic virulence factors and IL-18 as bait.

Introduction

The skin provides the body's outermost and first resistance to infectious, chemical, and physical insults. The obligate human pathogen Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) colonizes oropharyngeal mucosa and epidermal surfaces, specifically adhering to and invading skin epithelial cells and keratinocytes[1–4]. Beyond superficial infections like impetigo, further tissue invasion can lead to cellulitis, sepsis, and necrotizing fasciitis. Invasive infections, and immune-mediated sequelae like rheumatic heart disease, are responsible for an estimated half a million annual deaths globally[5]. Inflammation typically serves to recruit and activate an antimicrobial immune response that is host-protective, but excess inflammation can contribute to tissue damage and complicates the treatment of invasive infections[6]. GAS induces inflammation by several mechanisms, including dedicated virulence factors like superantigens, and several lines

of evidence suggest that at some body sites, inflammation can promote GAS replication and transmission[7–9]

Interleukin-18 (IL-18) is a proinflammatory cytokine that induces cell-mediated immunity[10]. IL-18 is detected by the IL-18R/IL-18RAP (IL18R1/IL1R7, IL-18Rα/IL-18Rβ) receptor complex and works with IL-12 to induce IFN-γ and Th1-type responses from T cells, NK cells, and dendritic cells[11–13]. Accordingly, IL-18 is important in the host defense against *Salmonella* Typhimurium[14], *Shigella flexneri*[15], *Yersinia enterocolitica*[16], *Listeria monocytogenes*[17], *Burkholderia pseudomallei*[18], *Mycobacterium tuberculosis*[19], *Streptococcus pneumoniae*[20], and group B *Streptococcus*[21]. Newly synthesized IL-18 (pro-IL-18) is inert and requires the removal of an amino-terminal autoinhibitory domain, which is canonically achieved by the host protease caspase-1[11,12]. Caspase-1 is regulated by the inflammasome complex, which in myeloid cells can also regulate IL-1β maturation and the cell death program pyroptosis[22]. Caspase-8[23], granzyme B[24], chymase[25], proteinase 3[26], and neutrophil elastase[27] also cleave pro-IL-18, though their physiologic relevance in IL-18 activation remains to be established. Human keratinocytes constitutively express and release pro-IL-18[28–32], suggesting the skin as an anatomical location where extracellular proteases participate in IL-18 activation.

We hypothesized that these early GAS interactions with human keratinocytes could contribute to the development of inflammation during infection. This study shows that keratinocytes release numerous proinflammatory cytokines during GAS infection, including pro-IL-18. During homeostasis, this form of IL-18 would not ordinarily be activated nor have proinflammatory activity. However, upon infection by GAS it is directly matured by the bacterial secreted cysteine protease SpeB, activating a proinflammatory cytokine cascade. This mechanism supports a model wherein SpeB acts as a "bacterial caspase" that proteolytically activates proinflammatory

cytokines of the IL-1 family, which in humans may act as an early sentinel to limit GAS invasive infection and may broadly be a form of effector-triggered immunity against proteases.

Results

GAS induces keratinocytes secretion of proinflammatory cytokines

The epithelium is one of the first body tissues GAS will contact. Therefore, resident cells provide one of the earliest responses to infection by producing antimicrobial effectors, proinflammatory cytokines, and chemokines that are necessary to effectively coordinate the immune response[33–36]. To assess the cytokine repertoire elicited by GAS, we infected immortalized and primary epithelial cells with M1T1 GAS 5448, a highly virulent strain associated with modern epidemic invasive infections[37]. Similar to previous observations[33,34,38], we observed robust secretion of macrophage migration inhibitory factor (MIF), IL-8 (CXCL8), and other proinflammatory cytokines and chemokines by infected HaCaT keratinocytes (**Figure 1A**). Detroit 562 human pharyngeal epithelial cells and A-431 human keratinocytes all secreted a similar, nonredundant cytokine profile. Hep-2 cells are one of the lines reported by the International Cell Line Authentication Committee (ICLAC) to contain characteristic HeLa cell markers and were apparently derived by HeLa contamination of human laryngeal adenocarcinoma cells[39]. We include it for context since extensive prior work has used it to model epithelial cell infection and found that it produces a cytokine repertoire partially overlapping other cell lines, as well as CCL2 (MCP-1), which is not characteristic of epithelial cells (**Figure 1A**).

Compared to each cell line, human primary keratinocytes (NHEK) expressed a greater diversity in CXC-family chemokines than any individual cell line and were the only ones that released IL-1α, a surface-tethered pro-inflammatory cytokine constitutively produced by healthy

human epithelial cells (**Figure 1A**). Human primary endothelial cells (HUVEC) secreted a different repertoire of cytokines and chemokines, showing a distinct response (**Figure 1A**). No cell tested produced detectable CCL1, CCL5, G-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-17, IL-17E, IL-21, IL-27, IL-32α, MIP-1, or TNF-α at a resting state or during infection with GAS (**S1 Figure**). Overall, primary cells formed distinct groups by multivariate analysis relative to all cell lines (**Figure 1B**) due to their distinct and expanded cytokine secretion profiles (**Figure 1C**) that diverged further during infection. Altogether, these results suggest that common cell lines do not fully recapitulate the cytokine response as it may happen *in vivo*, and caution should be used when modeling the inflammatory output of the host-pathogen interaction while using them.

GAS activates IL-18

IL-18, a cytokine of the IL-1 family that bridges the innate and adaptive immune responses by stimulating IFN-γ production from T lymphocytes and NK cells[10], stood out for its expression pattern. In immune cells, IL-18 maturation is conventionally regulated by the inflammasome protease caspase-1, which also regulates its release through the cell death program, pyroptosis[40]. Thus, we expected to observe release only during infection, not from resting cells. In contrast, prior work has observed that primary human keratinocytes express a high level of pro-IL-18 and release it in their resting state without infection or further stimulation[29,41]. Consistent with these prior studies and **Figure 1A**, we observed that GAS infection was not required for IL-18 secretion, and there was significant release by healthy, uninfected keratinocytes (**Figure 2A**). IL-18 release is typically thought to require cell death, and we recently have shown that GAS induces cell death in keratinocytes via activation of the cell death effector Gasdermin A[42]. However, this IL-18

release did not require cell lysis (**Figure 2B**). Furthermore, no significant release of the inflammasome-regulated cytokine IL-1β was observed in the absence of infection, consistent with inflammasome-independent secretion of pro-IL-18 (**Figure 2C**). By microscopy, no cell lysis nor activation of inflammasome caspase-1 was observed under these infection conditions, though was increased at a higher multiplicity of infection (MOI) (**Figure 2D**).

We next examined whether the IL-18 secreted by keratinocytes was active. For cytokines that are post-translationally regulated by proteolysis, as for IL-18, activation is not necessarily specific to a single protease or cleavage site. Instead, there is potential for plasticity in this process, and the determinant of activity is exclusively whether the cleavage product(s) can bind and stimulate signaling through the cognate receptor[43]. To measure whether keratinocyte-released IL-18 had biological activity, we used HEK-Blue IL-18 Reporter cells, which have been engineered to express the IL-18 receptor complex (IL-18R and IL-18RAP) and induce an alkaline phosphatase reporter in response to IL-18, but not other cytokines or pathogen-associated molecular patterns. None of the IL-18 released by uninfected keratinocytes had activity; however, it was rendered active during GAS infection (**Figure 2E**). This suggested a GAS-dependent mechanism for the activation of IL-18. To exclude the possibility of the inflammasome or other keratinocyte factors in this activation, we removed supernatants from keratinocytes and incubated them with GAS. GAS was able to stimulate IL-18R signaling from this cell-free conditioned media (**Figure 2F**), suggesting conventional cellular regulators (the inflammasome) were not essential. Since no activation was seen by GAS alone, it does not directly stimulate IL-18R signaling, but instead, one or more GAS factors may directly act on the inert pro-IL-18 secreted by keratinocytes to convert it to an active form.

GAS protease SpeB is required for the activation of keratinocyte IL-18

To determine the specific bacterial factors responsible for the direct maturation of pro-IL-18 by GAS, we screened a panel of defined virulence factor mutants. During keratinocyte infection, we found that the secreted cysteine protease SpeB was essential for generating active IL-18 (**Figure 3A**). Complementation of Δ*speB* GAS 5448 restored activation, while catalytically inactive $SpeB_{C192S}$ did not, indicating the enzymatic activity of SpeB was required for IL-18 conversion (**Figure 3B**). Furthermore, in the absence of infection or other treatment, active SpeB alone was sufficient to activate IL-18 in a dose-dependent manner (**Figure 3C**). Taken together, SpeB is the GAS virulence factor responsible for IL-18 activation.

Conventional IL18 activation occurs by Caspase-1 removing an N-terminal pro-domain[40], freeing an ~18 kDa active C-terminal product to bind and form a signaling complex with IL-18R and IL-18RAP[44]. In contrast, SpeB initially truncated recombinant pro-IL-18 to 22 kDa which was further truncated down to several products ranging down to ~17 kDa (**Figure 3D**). SpeB will cleave after a Glu, Thr, Ala, Gly, Ser, Asn, or Asp that is preceded by a hydrophobic amino acid (Ile, Val, Phe, Tyr, or Met), particularly in regions rich in negatively-charged amino acids (Asp or Glu)[45,46]. The N-terminus of pro-IL-18 contains several potential cleavage sites for, matching these observations by SDS-PAGE and N-terminal cleavages confirmed Edman sequencing (**Figure 3E**).

T cell activation by keratinocyte IL-18 requires SpeB

IL-18 was initially discovered for its ability to induce type II interferon (IFN-γ) from T cells[47]. Thus, we further examined whether the keratinocyte IL-18 processed by GAS is able to signal to induce IFN-γ production from human peripheral blood mononuclear cells (PBMCs)

(**Figure 4A**). Keratinocytes alone did not induce IFN-γ from PBMCs, consistent with an insufficiency of the pro-IL-18 they release to signal or be activated by PBMCs (**Figure 4B**). However, during infection with SpeB-expressing GAS, there was activation of IL-18 signaling and production of and a proportionate SpeB-dependent increase in the production of IFN-γ (**Figure 4B**). Purified SpeB was sufficient to induce IFN-γ (**Figure 4B**), which required both NHEKs and specifically T cells and/or NK cells, since IFN-γ signaling was lost when $CD2^+$ cells were depleted (**Figure 4C**). Consistent with an inflammasome-independent origin of this IL-18 signaling, IFN-γ was significantly decreased by anti-IL-18 neutralizing antibody, but not the inflammasome inhibitor drugs YVAD-cmk or VX765 (**Figure 4D**).

IL-18 secretion is species-restricted

IL-18 and IFN-γ are critical in human immunity and broadly protective against infection[48–50], therefore, they are commonly studied using primary human cells such as whole blood or PBMCs[6,51–54]. Mice are frequently used to model infection, but only a few instances have *il18*$^{-/-}$ mice are susceptible to infection[19,55–57]. This suggests that there could be differences in immune signaling between species. However, since IL-18 expression is elevated in experimental murine infections[43,52,58], we decided to examine whether IL-18 activation by SpeB could provide protective benefit in the typical skin infection model. No difference in GAS growth was observed between wild-type C57BL/6 and *il18*$^{-/-}$ mice (**Figure 5A**), suggesting no protective benefit in a model of acute infection. Since mice are not a natural host for GAS, we next sought to determine whether this was a species-dependent difference. Mouse pro-IL-18 has only 64.2% identity to human, with the greatest divergence in the N-terminal pro-domain. Nonetheless, SpeB-processed mouse pro-IL-18 to a biologically active product, albeit to a lesser extent than Caspase-1 (**Figure**

5B). However, when we examined primary mouse keratinocytes, we found that, unlike human cells, they did not secrete detectable IL-18 into the supernatant (**Figure 5C**), but they did contain intracellular stores that could be released by chemical lysis (**Figure 5D**). This intracellular IL-18 lacked activity but could be converted to the active form when incubated with SpeB (**Figure 5E**). Thus, SpeB-dependent activation of keratinocyte IL-18 in mice is limited by differences in IL-18 secretion between species.

