Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

____________________ ________________

Shyra-Lynn Wilde Date

Role of Interleukin-6 during restriction of acapsulated group A *Streptococcus*

By

Shyra-Lynn Wilde
Doctor of Philosophy
Graduate Division of Biological and Biomedical Science
Microbiology and Molecular Genetics

____

Christopher LaRock
Advisor

____

Joanna Goldberg
Committee Member

_

____

Shonna McBride
Committee Member

____

Philip Rather
Committee Member

____

Sarah Satola
Committee Member

____

William Shafer
Committee Member

Accepted:

____

Kimberly Jacob Arriola, Ph.D, MPH
Dean of the James T. Laney School of Graduate Studies

____

Date

Role of Interleukin-6 during restriction of acapsulated group A *Streptococcus*

By

Shyra-Lynn Wilde
B.S., Arizona State University, 2016

Advisor: Christopher N. LaRock, Ph.D.

An abstract of
A dissertation submitted to the faculty of the
James T. Laney School of Graduate Studies at Emory University
in partial fulfillment of the requirements for the degree of
Doctor of philosophy in
Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics
2022

Abstract

Streptococcus pyogenes (group A *Streptococcus*; GAS) is an exclusively human pathogen that causes roughly 600 million infections annually. Infection can be relatively mild in the form of 'strep throat' or pharyngitis, or manifest as an invasive infection such as bacteremia, streptococcal toxic shock syndrome, or necrotizing fasciitis. GAS infection can also result in post-infectious immune sequelae like rheumatic fever and rheumatic heart disease. GAS causes an estimated 18 million invasive infections and 600,000 annual deaths due to these diseases.

Invasive GAS in immunocompetent individuals is largely linked to hypervirulent strains, such as strain 5448, that have developed strategies to overcome host immune defenses. Congenital immunodeficiencies and those acquired from chronic disease or immunosuppressant drugs also increase risk of severe illness, specifically, drugs inhibiting Interleukin-1 or -6 (IL-1/6) signaling. We recovered a non-hypervirulent strain of GAS, M4C20, from the blood of a patient receiving a biologic inhibitor of IL-6. Survival of this strain and 5448 were markedly different in both *in vitro* and *in vivo* infection models. M4C20 was only virulent in the presence of IL-1 or IL-6 inhibitors, but 5448 was broadly virulent and resisted IL-6-mediated killing. These findings introduced IL-6 signaling deficiencies as a risk factor for invasive GAS infection, but the mechanism by which IL-6 contributes to GAS killing remained unclear.

Our work was later able to demonstrate that IL-6 acts against GAS by inducing the production of antimicrobial reactive oxygen species (ROS). GAS lacks catalase, a virulence factor used by many diverse species for detoxifying ROS, yet some strains of GAS can withstand ROS and cause severe disease. Through analysis of clinical isolates, we found that the capsule of GAS, composed of hyaluronic acid, also confers protection against ROS. We also showed that hyaluronic acid can act as a direct antioxidant against ROS *in vitro*. Nonetheless, we find that *in vivo* ROS is not essential for killing of GAS in an intradermal infection model. However, lesion size was significantly impacted by both the absence of ROS in host cells and production of capsule by GAS, supporting a model in which ROS and hyaluronic acid regulate pathology during invasive GAS skin infections.

Role of Interleukin-6 during restriction of acapsulated group A *Streptococcus*

By

Shyra-Lynn Wilde
B.S., Arizona State University, 2016

Advisor: Christopher N. LaRock, Ph.D.

A dissertation submitted to the faculty of the
James T. Laney School of Graduate Studies at Emory University
in partial fulfillment of the requirements for the degree of
Doctor of philosophy in
Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics
2022

Acknowledgements

Nobody can achieve great success alone, and a Ph.D. is no exception. I am immensely grateful to everyone who has contributed, whether directly or indirectly, to my academic achievements.

I am so grateful every day that I crossed paths with my advisor, Christopher LaRock, and that he was willing to take me under his wing and guide me through my Ph.D. It is no small feat to set up a lab and take on your first graduate student so early on in your independent research career. I genuinely would not have made it through grad school without Chris's constant support and encouragement. I hope that one day I can become even half of the scientist and person that Chris is, because I would still be a brilliant scientist and an equally wonderful person.

To my lab members, current and former, thank you for everything you have done for me no matter how small or large. I have not been a perfect lab citizen, but I am thankful that I have a great team to help me when I need it the most. To Doris, Anders, Ananya, Stephanie, Jenna, Emily, Rae, Summer, and Keya- I am forever grateful for your role in helping me cross the finish line.

I am indebted to my incredible and distinguished committee members: Drs. Joanna Goldberg, Shonna McBride, Sarah Satola, Philip Rather, and William Shafer. Everyone has contributed their unique knowledge and perspectives that have helped me to grow as a scientist and look beyond my small niche of research. I am so grateful for all the support I have received from each one of you.

I have made many friends along my journey, both inside and outside of Emory. There are too many to list here, but I am so appreciative of everyone who has helped me stay afloat through both the good and bad times. I will forever cherish the memories I have made during my time here. I am indebted to my lifelong friends who got to know me before I started graduate school, and

then stood witness to my evolution into the person I am today. I have come out of this a stronger, wiser, and more ambitious person thanks to you all. You are the true definition of "forever".

Most importantly, I would not be writing this if it wasn't for the unconditional love and support my family has shown me. My mother, Stephanie, sacrificed so many things to get me to where I am now- I hope you feel that the sacrifices you made were worth it. It takes an incredibly strong woman to provide for myself and the rest of the family in the way that you did. My sister, Shandra- my confidant, my best friend, my everything. I am forever grateful for the love and support you have shown me and others, even if we can't spend as much time together as we would like.

Lastly, I would like to give my most earnest thanks to those who I have lost along my journey. My father, Keli- losing you was my worst nightmare, an unfathomable pain that I will feel forever. You prepared me for this moment my entire life, even if I didn't realize it at the time. Thank you for teaching me how to be my true, authentic self and harness the tenacity that came to you so naturally. Even if we are worlds apart, you are the spark that keeps me going. I love you infinity.

Table of Contents

Abstract

Acknowledgements

Table of Contents

List of Tables and Figures

Chapter 1: Introduction…………………………………………………………………… **1**

Chapter 1 References...**21**

Chapter 1 Tables and Figures………………………………………………………...….**38**

Chapter 2: Opportunistic invasive infection by group a *Streptococcus* during anti-interleukin-6 immunotherapy………………………………………………………………………..…**42**

Chapter 2 References...**54**

Chapter 2 Tables and Figures…………………………………………………………....**56**

Chapter 3: Reactive oxygen species and hyaluronic acid capsule drive pathology during group A Streptococcal skin infections……………………………………………..……………..…**59**

Chapter 3 References...**80**

Chapter 3 Tables and Figures…………………………………………………………....**85**

Chapter 4: Concluding Remarks and Future Directions…………………………………......**94**

Chapter 4 References…………………………………………………………..…..….**104**

Chapter 4 Figures…………………...………………………………………………..**109**

List of Tables and Figures

Chapter 1

Table 1. Predominant GAS virulence factors, their known mechanisms and targets, and their essentiality in models of invasive (intradermal, subcutaneous, or intravenous) or nasopharyngeal (intranasal) infection.

Figure 1. Invasive GAS infections by drug target.

Figure 2. Proinflammatory virulence mechanisms of GAS and their targets.

Chapter 2

Figure 1. Impaired IL-6 signaling promotes growth of GAS M4(C20).

Figure 2. IL-6-mediated killing is strain-specific.

Chapter 3

Figure 1. IL-6 induces ROS.

Figure 2. Non-encapsulated strains of GAS are more susceptible to peroxide.

Figure 3. Hyaluronic acid directly protects against peroxide-mediated killing.

Figure 4. Lesion size, but not bacterial load, is both ROS- and capsule-dependent in *vivo*.

Figure 5. Wilde-type and gp91$^{phox-/-}$ mice produce similar levels of pro-inflammatory cytokines in skin lesions.

Figure 6. Skin lesions from gp91$^{phox-/-}$ mice infected with acapsulated GAS have decreased levels of myeloperoxidase compared to wild-type mice.

Chapter 4

Figure 1. Comparison of *in vivo* phenotypes during intradermal infection.

Chapter 1

Introduction

Shyra Wilde[1], Anders F. Johnson[1], and Christopher N. LaRock[1,2*]

Published in part as "Playing with fire: Proinflammatory virulence mechanisms of group A *Streptococcus*" in Frontiers in *Cellular and Infection Microbiology* July 2021, doi: 10.3389/fcimb.2021.704099

[1]Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, GA 30322, USA

[2]Department of Microbiology and Immunology, Division of Infectious Diseases, Department of Medicine, and Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30322, USA

I: Introduction – Group A *Streptococcus*

Streptococcus pyogenes (group A *Streptococcus*; GAS) is an exclusively human pathogen [1]. GAS specifically colonizes the upper respiratory mucosa, primarily the oropharynx, and tonsils and other associated lymphoid tissues as their primary site for carriage and dissemination to other body sites and between individuals. Humans, particularly children, are commonly transiently colonized without overt symptoms. Infection can be relatively mild (e.g., 'strep throat' pharyngitis, impetigo) or serious (including scarlet fever, puerperal sepsis, bacteremia, streptococcal toxic shock syndrome (STSS), necrotizing fasciitis, endocarditis and pneumonia) and result in post-infection immune sequelae (including acute poststreptococcal glomerulonephritis, rheumatic fever, rheumatic heart disease, and Sydenham chorea). GAS is a major health burden and the cause of an estimated 700 million pharyngeal and skin infections. Invasive infections account for approximately 18 million infections and 600,000 annual deaths [2]. Infection requires several virulence factors to subvert host immune processes; these are outlined as follows.

II: Fundamental virulence factors of GAS

SpeB, a broad-spectrum protease

The Streptococcal pyrogenic exotoxin B (SpeB) is a secreted cysteine protease with broad specificity, high turnover, and which can comprise up to 95% of the total secreted protein that can be detected from GAS [3]. In tissue, SpeB cleavage of occludin, E-cadherin, and desmoglein can disrupt tight junctions and may enhance tissue invasion and edema [4,5]. SpeB targets several components of the immune system, including cytokines and chemokines, which may inhibit their signaling [6]; and cathelicidin/LL-37, defensins, complement proteins, and immunoglobulins,

which may enhance GAS resistance to these immune effector molecules (Table 1). Cleavage by SpeB is generally assumed to be destructive, but SpeB cleavage activates bradykinin [7], the protease-activated receptor PAR-1 [8], matrix metalloprotease zymogens [9,10], and the proinflammatory cytokine IL-1β [11,12]. The pathogenic benefit GAS derives from activating proinflammatory cytokines such as IL-1β requires additional study, but may involve disrupting colonization resistance mediated by the microbiota [11]. Conversely, SpeB can cleave and potentially inactivate most GAS virulence factors [3], including those with proinflammatory activities such as Streptolysin O (SLO), superantigens, and M protein (all discussed in greater detail in following sections). However, this may occur only to a limited degree during infection since both SpeB and the virulence factors it cleaves are essential [1,3,13,14]. Nonetheless, SpeB is conserved and promotes GAS survival by intradermal [12], subcutaneous [14,15], intraperitoneal [16–18], and intranasal [11,19,20] routes in murine experimental infections (Table 1).

Some of the underlying cost-benefit of SpeB expression is clear from mutants recovered from natural and experimental infections. These include mutations in the two-component regulatory system CovRS (CsrRS) that lead to constitutive *speB* repression, increased expression of other virulence factors that promote tissue invasion, and shifts in cytokine responses [12,21,22]. Development of *covRS* mutations is variable between GAS serotypes, and more rarely, other mutations are found to disrupt *speB* expression, such as in the positive regulator *ropB* [23,24]. CovRS mutants are widely observed in human invasive infection [25–27] and murine models of invasive infection [28], and less frequently during human pharyngitis [29–31] or murine models of pharyngitis [12,11,32–34]. CovRS mutants are attenuated in transmission and colonization [35],

but can have pathogenic synergy in combination with non-mutant GAS that increases disease severity in murine models of invasive infection [34].

Superantigens

Superantigens are secreted virulence factors that act as T cell mitogens and have strong proinflammatory activity [36]. Superantigens bypass conventional processing by Antigen Presenting Cells (APCs) and directly bind Major Histocompatibility Complex class II (MHC-II) proteins to the variable β-chain of the T cell Receptor (TCR) [37]. The crosslinking allows for activation of up to 30% of human T cells, compared to the 0.01% typical of an antigen [38]. Mucosal associated invariant T (MAIT) cells are the most responsive to superantigens [39] and major contributors to the "cytokine storm" of pathological and proinflammatory IFNγ, TNF, IL-6, and IL-1β (Figure 1) during STSS [40].

Most GAS encode several superantigens (summarized in Table 1), which are typically encoded on mobile genetic elements like lysogenic phage [41]. This wide prevalence suggests there is a selective benefit to express superantigens. The other major superantigen-producing pathogen is *Staphylococcus aureus*, which can use superantigens to control bacterial number during nasal colonization of mice [42]. Superantigens and T cells clearly promote GAS growth in murine nasopharyngeal infections [43,44]. While additional work is required to delineate the pathogenic benefit for GAS, superantigens potentially dampen restrictive innate immune responses by inducing T cell anergy or to increase nutrient availability as a result of inflammation [45]. Recent research suggests this is true in humans, and shows that superantigens can also direct germinal center follicular helper T cells to kill B cells, and patients with recurrent tonsillitis have

smaller tonsil germinal centers and reduced antibody responses [46]. Thus, superantigens may not only promote infection but also induce host susceptibility to subsequent infection.

Pore-forming toxins

One of the first virulence factors identified in GAS was streptolysin O (SLO), a toxin that forms pores ~30 μm in diameter in the plasma membrane of target cells [47]. SLO targets immune cells and keratinocytes for translocation of the co-regulated NAD-glycohydrolase toxin Nga [48], which can promote the intracellular survival of GAS [49]. Like many other pore-forming toxins, SLO is a TLR4 agonist and can induce proinflammatory cytokine expression [50], and also activates the NLRP3 inflammasome to induce inflammatory cell death by pyroptosis of macrophages (Figure 1) and the maturation and release of proinflammatory IL-1β [51]. This inflammation may be beneficial for GAS during pharyngitis, where IL-1β promotes GAS growth [11], but may also work to eliminate cells that could restrict GAS growth in other circumstances. During interactions with neutrophils, SLO leads to release of azurocidin that induces edema [52], while in all cells, the released cytosolic contents are detected by pattern recognition receptors (PRRs) as damage-associated molecular patterns (DAMPs) that induce further inflammation (Reviewed in [53]). Other recent work indicates that sub-lethal quantities of SLO and Nga can be anti-inflammatory, leading to degradation of pro-IL-1β and suppress IL-1β activation, suggesting there are more complexities to this immunomodulation yet to discover [54–56].

GAS encodes a second pore-forming toxin, streptolysin S (SLS), which resembles bacteriocins in sequence and function [57]. SLS lyses a broad array of cells including erythrocytes, platelets, lymphocytes, and keratinocytes [58], and is responsible for β-hemolysis (Table 1). SLS-induced death of keratinocytes can disrupt tissue, promoting lesion formation and dissemination

[59]. SLS also activates the p38 MAPK and NF-kB pathways, broadly inducing of IL-1β, IL-6, and other proinflammatory cytokines [60] that can be beneficial for GAS at sites where inflammation is beneficial, such as the nasopharynx [11]. Lastly, SLS directly activates nociceptor neurons to release the neuropeptide calcitonin gene-related peptide (CGRP), which induces pain and increases necrotizing fasciitis severity [61].

M protein

M protein is the most abundant protein on the GAS surface, forming dimeric coil-coils that extend as hair-like fibrils from the cell wall [62]. There are over 250 allelic variants, each with the variable ability for binding different host factors which include fibrinogen, plasminogen, C4b-binding protein, Protein H, IgA, IgG, LL-37, and the histones contained within antimicrobial neutrophil extracellular traps (Table 1). Each of these interactions can promote virulence by both binding host immune effectors and coating the bacterial surface with a barrier of non-immunogenic endogenous proteins. Binding to each of these factors primarily occurs in the variable N-terminal region of M protein; the C-terminus is more conserved and thought to function as a stalk to project these functions distally from the surface [63]. Few M protein alleles have been comprehensively examined for their host factors they target, but the binding motifs mediating most of these interactions are widespread, with most M alleles predicted to bind several different host factors [64].

Surface-anchored M protein is not proinflammatory, however, some alleles of M protein can also be released and gain drastically altered activities [65–70]. Both neutrophil proteases [69] and SpeB [67] may be involved in M protein release. Soluble M protein has greater availability as an antigen, a PRR agonist, and can form toxic aggregates with fibrinogen to induce cell death,

neutrophil degranulation, and vasculature leakage responses [1,69,71–73]. Some M protein alleles may also have weak T cell mitogen activity [74]. Binding of M protein to platelets leads to their activation and thrombosis [75–77]. Lastly, M protein is a major agonist of TLR2, inducing expression of the numerous proinflammatory molecules regulated by NF-kB [78], and activates the NLRP3 inflammasome, resulting in proinflammatory cell death by pyroptosis [68]. As with pore-forming toxins, these proinflammatory activities contribute to the pathology and complications of infection, but it is not clear whether they are required for virulence.

Capsule

Capsule, the major focus of this work, is an extracellular polysaccharide made up of hyaluronic acid, which is structurally identical to the hyaluronic acid found in human connective tissue [79,80]. Hyaluronic acid production in GAS is regulated by the highly conserved *hasABC* operon [81]. Capsule is most known for its role in protecting GAS from opsonization by masking epitopes such as M protein [82,83]. Current evidence suggests that capsule can also promote dissemination into deeper tissues and is important for the development of systemic infection [84,85], as capsule promotes infection for closely related species such as *Streptococcus pneumoniae* [86]. Isolates of GAS from invasive infections frequently carry mutations in the *covRS* two-component system that result in clones that highly express capsule (hyper-encapsulated), further confirming a protective role of capsule for GAS during invasive infection [1,32,87]. Encapsulated GAS can also bind the host cell surface receptor CD44, a hyaluronic acid-binding protein that is expressed by both pharyngeal and epithelial cells [84,88]. This interaction results in epithelial cell movement and disruption of intercellular junctions, providing a mechanism for encapsulated GAS to penetrate deeper tissues to cause invasive infections [84]. Capsule appears

to be essential for long-term pharyngeal colonization [88,89], invasive pneumonia [1], invasive soft tissue and myositis infections [84,85,90], and systemic infections *in vivo* [15,85,91]. Altogether, this evidence suggests that capsule is important for the selection of hyperinvasive clones *in vivo*, which often occurs via mutation of the *covRS* two-component regulatory system [32].

Despite its high conservation across GAS strains, there are a select few serotypes (M4, M22, M28, and M89) that have lost the *hasABC* operon and therefore do not produce capsule [92]. Nonetheless, these strains are maintained withing the human population and retain virulence and are capable of causing invasive disease [93–98]. This is suggestive of compensatory virulence mechanisms, but these mechanisms remain largely unclear. Capsule-producing strains of GAS have a point mutation in the *hylA* gene that degrades hyaluronic acid [99]; acapsulated GAS have an intact copy of *hylA*, suggesting that these strains may either degrade hyaluronic acid for nutritional purposes or degrade host hyaluronan as a mechanism for dissemination [99,100]. Some, but not all, acapsulated GAS also carry mutations in other portions of their genome that result in enhanced cytotoxin production [95,97], which may confer an additional compensatory mechanism.

III: Correlation of virulence factors with invasive GAS infections

GAS is typically categorized into serotypes by its surface M protein, encoded by the *emm* gene, of which there are over 200 subtypes [101]. Yet, most invasive GAS infections are caused by a small number of serotypes, particularly the M1, M3, M12, and M28 serotypes [102]. Some serotypes have strong associations with specific disease pathologies; for instance, serotypes M1 and M3 are typically associated with necrotizing fasciitis, streptococcal toxic shock syndrome

(STSS), and acute rheumatic fever [38,103–105], and M28 GAS are generally associated with puerperal sepsis [106].

Since the 1980s there has been a resurgence in GAS infections that has largely been attributed to the emergence of a hyper-invasive clone of GAS. This clone, M1T1, has undergone significant changes to its genome that contribute to virulence and is now widely distributed worldwide [48,107–109]. M1T1 GAS has acquired multiple prophages that encode for virulence factors such as the DNase Sda1 and exotoxin SpeA2 [48,102]. This clone has also acquired a 36kb chromosomal region from an M12 GAS strain via horizontal gene transfer, giving rise to three distinct polymorphisms that resulted in increased expression of the virulence factors streptolysin O (SLO) and NAD-glycohydrolase (Nga) [110], the latter of which was not produced by M1 GAS prior to the 1980s [48]. Other serotypes of GAS, such as M12, have also acquired prophages encoding for superantigens Ssa and SpeC, as well as the DNase Spd1 that have been largely associated with scarlet fever outbreaks [111].

Most GAS strains isolated from severe infections have spontaneous mutations in the two-component system *covRS*, which regulates approximately 10-15% of the GAS core genome [30,112]. CovRS generally suppresses the production of virulence factors such as hyaluronic acid capsule, M protein, and the IL-8-degrading protease SpyCEP; frameshift mutations in *covS* result in increased expression of these virulence factors and abolishment of protease SpeB expression [113,114]. These transcriptional changes allow for superficial infections to disseminate and become life-threatening, systemic infections. The DNase Sda1 of M1T1 GAS strains has been shown to act as a selective pressure toward the generation of *covRS* mutations, since abolishing expression of SpeB would allow greater success for immune evasion tactics [13].

Other serotypes of GAS such as M4 and M89 have also been recovered from recent outbreaks. M89 GAS have been increasingly associated with invasive GAS infections since the early 2000s [95,96,115], and are characterized by a similar recombination event to M1T1 that resulted in increased production of Nga and SLO [97,110]. An increase in both pharyngitis and invasive diseases caused by M4 GAS has also been observed [94,98,100,116]. Interestingly, M4 GAS did not undergo the same recombination events as M1 and M89 strains as they produce lower levels of Nga and SLO [93]. A majority of M4 GAS in the United States appear to have generated a chimeric *emm* gene by fusing with the M-like gene, encoded by *enn*, which may have unique implications for M protein interactions with host cells [117]. What is perhaps most striking about the increase of infections caused by M89 and M4 type GAS is that these strains have lost the genetic locus that is responsible for capsule production [92]. Capsule production in GAS is regulated by the *hasABC* operon, which produces hyaluronic acid that is structurally identical to the hyaluronic acid found in human connective tissue [79,80]. Capsule has long been thought to be essential for virulence of GAS, as well as necessary for driving the generation of covRS mutations [32]. However, these strains are fully capable of causing invasive disease in humans and grow *ex vivo* in whole human blood efficiently [93,95,97], indicating that these strains may have acquired compensatory mechanisms in order to cause disease. While M89 GAS have increased toxin expression, it is unclear how M4 GAS has been able to cause an increasing amount of disease.

Although pathogenetic mechanisms vary between serotypes of GAS, it has become clear that GAS can cause life-threatening systemic diseases using an array of virulence factors that deliberately induce inflammation. Morbidity and mortality from invasive GAS infections like STSS and sepsis are driven by an exaggerated and unregulated host immune response, largely

driven by neutrophils and the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF.

IV: Host responses to GAS infection

The inflammatory symptoms of infection (fever, redness, and swelling) ultimately coordinate antimicrobial processes to act against the pathogen and resolve infection. Successful bacterial pathogens commonly work to avoid immune recognition and delay or prevent their clearance by antimicrobial immune effectors. Strategies include modifying pathogen-associated molecular patterns (PAMPs) agonists of Nod-like receptor (NLR) or Toll-like receptor (TLR) pattern recognition receptors (PRRs) to prevent their recognition (or restrict their accessibility), or interfering with signaling downstream of these receptors using toxins and effectors [53]. NLR and TLR PRRs detect numerous GAS PAMPs including lipopeptides, lipoteichoic acid, peptidoglycan, CpG-rich DNA, SLO (reviewed in [53]), SIC [118], and the C-type lectin agonist monoglucosyldiacylglycerol [119]. Although a specific toll-like receptor has not been identified as essential for recognition of GAS PAMPs, it is known that inflammatory responses to GAS are driven by the adaptor molecule MyD88 that is used by nearly all TLRs, suggesting that recognition of GAS requires multiple TLRs [120,121]. Multiple TLRs have been implicated in recognition of GAS PAMPs such as TLR2 [122], TLR4 [123,124], and TLR9 [125,126], further supporting this hypothesis.

Neutrophils

GAS is classically defined as a "pyogenic" bacterium, characterized by a robust infiltration of neutrophils during infection and subsequent formation of a large amount of pus relative to

infection by other microbes. Neutrophils, along with macrophages, are the most abundant cell type recruited during infections by GAS [127]. Neutrophils are the most abundant white blood cell in the human body, accounting for 40-80% of white blood cells [128]. Neutrophils are packed with numerous types of antimicrobial effectors that are sorted into granules, which are released upon encountering a pathogen by tightly controlled mechanisms. Primary (azurophilic) granules contain most of the bactericidal effectors including myeloperoxidase, neutrophil elastase, cathepsins, and defensins [128]; hence, neutrophils can cause extensive damage to not only invading pathogens, but to the host if not controlled properly [129]. Following intracellular stimuli such as the production of reactive oxygen species (ROS), neutrophils can rapidly decondense their chromatin and form neutrophilic extracellular traps (NETs) composed of cytosolic and granular proteins [130]. NETs are a cornerstone of neutrophil biology, and GAS have evolved ways to counteract their effects in the form of DNase Sda1, which degrade NETs to subvert innate immune responses [131]. Neutrophils also produce the human antimicrobial peptide LL-37 that directly interacts with bacterial membranes, as well as stimulates immune cell recruitment and function [132]. GAS can directly neutralize active LL-37 by binding to M1 protein, as well as inhibit proteolytic processing of the precursor form hCAP-18 by neutrophils [65].

Pro-inflammatory cytokines

Infections caused by GAS induce a large amount of pro-inflammatory cytokines, notably Interleukin-1β and Interleukin-6 [133]. Cytokines are small, soluble proteins that induce the stimulation of immune cells and the production of antimicrobial effectors through a relay of signaling processes that ultimately activate their transcription. Interleukin-1B (IL-1β) is critical for defense against pathogens, as it serves as a strong recruiter of both neutrophils and macrophages

that contribute to killing GAS [134]. IL-1β is typically released during the inflammatory cell death program known as pyroptosis, which serves to deprive intracellular pathogens such as GAS of a replicative niche [53]. However, GAS is able to induce IL-1β independent of this mechanism via the virulence factor SpeB [12].

