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Streptococcus pyogenes Superantigens: Studies Into Host Specificity and Functional Redundancy

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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Abstract

Streptococcus pyogenes is a human-specific globally prominent bacterial pathogen that secretes extremely potent exotoxins known as superantigens. Superantigens function to overstimulate T lymphocytes, capable of inducing excessive cytokine responses, potentially leading to toxic shock syndrome. Each strain of *S. pyogenes* encodes multiple distinct superantigens, yet the reasons why *S. pyogenes* retains multiple superantigens has remained elusive. Using a murine model of acute nasopharyngeal infection, the role of each superantigen encoded by *S. pyogenes* MGAS5005 was evaluated using isogenic superantigen-deletion or -complemented strains, and passive immunization with superantigen-neutralizing antibodies. The superantigen SpeG, and likely SpeJ, were not required for infection. However, SpeA and SmeZ were both required for infection of HLA-DQ8 transgenic mice, and thus, are not functionally redundant. This supports the theory that *S. pyogenes* superantigen expression varies depending on host factors, and provides insight into superantigen function in non-severe infections.

Keywords

Streptococcus pyogenes, superantigens, acute nasal infection, redundancy, pharyngitis, T cell, transgenic mouse

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Katherine Kasper constructed the following plasmids and bacterial strains that were used in this study and listed in Tables 1 and 2: MGAS8232 *speJ* complement plasmid (pG$^+$host5::*I-SceI*::IntMGAS8232::*speJ*), MGAS5005 *speA* complement plasmid (pG$^+$host5::*I-SceI*::IntMGAS5005::*speA*), MGAS5005 superantigen-deletion mutants MGAS5005Δ*speA*, MGAS5005Δ*speG*, both MGAS5005Δ*speJ* strains, MGAS5005Δ*smeZ*, the original MGAS5005Δ*speA*/*smeZ* strain, and MGAS5005Δ*speA*/*speJ*/*smeZ*. The MGAS5005, MGAS5005 single mutants, and MGAS8232 *in vivo* infections in FVB mice (which I assisted with) are previously published in Katherine's doctoral thesis. She also assisted in execution of the *in vivo* infections in this work with the MGAS5005 mutant strains in FVB and DR4/DQ8 mice.

Joseph Zeppa produced all recombinant superantigens used in the splenocyte and PBMC assays. He also assisted with *in vivo* infections for the DQ8 mice and C57Bl/6 mice, as well as passive immunization experiments.

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List of Abbreviations

°C – degrees Celsius
Δ – delta/deletion
× *g* – times gravity
μg – microgram
μL – microliter
μF – microfarad
Ω – ohm
Ab – antibody
ACK – ammonium-chloride-potassium
Amp – ampicillin
ARF – acute rheumatic fever
AP-1 – activator protein 1
APC – antigen presenting cell
APSGN – acute post-streptococcal glomerulonephritis
BHI – brain heart infusion
BSA – bovine serum albumin
B6 – c57Bl/6 mice
C4BP – C4b-binding protein
Ca^{2+} – calcium ion
Carma1 – caspase recruitment domain-containing and membrane-associated guanylate kinase-containing protein 1
CD – cluster of differentiation
CFU – colony forming units
cNT – complete nasal turbinates
CovR/S – control of virulence two-component system
CsrR/S – capsule synthesis regulator two-component system
Da – daltons
DAG – diacylglycerol
DMF – *N,N*-dimethylformamide
DNA – deoxyribonucleic acid
DNAse – deoxyribonuclease
ELISA – enzyme-linked immunosorbent assay
Erm – erythromycin
fg – femtogram
For – forward
HBSS – Hank's balanced saline solution
HLA-DR4/DQ8 – human leukocyte antigen genotype DR4/DQ8
HLA-DQ8 – human leukocyte antigen genotype DQ8
HP – human plasma
HRP – horseradish peroxidase
ICE – integrative and conjugative elements
IκB – inhibitor of NFκB
IKK – IκB kinase
IKKγ – regulatory subunit of IKK complex

