THE EFFECT OF A NATURAL covS MUTATION ON VIRULENCE FACTOR
EXPRESSION AND INNATE IMMUNE EVASION IN A HYPERVERULENT
STRAIN OF GROUP A STREPTOCOCCUS

by

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Zachary William Stetzner

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Group A *Streptococcus* (GAS) is a highly versatile pathogen that is able to colonize multiple locations on the body, resulting in numerous diseases such as mild pharyngitis and the potentially lethal necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS). The high mortality rates associated with severe invasive diseases are particularly concerning. GAS strains isolated from patients with severe invasive infections frequently display hypervirulence, but the basis for this hypervirulence is not fully understood. The objective of this project is to elucidate the underlying mechanism behind this hypervirulent phenotype in a serotype M3 STSS isolate, MGAS315. A comparative study of MGAS315 and a serotype M28 puerperal sepsis isolate, MGAS6180, revealed that MGAS315 has a substantially higher capacity to invade soft tissue and inhibit neutrophil recruitment than MGAS6180 in a murine model of subcutaneous infection. Deletion of the platelet-activating factor (PAF) acetylhydrolase gene *sse* reduced MGAS315 skin invasion and innate immune evasion. These results cannot be explained by the proposal that the hypervirulence of MGAS315 is due to the acquisition of additional prophage-encoding virulence factors. *SsE* is negatively regulated by the two-component regulatory system CovR/S, and MGAS315 has a CovS*G457V* mutation compared with the *covS* gene of the serotype M1 isolate MGAS2221. We hypothesize that the CovS*G457V* mutation is responsible for the hypervirulence of MGAS315. To test this hypothesis, the mutated *covS* gene in MGAS315 was replaced with wild-type *covS*, resulting in MGAS315wt*covS*. The mRNA levels of CovR/S-controlled capsule synthase gene *hasA* and the IL-8 peptidase gene *spyCEP* in MGAS315wt*covS* were 24% and 3% of those in MGAS315, respectively. Repairing the *covS* mutation in MGAS315 also reduced the PAF acetylhydrolase activity in the culture supernatant and SsE production as measured by western blotting analysis. These results indicate that the CovS*G457V* mutation enhanced the expression of CovR/S-controlled virulence factors. More importantly, repairing the CovS*G457V* mutation attenuated the innate immune evasion, skin evasion, and virulence during infection. Collectively, this work demonstrates the CovS*G457V* mutation increases virulence factor expression and enhances innate immune evasion, thereby contributing to the hypervirulence of MGAS315.
INTRODUCTION

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is an extracellular, Gram-positive bacterium responsible for a variety of diseases ranging from mild cases of pharyngitis and pyoderma to highly invasive necrotizing fasciitis and streptococcal toxic shock syndrome (STSS)\(^1\). GAS infection is also highlighted by post-streptococcal immune sequelae resulting in acute rheumatic fever and glomerulonephritis. The clinical impact is significant as GAS is exclusively a human pathogen and maintains a narrow ecological in a human reservoir. Fortunately, *S. pyogenes* has maintained susceptibility to penicillin likely due to the inability to express β-lactamase\(^2\). Still, even with effective antibiotics, GAS infections continue to plague a substantial proportion of the world’s population.

**Epidemiology of GAS**

Since the resurgence of severe GAS infections in the mid-1980s, there have been approximately 10,000 cases reported in the United States annually\(^3,4\). In 2005, approximately 18.1 million people suffered from severe GAS infection accounting for 517,000 deaths annually; however, this number is likely much higher as many GAS infections go unreported\(^5\). In addition to these severe cases, over 700 million cases of superficial and self-limiting infections such as pharyngitis and pyoderma occur each year further contributing to increases in health care costs\(^5\). The prevalence of GAS is attributable to several factors responsible for transmission. GAS is transmitted via direct skin contact, droplets from pharyngeal carriers, and contaminated fomites.
Overcrowding has a considerable impact on spread as many cases of GAS have been documented on military bases and crowded households in New Zealand have reported increases in rheumatic fever\textsuperscript{6,7}.

Transmission of GAS and the disease manifestations tend to differ between urbanized and developing countries. Traditionally, in industrialized countries with temperate climates, GAS infections are most commonly associated with pharyngeal carriage and droplet spread\textsuperscript{8}. In contrast, a disproportionately high rate of pyoderma (≥70% in children) has been observed in aboriginal communities of northern Australia, where up to 14 different strains of GAS may be circulating through an individual community at any one time\textsuperscript{9,10}. Moreover, the prevalence of rheumatic heart disease is highest (5-7 cases/1000) in regions such as Sub-Saharan Africa where poor hygiene is common and access to medical treatment is limited\textsuperscript{11}. However, the infrastructure needed to monitor and accurately classify differences in GAS disease is relatively uncommon in these developing countries suggesting these numbers may, in fact, be underestimates of the actual GAS burden. This disparity in disease between socioeconomic classes has led to increased surveillance of GAS to elucidate what molecular markers are associated with particular disease types and identify the populations most commonly associated with those GAS strains.

\textit{Emm}-Gene Serotyping

While economic and environmental factors are indicators of specific cases of GAS, they are certainly not the sole determinants. In order to effectively monitor the spread of GAS and identify the variations in disease both geographically and in body site
preference, epidemiologists began using the cell surface M-protein to track incidences of GAS across the globe. More than 200 different emm-types have been documented and it is evident that GAS emm serotypes differ significantly between developing and developed countries\textsuperscript{12,13}. In low-income regions such as India and Ethiopia, the diversity of circulating emm-types is high and no one emm-type appears to dominate\textsuperscript{14,15}. In industrialized nations; however, a fewer number of circulating emm-types have been reported with even less dominant\textsuperscript{16}. Only 25 emm-types accounted for 90\% of the GAS isolates from urbanized countries\textsuperscript{16}.

Differences in emm-types exist not only between geographic and socioeconomic regions but also in tissue tropism. This variation in emm-typing can be further classified based on emm chromosomal architecture or “emm patterns” which have been shown to be strong biological markers of tissue site preference\textsuperscript{17,18}. Emm patterns are based on the arrangement of emm and emm-like genes and have been shown to directly correlate with the emm-type of a specific GAS strain\textsuperscript{17,19}. GAS can be categorized into three major patterns: A-C, D, and E. Pattern A-C isolates are commonly associated with throat colonization, pattern D strains correspond to isolates recovered from skin infections, and pattern E consists of individuals associated with both throat and skin sites\textsuperscript{20}. Emm-pattern A-C includes a wide range of GAS strains such as the “invasive” (emm 1, 3, 12, and 18) and the “rheumatogenic” isolates (emm 1, 2, 6, 12, 18, 28, 75, and 89)\textsuperscript{3,21}. It appears differences in emm type, pattern, and tissue preference play a pivotal role in GAS pathogenesis and disease presentation.
Pharyngeal GAS Disease

GAS is responsible for 15% to 36% of pharyngitis-related cases that occur each year in the United States resulting in a significant economic burden of approximately $2 billion annually in direct medical costs\textsuperscript{22,23}. A major contributor to this pronounced number of pharyngeal GAS cases is the carrier state. During carriage, GAS poses no threat to the host; however, it serves as a critical reservoir for transmission through contact\textsuperscript{24}. The carrier state further complicates the estimates of acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (ASPGN) by misappropriating asymptotic carriers as possible infectious cases\textsuperscript{25}.

Pharyngitis is a seemingly benign disease that subsequently leads to relatively mild illnesses such as scarlet fever but also results in more severe, life-threatening complications such as STSS and ARF. Scarlet fever is associated with illness in school-aged children ranging from 5-12 years old and characterized by a red rash and strawberry tongue\textsuperscript{26}. The streptococcal pyrogenic exotoxins or superantigens A, B, and C have been identified as the key factors of the disease\textsuperscript{27}. Since the introduction of antibiotics, however, the disease is easily managed and has become less common. Rheumatic fever, on the other hand, is a particularly devastating illness responsible for irreversible heart damage. ARF, a nonsuppurative sequelae of pharyngitis, is estimated to affect 1 in 1,000 people with a significant proportion being children, adolescents, and young adults\textsuperscript{11,28}. During ARF pathogenesis, an autoimmune response is generated when cross-reactive antibodies to the GAS M protein also target cardiac myosin and heart valve glycoproteins\textsuperscript{28}. In addition, the hyaluronic acid capsule of GAS which is also present in
the host extracellular matrix may produce an autoimmune response by activating B cells via cross-linking of the B cell receptors\textsuperscript{29}.