Along with IL-1β, interleukin-6 (IL-6) is a pleiotropic cytokine with both pro-inflammatory functions phenotype during infection. IL-6 signals through two mechanisms: classical signaling, which involves IL-6 binding to membrane-bound IL-6R present on hepatocytes and a small number of leukocytes, and trans-signaling, which involves a soluble IL-6/IL-6R complex that can bind to a large number of immune cells via gp130 [135]. IL-6 trans-signaling has been shown to be the pro-inflammatory form of IL-6 signaling [136]; neutrophils that undergo apoptosis actively shed IL-6R, which further promote the inflammatory response [137]. IL-6 was initially discovered as a B-cell differentiation factor but is also responsible for the recruitment of neutrophils and other lymphocytes to the site of infection [136], as well as regulation of neutrophil apoptosis and clearance. IL-6 trans-signaling induces the phosphorylation and subsequent activation of the JAK/STAT, MAPK, and Erk pathways that further regulate the production of antimicrobial effectors [136].

Reactive oxygen species (ROS)

Expression of both IL-1β and IL-6 during infection, as well as other inflammatory processes, ultimately lead to the production of reactive oxygen species (ROS). Also referred to as “free radicals”, ROS are a group of highly unstable intermediate molecules that are directly antimicrobial. ROS are utilized by professional phagocytes to kill invading pathogens through direct damage to cell membranes as well as DNA and protein damage (reviewed in [138] and

[139]). ROS are also highly toxic to host cells, hence mechanisms have been developed to counteract their effects, such as superoxide dismutase and catalase (reviewed in [140]). A number of bacterial pathogens employ similar mechanisms to withstand oxidative stress and subsequently, humans with Chronic Granulomatas Disease (CGD) that are deficient in phagocytic ROS production, are more susceptible to catalase-positive pathogens and certain types of fungal species [141–143]. ROS are also an important class of signalling molecules that orchestrate the innate immune response to pathogens through multiple mechanisms: regulation of redox-sensitive transcription factors such as NF-kB [144], regulation of neutrophil chemoattraction [145], and neutrophil apoptosis and clearance [146,147].

V: Host susceptibility factors for invasive GAS infections

Proinflammatory pathways have evolved to initiate and coordinate the immune response against pathogens to protect against infection. Correspondingly, transgenic mice with deficiencies in inflammatory signaling are generally more susceptible to infection, as are humans with immunodeficiencies due to genetic disorders, preexisting conditions, or anti-inflammatory drugs[148–150]. The study of when pathogens instead benefit from inflammation is an emergent field that runs counter to this prevailing paradigm.

During GAS infection of the murine nasopharynx (a model of strep throat), neutrophils, T cells, and the proinflammatory cytokine IL-1β all promote GAS growth [11,44], despite conventionally having vital antimicrobial roles in immunity and acting to counter GAS invasion at other body sites [12,61]. In antibiotic-treated mice, neutrophils and IL-1β are no longer essential for GAS colonization of the nasopharynx, suggesting this immune axis may be necessary for overcoming interference from antibiotic-sensitive members of the resident microbiota [11].

Diverse pathogens including *Salmonella enterica* serovar Typhimurium [151,152], *Helicobacter pylori* [153], *Pseudomonas aeruginosa* [154,155], and *Candida albicans* [156] also subvert inflammation to their advantage, either to disrupt membrane barrier function, promote dissemination, acquire nutrients, or antagonize competing microbes. For the best-characterized example closely related to GAS, *Streptococcus pneumoniae*, inflammation broadly promotes growth and transmission, though T cells, neutrophils, and IL-1β each have specifically antagonistic effects on *S. pneumoniae* growth [157]. Thus, while several pathogens have a common strategy of subverting inflammation for their benefit, the specific mechanisms and benefits can greatly differ.

The molecular basis underlying the requirement for T cells at the earliest stages of GAS infection is more enigmatic, but later T cells responses are uncoordinated, non-specific, and potentially less effective [44]. Excessive inflammation can promote epitope spreading, whereby increased activation of antigen-presenting cells and T cells leads to broader specificities and increased chance of recognizing self-antigen [158,159]. Recurrent GAS infections drive the generation of autoreactive antibodies that cross-react with heart valve endothelium, lysogangliosides, dopamine receptors, and other human tissues [160]. These antibodies may give rise to immune sequelae such as acute rheumatic fever (ARF) and rheumatic heart disease (RHD), which account for a majority of annual deaths from GAS [2]; however, the pathogenesis of these conditions remains controversial. A neurological manifestation of these autoreactive antibodies is Sydenham chorea (SC), characterized by an uncontrolled movement of the arms, legs, and facial muscles [161].

The aberrant immune activation GAS has evolved to promote nasopharyngeal infection drives morbidity and mortality when GAS is found in other body sites. Excessive systemic inflammation directly induces death through sepsis, organ failure, disseminated intravascular

coagulation, thrombosis, and edema [162]. During invasive skin infections like necrotizing fasciitis, inflammation drives localized microvascular thrombosis, tissue hemorrhage, and cell infiltrate, all which further the proinflammatory cycle [163]. Through these mechanisms, which can limit the perfusion of antibiotics and provide a protective intracellular niche within macrophages, inflammation may also contribute to antibiotic failure [164]. These observations highlight the necessity of a carefully balanced immune response towards a pathogen.

In humans, post-marketing surveillance shows an association between some immunosuppressive drugs and an increased incidence of invasive GAS infections (Figure 1). There is a strong association between IL-1-inhibiting biologics (i.e., Anakinra, anti-IL-1R) and necrotizing skin infections caused by GAS, as patients taking Anakinra are approximately ~330-fold more likely to develop an invasive GAS infection relative to other immunosuppressive drugs [12]. Anakinra-treated patients also experience higher rates of mortality when presenting with a necrotizing GAS infection, compared to patients taking other immunosuppressants or immunocompetent individuals [1,12]. There was no increased risk of developing an infection caused by other pathogens such a *Staphylococcus aureus*, indicating that IL-1β is necessary to protect against GAS infection [12]. Further studies revealed a mechanism by which GAS activates IL-1β independently of the canonical caspase-1 mechanism: pro-IL-1β can be proteolytically activated by the GAS protease SpeB, further highlighting the importance of IL-1β during GAS infections [12].

Post-marketing surveillance data has also shown an association between IL-6-inhibiting therapeutics and invasive GAS infections (Figure 1)[12,165]. Though patients taking IL-6-inhibiting biologics (i.e., Tocilizumab; IL-6R) are not as disproportionately affected by invasive GAS infections as patients taking Anakinra (Figure 1), these individuals are still ~50-fold more

likely to develop an invasive GAS infection compared to patients not taking this class of immunosuppressant. These infections have manifested as both necrotizing skin infections [166], as well as bloodstream infections and sepsis [165]. A molecular mechanism has yet to be identified for the role of IL-6 in the immune restriction of invasive infection by GAS.

VI: Potential for therapeutics to manage infection

There is no GAS vaccine after 100 years of research. Ongoing preclinical and clinical work focus on the immunodominant, but variable, M protein, the conserved group A carbohydrate, and multi-valent vaccines against major GAS virulence factors (reviewed in [167]). Development of a safe and effective vaccine is important, because invasive infections have a mortality rate upwards of 20% within seven days of the onset, even with antibiotic therapy and surgical debridement of infected necrotic tissue [168]. GAS remains penicillin sensitive [169], and clindamycin is recommended for severe infections and patients with penicillin allergies [170–172]. Macrolide resistance is increasingly prevalent [173–175], but tedizolid and linezolid may be used instead [176]. Despite the availability of antibiotics to treat infection, antibiotic monotherapy can fail to eradicate GAS during pharyngitis or invasive disease [170,177]. Antibiotic inefficacy is multifactorial and can be due to bacterial tolerance, reservoirs of protected intracellular bacteria, failure of the drug to reach the infection site due to tissue necrosis and thrombosis, and the rapid progression of disease [178–180]. Below, we will discuss recent developments in therapeutics based on targeting inflammation and proinflammatory virulence factors.

Intravenous immunoglobulin (IVIG) are non-specific antibodies pooled from human donors. As an adjunctive therapy, IVIG may decrease morbidity by not only opsonizing the bacteria, but neutralizing GAS exotoxins [181,182]. In murine models, IVIG treatment in

conjunction with penicillin and clindamycin was successful at modulating the systemic inflammatory response as well as increasing bacterial killing [183]. In human trials, IVIG treatment is associated with a 20-30% increase in survival during STSS [184], but this may have lessor benefit in children [185]. The use of IVIG for the treatment of GAS disease is an area of active research, but shows promise in safety and efficacy, though may be cost-prohibitive in many regions where the health burden is highest [186].

Several FDA-approved drugs have potential for off-label use during GAS disease. The HIV protease inhibitor nelfinavir inhibits streptolysin S, limiting its pro-virulence and proinflammatory activities [187]. Excessive inflammation during STSS and other conditions is harmful, and therapeutics targeting inflammation may also have therapeutic benefit. During invasive infections, the opioid-derivative cough suppressant dextromethorphan may prolong survival through its anti-inflammatory activities [188–190]. Drugs that block nociceptor signaling also promote beneficial immune responses [61]. Lastly, inhibiting protein synthesis can block production of SpeA, the nuclease Sda1, SLO, and other toxins [172,191–193]. Thus, antibiotics like clindamycin may have therapeutic benefits in addition to direct killing of GAS.

VII: Concluding remarks

As an obligate human pathogen GAS is highly adept at manipulating human innate and adaptive immune responses. This often serves to resist immune effectors, mask the pathogen, and subvert immune signaling (Table 1), but GAS also induces and thrives off the activation of other immune processes (Figure 1). New insights into the molecular mechanisms involved may come from forward genetic screens based on high-throughput sequencing of transposon mutant libraries, which have already provided new insights into regulatory, metabolic, and antimicrobial resistance mechanisms that contribute to fitness during infection [194,195]. The complicated relationship of

GAS with inflammation can underlie infectious risk with some anti-inflammatory therapeutics, like inhibitors of IL-1β, IL-6, or TNF, but may provide opportunities for treatments with other immune-targeted drugs.

Host-directed molecules based on a better understanding of the role of inflammation in GAS pathogenesis hold hope for future therapeutics. Debilitating and difficult-to-treat disease manifestations following GAS infection like rheumatic heart disease, scarlet fever, toxic shock syndrome, and post-streptococcal glomerulonephritis result from aberrant and excessive immune responses during and after infection and can potentially be treated through immunomodulation. Since many GAS deaths are from immune-mediated complications, knowledge of which GAS factors induce inflammation, and which inflammatory pathways drive pathology in immune disease, will be essential for these strategies. Careful dissection of these processes will be essential to develop adjunctive therapies to reduce the significant morbidity and mortality caused by GAS.

In this work, I dissect the molecular interactions of a naturally capsule-deficient strain of GAS that infected a patient taking IL-6 inhibiting immunotherapeutics. This was with the goal of defining whether IL-6 suppression is a risk factor for invasive GAS infection, whether it would be true for all strains of GAS, and what the molecular basis was. I found that inhibition of IL-6 signaling *in vivo* was sufficient to increase pathogenesis of this specific isolate, and addition of exogenous IL-6 was sufficient to restrict intracellular growth of this strain within macrophages. However, exogenous IL-6 was not sufficient to kill traditionally invasive GAS strain 5448 *in vitro*, indicating differences in susceptibility to IL-6-mediated killing by GAS.

In subsequent work, I found that resistance to IL-6-mediated killing is due in part to the production of hyaluronic acid capsule by most GAS strains, including 5448. I also found that IL-6 directly induces the production of phagocytic ROS, which phagocytes use to kill GAS.

Encapsulated strains of GAS were more resistant to peroxide-mediated killing *in vitro*, and acapsulated GAS were able to effectively grow within phagocytes and withstand neutrophil killing when supplemented with N-acetylcysteine, a ROS-scavenging drug. *In vivo*, encapsulated and acapsulated GAS were able to grow to similar levels in an intradermal model of infection. This was also true in ROS-deficient ($gp91^{phox-/-}$) mice, indicating that ROS is not essential for killing GAS in a skin infection model. However, $gp91^{phox-/-}$ mice infected with acapsulated GAS had visibly worse histopathological effects in comparison to wild-type mice infected with the same strains, indicating a role for ROS and hyaluronic acid in wound healing during infection. With this increased understanding of the molecular interactions of GAS with its human host, we are better poised to develop novel therapeutics and prophylaxis strategies to combat this challenging pathogen.

References

1. Walker MJ, Barnett TC, McArthur JD, et al. Disease Manifestations and Pathogenic Mechanisms of Group A Streptococcus. Clin Microbiol Rev. **2014**; 27(2):264–301.

2. Ralph AP, Carapetis JR. Group A Streptococcal Diseases and Their Global Burden. Curr Top Microbiol Immunol. **2012**; :1–27.

3. Aziz RK, Pabst MJ, Jeng A, et al. Invasive M1T1 group A Streptococcus undergoes a phase-shift in vivo to prevent proteolytic degradation of multiple virulence factors by SpeB. Mol Microbiol. **2004**; 51(1):123–134.

4. Sumitomo T, Nakata M, Higashino M, Terao Y, Kawabata S. Group A streptococcal cysteine protease cleaves epithelial junctions and contributes to bacterial translocation. J Biol Chem. **2013**; 288(19):13317–13324.

5. Sumitomo T, Mori Y, Nakamura Y, et al. Streptococcal Cysteine Protease-Mediated Cleavage of Desmogleins Is Involved in the Pathogenesis of Cutaneous Infection. Front Cell Infect Microbiol. **2018**; 8:524.

6. Egesten A, Olin AI, Linge H, et al. SpeB of Streptococcus pyogenes Differentially Modulates Antibacterial and Receptor Activating Properties of Human Chemokines. PLoS ONE. **2009**; 4(3):e4769.

7. Herwald H, Collin M, Müller-Esterl W, Bjorck L. Streptococcal cysteine proteinase releases kinins: a virulence mechanism. J Exp Med. **1996**; 184(2):665–673.

8. Ender M, Andreoni F, Zinkernagel AS, Schuepbach RA. Streptococcal SpeB Cleaved PAR-1 Suppresses ERK Phosphorylation and Blunts Thrombin-Induced Platelet Aggregation. PLoS ONE. **2013**; 8(11):e81298.

9. Burns EH, Marciel AM, Musser JM. Activation of a 66-kilodalton human endothelial cell matrix metalloprotease by Streptococcus pyogenes extracellular cysteine protease. Infect Immun. **1996**; 64(11):4744–4750.

10. Tamura F, Nakagawa R, Akuta T, et al. Proapoptotic effect of proteolytic activation of matrix metalloproteinases by Streptococcus pyogenes thiol proteinase (Streptococcus pyrogenic exotoxin B). Infect Immun. **2004**; 72(8):4836–4847.

11. LaRock DL, Russell R, Johnson AF, Wilde S, LaRock CN. Group A Streptococcus Infection of the Nasopharynx Requires Proinflammatory Signaling Through the Interleukin-1 Receptor. Infect Immun [Internet]. American Society for Microbiology Journals; **2020** [cited 2020 Sep 12]; . Available from: http://iai.asm.org/content/early/2020/07/22/IAI.00356-20

12. LaRock CN, Todd J, LaRock DL, et al. IL-1β is an innate immune sensor of microbial proteolysis. Sci Immunol. **2016**; 1(2):eaah3539.

13. Walker MJ, Hollands A, Sanderson-Smith ML, et al. DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med [Internet]. **2007**; . Available from: http://www.nature.com/nm/journal/v13/n8/abs/nm1612.html

14. Cole J, McArthur JD, McKay FC, et al. Trigger for group A streptococcal M1T1 invasive disease. FASEB J [Internet]. **2006**; . Available from: http://www.fasebj.org/content/20/10/1745.short

15. Ashbaugh CD, Warren HB, Carey VJ, Wessels MR. Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection. J Clin Invest [Internet]. **1998** [cited 2019 Aug 12]; . Available from: https://www.jci.org/articles/view/3065/pdf

16. Kuo CF, Wu JJ, Lin KY, et al. Role of streptococcal pyrogenic exotoxin B in the mouse model of group A streptococcal infection. Infect Immun. **1998**; 66(8):3931–3935.

17. Lukomski S, Sreevatsan S, Amberg C, et al. Inactivation of Streptococcus pyogenes extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains. J Clin Invest. **1997**; 99(11):2574–2580.

18. Lukomski S, Burns EH, Wyde PR, et al. Genetic inactivation of an extracellular cysteine protease (SpeB) expressed by Streptococcus pyogenes decreases resistance to phagocytosis and dissemination to organs. Infect Immun. **1998**; 66(2):771–776.

19. Lukomski S, Montgomery CA, Rurangirwa J, et al. Extracellular Cysteine Protease Produced by Streptococcus pyogenes Participates in the Pathogenesis of Invasive Skin Infection and Dissemination in Mice. Infect Immun. **1999**; 67(4):1779–1788.

20. Makthal N, Do H, VanderWal AR, Olsen RJ, Musser JM, Kumaraswami M. Signaling by a Conserved Quorum Sensing Pathway Contributes to Growth Ex Vivo and Oropharyngeal Colonization of Human Pathogen Group A Streptococcus. Infect Immun [Internet]. **2018** [cited 2020 Sep 18]; 86(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5913841/

21. Li J, Liu G, Feng W, et al. Neutrophils Select Hypervirulent CovRS Mutants of M1T1 Group A Streptococcus during Subcutaneous Infection of Mice. Infect Immun. **2014**; 82(4):1579–1590.

22. Williams JG, Ly D, Geraghty NJ, et al. Streptococcus pyogenes M1T1 variants activate caspase-1 and induce an inflammatory neutrophil phenotype. Front Cell Infect Microbiol [Internet]. **2021** [cited 2020 Mar 20]; . Available from: http://biorxiv.org/lookup/doi/10.1101/2020.03.02.972240

23. Feng W, Minor D, Liu M, et al. Null Mutations of Group A Streptococcus Orphan Kinase RocA: Selection in Mouse Infection and Comparison with CovS Mutations in Alteration of in vitro and in vivo Protease SpeB Expression and Virulence. Infect Immun. **2016**; :IAI.00790-16.

24. Friaes A, Pato C, Melo-Cristino J, Ramirez M. Consequences of the variability of the CovRS and RopB regulators among Streptococcus pyogenes causing human infections. Sci Rep. **2015**; 5(1):1–12.

25. Chatellier S, Ihendyane N, Kansal RG, et al. Genetic Relatedness and Superantigen Expression in Group A Streptococcus Serotype M1 Isolates from Patients with Severe and Nonsevere Invasive Diseases. Infect Immun. **2000**; 68(6):3523–3534.

26. Kansal RG, McGeer A, Low DE, Norrby-Teglund A, Kotb M. Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A Streptococcal Infection Cases. Infect Immun. **2000**; 68(11):6362–6369.

27. Olsen RJ, Raghuram A, Cantu C, et al. The Majority of 9,729 Group A Streptococcus Strains Causing Disease Secrete SpeB Cysteine Protease: Pathogenesis Implications. Infect Immun. **2015**; 83(12):4750–4758.

28. Engleberg NC, Heath A, Miller A, Rivera C, DiRita VJ. Spontaneous mutations in the CsrRS two-component regulatory system of Streptococcus pyogenes result in enhanced virulence in a murine model of skin and soft tissue infection. J Infect Dis. **2001**; 183(7):1043–1054.

29. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM. Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. PLoS Pathog. **2006**; 2(1):e5.

30. Ikebe T, Ato M, Matsumura T, et al. Highly Frequent Mutations in Negative Regulators of Multiple Virulence Genes in Group A Streptococcal Toxic Shock Syndrome Isolates. PLOS Pathog. **2010**; 6(4):e1000832.

31. Shea PR, Beres SB, Flores AR, et al. Distinct signatures of diversifying selection revealed by genome analysis of respiratory tract and invasive bacterial populations. Proc Natl Acad Sci. **2011**; 108(12):5039–5044.

32. Cole J, Pence MA, Köckritz-Blickwede M von, et al. M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A Streptococcus. mBio [Internet]. **2010**; 1(4). Available from: http://mbio.asm.org/content/1/4/e00191-10.long

33. Fiebig A, Loof TG, Babbar A, et al. Comparative Genomics of Streptococcus pyogenes M1 isolates differing in virulence and propensity to cause systemic infection in mice. Int J Med Microbiol IJMM. **2015**; .

34. Horstmann N, Tran CN, Brumlow C, et al. Phosphatase activity of the control of virulence sensor kinase CovS is critical for the pathogenesis of group A streptococcus. Wessels MR, editor. PLOS Pathog. **2018**; 14(10):e1007354.

35. Hollands A, Pence MA, Timmer AM, et al. Genetic switch to hypervirulence reduces colonization phenotypes of the globally disseminated group A streptococcus M1T1 clone. J Infect Dis. **2010**; 202(1):11–19.

36. Proft T, Fraser JD. Streptococcal Superantigens: Biological properties and potential role in disease. **2016**; :41.

37. Spaulding AR, Salgado-Pabón W, Kohler PL, Horswill AR, Leung DYM, Schlievert PM. Staphylococcal and Streptococcal Superantigen Exotoxins. Clin Microbiol Rev. American Society for Microbiology Journals; **2013**; 26(3):422–447.

38. Lappin E, Ferguson AJ. Gram-positive toxic shock syndromes. Lancet Infect Dis. **2009**; 9(5):281–290.

39. Shaler CR, Choi J, Rudak PT, et al. MAIT cells launch a rapid, robust and distinct hyperinflammatory response to bacterial superantigens and quickly acquire an anergic phenotype that impedes their cognate antimicrobial function: Defining a novel mechanism of superantigen-induced immunopathology and immunosuppression. PLoS Biol. **2017**; 15(6):e2001930.

40. Emgård J, Bergsten H, McCormick JK, et al. MAIT Cells Are Major Contributors to the Cytokine Response in Group A Streptococcal Toxic Shock Syndrome. Proc Natl Acad Sci. **2019**; :201910883.

41. Ben Zakour NL, Davies MR, You Y, et al. Transfer of scarlet fever-associated elements into the group A *Streptococcus* M1T1 clone. Sci Rep. **2015**; 5:15877.

42. Xu SX, Kasper KJ, Zeppa JJ, McCormick JK. Superantigens Modulate Bacterial Density during Staphylococcus aureus Nasal Colonization. Toxins. **2015**; 7(5):1821–1836.

43. Kasper KJ, Zeppa JJ, Wakabayashi AT, et al. Bacterial Superantigens Promote Acute Nasopharyngeal Infection by Streptococcus pyogenes in a Human MHC Class II-Dependent Manner. DeLeo FR, editor. PLoS Pathog. **2014**; 10(5):e1004155.

44. Zeppa JJ, Kasper KJ, Mohorovic I, Mazzuca DM, Haeryfar SMM, McCormick JK. Nasopharyngeal infection by Streptococcus pyogenes requires superantigen-responsive Vβ-specific T cells. Proc Natl Acad Sci. **2017**; 114(38):10226–10231.

45. Sriskandan S, Faulkner L, Hopkins P. Streptococcus pyogenes: Insight into the function of the streptococcal superantigens. Int J Biochem Cell Biol. **2007**; 39(1):12–19.

46. Dan JM, Havenar-Daughton C, Kendric K, et al. Recurrent group A Streptococcus tonsillitis is an immunosusceptibility disease involving antibody deficiency and aberrant TFH cells. Sci Transl Med. **2019**; 11(478):eaau3776.

47. Herbert D, Todd EW. Purification and properties of a haemolysin produced by group A haemolytic streptococci (streptolysin O). Biochem J. **1941**; 35(10–11):1124–1139.

48. Sumby P, Porcella SF, Madrigal AG, et al. Evolutionary Origin and Emergence of a Highly Successful Clone of Serotype M1 Group A Streptococcus Involved Multiple Horizontal Gene Transfer Events. J Infect Dis. **2005**; 192(5):771–782.

49. Bastiat-Sempe B, Love JF, Lomayesva N, Wessels MR. Streptolysin O and NAD-glycohydrolase prevent phagolysosome acidification and promote group a streptococcus survival in macrophages. mBio. **2014**; 5(5):e01690-14.

50. Park JM, Ng VH, Maeda S, Rest RF, Karin M. Anthrolysin O and Other Gram-positive Cytolysins Are Toll-like Receptor 4 Agonists. J Exp Med. **2004**; 200(12):1647–1655.

51. Timmer AM, Timmer JC, Pence MA, et al. Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. J Biol Chem. **2009**; 284(2):862–871.

52. Nilsson M, Sørensen OE, Mörgelin M, Weineisen M, Sjöbring U, Herwald H. Activation of human polymorphonuclear neutrophils by streptolysin O from Streptococcus pyogenes leads to the release of proinflammatory mediators. Thromb Haemost. **2006**; 95(6):982–990.

53. LaRock CN, Nizet V. Inflammasome/IL-1 β responses to streptococcal pathogens. Front Immunol. **2015**; :1–12.

54. Hancz D, Westerlund E, Bastiat-Sempe B, et al. Inhibition of Inflammasome-Dependent Interleukin 1β Production by Streptococcal NAD+-Glycohydrolase: Evidence for Extracellular Activity. mBio. **2017**; 8(4):e00756-17.

55. Hancz D, Westerlund E, Valfridsson C, et al. Streptolysin O Induces the Ubiquitination and Degradation of Pro-IL-1β. J Innate Immun. **2019**; :1–12.

56. Westerlund E, Valfridsson C, Yi DX, Persson JJ. The Secreted Virulence Factor NADase of Group A Streptococcus Inhibits P2X7 Receptor-Mediated Release of IL-1β. Front Immunol [Internet]. **2019** [cited 2019 Jul 21]; 10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6591467/

57. Nizet V, Beall B, Bast DJ, et al. Genetic locus for streptolysin S production by group A streptococcus. Infect Immun. **2000**; 68(7):4245–4254.

58. Ofek I, Bergner-Rabinowitz S, Ginsburg I. Oxygen-stable hemolysins of group A streptococci. VII. The relation of the leukotoxic factor to streptolysin S. J Infect Dis. **1970**; 122(6):517–522.

59. Sumitomo T, Nakata M, Higashino M, et al. Streptolysin S contributes to group A streptococcal translocation across an epithelial barrier. J Biol Chem. **2011**; 286(4):2750–2761.

60. Goldmann O, Sastalla I, Wos-Oxley M, Rohde M, Medina E. Streptococcus pyogenes induces oncosis in macrophages through the activation of an inflammatory programmed cell death pathway. Cell Microbiol. **2009**; 11(1):138–155.

61. Pinho-Ribeiro FA, Baddal B, Haarsma R, et al. Blocking Neuronal Signaling to Immune Cells Treats Streptococcal Invasive Infection. Cell. **2018**; 173(5):1083-1097.e22.

62. Smeesters P, McMillan DJ, Sriprakash KS. The streptococcal M protein: a highly versatile molecule. Trends Microbiol. **2010**; 18(6):275–282.