IL-2 – interleukin-2
IM – intramuscular
i.p. - intraperitoneally
IP_3 – inositol triphosphate
IPTG – isopropyl β-D-1-thiogalactopyranoside
ITAM – immunoreceptor tyrosine-based activation motif
LAT – linker for the activation of T cells
LB – Luria Bertani
kb – kilobase
kDa - kilodaltons
M cell – membranous cell
M1 – *emm*1 type
M18 – *emm*18 type
MAM – *M. arthritidis* T cell mitogen
MCS – multiple cloning site
MGE – mobile genetic elements
MHC – major histocompatibility complex
mL – milliliter
MMTV – mouse mammary tumour viruses
NALT – nasal associated lymphoid tissue
Neo – neomycin
NFAT – nuclear factor of activated T cells
NF-κB – nuclear factor κ-light-chain-enhancer of B cells
ng – nanogram
OCD – obsessive-compulsive disorder
PBMC – peripheral blood mononuclear cell
PBS – phosphate buffered saline
PBS-T – phosphate buffered saline with 0.05% (v/v) tween-20
pg - picogram
PIP_2 – phosphatidylinositol 4,5-bisphosphate
PKC-θ – protein kinase C θ
PLCγ1 – phospholipase C γ 1
PLCβ – phospholipase C β
pMHC – peptide-MHC complex
PVDF – polyvinylidine difluoride
Rev – reverse
RHD – rheumatic heart disease
RNA – ribonucleic acid
RNAse – ribonuclease
rSAg – recombinant superantigen
SAg – superantigen
SDS – sodium dodecyl sulphate
SEM – standard error of the mean
SLO – streptolysin O
SLP-76 – Src-homology 2 domain-containing linker protein of 76 kDa
SLS – streptolysin S
SmeZ – streptococcal mitogenic exotoxin Z
Spe – streptococcal pyrogenic exotoxin

SpeA – streptococcal pyrogenic exotoxin A
SpeG – streptococcal pyrogenic exotoxin G
SpeJ – streptococcal pyrogenic exotoxin J
STSS – streptococcal toxic shock syndrome
TCA – trichloroacetic acid
TBS – tris-buffered saline
TCR – T cell receptor
THY – Todd Hewitt broth + 1% yeast extract
TMB – 3-3'-5-5'-tetramethybenzidine
TSA + 5% SB – Trypticase
TSS – toxic shock syndrome
V – volts
v/v – volume per volume
Vβ – β chain variable region
w/v – weight per volume
X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPM – *Y. pseudotuberculosis* mitogen

Chapter 1 : Introduction

1 Introduction

1.1 *Streptococcus pyogenes*

Streptococcus pyogenes, also known as Group A streptococci by Lancefield designation, is a Gram-positive bacterial species known for growing in pairs or chains of spherical (coccoid) cells and for producing β-haemolysis on blood agar plates [1]. Although humans are the only natural reservoir and host of *S. pyogenes*, the species is truly a versatile pathogen that is able to live and cause infections in a variety of tissue types [2]. Currently, there is no accepted vaccine for *S. pyogenes* [3-5]. Though several candidates targeting various *S. pyogenes* virulence factors have been tried, only two vaccine candidates have progressed to human clinical trials. Both vaccines were based on the M protein (discussed below) [6].

1.2 *S. pyogenes* genome

The *S. pyogenes* genome exists as a single circular chromosome approximately 1.85 Mb in length [2]. Interspersed in the core chromosome are a number of mobile genetic elements (MGE) such as integrative and conjugative elements (ICE) and prophage DNA, the locations and composition of which may differ from strain to strain [7]. Although MGEs in *S. pyogenes* account for ~10% of total DNA, these element account for ~75% of the genetic variation between individual strains [7]. *S. pyogenes* also contains 13 two-component systems and several stand-alone response regulators which collectively regulate expression of the genome [8].