**Invasive GAS Disease**

Invasive GAS infections such as necrotizing fasciitis (NF) and STSS display a rapid onset of severe symptoms including accelerated deterioration of tissue and systemic dissemination resulting in a high number of fatalities. From 1994-1995, 556 Swedish patients diagnosed with invasive GAS had a 16% mortality rate which rose to 37% among those identified with STSS\textsuperscript{30}. In 1999, the United States reported 9,500 cases of invasive GAS contributing to 1,100 deaths\textsuperscript{31}. These high mortality rates demonstrate the virulence of invasive GAS and the need for effective treatments to combat this manifestation of the pathogen.

Necrotizing fasciitis is a particularly devastating disease and is often deceptive in its presentation. NF often begins as a harmless skin lesion but progresses rapidly to a highly lethal disease. Invading GAS penetrates subcutaneous and deep soft tissue and spreads along the fascial planes of the muscle inducing a potent proinflammatory response\textsuperscript{32}. The combination of GAS virulence factors and degradative host enzymes causes significant tissue damage as well as increased bacteremia and systemic dissemination\textsuperscript{33}.

STSS can begin with infection at any site but is often associated with a pre-existing skin lesion. Symptoms of STSS include pain which can be found in an extremity or the peritoneal cavity, fever, and localized swelling or erythema which is followed by shock and multiple organ failure a few hours later\textsuperscript{34,35}. Superantigens of \textit{S. pyogenes}
have been identified as the primary culprits responsible for STSS. These superantigens bind to the T-cell receptor and major histocompatibility complex class II (MHCII) simultaneously. Subsequent activation of T-cells and antigen-presenting cells leads to production of dangerously high levels of cytokines such as tumor necrosis factor alpha (TNF-α) and beta (TNF-β), interleukin-1 (IL-1) and IL-2. Streptococcal pyrogenic exotoxin (Spe) A and C as well as the streptococcal mitogenic exotoxin Z (SmeZ) have been reported to be involved in STSS. The resurgence of invasive GAS infections is unsettling and, although, the probability of contracting NF and STSS is low, the mortality rates are high warranting further examination into the molecular mechanisms responsible for the severity of the disease.

**Virulence Factors**

Several GAS virulence factors have been identified that are responsible for the severe pathogenesis of invasive GAS disease. These include but are not limited to: the hyaluronic acid capsule, M protein, streptococcal secreted esterase, streptococcal pyrogenic toxin B (SpeB), streptolysin S, DNases, and SpyCEP, an interleukin-8 (IL-8) protease (Fig. 1). This array of proteins allows GAS to circumvent the host response and survive in an otherwise hostile environment. This thesis will focus only on virulence factors specific to the work presented.
Figure 1. GAS has a variety of virulence factors that contribute to disease pathogenesis. Virulence factors such as the hyaluronic acid capsule and M protein prevent opsonophagocytosis whereas SpyCEP and SsE inhibit neutrophil infiltration. The broad-spectrum protease SpeB targets both host and bacterial proteins.

**Hyaluronic Acid Capsule**

The GAS capsule consists of a polymer of hyaluronic acid containing repeating units of glucoronic acid and N-acetylglucosamine\(^4^2\). Synthesis of the capsule depends on coordinated transcription of three genes contained in the *has* operon (Fig. 1): *hasA*, *hasB*, and *hasC*\(^4^3\). The *hasA* gene encodes hyaluronate synthase, *hasB* encodes UDP-glucose dehydrogenase, and *hasC* encodes UDP-glucose pyrophosphorylase\(^4^4-4^6\). The hyaluronic
acid capsule promotes adherence to CD44 found on keratinocytes allowing for penetration into deeper tissues and subsequent dissemination\(^47\). However, the primary function of the capsule is to prevent phagocytosis. This is most likely accomplished by inhibiting access of phagocytes to complement found on the surface of GAS\(^27\). Increased capsule production has also been associated with hypervirulent GAS strains as well as prolonged post-infection sequelae of rheumatic fever\(^48,49\).

**Streptococcal Secreted Esterase**

The carboxylic esterase identified in GAS (SsE) contributes to skin invasion and systemic dissemination and immunization with SsE has been shown to provide protection against invasive infection\(^50-52\). SsE regulates virulence by modulating the host immune response. Esterase reduces neutrophil recruitment to the infection site by hydrolyzing the chemotactic signal, platelet-activating factor (PAF) to its biologically inactive form, lyso-PAF (Fig. 1)\(^53\).

**SpyCEP/ScpC**

SpyCEP/ScpC is a cell wall anchored protein which cleaves the chemokine IL-8/CXC at the C terminus (Fig. 1)\(^54\). IL-8 promotes neutrophil recruitment by adhering to the vasculature effectively slowing down rolling neutrophils and increasing transmigration into infected tissue\(^55,56\). IL-8 also induces the formation of neutrophil extracellular traps (NETs). Consequently, the degradation of IL-8 by SpyCEP prevents the formation of these NETs\(^57\).
Streptococcal Pyrogenic Toxin B (SpeB)

SpeB is an extracellular, broad-spectrum cysteine protease that degrades both host and bacterial factors (Fig. 1). This protease degrades fibronectin and vitronectin, converts IL-1β precursor to mature IL-1β, and cleaves a range of chemokines such as CXCL10 and CXCL11 eliminating their antibacterial effects\(^{58-60}\). SpeB also cleaves the M protein and the C5a peptidase from the cell wall surface and degrades the DNase, Sda1\(^{61-63}\).

**Transcriptional Regulation of Virulence Factors**

GAS pathogenesis requires a sophisticated transcriptional network to regulate gene expression of these virulence factors. *S. pyogenes* gene transcription consists of stand-alone regulators (RRs) and two-component systems (TCSs). The RRs include Mga, RofA-like protein, and Rgg/RopB which control expression of several virulence genes\(^{64-66}\). Mga is, perhaps, best known as a positive regulator of the *emm* gene but it also mediates *scpA* (C5a peptidase), *sclA* (collagen-like protein), and *sic* (secreted inhibitor of complement)\(^{67,68}\). Because Mga regulates numerous GAS virulence factors, deletion of *mga* leads to considerable attenuation of *S. pyogenes* in mouse models of infection\(^69\). RopB, another RR, is the major regulator of the non-specific cysteine protease, SpeB, and is necessary for *speB* transcription\(^{70}\). Mutations in *ropB* are frequently identified in M3 GAS strains and these single nucleotide polymorphisms (SNPs) in *ropB* display a SpeB-negative phenotype and exacerbate disease\(^{71}\). In addition to the stand-alone regulators, *S. pyogenes* also modulates transcription via two-
component systems. GAS has many TCSs but the CovR/S (control of virulence) system is of particular interest because of the prominent role it plays in regulation of virulence factors. The CovR/S transcriptional network is complex and is mediated by a variety of signals within the bacterium as well as the host.

**CovR/S Gene Regulation**

The CovR/S TCS which regulates directly or indirectly nearly 15% of the GAS genome is the primary mediator of virulence factor expression. The response regulator, CovR, represses a variety of virulence factors such as the hyaluronic acid capsule, streptokinase, and the DNase sda. The sensor kinase, CovS, regulates CovR repression of virulence factors by phosphorylation thereby altering CovR binding to the respective promoters. However, it appears CovR phosphorylation is independent of CovS as other molecules are capable of phosphotransfer to CovR such as acetyl phosphate. In addition to the kinase activity of CovS, environmental signals such as increased temperate and low pH can switch CovS to a phosphatase subsequently inactivating CovR-mediated repression. Many studies have shown that changes to CovR or CovS as well as the route of infection alter GAS pathogenesis significantly leading to either attenuated or increased virulence.