IL-18 activation is microbial species-restricted

GAS with mutations in the CovS component of the CovRS (CsrRS) two-component regulator naturally arise in human invasive infections and in murine models[59–61]. *covS* mutants no longer express SpeB, enter keratinocytes, or activate IL-1β or GSDMA[42,43]. Similarly, *covS* mutants do not activate keratinocyte-secreted pro-IL-18 (**Figure 6A**), and variable expression between clinical isolates leads to heterogeneity in whether any single clone activates IL-18 (**Figure 6B**). Many bacteria other than GAS secrete proteases, they are one of the most common protein classes and are frequently employed by pathogens as virulence factors. IL-1β, for example, is directly activated not only by SpeB, but also by *Pseudomonas aeruginosa* LasB and *Staphylococcus epidermidis* Esp[43,62,63]. These proteases potentially represent a group of "bacterial caspases" that may share the activation of these two related IL-1 family cytokines. Proteases such as these are often cell-density regulated and accumulate in overnight cultures of bacteria. Of these species and other major skin bacteria, under these conditions only GAS released proteases that activated the inert pool of pro-IL-18 secreted by keratinocytes (**Figure 6C)**. This suggests that constitutive pro-IL-18 secretion by human keratinocytes does not result in chronic inflammation, in part, because few proteases extracellularly convert IL-18 into an active form. In support of this,

we tested gain-of-function using *Lactococcus lactis* made to express SpeB and found that the expression of catalytically active SpeB was sufficient for *L. lactis* to activate IL-18 (**Figure 6D**). Suggesting that targeting bacterial virulence factors can impact this process, treatment with E-64, a cysteine protease inhibitor active against SpeB, repressed GAS activation of IL-18 (**Figure 6E**).

Discussion

SpeB is essential for GAS colonization throughout the body and penetrating deeper into tissue during invasive infection[43,64]. Skin keratinocytes are thus poised to mount some of the earliest immune responses against this important pathogen. Still, they must safeguard against aberrant activation by the microbiota since they are in constant contact with numerous other species they must tolerate. Consistent with prior observations, we observed constitutive pro-IL-18 secretion by primary human keratinocytes[28–31], (**Figure 1A** and **2A**) and discovered that it could be directly activated by SpeB (**Figure 3D**). Other bacterial species relevant for colonizing the skin do not similarly activate immature pro-IL-18 (**Figure 6C**), so we hypothesize that this pool of cytokine is poised for activation to serve as an early sentinel for infection by potentially invasive pathogens that make use of proteolytic virulence factors. By directly sensing specific proteases required for infection, IL-18 allows keratinocytes to discriminate between numerous species with high virulence potential and non-pathogenic commensal bacterial species. Furthermore, there may be pathogens from genera other than *Streptococcus* with this activity since secreted proteases are broadly important for human pathogens and microbiota alike[65].

Diverse proteases generate a variety of IL-18 truncations, but how this impacts signaling activity has been unclear[24–27]. Recent NMR studies show that mature (caspase-1-cleaved) IL-18

has distinct spectra from pro-IL-18, suggesting that intramolecular interactions inhibit receptor binding from the pro-domain inducing significant structural changes in the cytokine[44]. Amino-terminal truncations showed removal of the first eight amino acids gave folding similar to pro-IL-18, the first ten an intermediate form, and 12, 13, or 22 a form resembling the mature protein[44]. Our observations, with those of Tsutsumi et al.[44], suggest that removing variable portions of N-terminus is still sufficient for cytokine activation. Additional factors may regulate IL-18 activation beyond the primary sequence in this region, such as stabilizing reactions in the secondary structure burying side chains recognized by LasB or other promiscuous proteases in the environment.

We further observed that primary keratinocytes secrete a more diverse set of cytokines relative to immortalized cell lines (**Figure 1**). One of them is IL-18, a cytokine that serves as a crucial bridge from the innate immune responses to the adaptive immune cells. However, all cytokine profiles were variable between cell lines, which are commonly used or interpreted interchangeably. Therefore, caution should be exercised when immortalized cells are used for modeling host-pathogen interactions, and autocrine or paracrine effects of these cytokines should be considered when there is potential to impact experimental findings. Furthermore, antimicrobial effectors are commonly co-regulated by many of the same signaling transduction networks, and these potential defects in each line can mask pathogen phenotypes. Furthermore, unlike keratinocytes, endothelial cells did not release IL-18. Therefore, extracellular activation of IL-18 by SpeB may be most important within the skin, giving an implicit role in priming early innate and adaptive immune responses to infection.

Materials and Methods

Bacterial Strains

GAS strain 5448, representative of the pandemic M1T1 clone, and its isogenic *ΔspeB, Δemm1, Δslo, ΔspyA, ΔcepA, Δmac, ΔcovRS,* JRS4, M4C20, *Pseudomonas aeruginosa* PA01, *Staphylococcus aureus* USA 300, and *Lactococcus lactis* carrying pSpeB have been previously described [36,43,62,66]. HKU16, a M12 scarlet fever isolate, was provided by Mark Walker and previously described [67]. *Streptococcus anginosus* F0211, *Streptococcus intermedius* F0413, and *Streptococcus mitis* F0392 were obtained through BEI Resources, NIAID, NIH. *Streptococcus constellatus, Staphylococcus hominus, Staphylococcus hominus,* and additional GAS clones are all clinical isolates provided through the Emory University Investigational Clinical Microbiology Core. All bacteria were statically grown overnight at 37 ℃ with 5% CO_2 in Todd Hewitt broth (THB, Difco), washed two times with phosphate-buffered saline (PBS), and diluted to a multiplicity of infection (MOI) of 10 for *in vitro* infections. Selection for bacteria carrying pSpeB was maintained with spectinomycin (Sigma) 100 μg/ml and expression was controlled with titrations of anhydrotetracycline (Cayman Chemical) as previously[66].

Cell Culture

A-431 lung epidermal cells (ATCC), Detroit 562 pharyngeal cells (ATCC), HaCat cells (unavailable from a repository, provided by C. Quave, Emory), and HEp-2 laryngeal cells (ATCC) were cultured in Eagle's Minimum Essential Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (hiFBS, Atlanta Biologicals). HEK-Blue™ IL-18 Reporter cells (Invivogen) were cultured in Dulbecco's Modified Eagle's Medium (Gibco) with 10% hiFBS. 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL normocin (all Invivogen) were

supplemented during routine culture and omitted during experimental infections, unless otherwise noted.

Primary Cell Culture

Primary human keratinocytes were supplied by Lonza and cultured in Growth Medium 2 with supplement (C-20011, C-39011; PromoCell). Primary umbilical vein endothelial cells were supplied by Lifeline Cell Technology and cultured in endothelial cell growth medium (C-22010; PromoCell). Mouse primary keratinocytes were isolated from the tails of C57Bl/6 mice and cultured as previously [42]. Peripheral blood mononuclear cells (PBMCs) were isolated from donor blood by Ficoll Histopaque 1077, then frozen at $5x10^6$/mL in FBS/DMSO until use. Cells were thawed and cultured in RPMI + 10% hiFBS. T lymphocytes were depleted from PBMCs using positive selection for CD2 by MACS standard protocol (Militenyi). All cells were maintained at 37 ℃ and 5% CO_2. For PBMC-NHEK coculture, NEHKs were plated at $4x10^4$/mL in keratinocyte growth media in TC-treated 96 well plates and grown for 48 h. The media was then aspirated and replaced with 100uL $1x10^6$/mL PBMCs in RPMI + 10% FBS. For cell lysis, 0.05 % triton X-100 (Sigma) was added 5 min. Other cell treatments are 5 μM caspase-1 inhibitor YVAD-fmk (R&D Systems), 50 μM SpeB inhibitor E-64 (Sigma), 20 μg/mL anti-IL-18 IgG (Abcam), or 10 μM caspase-1 inhibitor VX-765 (Invivogen), for the full duration of the experiment.

Cell Measurements

The relative levels of selected human cytokines and chemokines were determined in parallel using a membrane-based antibody array (ARY005B, R&D Systems). Cells were seeded at 7.5 x 10^5 cells per well in a tissue-culture treated six-well plate. Cells were infected at an MOI

of 10, and bacteria spun onto cells at 160 x g for three minutes. 1 mL of cell supernatant was collected after six hours of infection, incubated on the membrane overnight at 4 ℃ and developed following the manufacturer's protocol. Chemiluminescence was measured using the ChemiDoc MP imaging system, and mean pixel density was quantified using ImageJ. Only proinflammatory cytokines made by at least one of the cells lines under study are presented graphically. IL-18 signaling activity was measured in 50 μL volumes of cell supernatant using HEK-Blue™ IL-18 Reporter cells (Invivogen) and secreted alkaline phosphatase activity measured after 18 h incubation as previously described [68]. For murine cells, supernatants were concentrated 4x due to the lower sensitivity of detection of mouse IL-18. Both were normalized to known quantities of recombinant mature IL-18 (Invivogen). Total IL-18, IL-1β, and IFN-γ were quantified by ELISA following the dilutions, standards, and protocol of the manufacturer (R&D Systems). Cell lysis was measured by quantifying released LDH, compared to untreated and Triton X-100 treated controls, following the manufacturer's protocol (CytoTox 96; Promega). Cell lysis and caspase-1 activation was determined by propidium iodide and Fam-YVAD-FMK staining by the manufacturer's protocol (Immunochemistry Technologies). Immunofluorescence and brightfield images were collected using an AxioObserver D1 microscope (Zeiss) and exported with Zen Pro 2 (Zeiss). All images were acquired with the same acquisition settings, and brightness, contrast, and all other parameters were identical between samples.

Protein Purification

The fully spliced coding sequence for human *il18* and murine *il18*, encoding pro-IL-18 for each species, was generated by gene synthesis for expression from pETPP [36] to generate pETPP-hIL18 and pETPP-mIL18. Expression was induced from the plasmids in BL21 cell induced with

0.2 mM IPTG (Sigma) overnight at 18°C. Bacterial cell pellets were suspended in 10 mL of phosphate-buffered saline (PBS, pH 7.4). Cells were completely lysed by sonication at 40% amplitude for two minutes for 30 seconds at 10 second intervals and centrifugation at 7500 x g for 10 minutes. Lysate was through Talon gravity columns loaded with HisPur™ Cobalt Resin (Thermo Scientific), washed with PBS, and eluted in PBS supplemented with 300 mM imidazole. SpeB was purified as previously [43] and found to be >99% pure by SDS-PAGE. The specific activity of SpeB was measured by incubation with the specific FRET peptide Mca-IFFDTWDNE-Lys-Dnp (CPC Scientific) in PBS with 1 mM DTT and measuring the change in fluorescence, as previously [43]. Cleavage of purified human and murine pro-IL-18 was performed as previously with pro-IL-1β [43], with SpeB (100 ng) and Caspase-1 (1U, Novus) in PBS with 2 mM dithiothreitol 2 h at 37 ℃. Reactions were stopped by the addition of Laemmli buffer, 10% β-ME, and 1 x SDS loading buffer solution, then boiled at 95 ℃ for 5 min. Samples were analyzed by SDS-PAGE on Tris-Glycine gels (Invitrogen) and visualized with AquaStain (Bulldog Bio). Samples for Edman Degradation were wet transferred to PVDF membrane and visualized with SimplyBlue Safe Stain (Invitrogen), then sequenced on an ABI 494 Protein Sequencer by the Tufts University Core Facility.

Mouse Protocols

GAS cultures were grown statically overnight at 37 °C in Todd-Hewitt broth, washed 2x in PBS, and $1x10^8$ colony-forming units (CFU) in 100 µL of PBS injected subcutaneously into seven-week-old C57BL/6 or *il18*$^{-/-}$ male or female mice (Jackson Laboratories) as previously [43]. After 72 h, mice were euthanized by CO_2 asphyxiation, lesions removed, homogenized, and CFU enumeration by dilution plating on blood agar plates.

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Blood was collected from healthy adult volunteers under informed consent and approved by the Institutional Review Board of Emory University. Animal experiments were approved by the Institutional Animal Care and Use Committees of Emory University.

Statistics and Data Analysis

Values are expressed as mean ± standard deviation. Differences between groups were analyzed using a 1-way analysis of variance with Dunnett multiple comparisons analysis unless otherwise indicated. Differences are considered statistically significant at a P value of <0.05 using GraphPad Prism v9 software. Principal Component Analysis (PCA) was used to reduce the number of variables needed to adequately describe differences in cytokine profiles; each condition was treated as an independent variable, and multivariate analysis was performed in Prism v9. Protein models were visualized using PyMol 2.3.3. Diagrams created with BioRender.com.

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Competing Interests

The authors declare that no competing interests exist.