63. Ghosh P. Variation, Indispensability, and Masking in the M protein. Trends Microbiol. **2018**; 26(2):132–144.

64. Sanderson-Smith M, De Oliveira DMP, Guglielmini J, et al. A systematic and functional classification of Streptococcus pyogenes that serves as a new tool for molecular typing and vaccine development. J Infect Dis. **2014**; 210(8):1325–1338.

65. LaRock CN, Döhrmann S, Todd J, et al. Group A Streptococcal M1 Protein Sequesters Cathelicidin to Evade Innate Immune Killing. Cell Host Microbe. **2015**; 18(4):471–477.

66. Dohrmann S, LaRock CN, Anderson EL, et al. Group A Streptococcal M1 Protein Provides Resistance against the Antimicrobial Activity of Histones. Sci Rep. **2017**; 7:43039.

67. Berge A, Bjorck L. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. J Biol Chem [Internet]. **1995**; . Available from: http://www.jbc.org/content/270/17/9862.short

68. Valderrama JA, Riestra AM, Gao NJ, et al. Group A streptococcal M protein activates the NLRP3 inflammasome. Nat Microbiol. **2017**; 27(Pt 3):264.

69. Herwald H, Cramer H, Morgelin M, et al. M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. Cell [Internet]. **2004**; . Available from: http://www.sciencedirect.com/science/article/pii/S0092867404000571

70. Elliott SD. A PROTEOLYTIC ENZYME PRODUCED BY GROUP A STREPTOCOCCI WITH SPECIAL REFERENCE TO ITS EFFECT ON THE TYPE-SPECIFIC M ANTIGEN. J Exp Med. **1945**; 81(6):573–592.

71. Soehnlein O, Oehmcke S, Ma X, et al. Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. Eur Respir J. **2008**; 32(2):405–412.

72. Macheboeuf P, Buffalo C, Fu C, et al. Streptococcal M1 protein constructs a pathological host fibrinogen network. Nature. **2011**; 472(7341):64–68.

73. Hertzén E, Johansson L, Kansal R, et al. Intracellular Streptococcus pyogenes in human macrophages display an altered gene expression profile. PloS One. **2012**; 7(4):e35218.

74. Påhlman LI, Olin AI, Darenberg J, et al. Soluble M1 protein of Streptococcus pyogenes triggers potent T cell activation. Cell Microbiol. **2007**; 0(0):070928215112001-???

75. Palm F, Sjöholm K, Malmström J, Shannon O. Complement Activation Occurs at the Surface of Platelets Activated by Streptococcal M1 Protein and This Results in Phagocytosis of Platelets. J Immunol. **2019**; 202(2):503–513.

76. Shannon O, Hertzén E, Norrby-Teglund A, Mörgelin M, Sjöbring U, Björck L. Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. Mol Microbiol. **2007**; 65(5):1147–1157.

77. Horváth A, Olive C, Karpati L, Sun HK, Good M, Toth I. Toward the development of a synthetic group a streptococcal vaccine of high purity and broad protective coverage. J Med Chem. **2004**; 47(16):4100–4104.

78. Pahlman L, Morgelin M, Eckert J, et al. Streptococcal M protein: a multipotent and powerful inducer of inflammation. J Immunol. **2006**; 177(2):1221–1228.

79. Markovitz A, Cifonelli JA, Dorfman A. The biosynthesis of hyaluronic acid by group A Streptococcus. VI. Biosynthesis from uridine nucleotides in cell-free extracts. J Biol Chem. **1959**; 234:2343–2350.

80. Stoolmiller AC, Dorfman A. The biosynthesis of hyaluronic acid by Streptococcus. J Biol Chem. **1969**; 244(2):236–246.

81. Wessels MR, Goldberg JB, Moses AE, DiCesare TJ. Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci. Infect Immun. **1994**; 62(2):433–441.

82. Dale JB, Washburn RG, Marques MB, Wessels MR. Hyaluronate capsule and surface M protein in resistance to opsonization of group A streptococci. Infect Immun. **1996**; 64(5):1495–1501.

83. Foley MJ, Wood WB. STUDIES ON THE PATHOGENICITY OF GROUP A STREPTOCOCCI. J Exp Med. **1959**; 110(4):617–628.

84. Cywes C, Wessels MR. Group A Streptococcus tissue invasion by CD44-mediated cell signalling. Nature. **2001**; 414(6864):648–652.

85. Saito M, Kajiwara H, Ishikawa T, et al. Delayed Onset of Systemic Bacterial Dissemination and Subsequent Death in Mice Injected Intramuscularly with Streptococcus pyogenes Strains. Microbiol Immunol. **2001**; 45(11):777–786.

86. Brissac T, Martínez E, Kruckow KL, et al. Capsule Promotes Intracellular Survival and Vascular Endothelial Cell Translocation during Invasive Pneumococcal Disease. mBio. 12(5):e02516-21.

87. Levin JC, Wessels MR. Identification of csrR/csrS, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. Mol Microbiol. **1998**; 30(1):209–219.

88. Cywes C, Stamenkovic I, Wessels MR. CD44 as a receptor for colonization of the pharynx by group A Streptococcus. J Clin Invest. **2000**; 106(8):995–1002.

89. Ashbaugh CD, Moser TJ, Shearer MH, White GL, Kennedy RC, Wessels MR. Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. Cell Microbiol. **2000**; 2(4):283–292.

90. Schrager HM, Rheinwald JG, Wessels MR. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. J Clin Invest. **1996**; 98(9):1954–1958.

91. Wessels MR, Moses AE, Goldberg JB, DiCesare TJ. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc Natl Acad Sci U S A. **1991**; 88(19):8317–8321.

92. Flores AR, Jewell BE, Fittipaldi N, Beres SB, Musser JM. Human disease isolates of serotype m4 and m22 group a streptococcus lack genes required for hyaluronic acid capsule biosynthesis. mBio. **2012**; 3(6):e00413-00412.

93. DebRoy S, Sanson M, Shah B, et al. Population Genomics of emm4 Group A Streptococcus Reveals Progressive Replacement with a Hypervirulent Clone in North America. mSystems. **2021**; 6(4):e0049521.

94. Flores AR, Chase McNeil J, Shah B, Van Beneden C, Shelburne SA. Capsule-Negative emm Types Are an Increasing Cause of Pediatric Group A Streptococcal Infections at a Large Pediatric Hospital in Texas. J Pediatr Infect Dis Soc. **2019**; 8(3):244–250.

95. Zhu L, Olsen RJ, Nasser W, Riva Morales I de la, Musser JM. Trading Capsule for Increased Cytotoxin Production: Contribution to Virulence of a Newly Emerged Clade of emm89 Streptococcus pyogenes. mBio. **2015**; 6(5):e01378-01315.

96. Turner CE, Abbott J, Lamagni T, et al. Emergence of a New Highly Successful Acapsular Group A Streptococcus Clade of Genotype emm89 in the United Kingdom. mBio. **2015**; 6(4):e00622.

97. Turner CE, Holden MTG, Blane B, Horner C, Peacock SJ, Sriskandan S. The Emergence of Successful Streptococcus pyogenes Lineages through Convergent Pathways of Capsule Loss and Recombination Directing High Toxin Expression. mBio. **2019**; 10(6):e02521-19.

98. Whitehead BD, Smith HV, Nourse C. Invasive group A streptococcal disease in children in Queensland. Epidemiol Infect. **2011**; 139(4):623–628.

99. Wessels MR. Capsular polysaccharide of group A Streptococcus. Microbiol Spectr. **2019**; 7(1):10.1128/microbiolspec.GPP3-0050–2018.

100. Henningham A, Yamaguchi M, Aziz RK, et al. Mutual Exclusivity of Hyaluronan and Hyaluronidase in Invasive Group A Streptococcus. J Biol Chem. **2014**; 289(46):32303–32315.

101. Metzgar D, Zampolli A. The M protein of group A Streptococcus is a key virulence factor and a clinically relevant strain identification marker. Virulence. **2011**; 2(5):402–412.

102. Barnett TC, Bowen AC, Carapetis JR. The fall and rise of Group A Streptococcus diseases. Epidemiol Infect. **2018**; 147:e4.

103. Shea PR, Ewbank AL, Gonzalez-Lugo JH, et al. Group A Streptococcus emm Gene Types in Pharyngeal Isolates, Ontario, Canada, 2002–2010. Emerg Infect Dis. **2011**; 17(11):2010–2017.

104. Olsen RJ, Musser JM. Molecular pathogenesis of necrotizing fasciitis. Annu Rev Pathol. **2010**; 5:1–31.

105. Wessels MR. Clinical practice. Streptococcal pharyngitis. N Engl J Med. **2011**; 364(7):648–655.

106. Hamilton SM, Stevens DL, Bryant AE. Pregnancy-Related Group A Streptococcal Infections: Temporal Relationships Between Bacterial Acquisition, Infection Onset, Clinical Findings, and Outcome. Clin Infect Dis Off Publ Infect Dis Soc Am. **2013**; 57(6):870–876.

107. Cleary PP, Kaplan EL, Handley JP, et al. Clonal basis for resurgence of serious Streptococcus pyogenes disease in the 1980s. Lancet Lond Engl. **1992**; 339(8792):518–521.

108. Aziz RK, Kotb M. Rise and Persistence of Global M1T1 Clone of Streptococcus pyogenes. Emerg Infect Dis. **2008**; 14(10):1511–1517.

109. Nasser W, Beres SB, Olsen RJ, et al. Evolutionary pathway to increased virulence and epidemic group A Streptococcus disease derived from 3,615 genome sequences. Proc Natl Acad Sci U S A. **2014**; 111(17):E1768–E1776.

110. Zhu L, Olsen RJ, Nasser W, et al. A molecular trigger for intercontinental epidemics of group A Streptococcus. J Clin Invest. **2015**; 125(9):3545–3559.

111. Brouwer S, Barnett TC, Ly D, et al. Prophage exotoxins enhance colonization fitness in epidemic scarlet fever-causing Streptococcus pyogenes. Nat Commun. **2020**; 11(1):5018.

112. Sarkar P, Sumby P. Regulatory gene mutation: a driving force behind group A Streptococcus strain- and serotype-specific variation. Mol Microbiol. **2017**; 103(4):576–589.

113. Treviño J, Perez N, Ramirez-Peña E, et al. CovS Simultaneously Activates and Inhibits the CovR-Mediated Repression of Distinct Subsets of Group A Streptococcus Virulence

Factor-Encoding Genes. Infect Immun. American Society for Microbiology; **2009**; 77(8):3141–3149.

114. Graham MR, Smoot LM, Migliaccio CAL, et al. Virulence control in group A Streptococcus by a two-component gene regulatory system: Global expression profiling and in vivo infection modeling. Proc Natl Acad Sci U S A. **2002**; 99(21):13855–13860.

115. Chochua S, Metcalf BJ, Li Z, et al. Population and Whole Genome Sequence Based Characterization of Invasive Group A Streptococci Recovered in the United States during 2015. mBio. **2017**; 8(5):e01422-17.

116. Kim S, Lee S, Park H, Kim S. Predominance of emm4 and antibiotic resistance of Streptococcus pyogenes in acute pharyngitis in a southern region of Korea. J Med Microbiol. **2019**; 68(7):1053–1058.

117. DebRoy S, Li X, Kalia A, et al. Identification of a chimeric emm gene and novel emm pattern in currently circulating strains of emm4 Group A Streptococcus. Microb Genomics. **2018**; 4(11):e000235.

118. Neumann A, Happonen L, Karlsson C, Bahnan W, Frick I-M, Björck L. Streptococcal protein SIC activates monocytes and induces inflammation. iScience. **2021**; 24(4):102339.

119. Imai T, Matsumura T, Mayer-Lambertz S, et al. Lipoteichoic acid anchor triggers Mincle to drive protective immunity against invasive group A Streptococcus infection. Proc Natl Acad Sci. **2018**; 18:201809100.

120. Loof TG, Goldmann O, Medina E. Immune recognition of Streptococcus pyogenes by dendritic cells. Infect Immun. **2008**; 76(6):2785–2792.

121. Joosten LAB, Koenders MI, Smeets RL, et al. Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J Immunol Baltim Md 1950. **2003**; 171(11):6145–6153.

122. Fieber C, Janos M, Koestler T, et al. Innate Immune Response to Streptococcus pyogenes Depends on the Combined Activation of TLR13 and TLR2. PLoS ONE. **2015**; 10(3):e0119727.

123. Bhattacharjee P, Keyel PA. Cholesterol-dependent cytolysins impair pro-inflammatory macrophage responses. Sci Rep. **2018**; 8:6458.

124. Park JM, Ng VH, Maeda S, Rest RF, Karin M. Anthrolysin O and Other Gram-positive Cytolysins Are Toll-like Receptor 4 Agonists. J Exp Med. **2004**; 200(12):1647–1655.

125. Uchiyama S, Andreoni F, Schuepbach RA, Nizet V, Zinkernagel AS. DNase Sda1 Allows Invasive M1T1 Group A Streptococcus to Prevent TLR9-Dependent Recognition. PLoS Pathog. **2012**; 8(6):e1002736.

126. Zinkernagel AS, Hruz P, Uchiyama S, et al. Importance of Toll-Like Receptor 9 in Host Defense against M1T1 Group A Streptococcus Infections. J Innate Immun. **2012**; 4(2):213–218.

127. Chiappini N, Seubert A, Telford JL, et al. Streptococcus pyogenes SpyCEP Influences Host-Pathogen Interactions during Infection in a Murine Air Pouch Model. PLoS ONE. **2012**; 7(7):e40411.

128. Lacy P. Mechanisms of Degranulation in Neutrophils. Allergy Asthma Clin Immunol Off J Can Soc Allergy Clin Immunol. **2006**; 2(3):98–108.

129. Wang J. Neutrophils in tissue injury and repair. Cell Tissue Res. **2018**; 371(3):531–539.

130. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. Nature Publishing Group; **2018**; 18(2):134–147.

131. Walker MJ, Hollands A, Sanderson-Smith ML, et al. DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med. **2007**; 13(8):981–985.

132. Wong JH, Ye XJ, Ng TB. Cathelicidins: peptides with antimicrobial, immunomodulatory, anti-inflammatory, angiogenic, anticancer and procancer activities. Curr Protein Pept Sci. **2013**; 14(6):504–514.

133. Goldmann O, Köckritz-Blickwede M von, Höltje C, Chhatwal GS, Geffers R, Medina E. Transcriptome Analysis of Murine Macrophages in Response to Infection with Streptococcus pyogenes Reveals an Unusual Activation Program. Infect Immun. **2007**; 75(8):4148–4157.

134. LaRock CN, Nizet V. Inflammasome/IL-1β Responses to Streptococcal Pathogens. Front Immunol [Internet]. **2015** [cited 2021 Jun 7]; 6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4597127/

135. Rose-John S, Winthrop K, Calabrese L. The role of IL-6 in host defence against infections: immunobiology and clinical implications. Nat Rev Rheumatol. **2017**; 13(7):399–409.

136. Rose-John S. IL-6 Trans-Signaling via the Soluble IL-6 Receptor: Importance for the Pro-Inflammatory Activities of IL-6. Int J Biol Sci. **2012**; 8(9):1237–1247.

137. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta BBA - Mol Cell Res. **2011**; 1813(5):878–888.

138. Herb M, Schramm M. Functions of ROS in Macrophages and Antimicrobial Immunity. Antioxidants [Internet]. **2021** [cited 2021 May 14]; 10(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7923022/

139. Winterbourn CC, Kettle AJ, Hampton MB. Reactive Oxygen Species and Neutrophil Function. Annu Rev Biochem. **2016**; 85:765–792.

140. Sen A, Imlay JA. How Microbes Defend Themselves From Incoming Hydrogen Peroxide. Front Immunol [Internet]. Frontiers; **2021** [cited 2021 May 22]; 12. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2021.667343/full

141. Winkelstein JA, Marino MC, Johnston RB, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). **2000**; 79(3):155–169.

142. Berg JM van den, Koppen E van, Åhlin A, et al. Chronic Granulomatous Disease: The European Experience. PLoS ONE. **2009**; 4(4):e5234.

143. Gallin JI, Alling DW, Malech HL, et al. Itraconazole to Prevent Fungal Infections in Chronic Granulomatous Disease. N Engl J Med. Massachusetts Medical Society; **2003**; 348(24):2416–2422.

144. Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radic Biol Med. **1996**; 21(3):335–348.

145. Jones SA, Wolf M, Qin S, Mackay CR, Baggiolini M. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. Proc Natl Acad Sci U S A. **1996**; 93(13):6682–6686.

146. Coxon A, Rieu P, Barkalow FJ, et al. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. Immunity. **1996**; 5(6):653–666.

147. Fadeel B, Kagan VE. Apoptosis and macrophage clearance of neutrophils: regulation by reactive oxygen species. Redox Rep Commun Free Radic Res. **2003**; 8(3):143–150.

148. Broderick L, Hoffman HM. IL-1 and autoinflammatory disease: biology, pathogenesis and therapeutic targeting. Nat Rev Rheumatol. **2022**; .

149. Broderick L, Tourangeau LM, Kavanaugh A, Wasserman SI. Biologic Modulators in Allergic and Autoinflammatory Diseases. Curr Opin Allergy Clin Immunol. **2011**; 11(4):355–360.

150. Hoffman HM, Broderick L. JAK inhibitors in autoinflammation. J Clin Invest. 128(7):2760–2762.

151. Winter SE, Thiennimitr P, Winter MG, et al. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature. Nature Publishing Group; **2010**; 467(7314):426–429.

152. Arpaia N, Godec J, Lau L, et al. TLR Signaling Is Required for Salmonella typhimurium Virulence. Cell. Elsevier; **2011**; 144(5):675–688.

153. Kusters JG, Vliet AHM van, Kuipers EJ. Pathogenesis of Helicobacter pylori Infection. Clin Microbiol Rev. American Society for Microbiology Journals; **2006**; 19(3):449–490.

154. Palomo J, Marchiol T, Piotet J, et al. Role of IL-1β in Experimental Cystic Fibrosis upon P. aeruginosa Infection. PLoS ONE. **2014**; 9(12):e114884.

155. Sun J, LaRock DL, Skowronski EA, et al. The Pseudomonas aeruginosa protease LasB directly activates IL-1β. EBioMedicine [Internet]. Elsevier; **2020** [cited 2020 Sep 23]; 60. Available from: https://www.thelancet.com/journals/ebiom/article/PIIS2352-3964(20)30360-1/abstract

156. Jawhara S, Thuru X, Standaert-Vitse A, et al. Colonization of mice by Candida albicans is promoted by chemically induced colitis and augments inflammatory responses through galectin-3. J Infect Dis. **2008**; 197(7):972–980.

157. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nat Rev Microbiol. **2018**; 16(6):355–367.

158. Brocke S, Gaur A, Piercy C, et al. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. Nature. Nature Publishing Group; **1993**; 365(6447):642–644.

159. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature. Nature Publishing Group; **1992**; 358(6382):155–157.

160. Cunningham MW. Molecular Mimicry, Autoimmunity and Infection: The Cross-reactive Antigens of Group A Streptococci and their Sequelae. Microbiol Spectr [Internet]. **2019** [cited 2020 Jun 30]; 7(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6684244/

161. Joshi A, Shrestha RPB, Shrestha PS, et al. Sydenham's Chorea as Presentation of Rheumatic Heart Disease. Kathmandu Univ Med J KUMJ. **2015**; 13(51):271–273.

162. Cohen J, Vincent J-L, Adhikari NKJ, et al. Sepsis: a roadmap for future research. Lancet Infect Dis. **2015**; 15(5):581–614.

163. Keller N, Andreoni F, Reiber C, et al. Human Streptococcal Necrotizing Fasciitis Histopathology Mirrored in a Murine Model. Am J Pathol. **2018**; 188(7):1517–1523.

164. Thulin P, Johansson L, Low DE, et al. Viable group A streptococci in macrophages during acute soft tissue infection. PLoS Med. **2006**; 3(3):e53.

165. Wilde S, Olivares KL, Nizet V, Hoffman HM, Radhakrishna S, LaRock CN. Opportunistic Invasive Infection by Group A Streptococcus During Anti–Interleukin-6 Immunotherapy. J Infect Dis. **2020**; 223(7):1260–1264.

166. Hamilton SM, Bayer CR, Stevens DL, Bryant AE. Effects of Selective and Nonselective Nonsteroidal Anti-inflammatory Drugs on Antibiotic Efficacy of Experimental Group A Streptococcal Myonecrosis. J Infect Dis. **2014**; 209(9):1429–1435.

167. Dale JB, Batzloff MR, Cleary PP, et al. Current Approaches to Group A Streptococcal Vaccine Development. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus Pyogenes Basic Biol Clin Manif [Internet]. Oklahoma City (OK): University of Oklahoma Health Sciences Center; 2016 [cited 2020 Sep 15]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK333413/

168. Wilkins AL, Steer AC, Smeesters PR, Curtis N. Toxic shock syndrome – the seven Rs of management and treatment. J Infect. **2017**; 74:S147–S152.

169. Vannice KS, Ricaldi J, Nanduri S, et al. Streptococcus pyogenes pbp2x Mutation Confers Reduced Susceptibility to β-Lactam Antibiotics. Clin Infect Dis Off Publ Infect Dis Soc Am. **2020**; 71(1):201–204.

170. Steer AC, Lamagni T, Curtis N, Carapetis JR. Invasive group a streptococcal disease: epidemiology, pathogenesis and management. Drugs. **2012**; 72(9):1213–1227.

171. Sriskandan S, McKee A, Hall L, Cohen J. Comparative effects of clindamycin and ampicillin on superantigenic activity of Streptococcus pyogenes. J Antimicrob Chemother. **1997**; 40(2):275–277.

172. Mascini EM, Jansze M, Schouls LM, Verhoef J, Van Dijk H. Penicillin and clindamycin differentially inhibit the production of pyrogenic exotoxins A and B by group A streptococci. Int J Antimicrob Agents. **2001**; 18(4):395–398.

173. Michos A, Koutouzi FI, Tsakris A, et al. Molecular analysis of Streptococcus pyogenes macrolide resistance of paediatric isolates during a 7 year period (2007-13). J Antimicrob Chemother. **2016**; 71(8):2113–2117.

174. Silva-Costa C, Friães A, Ramirez M, Melo-Cristino J. Macrolide-resistant Streptococcus pyogenes: prevalence and treatment strategies. Expert Rev Anti Infect Ther. **2015**; 13(5):615–628.

175. Fyfe C, Grossman TH, Kerstein K, Sutcliffe J. Resistance to Macrolide Antibiotics in Public Health Pathogens. Cold Spring Harb Perspect Med. **2016**; 6(10).

176. Bryant AE, Bayer CR, Aldape MJ, McIndoo E, Stevens DL. Emerging erythromycin and clindamycin resistance in group A streptococcus: Efficacy of linezolid and tedizolid in experimental necrotizing infection. J Glob Antimicrob Resist. **2020**; .

177. Kim KS, Kaplan EL. Association of penicillin tolerance with failure to eradicate group A streptococci from patients with pharyngitis. J Pediatr. **1985**; 107(5):681–684.

178. Fischetti VA, Dale JB. One More Disguise in the Stealth Behavior of Streptococcus pyogenes. mBio. **2016**; 7(3).

179. Hertzén E, Johansson L, Wallin R, et al. M1 protein-dependent intracellular trafficking promotes persistence and replication of Streptococcus pyogenes in macrophages. J Innate Immun. **2010**; 2(6):534–545.

180. Spinaci C, Magi G, Varaldo PE, Facinelli B. Persistence of erythromycin-resistant group a streptococci in cultured respiratory cells. Pediatr Infect Dis J. **2006**; 25(10):880–883.

181. Low DE. Toxic shock syndrome: major advances in pathogenesis, but not treatment. Crit Care Clin. **2013**; 29(3):651–675.

182. Norrby-Teglund A, Muller MP, Mcgeer A, et al. Successful management of severe group A streptococcal soft tissue infections using an aggressive medical regimen including intravenous polyspecific immunoglobulin together with a conservative surgical approach. Scand J Infect Dis. **2005**; 37(3):166–172.

183. Sriskandan S, Ferguson M, Elliot V, Faulkner L, Cohen J. Human intravenous immunoglobulin for experimental streptococcal toxic shock: bacterial clearance and modulation of inflammation. J Antimicrob Chemother. **2006**; 58(1):117–124.

184. Linnér A, Darenberg J, Sjölin J, Henriques-Normark B, Norrby-Teglund A. Clinical efficacy of polyspecific intravenous immunoglobulin therapy in patients with streptococcal toxic shock syndrome: a comparative observational study. Clin Infect Dis Off Publ Infect Dis Soc Am. **2014**; 59(6):851–857.

185. Shah SS, Hall M, Srivastava R, Subramony A, Levin JE. Intravenous immunoglobulin in children with streptococcal toxic shock syndrome. Clin Infect Dis Off Publ Infect Dis Soc Am. **2009**; 49(9):1369–1376.

186. Williams KA, Swedo SE, Farmer CA, et al. Randomized, Controlled Trial of Intravenous Immunoglobulin for Pediatric Autoimmune Neuropsychiatric Disorders Associated With Streptococcal Infections. J Am Acad Child Adolesc Psychiatry. **2016**; 55(10):860-867.e2.

187. Maxson T, Deane CD, Molloy EM, et al. HIV protease inhibitors block streptolysin S production. ACS Chem Biol. **2015**; 10(5):1217–1226.

188. Li M-H, Luo Y-H, Lin C-F, et al. Dextromethorphan efficiently increases bactericidal activity, attenuates inflammatory responses, and prevents group a streptococcal sepsis. Antimicrob Agents Chemother. **2011**; 55(3):967–973.

189. Chen C-L, Cheng M-H, Kuo C-F, et al. Dextromethorphan Attenuates NADPH Oxidase-Regulated Glycogen Synthase Kinase 3β and NF-κB Activation and Reduces Nitric Oxide Production in Group A Streptococcal Infection. Antimicrob Agents Chemother [Internet]. **2018** [cited 2020 Jul 3]; 62(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5971618/

190. Zhou R, Chen S-H, Li G, et al. Ultralow Doses of Dextromethorphan Protect Mice from Endotoxin-induced Sepsis-like Hepatotoxicity. Chem Biol Interact. **2019**; 303:50–56.

191. Herbert S, Barry P, Novick RP. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in Staphylococcus aureus. Infect Immun. **2001**; 69(5):2996–3003.

192. Goscinski G, Tano E, Thulin P, Norrby-Teglund A, Sjölin J. Release of SpeA from Streptococcus pyogenes after exposure to penicillin: dependency on dose and inhibition by clindamycin. Scand J Infect Dis. **2006**; 38(11–12):983–987.

193. Andreoni F, Zürcher C, Tarnutzer A, et al. Clindamycin Affects Group A Streptococcus Virulence Factors and Improves Clinical Outcome. J Infect Dis. **2017**; 215(2):269–277.

194. Le Breton Y, Belew AT, Valdes KM, et al. Essential Genes in the Core Genome of the Human Pathogen Streptococcus pyogenes. Sci Rep [Internet]. **2015** [cited 2020 Mar 27]; 5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4440532/

195. Zhu L, Olsen RJ, Beres SB, et al. Gene fitness landscape of group A streptococcus during necrotizing myositis. J Clin Invest. **2019**; 129(2):887–901.