Comparison of pharyngeal and invasive GAS isolates revealed that the pharyngeal transcriptome varied by 10% from the invasive transcriptome which included 23 proven virulence factor genes. Animal passage of the pharyngeal isolate MGAS2221 produced an invasive derivative strain which contained a 7-bp frameshift mutation in the covS gene suggesting mutations regulate CovR binding and the ability of GAS to cycle...
between pharyngeal and invasive phenotypes during infection\textsuperscript{75,78}. This phenotypic heterogeneity observed \textit{in vivo} is an advantageous defense mechanism and enables the bacteria to survive the hostile host environment. To better elucidate the mechanism behind cycling of pharyngeal and invasive phenotypes, a considerable effort has focused on the genetic differences in transcriptional regulators such as CovR/S and how mutations in these genes affect GAS pathogenesis.

**CovR/S Mutations in Hypervirulent Strains**

It is apparent that mutation in \textit{covR/S} plays a role in altering disease presentation; however, the extent of that role is not fully understood. Whole genome sequencing of GAS has been used to characterize the frequency of nucleotide variation in the \textit{covR/S} locus in invasive and pharyngeal strains providing evidence linking specific serotypes to an increase in \textit{covR/S} mutations\textsuperscript{79}. For example, an excess of \textit{covS} polymorphisms has been reported in invasive M3 isolates relative to pharyngeal strains that are expected to result in a truncated form of the CovS protein\textsuperscript{79}. Consequently, divergence in the rate of acquisition of \textit{covS} mutations suggests host selection pressures influence invasive and pharyngeal genomes differently\textsuperscript{79}. Furthermore, a substantial proportion of \textit{covR} mutations have been observed in numerous invasive and pharyngeal isolates of different serotypes and the nonsynonymous CovR\textsuperscript{Q216P} mutation has been implicated in the hypervirulence of an M3 isolate\textsuperscript{77,79-81}. The high frequency of \textit{covR/S} polymorphisms during invasive infection suggests mutations of the \textit{covR/S} genes contribute directly to the severity of disease.
Project Objective

MGAS315, an invasive serotype M3 isolate, is hypervirulent. It has been proposed that the hypervirulence of MGAS315 is due to the acquisition of additional prophage-encoding virulence factors\textsuperscript{82}. However, the basis for MGAS315 hypervirulence has not been established. Preliminary sequence analysis of the invasive and pharyngeal GAS isolates, MGAS315 and MGAS2221, respectively, revealed the covS gene of MGAS315 encodes a valine residue at position 457 whereas the MGAS2221 covS gene encodes a glycine at the same position. We hypothesize the CovS\textsuperscript{G457V} mutation in MGAS315 causes its hypervirulence. The objective of this project is to test this hypothesis in order to elucidate the basis for the hypervirulence of MGAS315.
MATERIALS AND METHODS

Bacterial Strains and Growth

MGAS315 and MGAS2221 are representative M3 and M1 serotypes isolated from a patient with streptococcal toxic shock syndrome in Texas and a scarlet fever patient in Australia, respectively\textsuperscript{75,82}. MGAS6180 is a serotype M28 puerperal fever isolate\textsuperscript{83}. MGAS2221ΔcovS and MGAS2221ΔcovS complemented with the wild-type covS gene have been described previously\textsuperscript{75}. These strains and their derivatives were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37°C in 5% CO\textsubscript{2}.

Generation of MGAS315 sse Deletion Mutant

The plasmid used to generate the MGAS315Δsse isogenic mutant (pΔsse) was generated by sequentially PCR cloning the downstream and upstream flanking fragments of the sse gene from MGAS315 chromosomal DNA into pGRV at the HindIII/HincII and HindIII sites using primer pairs

\begin{align*}
\text{GCTGAGCTTCCACCAATTACTTTATTTACTGGGACAC} & \quad \text{and} \quad \text{CGTCAGGTTGACGATACTATGGAAGCCTATATTATGG} \\
\text{GCTGAGCTTTAACCCATTATGGGTAATATGACG} & \quad \text{and} \quad \text{CGTCAGGCTTGTTCATCTTTAGAATAGTAACTC},
\end{align*}

respectively\textsuperscript{51,84}. This plasmid was introduced into MGAS315 using electroporation as described previously\textsuperscript{51}. The plasmid was integrated into the MGAS315 genome through two homologous
recombination events. The first crossover event occurred between the flanking fragment on pΔsse and the homologous region in the MGAS315 genome. The strain was selected with 150 μg/mL of spectinomycin. Transformants were passaged eight times in THY without spectinomycin to allow for the second crossover event to occur, generating an isogenic mutant which contained an in-frame deletion of sse in MGAS315 which was spectinomycin sensitive.

**Generation of MGAS315 covS Deletion Mutant and Replacement with MGAS2221 covS**

The covS gene of MGAS315 was deleted and then subsequently replaced with the wild-type covS gene from MGAS2221 as described previously. To generate an in-frame deletion of the covS gene in MGAS315, we constructed a plasmid (pΔcovS) by amplifying the upstream and downstream flanking fragments of the 1,290-bp internal fragment of MGAS315 covS into pGRV at the BglII/XhoI and XhoI/BamHI restriction enzyme sites using primer pairs [GGACAAGCTTTGAAATAGTCAGGATATGAG and GCGGATCCGGCAATCAGTGTAAAGGCAGA; GCGGATCCGTAGATGGGTATCATTTACAG and GCGGATCCTGGTAGATAGAGACCAGTC, respectively. This plasmid was introduced into MGAS315 by electroporation. Chloramphenicol-resistant transformants were selected on THY agar plates with 10 μg/ml chloramphenicol. Transformants obtained were passaged several times on THY plates to allow the second crossover event to occur, generating an in-frame deletion mutant of covS, which was chloramphenicol sensitive and identified by diagnostic PCR using primers.
CTCTAACTCTCTTTAGACTG and GATTTCTCTCACTAAACGTG.

Next, the wild-type covS gene was inserted into the MGAS315ΔcovS genome. A DNA fragment containing covS and the flanking sequences from MGAS2221 was PCR amplified. The PCR product was cloned into pGRV at the BglII and BamHI sites, yielding pcovS, which was introduced into MGAS315ΔcovS by electroporation. The ΔcovS locus in MGAS315ΔcovS was replaced with the wild-type covS gene in pcovS through two recombination events, yielding an isogenic mutant of MGAS315 that carried the wild-type covS gene (MGAS315wtcovS), which was confirmed by diagnostic PCR using primers CTCTAACTCTCTTTAGACTG and GATTTCTCTCACTAAACGTG. DNA sequencing was used to confirm the CovSV457 mutation was replaced by the wild-type CovSG457.

Generation of MGAS315 ropB Deletion Mutant and Replacement with MGAS2221 ropB

An in-frame ropB deletion mutant of MGAS315 missing amino acids 30–223 of RopB was created as described above. First, the 3’ and 5’ ~ 700-bp flanking fragments of the ropB fragment were amplified from the MGAS315 chromosomal DNA using primer pairs

\[
\text{GGGGACAAGTTTGTACAAAAAAGCAGGCTAGATGATATGGGACCGTTCTC} \\
\text{and ATGTCTTGACATCAGTAGCAA} \\
\text{TTGCCTAGTGAG} \\
\text{ATAATCACC; } \\
\text{TTGCTACTGATGTCAAGACAT} \\
\text{TATGAACGGTGTTGTGTGTC and } \\
\text{GGGGACCACTTTGTACAAAGGACTGATGATATGGGACCGTTCTC}
\]

and

\[
\text{ATGTCTTGACATCAGTAGCAA} \\
\text{TTGCCTAGTGAG} \\
\text{ATAATCACC; } \\
\text{TTGCTACTGATGTCAAGACAT} \\
\text{TATGAACGGTGTTGTGTGTC and } \\
\text{GGGGACCACTTTGTACAAAGGACTGATGATATGGGACCGTTCTC}
\]

respectively. Crossover PCR was used to fuse together the two PCR products via the 21-
bp complementary tag sequence that is underlined in the primer sequences. Using Gateway® technology, the fused PCR product was subcloned into the donor vector pDONR221 resulting in the plasmid pDONR221-ΔropB. The ΔropB DNA fragment was then transferred to the destination vector pBBL740 vector, yielding the suicide plasmid BBL740-ΔropB. This plasmid was then introduced into MGAS315 using electroporation. The first crossover between BBL740-ΔropB and the homologous region in MGAS315 was selected for on THY agar plates containing 10 µg/ml chloramphenicol. Chloramphenicol-resistant transformants were then passaged several times in THY media to allow the second crossover event to occur, generating an in-frame deletion mutant of ropB, which was chloramphenicol sensitive and identified by diagnostic PCR using primers ATCAACTAGGAAGGCTTGAC and CAAAAGGCTAGACCTCTGC.