1.3 *S. pyogenes* virulence factors

The *S. pyogenes* spectrum of infections can be attributed to its wide range of virulence factors, which lead to adherence, immune system evasion, deliberate stimulation or degradation of host components, and direct cell lysis. The virulence factors discussed below do not represent an exhaustive list, but represent a selection of well-studied factors.

1.3.1 M protein

Serological specificities between *S. pyogenes* strains are based on differences in M protein [9]. Encoded by the *emm* gene, that is located on an ancient pathogenicity island (acquired before speciation) [10], the surface-bound M protein is made of long fibrils formed into a relatively-conserved C-terminal coiled-coil with an N-terminal hypervariable non-helical region [10-13]. M proteins have been shown to have binding sites for a number of human proteins including fibronectin, glycosaminoglycans, fibrinogen, C4b-binding protein (C4BP), plasminogen, and factor H [14-20]. By binding these different molecules, M proteins contribute to bacterial aggregation, host cell adherence, as well as evasion of complement and phagocytosis [11]. Through N-terminal hypervariable region sequencing, it has been determined that there are more than 200 *emm* types, which vary in sequence and size [11, 21, 22]. Certain *emm* types have been associated with specific streptococcal diseases including *emm1* (M1) strains which have been associated with invasive infections; *emm* types 3, 5, 6, 18, and 89 have been associated with pharyngitis; and *emm* types 1, 5, 6, 14, 18, and 24 which have been referred to as "rheumatogenic" for their association with acute rheumatic fever (ARF) [6, 23-27]. *emm* types can also have certain geographic associations and may be prevalent in specific areas of the world [28], while others are distributed globally [29].

1.3.2 Hyaluronic acid capsule

S. pyogenes produces a hyaluronic capsule, which has been demonstrated to provide the bacteria with increased resistance to phagocytosis [30, 31]. The repeating units of β1,4-linked glucuronic acid connected via β1,3-linked *N*-acetylglycosamine form a glycosaminoglycan fiber which is indistinguishable from those produced in human connective tissue [32]. The genes for the enzymes responsible for capsule production are highly conserved, and are as follows: *hasA* encodes the hyaluronate synthase, *hasB* encodes UDP-glucose dehydrogenase, and *hasC* encodes UDP-glucose pyrophosphorylase [30, 32]. These genes constitute a three gene operon regulated by a promoter located upstream of *hasA*. Difference in capsule production can be attributed to *cis*-acting promoter elements, growth phase, and environmental signals, but ultimately, this operon is regulated by the control of virulence (CovR/S; formerly CsrR/S) two-

component system [30, 32, 33]. It has been suggested that downregulation of capsule synthesis leads to an asymptomatic carriage state [34] while increased capsule production leads to symptomatic infections. While *emm*18 (M18) strains are typically associated with the "less severe" rheumatic fever and pharyngeal infections [30], the *has* operon promoter in the M18 strain was three times more active compared to the *has* operon promoter of a poorly-encapsulated strain [33]. This hyper-encapsulation by the M18 strain is due to premature truncation in the RocA protein [35]; interestingly, the RocA truncation was also associated with increased capsule expression in an M3 strain [36]. In addition to the link between capsule expression and "less severe" infections, Turner *et al.* noticed increased capsule expression by invasive *S. pyogenes* isolates [37], and Levin *et al.* demonstrated an increase in lethality using a hyper-encapsulated strain in a murine model of systemic infection [30]. This strain had a deletion in the *covR* gene to increase capsule expression, and since the CovR/S two-component system is now known to regulate more genes than the hyaluronic acid capsule production, it is possible that the extreme phenotype observed was due to effects from other virulence factors as well.