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Figures

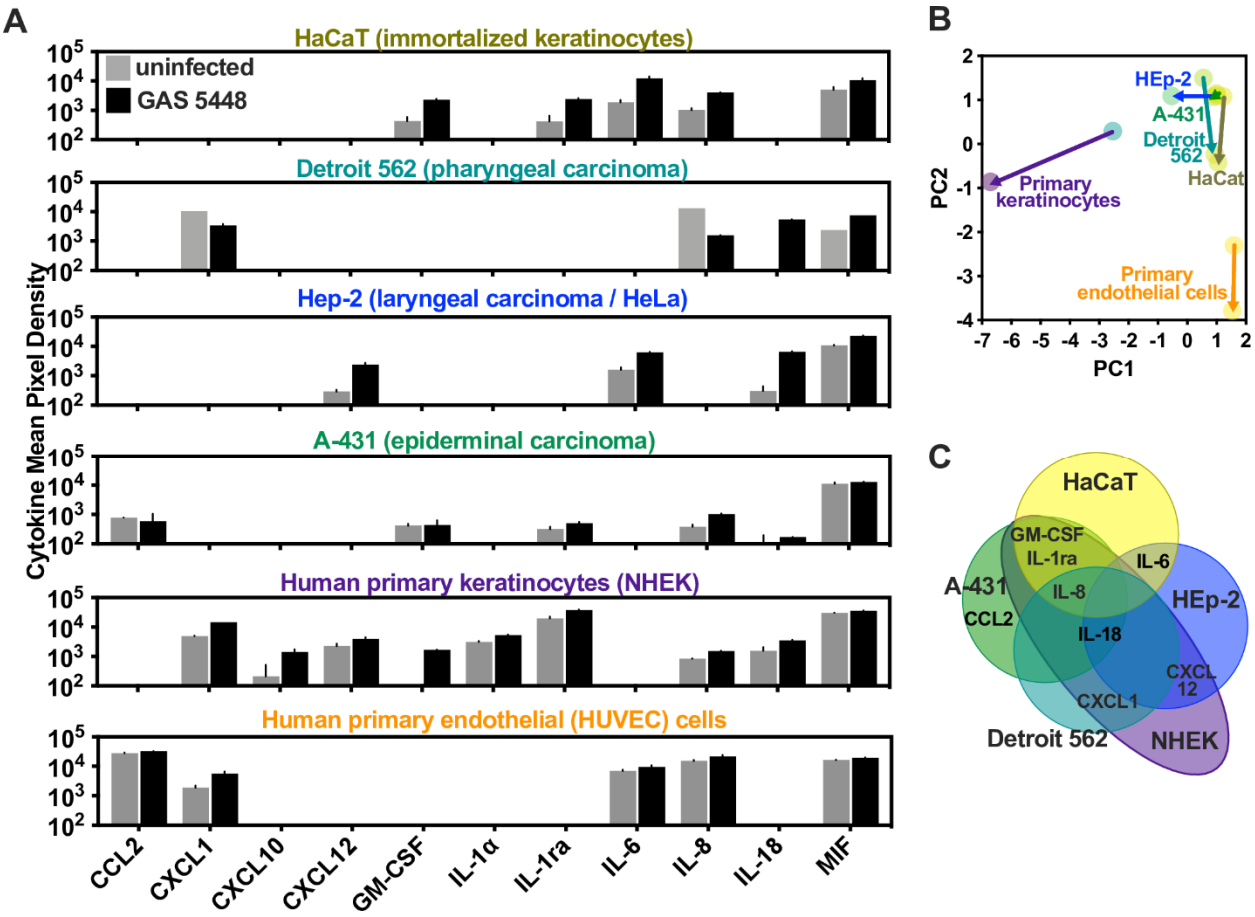


Figure 1. Cytokine profiles of keratinocytes and related cell lines.

HaCaT, Detroit 562, HEp-2, A-431, primary keratinocytes, or HUVEC cells, were infected with 7.5 x 10^6 colony-forming units (CFU) of GAS for 6 h. (A) Relative abundance of select cytokines was examined by membrane-based antibody array. (B) Cytokine profiles of each cell were examined by multivariate (principal component analysis), of the total variance, PC1 explains 48.67% and PC2 20.87%, from the raw cytokine quantities tabulated in (A). Arrows indicate change in cells from uninfected to 6 h infection. (C) Graphical representation of the congruent cytokine profiles between cell types.

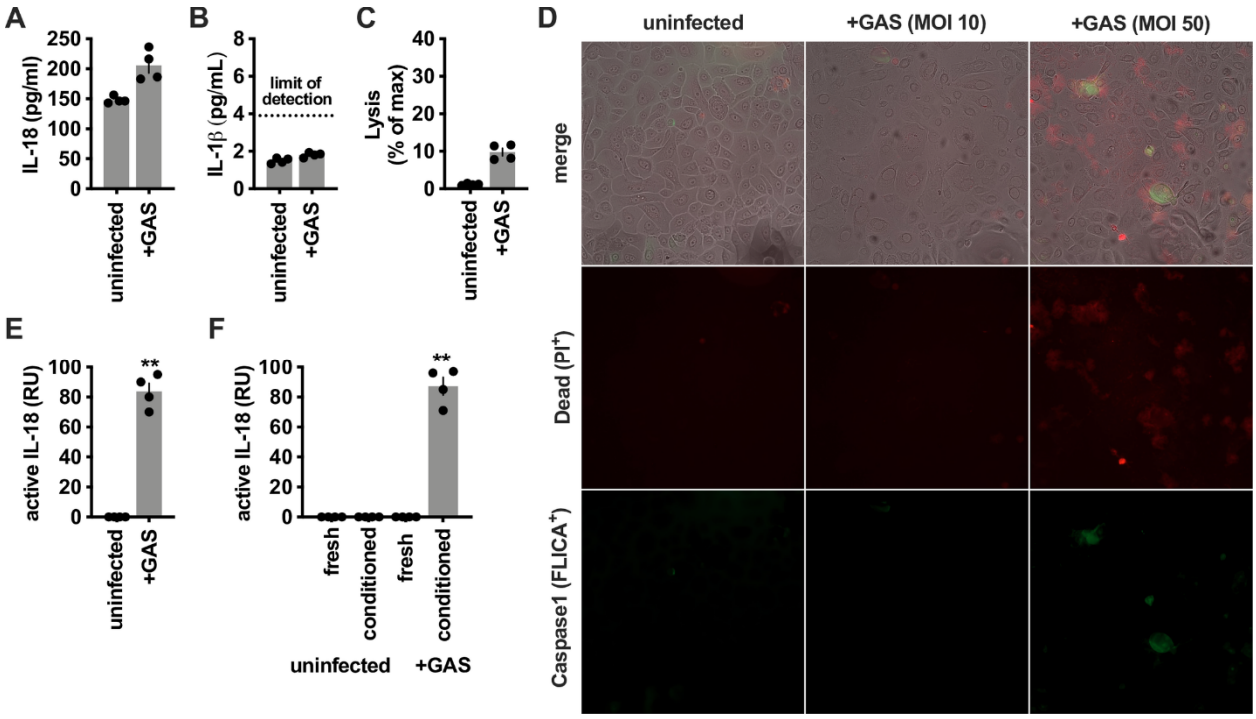


Figure 2. Examination of IL-18 activation in GAS-infected keratinocytes.

Primary human keratinocytes were infected with GAS at a multiplicity of infection (MOI) of 10 for 4 h and (A) secreted IL-18 (total, pro- and mature forms) was measured by ELISA, (B) cell lysis measured by LDH release, (C) total IL-1β measured by ELISA, or (D) cells visualized by immunofluorescent microscopy with staining for cell death by propidium iodide uptake (red) and caspase-1 activation (green). (E) Secreted bioactive IL-18 measured with HEK-Blue IL18 reporter cells during infection as in (A). (F) Fresh or conditioned media were removed from primary human keratinocytes and incubated with GAS, then IL-18 activity was measured as in (E). Data were analyzed by 1-way ANOVA using Dunnett multiple comparisons analysis. All data represent at least 3 independent experiments with 4 replicates. Bars show median values ± standard deviation. $^{**}P < 0.005$; ns, not significant.

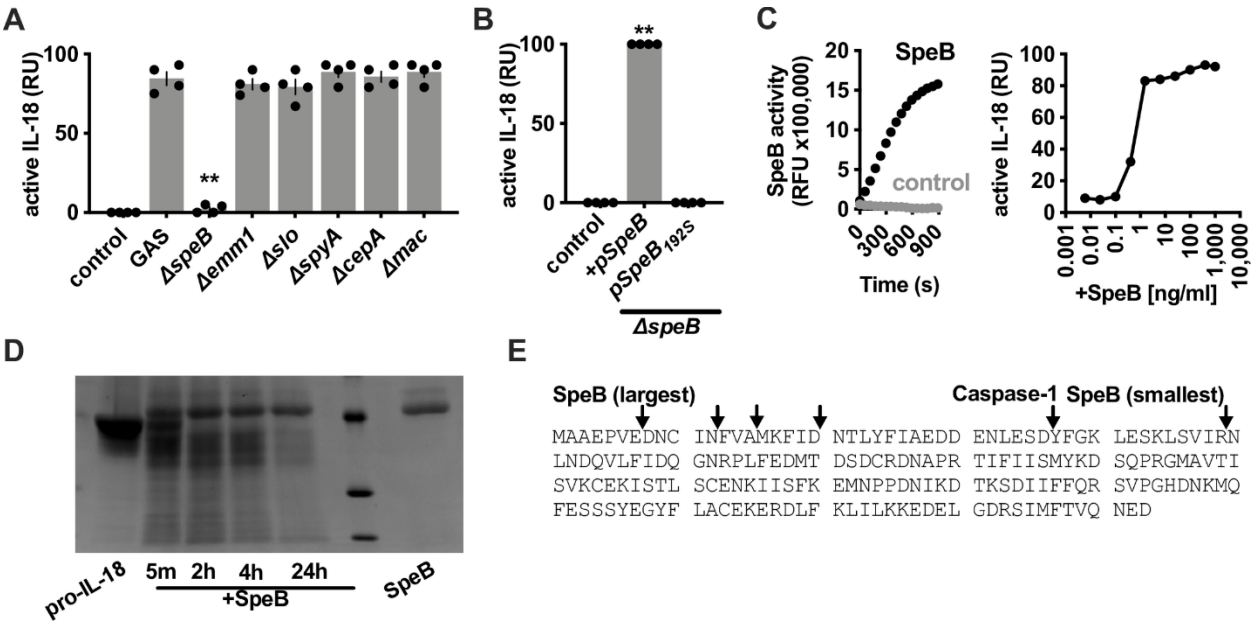


Figure 3. Examination of GAS requirements for IL-18 activation.

Primary human keratinocytes were infected with GAS at MOI 10 for 4 h, then (A, B) bioactive IL-18 was measured with HEK-Blue IL18 reporter cells. (C) SpeB activity was measured using specific substrate sub103 and IL-18 activity measured in the supernatants from keratinocytes treated with titrations of purified SpeB protein. (D) Recombinant human pro-IL-18 was purified and incubated with purified, active SpeB, then cleavage products were separated by SDS-PAGE and visualized by staining. (E) Coding sequence of human IL-18 with probable and known cleavage sites indicated; the largest and smallest confirmed by Edman sequencing. Data were analyzed by 1-way ANOVA using Dunnett multiple comparisons analysis. All data represent at least 3 independent experiments with 4 replicates. Bars show median values ± standard deviation. ** P <0.005; ns, not significant.