Next, the wild-type ropB gene from MGAS2221 was inserted into the MGAS315ΔropB genome via electroporation. Generation of the plasmid containing the wild-type ropB (BBL740-wtropB) gene was generated as described above. Two homologous recombination events between the wtropB plasmid and the ΔropB locus in MGAS315ΔropB resulted in the derivative MGAS315 strain containing the wild-type ropB gene (MGAS315wtropB) which was confirmed by diagnostic PCR using primers ATCAACTAGGAAGGCTTGAC and CAAAAGGCTAGACCTCTGC. DNA sequencing was used to confirm the RopBP103 mutation was replaced by RopBS103.
In trans Expression of the MGAS315 covS Gene in MGAS2221ΔcovS

The covS gene of MGAS315 was PCR cloned into pDCBB-RFA using Gateway® Technology as described above. pDCBB was modified into pDCBB-RFA by inserting the blunt-ended reading frame cassette A into pDCBB at the EcoRV site\textsuperscript{75}. The covS gene was PCR amplified from MGAS315 using primer pairs

\begin{align*}
\text{GGGGACAAGTTTGTACAAAAAAGCAGGCT} & \text{ACGTTATTCGTGAGAAATAAGTC} \\
\text{GGGGACCACTTTGTACAAGAAAGCTGGGT} & \text{TACCTGTCACATTAACAATGCGC.}
\end{align*}

The PCR product was cloned into the donor vector pDONR221 resulting in pDONR221-covS. The covS gene in pDONR221 was then transferred to the destination vector DCBB-RFA yielding pDCBB-covS\textsuperscript{G1370T} which was confirmed by PCR. pDCBB-covS\textsuperscript{G1370T} was introduced into MGAS2221ΔcovS via electroporation.

Measurement of Esterase Production and Acetylhydrolase Activity

Esterase activity was measured using a colorimetric assay using 2-thio PAF as described previously\textsuperscript{53}. MGAS315 and mutant strains were grown to mid-exponential phase in THY and the supernatants collected. 100 µL of supernatant was mixed with 30 µL of a solution containing 0.88 mM 2-thio PAF and 1.26 mM 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB). The absorbance at 414 nm of the reactions was recorded every 10 s for 6 min using a SPECTRA\textsuperscript{Max} 384 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

To obtain esterase in culture supernatant, MGAS315 was grown in protein-
reduced THY (PR-THY) to an OD$_{600}$ of 0.20, and the cultures were centrifuged to obtain the culture supernatant. Proteins in 8-13 mL of culture supernatant were precipitated with 3 volumes of cold ethanol, and the precipitates were pelleted by centrifugation and dissolved in 200 µL of saturated urea, followed by adjustment with 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer to 1X. PR-THY was prepared by passing THY through a membrane filter with a 10,000 molecular-weight cutoff as described previously$^{86}$. For Western blotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes with Towbin transfer buffer using a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories) at 15 V for 40 min. The membrane was treated with 10 mL of block solution (1:20 Amersham Liquid Block in 150 mM NaCl and 100 mM Tris-HCl, pH 7.4) for 1 h and incubated for 1 h with primary antibodies added to the block solution. The membrane was then rinsed twice and washed three times for 10 min each with 0.1% Tween 20 in PBS. The membrane was incubated with goat anti-mouse (heavy plus light chains) horseradish peroxidase-conjugated secondary antibodies (GenScript, Corp.) for 1 h and rinsed and washed as described above. Antigen-antibody reactivity was visualized by enhanced chemiluminescence.

**Mouse Infections**

GAS was grown to the mid-exponential phase or an optical density at 600 nm (OD$_{600}$) of 0.30 in THY. The bacteria were harvested by centrifugation and washed three times in pyrogen-free Dulbecco phosphate-buffered saline (DPBS) and then resuspended in DPBS to the desired doses. Groups of female CD1 mice (5 weeks old; Charles River
Laboratory) were subcutaneously infected with 0.2 ml of GAS suspension in DPBS at an OD$_{600}$ of 0.08 or 1.1, and actual inocula were determined by plating. For survival studies, mice were monitored daily for 12 or 14 days to determine mortality rates. For other studies, mice were sacrificed at 24 h after inoculation to collect skin for measurement of the infected lesion size and neutrophil infiltration. Spleen and liver samples were homogenized in 0.4 mL DPBS using a Kontes pestle. Homogenates were serially diluted in DPBS and plated to quantify the number of viable GAS.

**Quantification of Neutrophil Infiltration**

Numbers of recruited neutrophils in the infected skin samples were determined by the myeloperoxidase assay, as described previously$^{53,87}$. First, the skin around the infection site was peeled off, and the infection area was recognized by the boundary of inflammation in its picture and measured using the area measurement tool of the ImageJ software program (version 1.46, rsbweb.nih.gov/ij/). The infection area was excised and homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium and sonicated on ice for 90 s to extract myeloperoxidase. The samples were centrifuged at 3,500 RPM for 15 min. The myeloperoxidase activity in the supernatant was measured in 0.2 ml of HTAB, 0.167 mg of o-dianisidine dihydrochloride/mL, and 0.001% hydrogen peroxide. The change in absorbance at 460 nm ($\Delta A_{460}$) was recorded over time with a SPECTRA$^{\text{Max}}$ 384 Plus spectrophotometer. A standard curve of myeloperoxidase activity, $\Delta A_{460}$/min, versus known numbers of murine neutrophils was used to convert the measured myeloperoxidase activity to the number of neutrophils.
Quantitative RT-PCR Analysis

GAS strains were grown in THY at 37°C in 5% CO$_2$ to an OD$_{600}$ of 0.30 and centrifuged at 3,500 RPM for 15 min. Total RNA was isolated by RNeasy minikit (Qiagen). TaqMan real-time PCR was used to analyze the relative expression levels of has$A$, spy$CEP$, and $emm3$ mRNA. Changes in the transcript levels were compared using the $\Delta\Delta^{CT}$ method and normalized to the control gene $gyrA^{51}$.

SpeB Activity Assay

GAS strains were grown overnight in THY at 37°C in 5% CO$_2$ to stationary phase and centrifuged at 3,500 RPM for 15 min to collect the supernatants. SpeB activity was assessed by mixing 10 µL GAS supernatant with 5 µL $\beta$-mercaptoethanol and plating 5 µL of the mixture on a casein plate$^{88}$. The plate was incubated at 37°C for 2 hours. SpeB-positive GAS strains were identified by the presence of an opaque, white color indicating casein hydrolysis.