196. Turner CE, Sommerlad M, McGregor K, et al. Superantigenic Activity of emm3 Streptococcus pyogenes Is Abrogated by a Conserved, Naturally Occurring smeZ Mutation. PLoS ONE [Internet]. **2012** [cited 2020 Sep 12]; 7(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3462185/

197. Ji Y, Carlson B, Kondagunta A, Cleary PP. Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization of mice by the group A Streptococcus. Infect Immun. **1997**; 65(6):2080–2087.

198. Sun H, Ringdahl U, Homeister J, et al. Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. Science [Internet]. **2004**; . Available from: http://www.sciencemag.org/content/305/5688/1283.short

199. Zinkernagel AS, Timmer AM, Pence MA, et al. The IL-8 Protease SpyCEP/ScpC of Group A \emphStreptococcus Promotes Resistance to Neutrophil Killing. Cell Host Microbe [Internet]. **2008**; . Available from: http://www.sciencedirect.com/science/article/pii/S1931312808002199

200. Okumura CYM, Anderson EL, Döhrmann S, et al. IgG Protease Mac/IdeS Is Not Essential for Phagocyte Resistance or Mouse Virulence of M1T1 Group A Streptococcus. mBio [Internet]. **2013** [cited 2020 Sep 13]; 4(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3735186/

201. Tanaka M, Kinoshita-Daitoku R, Kiga K, et al. Group A Streptococcus establishes pharynx infection by degrading the deoxyribonucleic acid of neutrophil extracellular traps. Sci Rep [Internet]. **2020** [cited 2020 Sep 12]; 10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7039874/

202. Soh KY, Loh JMS, Proft T. Cell wall-anchored 5'-nucleotidases in Gram-positive cocci. Mol Microbiol. **2020**; 113(4):691–698.

203. Betschel SD, Borgia SM, Barg NL, Low DE, De Azavedo JCS. Reduced Virulence of Group A Streptococcal Tn916 Mutants That Do Not Produce Streptolysin S. Infect Immun. **1998**; 66(4):1671–1679.

204. Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR. Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis. **2009**; 9(10):611–616.

205. Chen Y-H, Li S-H, Yang Y-C, Hsu S-H, Nizet V, Chang Y-C. T4 Pili Promote Colonization and Immune Evasion Phenotypes of Nonencapsulated M4 Streptococcus pyogenes. mBio [Internet]. **2020** [cited 2020 Sep 12]; 11(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7374061/

206. Wierzbicki IH, Campeau A, Dehaini D, et al. Group A Streptococcal S Protein Utilizes Red Blood Cells as Immune Camouflage and Is a Critical Determinant for Immune Evasion. Cell Rep. **2019**; 29(10):2979-2989.e15.

207. Frick I-M, Åkesson P, Rasmussen M, Schmidtchen A, Björck L. SIC, a Secreted Protein of Streptococcus pyogenesThat Inactivates Antibacterial Peptides. J Biol Chem. American Society for Biochemistry and Molecular Biology; **2003**; 278(19):16561–16566.

208. Lukomski S, Hoe NP, Abdi I, et al. Nonpolar Inactivation of the Hypervariable Streptococcal Inhibitor of Complement Gene (sic) in Serotype M1 Streptococcus pyogenes Significantly Decreases Mouse Mucosal Colonization. Infect Immun. **2000**; 68(2):535–542.

209. Sjögren J, Okumura CY, Collin M, Nizet V, Hollands A. Study of the IgG endoglycosidase EndoS in group A streptococcal phagocyte resistance and virulence. BMC Microbiol. **2011**; 11:120.

210. Hoff JS, DeWald M, Moseley SL, Collins CM, Voyich JM. SpyA, a C3-Like ADP-Ribosyltransferase, Contributes to Virulence in a Mouse Subcutaneous Model of Streptococcus pyogenes Infection ▿. Infect Immun. **2011**; 79(6):2404–2411.

211. Ricci S, Janulczyk R, Björck L. The Regulator PerR Is Involved in Oxidative Stress Response and Iron Homeostasis and Is Necessary for Full Virulence of Streptococcus pyogenes. Infect Immun. **2002**; 70(9):4968–4976.

212. Janulczyk R, Ricci S, Björck L. MtsABC Is Important for Manganese and Iron Transport, Oxidative Stress Resistance, and Virulence of Streptococcus pyogenes. Infect Immun. **2003**; 71(5):2656–2664.

Name	Function	Target(s)	*In vivo* requirement?	
			Invasive	Intranasal
Streptococcal pyrogenic exotoxin (Spe A, C, G, H, I, J, K, L, M, Q, R); SmeZ; SSA	Superantigen (binding activity)	TCR, MHC, potentially co-receptors	N	Y [43,196]
Streptococcal pyrogenic exotoxin B (SpeB)	Protease	IL-1β, 100+ other host & GAS proteins	N [12]	Y [11]
ScpA	Protease	C5a	N [1]	Y [197]
Streptokinase (Ska)	Protease	Plasminogen activator	Y [198]	?
SpyCEP/ScpC	Protease	IL-8	Y [199]	?
IdeS/Mac	Mimicry, Protease	FcγRIIIB; IgG opsonophagocytis	N [200]	?
Sda1	DNase	NETS	Y [13]	Y [201]
Nga	Glycohydrolase	NAD^+, unknown other	N [32]	?
Streptococcal 5′-nucleotidase A (S5nA)	Hydrolase	Nucleosides (AMP, ADP, ATP)	Y [202]	?
Streptolysin O (SLO)	Pore-forming toxin	Epithelial & leukocyte membranes	N (Steer et al., 2009) Y (Timmer et al., 2009)	?
Streptolysin S (SLS)	Pore-forming toxin	Erythrocyte membranes	N (Hall et al., 2004) Y (Betschel et al., 1998)	?
M protein	Adhesion, sequestration	LL-37, histones, albumin, plasminogen, fibrinogen, CD46, Ig, C3b, factor H, C4b-BP	N [32] Y [15]	Y (Anderson et al., 2014)
T antigen	Adherence	Fibronectin, collagen	Y [205]	?
Protein S	Sequestration	Erythrocyte fragments	Y [206]	?
SIC	Sequestration	Lysozyme, kininogen, defensins, LL-37, C5b	Y [207]	Y (Lukomski et al., 2000)

EndoS	Endo-β-N-acetylglucosaminidase	IgG	N (Sjögren et al., 2011)	?
SpyA	ADP ribosyltransferase	Actin, vimentin, others?	Y (Hoff et al., 2011)	?
SodA	Superoxide dismutase	Reactive oxygen species	Y (Ricci et al., 2002; Janulczyk et al., 2003)	?
HasABC	Hyaluronic acid capsule	Antimicrobials, CD44	N (Henningham et al., 2014)	Y [88]

Table 1. Predominant GAS virulence factors, their known mechanisms and targets, and their essentiality in models of invasive (intradermal, subcutaneous, or intravenous) or nasopharyngeal (intranasal) infection. Abbreviations as follows: TCR, T cell receptor; MHC, Major histocompatibility complex; NET; neutrophilic extracellular trap; IL, interleukin; FcγRIIIB, Fcγ receptor type III, also referred to as CD16.

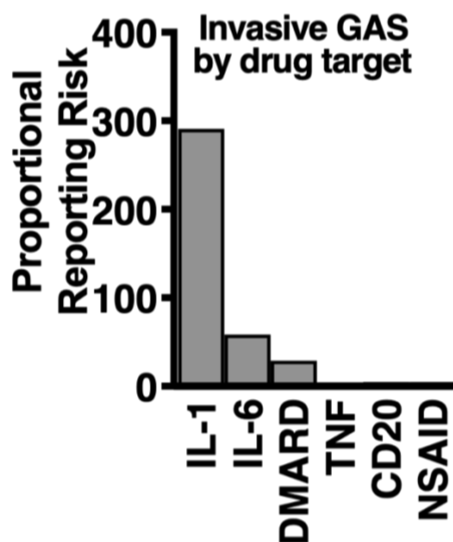

Figure 1. Invasive GAS infections by drug target. Post-marketing surveillance of immunosuppressive drugs reveals a disproportionate frequency of invasive GAS infections associated with IL-1 and IL-6-inhibiting biologics. Risk is expressed as the proportional reporting risk (PRR), the ratio to which a specific adverse event is reported for a specific drug, compared to the frequency of the same adverse event being reported for other drugs (or classes of drugs). Figure is adapted from LaRock et al., *Sci Immunol* (2016).

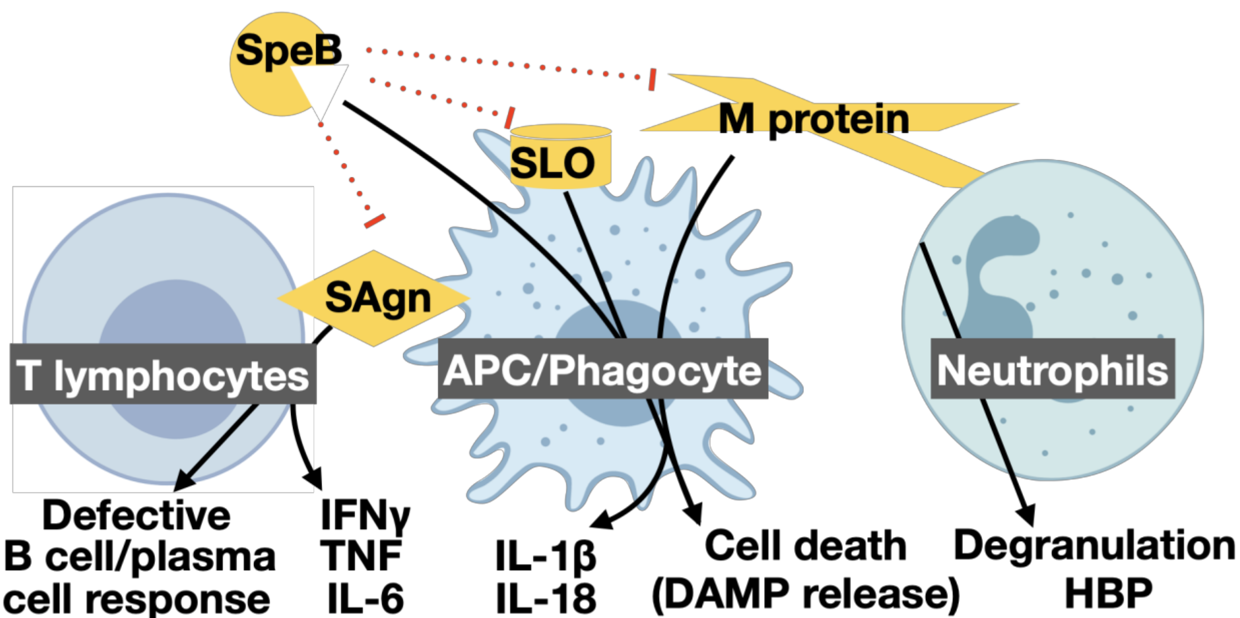

Figure 2. Proinflammatory virulence mechanisms of GAS and their targets. The GAS protease SpeB is directly proinflammatory by activating pro-IL-1β, other host substrates, and inactivating anti-inflammatory GAS effectors. SpeB cleavage of other proinflammatory cytokines, and proinflammatory virulence factors such as superantigens (SAgn), streptolysin O (SLO), and M protein can lead to their inactivation and have anti-inflammatory contributions. Superantigens forcibly bind T lymphocytes and APCs, leading to excessive T cell activation. Activated T cells kill other immune cells and release a "cytokine storm" of IFNγ, TNF, and IL-6, hallmark of STSS. The pore-forming toxins SLO and streptolysin S (SLS) form large pores in host cells that can lead to the passive release DAMPs, and other cytosolic or organelle-associated proinflammatory compounds, or be detected by the inflammasome to further activate inflammatory cell death by pyroptosis. Proinflammatory effects of SLO can include aiding translocation of the virulence factors Nga. M protein proteolytically released from the GAS surface can similarly form complexes that induce pyroptosis in macrophages or hyper-degranulation by neutrophils. Like other microbes, GAS has numerous TLR agonists that activate proinflammatory regulatory programs (reviewed in [53]).

Chapter 2

Opportunistic Invasive Infection by Group A *Streptococcus* During Anti-Interleukin-6 Immunotherapy

Shyra Wilde[1], Katherine L. Olivares[1,4], Victor Nizet[5,6], Hal M. Hoffman[,6], Suhas Radhakrishna[6] & Christopher N. LaRock[1,2,3]

Published in *Journal of Infectious Diseases* 2021 Aug, doi: 10.1093/infdis/jiaa511

[1]Department of Microbiology and Immunology, [2]Division of Infectious Diseases, Department of Medicine, [3]Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30322, USA
[4]Wayne State University, Detroit, MI, 48202, USA
[5]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093, USA
[6]Rady Children's Hospital, San Diego, CA 92123, USA and Department of Pediatrics, University of California San Diego, CA 92093

ABSTRACT

Invasive group A *Streptococcus* (GAS) in immunocompetent individuals is largely linked to hypervirulent strains. Congenital immunodeficiencies and those acquired from chronic disease or immunosuppressant drugs also increase risk of severe illness. We recovered GAS from the blood of a patient receiving a biologic inhibitor of IL-6. Growth of this serotype M4 isolate in human blood or a murine bacteremia model was promoted by IL-1 or IL-6 inhibition. Hyperinvasive M1T1 GAS was unaffected by IL-6 in both models. These findings based on a natural experiment introduce IL-6 signaling deficiencies as a risk factor for invasive GAS.

BACKGROUND

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a top cause of infectious mortality responsible for over 500,000 annual deaths worldwide (reviewed in (1)). Necrotizing fasciitis, toxic shock syndrome, and other invasive GAS (iGAS) infections have been associated with a globally-disseminated clone of serotype M1T1 that emerged in the early 1980s upon acquiring additional virulence determinants that antagonize immune signaling and innate antimicrobial defenses (2). Host factors contributing to iGAS are largely unknown, but infections have been associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (3) and Anakinra (recombinant human interleukin-1 receptor antagonist, IL-1Ra) (4). These infectious adverse events suggest that specific pro-inflammatory signals are needed to coordinate an effective immune response to iGAS, leaving the possibility that other immunodeficiencies may increase the risk of infection.

The pro-inflammatory cytokine interleukin-6 (IL-6) is a key component of the acute phase response (reviewed in (5)). Elevated IL-6 contributes to inflammatory pathology in numerous chronic disorders including rheumatoid arthritis, Castleman's disease, and juvenile idiopathic arthritis (5). Inhibition of IL-6 signaling can be therapeutic in these diseases and may be accomplished with antibodies that block the IL-6 receptor (Tocilizumab) or IL-6 itself (Siltuximab). Primary adverse events observed during clinical trials were infections (6–9), and several case reports have since described necrotizing fasciitis caused by GAS in individuals taking IL-6 signaling inhibitors for the treatment of rheumatoid arthritis (10, 11). Anti-cytokine drugs are often associated with infectious adverse events, e.g. the association of *Mycobacterium tuberculosis*

with TNF inhibitors (4), but the extent to which any particular pathogen is associated with any targeted pathway is uncertain.

This study examines an M4 iGAS isolate from a pediatric patient with systemic juvenile idiopathic arthritis who developed sepsis while under treatment with inhibitors of IL-1 and IL-6. The patient began immunotherapy with biologic IL-1 inhibitors. When IL-1 therapy failed, the patient was switched to IL-6 inhibitors, after which they developed sepsis. We show that anti-IL-6 immunotherapeutics promote iGAS, as previously observed with IL-1 inhibition. However, this effect is not extrapolated to hyperinvasive strains, which are already resistant to the effects of IL-6. Infectious adverse events are common with broad action immunosuppressants, but highly targeted pharmaceuticals like biologics may have a more limited risk profile. Our results find these risks depend on the immune pathway targeted and intrinsic differences between bacterial strains.

METHODS

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 UCSD or Emory University.

Bacterial Characterization and Culture. Blood culture from the patient yielded colonies with β-hemolytic activity on 5% blood agar and confirmed to express the group A antigen by Streptex* Rapid Latex Agglutination Test (Remel Inc., Lenexa, KS). Genomic DNA was isolated with

DNAzol (MRC, Inc) and the *emm*-type identified by PCR amplification with primers targeting the *emm* hypervariable region (TATT(C/G)GCTTAGAAAATTAA and GCAAGTTCTTCAGCTTGTTT) and Sanger sequenced. The sequence was submitted to the CDC Streptococci Group A Subtyping Blast Server and found to be a 100% match to the M4.0 serogroup. The strain was named M4(C20). Consistent with other M4 serotype GAS (12), M4(C20) produced no capsule when examined by hyaluronic acid enzyme-linked immunosorbent assay (Corgenix). All colonies cultured had the ability to hydrolyze azocasiene, indicating SpeB was functional and the *covRS* operon was not inactivated, as has been describe for some isolates from invasive infections (4). Bacteria were routinely grown in Todd-Hewitt broth with 5% yeast extract (Difco Laboratories) statically at 37°C and 5% CO_2.

Animal infection model. 8 to 10-week-old C57BL/6 (Jackson labs) mice of both sexes were treated intravenously 4 h pre-infection with Anakinra (Kineret; 50 mg/kg; inhibits both human and murine IL-1R1(4)), or a monoclonal antibody targeting murine IL-6R (BioXcell; 50 mg/kg; Tocilizumab does not inhibit murine IL-6R), or IgG isotype control (BioXcell; 50 mg/kg). IL6$^{-/-}$ mice (M. Karin) were not pre-treated with any monoclonal antibodies. All mice were inoculated intravenously with 10^8 CFU of either GAS strain 5448 or M4(C20). Mice were monitored for 5 days post-infection.

***Ex vivo* infection models.** Heparinized whole human blood treated with Anakinra (Kineret; 50 µg/ml), Tocilizumab (Actemra; 50 µg/ml), or IgG isotype control (BioXcell; 50 µg/ml) was inoculated with 10^7 colony forming units (CFU) of clinical isolate M4(C20). Bacterial growth was monitored by dilution plating 3 h post-infection. THP-1 macrophages were infected as detailed

previously (4). Briefly, THP-1 cells (ATCC) were differentiated with 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72 h, then media exchanged with RPMI 1640 (Gibco) supplemented with 10% pooled human serum and no antibiotics 1 h prior to infection. Recombinant human IL-6 (Invivogen) was added at this time in experiments featuring its use. Subcultured GAS grown to OD_{600} ~0.4 (log-phase) were washed and resuspended in PBS and diluted for a multiplicity of infection of 4. Plates were centrifuged 3 min at 160 x g to promote bacteria-macrophage contact. 100 µg/mL gentamicin was added at 90 min to prevent overgrowth of extracellular bacteria. THP-1 cells were washed with PBS, lysed with 0.05% Triton X-100 (Sigma), and bacteria quantified by dilution plating onto THY agar. Supernatants were used for cytokine measurement using an IL-6 reporter cells (HEK-Blue IL-6; InvivoGen) according to the manufacturer's protocol. Briefly, cells were seeded at $8x10^5$ cell/mL in a 96-well plate, 20 µL of sample or dilutions of recombinant human IL-6 (Invivogen) added, and after 24 h, IL-6-induced secreted alkaline phosphatase measured with QUANTI-Blue (Invivogen). Concentrations were determined relative to a standard curve of recombinant IL-6; IL-6-neutralizing antibodies abolish all activity, confirming the receptor specificity of signaling.

Statistical Analysis. Values are expressed as means ± standard error of the mean. Differences between groups were analyzed using one-way ANOVA with Dunnett's multiple comparisons analysis unless otherwise indicated. Differences are considered statistically significant at a *P* value of $< .05$ using GraphPad Prism v8.4.1. Data include a minimum of three biological replicates to ensure reproducibility. All figures were generated using Prism v8.4.1.

RESULTS

IL-6 Inhibition Promotes Group A *Streptococcus* Replication

We previously reported that Anakinra-treated and IL-1R$^{-/-}$ mice are more susceptible to iGAS (4). Post-marketing surveillance of adverse events revealed a correlation with not only anti-IL-1 immunotherapeutics, but also anti-IL-6, with multiple reports of sepsis, necrotizing fasciitis, and toxic shock syndrome caused by GAS in individuals taking Tocilizumab (4). We therefore sought to confirm if IL-6 repression promoted host susceptibility to GAS using the patient isolate M4(C20). We examined the role of anti-IL-1 and anti-IL-6 therapeutics in promoting GAS growth using a modified Lancefield assay, wherein heparinized whole blood from healthy human donors was inoculated with a known quantity of GAS and bacterial replication monitored. Blood was treated with either Anakinra (Kineret; 50 ug/ml), Tocilizumab (Actemra; 50 ug/ml), both Anakinra and Tocilizumab, or IgG isotype control (BioXcell; 50 ug/ml). Inhibition of signaling by either anti-IL-1 or anti-IL-6 significantly promoted GAS replication (Figure 1A).

C57BL/6 mice treated with anti-IL-1R, anti-IL-6R, both, or an isotype IgG control were intravenously inoculated with $1x10^8$ CFU of the clinical isolate M4(C20). Each of the neutralizing antibodies was sufficient to induce a significantly shortened survival time (Figure 1B). Recapitulating the anti-IL-6 phenotype, IL-6$^{-/-}$ C57BL/6 mice infected with M4(C20) also experienced a significant increase in mortality compared to wild-type C57BL/6 mice (Figure 1C). In contrast, M1(5448), a clone of the epidemic strain contributing to the resurgence in invasive infections, resulted in rapid death of both wild-type and IL6$^{-/-}$ mice. In order to recapitulate the slower mortality kinetics of M4(C20), we also infected mice with $6x10^6$ CFU of M1(5448). Both wild-type and IL-6$^{-/-}$ mice were equally susceptible to M1(5448) (Figure 1C, 1D). These data

suggest GAS is not universally restricted by IL-6, but the severity of infection by some strains is enhanced by its inhibition.

Group A *Streptococcus* Evasion of IL-6 Restriction

To examine whether IL-6 is differentially induced by GAS strains, THP-1 macrophages were infected with M4(C20), strain M1(5448), or other isolates collected from iGAS infection of either serotype, 87160 (M4) and 74553 (M1). IL-6 levels did not vary significantly (Figure 2A), suggesting that strains may vary in their resistance to IL-6-regulated immune effectors through a mechanism that does not involve changes in IL-6 expression. We next examined whether clinical isolate M4(C20) was more susceptible to IL-6-mediated killing than M1(5448), and if this trend was recapitulated in other M1 and M4 clinical isolates. For this, we preincubated THP-1 macrophages with 500 ng/mL of recombinant IL-6 and infected them with M4(C20), M1(5448), 87160 (M4), or 74553 (M1). IL-6 attenuated the growth of both M4 strains, but neither M1 strain (Figure 2B). The concentration required for increased restriction is less than 100 ng/ml (Figure 2C). Together these data show that IL-6-induced defenses have a direct role in restricting growth of some strains of GAS, and that M1 GAS may possess a mechanism to evade these effects.

DISCUSSION

Immunosuppression is a risk factor for severe infections in humans and animal models of disease. How a pathogen gains virulence from any specific defect in immune signaling is not always well-defined, and this can be particularly complicated in the cytokine cascade of sepsis. IL-6 knockout mice, for example, can have increased pathogen burden that shorten the time-to-death in

experimental infection models [5]. In some sepsis models, the failure to control pathogen replication without IL-6 leads to earlier death, while in others, the absence of IL-6 in the cytokine storm prolongs survival (5, 13). Here, we demonstrate that anti-IL-6 immunotherapy can promote iGAS infection. It is well-documented that some strains like M1(5448) have a greater propensity for invasion and can produce iGAS in otherwise healthy individuals [2, 4]. This finding is consistent with our observation that M1(5448) is completely unaffected by IL-6 repression, suggesting it has virulence mechanisms to resist the immune effects of IL-6 signaling. However, M4(C20) shows that some strains may be more opportunistic in nature and produce disease of increased severity upon immunosuppression by drugs such as Tocilizumab. Our prior identification of greater iGAS reporting risk associated with IL-1 inhibitors compared to IL-6 inhibitors (4) may reflect these observations that some strains are not restricted by IL-6. Thus, we note that infectious risks from immunomodulation are not uniform - just as immune pathways are robust but independent, bacterial pathogens can dissimilarly be impacted by these treatments, even within the same species.

Co-incidence of GAS and IL-6 immunotherapy is expected to be generally low; GAS carriage is primarily in the young (14) and use of immunotherapeutics is most common in the elderly. Use of Tocilizumab in other populations for indications other than rheumatoid arthritis, such as juvenile idiopathic arthritis, cytokine release syndrome, or COVID-19, may carry greater risk of iGAS due to elevated exposure to the pathogen, and likely different risks for other opportunistic bacterial infections as well. Furthermore, immunotherapeutics may act to not only impair immune function, but to mask symptoms and alter inflammatory laboratory findings, leading to delays in treatment. Additional clinical studies will be required to examine the molecular mechanisms of how

immunotherapeutics may impact infection severity and incidence in different populations. There is currently no vaccine against GAS, but if specific high-risk patient groups are identified, they could benefit from screening for asymptomatic carriage and the use of antibiotic prophylaxis to preclude severe disease, which has been successful for preventing epidemics in at-risk military populations (15).

NOTES

- ***Potential conflicts of interest.*** All authors: No reported conflicts.

- ***Acknowledgments.*** We thank the Clinical and Translational Discovery Core of Children's Healthcare of Atlanta and Emory University for support and coordination of blood collection. IL6$^{-/-}$ mice were generously provided by M. Karin (UCSD). This study was supported in part by the Investigational Clinical Microbiology Core (ICMC), which was supported by the Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine.
- ***Financial support.*** This work was supported by startup funds from Emory University and the National Institutes of Health (grant number K22 AI130223; awarded to C.L.).
- ***Presented in part***: ASM Microbe, San Francisco, CA, June 2019

- ***For correspondence***: Christopher.LaRock@emory.edu

Author Contributions- Chapter 2

Conceptualization: Christopher LaRock

Methodology: Christopher LaRock, Shyra Wilde

Validation: Christopher LaRock, Shyra Wilde, Katherine Leigh Olivares

Analysis: Christopher LaRock, Shyra Wilde

Investigation: Christopher LaRock, Shyra Wilde, Katherine Leigh Olivares

Resources: Christopher LaRock, Hal Hoffman, Suhas Radhakrishna, Victor Nizet

Writing- draft: Christopher LaRock, Shyra Wilde

Writing- revisions: Christopher LaRock, Shyra Wilde, Katherine Leigh Olivares, Victor Nizet, Hal Hoffman, Suhas Radhakrishna

Visualization: Christopher LaRock, Shyra Wilde

Supervision: Christopher LaRock

Project Administration: Christopher LaRock

Funding Acquisition: Christopher LaRock

REFERENCES

1. Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. Lancet Infect Dis 5:685–694.