Interestingly, the hyaluronic acid capsule has been shown to readily interact with human CD44, in turn inducing membrane ruffling, actin cytoskeleton rearrangement, and disruption of tight junctions [38]. Similarly, in a cynomolgous macaque model of *S. pyogenes* pharyngitis, expression of the *has* operon positively correlated to expression of vesicle formation and clathrin adaptor genes, downstream of CD44, for endocytosis [39]. It has also been proposed that *S. pyogenes* uses its capsule to directly invade keratinocytes [40]. These studies support the belief that *S. pyogenes* employs its capsule to evade phagocytosis, invade human tissues, potentially persist intracellularly, and promote infection.

1.3.3 Streptococcal pyrogenic exotoxin B

Streptococcal pyrogenic exotoxin B (SpeB) is a secreted cysteine protease that is known to cleave a number of *S. pyogenes* factors and host molecules. SpeB is secreted as a zymogen, then transitions through several intermediates to its active form via autocatalysis at multiple cleavage sites in the zymogen pro-domain [41]. Mature SpeB then cleaves more zymogen to its active form. SpeB has been shown to cleave E-cadherin

and occludin, two components of tight junctions, potentially facilitating translocation of the bacteria across the epithelial barrier and contributing to an invasive disease phenotype [42]. SpeB is able to degrade the *S. pyogenes* virulence factors M1, streptolysin O (SLO), SpeF DNAse (also known as DNAse B), glycolytic enzymes, secreted inhibitor of complement (SIC), streptokinase, and host immune mediators including C1 inhibitor, as well as several complement proteins (C3b and the membrane attack complex) [43-45], presumably as a method of modulating host inflammatory responses *in vivo* [43]. Aziz and colleagues [43] propose that *S. pyogenes* alters SpeB expression upon infection *in vivo* and therefore modulates virulence factor degradation. Carroll and Musser [41] suggest that SpeB is regulated in a complex manner with both environmental signals such as pH and salt concentration, as well as intrinsic regulators of transcription, translation, and post-transcriptional modifications; for example, at the level of transcription alone there are at least 12 regulatory factors involved including regulator of protease B (RopB), the multiple gene regulator protein Mga, and the CovR/S two-component system [46].

1.3.4 CovR/S and virulence factor regulation

The control of virulence response regulator and sensor kinase (CovR/S) two-component system regulates up to 15% of *S. pyogenes* genes, including virulence factors [47]. Originally identified as capsule synthesis regulator regulator component and sensor component (CsrR/CsrS) [30], CovR/S contains the canonical parts of a two component system including a cytoplasmic response regulator (CovR) and a membrane-located sensor kinase (CovS) [47, 48]. One proposed model indicates that CovS senses environmental signals such as pH, osmolarity, and temperature [49]. Under normal conditions, CovS phosphorylates CovR, leading to the repression of the expression of virulence genes such as the hyaluronic capsule, SpeB, streptokinase, and streptolysin S (SLS) during exponential growth [49, 50]. Under mild stress conditions, CovR is inactivated by CovS, leading to the de-repression (and thus, expression) of genes required for virulence and growth under stress [49]. Strains with a mutation in *covR* have been associated with invasive infections, and may even be selected for *in vivo* [51]. *covR* mutations are typically amino acid substitutions where the final protein loses CovS binding activity but still retains some DNA binding activity [51, 52]. *covS* mutations

acquired *in vivo* can be separated into two categories: mutations from mouse-passaged strains are typically frameshift mutations that remove function of the resulting protein, while mutations from clinical isolates are usually point mutations in which the resulting CovS protein retains some function [52]. Interestingly, strains that have a mutation in *covS* do not produce SpeB, while strains with mutations in both *covR* and *covS* do, indicating that CovS attenuates the repression of SpeB in a process facilitated by CovR [51]. M1 strains that cause invasive disease have also been associated with mutations in *covS* [52]. It should be noted, however, that different mutations within the same gene can produce differing effects with regards to virulence factor secretion profiles [51].