Statistical Analyses

All data sets were analyzed using GraphPad Prism, version 6 for Macintosh (GraphPad Software, San Diego, CA). Survival data were analyzed using the Log-rank (Mantel-Cox) test. Other data sets were analyzed using a two-tailed Student’s t-test, one-way ANOVA, or Kruskal-Wallis test as indicated.
RESULTS

Hypervirulent MGAS315 has a High Capacity to Invade Soft Tissue and Evade Innate Immunity

Different clinical GAS isolates can display different manifestations in infections. Mice subcutaneously infected with an invasive GAS isolate, MGAS315, could not survive (Fig. 2A), confirming its hypervirulence in previous studies\textsuperscript{39,77,82}. In contrast, all mice infected with MGAS6180 survived (Fig. 2A). These strains must have dramatic differences in skin invasion, systemic dissemination, and innate immune evasion. Near the endpoint in the survival study, lesion size in MGAS315-infected mice (1437 ± 42 mm\textsuperscript{2}) was 85-fold higher compared to that in the MGAS6180 infection (16.9 ± 2.5 mm\textsuperscript{2}) (Fig. 2B). Concomitantly, dissemination to the spleen and liver increased drastically over the course of infection. Near the endpoint, MGAS315-treated mice had (8.1 ± 1.8) \times 10^8 CFU/g and (8.9 ± 2.7) \times 10^7 CFU/g GAS in the spleen and liver, respectively (Fig. 2C-D, closed symbols). However, mice inoculated with MGAS6180 had 5495 ± 5163 CFU/g and 7180 ± 3955 CFU/g GAS in the spleen and liver, respectively (Fig. 2C-D, closed symbols). These GAS load data suggest that MGAS315 has a higher capacity to invade the skin and become systemic in the subcutaneous infection.

To further compare the systemic dissemination of the two strains, we measured the GAS load in the spleen and liver at day 1 after inoculation. Mice infected with MGAS315 had (6.2 ± 4.8) \times 10^5 CFU/g and (9.4 ± 5.3) \times 10^4 CFU/g in the spleen and liver, respectively, which were 120- and 37-fold higher than the bacterial load in the spleen (4864 ± 4484 CFU/g) and liver (2484 ± 1182 CFU/g) of MGAS6180-infected
mice, respectively (Fig. 2C-D, open symbols). The MGAS315 bacterial load increased from 24 hours post-infection to the endpoint by >1,000 fold; however, the MGAS6180 bacterial load from day 1 to day 12 after inoculation (MGAS6180 CFU in spleen: day 1, 4864 ± 4484; day 12, 5495 ± 5163. CFU in liver: day 1, 2484 ± 1182; day 12, 7180 ± 3955) was not nearly as dramatic as MGAS315.

This difference in bacterial load was accompanied by distinct neutrophil responses to MGAS315 and MGAS6180 infection. At 24 h post-inoculation (p.i.), neutrophil infiltration at the lesion site was 3.7-fold lower in mice inoculated with MGAS315 (4.15 ± 3.22 x 10^6 neutrophils/mm^2) than MGAS6180-treated mice (15.2 ± 2.02 x 10^4 neutrophils/mm^2) (Fig. 2E). Visual observation of the lesions revealed mice treated with MGAS315 had larger lesions with less pus-like infiltrate than MGAS6180 sites (Fig. 2F-G). MGAS315 has a high capacity to invade skin tissue, to disseminate, and to cause lethal systemic infection whereas MGAS6180 causes a persistent infection with low bacterial load and lower virulence. From this data, it is apparent MGAS315 is associated with increased systemic dissemination accompanied by reduced neutrophil recruitment.
Figure 2. MGAS315 is a hypervirulent isolate associated with reduced neutrophil recruitment. (A) Mice inoculated subcutaneously with MGAS315 (\( \sim 1.7 \times 10^8 \) CFU) had significantly higher mortality compared to mice treated with MGAS6180 (\( \sim 1 \times 10^8 \) CFU). At the time of death, mice treated with MGAS315 had larger lesions (B), higher bacterial load in the spleen (C, closed), and liver (D, closed). At 24 h p.i., mice treated with MGAS315 (\( \sim 4 \times 10^7 \) CFU) had higher bacterial load in the spleen (C, open) and liver (D, open) compared to MGAS6180 (\( \sim 5.8 \times 10^7 \) CFU). At 24 h p.i., mice treated with MGAS315 (\( \sim 1.5 \times 10^8 \) CFU) had lower neutrophil influx (E) compared to MGAS6180 (\( \sim 2.3 \times 10^8 \) CFU). Visual observation of the lesions also revealed more severe spread and less pus-like infiltrate in mice inoculated with MGAS315 (F) compared to MGAS6180 (G). *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \), ****\( P<0.0001 \) as determined by a two-tailed Student’s t-test and Log-rank (Mantel-Cox) test. Data graphed are mean ± standard error of mean (SEM) for replicate biological samples.
A Critical Role for the Esterase SsE in Skin Invasion and Innate Immune Evasion by MGAS315

The streptococcal secreted esterase has been shown to play an active role in the virulence of an invasive serotype M1 strain MGAS5005 specifically through innate immune evasion. Hydrolysis of PAF by esterase impeded neutrophil recruitment and thus, enhanced virulence\textsuperscript{53}. Additionally, immunization with the recombinant SsE protein was shown to be protective in a mouse model of subcutaneous infection with MGAS5005\textsuperscript{50}. Furthermore, deletion of the sse gene in MGAS5005 revealed significant attenuation of GAS following subcutaneous infection\textsuperscript{51}. We hypothesize that SsE also critically contributes to the capability of MGAS315 to invade soft tissues and evade neutrophil responses. To test this hypothesis, we deleted the sse gene in MGAS315 and evaluated the effects of the sse deletion on MGAS315 invasion of the skin and inhibition of neutrophil recruitment in subcutaneous infection of mice. The lesion size caused by MGAS315\textsuperscript{Dsse}, 286 ± 32 mm\textsuperscript{2}, was reduced by 71\% compared with that caused by MGAS315 (997.4 ± 72.5 mm\textsuperscript{2}) (Fig. 3A). The level of neutrophils at the MGAS315\textsuperscript{Dsse} infection site were (27.7 ± 6.58) \times 10\textsuperscript{4} neutrophils/mm\textsuperscript{2}, a 7.3-fold increase relative to the parental strain (3.77 ± 1.3 \times 10\textsuperscript{4} neutrophils/mm\textsuperscript{2}) (Fig. 3B). Thus, SsE plays a critical role in the skin invasion and innate immune evasion by MGAS315.
Figure 3. Esterase is required for virulence in MGAS315. At 24 h p.i., mice treated with MGAS315 had larger lesions (A) and decreased neutrophil infiltration (B) compared to treatment with MGAS315Δsse. **P<0.01, ***P<0.001 as determined by a two-tailed Student’s t-test. Data graphed are mean ± standard error of mean (SEM) for replicate biological samples.

Repairing the covS\textsuperscript{G1370T} Mutation of MGAS315

The serotype M1 strain MGAS5005 contains a natural 1-bp deletion in the covS gene, and this null covS mutation is responsible for the high capacity of MGAS5005 to evade neutrophil responses and invade the skin tissue\textsuperscript{85}. The MGAS315 skin invasion, inhibition of neutrophil recruitment, and hypervirulence observed in subcutaneous mouse infection are very similar to that of MGAS5005. The MGAS315 covS gene does not have a deletion or nonsense mutation but, rather, a covS\textsuperscript{G1370T} missense mutation, resulting in the CovS\textsuperscript{G457V} substitution. We hypothesize that the covS\textsuperscript{G1370T} mutation enhanced the skin invasion, innate immune evasion, and hypervirulence of MGAS315. To test our hypothesis, we generated a derivative strain from MGAS315 in which the
mutated covS gene was replaced by the functional covS gene from MGAS2221. Repairing the MGAS315 covS gene was achieved by first knocking out the mutated covS gene in MGAS315 and then knocking in the MGAS2221 covS gene. The entire procedure consisted of four steps (Fig. 4A). In step 1, the suicide plasmid pΔcovS containing a chloramphenicol resistance gene in addition to the two flanking regions of the covS gene was introduced into MGAS315 and was integrated into the MGAS315 genome through a single crossover event between one flanking fragment on the plasmid and the homologous region in the MGAS315 genome resulting in a strain that was resistant to chloramphenicol. In step 2, this chloramphenicol-resistant derivative was grown on THY agar plates for at least 10 serial passages to facilitate the second crossover event between the other covS flanking fragment on the plasmid and the homologous sequence in the chromosome resulting in the MGAS315ΔcovS strain. In step 3, the suicide plasmid pcovSwt containing the covS gene from MGAS2221 and its two flanking sequences was integrated into the MGAS315ΔcovS genome. A single crossover event between the flanking sequence on the plasmid and corresponding region in the chromosome yielded a chloramphenicol-resistant strain. In step 4, this strain was passaged on THY agar plates to produce the second crossover event between the other flanking sequence on the plasmid and the homologous region in the genome, resulting in the replacement of the MGAS315 covSG1370T with the wild-type covS gene.