2. Sumby P, Porcella SF, Madrigal AG, Barbian KD, Virtaneva K, Ricklefs SM, Sturdevant DE, Graham MR, Vuopio-Varkila J, Hoe NP, Musser JM. 2005. Evolutionary Origin and Emergence of a Highly Successful Clone of Serotype M1 Group A Streptococcus Involved Multiple Horizontal Gene Transfer Events. J Infect Dis 192:771–782.

3. Hamilton SM, Bayer CR, Stevens DL, Bryant AE. 2014. Effects of selective and nonselective nonsteroidal anti-inflammatory drugs on antibiotic efficacy of experimental group A streptococcal myonecrosis. J Infect Dis 209:1429–1435.

4. LaRock CN, Todd J, LaRock DL, Olson J, O'Donoghue AJ, Robertson AAB, Cooper MA, Hoffman HM, Nizet V. 2016. IL-1β is an innate immune sensor of microbial proteolysis. Sci Immunol 1:eaah3539.

5. Rose-John S, Winthrop K, Calabrese L. 2017. The role of IL-6 in host defence against infections: immunobiology and clinical implications. Nat Rev Rheumatol 13:399–409.

6. Smolen JS, Beaulieu A, Rubbert-Roth A, Ramos-Remus C, Rovensky J, Alecock E, Woodworth T, Alten R. 2008. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. The Lancet 371:987–997.

7. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T. 2004. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: A multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 50:1761–1769.

8. Diao H, Kohanawa M. 2005. Endogenous Interleukin-6 Plays a Crucial Protective Role in Streptococcal Toxic Shock Syndrome via Suppression of Tumor Necrosis Factor Alpha Production. Infect Immun 73:3745–3748.

9. Dileepan T, Linehan JL, Moon JJ, Pepper M, Jenkins MK, Cleary PP. 2011. Robust Antigen Specific Th17 T Cell Response to Group A Streptococcus Is Dependent on IL-6 and Intranasal Route of Infection. PLoS Pathog 7.

10. Rosa-Gonçalves D, Bernardes M, Costa L. 2018. Necrotizing fasciitis in a patient receiving tocilizumab for rheumatoid arthritis • Case report. Reumatol Clínica Engl Ed 14:168–170.

11. van de Sande MGH, van Slobbe-Bijlsma ER. 2012. Necrotizing fasciitis in a rheumatoid arthritis patient treated with tocilizumab. Rheumatol Oxf Engl 51:577–578.

12. Beres SB, Richter EW, Nagiec MJ, Sumby P, Porcella SF, DeLeo FR, Musser JM. 2006. Molecular genetic anatomy of inter- and intraserotype variation in the human bacterial pathogen group A Streptococcus. Proc Natl Acad Sci U S A 103:7059–7064.

13. Riedemann NC, Neff TA, Guo R-F, Bernacki KD, Laudes IJ, Sarma JV, Lambris JD, Ward PA. 2003. Protective Effects of IL-6 Blockade in Sepsis Are Linked to Reduced C5a Receptor Expression. J Immunol 170:503–507.

14. Roberts AL, Connolly KL, Kirse DJ, Evans AK, Poehling KA, Peters TR, Reid SD. 2012. Detection of group A Streptococcus in tonsils from pediatric patients reveals high rate of asymptomatic streptococcal carriage. BMC Pediatr 12:3.

15. Webber BJ, Kieffer JW, White BK, Hawksworth AW, Graf PCF, Yun HC. 2019. Chemoprophylaxis against group A streptococcus during military training. Prev Med 118:142–149.

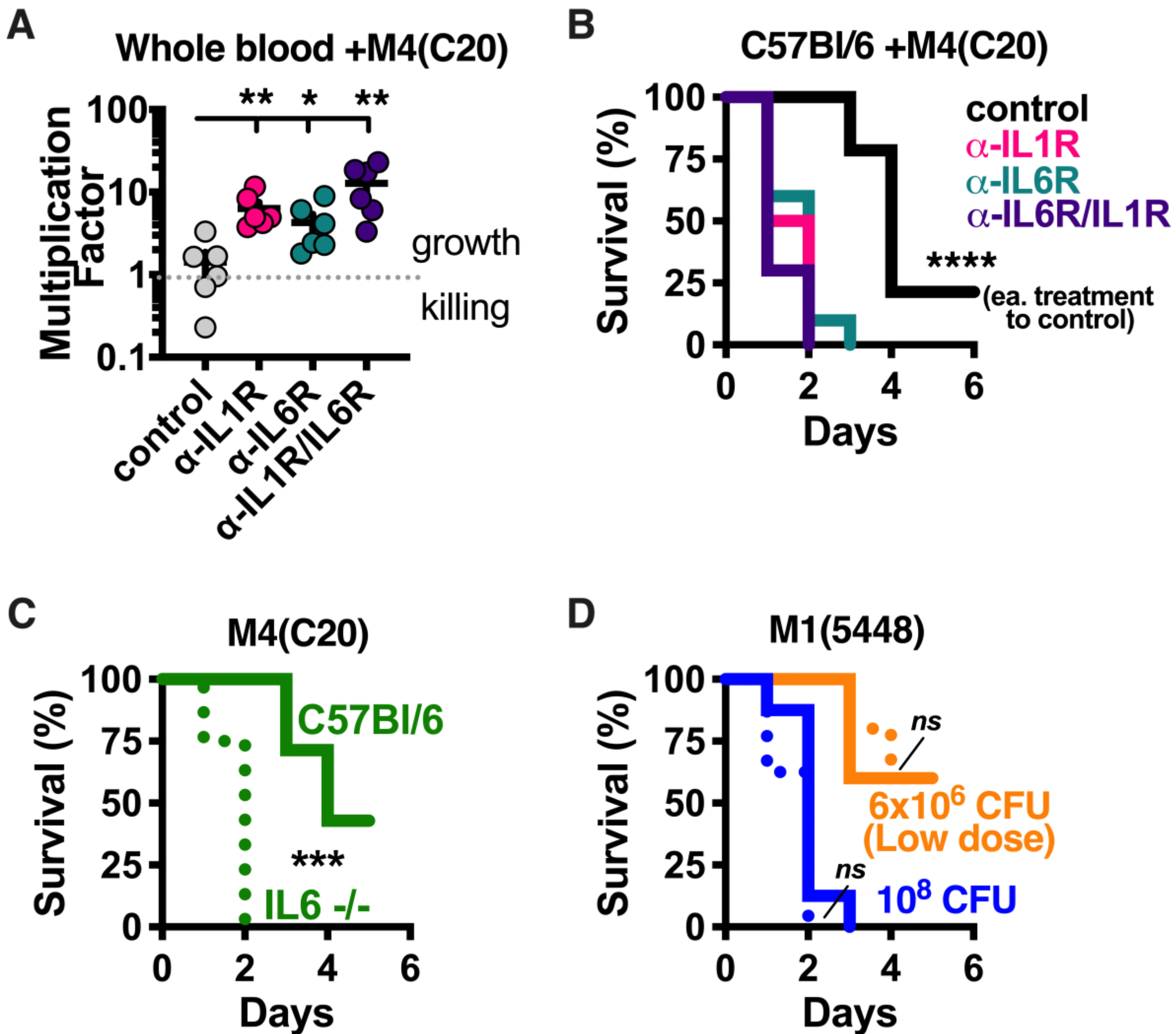

Figure 1. Impaired IL-6 signaling promotes growth of GAS M4(C20). (A). Heparinized whole human blood was treated with Kineret (α-IL1R through rIL1RA; 50 μg/ml) and/or Tocilizumab (α-IL6R neutralizing antibody; 50 μg/ml) with isotype antibody control (50 μg/ml) was inoculated with 10^7 CFU of GAS M4(C20). After 24 h, CFU were enumerated for each group and growth determined relative to starting inoculum (Multiplication Factor); values >1 denote growth. Statistical significance between groups (n=6) was determined was measured by one-way ANOVA using Dunnett's multiple comparisons analysis, using the IgG control group as the reference. Data are representative of three independent donors. **(B).** Wild-type C57BL/6 mice were treated with a

neutralizing monoclonal antibody against either IL-1R (red; n=8), IL-6 (blue; n=8), both (purple; n=8), or an IgG (black; n=8) isotype then inoculated intravenously with 10^8 CFU of GAS strain M4(C20) and monitored for the given time intervals. Statistics were calculated by Log-rank (Mantel-Cox) test in comparison to control mouse group. **(C** and **D).** C57BL/6 wild-type (solid lines) or IL6-knockout ($IL6^{-/-}$; dotted lines) mice were inoculated intravenously with 10^8 CFU of GAS strain M4(C20) (clinical isolate, green), 10^8 CFU M1(5448) (blue), or $6x10^6$ CFU M1(5448) (orange; lower dose to more closely match M4(C20) kinetics) and monitored for the given time intervals. Statistics were calculated by Log-rank (Mantel-Cox) test. Data represent at least 2 independent experiments with 8 mice each. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$; ****, $p<0.00005$; *ns*, not significant.

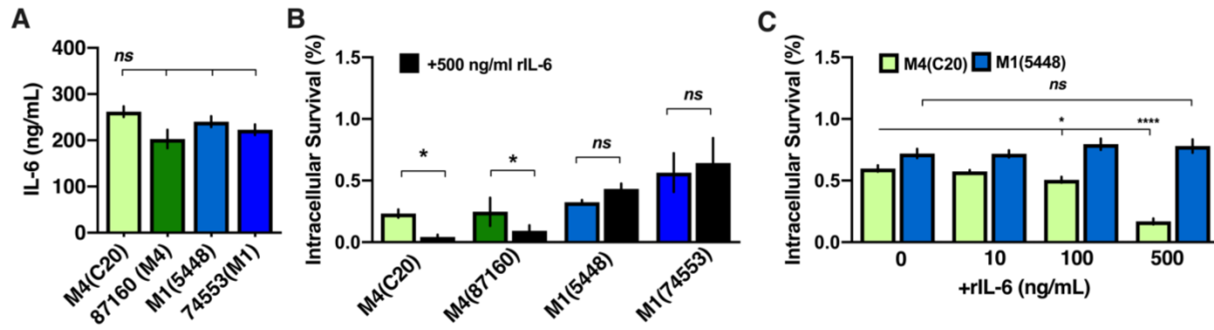

Figure 2. IL-6-mediated killing is strain-specific. (A). Differentiated THP-1 cells were infected with M4(C20), M1(5448), or additional control M1 (74553) and M4 (87160) iGAS isolates (MOI=4) for 2 h and IL-6 release measured. Statistical significance was measured by one-way ANOVA using Dunnett's multiple comparisons analysis, with M4(C20) as the reference strain. **(B** and **C).** THP-1 cells were treated 1 h with 500 ng/mL (B) or titrations of exogenous recombinant human IL-6 (C), and CFU enumerated 2 h post-infection. Data were analyzed by one-way ANOVA using Dunnett's multiple comparisons analysis. All data represent at least three independent experiments with four replicates. Bars show median values ± SEM. *, p<0.05; **, p<0.005; ***, p<0.0005; ****, p<0.00005; *ns*, not significant.

Chapter 3

Reactive oxygen species and hyaluronic acid capsule drive pathology during group A Streptococcal skin infections

Shyra Wilde[1], Anders Johnson[1], and Christopher LaRock[1,2]

[1]Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, GA 30322, USA

[2]Department of Microbiology and Immunology, Division of Infectious Diseases, Department of Medicine, and Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30322, USA

Abstract

Immune requirements for killing of group A *Streptococcus* (GAS) during infection have remained largely unknown. We have previously identified the pro-inflammatory cytokine IL-6 as an important signaling molecule for the initiation of an immune response that can kill some strains of GAS, but specific mediators of this response are unclear. Our work demonstrates that IL-6 acts against GAS by inducing the production of antimicrobial reactive oxygen species (ROS). GAS lacks catalase, a virulence factor used by many diverse species for detoxifying ROS, yet some strains of GAS associated with hypervirulence are still able to withstand ROS and cause severe disease. Through analysis of clinical isolates, we have found that the capsule of GAS, which has a well-characterized role as a physical barrier against opsonization, also confers protection against ROS. We also show that the hyaluronic acid the capsule is composed of can act as a direct antioxidant against ROS *in vitro*. Nonetheless, we find that *in vivo* ROS is not essential for killing of GAS in an intradermal infection model. However, lesion size is significantly impacted by both the absence of ROS in host cells and production of capsule by GAS. Although the number of bacteria found within skin lesions was the same between wild-type and gp91$^{phox-/-}$ mice (deficient in ROS production), gp91$^{phox-/-}$ mice had significantly larger skin lesions. This phenotype was only observed when infected with acapsulated strains of GAS, indicating a contribution by both ROS and hyaluronic acid to wound healing.

Introduction

Group A Streptococcus (GAS; *Streptococcus pyogenes*) is the etiological cause of common and relatively mild infections such as strep throat and impetigo but can occasionally progress towards life-threatening infections such as necrotizing fasciitis and sepsis. There are an estimated 500,000 annual deaths from invasive GAS infections and approximately 18 million people suffer from invasive GAS infections or other post-infectious immune sequelae like rheumatic fever [1,2]. Although conventional antibiotics such as penicillin can kill GAS, antibiotic treatment failure remains high due to a variety of mechanisms [3]. Invasive GAS infections progress rapidly and overwhelm the host immune system, the primary contributor to morbidity and mortality associated with such infections.

How specific immune processes act to counteract invasive infection by GAS is largely unknown. IL-1β has previously been shown to be important for activating and recruiting neutrophils to the site of infection [4,5]. IL-6 has also been implicated in the restriction of GAS, as mice treated with IL-6-inhibiting biologic drugs or that have *il6* gene knockout ($IL6^{-/-}$) succumb to systemic GAS infections faster than wild-type mice [6]. While IL-1β mediated restriction appears to be universal, IL-6 is conditional; both wild-type and $IL6^{-/-}$ mice infected with M1T1 GAS strain 5448 experienced similar times to death, but mice infected with the clinical isolate M4C20 displayed significantly different susceptibilities to infection. *In vitro* studies revealed that M4C20, but not 5448, was killed by macrophages when stimulated with exogenous IL-6 in a dose-dependent manner [6]. Therefore, the role of IL-6 during killing of GAS appears to be dependent on both host and bacterial genetic factors. In this study, we investigate why IL-6 is important against only some strains of GAS and identify a GAS virulence factor protective against IL-6-mediated killing.

IL-6 is strongly upregulated during GAS infection [7]. IL-6 is part of the acute-phase proinflammatory response, alongside TNF and IL-1β, with pleotropic roles in immunity that include direction of B-cell differentiation and the recruitment of monocytic cells [8]. Much of this signaling occurs though soluble IL-6-receptor complexes, the dominant mechanism of IL-6 signaling during infection, and has been shown to have important roles in combatting microbial infection and modulating immune responses during sepsis [9]. Within phagocytes, IL-6 signaling induces phosphorylation and subsequent activation of multiple pro-inflammatory transcriptional regulators, including STAT3 and NF-kB [10]. Excessive IL-6 can have pathological effects that result in autoimmune disorders such as rheumatoid arthritis and Castleman's disease, which are typically managed using IL-6-inhibiting biologics [11]. Inhibition of IL-6 signaling can increase susceptibility to bacterial infections, including infections by GAS, that manifest as either bloodstream infections [6] or necrotizing skin infections [12].

Although IL-6 appears to be important for killing GAS, a specific mechanism by which IL-6 kills GAS has not been identified. In this work, we show that the production of reactive oxygen species (ROS) induced by IL-6 is sufficient to kill some strains of GAS in both human macrophages and neutrophils, as the addition of ROS scavengers enhances growth of GAS. Additionally, we demonstrate that the hyaluronic acid capsule produced by some strains of GAS serves to protect against ROS by acting as a direct antioxidant. *In vitro*, acapsulated GAS are more susceptible to hydrogen peroxide, but growth is rescued when exogenous hyaluronic acid is added. Capsule has been shown to be broadly important for full virulence of GAS in multiple *in vivo* models of infection [13–16]. Discordant with our *in vitro* findings, we find that capsule is not essential for protecting against GAS killing in the skin *in vivo*. We also find that ROS is not essential for direct killing of GAS in our infection model. However, we demonstrate that wound

healing *in vivo* is dependent on both ROS production by the host and capsule production by GAS. This work enhances our understanding of the immune processes that drive invasive skin infection caused by GAS.

Materials and methods

Measurement of intracellular ROS. Intracellular ROS was measured by reduction of 2',7' – dichlorofluorescin diacetate (DCFDA; Abcam). THP-1 monocytes were differentiated in a black 96-well tissue culture plate using 200nM phorbol 12-myristate 13-acetate (PMA) for 48 hours prior to the experiment [6]. On the day of experiment, cells were stained with DCFDA for 45 minutes according to the manufacturers protocol and then stimulated with either 1μM hydrogen peroxide (H_2O_2) or 500ng/mL of recombinant IL-6 protein (Invitrogen). Measurements were taken using a PerkinElmer VICTOR Nivo multimode microplate reader, measuring absorbance at ex/em 485/535nm every 10 minutes at 37ºC for 180 minutes total. ROS induction was measured by calculating the V_{max} of each condition, which we defined as the largest change in relative units (RU) per minute.

Fluorescent microscopy. 2 x 10^4 THP-1 monocytes were seeded into each well of an 8-well Millicell EZ SLIDE (EMD Millipore) and terminally differentiated as stated previously. Cell treatments included recombinant IL6 (Invitrogen; 500ng/mL) and tert-butyl hydrogen peroxide (TBHP; 200μM, Abcam). Following a 30-minute pre-treatment with IL-6 or TBHP, cells were stained with CellROX Deep Red (to visualize oxidative burst, 5μM; Invitrogen) and anti-tubulin conjugated to Alexa Fluor 488 (Novus biologicals, clone YOL1/34, 1:200) for 30 minutes. Cells were then fixed for 20 minutes in BD CytoFix, washed in PBS 3 times, then mounted using SlowFade Diamond Antifade Mountant (Thermo). Samples were visualized on a Zeiss

AxioObserver Z1 microscope using filters for AlexaFluor 488 and AlexaFluor 647. Images were processed using ImageJ to merge channels and adjust intensity, with consistent settings held between all samples.

Bacterial culturing and strains. GAS strains 5448, 5448Δ*hasA*, and M4C20 have been studied and characterized previously [6,14,17]. All other GAS clinical isolates, both encapsulated and acapsulated, were obtained through the Georgia Emerging Infections Program. Bacteria were routinely grown overnight in Todd-Hewitt broth at 37ºC in a 5% CO2 incubator. For SpeB expression assays, supernatants were taken from freshly grown overnight cultures. For *in vitro* experiments, cultures were washed in PBS and resuspended in PBS+20% glycerol, then stored in single-use aliquots at –80C for later use. Bacterial aliquots were diluted to the appropriate multiplicity of infection at the time of the experiment.

SpeB expression tests. SpeB protease activity was measured by hydrolysis of azocasein as previously demonstrated [18] with minor modifications previously detailed [4]. Sodium acetate activation buffer was comprised of 0.1M sodium acetate-acetic acid, 1mM EDTA, 20mM DTT, pH=8. Supernatants from overnight cultures of GAS were added 1:1 with activation buffer, then added 1:2 with activation buffer including 2% (w/v) azocasein and incubated overnight. Hydrolysis was visually assessed by turbidity of each well.

Hydrogen peroxide susceptibility assays. Susceptibility to hydrogen peroxide was assessed using both solid and liquid media. For the solid media method, 0.1mL of GAS in THY broth was spread onto a THY agar plate and left to dry completely. When plates were sufficiently dried, 1uL of 30% hydrogen peroxide was spotted onto the plate in triplicate and plates were incubated overnight. The following day, susceptibility was measured as the zone of inhibition surrounding the peroxide. A second iteration of this assay was performed using paper filter discs soaked in either distilled

water (control), 30% hydrogen peroxide, or a 1:1 solution of 30% hydrogen peroxide and 1mg/mL hyaluronic acid (HA). Zones of inhibition for each condition were quantified after imaging.

Minimum inhibitory concentration. Minimum inhibitory concentration (MIC) assays using liquid media were carried out in 96-well plates using a method adapted from Ayala and Shafer [19]. Briefly, dilutions of peroxide were made in RPMI 1640 media (no FBS, no antibiotics, no phenol red; Gibco) and added to each well. 5×10^5 CFU of GAS were added to each dilution of peroxide, and the final volume of each well was brought to 200µL using RPMI. Plates were incubated at 37ºC, 5% CO_2 overnight. The following day, AlamarBlue (Bio-Rad) was added at a 1:10 dilution and incubated at 37ºC for 2-4 hours. The MIC was determined to be the lowest concentration that inhibited growth of GAS (media remained blue after addition of AlamarBlue). Each strain was tested three times to ensure reproducibility.

Capsule protection assays. Assays were performed as described in Brissac et al. with slight modifications [20]. 5×10^7 CFU of GAS were washed in 1mL PBS and resuspended in RPMI containing no FBS or antibiotics with 0.05% TWEEN-20. Hyaluronic acid isolated from *Streptococcus equi* (Sigma) was added to bacterial suspensions to a final concentration of 0.1, 1, 2, or 5mg/mL, from a stock solution of 5mg/mL in PBS. After mixing by vortexing, H_2O_2 was added to a final concentration of 10mM to each tube. Tubes were incubated for 30 minutes at 37ºC in parallel with an H_2O_2-only (0mg/mL HA) and an untreated control group, and CFU were enumerated by dilution plating.

NBT reduction. Reduction of nitrotetrazoleum blue (NBT), coupled with phenazine methosulfate (PMS) and NADH, was performed as described elsewhere [20,21]. Reactions were performed in 96-well plates in a final volume of 200ul per well. A mix of NADH (166 µM), NBT (43 µM), and HA (0, 0.1, 1, 2, or 5mg/mL) were freshly prepared in phosphate buffer (40 mM, pH 7.6) and

incubated for 2 min at room temperature. NBT reduction was started by the addition of 2.7μM PMS. Plates were read in a VICTOR Nivo (PerkinElmer) plate reader at 37°C. The optical density was monitored at 560/10nm every 30s for 30 min, with orbital shaking between readings. The antioxidant capabilities of HA were determined as a means of protection of NBT from reduction by PMS compared to the controls with no capsule.

Cell culture. THP-1 monocytes (human; ATCC, TIB-202) were cultured at 37ºC in 5% CO2 using RPMI with phenol red supplemented with 10% FBS and penicillin/streptomycin. Monocytes were terminally differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA; 200nM) for 48 hours prior to experiments as done previously [6].

Gentamicin protection assays. THP-1 monocytes were seeded as 0.1mL at 2 x 10^5 cells/ml in a 96-well plate and terminally differentiated with 200nM PMA for 48 hours. On the day of infection, cell culture media was replaced with RPMI (+FBS/-Antibiotics; Gibco) supplemented with 10% pooled human serum (Lot # C16037, Atlanta Biologicals). At this time, wells were treated with N-acetylcysteine (NAC; 20mM, Sigma) to scavenge free radicals. After 1 hour, cells were infected with GAS (MOI=4) and plates were spun down to promote bacterial-macrophage interaction. After 20 minutes, gentamicin was added to a final concentration of 100ug/mL to kill extracellular bacteria. At indicated time points, culture media was aspirated, and cells were washed twice in PBS. Cells were then lysed in 0.05% Triton X-100, diluted in PBS, and plated using the drip method onto THY agar plates. Intracellular CFU were enumerated after overnight incubation at 37ºC.

Neutrophil killing assays. Primary neutrophils were isolated from whole human blood. Blood was collected from healthy adult donors under informed consent and with approval from the Institutional Review Board at Emory University. Blood was collected into heparinized Vacutainer

tubes and isolated from whole human blood using Polymorphprep (Axis-shield). Neutrophil killing assays were performed as described previously with minor modifications [5]. Neutrophils were diluted to 1 x 10^5 cells/mL in RPMI containing 10% FBS with no antibiotic, and 1mL was seeded into each well of a 24-well plate. Neutrophils were either pre-treated with N-acetylcysteine (NAC; 20mM, Sigma) for one hour or left untreated. 1 x 10^6 CFU of GAS were added to each well, and samples were taken for CFU enumeration at 30 and 90-minutes post-infection. CFU were enumerated after overnight incubation of THY agar plates at 37ºC.

In vivo experiments. All animal experiments were performed with prior approval from Emory University's Institutional Animal Care and Use Committee. 6–8-week-old male wild-type C57BL/6 mice were ordered from Jackson Laboratories as needed. Male CGD (gp91$^{phox-/-}$) breeder mice were ordered from Jackson laboratories and breeding was maintained on-site using Emory's Managed Breeding Services. Mice were housed under pathogen-free conditions until infection, then moved to an ABSL2 facility for the duration of the experiment,

Intradermal infection model. The day prior to infection, fur was removed from the back using an electric razor and hair removal cream. The next day, the infection site on the lower back was surface sterilized by swabbing with an isopropanol wipe, then injected intradermally with 1 x 10^8 CFU of GAS. After 48 hours, mice were euthanized, and skin lesions were imaged prior to removal. CFU from lesions were enumerated after the tissue was homogenized in 1mL of sterile PBS. Lesion surface area was quantified using ImageJ.

Histology. Skin lesions collected during *in vivo* experiments that were not used for CFU enumeration were fixed in 4% paraformaldehyde. Lesions were paraffinized and sectioned at a width of 5μm. Slide preparation and myeloperoxidase (MPO) staining was performed by the Cancer Tissue and Pathology shared resource of Winship Cancer Institute of Emory University

using Leica Bond RXM automated immunohistochemistry staining platform (Leica Biosystems). Slides were heated for 30 minutes at 60 degrees Celsius, deparaffinized with the Bond Dewax Solution, and rinsed with Leica Wash Buffer. Following deparaffination the slides were heated to 100 degrees Celsius, and antigen retrieved for 20 minutes with Leica ER2(high pH) antigen retrieval buffer and then rinsed with Leica Wash Buffer. Peroxidase block was applied at room temperature for 5 minutes and the sections were washed with three rinses of Wash Buffer. The anti-Myeloperoxidase (Abcam, 1:1,000) was applied and incubated for 30 min at room temperature followed by three rinses of Wash Buffer. Leica Bond anti-Rb HRP secondary was applied and incubated for 8 min and the detection was completed in combination with Leica Refine DAB kit, as per manufacturer recommendations. Slides were counterstained with hematoxylin for 5 min. Slides were then dehydrated, cover-slipped, and evaluated by light microscopy with scanned images of the slides. The slides were scanned on a Hamamatsu Nanozoomer HT 2.0 at 40x. For slides stained using DAB and hematoxylin, MPO expression was quantified using ImageJ using color deconvolution and determining the ratio of MPO-producing cells (brown) to total cells (blue).