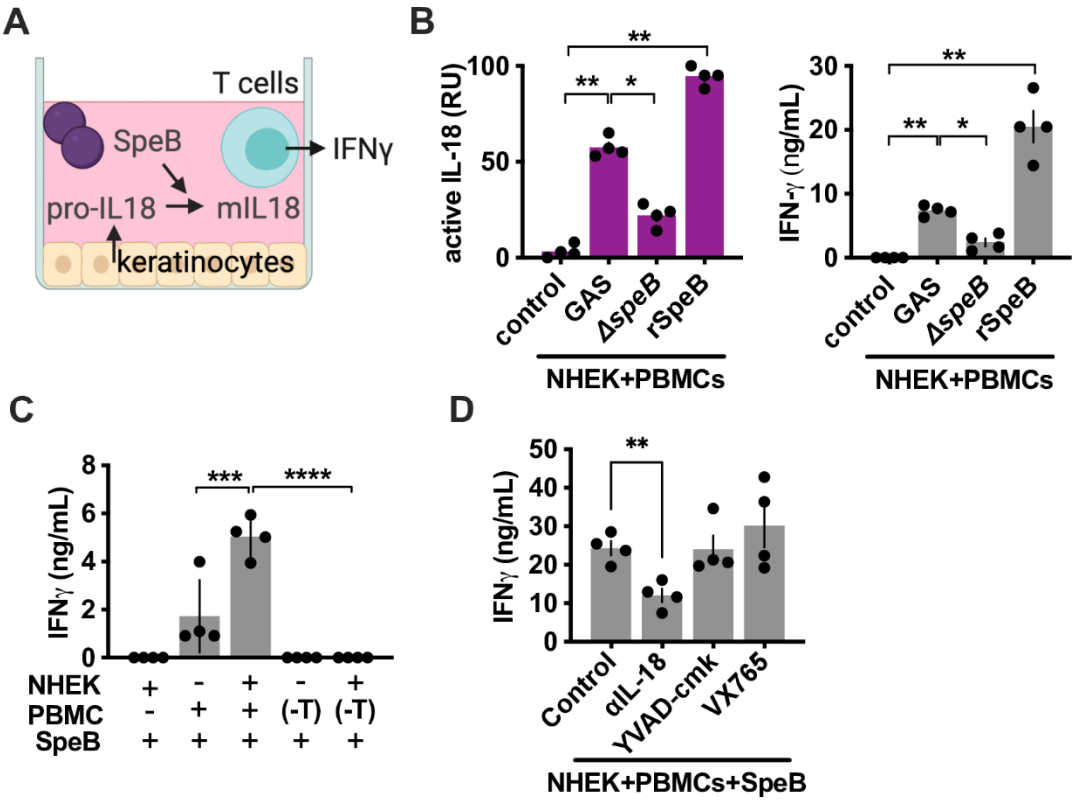


Figure 4. SpeB activation of IL-18 promotes antimicrobial IFN-γ responses.

(A) Diagram of primary keratinocyte/PBMC co-culture model; IFN-γ is a reporter of T cell activation, which can occur via IL-18 and additional mechanisms during infection. (B) Cocultured keratinocyte/PBMCs were infected with 10^5 CFU of GAS or treated with rSpeB. After 4 h, active IL-18 was quantified with HEK-Blue IL-18 reporter cells and IFN-γ by ELISA. (C, D) Cocultured cells were treated with SpeB as in (B), in combination or post-depletion of $CD2^+$ T cells, or with treatments with IL-18 neutralizing antibody or 5 μM YVAD-cmk or 10 μM VX765 to inhibit inflammasome function. Data represents at least 3 independent experiments with 4 replicates. Data were analyzed by 1-way ANOVA using Dunnett multiple comparisons analysis. Bars show median values ± standard deviation. **P* <0.05; ***P* <0.005; ns, not significant.

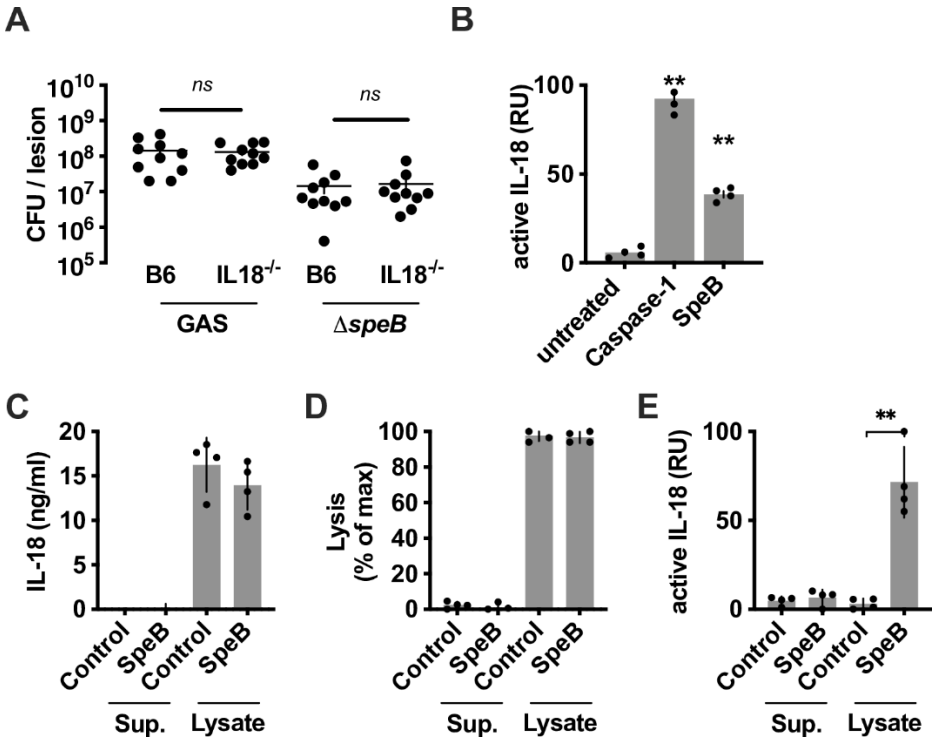


Figure 5. Mouse IL-18 can be activated by SpeB but is not secreted under normal inert conditions.

(A) C57BL/6 wild-type or IL-18-knockout (*il18*$^{-/-}$) mice were inoculated intradermally with 10^8 CFU of GAS 5448 or its D*speB* mutant. After 72 h, mice were euthanized, and GAS CFU was enumerated at the infection site. Results are from 2 independent experiments with 5 mice in each. (B) Recombinant mouse pro-IL-18 was incubated with human Caspase-1 or SpeB and activation measured with HEK-Blue IL18 reporter cells. (C) Supernatants or lysates from mouse primary keratinocytes were examined for IL-18 by ELISA and (D) cell lysis confirmed by LDH release assay, or (E) incubated 4 h with SpeB, and active IL-18 was quantified with HEK-Blue IL-18 reporter cells. Data represents at least 3 independent experiments with 4 replicates. Data were analyzed by 1-way ANOVA using Dunnett multiple comparisons analysis. Bars show median values ± standard deviation. **P* <0.05; ***P* <0.005; ns, not significant.

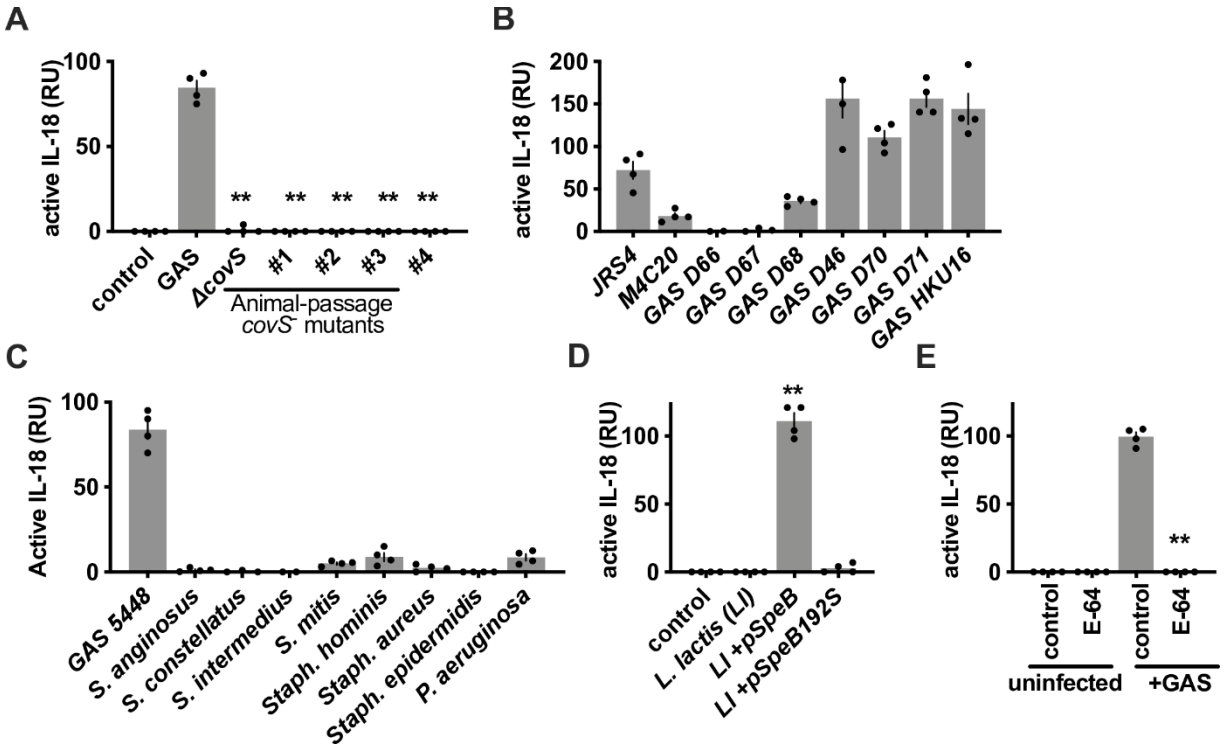
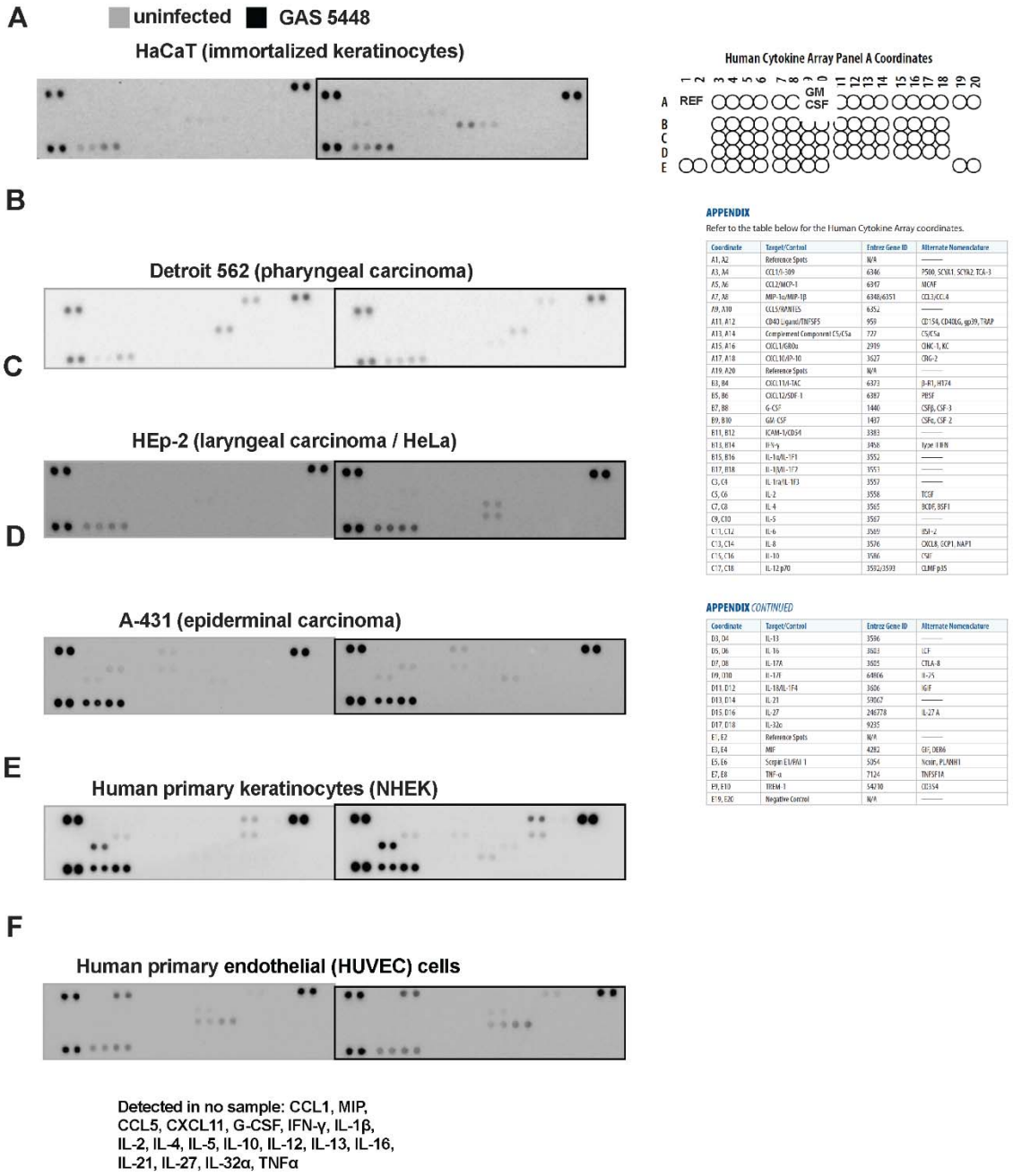


Figure 6. Bacterial activation of IL-18.