MGAS315ΔcovS mutants in step 2 and MGAS315wtcovS derivatives in step 4 were obtained by first identifying chloramphenicol-sensitive colonies and then by analyzing chloramphenicol-sensitive colonies via PCR using primers that flank the
deleted covS sequence. In the PCR analysis, the PCR product from MGAS315ΔcovS should be smaller than that from MGAS315 while the size of the PCR product from MGAS315wtcovS should be restored to that of MGAS315. Accordingly, we obtained strains with the smaller PCR product in step 2 and strains with the PCR product size of the full covS gene in step 4 (Fig. 4B, upper). DNA sequencing confirmed the replacement of the thymine at position 1370 of covS with a guanine in MGAS315wtcovS. (Fig. 4B, lower).

Some of our previous GAS mutants displayed down-regulation of the major virulence factor M protein due to a secondary spontaneous mutation during the construction of mutants, and M protein down-regulating mutations can cause virulence attenuation and most likely confound the interpretation of effects attributable to the function of the gene being investigated. To confirm normal expression of the M protein gene in the MGAS315wtcovS strain, we measured the mRNA levels of the M3 protein gene, emm3, in MGAS315, MGAS315ΔcovS, and MGAS315wtcovS by qRT-PCR. The relative levels of the emm3 transcript in MGAS315ΔcovS, and MGAS315wtcovS normalized to that of gyrA was decreased by less than 0.5-fold compared with that in MGAS315 whereas previously a >25-fold decrease in emm transcripts was observed in secondary mutations-caused M protein down-regulating mutants. Furthermore, the cycle numbers reaching threshold in RT-PCR signal for MGAS315ΔcovS and MGAS315wtcovS were actually lower than that in MGAS315. Thus, the MGAS315wtcovS strain had normal M protein expression.
Figure 4. Replacement of CovS^{G457V} in MGAS315 with wild-type CovS. (A) Schematic of deletion and subsequent replacement of MGAS315 covS with covS from MGAS2221.
Figure 4. Replacement of Cov$^{GV57V}$ in MGAS315 with wild-type CovS. (B) Upper, PCR confirmation of the covS deletion and replacement with wild-type covS. The picture shows agarose gel analysis of PCR reactions using MGAS315 (lane 1), MGAS315ΔcovS (lane 2), and MGAS315wtcovS (lane 3) genomic DNA. Lower, DNA sequencing verified replacement of the thymine at position 1370 with the guanine in the wild-type covS gene resulting in the subsequent valine to glycine substitution at position 457. (C) Differences in relative emm transcript levels between MGAS315, MGAS315ΔcovS, and MGAS315wtcovS. MGAS315ΔcovS and MGAS315wtcovS emm transcript expression was reduced by less than 0.5-fold compared to MGAS315. **P<0.01 as determined by ANOVA with Tukey’s post-test. Data graphed are mean ± standard error of mean (SEM) for 2 replicate measurements.
Effects of the Replacement of CovS\textsuperscript{G457V} with CovS\textsuperscript{G457G} in MGAS315 on Expression of Multiple Virulence Genes

The two-component system CovR/S negatively regulates multiple virulence genes, including the capsule synthase gene \textit{hasA}, IL-8 degrading peptidase \textit{spyCEP}, and the PAF acetylhydrolase gene \textit{sse}\textsuperscript{51,72,75,90}. If the CovS\textsuperscript{G457V} mutation is the basis for the high skin invasion, systemic dissemination, innate immune evasion, and virulence, it must relieve the CovR/S suppression of multiple virulence genes. If true, the replacement of CovS\textsuperscript{G457V} with CovS\textsuperscript{G457G} should reduce the expression of \textit{hasA}, \textit{spyCEP}, and \textit{sse}. To test this idea, MGAS315\textit{wt}cov\textit{S} was compared with MGAS315 and MGAS315\textit{Δ}cov\textit{S} in \textit{hasA}, \textit{spyCEP} expression as well as esterase production. We grew MGAS315, MGAS315\textit{Δ}cov\textit{S}, and MGAS315\textit{wt}cov\textit{S} to mid-exponential phase and measured transcript abundance of \textit{hasA} and \textit{spyCEP}. The level of \textit{hasA} mRNA in MGAS315\textit{wt}cov\textit{S} was reduced by 4.2-fold compared with MGAS315 (Fig. 5A) while \textit{spyCEP} expression was down-regulated by 32.8-fold (Fig. 5B). The difference in the down-regulation between \textit{hasA} and \textit{spyCEP} expression suggest that CovR/S with the CovS\textsuperscript{G457V} mutation could still negatively control \textit{hasA} to some extent but fully relieve the repression of \textit{spyCEP}. Consequently, deletion of the \textit{covS} gene from MGAS315 further increased the expression of \textit{hasA} but not \textit{spyCEP}.

To provide additional support for the effect of the CovS\textsuperscript{G457V} mutation on virulence gene expression, we transformed an MGAS2221\textit{Δ}cov\textit{S} strain with a plasmid containing the MGAS315 \textit{covS}\textsuperscript{G1370T} gene or the MGAS2221 \textit{covS}\textit{wt} gene and measured \textit{hasA} and \textit{spyCEP} expression. The transcript abundance of \textit{hasA} was 94- and 98-fold
lower in MGAS2221 and MGAS2221ΔcovS complemented with covS\textsuperscript{wt}, respectively, compared to that in MGAS2221ΔcovS, which was similar to hasA expression observed in MGAS315 (Fig. 5C). Additionally, there was a 178- and 135-fold decrease in the expression of spyCEP in MGAS2221 and the covS\textsuperscript{wt} complement MGAS2221ΔcovS strain, respectively, relative to MGAS2221ΔcovS (Fig. 5D). However, in trans expression of covS\textsuperscript{G1370T} in MGAS2221ΔcovS resulted in hasA and spyCEP expression levels similar to those seen in MGAS2221ΔcovS (Fig. 5C-D). This data further demonstrates that the CovS\textsuperscript{G457V} mutation loses the repressive function of covS in regulation of multiple virulence genes.

To determine the effect of covS mutation on sse expression, we compared the activity levels of the PAF acetylhydrolase in the culture supernatant of MGAS315, MGAS315ΔcovS, and MGAS315wtcovS using the colorimetric 2-thio PAF assay\textsuperscript{53}. The PAF acetylhydrolase activity of esterase was markedly lower in the MGAS315wtcovS strain (Fig. 5E) and was accompanied by a significant reduction in the amount of esterase produced as shown in the western blotting analysis (Fig. 5F). These results validate the contribution of this CovS\textsuperscript{G457V} mutation to high levels of CovR/S-controlled virulence factors in MGAS315.
Figure 5. CovS$^{G457V}$ mutation in MGAS315 increases expression of multiple virulence factors. Relative quantification of hasA and spyCEP in MGAS315, MGAS2221 and isogenic mutants. MGAS315wtcovS had decreased hasA (A) and spyCEP (B) expression compared to wild-type MGAS315. MGAS315ΔcovS had higher expression of hasA (A) but not spyCEP (B) compared to MGAS315. In trans expression of covS$^{G1370T}$ in MGAS2221ΔcovS restored hasA (C) and spyCEP (D) expression levels to those observed in MGAS2221ΔcovS. Gene expression is normalized to gyrA expression. ***P<0.001, ****P<0.0001 as determined by ANOVA with Tukey’s post-test when compared to MGAS315. Data graphed are mean ± standard error of mean (SEM) from 2 replicate measurements.
Figure 5 Continued.

Figure 5. CovS\textsuperscript{G457V} mutation in MGAS315 increases expression of multiple virulence factors. (E) GAS strains were grown to an OD\textsubscript{600} of 0.30 and acetylhydrolase activity of esterase was measured using the 2-thio PAF colorimetric assay. MGAS315 and MGAS315\textDelta covS had considerably higher esterase activity compared to the THY control and MGAS315\textcopyright covS. (F) Western blot revealed a considerable decrease in the amount of esterase produced by MGAS315\textcopyright covS.