Immunohistochemistry. Additional sections were prepared for immunohistochemistry (IHC) in-house by clearing using Histo-Clear II (Electron Microscopy Sciences) and ethanol. Mounted sections were then permeabilized using 0.5% TWEEN-20 (Sigma) in PBS, and antigen retrieval was carried out at 95C using Dako target retrieval solution, pH9 (Agilent). Sections were blocked in 1% BSA and stained using primary antibodies probing for myeloperoxidase (Abcam; rabbit anti-mouse 1:1,000) or the *Streptococcus* group A carbohydrate (Fitzgerald; goat 1:500) in 1% BSA at 4°C overnight. Sections were then stained stepwise using secondary antibodies for donkey anti-goat 555 (1:1,000, Sigma) and goat-anti rabbit 488 (1:1,000, Invitrogen), each for 2 hours at

4ºC. Sections were stained with Hoechst 33342 (Immunochemistry, 1:500) for 30 minutes, washed, and mounted using SlowFade Diamond Antifade Mountant (Thermo). The fluorescent slides were scanned at 20x on Akoya's Vectra Polaris with the following filters: DAPI, FITC, and Cy3. Images were processed using ImageJ, with consistent processing conditions maintained between images.

Cytokine arrays. Cytokine levels in lesion tissue were measured using the V-PLEX Proinflammatory Panel 1 Mouse Kit (Meso Scale Diagnostics). This kit measured expression of the cytokines IFN-y, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and TNF-a. Ten samples from each experimental group were randomly selected for analysis. Homogenized tissue in PBS was centrifuged, and total protein content from cell-free supernatants was quantified using Pierce Coomassie Plus (Bradford) Assay Reagent (Thermo). For cytokine arrays, total protein was normalized to 40ug per sample. Plate setup, reading, and analysis was performed by the Emory Multiplexed Immunoassay Core using a Meso QuickPlex SQ120.

Results and Discussion

IL-6 induces ROS.

To explicitly establish the connection between IL-6 and ROS production within phagocytes, we utilized a dye (DCFDA) that fluoresces when oxidized by ROS, and measured changes in ROS production upon stimulation by exogenous IL-6. The rate of oxidation of DCFDA is significantly increased upon stimulation of human macrophages with IL-6 (Fig 1A), demonstrating that IL-6 can specifically induce ROS. Oxidation of DCFDA by ROS was highest at 30 minutes post-stimulation. We also employed fluorescent microscopy using CellROX deep red to detect phagocytic ROS production within THP-1 macrophages. CellROX fluorescence was

increased when stimulated with either exogenous IL-6 (500ng/mL; Fig 1B) or Tert-butyl hydrogen peroxide (TBHP; Fig 1C) relative to untreated cells (Fig. 1D), confirming that IL-6 is sufficient to induce the production of phagocyte ROS.

Hyaluronic acid capsule protects GAS against ROS.

Defense against reactive oxygen species (ROS) is of particular interest for GAS. Neutrophils, which produce large amounts of ROS upon encountering a pathogen, are critical for clearance of GAS during infection [5,22–24]. GAS does not produce its own catalase but has evolved both direct and indirect mechanisms to withstand oxidative burst [25]; yet, different strains of GAS have varying susceptibility to hydrogen peroxide [22,26]. Thus, we suspected that a virulence factor that is not universally conserved across GAS strains may contribute to additional protection against ROS, and we hypothesized that this is mediated by the hyaluronic acid capsule. Capsule has been previously explored as a protective mechanism against ROS by forming aggregates and physically blocking peroxide from damaging the bacterium [26].

In order to test the hypothesis that capsule protects against ROS-mediated killing, we measured susceptibility to hydrogen peroxide on both solid and liquid media. The widely studied M1T1 GAS strain 5448 hyperinvasive strain was included as a control [17]. Each strain was tested for capsule production by ELISA against hyaluronan [27,28] and azocaseine hydrolysis to define *covRS* mutants, which are typically hyper-encapsulated [29]. GAS clinical isolates that were naturally acapsulated had significantly larger zones of inhibition than encapsulated GAS on THY plates spotted with hydrogen peroxide, indicating enhanced susceptibility. This observation was not specific to one serotype (Fig. 2A). An isogenic capsule mutant of reference GAS strain 5448

(5448Δ*hasA*) also displayed enhanced susceptibility to peroxide, confirming that the loss of hyaluronic acid capsule is sufficient to increase susceptibility to hydrogen peroxide.

We also employed a modified MIC assay using AlamarBlue to determine each strain's specific susceptibility to hydrogen peroxide [19]. The MIC of strain 5448Δ*hasA* was approximately half of the MIC of wild-type 5448 with an MIC of 375µM, and the MIC of M4C20 was 500µM. Interestingly, a hyper-encapsulated *covRS* mutant of 5448 (5448AP) was even more resistant to peroxide than the wild-type strain, with an MIC nearly double that of the wild-type 5448 strain (Fig. 2B). Consistent with the data in Figure 2A, several of the acapsulated clinical isolates tested in Figure 2A also had lower MICs toward peroxide (median 375µM) compared to 5448 and other encapsulated clinical isolates of GAS. Together, these data support the hypothesis that the hyaluronic acid capsule produced by some strains of GAS provides a mechanism to evade killing by reactive oxygen species *in vitro.*

Recent research has revealed a direct antioxidant role for capsule in the closely related species *Streptococcus pneumoniae* [20]. While the capsules between these species have different structures, we hypothesized that hyaluronic acid produced by some strains of GAS could similarly act as an antioxidant. To test this hypothesis, we devised a disc diffusion assay to observe changes in susceptibility of GAS to peroxide in the presence or absence of hyaluronic acid. When peroxide was added to a paper disc, there was a clear zone of clearance for acapsulated GAS (Fig. 3A). When a disc soaked in 1:1 solution of peroxide and hyaluronic acid was added to the plate, the zone of clearance decreased, suggesting that exogenous HA was sufficient to rescue acapsulated GAS from killing, potentially by detoxifying the peroxide as an antioxidant. Encapsulated GAS only had modest changes in the zone of clearance, indicating that the capsule on the surface of GAS is sufficient to protect from ROS-mediated killing.

We next examined the ability of exogenous hyaluronic acid to rescue growth of GAS in liquid media containing hydrogen peroxide. In these assays, 5 x 10^7 CFU of GAS were incubated with 10mM hydrogen peroxide and varying concentrations of hyaluronic acid. The addition of exogenous hyaluronic acid in concentrations above 1mg/mL was sufficient to restore the growth of the isogenic Δ*hasA* mutant of 5448, as well as the non-encapsulated GAS strain M4C20 (Fig. 3B). Growth of encapsulated strain 5448 was unaffected by exogenous hyaluronic acid in the presence of hydrogen peroxide. These observations are consistent with our hypothesis that hyaluronic acid can act as a redox sink and suggest that the capsule of the hypervirulent strain 5448 is sufficient to provide protection against ROS that cannot be enhanced with exogenous hyaluronic acid.

To further probe the direct mechanism of protection from ROS by capsule, we also measured oxidation of nitroterazoleum blue (NBT) paired with phenazine methosulfate (PMS) and NADH in the presence or absence of HA [20,21]. In this assay, hyaluronic acid was added in varying concentrations to NBT in phosphate buffer with NADH. Upon activation by PMS, NBT will either oxidize, resulting in a colorimetric change, or remain colorless if in the presence of an antioxidant. HA at a concentration of at least 1mg/mL was sufficient to prevent the oxidation of NBT by PMS (Fig. 3C), indicating that HA produced by GAS can act as an antioxidant toward ROS, and this occurred in a dose-dependent manner.

ROS kills GAS *in vitro*.

To expand on these findings and determine their relevance during infection, we developed an *in vitro* infection model in both human macrophages and primary neutrophils isolated from whole human blood. Neutrophils, monocytes, and macrophages have been shown to be the primary

cells recruited during GAS infection [30] and can produce large amounts of ROS in response to pathogens [31]. Intracellular survival of GAS within phagocytes, specifically macrophages, has been shown to be important for GAS pathogenesis [6,32,33]. THP-1 monocytes were terminally differentiated into macrophages and treated with either N-acetyl-L-cysteine (NAC; Sigma, 20mM) or left untreated, then infected with 8 x 10^4 CFU (MOI=4) of GAS strains 5448 (M1T1 pandemic strain), 5448ΔhasA (isogenic capsule mutant), or M4C20 (*emm*4, naturally deficient in capsule production). Intracellular bacteria were harvested at both 30 and 90-minutes post-infection and survival was determined by CFU plating. Consistent with our findings in Figure 3B, intracellular growth of acapsulated GAS (5448Δ*hasA* or M4C20) was significantly increased in macrophages at 90 minutes post infection when treated with NAC when compared to untreated macrophages (Fig. 3D). Encapsulated GAS (5448) survival was not affected by the addition of NAC, confirming the protective role of capsule against ROS *in vitro*.

Primary neutrophils from whole human blood were treated with NAC under the same conditions as our macrophage infection model and infected at MOI=10 [5]. Killing was measured as a percentage of bacteria surviving from 30 min to 90 min post-infection. Consistent with our macrophage infection model, acapsulated GAS survived significantly better in neutrophils treated with NAC (Fig. 3D). Encapsulated GAS experienced no statistically significant differences in growth in either condition, consistent with prior observations that exogenous IL-6 does not enhance intracellular killing of encapsulated GAS, as well as our hypothesis that capsule protects against ROS. We conclude that *in vitro*, IL-6 can kill acapsulated GAS through the production of ROS, and that this is applicable to both intracellular and extracellular GAS.

ROS is not essential for restricting GAS in the skin.

Based on our findings that implicate capsule on the surface of GAS as a direct antioxidant, we next evaluated the effect of capsule on survival in both wild-type and gp91$^{phox-/-}$ (chronic granulomatas disease model; deficient in phagocytic ROS production) C57BL6 mice. Mice were inoculated intradermally with 1 x 10^8 CFU of one of three GAS strains: wild-type strain 5448, 5448Δ*hasA*, or M4C20. Infections proceeded for 48 hours, after which skin lesions were harvested and processed for CFU enumeration. Based on our observation that acapsulated bacteria were more susceptible to peroxide *in vitro*, we hypothesized that acapsulated bacteria (5448Δ*hasA* and M4C20) would be more effectively cleared from skin in wild-type mice, and that this phenotype would be reversed in gp91$^{phox-/-}$ mice. Prior studies using a subcutaneous infection model have not established an NADPH oxidase-dependent mechanism of M1T1 GAS killing in the skin [23], but no studies have been undertaken to determine if capsule influences this phenotype. In contrast to our *in vitro* data, bacterial load between wild-type and gp91$^{phox-/-}$ mice was nearly identical for all strains tested (Fig. 4), including the acapsulated strains of GAS. After 48 hours the bacterial load in skin lesions was similar to the starting inoculum in each condition, suggesting that ROS is not essential for direct killing of GAS at this site of infection.

Pathology during GAS skin infections is influenced by ROS and hyaluronic acid capsule.

We next evaluated the severity of infection in our model by measuring the area of the lesion developed. In contrast to bacterial load, lesion size was significantly affected by both encapsulation status of the bacterium and production of ROS in the host (Figure 4). Acapsulated bacteria produced the smallest lesions in wild-type mice, with an average lesion size of 45mm^2 and 13mm^2 for 5448Δ*hasA* and M4C20, respectively (Fig 4B and Fig. 4C), compared to encapsulated strain

5448 which had an average lesion size of $63mm^2$ (Fig. 4A). In $gp91^{phox-/-}$ mice, the lesions in 5448Δ*hasA*-infected mice were approximately twice as large, and in M4C20-infected mice were four times as large, when compared to the wild-type mice infected with the same strains (Fig. 4B and 4C). Lesions on $gp91^{phox-/}$- mice from acapsulated GAS were nearly equivalent in size to the lesions produced by encapsulated GAS (Fig. 4). Taken together, we can conclude that ROS may not be acting in a directly antimicrobial during skin infection but appears to be important in modulating wound formation during infection.

ROS has been shown to have important roles in regulating inflammation, acting as signaling molecules that activate redox-sensitive transcriptional factors such as NF-kB [34]. ROS can also regulate chemotaxis and clearance of neutrophils from infection sites [35]. Thus, we hypothesized that the change in lesion size in CGD mice was a result of unregulated inflammation at the site of infection. To investigate this hypothesis, we characterized the inflammatory response at the site of infection in each group by measuring pro-inflammatory cytokine levels within lesions. Only one of the cytokines tested, IL-10, was statistically significantly different between genotypes, having increased induction in some $gp91^{phox-/-}$ mice (Fig. 5). IL-10 is generally thought to have a paradoxical relationship with IL-6- that is, the pro-inflammatory IL-6 drives production of the anti-inflammatory IL-10 during inflammation [36,37]. This was only observed for M4C20-infected mice, so it is likely not capsule-dependent, since 5448Δ*hasA*-infected mice did not have differences in IL-10 induction.

The importance of neutrophils during GAS infection is well-established in both *in vitro* and *in vivo* infection models [5,22,24,38,39] . Exacerbated inflammatory responses from neutrophils can contribute substantially to tissue damage [40], so we also identified neutrophil activation as a potential cause for exaggerated wound formation in $gp91^{phox-/-}$ mice. To measure

neutrophil activation, we next performed immunohistochemistry on lesions isolated from our *in vivo* infections. Lesions were stained for the GAS group A carbohydrate or myeloperoxidase (MPO), which is only produced by active neutrophils. Our analysis revealed that both encapsulated and acapsulated GAS were able to co-localize with MPO-producing neutrophils (Fig 6) in both wild-type and gp91$^{phox-/-}$ mice, confirming the significance of neutrophils in this model. MPO staining was robust in wild-type mice infected with either encapsulated or acapsulated GAS. However, MPO (green) staining appeared to be less pronounced in gp91$^{phox-/-}$ mice infected with acapsulated GAS, indicating that neutrophil activation may be compromised.

We next performed a quantitative analysis of MPO production in lesions using an anti-MPO antibody in conjunction with DAB staining. MPO-positive neutrophils appear brown upon imaging. We measured the ratio of MPO-positive (brown) cell density to hematoxylin-stained (blue) cells. Figure 6 shows representative sections from each group. In wild-type mice, lesions from both encapsulated and acapsulated GAS had similar ratios of MPO+ to total cells, corroborating our immunohistochemical analysis (Figure 6). However, in CGD mice, neutrophils within lesions had significantly lower MPO+ cell ratios when infected with acapsulated GAS (5448Δ*hasA* or M4C20). Wild-type or gp91$^{phox-/-}$ mice infected with GAS strain 5448 did not have any statistically significant differences in MPO+ ratios (Fig. 6). These data support the hypothesis that capsule production by GAS, as well as ROS production within the host, contribute to wound healing during GAS infection, and this may be largely modulated by neutrophils.

Conclusions

In this study we aimed to determine the molecular mechanisms by which IL-6 mediates restriction of acapsulated GAS, and we conclude that this occurs through the production of ROS

resulting from IL-6 induction; conversely, we also identify the hyaluronic acid capsule as a mechanism by which some strains of GAS can evade this killing strategy and reveal its role as a direct antioxidant *in vitro*. Acapsulated GAS are significantly more susceptible to peroxide *in vitro*, and the addition of ROS scavengers was sufficient to rescue growth of acapsulated GAS from killing by phagocytes.

ROS-mediated killing of GAS is of great interest since GAS does not produce catalase, which detoxifies hydrogen peroxide. Some strains of GAS even produce their own hydrogen peroxide, suggesting that GAS likely has independent mechanisms to protect against ROS like hydrogen peroxide [22]. GAS possesses genes that encode for alkyl hydroperoxide reductase *ahpC,* glutathione peroxidase *gpoA,* and NADH oxidase *noxA* that appear to be induced by oxidative stress and may be important for full virulence of GAS [41,42]. We hypothesized that an additional virulence factor contributed to oxidative stress resistance since different strains of GAS had varying susceptibility to hydrogen peroxide. The hyaluronic acid capsule produced by some strains of GAS is structurally identical to the hyaluronic acid in human connective tissue and is mediated by the *hasABC* operon [43]. Capsule has clear implications in GAS virulence, as acapsulated strains of GAS have decreased virulence *in vitro* and *in vivo* [13–16,44,45]. Capsule has previously been implicated in resistance to ROS [26], and we confirm that capsule can protect against ROS. We also further define the role of capsule during resistance and reveal that capsule also acts as a direct antioxidant.

It has been documented in humans that have Chronic Granulomatous Disease, a condition that significantly impairs ROS production (like gp91$^{phox-/-}$ mice), display irregular immune responses to infection characterized by persistent inflammation [46]. Additionally, ROS has been shown to have important roles in regulating inflammation, such as activating the transcriptional

factor NF-kB [47], as well as regulation of chemotaxis and clearance of neutrophils from infection sites [35]. Our data show that pro-inflammatory cytokine production in skin lesions between wild-type and gp91$^{phox-/-}$ mice was equal, indicating that impaired wound healing was not due to exacerbated immune responses at the site of infection. Myeloperoxidase (MPO) is exclusively produced by active neutrophils, and neutrophils are known to contribute substantially to host restriction of GAS. After measuring MPO production in skin lesions, we concluded that the ratio of MPO-positive neutrophils to total cells was equal between wild-type mice infected with encapsulated or acapsulated GAS. However, MPO-positive ratios were significantly decreased in gp91$^{phox-/-}$ mice infected with acapsulated GAS, suggesting that neutrophil activation could be impaired.

In this study, we found a striking contrast between our *in vitro* and *in vivo* phenotypes. Although ROS scavengers were sufficient to rescue growth of acapsulated GAS *in vitro*, gp91$^{phox-/-}$ mice that are deficient in phagocytic ROS production did not have increased bacterial loads within skin lesions when infected with acapsulated GAS. Bacterial load was similar between strains 5448, capsule mutant 5448Δ*hasA*, and non-M1T1 strain M4C20, indicating that gp91^{phox}-dependent ROS was not essential for GAS killing in this infection model, nor was virulence capsule-dependent. GAS may be able to survive better within niches such as the skin due to the decreased levels of ROS in comparison to other infection sites such as the lungs and blood [23]. However, this mechanism still does not explain why lesion sizes were not consistent between wild-type and gp91$^{phox-/-}$ mice; acapsulated GAS produced small lesions in wild-type mice, but lesion size was at least doubled in gp91$^{phox-/-}$ mice infected with the same strains. This data reveals that ROS production and capsule have effects on wound healing *in vivo* during GAS infections. Potentially, part of why acapsulated strains of GAS persist in the human population is because

while they are more sensitive to ROS, they induce less of it during infection due to differences in neutrophil recognition of the bacterium. While this work has expanded our fundamental knowledge of how the host kills GAS and how invasive strains of GAS evade killing, there are still many questions regarding the immune processes that drive pathology during invasive skin infections. This work serves as an important first step in guiding treatment strategies for invasive GAS infections by mitigating severe immune responses during infection.

References

1. Chamat-Hedemand S, Dahl A, Østergaard L, et al. Prevalence of Infective Endocarditis in Streptococcal Bloodstream Infections Is Dependent on Streptococcal Species. Circulation. American Heart Association; **2020**; 142(8):720–730.

2. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis. Elsevier; **2005**; 5(11):685–694.

3. Johnson AF, LaRock CN. Antibiotic Treatment, Mechanisms for Failure, and Adjunctive Therapies for Infections by Group A Streptococcus. Front Microbiol. **2021**; 12:760255.

4. LaRock CN, Todd J, LaRock DL, et al. IL-1β is an innate immune sensor of microbial proteolysis. Sci Immunol. **2016**; 1(2):eaah3539.

5. LaRock DL, Russell R, Johnson AF, Wilde S, LaRock CN. Group A Streptococcus Infection of the Nasopharynx Requires Proinflammatory Signaling through the Interleukin-1 Receptor. Infect Immun [Internet]. **2020** [cited 2021 Jun 7]; 88(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7504964/

6. Wilde S, Olivares KL, Nizet V, Hoffman HM, Radhakrishna S, LaRock CN. Opportunistic Invasive Infection by Group A Streptococcus During Anti–Interleukin-6 Immunotherapy. J Infect Dis. **2020**; 223(7):1260–1264.

7. Goldmann O, Köckritz-Blickwede M von, Höltje C, Chhatwal GS, Geffers R, Medina E. Transcriptome Analysis of Murine Macrophages in Response to Infection with Streptococcus pyogenes Reveals an Unusual Activation Program. Infect Immun. **2007**; 75(8):4148–4157.

8. Rose-John S, Winthrop K, Calabrese L. The role of IL-6 in host defence against infections: immunobiology and clinical implications. Nat Rev Rheumatol. **2017**; 13(7):399–409.

9. Akoumianaki T, Vaporidi K, Diamantaki E, et al. Uncoupling of IL-6 signaling and LC3-associated phagocytosis drives immunoparalysis during sepsis. Cell Host Microbe [Internet]. **2021** [cited 2021 Jul 5]; . Available from: https://www.sciencedirect.com/science/article/pii/S1931312821002808

10. Luo Y, Zheng SG. Hall of Fame among Pro-inflammatory Cytokines: Interleukin-6 Gene and Its Transcriptional Regulation Mechanisms. Front Immunol. **2016**; 7:604.

11. Kang S, Tanaka T, Narazaki M, Kishimoto T. Targeting Interleukin-6 Signaling in Clinic. Immunity. Elsevier; **2019**; 50(4):1007–1023.

12. Hamilton SM, Bayer CR, Stevens DL, Bryant AE. Effects of Selective and Nonselective Nonsteroidal Anti-inflammatory Drugs on Antibiotic Efficacy of Experimental Group A Streptococcal Myonecrosis. J Infect Dis. **2014**; 209(9):1429–1435.

13. Cole JN, Pence MA, Köckritz-Blickwede M von, et al. M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A Streptococcus. mBio. **2010**; 1(4):e00191-10.

14. Wessels MR, Moses AE, Goldberg JB, DiCesare TJ. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc Natl Acad Sci U S A. **1991**; 88(19):8317–8321.

15. Ashbaugh CD, Moser TJ, Shearer MH, White GL, Kennedy RC, Wessels MR. Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. Cell Microbiol. **2000**; 2(4):283–292.

16. Saito M, Kajiwara H, Ishikawa T, et al. Delayed Onset of Systemic Bacterial Dissemination and Subsequent Death in Mice Injected Intramuscularly with Streptococcus pyogenes Strains. Microbiol Immunol. **2001**; 45(11):777–786.

17. Fiebig A, Loof TG, Babbar A, et al. Comparative Genomics of Streptococcus pyogenes M1 isolates differing in virulence and propensity to cause systemic infection in mice. Int J Med Microbiol. **2015**; 305(6):532–543.

18. Collin M, Olsén A. Generation of a mature streptococcal cysteine proteinase is dependent on cell wall-anchored M1 protein. Mol Microbiol. **2000**; 36(6):1306–1318.

19. Ayala JC, Shafer WM. Transcriptional regulation of a gonococcal gene encoding a virulence factor (L-lactate permease). PLoS Pathog [Internet]. **2019** [cited 2021 Jun 11]; 15(12). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6957213/

20. Brissac T, Martínez E, Kruckow KL, et al. Capsule Promotes Intracellular Survival and Vascular Endothelial Cell Translocation during Invasive Pneumococcal Disease. mBio. 12(5):e02516-21.

21. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant Activity of *Centaurium erythraea* Infusion Evidenced by Its Superoxide Radical Scavenging and Xanthine Oxidase Inhibitory Activity. J Agric Food Chem. **2001**; 49(7):3476–3479.

22. Saito M, Ohga S, Endoh M, et al. H2O2-nonproducing Streptococcus pyogenes strains: survival in stationary phase and virulence in chronic granulomatous disease. Microbiology. Microbiology Society,; 147(9):2469–2477.

23. Lei B, Minor D, Feng W, et al. Tissue Tropism in Streptococcal Infection: Wild-Type M1T1 Group A Streptococcus Is Efficiently Cleared by Neutrophils Using an NADPH Oxidase-Dependent Mechanism in the Lung but Not in the Skin. Infect Immun [Internet]. **2019** [cited 2021 May 21]; 87(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6759303/

24. Li J, Liu G, Feng W, et al. Neutrophils select hypervirulent CovRS mutants of M1T1 group A Streptococcus during subcutaneous infection of mice. Infect Immun. **2014**; 82(4):1579–1590.

25. Henningham A, Döhrmann S, Nizet V, Cole JN. Mechanisms of group A Streptococcus resistance to reactive oxygen species. FEMS Microbiol Rev. **2015**; 39(4):488–508.

26. Cleary PP, Larkin A. Hyaluronic acid capsule: strategy for oxygen resistance in group A streptococci. J Bacteriol. **1979**; 140(3):1090–1097.

27. Hollands A, Pence MA, Timmer AM, et al. A Genetic Switch to Hypervirulence Reduces Colonization Phenotypes of the Globally Disseminated Group A Streptococcus M1T1 Clone. J Infect Dis. **2010**; 202(1):11–19.

28. Henningham A, Yamaguchi M, Aziz RK, et al. Mutual Exclusivity of Hyaluronan and Hyaluronidase in Invasive Group A Streptococcus. J Biol Chem. **2014**; 289(46):32303–32315.

29. Levin JC, Wessels MR. Identification of csrR/csrS, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. Mol Microbiol. **1998**; 30(1):209–219.

30. Chiappini N, Seubert A, Telford JL, et al. Streptococcus pyogenes SpyCEP Influences Host-Pathogen Interactions during Infection in a Murine Air Pouch Model. PLoS ONE. **2012**; 7(7):e40411.

31. Lee H-J, Woo Y, Hahn T-W, Jung YM, Jung Y-J. Formation and Maturation of the Phagosome: A Key Mechanism in Innate Immunity against Intracellular Bacterial Infection. Microorganisms [Internet]. **2020** [cited 2021 May 14]; 8(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7564318/

32. Thulin P, Johansson L, Low DE, et al. Viable Group A Streptococci in Macrophages during Acute Soft Tissue Infection. PLoS Med. **2006**; 3(3):e53.

33. Goldmann O, Rohde M, Chhatwal GS, Medina E. Role of Macrophages in Host Resistance to Group A Streptococci. Infect Immun. **2004**; 72(5):2956–2963.

34. Segal BH, Grimm MJ, Khan ANH, Han W, Blackwell TS. Regulation of innate immunity by NADPH oxidase. Free Radic Biol Med. **2012**; 53(1):72–80.

35. Coxon A, Rieu P, Barkalow FJ, et al. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. Immunity. **1996**; 5(6):653–666.

36. Wanidworanun C, Strober W. Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis. J Immunol Baltim Md 1950. **1993**; 151(12):6853–6861.

37. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta BBA - Mol Cell Res. **2011**; 1813(5):878–888.

38. Lin A, Loughman JA, Zinselmeyer BH, Miller MJ, Caparon MG. Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes infection. Infect Immun. **2009**; 77(11):5190–5201.

39. Sjölinder H, Lövkvist L, Plant L, et al. The ScpC protease of Streptococcus pyogenes affects the outcome of sepsis in a murine model. Infect Immun. **2008**; 76(9):3959–3966.