(A, B) IL-18 activation was measured in the supernatants from keratinocytes infected with the indicated gene knockouts of GAS strain 5448 or wild-type GAS, and after 4 h, bioactive IL-18 was measured with HEK-Blue IL18 reporter cells. (C) IL-18 activation was measured in the supernatants from keratinocytes infected with the indicated bacterial species and after 4 h, bioactive IL-18 was measured with HEK-Blue IL18 reporter cells. (D, E) IL-18 activation was measured in the supernatants from keratinocytes infected with the indicated gene knockouts of GAS strain 5448, *L. lactis*, or during treatment with 5 μM E-64. Spectinomycin and anhydrotetracycline to maintain SpeB expression from the indicated plasmids. Data represents at least 3 independent experiments with 4 replicates. Data were analyzed by 1-way ANOVA using Dunnett multiple comparisons analysis compared to uninfected/untreated keratinocytes. Bars show median values ± standard deviation. **P* <0.05; ***P* <0.005; ns, not significant.

Supplementary Figures



APPENDIX

Refer to the table below for the Human Cytokine Array coordinates.

Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
A1, A2	Reference Spots	N/A	———
A3, A4	CCL1/I-309	6346	P500, SCYA1, SCYA2, TCA-3
A5, A6	CCL2/MCP-1	6347	MCAF
A7, A8	MIP-1α/MIP-1β	6348/6351	CCL3/CCL4
A9, A10	CCL5/RANTES	6352	———
A11, A12	CD40 Ligand/TNFSF5	959	CD154, CD40LG, gp39, TRAP
A13, A14	Complement Component C5/C5a	727	C5/C5a
A15, A16	CXCL1/GRO α	2919	CINC-1, KC
A17, A18	CXCL10/IP-10	3627	CRG-2
A19, A20	Reference Spots	N/A	———
B3, B4	CXCL11/I-TAC	6373	β-R1, H174
B5, B6	CXCL12/SDF-1	6387	PBSF
B7, B8	G-CSF	1440	CSFβ, CSF-3
B9, B10	GM-CSF	1437	CSFα, CSF-2
B11, B12	ICAM-1/CD54	3383	———
B13, B14	IFN-γ	3458	Type II IFN
B15, B16	IL-1α/IL-1F1	3552	———
B17, B18	IL-1β/IL-1F2	3553	———
C3, C4	IL-1ra/IL-1F3	3557	———
C5, C6	IL-2	3558	TCGF
C7, C8	IL-4	3565	BCDF, BSF1
C9, C10	IL-5	3567	———
C11, C12	IL-6	3569	IFN-β2
C13, C14	IL-8	3576	CXCL8, GCP1, NAP1
C15, C16	IL-10	3586	CSIF
C17, C18	IL-12 p70	3592/3593	CLMF p35

APPENDIX *CONTINUED*

Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
D3, D4	IL-13	3596	———
D5, D6	IL-16	3603	LCF
D7, D8	IL-17A	3605	CTLA-8
D9, D10	IL-1F7	64806	IL-25
D11, D12	IL-18/IL-1F4	3606	IGIF
D13, D14	IL-21	59067	———
D15, D16	IL-27	246778	IL-27 A
D17, D18	IL-32α	9235	
E1, E2	Reference Spots	N/A	———
E3, E4	MIF	4282	GIF, DER6
E5, E6	Serpin E1/PAI-1	5054	Neuin, PLANH1
E7, E8	TNF-α	7124	TNFSF1A
E9, E10	TREM-1	54210	CD354
E11, E12	Negative Control	N/A	———

Figure S1. Images of cytokine arrays for quantification of secreted cytokines

Chapter 3

The synergy of SpeB with streptococcal superantigens

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Abstract

Group A streptococcus (GAS) is major cause of human infectious disease mortality. More severe manifestations, such as scarlet fever and streptococcal toxic shock syndrome, are driven by streptococcal pyogenic exotoxins that act as superantigens. GAS also makes a streptococcal pyogenic exotoxin that is a protease, SpeB, described to broadly degrade host and microbial factors alike. We describe a range of superantigen lability to SpeB, not specific to structure, conservation, or when compared to superantigens from *Staphylococcus aureus*, species of origin. Prior work shows that SpeB activates proinflammatory signaling, including the cytokines IL-1β and IL-18, that has the potential to modify the cellular response to superantigens. We further show that rather than strictly antagonizing superantigen activity through degradation, SpeB can enhance the activity of superantigens in a synergistic manner. For SpeB-labile superantigens such as SmeZ, this is limited by the rate of degradation, but for highly resistant superantigens like SpeA, activity remains synergistic up to supra-physiological concentrations. Taken together, these findings show that SpeB can cooperate with superantigens, and reveal a potential regulatory mechanism to limit excessive immune activation by some superantigens through their degradation.

Introduction

Group A streptococcus (GAS) is an obligate human pathogen responsible for at least an estimated 600,000 deaths annually[1]. Beyond pharyngitis, GAS is also responsible for serious infections such as scarlet fever (SF), necrotizing fasciitis, and streptococcal toxic shock syndrome (STSS). These diseases can require rapid and serious measures of treatment[2], such as heavy antibiotic regiments[3], experimental immunotherapeutics such as intravenous immunoglobulin (IVIG)[4], and surgical interventions[5]. Prevention is further complicated due to the lack of an

approved vaccine[6]. Greater understanding of the bacterial factors involved in disease progression will give targets for therapeutic strategies and vaccine development.

The GAS virulence factors responsible for SF and STSS are secreted streptococcal pyogenic exotoxins (Spe) that act as superantigens[1]. Superantigens are non-specific mitogens that bypass the antigen processing pathway to directly bind T cell receptors with MHC-II receptors found on antigen presenting cells[7]. Consequently, this non-specific immune cell activation induces proinflammatory cytokines such as IL-1β, IL-6, and IFNγ, resulting in a cytokine storm[8,9]. Mucosal-associated invariant T (MAIT) cells drive inflammation[10]. Evidence that inflammation from these toxins necessary for colonization and dissemination[11–13]. SpeA has 4 alleles, with SpeA2 and SpeA3 being linked to STSS[14].

Another virulence factor necessary for many invasive diseases is the cysteine protease SpeB[15]. SpeB has been shown to cleave and degrade many proteins from both the host and bacteria, as well as activate proinflammatory immune factors[16,17]. While SpeB and superantigens were originally isolated as the causative agents behind scarlet fever[18], how these proteins interact with each other remains unclear. Prior work has shown that SmeZ is readily degraded by SpeB[19], and SpeA is resistant to degradation[20]. In addition, SpeB has been shown prior to cleave most proteins in the GAS proteome[21], however the strain chosen did not cover the full breadth of GAS superantigens. In this report, we show that not all superantigens are degraded by SpeB, and some are resistant to degradation. In addition, we show superantigens synergize with SpeB to enhance the proinflammatory response *in vitro*. Altogether, we propose that while some superantigens may be degraded *in vitro*, SpeB may still enhance their efficacy at inducing a cytokine storm.

Results

Degradation of streptococcal superantigens by SpeB is a variable phenotype

The abundant secreted protease SpeB has previously been indicated to have little proteolytic activity toward SpeA2, and to degrade SmeZ [19,20]. The action of SpeB on other streptococcal superantigens remains unknown. To better identify whether SpeB broadly degrades superantigens, or if they are more typically resistant, we incubated eleven superantigens produced by GAS individually with SpeB, then visualized the changes in their mass by SDS-PAGE. Surprisingly, we found that the degradation by SpeB was highly variable between superantigens (**Figure 1**). SpeH, SpeK, and SmeZ were fully degraded, SpeC and SpeG partially, and no change in SpeA, SpeI, SpeJ, or SSA was observed under the same conditions. Both SpeL and SpeM were cleaved to generate distinct cleavage products that were not further degraded. We verified the product of SpeM cleavage by Edman Degradation, with the cleavage site occurring at R122 (**Figure 2**). Altogether, this shows that there is no universal pattern to SpeB degrading superantigens produced by GAS.

Degradation of staphylococcal superantigens by SpeB is a variable phenotype

We were curious if this phenotype of resistance was exclusive to superantigens produced by GAS. Because the superantigens produced by *S. aureus* did not evolve in the presence of SpeB, they might be more susceptible to degradation by SpeB, as there was no selective pressure to minimize accessible SpeB cleavage sites. Under the same conditions as with GAS superantigens, TSST-1 resisted degradation, while SEA had some degradation, and SEB had distinct cleavage

products form (**Figure 3**). This confirms that resistance to cleavage by SpeB is not exclusive to those produced by GAS.

Of interest, we observed cleavage of SEB and not SpeA, though these proteins share significant sequence and structural homology[22]. They are both group II superantigens and are historically the most linked superantigen to toxic shock caused by their respective pathogen[8]. Using Edman Degradation on cleavage fragments identified by SDS-PAGE, the SpeB cleavage site within SEB was confirmed at N131 (**Figure 4a**). This site is located within an extended cysteine loop thought to be responsible for the emetic ability of SEB to cause food poisoning[23,24]. SpeA is not emetic[25], and has the equivalent cysteine loop truncated by 9 amino acids relative to SEB (**Figure 4b**). Thus, the absence of this extended loop may protect SpeA from degradation by SpeB, allowing coexistence of these virulence factors during infection.

Superantigen degradation by SpeB is not phylogenetically linked

There have been 5 distinct groups for superantigens proposed based on function and structural makeup[26]. Since we observed a trend in the cleavage of the Staphylococcal superantigen SEB but not the related Streptococcal superantigen SpeA, we next sought to determine if SpeB degradation followed these grouping patterns. We aligned the full protein sequences of superantigens produced by GAS and selected *S. aureus* superantigens by MAFFT, then generated a dendrogram using BIONJ. Results showed SpeB cleavage did not align with superantigen group (**Figure 5)**. SEB and SpeH stand out as group II superantigens that are degraded by SpeB either partially or fully. All the group IV superantigens except for SpeJ showed some degradation. Only single superantigens from groups III and V were available for testing, as the remaining group

members are produced by *S. aureus*, therefore broader trends among these groups will not be made. TSST-1, the only member of group I, was not degraded. These data suggest that degradation of superantigens by SpeB is independent of superantigen grouping.

SpeB has a synergistic effect on superantigen activity

With the knowledge that some superantigens are resistant to degradation by SpeB, and that SpeB is proinflammatory, we wanted to determine whether SpeB would impact the activity of superantigens. Specifically, we hypothesized SpeB activation of IL-1b, IL-18, and other factors could prime cells for superantigen responses, and overlap with the proinflammatory cytokine storm generated by superantigens. Using a human PBMC model, we incubated SpeB with either SpeA2, a superantigen resistant to degradation, or SmeZ, a superantigen susceptible to degradation, at varying concentrations in a checkerboard format to model synergy. Superantigen activity was quantified by ELISA using IL-2 as a measurement of T-cell activity. We observed a dose response with superantigen alone that was amplified by the presence of SpeB. Higher concentrations of SpeB paired with SpeA2 lead to a 431% increase in IL-2, resulting in values 138% higher than expected (**Figure 6**). With SmeZ, this increase in IL-2 was 582%, resulting in values 160% higher compared to what was expected. However, the maximum increase for SmeZ was seen with only 5 ng/mL of SpeB, where there was a 36,773% increase in IL-2, resulting in values 240% higher than expected. This suggests that even though degradation of SmeZ is occurring, SpeB is still able to synergize and enhances activity. Taken together, this shows that SpeB acts synergistically with superantigens during infection.