**SpeB Activity is not Fully Dependent on CovS\textsuperscript{G457V} Mutation**

SpeB is known to degrade several host proteins as well as bacterial virulence factors and, its down-regulation is critical for GAS to transition from superficial to severe, invasive infections\textsuperscript{63}. Epidemiological studies have revealed an inverse
relationship between SpeB production and disease severity. Isolates from severe cases such as STSS and NF had considerably lower SpeB activity compared to those from non-invasive infections. Alterations in SpeB are often attributed to covR/S polymorphisms and depending on the mutation may differentially regulate SpeB. For example, deletions in covS abrogate SpeB production while null covR mutations significantly enhance SpeB expression. Furthermore, mutations in the SpeB regulator gene ropB also alter SpeB expression. Previously, a serine to proline substitution at position 103 has been identified in the ropB gene of MGAS315 and this mutation accounts for the lack of SpeB as it prevents RopB from binding to the SpeB promoter. To determine the effect of both ropB and covS mutation on SpeB activity, we used the caseinolytic plate assay as described previously to compare differences among strains. SpeB production was not present in MGAS315wtcovS and, in fact, was only restored when MGAS315ΔropB was corrected with the wild-type ropB gene (Fig. 6). From this data, it appears in M3 GAS strains SpeB production is dependent on RopB, not CovS, for proper expression; however further analysis is required to determine the effect of covS mutation on SpeB.

Figure 6. SpeB activity is absent in MGAS315wtcovS. MGAS315 and isogenic mutants were grown to stationary phase and SpeB activity measured using the caseinolytic plate assay. Restoring MGAS315 covS with wild-type covS has no effect on SpeB activity; however, when MGAS315 ropB is replaced by MGAS2221 ropB activity is restored.
Repairing the CovS$^{G457V}$ Mutation Attenuates MGAS315 Skin Invasion, Innate Immune Evasion, and Virulence

Since the replacement of CovS$^{457V}$ with CovS$^{457G}$ reduced the expression of multiple virulence genes in MGAS315, we hypothesize that repairing this CovS$^{457V}$-to-CovS$^{457G}$ mutation reduces the capacity of MGAS315wtcov$S$ to invade soft tissue and evade innate immunity, thereby attenuating MGAS315 virulence. MGAS315wtcov$S$ was compared with MGAS315 and MGAS315Δcov$S$ in skin lesion size, neutrophil recruitment, and survival during subcutaneous infection of mice. 93% of mice infected with MGAS315wtcov$S$ survived whereas mice infected with MGAS315 and MGAS315Δcov$S$ had an 80% and 100% mortality rate, respectively, ($P$ values: MGAS315wtcov$S$ versus MGAS315, <0.0001; MGAS315wtcov$S$ versus MGAS315Δcov$S$, <0.0001), indicating that repairing the CovS$^{G457V}$ mutation significantly reduced virulence of MGAS315 (Fig. 7A). MGAS315Δcov$S$ was more virulent than MGAS315 ($P = 0.0009$), and this result was consistent with the higher expression levels of has$A$ in MGAS315Δcov$S$ relative to MGAS315 as shown in Figure 5A. These results suggest that the CovS$^{G457V}$ dramatically increases virulence but not to the full extent of null cov$S$ mutation caused increases in virulence. Repairing the cov$S$ mutation in MGAS315 also reduced skin invasion and innate immune evasion by MGAS315. At 24 h post-inoculation, lesion size and neutrophil recruitment did not vary significantly between MGAS315 (146.2 ± 12.7 mm$^2$; 7.49 ± 1.05 x 10$^4$ neutrophils/mm$^2$) and MGAS315Δcov$S$ (161.5 ± 12.4 mm$^2$; 7.49 ± 1.53 x 10$^4$ neutrophils/mm$^2$) groups; however, mice inoculated with MGAS315wtcov$S$ caused significantly smaller lesions
(37.8 ± 2.74 mm²) and induced greater neutrophil infiltration (2.68 ± 1.78 x 10⁶ neutrophils/mm²) compared to both MGAS315 and MGAS315ΔcovS groups (Fig. 7B-C). Collectively, these results demonstrate that the CovS^{G457V} mutation in MGAS315 leads to reduced neutrophil recruitment accompanied by enhanced skin invasion and virulence.
Figure 7. MGAS315wtcovS virulence is significantly attenuated *in vivo*. Mice were inoculated subcutaneously MGAS315 (~3x10^8 CFU), MGAS315ΔcovS (~4x10^8 CFU), and MGAS315wtcovS (~3x10^8 CFU). (A) Survival analysis over 14 days showed a significant increase in survival in mice treated with MGAS315wtcovS. (B) Lesion size was significantly greater in wild-type MGAS315 compared to MGAS315wtcovS. (C) Neutrophil infiltration at the lesion site was significantly less in wild-type MGAS315 compared to MGAS315wtcovS. *P<0.05, ****P<0.0001 as determined by ANOVA with Tukey’s post-test or Kruskal-Wallis with Dunn’s post-test and Log-rank (Mantel-Cox) test. Data graphed are mean ± SEM for 4 replicate biological samples.
DISCUSSION

This project aims at understanding the basis for the hypervirulence of MGAS315. MGAS315 was recovered from a case of severe invasive GAS infection in the late 1980s and has been shown to display hypervirulence in mouse models of infection. Prophages encoding superantigens SpeA and SpeK and the extracellular phospholipase A2 (Sla) have been documented in MGAS315, and what’s more, recombination of these phages produced chimeras which may be responsible for the unusual rise in these hypervirulent strains of GAS. Acquisition of the virulence factor Sla is a crucial molecular event in the evolution, rapid emergence, and widespread dissemination of a distinct GAS clone that causes unusually severe human infections. Then again, a high frequency of mutations in covR/S have been identified in invasive GAS isolates which may offer an alternative explanation for this hypervirulence. Previously, null covS mutations have been shown to enhance expression of multiple CovR/S-controlled virulence factors, skin invasion, innate immune evasion and abolish SpeB production. Our results suggest the CovS^{G457V} mutation is responsible for the hypervirulence of MGAS315, the importance of which is two-fold. First, we identified an additional event that is critical for MGAS315 hypervirulence. Second, this finding illustrates covS mutations are also associated with invasive isolates of serotype M3.

The role of the CovS^{G457V} mutation in the hypervirulence of MGAS315 is apparently mediated by the mutation-caused enhancement of multiple virulence factors. Mutation in covS is associated with invasive GAS strains and is known to alter virulence factor expression. We observed a significant reduction in hasA and spyCEP when
wild-type MGAS315 \textit{covS} was replaced with the \textit{covS} gene from MGAS2221 as well as diminished esterase production and activity. These findings correlate with a decrease in lesion size and enhanced neutrophil infiltration, suggesting that circumventing the innate immune response is critical for hypervirulent GAS strains. GAS produces a variety of virulence factors that aid in evading the innate immune response and are critical for GAS survival in the host. The hyaluronic acid capsule, esterase, and SpyCEP, for example, are key bacterial components that are critical for establishing and sustaining a persistent infection. Under basal conditions, the genes encoding these proteins are tightly regulated by the CovR/S TCS but within the hostile environment of the host, CovR-mediated repression is released and these genes become activated. In invasive cases, the CovR/S TCS becomes dysregulated leading to uncontrolled transcription of virulence factors resulting in severe disease. Hypervirulent GAS strains are associated with a high frequency of \textit{covR/S} mutations and null \textit{covS} mutations increase \textit{hasA} and \textit{spyCEP} expression in an invasive M1 isolate\textsuperscript{79,85}. However, these findings have yet to be documented in a M3 invasive isolate and the subsequent effect on innate immune evasion is unknown.