40. Wang J. Neutrophils in tissue injury and repair. Cell Tissue Res. **2018**; 371(3):531–539.

41. King KY, Horenstein JA, Caparon MG. Aerotolerance and Peroxide Resistance in Peroxidase and PerR Mutants of Streptococcus pyogenes. J Bacteriol. **2000**; 182(19):5290–5299.

42. Brenot A, King KY, Janowiak B, Griffith O, Caparon MG. Contribution of Glutathione Peroxidase to the Virulence of Streptococcus pyogenes. Infect Immun. **2004**; 72(1):408–413.

43. Crater DL, Rijn I van de. Hyaluronic acid synthesis operon (has) expression in group A streptococci. J Biol Chem. **1995**; 270(31):18452–18458.

44. Stollerman GH, Dale JB. The importance of the group a streptococcus capsule in the pathogenesis of human infections: a historical perspective. Clin Infect Dis Off Publ Infect Dis Soc Am. **2008**; 46(7):1038–1045.

45. Vega LA, Sanson MA, Shah BJ, Flores AR. Strain-Dependent Effect of Capsule on Transmission and Persistence in an Infant Mouse Model of Group A Streptococcus Infection. Infect Immun. **2020**; 88(4).

46. Gallin JI, Buescher ES. Abnormal regulation of inflammatory skin responses in male patients with chronic granulomatous disease. Inflammation. **1983**; 7(3):227–232.

47. Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radic Biol Med. **1996**; 21(3):335–348.

Author Contributions- Chapter 3

Conceptualization: Christopher LaRock, Shyra Wilde

Methodology: Christopher LaRock, Shyra Wilde

Validation: Christopher LaRock, Shyra Wilde, Anders Johnson

Analysis: Christopher LaRock, Shyra Wilde

Investigation: Christopher LaRock, Shyra Wilde, Anders Johnson

Resources: Christopher LaRock

Writing- draft: Christopher LaRock, Shyra Wilde

Writing- revisions: Christopher LaRock, Shyra Wilde

Visualization: Christopher LaRock, Shyra Wilde

Supervision: Christopher LaRock

Project Administration: Christopher LaRock

Funding Acquisition: Christopher LaRock

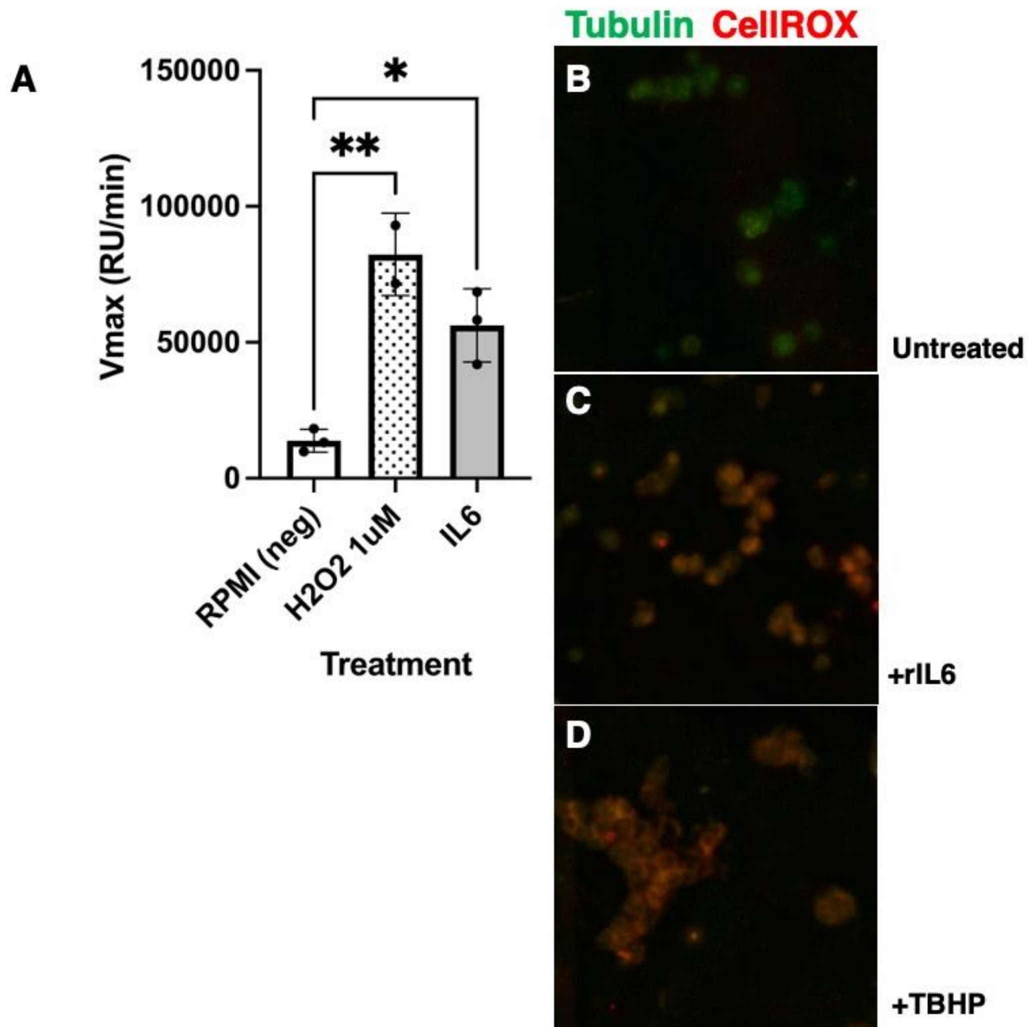

Figure 1. IL-6 induces ROS. (A). Exogenous IL-6 is sufficient to induce ROS production in human macrophages. V_{max} indicates the highest change in oxidation between readings, which occurred at 30min post infection. Statistical significance was measured by one-way ANOVA with _ multiple comparisons. (B-D). THP-1 monocytes were treated with either IL6 (C) or TBHP (D) and compared to an untreated control (B) using fluorescent microscopy. Cells were stained with anti-tubulin (control; green) and CellROX deep red to measure phagocytic ROS production.

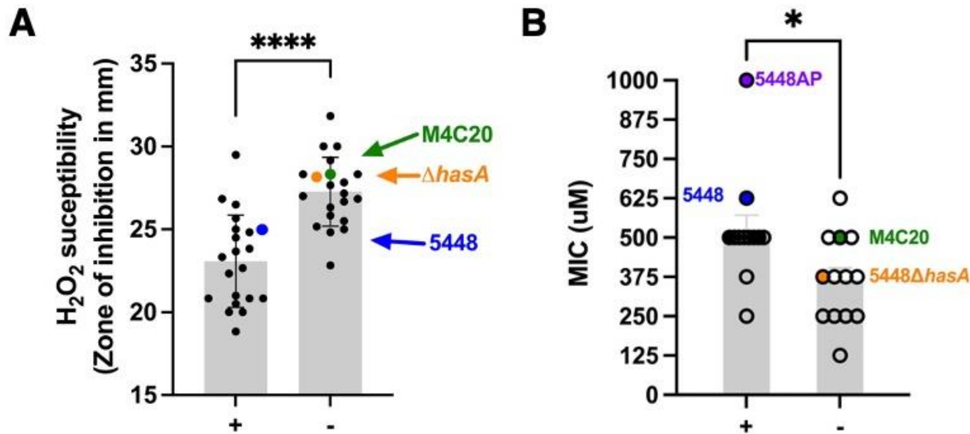

Figure 2. Non-encapsulated strains of GAS are more susceptible to peroxide. (A). Hydrogen peroxide shows greater zones of inhibition for non-encapsulated (-) strains of GAS (n=21) than encapsulated (+) strains of GAS (n=20). Each data point represents a single clinical isolate and is an average of two experiments with three technical replicates. (B). Encapsulated strains of GAS have an increased minimum inhibitory concentration against peroxide. Statistical significance was determined using an unpaired t-test. * = $p<0.05$, **** = $p<0.0001$.

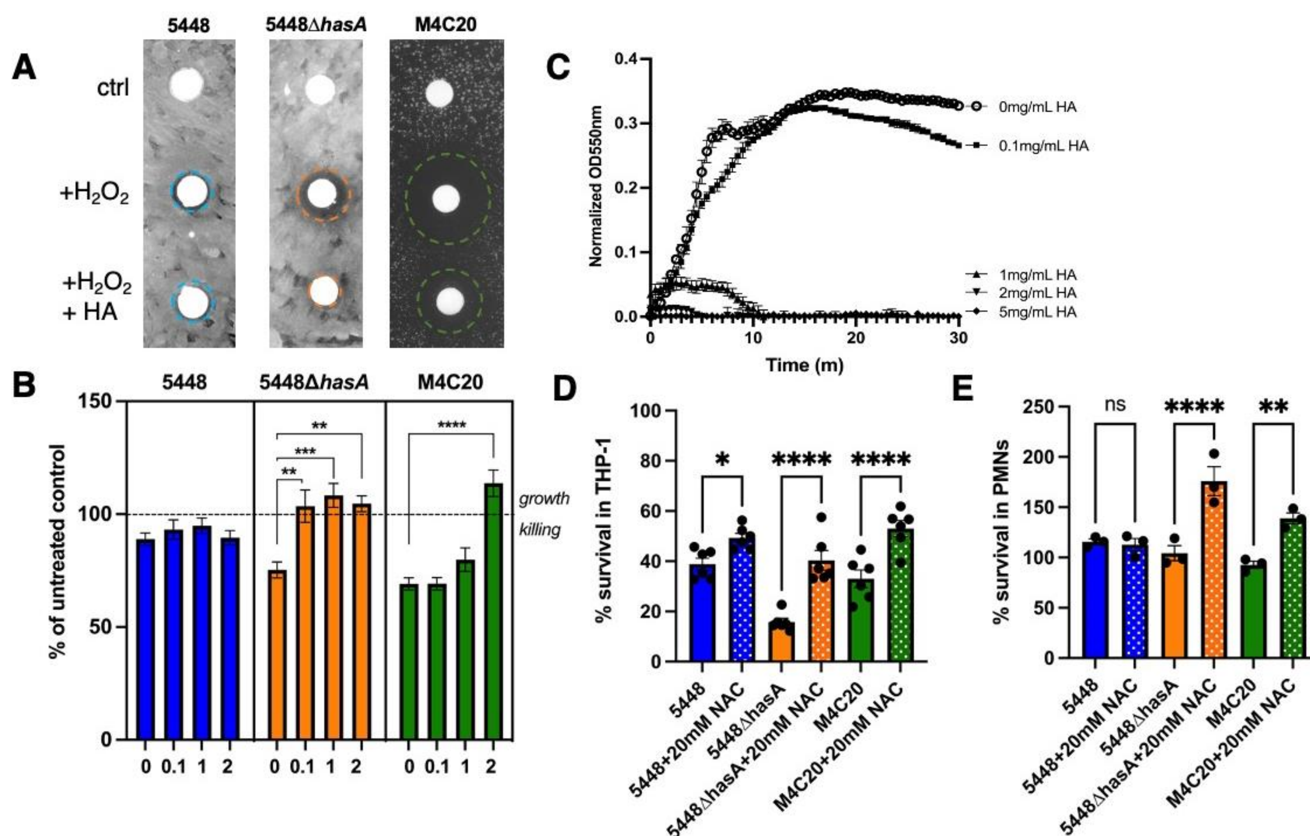

Figure 3. Hyaluronic acid directly protects against peroxide-mediated killing. (A). Addition of 1mg/ml hyaluronic acid to hydrogen peroxide is sufficient to reduce killing of acapsulated GAS on THY plates. Images are representative of three separate experiments. Dashed lines outline the zone of clearance. (B). Exogenous capsule at concentrations of 0.1mg/mL or greater were sufficient to confer protection against peroxide in non-encapsulated strains of GAS (5448Δ*hasA*; orange and M4C20; green). Wild-type strain 5448 (blue) was unaffected by exogenous hyaluronic acid. Data are expressed as the mean +/- SEM. Statistical analysis was done by one-way ANOVA with Tukey's multiple comparisons posttest. **$p<0.005$; ***$p<0.0005$; ****$p<0.0001$ (C). Hyaluronic acid solutions of at least 1mg/mL were sufficient to protect NBT from oxidation. Data were normalized by subtracting the OD550 of parallel reactions with no PMS added to account for background colorimetric changes. Data represent three separate experiments with three technical

replicates per experiment. Data are shown as mean +/-SEM. (D). Addition of 20mM NAC, a ROS scavenger, rescues growth of acapsulated GAS within human macrophages. Statistical significance was measured by ordinary one-way ANOVA with Sidak's multiple comparisons. * = $p<0.05$; **** = $p<0.0001$ (E). Addition of 20mM NAC decreases killing of acapsulated GAS by primary human neutrophils. Statistical significance was measured using ordinary one-way ANOVA with Sidak's multiple comparisons. ** = $p<0.005$, **** = $p<0.0001$, ns = no significance. Data are expressed as the mean +/- SEM.

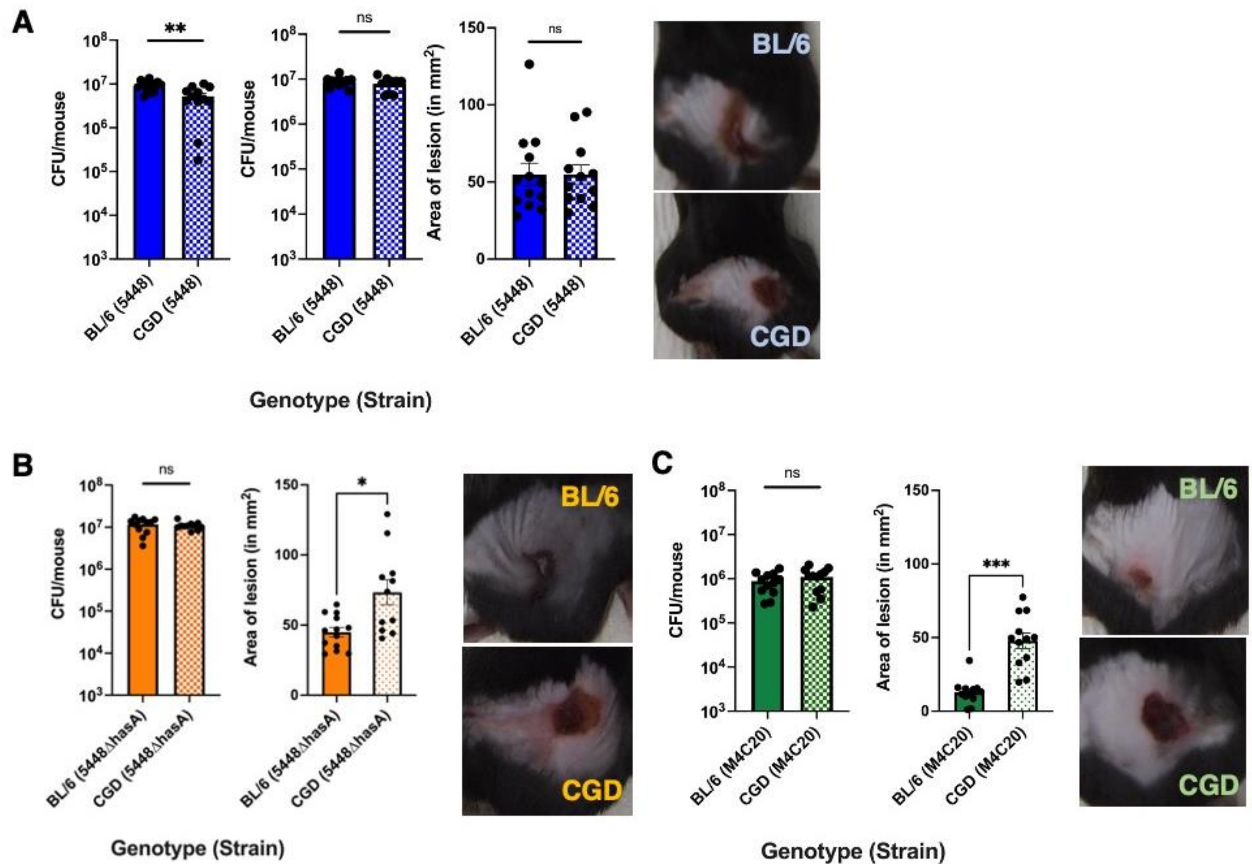

Figure 4. Lesion size, but not bacterial load, is both ROS- and capsule-dependent *in vivo*. Wild-type C57BL/6 or gp91$^{phox-/-}$ (CGD) mice were injected intradermally with 10^8 CFU of GAS strain 5448 (A), 5448Δ*hasA* (B), or M4C20 (C). Infections proceeded for 48h and CFU was enumerated from homogenized whole lesions by dilution plating. Lesion size was quantified using ImageJ. Each data point represents a single mouse. Data are expressed as mean +/- SEM. Statistical significance was determined by unpaired two-tailed t-test. *= p<0.05, ns= no significance. Representative images of lesions from each group are also included.

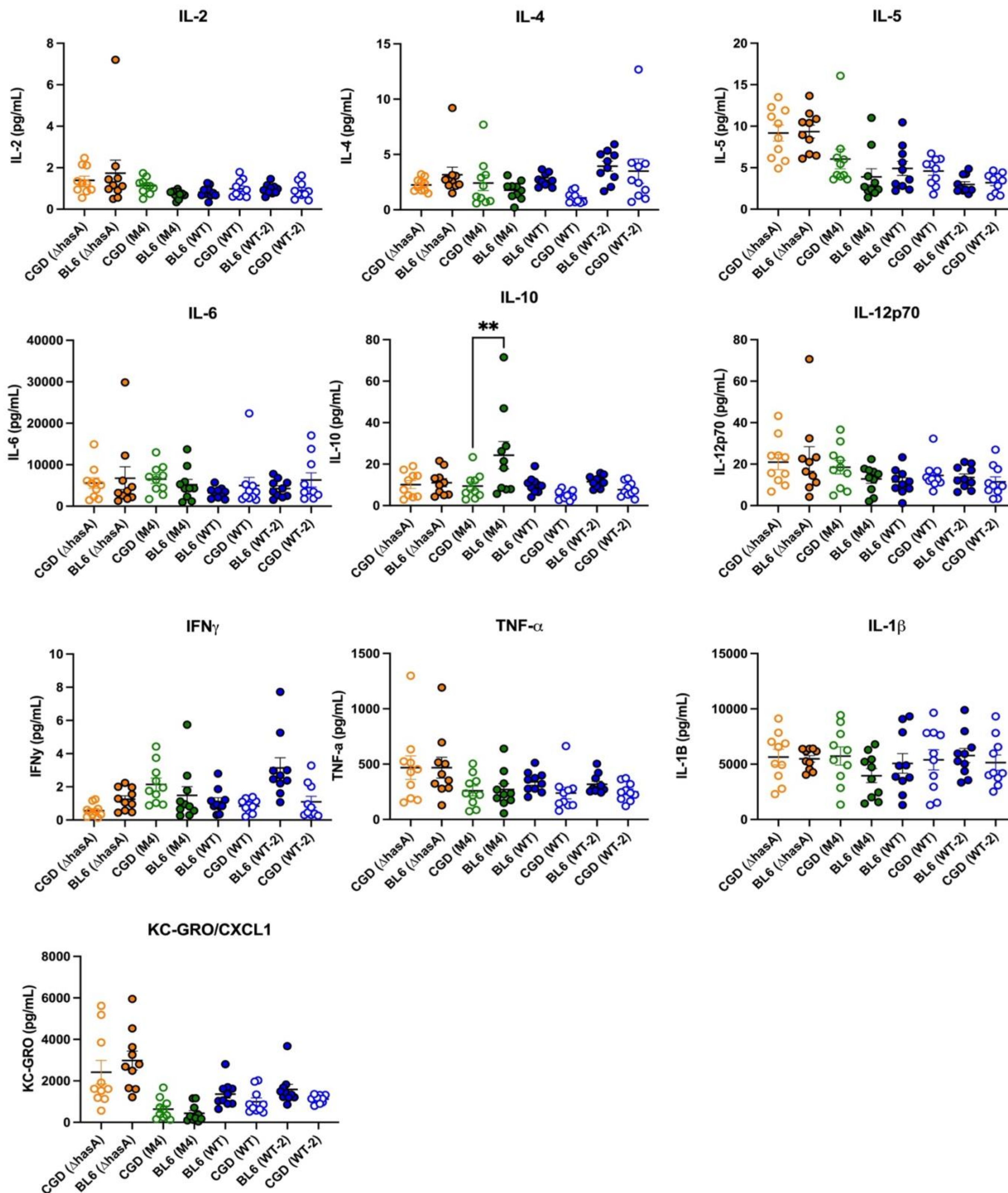
IL-2
IL-2 (pg/mL)
IL-4
IL-4 (pg/mL)
IL-5
IL-5 (pg/mL)
IL-6
IL-6 (pg/mL)
IL-10
**
IL-10 (pg/mL)
IL-12p70
IL-12p70 (pg/mL)
IFNγ
IFNy (pg/mL)
TNF-α
TNF-a (pg/mL)
IL-1β
IL-1B (pg/mL)
KC-GRO/CXCL1
KC-GRO (pg/mL)
CGD (ΔhasA)
BL6 (ΔhasA)
CGD (M4)
BL6 (M4)
BL6 (WT)
CGD (WT)
BL6 (WT-2)
CGD (WT-2)

Figure 5. Wilde-type and gp91$^{phox-/-}$ mice produce similar levels of pro-inflammatory cytokines in skin lesions. Cell-free supernatant was taken from each homogenized tissue sample used in figure 4 and pro-inflammatory cytokine levels were measured using the MSD V-plex pro-inflammatory cytokine panel 1. Each point represents a lesion from a single mouse. Data are expressed as the mean +/- SEM. Statistical analysis was performed using ordinary one-way ANOVA with Sidak's multiple comparisons test. ** = $p<0.005$

A

Control 5448 5448Δ*hasA* M4C20

BL/6 (IHC)

Hoechst αMPO αGAS

BL/6 (DAB)

MPO+ MPO-

B

Control 5448 5448Δ*hasA* M4C20

CGD (IHC)

Hoechst αMPO αGAS

CGD (DAB)

MPO+ MPO-

C

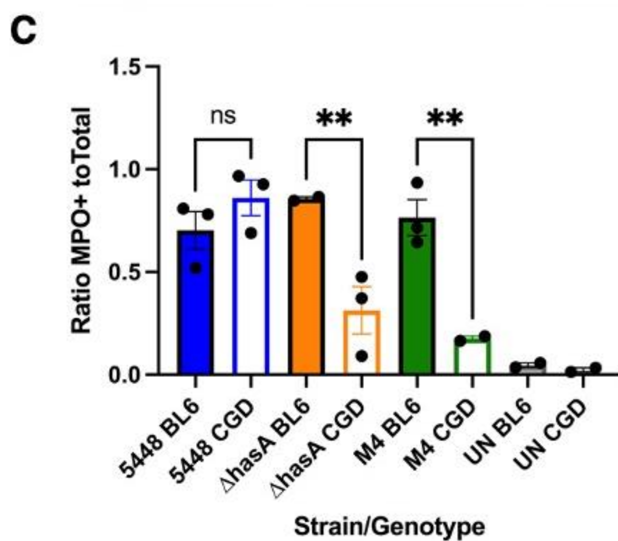

Figure 6. Skin lesions from gp91$^{phox-/-}$ mice infected with acapsulated GAS have decreased levels of myeloperoxidase compared to wild-type mice. Myeloperoxidase (MPO) production was determined using immunohistochemistry with antibodies probing for MPO (Abcam). [DAB stain, get protocol from CTPSR]. (A). Representative images of lesions stained with anti-MPO (n=3 for each group). (B). MPO levels were quantified using color deconvolution in ImageJ. Values represent the ratio of the intensity of DAB-stained cells to the intensity of total cells and are plotted as the mean +/- SEM. Each data point represents a single lesion. Statistical significance was determined using one-way ANOVA with Sidak's multiple comparisons test. ns=no significance, **p=<0.01.

Chapter 4

Concluding Remarks and Future Directions

Shyra Wilde

Concluding Remarks

Group A *Streptococcus* (GAS) remains a global threat to human health as severe infections from GAS cause approximately 500,000 deaths annually [1]. The current standard of care for invasive GAS skin infections includes early and aggressive antibiotic therapy, surgical debridement of necrotic or infected tissue, or treatments like intravenous immunoglobulin (IVIG) that have seen varying levels of success [2,3]. Yet, invasive GAS infections still carry a high mortality rate. There have been many confounding variables regarding the development of vaccines against GAS [4], making vaccination against GAS an infeasible option; thus, effective treatment options are critical for reducing morbidity and mortality. Most severe diseases caused by GAS such as toxic shock, scarlet fever, and sepsis are characterized by exacerbated and highly inflammatory immune responses to infection [5] , highlighting the importance of identifying the immune factors necessary to restrict GAS infection.

This work has advanced our fundamental understanding of how our immune system responds to and kills GAS. Previous work has highlighted IL-1-inhibiting biologics as a risk factor for the development of severe GAS infections, particularly necrotizing fasciitis [8]; IL-6-inhibiting biologics have also been implicated as a risk factor, but this could not be recapitulated in mice infected with GAS strain 5448. Serotype *emm*4 GAS strain M4C20 was isolated from a pediatric patient with a GAS bloodstream infection, and the patient, rather unusually, was also taking IL-6-inhibiting biologics to manage an autoimmune disorder. Further analysis of post-marketing surveillance data revealed an association between IL-6-inhibiting biologics and incidence of invasive GAS infections. In chapter 2 of this dissertation, we were able to demonstrate that blocking IL-6 receptors both *in vitro* and *in vivo* was sufficient to increase the pathogenicity of strain M4C20, but these conditions were not able to increase the pathogenicity of the hyperinvasive

GAS strain 5448. Addition of exogenous IL-6 *in vitro* was also sufficient to enhance killing of strain M4C20 and other *emm*4 GAS but was not sufficient for killing strain 5448, as well as other *emm*1 GAS clinical isolates. This observation highlighted new insights into how GAS is killed within the host, as well as how hypervirulent strains of GAS can evade host killing.

Chapter 3 of this dissertation further probed the molecular mechanisms at play during IL-6-mediated killing of GAS, as well as the mechanism by which some strains of GAS can evade killing. We identified that IL-6-mediated killing occurs through the production of ROS. We also identify the hyaluronic acid capsule as a mechanism by which some strains of GAS can evade ROS-mediated killing and reveal a role for hyaluronic acid capsule as a direct antioxidant *in vitro*. Acapsulated GAS were significantly more susceptible to peroxide *in vitro*, and the addition of ROS scavengers was sufficient to rescue acapsulated GAS from killing by phagocytes.