Discussion

We present a characterization of SpeB interaction with a panel of streptococcal and staphylococcal superantigens. We then go on to show that SpeB can enhance the activity of superantigens, and that this increase is beyond additive. Even with SmeZ, a superantigen established as very susceptible to degradation by SpeB[19], a synergistic effect is observed, but is lost with higher quantities of SpeB. Our findings are summarized in a model figure (**Figure 5**). We propose these findings show another method that SpeB is used by GAS to modulate the immune response to its own benefit. Prior work has already shown cytokines activated by SpeB enhance dissemination of GAS[27]. In addition, IL-18 is a key component in cytokine mediated activation of MAIT cells, known to be heavily upregulated in STSS[10]. Taken together, cytokines activated by SpeB are feeding into the superantigen cytokine storm, driving inflammation to promote GAS dissemination.

Another mechanism to consider is GAS utilizing SpeB to degrade superantigens to prevent overstimulation of the host immune system. This would provide a mechanistic reason for why SpeA, SSA, and SpeC are linked to isolates responsible for outbreaks of scarlet fever[28]. SpeA and SSA are not degraded, and SpeC is degraded at a slow rate. These superantigens being degradation-resistant may be a component of why they are linked to GAS isolates responsible for scarlet fever outbreaks. The new strain M1$_{UK}$ responsible for increasing scarlet fever outbreaks has been characterized for its increased SpeA production[29]. With more SpeA being present and not being degraded, this may be in part why M1$_{UK}$ is more likely to cause SF in humans. This also complements prior work showing that *S. aureus* downregulates the human response to cytokine storms using molecules in the peptidoglycan[30], showing that pathogens work to manage inflammation at a rate that promotes bacterial survival without host mortality. Further work

investigating if *S. aureus* uses proteases to regulate and synergize with superantigens should be performed to complement these findings.

Current literature featuring superantigen-susceptible mice suggests SpeA and other superantigens play a role in colonization[11–13]. This offers an explanation to why GAS has such a variety of superantigens it can produce. Because each superantigen has a Vβ chain of MHC-II for optimal binding, producing a variety of superantigens allows for infecting the most possible hosts. In GAS, superantigens are either encoded on the chromosome or prophage, and no strain contains all at the same time[31]. There was no consistent trend in superantigens encoded on the chromosome compared to those from prophage for being degraded.

Treatment of invasive GAS infections often calls for rapid therapeutics to prevent mortality. By better understanding the components of the superantigen cytokine storm, we can target each component with preexisting inhibitors. When used in tandem with standard antibiotic therapies and experimental IVIG, it may help to reduce symptoms and promote survival. These data will help guide future research into the superantigen cytokine storm, and how we can better inhibit it to prevent mortality.

Materials and Methods

Protein Purification

SpeB was purified as previously described[16], with >99% purity shown by SDS-PAGE and quantified by Bradford Assay. Recombinant streptococcal superantigen SpeA2 was cloned from GAS M1T1 5448. The coding sequence, minus the signal peptide, for SpeA2 was generated for expression in pET-SUMO. Expression was induced from BL21 *E. coli* with 1mM IPTG (Research

Product International) for 3 hours at 37 °C. Cells were pelleted at 8,000g for 10 minutes, then frozen at -20°C until use. Cell pellets were thawed and resuspended in PBS, then lysed by sonication at 15% amplitude (Sonic Dismembrator Model 500, Fisher Scientific) for 4 minutes at 30 second intervals on ice, then centrifuged at 21,000 g for 30 minutes at 4 °C. Lysate was run through Talon gravity columns with Cobalt resin (Thermo Scientific), washed with PBS, and eluted in PBS with 300mM imidazole (Sigma). Protein was dialyzed overnight at 4 °C in PBS with ulp1 protease to remove the SUMO tag and imidazole, then purified by reverse nickel column. The ulp1 protease was generated previously[17]. Recombinant streptococcal superantigens SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SmeZ, and SSA were provided by John McCormick from prior studies that describe their expression and purification[11]. Recombinant staphylococcal superantigens SEA, SEB, and TSST-1 were purchased lyophilized (Toxin Technologies, Florida) and resuspended at 1 mg/mL in PBS.

Primary Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donor blood by centrifugation over Ficol Histopaque 1077 (Sigma), then frozen at $5x10^6$ cells/mL in 90% FBS/10% DMSO until use. Cells were thawed and cultured in RPMI + 10% heat inactivated FBS (Atlanta Biologicals). Cells were maintained at 37 °C, 5% CO_2. PBMCs were cultured in 100µL volumes at $1x10^5$ cells/well in tissue culture treated 96 well plates. Superantigens and SpeB were added from 100X stocks, then incubated at 37 °C, 5% CO_2 for 24 hours. Supernatants were then collected, quantified, and analyzed by ELISA for IL-2, using the manufacturers protocol and recommendations for dilutions (R&D Biosciences).

SDS-PAGE

Cleavage of superantigens was performed as described in chapter 2. Briefly, superantigens (10 μg) were incubated with SpeB (1 μg) in PBS with 2mM Dithiothreitol (Sigma) at 37 °C. Reactions were stopped by freezing at -20 °C, then tricine SDS sample buffer and sample reducing agent (Invitrogen) were added, then heated at 85 °C for 2 minutes. Samples were analyzed by SDS-PAGE on 16% Tricine gels (Invitrogen), protein stained with AquaStain (Bulldog Bio), and visualized. New amino termini generated by SpeB cleavage were identified by Edman Degradation as previously[16]. Briefly, after SDS-PAGE, proteins were wet transferred to PVDF membrane, visualized with SimplyBlue Safe Stain (Invitrogen), then sequenced on an ABI 494 protein sequencer by the Tufts University Core Facility.

Sequence Alignment

Non-redundant full length protein sequences were collected from NCBI. Protein alignment was generated in MegAlign Pro using a MAFFT7 l-ins-i algorithm for one conserved domain with a BLOSUM62 scoring matrix. The alignment was 337 amino acids long, while original protein lengths ranged from 225 to 266 amino acids. The dendrogram based on this alignment was generated using the BIONJ version of the neighbor-joining algorithm. Protein structures were collected from PDB and aligned in Pymol 2.3.3.

Ethics Statement

This study was conducted according to principles expressed in the Declaration of Helsinki. Blood was collected from health adult volunteers under informed consent and approved by the Institutional Review Board of Emory University.

Statistics and Data Analysis

Values shown are expressed as mean ± standard deviation. Differences between groups were analyzed by 1-way analysis of variance with Dunnett multiple comparisons analysis unless otherwise noted. Significance assigned to p values of <0.05, calculated with GraphPad Prism v9.

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Figures

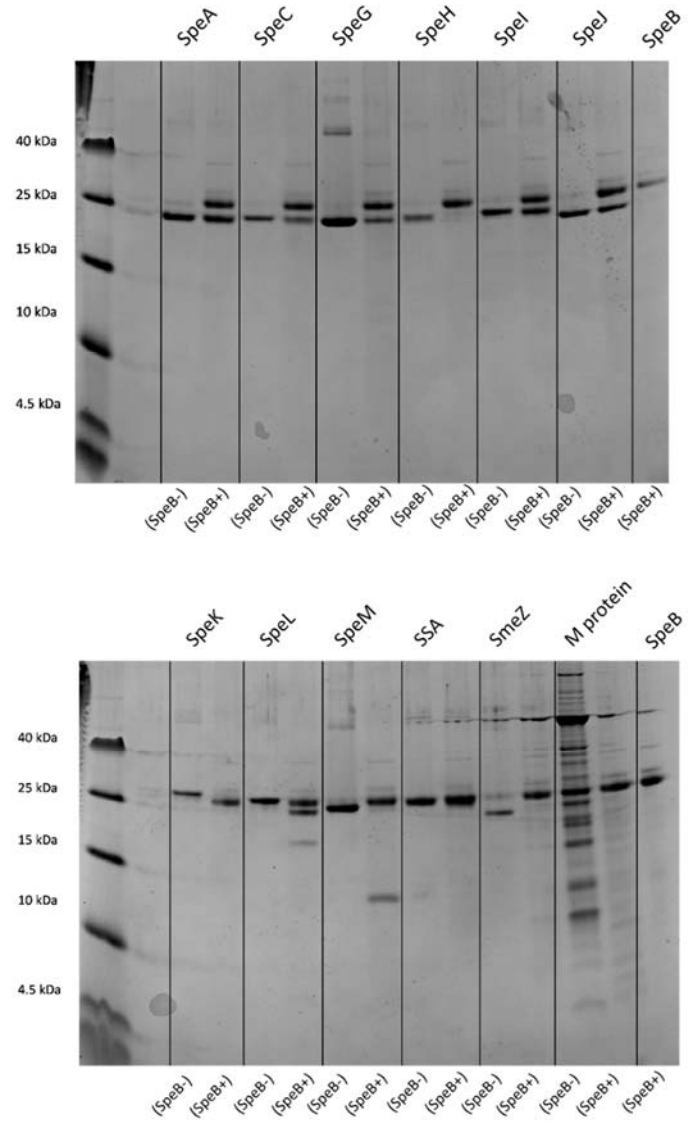

Figure 1. SpeB degradation of GAS superantigens is variable. 1 μg of SpeA2, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SSA, or SmeZ was incubated with 1 ug of SpeB for 24 hours at 37 °C, with $SpeB^-$ negative control. M protein was included as a control for SpeB activity. Data are representative of 3 independent repeats.

MKKNTLTLLF LVCVSLALYT TESVFSDAVL VNSELKNIYT KDVINRTNMK ITKKIGTQLI
FNTNEKTRVW DDDNYNKVIS SNVSPAQERR FKEEEVDIYA LIKSYSVICK EQYNYVDGGL
SpeB▼
IRTSDREKLD STIYMNIFGE QIPLKEQSKY KITFQNKFVT FQEIDVRLRK SLMSDNRIKL
YEHNSICKKG YWGIHYKDNT TKFTDLFTHP NYTDNETIDM SKVSHFDVYL NEDFSKN

Figure 2. SpeB cleavage of SpeM. Full coding sequence of SpeM with SpeB cleavage site confirmed by Edman Degradation.

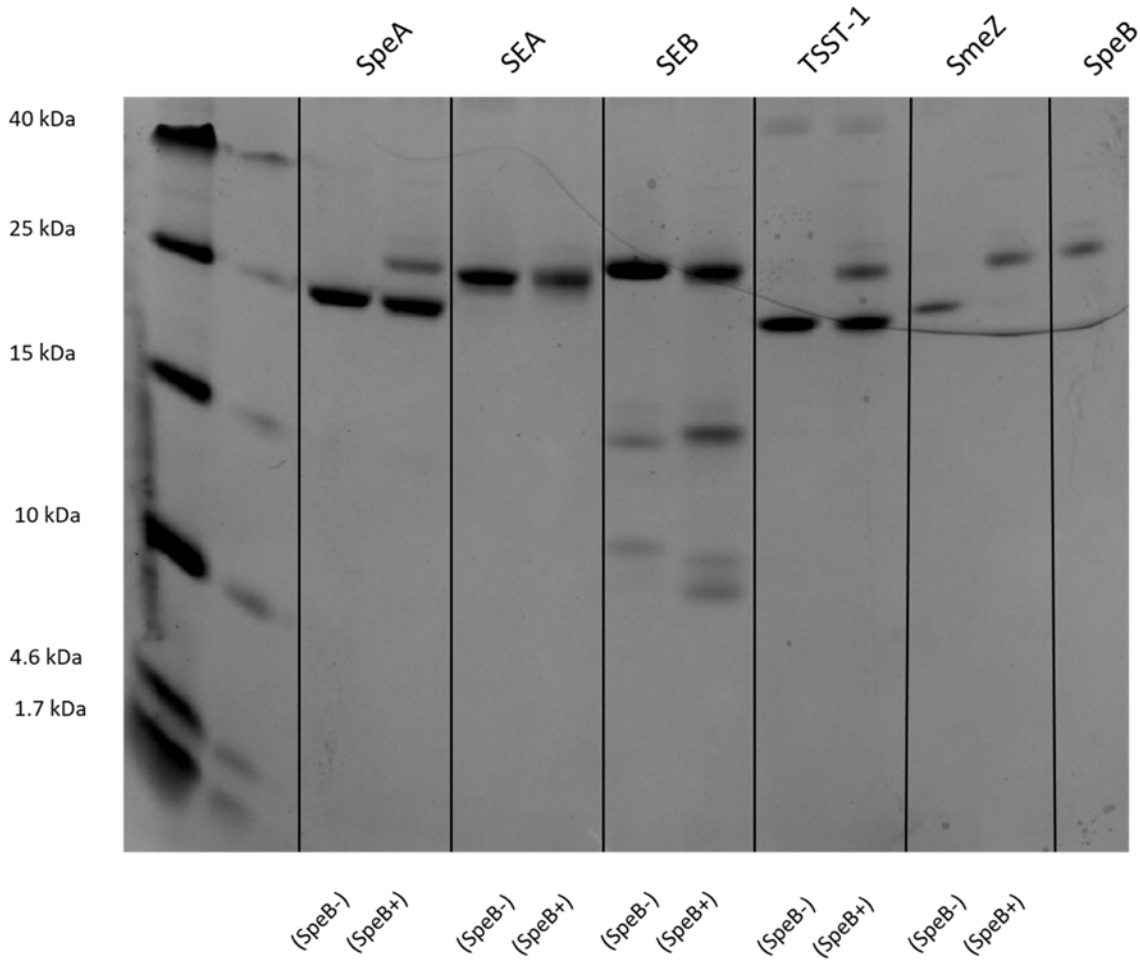

Figure 3. SpeB degradation of staphylococcal superantigens is variable. 1 μg of SpeA2, SEA, SEB, TSST-1, or SmeZ was incubated with 1 ug of SpeB for 24 hours at 37 °C, with $SpeB^-$ negative control. Data are representative of 3 independent repeats.