Repairing the Cov\textsuperscript{G457V} mutation in MGAS315 lowered \textit{hasA} and \textit{spyCEP} expression significantly. Several studies have suggested a role for both SpyCEP and the capsule in innate immune evasion. SpyCEP has been shown to inhibit neutrophil recruitment by degradation of the chemokine IL-8 while the hyaluronic acid capsule enhances resistance to NET-mediated extracellular killing\textsuperscript{57,90,97}. Notably, expression of the capsule was absolutely necessary for the acquisition of \textit{covR/S} mutations and the
switch to an invasive phenotype \textit{in vivo}\textsuperscript{97}. Our work demonstrates partial relief of the CovR repression of \textit{spyCEP} and \textit{hasA} as a result of the CovS\textsuperscript{G457V} mutation in MGAS315 contributes to decreased neutrophil ingress at the lesion site. Additionally, deletion of \textit{sse} in MGAS315 displayed decreased lesion size and increased neutrophil infiltration (Fig. 3). This is not surprising given that previous work has shown that SsE is important for inhibition of neutrophil recruitment by hydrolysis of platelet-activating factor\textsuperscript{53}. Our data showed repairing the \textit{covS} mutation reduced esterase production and activity considerably. Thus, it appears \textit{covS} mutation in hypervirulent isolates removes repression of \textit{sse} further preventing neutrophil influx to the lesion site.

SpeB activity, or lack thereof, is a defining characteristic of invasive isolates and epidemiological studies have linked polymorphisms in \textit{covR/S} to a loss in SpeB production\textsuperscript{75,91}. Interestingly, while the CovS\textsuperscript{G457V} mutation appears to be responsible for the increase in virulence factor expression, it doesn’t appear to affect SpeB production in MGAS315 as repairing the CovS\textsuperscript{G457V} mutation in MGAS315 did not restore SpeB activity. This result is consistent with the previous finding that the RopB\textsuperscript{S103P} mutation is responsible for the SpeB production-negative phenotype\textsuperscript{94}. The MGAS315 derivative strain with wild-type RopB/CovS\textsuperscript{G457V} produces SpeB, indicating that the CovS\textsuperscript{G457V} mutation does not abolish SpeB production. This is the first identified \textit{covS} mutation that enhances virulence factor expression but does not affect SpeB production. If the enhanced expression of multiple virulence factors and abolishment of SpeB production both contribute to the hypervirulence of invasive GAS strains, the CovS\textsuperscript{G457V} mutation of MGAS315 was likely acquired after the RopB\textsuperscript{S103P} mutation occurred. This speculation
is consistent with the finding that \textit{ropB} polymorphisms in serotype M3 GAS strains are significantly associated with pharyngeal infections relative to invasive isolates such as MGAS315\textsuperscript{98}. Future work may focus on characterizing the interaction between RopB and CovR/S and how combinatorial mutation in these genes dictates virulence factor expression and subsequent innate immune evasion \textit{in vivo}.

In summary, the CovS\textsuperscript{G457V} mutation is required to relieve repression of virulence genes \textit{hasA}, \textit{spyCEP}, and \textit{sse} leading to enhanced skin invasion, inhibition of neutrophil recruitment, and GAS dissemination. Although, RopB\textsuperscript{S103P} mutation is necessary for the abrogation of SpeB, CovS\textsuperscript{G457V} mutation doesn’t appear to have an effect. These results indicate that an intact CovR/S system are critical for preventing GAS from causing invasive infections and the introduction of mutations in the \textit{covS} and \textit{ropB} genes contributes to a reduced neutrophil response leading to a hypervirulent phenotype.
CONCLUSION

The basis for hypervirulence of serotype M3 GAS strains has been attributed to the acquisition of prophages encoding virulence factors and the subsequent recombination of phages. Herein, we describe a previously unrecognized mechanism for this hypervirulence in an M3 GAS isolate, MGAS315. Epidemiological studies have shown a considerable proportion of invasive GAS strains contain covR/S mutations such as MGAS315 which contains a glycine to valine substitution at position 457. By generating an isogenic derivative strain of MGAS315 where the mutated covS gene was restored to wild-type, we gained further insight into the effect of covS mutation on hypervirulence in MGAS315. First, MGAS315 has a high capacity to invade the skin, evade innate immune responses, and accumulate high systemic GAS loads, and we identified the virulence factor SsE as a major contributor in MGAS315 skin invasion and innate immune evasion. Second, replacement of the MGAS315 covS\textsuperscript{1370T} gene with the MGAS2221 covS\textsuperscript{1370G} gene reduced skin invasion as well as the expression of the virulence genes hasA, spyCEP, and sse leading to enhanced neutrophil recruitment, attenuating the hypervirulence of MGAS315. Third, in \textit{trans} expression of covS\textsuperscript{wt} but not covS\textsuperscript{1370T} in MGAS2221ΔcovS reversed the effect of the covS deletion on the virulence gene expression in MGAS2221. Lastly, repairing the RopB\textsuperscript{S103P} mutation in MGAS315 also reduced the expression of hasA, spyCEP, and sse (data not shown) and restored SpeB production even in the presence of the CovS\textsuperscript{G457V} mutation. These observations are summarized in Table 1. We conclude that both the CovS\textsuperscript{G457V} and RopB\textsuperscript{S103P} mutations contribute to the hypervirulence of MGAS315.
Null \textit{covS} mutations in invasive serotype M1 strains enhance the expression of multiple virulence genes and abolish SpeB production\textsuperscript{95}. We show here a \textit{covS} missense mutation that enhances expression of multiple virulence genes but has no effect on SpeB production in an M3 strain. These findings are unusual for a hypervirulent GAS strain as mutations within \textit{covR/S} are often linked to the lack of SpeB in invasive GAS isolates\textsuperscript{91}. Our data suggest that in MGAS315 the SpeB-negative phenotype can be attributed to the mutation in \textit{ropB} as replacement of RopB\textsuperscript{P103} with wild-type RopB\textsuperscript{S103} restored SpeB production. Surprisingly, it appears that both RopB\textsuperscript{S103P} and CovS\textsuperscript{G457V} mutations are required for enhanced expression of \textit{hasA}, \textit{spyCEP}, and \textit{sse} in MGAS315; however, more detailed experiments are needed to verify this possibility. It is known that the functional RopB is required for SpeB production but is not involved in regulation of \textit{hasA}, \textit{spyCEP}, and \textit{sse} in M1 strains, and, therefore, our results indicate that the regulation of virulence genes in MGAS315 is different from that in M1 strains. To our knowledge this is the first example of an invasive GAS isolate that is dependent on simultaneous \textit{covR/S} and \textit{ropB} mutations for increased virulence factor expression.

Table 1. Gene expression patterns and SpeB production in MGAS2221, MGAS315, and MGAS315 isogenic Mutants.

\begin{center}
\begin{tabular}{|c|c|c|c|c|}
\hline
 & MGAS2221 & MGAS315 & MGAS315 wtCovS & MGAS315 CovS\textsuperscript{G457V} \\
\hline
\textit{hasA}, \textit{spyCEP}, \textit{sse} & ↓ & ↑ & ↓ & ↓ \\
\hline
SpeB & + & − & − & + \\
\hline
\end{tabular}
\end{center}
As outlined below, our findings are consistent with a model wherein the introduction of point mutations in both \textit{covS} and \textit{ropB} are required to relieve repression of the virulence genes \textit{hasA}, \textit{spyCEP}, and \textit{sse}; however, only mutation in \textit{ropB} is necessary for the abolishment of SpeB production (Fig. 8). The synergistic combination of enhanced virulence factor expression and absence of SpeB in MGAS315 leads to increased skin invasion, reduced neutrophil infiltration, and systemic GAS survival (Fig. 8). The work presented here offers an alternative explanation for the hypervirulence exhibited by MGAS315 as well as illustrates a complex regulatory relationship between RopB and CovR/S.
Figure 8. Model of the effect of covS and ropB mutations on MGAS315 infection. Both the CovS$^{457V}$ and RopB$^{S103P}$ mutations are necessary for increased expression of virulence factors hasA, spyCEP, and sse in MGAS315. Only RopB$^{S103P}$ mutation is required for the abolishment of SpeB. The combination of increased virulence factor expression and the lack of SpeB enhances skin invasion and innate immune evasion by MGAS315.
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