In vivo, gp91$^{phox-/-}$ mice that were deficient in phagocytic ROS production did not have increased bacterial loads within skin lesions when infected with acapsulated GAS, which contrasted with our *in vitro* findings. Bacterial load was similar for infections with strains 5448, capsule mutant 5448Δ*hasA*, and non-M1T1 strain M4C20, indicating that gp91^{phox}-dependent ROS was not essential for GAS killing in this infection model, nor was virulence capsule-dependent (Figure 1). acapsulated GAS produced small lesions in wild-type mice, but lesion size was at least doubled in gp91$^{phox-/-}$ mice infected with the same strains (Figure 1). This data reveals that ROS production and capsule have effects on wound healing *in vivo* during GAS infections.

Immunohistochemical analysis showed that GAS were still able to colocalize with activated neutrophils in both wild-type and gp91$^{phox-/-}$ mice. Neutrophil restriction of GAS prevents bacterial dissemination that would cause systemic infections [6]. Interestingly, acapsulated GAS induced lower levels of myeloperoxidase (MPO) in gp91$^{phox-/-}$ mice, which is indicative of

activated neutrophils (Figure 1). Activation and degranulation of neutrophils release many effectors that could potentially contribute to tissue damage and histopathological effects [7]. It is unclear whether the decreased number of MPO-positive neutrophils is due to larger numbers of dead neutrophils, a consequence of deficient ROS production by gp91$^{phox-/-}$ mice, or if acapsulated GAS prevent neutrophil activation through an unknown mechanism. Further studies are required to elucidate this mechanism, which are outlined below.

Scientific Implications and Future Directions

Many questions remain unanswered about the specific impact of IL-6 and ROS on killing of GAS *in vivo*. Scavenging ROS is sufficient to rescue growth of acapsulated GAS *in vitro*, but these data are discordant with the phenotype observed *in vivo*. Instead, we find that ROS is not essential for killing GAS during *in vivo* skin infections. However, we demonstrate that both ROS and capsule production by GAS influence wound healing in our skin infection model; in the absence of ROS, wound healing is impaired when gp91$^{phox-/-}$ mice are infected with acapsulated GAS as these mice have significantly larger lesions at the site of infection. This data points toward a model that suggests ROS is not essential for killing GAS in the skin but does have an important role in wound formation and healing, which is further influenced by capsule production in some strains of GAS.

In order to better understand the immune responses that drive pathology *in vivo*, continuing studies will use fluorophore-activated cell sorting (FACS) to examine the cellular response in skin lesions. More specifically, we hope to identify any differences in cell population composition and their activity between wild-type and gp91$^{phox-/-}$ mice, as well as any differences between mice infected with encapsulated or acapsulated GAS. FACS utilizes the light-scattering patterns of

fluorescently labeled cell populations to sort heterogeneous populations of cells into individual groups. These groups can be further sorted and characterized using carefully selected cell surface markers, conjugated to a unique fluorescent dye, that reveal cell identities or the activities of a particular cell type. We will begin by identifying leukocytes using an anti-CD45 antibody, then further separating this population into macrophages (F4/80), neutrophils (Ly6G, CD11b), dendritic cells (CD11c), and T-cells (CD3). Based on prior literature, we expect to find large amounts of neutrophils within skin lesions, but we will likely also recover large numbers of macrophages [8,9].

We are primarily interested in characterizing the neutrophil population within skin lesions. Neutrophils play a critical role in the killing of GAS and contribute substantially to phenotypes observed during infection [7]. Within our neutrophil subset, we will first use a viability stain to quantify levels of live and dead cells; based on prior literature, it is possible that we will see many necrotic neutrophils at the site of infection [10]. Since MPO levels were decreased in gp91$^{phox-/-}$ mice infected with acapsulated GAS, it is possible that this is due to an increase in dead cell populations within the lesion. We will also use markers for MPO (neutrophil activation), CD63 (to measure neutrophil degranulation), and CXCR1 (neutrophil chemotaxis/recruitment). Primary (or azurophilic) granules contain most of the antimicrobial effectors contained within neutrophils, including myeloperoxidase [11]. Secondary granules release lactoferrin [11]. Release of these effectors could cause serious histopathological effects and explain differences between our experimental groups [7].

To further strengthen our *in vitro* observations, we will clone the *hasABC* operon, which controls capsule production in GAS, to allow expression from a plasmid. This plasmid will be used to restore capsule production in the 5448Δ*hasA* mutant strain, as well as the naturally acapsulated

M4C20 strain. While both these strains are fully sequenced, and the Δ*hasA* mutant has previously been characterized and widely published [12–19], complementation will help exclude the possibility of off-target mutations. Capsule-complemented *emm*4 strains have been successfully made [20], but not with the locus from 5448, or complementation within 5448Δ*hasA* or M4C20. These complemented strains will be tested *in vitro* for susceptibility to phagocyte ROS, and we expect that complementation will enhance survival of acapsulated GAS strains, potentially above wide-type strains if expression is higher. These strains can also be used *in vivo* to determine whether the level of capsule production impacts wound healing in an intradermal infection model.

Capsule is most known for its protection against opsonization by phagocytes [21]. Encapsulated GAS can also survive more efficiently within phagocytes [22–25], promoting long-term colonization. Capsule also engages CD44 receptors on epithelial and some leukocytes [18,19,26], which may advance dissemination of encapsulated GAS into deeper tissues to cause systemic infection. In our *in vitro* infection model, we anticipate that the addition of capsule to acapsulated GAS will decrease the number of phagocytosed bacteria but prolong the survival of bacteria that do become internalized. We also anticipate that the addition of capsule to acapsulated GAS will have a less profound phenotype in the presence of ROS-scavenging drugs like NAC. *In vivo*, we expect that *hasABC*-complemented GAS will produce similarly sized lesions as the wild-type 5448 strain in wild-type mice; we do not expect any significant changes in bacterial load at the site of infection, since this did not appear to be capsule-dependent in our initial studies. In gp91$^{phox-/-}$ mice, we anticipate further delayed wound healing.

Clinical Implications

The emergence of acapsulated GAS strains capable of causing severe infections has challenged the essentiality of capsule during infection [27–32]. Although some serotypes, such as M89, have acquired compensatory mechanisms such as increased cytotoxin production [27,30], others, such as M4, have not developed these mutations or acquired virulence factors. Thus, how acapsulated GAS are able to persist in the population is a major question regarding GAS pathogenesis. M4 GAS have been increasingly associated with pharyngeal colonization [31]; decades of research have shown that GAS isolated from asymptomatic carriers is often acapsulated and is either completely incapable of producing capsule or only produces small amounts [33]. Thus, the loss of capsule production may be beneficial for pharyngitis-causing strains of GAS such as M4, as it can be more effectively internalized and persist within host cells [34,35]. This may also explain how acapsulated GAS can survive despite being extraordinarily sensitive to ROS- they simply induce less ROS during infection, and capsule would not be essential for protecting against ROS-mediated damage in that instance.

One major question that remains regarding GAS pathogenesis is why some strains are associated with a specific set of disease manifestations- that is, which genetic factors in both the bacterium and host determine tropism for GAS? Recent studies identify ROS as a potential factor for determining cell tropism [10]. The skin has been shown to be a favorable niche for GAS, which produce less ROS than other sites such as the lungs [10], ultimately providing a source of replication and dissemination for GAS. The lungs, however, induce a large influx of neutrophils and effectively clear GAS by the production of ROS [10]. This, potentially, is one of the factors that makes the lung a site that GAS does not ordinarily colonize or infect, despite its ability to grow in many other sites of the human body. This research confirms this hypothesis, as clearance

of GAS from skin infections does not appear to be ROS-dependent (Figure 1). The encapsulated GAS strains that typically produce devastating invasive infections are more equipped to do so with the production of capsule and the other many virulence factors that acapsulated serotypes of GAS have not acquired; hence, acapsulated GAS may opt for long-term pharyngeal colonization.

Acapsulated GAS such as M4 may be able to colonize the pharynx even if they are more susceptible to ROS-mediated killing, if the pharynx does not have the ability to support a local accumulation of ROS like the lung [14,18,19]. Since the pharynx would provide an effective niche to colonize long-term, this could explain an expansion in the frequency of M4-type GAS: acapsulated bacteria are better internalized and are thereby shielded from killing by penicillin, which in the form of amoxicillin is one of the more commonly used antibiotics for the treatment of GAS pharyngitis [36,37]. Capsule can promote adherence to CD44-expressing cells [26,38], so there appears to be redundant, possibly competing, mechanisms for gaining cell entry [39–44]. Nonetheless, our recent results with a large panel of isolates find that the degree of encapsulation negatively correlates with internalization, and subsequent induction of GSDMA-dependent pyroptosis within keratinocytes [45]. Altogether, the ability of capsule to modulate internalization in the pharynx is likely to have a major role in promoting antibiotic failure, persistent colonization, and asymptomatic transmission.

Therapeutic Considerations

Despite advances in medical treatment of invasive GAS infections, mortality is still alarmingly high, approximately 20% [46]. Death from invasive GAS infections occurs rapidly, often within seven days of diagnosis [46]. Treatment of invasive infections uses a combination of approaches, typically including antibiotic therapies and early surgical interventions [36].

Antibiotic therapies typically consist of either penicillin or amoxicillin in conjunction with clindamycin to neutralize the toxin activity of GAS [47]. Antibiotic resistance has traditionally not been an issue for GAS, although reports of isolates with reduced susceptibility to beta-lactams have been noted via mutations in the pbp2x protein [48,49]. Penicillin has been a cornerstone of GAS treatment for some time, but antibiotic therapies alone do not suffice, partially due to intracellular niches of bacteria as reviewed in previous sections (also reviewed in [50]). Treatment is further complicated due to occlusion of blood vessels during soft tissue infections, preventing intravenous antibiotics from fully penetrating the infected area [51,52].

Advances in diagnostic medicine have allowed us to identify both risk factors and biomarkers that may be indicative of prognoses during necrotizing soft tissue infections (NSTIs). A recent study identified multiple elevated serum biomarkers that were indicative of a Type II NSTI (caused by beta-hemolytic streptococci), but CXCL10/IP-10, a chemoattractant for macrophages, appeared to be the most useful for identifying a Type II NSTI [53]. This study was also able to identify markers of Type II NSTIs that were associated with septic shock outcomes, and IL-6 was the most highly associated with septic shock [53]. This information can be used to inform physicians and provide more rapid treatment options to those who exhibit serum profiles reflective of Type II NSTIs and give a more accurate prognosis. This work may also serve as an aid in symptom management, as mortality during GAS infections is associated with aberrant immune responses (reviewed in [54]). Risk factors for NSTIs caused by GAS remain unclear, as GAS can cause necrotizing infections in previously healthy individuals [48,49].

An ideal treatment for Type II NSTIs would consist of antibiotic therapy, to eradicate a majority of bacteria present, paired with a less invasive therapeutic option; approximately 20% of survivors of such infections require amputations that drastically alter the quality of life for the

patient [46]. The antibiotic rifampin has been used due to is ability to target intracellular bacteria and is often used for antibiotic treatment of *Mycobacterium tuberculosis* [55]; a recent case study has shown success with amoxicillin and adjunctive rifampin treatment to treat an NSTI caused by an M1 strain of GAS [56]. This therapy was adapted to an *in vitro* organotypic skin tissue model and was shown to be successful with multiple M types of GAS [56].

Hyperbaric oxygen therapy (HBOT) has been used to treat some NSTIs with varying levels of success [57–60]. The premise of HBOT is to administer pure oxygen at higher-than-average atmospheric pressures to supply the blood with oxygen and advance wound healing. The research in my dissertation further provides a case for HBOT: since gp91$^{phox-/-}$ mice experienced increases in wound size when infected with GAS (Figure 1), this points toward ROS as having a critical role in tissue healing and wound formation. The regeneration of oxygen molecules may provide aid by limiting pathology during infection and potentially aiding in the administration of other therapeutics, especially since blood vessel occlusion has been observed during necrotizing GAS infections [51,52]. In one study, mortality from NSTIs was nearly half in those treated with HBOT when compared to those who did not receive HBOT [59].

Summary

In conclusion, we have identified a molecular mechanism for IL-6-mediated killing of GAS, the production of ROS, as well as a mechanism for GAS to evade this strategy, via production of capsule. This study highlights i) new risk factors for invasive GAS infections, ii) a new mechanism by which hyaluronic acid capsule is protective for GAS, and iii) a new understanding of which immune factors drive pathology during invasive GAS skin infections. At minimum, we can now identify at least in part the root cause of histopathological effects seen during infection. We are

hopeful that this research can be used to guide the design of new therapeutics or combination therapies that will reduce the morbidity associated with invasive GAS skin infections.

References

1. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis. Elsevier; **2005**; 5(11):685–694.

2. Linnér A, Darenberg J, Sjölin J, Henriques-Normark B, Norrby-Teglund A. Clinical Efficacy of Polyspecific Intravenous Immunoglobulin Therapy in Patients With Streptococcal Toxic Shock Syndrome: A Comparative Observational Study. Clin Infect Dis. **2014**; 59(6):851–857.

3. Shah SS, Hall M, Srivastava R, Subramony A, Levin JE. Intravenous Immune Globulin in Children with Streptococcal Toxic Shock Syndrome. Clin Infect Dis Off Publ Infect Dis Soc Am. **2009**; 49(9):1369–1376.

4. Beall B, Van Beneden C. Challenges to Vaccine Development: The Diversity of Group A Streptococcal Strains Among Varied Climates and Global Regions. J Infect Dis. **2020**; 221(9):1394–1397.

5. Waddington CS, Snelling TL, Carapetis JR. Management of invasive group A streptococcal infections. J Infect. Elsevier; **2014**; 69:S63–S69.

6. Storisteanu DML, Pocock JM, Cowburn AS, et al. Evasion of Neutrophil Extracellular Traps by Respiratory Pathogens. Am J Respir Cell Mol Biol. **2017**; 56(4):423–431.

7. Wang J. Neutrophils in tissue injury and repair. Cell Tissue Res. **2018**; 371(3):531–539.

8. LaRock DL, Russell R, Johnson AF, Wilde S, LaRock CN. Group A Streptococcus Infection of the Nasopharynx Requires Proinflammatory Signaling through the Interleukin-1 Receptor. Infect Immun [Internet]. **2020** [cited 2021 Jun 7]; 88(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7504964/

9. Chiappini N, Seubert A, Telford JL, et al. Streptococcus pyogenes SpyCEP Influences Host-Pathogen Interactions during Infection in a Murine Air Pouch Model. PLoS ONE. **2012**; 7(7):e40411.

10. Lei B, Minor D, Feng W, et al. Tissue Tropism in Streptococcal Infection: Wild-Type M1T1 Group A Streptococcus Is Efficiently Cleared by Neutrophils Using an NADPH Oxidase-Dependent Mechanism in the Lung but Not in the Skin. Infect Immun [Internet]. **2019** [cited 2021 May 21]; 87(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6759303/

11. Lacy P. Mechanisms of Degranulation in Neutrophils. Allergy Asthma Clin Immunol Off J Can Soc Allergy Clin Immunol. **2006**; 2(3):98–108.

12. Falaleeva M, Zurek OW, Watkins RL, et al. Transcription of the Streptococcus pyogenes Hyaluronic Acid Capsule Biosynthesis Operon Is Regulated by Previously Unknown Upstream Elements. Infect Immun. **2014**; 82(12):5293–5307.

13. Saito M, Kajiwara H, Ishikawa T, et al. Delayed Onset of Systemic Bacterial Dissemination and Subsequent Death in Mice Injected Intramuscularly with Streptococcus pyogenes Strains. Microbiol Immunol. **2001**; 45(11):777–786.

14. Husmann LK, Yung DL, Hollingshead SK, Scott JR. Role of putative virulence factors of Streptococcus pyogenes in mouse models of long-term throat colonization and pneumonia. Infect Immun. **1997**; 65(4):1422–1430.

15. Jadoun J, Eyal O, Sela S. Role of CsrR, hyaluronic acid, and SpeB in the internalization of Streptococcus pyogenes M type 3 strain by epithelial cells. Infect Immun. **2002**; 70(2):462–469.

16. Cole JN, Pence MA, Köckritz-Blickwede M von, et al. M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A Streptococcus. mBio. **2010**; 1(4):e00191-10.

17. Cole JN, Aziz RK, Kuipers K, Timmer AM, Nizet V, Sorge NM van. A conserved UDP-glucose dehydrogenase encoded outside the hasABC operon contributes to capsule biogenesis in group A Streptococcus. J Bacteriol. **2012**; 194(22):6154–6161.

18. Ashbaugh CD, Moser TJ, Shearer MH, White GL, Kennedy RC, Wessels MR. Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. Cell Microbiol. **2000**; 2(4):283–292.

19. Wessels MR, Bronze MS. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. Proc Natl Acad Sci U S A. **1994**; 91(25):12238–12242.

20. Henningham A, Yamaguchi M, Aziz RK, et al. Mutual Exclusivity of Hyaluronan and Hyaluronidase in Invasive Group A Streptococcus. J Biol Chem. **2014**; 289(46):32303–32315.

21. Dale JB, Washburn RG, Marques MB, Wessels MR. Hyaluronate capsule and surface M protein in resistance to opsonization of group A streptococci. Infect Immun. **1996**; 64(5):1495–1501.

22. Bastiat-Sempe B, Love JF, Lomayesva N, Wessels MR. Streptolysin O and NAD-Glycohydrolase Prevent Phagolysosome Acidification and Promote Group A Streptococcus Survival in Macrophages. mBio. **2014**; 5(5):e01690-14.

23. Wilde S, Olivares KL, Nizet V, Hoffman HM, Radhakrishna S, LaRock CN. Opportunistic Invasive Infection by Group A Streptococcus During Anti–Interleukin-6 Immunotherapy. J Infect Dis. **2020**; 223(7):1260–1264.

24. Thulin P, Johansson L, Low DE, et al. Viable Group A Streptococci in Macrophages during Acute Soft Tissue Infection. PLoS Med. **2006**; 3(3):e53.

25. O'Neill AM, Thurston TLM, Holden DW. Cytosolic Replication of Group A Streptococcus in Human Macrophages. mBio. **2016**; 7(2):e00020-16.

26. Cywes C, Wessels MR. Group A Streptococcus tissue invasion by CD44-mediated cell signalling. Nature. **2001**; 414(6864):648–652.

27. Zhu L, Olsen RJ, Nasser W, Riva Morales I de la, Musser JM. Trading Capsule for Increased Cytotoxin Production: Contribution to Virulence of a Newly Emerged Clade of emm89 Streptococcus pyogenes. mBio. **2015**; 6(5):e01378-01315.

28. Whitehead BD, Smith HV, Nourse C. Invasive group A streptococcal disease in children in Queensland. Epidemiol Infect. **2011**; 139(4):623–628.

29. Turner CE, Abbott J, Lamagni T, et al. Emergence of a New Highly Successful Acapsular Group A Streptococcus Clade of Genotype emm89 in the United Kingdom. mBio. **2015**; 6(4):e00622.

30. Turner CE, Holden MTG, Blane B, Horner C, Peacock SJ, Sriskandan S. The Emergence of Successful Streptococcus pyogenes Lineages through Convergent Pathways of Capsule Loss and Recombination Directing High Toxin Expression. mBio. **2019**; 10(6):e02521-19.

31. Flores AR, Jewell BE, Olsen RJ, et al. Asymptomatic carriage of group A streptococcus is associated with elimination of capsule production. Infect Immun. **2014**; 82(9):3958–3967.

32. Flores AR, Chase McNeil J, Shah B, Van Beneden C, Shelburne SA. Capsule-Negative emm Types Are an Increasing Cause of Pediatric Group A Streptococcal Infections at a Large Pediatric Hospital in Texas. J Pediatr Infect Dis Soc. **2019**; 8(3):244–250.

33. Seastone CV. THE OCCURRENCE OF MUCOID POLYSACCHARIDE IN HEMOLYTIC STREPTOCOCCI OF HUMAN ORIGIN. J Exp Med. **1943**; 77(1):21–28.

34. Molinari G, Chhatwal GS. Invasion and survival of Streptococcus pyogenes in eukaryotic cells correlates with the source of the clinical isolates. J Infect Dis. **1998**; 177(6):1600–1607.

35. O'Seaghdha M, Wessels MR. Streptolysin O and its Co-Toxin NAD-glycohydrolase Protect Group A Streptococcus from Xenophagic Killing. PLOS Pathog. Public Library of Science; **2013**; 9(6):e1003394.

36. Stevens DL, Bisno AL, Chambers HF, et al. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. Clin Infect Dis Off Publ Infect Dis Soc Am. **2014**; 59(2):e10-52.

37. Mustafa Z, Ghaffari M. Diagnostic Methods, Clinical Guidelines, and Antibiotic Treatment for Group A Streptococcal Pharyngitis: A Narrative Review. Front Cell Infect Microbiol. **2020**; 10:563627.

38. Schrager HM, Rheinwald JG, Wessels MR. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. J Clin Invest. **1996**; 98(9):1954–1958.

39. Abbot EL, Smith WD, Siou GPS, et al. Pili mediate specific adhesion of Streptococcus pyogenes to human tonsil and skin. Cell Microbiol. **2007**; 9(7):1822–1833.

40. Tsai P-J, Chen Y-H, Hsueh C-H, et al. Streptococcus pyogenes induces epithelial inflammatory responses through NF-κB/MAPK signaling pathways. Microbes Infect. **2006**; 8(6):1440–1449.

41. Takahashi R, Radcliff FJ, Proft T, Tsai CJ-Y. Pilus proteins from Streptococcus pyogenes stimulate innate immune responses through Toll-like receptor 2. Immunol Cell Biol. **2022**; 100(3):174–185.

42. Darmstadt GL, Fleckman P, Rubens CE. Tumor necrosis factor-alpha and interleukin-1alpha decrease the adherence of Streptococcus pyogenes to cultured keratinocytes. J Infect Dis. **1999**; 180(5):1718–1721.

43. Darmstadt GL, Mentele L, Fleckman P, Rubens CE. Role of keratinocyte injury in adherence of Streptococcus pyogenes. Infect Immun. **1999**; 67(12):6707–6709.

44. Darmstadt GL, Fleckman P, Jonas M, Chi E, Rubens CE. Differentiation of cultured keratinocytes promotes the adherence of Streptococcus pyogenes. J Clin Invest. **1998**; 101(1):128–136.

45. LaRock DL, Johnson AF, Wilde S, Sands JS, Monteiro MP, LaRock CN. Group A Streptococcus induces GSDMA-dependent pyroptosis in keratinocytes. Nature. **2022**; 605(7910):527–531.

46. Madsen MB, Skrede S, Perner A, et al. Patient’s characteristics and outcomes in necrotising soft-tissue infections: results from a Scandinavian, multicentre, prospective cohort study. Intensive Care Med. **2019**; 45(9):1241–1251.

47. Andreoni F, Zürcher C, Tarnutzer A, et al. Clindamycin Affects Group A Streptococcus Virulence Factors and Improves Clinical Outcome. J Infect Dis. **2017**; 215(2):269–277.

48. Vannice KS, Ricaldi J, Nanduri S, et al. Streptococcus pyogenes pbp2x Mutation Confers Reduced Susceptibility to β-Lactam Antibiotics. Clin Infect Dis Off Publ Infect Dis Soc Am. **2020**; 71(1):201–204.

49. Musser JM, Beres SB, Zhu L, et al. Reduced In Vitro Susceptibility of Streptococcus pyogenes to β-Lactam Antibiotics Associated with Mutations in the pbp2x Gene Is Geographically Widespread. J Clin Microbiol. **2020**; 58(4):e01993-19.

50. Johnson AF, LaRock CN. Antibiotic Treatment, Mechanisms for Failure, and Adjunctive Therapies for Infections by Group A Streptococcus. Front Microbiol. **2021**; 12:760255.

51. Bryant AE, Bayer CR, Chen RYZ, Guth PH, Wallace RJ, Stevens DL. Vascular Dysfunction and Ischemic Destruction of Tissue in Streptococcus pyogenes Infection: The Role of Streptolysin O–Induced Platelet/Neutrophil Complexes. J Infect Dis. **2005**; 192(6):1014–1022.

52. Lukomski S, Montgomery CA, Rurangirwa J, et al. Extracellular Cysteine Protease Produced by Streptococcus pyogenes Participates in the Pathogenesis of Invasive Skin Infection and Dissemination in Mice. Infect Immun. **1999**; 67(4):1779–1788.

53. Palma Medina LM, Rath E, Jahagirdar S, et al. Discriminatory plasma biomarkers predict specific clinical phenotypes of necrotizing soft-tissue infections. J Clin Invest. 131(14):e149523.

54. Tsatsaronis JA, Walker MJ, Sanderson-Smith ML. Host responses to group a streptococcus: cell death and inflammation. PLoS Pathog. **2014**; 10(8):e1004266.

55. Zaw MT, Emran NA, Lin Z. Mutations inside rifampicin-resistance determining region of rpoB gene associated with rifampicin-resistance in Mycobacterium tuberculosis. J Infect Public Health. **2018**; 11(5):605–610.

56. Bergsten H, Palma Medina LM, Morgan M, et al. Adjunctive Rifampicin Increases Antibiotic Efficacy in Group A Streptococcal Tissue Infection Models. Antimicrob Agents Chemother. 65(11):e00658-21.

57. Levett D, Bennett MH, Millar I. Adjunctive hyperbaric oxygen for necrotizing fasciitis. Cochrane Database Syst Rev. **2015**; 1:CD007937.

58. Jallali N, Withey S, Butler PE. Hyperbaric oxygen as adjuvant therapy in the management of necrotizing fasciitis. Am J Surg. Elsevier; **2005**; 189(4):462–466.

59. Devaney B, Frawley G, Frawley L, Pilcher DV. Necrotising soft tissue infections: the effect of hyperbaric oxygen on mortality. Anaesth Intensive Care. **2015**; 43(6):685–692.

60. Nedrebø T, Bruun T, Skjåstad R, Holmaas G, Skrede S. Hyperbaric oxygen treatment in three cases of necrotizing infection of the neck. Infect Dis Rep. **2012**; 4(1):e21.

	5448	5448Δ*hasA*	M4C20
CFU	=	=	=
Lesion area	=	↑	↑
MPO	=	↓	↓

(BL6 vs. CGD mice)

Figure 1. Comparison of *in vivo* phenotypes during intradermal infection. Wild-type or gp91$^{phox-/-}$ (CGD) C57BL6 mice infected with either encapsulated (5448) or acapsulated (5448Δ*hasA*, M4C20) GAS had similar bacterial load within lesions (denoted by = sign). However, lesion area was significantly increased in gp91$^{phox-/-}$ mice infected with acapsulated GAS. Lesion area remained the same size when infected with encapsulated GAS, indicating that encapsulated GAS may promote less excessive pathology. Myeloperoxidase (MPO) production, which is an indicator of activated neutrophils, was decreased within lesions from gp91$^{phox-/-}$ mice infected with acapsulated GAS. Pathological effects were similar between wild-type and gp91$^{phox-/-}$ mice infected with encapsulated GAS strain 5448, highlighting the essentiality of both ROS production and capsule for wound healing.