A)

```
MYKRLFISHV ILIFALILVI STPNVLAESQ PDPKPDELHK SSKFTGLMEN
MKVLYDDNHV SAINVKSIDQ CLYFDLIYSI KDTKLGNYDN VRVEFKNKDL
                              SpeB▼
ADKYKDKYVD VFGANYYYQC YFSKKTNDIN SHQTDKRKTC MYGGVTEHNG
NQLDKYRSIT VRVFEDGKNL LSFDVQTNKK KVTAQELDYL TRHYLVKNKK
LYEFNNSPYE TGYIKFIENE NSFWYDMMPA PGDKFDQSKY LMMYNDNKMV
DSKDVKIEVY LTTKKK
```

B)

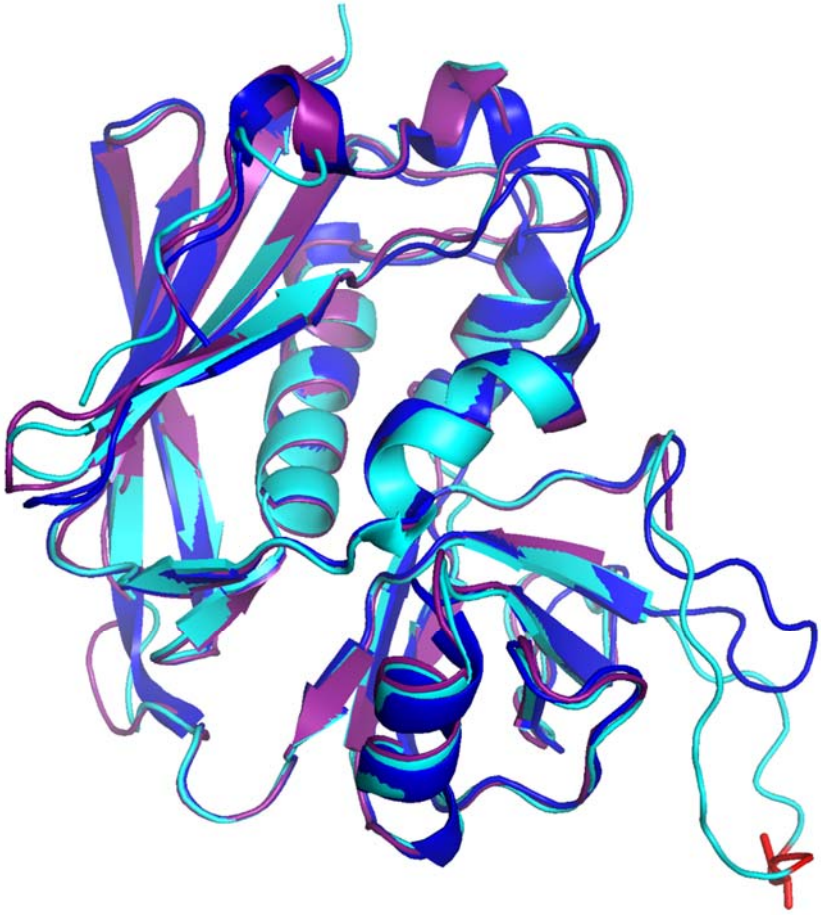

Figure 4. SEB is cleaved by SpeB at N131. A) Full coding sequence of SEB with SpeB cleavage site confirmed by Edman Degradation. **B)** SEB (2seb, dark blue), SpeA (1uup, teal), and SSA (1bxt, purple) aligned in Pymol. SpeB cleavage site in SEB highlighted in red.

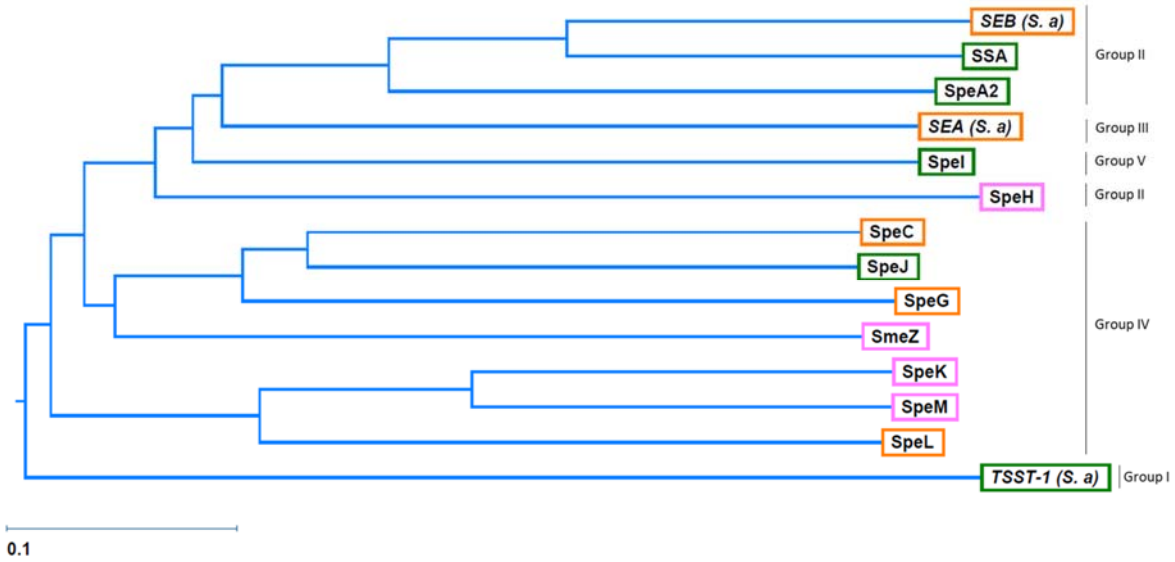

Figure 5. SpeB degradation of superantigens is not correlated with group classification. Dendrogram of all superantigens presented in Figure 1 and Figure 3, and their respective cleavage patterns by SpeB. Green is resistant to degradation, orange is susceptible to slow degradation or distinct cleavage products are formed, pink is total degradation. Sequences aligned in MegAlign Pro with MAFFT, dendrogram generated using BIONJ.

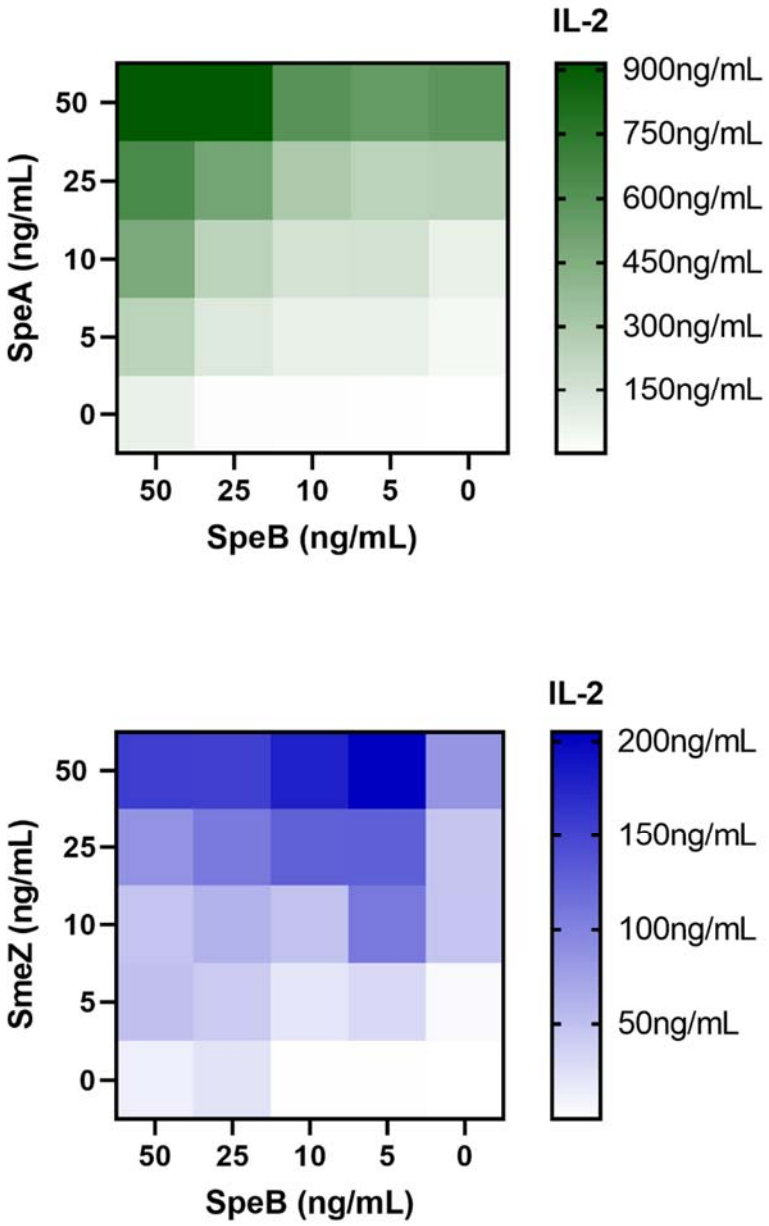

Figure 6. SpeB synergizes with superantigens to enhance activity. Superantigens and SpeB were incubated together at listed concentrations with PBMCs for 24 hours. Heatmap shows concentration of IL-2 in supernatant, quantified by ELISA. Data are pooled from three independent replicates performed in triplicate for SmeZ and two independent replicates performed in triplicate for SpeA2.

Chapter 4

Discussion

Conclusion

With continuing outbreaks of severe disease, GAS remains a worldwide pathogen of concern. Improved treatment will require a better understanding of how GAS virulence factors interact both with the host and each other. Controlling the pathological proinflammatory activities of GAS virulence factors has remained a challenge. This work has sought to better understand the molecular mechanisms underlying the interaction of SpeB with host and other bacterial factors, with the goal of utilizing these findings to shape research into therapeutics beyond antibiotics. Findings from this dissertation have been summarized in **Figure 1**.

In Chapter 2, we provide insights into the interactions of the GAS protease SpeB with the hosts first line of defense against pathogens. We show that pro-IL-18 constitutively secreted by keratinocytes is matured by SpeB into active form. We also identify the SpeB cleavage site is on the N-terminus near the canonical site of caspase-1 activity. This active IL-18 can proceed then promote T cell activation, shown by the induction of IFN-γ. SpeB activation of keratinocyte secreted IL-18 is unique to humans, as keratinocytes isolated from mice do not constitutively secrete IL-18. Finally, activation of extracellular IL-18 is not shown by other commensal skin microbiota, showing the uniqueness of this response.

This research offers several technical contributions to the field. We showed the cytokine response to GAS infection in other primary and immortalized cell lines beyond NHEKs. These data reinforce the importance of using primary cell lines as models of infection because of the breadth of cytokines that can be lost during the immortalization process. We also highlighted the limitations of using animal models for obligate human pathogens. While it has been shown prior that mice models require genetic modifications to properly model GAS pathogenesis in regards to superantigens[1,2], SpeB is unable to cleave extracellular pro-IL-18 if it is not being secreted. This

could suggest a potential limitation to modeling GAS skin infections in mouse models that should be accounted for.

Second, we have shown an additional target of SpeB activation, and how it continues to act as a bacterial caspase. Previous work done by the lab has shown the activation of IL-1β and GSDMA by SpeB[3,4]. GSDMA is primarily produced by cells in the skin, including keratinocytes. Since keratinocytes are constitutively secreting proIL-18, and active IL-18 goes on to promote cytokine-dependent T-cell activation, it can be proposed that the pro-IL-18 secreted is acting as a sensor of bacterial pathogens. This is supported by the absence of IL-18 activation by commensal microbes. Therefore IL-18 secretion by keratinocytes is another example of the human immune system laying traps for bacterial proteases such as SpeB.

One question that was regularly asked when presenting our findings is whether the activation of IL-18 by bacterial proteases is exclusive to GAS. After additional investigation, we found that *Staphylococcus aureus* was also capable of independent pro-IL-18 activation. However, the mechanism of activation is unknown, and activation is not found in all strains of *S. aureus*. We examined the pro-IL-18 activation abilities of 191 strains of clinically isolated MRSA and found that ~60 had the potential for activation of pro-IL-18. When mapped phylogenetically (**Figure 2**), these strains did not group by clonal complex, site of isolation, or disease manifestation.

The next steps of investigating *S. aureus* will be to investigate protease expression of these strains, to identify a virulence factor responsible for activation. The cysteine protease staphopain is a major proinflammatory virulence factor secreted by *S. aureus* already known to interact with host epithelium[5], and is possibly the activating factor. The contribution of this protease, and others, will be investigated further. Once the protease is confirmed, we will work to verify findings with knockout and complement strains, as well as recombinant proteins to show necessity and

sufficiency for this activity. It is also notable that most other pathogens did not activate IL-18 in this manner, and *S. aureus* is the only other major pathogen to make superantigens. These results suggest there could be beneficial dynamics between these processes. With recent findings showing the importance of cytokine mediated T cell activation during cytokine storms[6], it is possible that *S. aureus* is also using IL-18 to enhance superantigen activity.

In Chapter 3, we show that the interaction of SpeB with streptococcal superantigens is not as simple as universal superantigen degradation. We present a panel showing variable degradation by SpeB of 11 superantigens from GAS and 3 superantigens from *S. aureus*. Degradation by SpeB does not follow any superantigen grouping, nor is it species restricted. In SEB, another group II superantigen like SpeA[7], we identify the SpeB cleavage site within SEB is found in the cysteine loop responsible for emesis. We also show that synergy is present in SpeB-superantigen interactions, as the cytokines being activated by SpeB may work to enhance the cytokine storm produced by superantigens. Therefore, SpeB may be used by GAS to regulate superantigen activity, by enhancing the cytokine storm while also degrading superantigens to prevent overstimulation.

The research presented here helps to characterize the direct interaction of SpeB with streptococcal superantigens. Degradation of superantigens by SpeB may be a regulatory mechanism to modulate the host immune response during invasion. SpeB is known to degrade other virulence factors during infection[8], and *S. aureus* has been shown to modulate the cytokine storm it produces[9]. While prior work has shown that SpeB degrades some superantigens, the resistance to degradation seen in SpeA and SSA is interesting regarding pandemic isolates. With the link of SpeA and SSA to isolates responsible for pandemic outbreaks of scarlet fever[10,11], resistance to degradation by SpeB may play a role in why these superantigens specifically are

linked to these outbreaks. Recently it was shown the $M1_{UK}$ clade of isolates has a point mutation resulting in increased production of SpeA[12]. With increased expression of SpeA being linked to scarlet fever-causing strains of GAS[11], the fact that SpeB is unable to readily degrade SpeA may play a role in increased cases of scarlet fever.

While we have shown the direct interaction of SpeB with superantigens, the mechanism of synergy remains to be characterized. The cytokine storm has many different components, and how the cytokines activated by SpeB insert into this mechanism needs to be clarified. IL-18 has been shown to be part of the mechanism for cytokine-mediated activation of T cells[6,13]. IL-1β may be contributing through recruitment and activation of immune cells[14], or synergizing with IL-18 to enhance production of IFN-γ[15]. GSDMA activation promotes cell death, releasing more IL-1β and IL-18 that may feed into the cytokine storm[16]. These cytokines may enhance the storm leading to further activation of cells.

With a whole family of toxins that purposely induce inflammation, the natural question is can we target these mechanisms for potential therapeutics. Superantigens have been shown as beneficial for colonization[1,2,17], so it stands that developing inhibitors would be protective. While there have been efforts to develop inhibitors for superantigens[18], they have limited efficacy at preventing an already progressing cytokine storm. GAS requires IL-1 signaling to colonize the nasopharynx[19], conversely, those taking inhibitors of IL-1 family cytokines are at greater risk of invasive disease[3]. TNFα has also been shown to be a major component of the cytokine storm, as pretreatment with inhibitors has been found to be protective against toxic shock in mice[20]. However efficacy was reduced when used after exposure to superantigens. As it stands, more work remains to be done to identify potential therapeutics to help treat superantigen-mediated diseases.

In conclusion, this work advances the understanding of how GAS interacts with the host immune system. The discovery that IL-18 is matured by SpeB offers another method for the host to detect and respond to bacterial infections. Continuing to find cytokine targets of SpeB, and how those targets go on to induce inflammation will allow us to understand host surveillance for pathogens. We further show how SpeB controls its own virulence factors to enhance and control inflammation by synergizing and degrading superantigens. A better understanding of SpeA and other superantigens will help us better understand the mechanisms that lead to the evolution of pandemic-causing isolates. Through understanding how SpeB interacts with itself and the host, we can find targets to exploit to provide better treatment for GAS infections.

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Figures

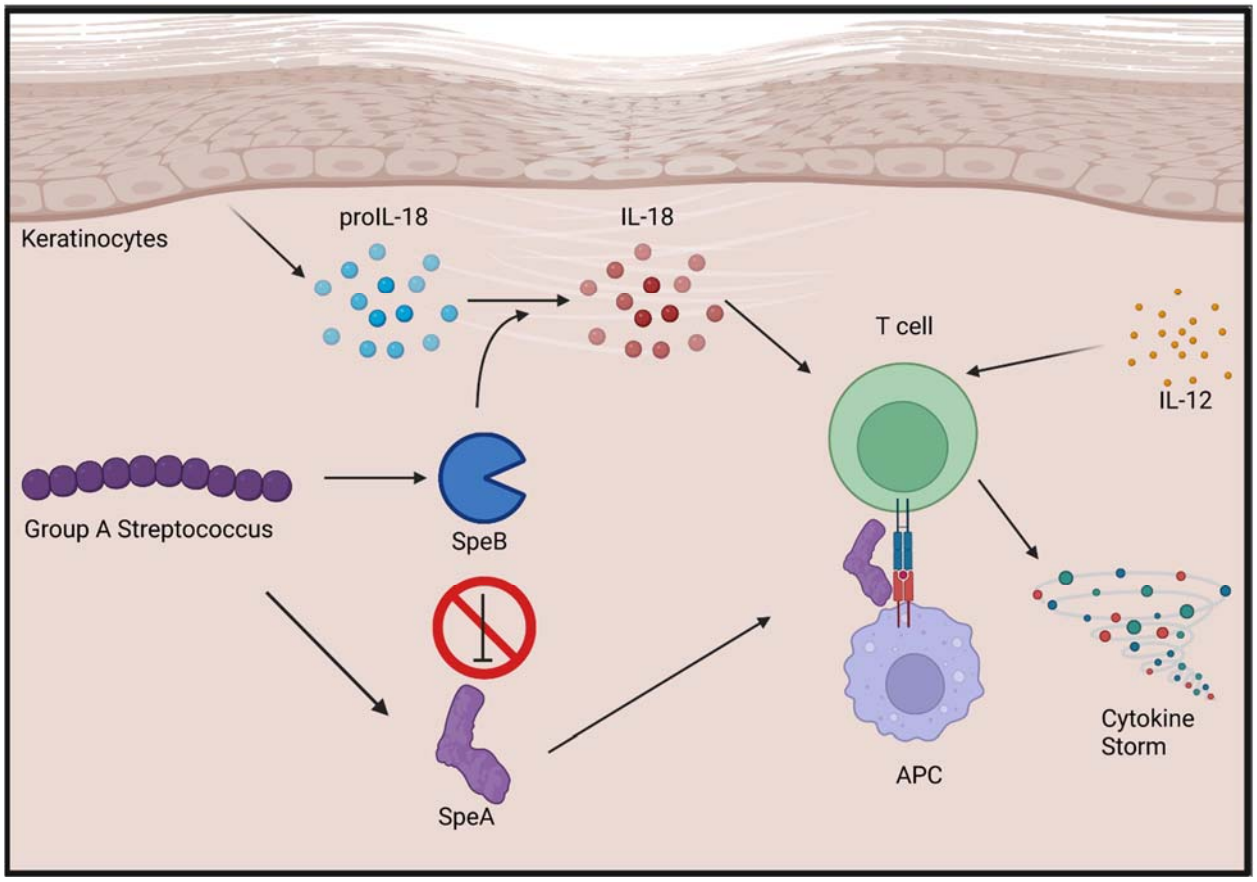

Figure 1. Graphical summary. Graphical summary of findings presented in Chapter 2 and Chapter 3. Created with Biorender.com.

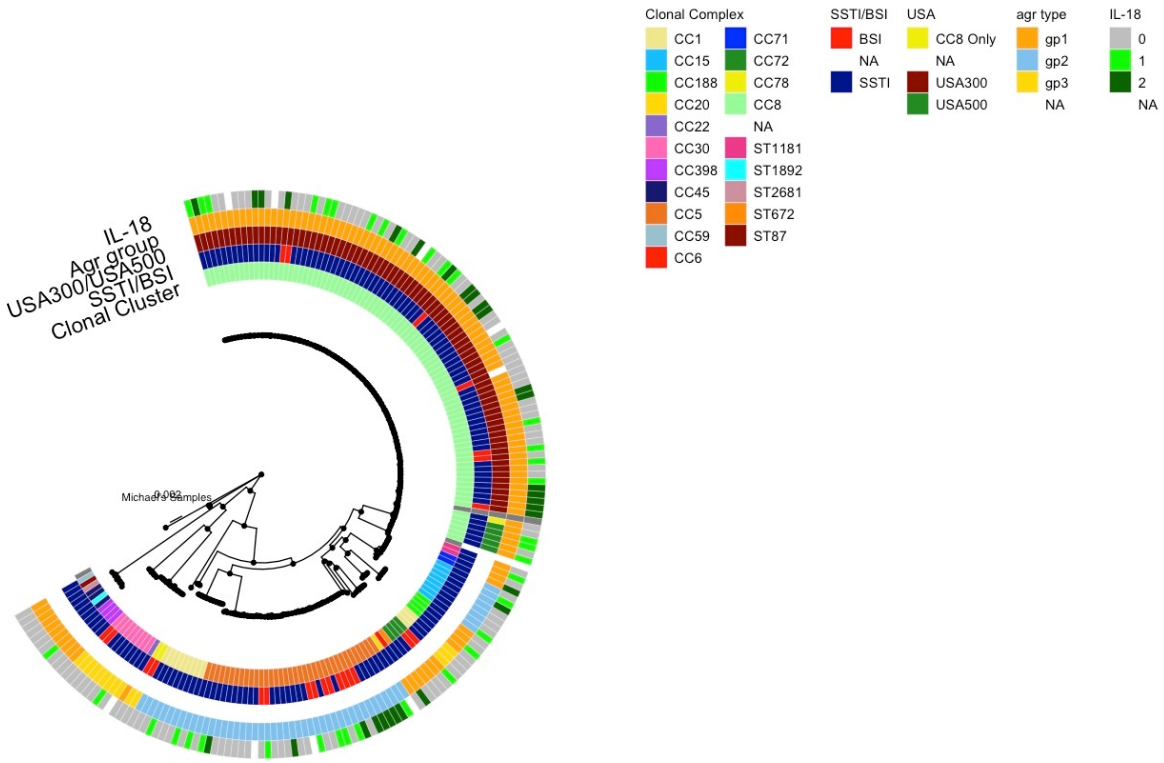

Figure 2. IL-18 activation by *S. aureus*. Alignment of 191 strains of MRSA. Noted for Clonal cluster, isolated from soft tissue or bloodstream, if USA300 or USA500 strain, agr type, and ability to mature proIL-18 independent of infection. Figure was generated by Brooke Talbot, Katrina Hofstetter, and Timothy Read.