The other main participant in virulence factor regulation is the Mga regulator protein. Gene regulation by Mga can be either direct or indirect [46]. Regulation of *mga* itself is growth phase dependent as its expression is active during early and late exponential phases and turned off in stationary phase, indicating its importance in establishment of infection [53]. Its gene product, Mga, regulates a number of iron and amino acid uptake genes and genes for ribosomal proteins, as well as virulence genes including the *emm* gene, M protein expression, *scpA* which encodes C5a peptidase (discussed below), *fba* encoding fibronectin binding protein, and *sic* (encoding SIC) in response to environmental conditions [46, 53]. Although typically thought to be solely an activator of transcription, its repressor function has also been described, particularly with respect to transport, binding, and metabolism of sugar substrates [46].

Interestingly, CovR represses the gene for the response regulator TrxR of the TrxR/S two-component system, which typically activates the Mga regulon [54]. This link highlights the complexity of *S. pyogenes* virulence gene regulation, the networks of which are just beginning to be understood.

1.3.5 Other *S. pyogenes* virulence factors

Streptokinase, regulated by the CovR/S two-component system [55], is an enzyme secreted by *S. pyogenes* that cleaves plasminogen to plasmin [56]. It has found alternative use as an anticoagulant in supplemental therapy for patients with myocardial infarction [56]. Mice transgenic for human plasminogen are more susceptible to, and have higher

mortality from skin infections by *S. pyogenes* than non-transgenic mice [18]. Because of the expression of streptokinase by *S. pyogenes*, plasminogen has been suggested to be an essential host factor for establishment of *S. pyogenes* infection [18]. *S. pyogenes* is thought to use streptokinase in order to increase plasminogen cleavage to plasmin, which then results in increased degradation of the extracellular matrix as well as fibrin deposits to enhance dissemination of *S. pyogenes*. Interestingly, *S. pyogenes* also carries other plasminogen-binding proteins which aid in virulence by promoting adhesion and invasion into host epithelial cells, as well as preventing phagocytic engulfment and killing in blood [57].

Sda1 is a deoxyribonuclease (DNAse) utilized solely by the M1T1 globally disseminated clone of *S. pyogenes* [58]. While most *S. pyogenes* strains carry at least one DNAse, Sda1 is encoded by a unique allele (*sda1*) in a bacteriophage element, and seems to have a chimeric nature when compared to other similar DNAses [58]. Although the longer amino acid sequence of Sda1 correlates with increased DNAse activity [58] and escape from neutrophil extracellular traps [59], transfer of the bacteriophage containing *sda1* to a strain that originally lacked it did not lead to increased virulence in an invasive model of *S. pyogenes* infection [60].

S. pyogenes uses C5a peptidase to degrade host C5a, a chemokine produced in the complement cascade and used to recruit neutrophils to the site of infection [61, 62]. C5a peptidase is encoded by the *scpA* gene and is a surface protein which cleaves C5a between the lysine at position 68 and the asparagine at position 69, interfering with the epitope required for recognition by neutrophil C5a receptors [63]. Similar to other virulence factors of *S. pyogenes*, C5a peptidase demonstrates strict specificity for its substrate C5a [63, 64].

S. pyogenes cell envelope proteinase (SpyCEP) is a surface-associated proteinase produced by *S. pyogenes* that cleaves interleukin (IL)-8 at its C-terminus, thus reducing the number of neutrophils recruited to the site of infection [65]. Invasive disease isolates of *S. pyogenes* have been found to have higher SpyCEP expression compared to

pharyngeal isolates [65]; this may be due to its regulation by the CovR/S two-component system [37].

S. pyogenes employs two cytolysins: streptolysin O (SLO) and SLS. SLO is a secreted, oxygen-labile, thiol-activated toxin that binds cholesterol in cell membranes [66]. SLO aids in pathogenesis of *S. pyogenes* infections by polymerizing in membranes to form pores that lyse erythrocytes, macrophages, lymphocytes, and platelets [66-68]. Alternatively, the classic β-haemolysis, or complete lysis, of red blood cells produced by *S. pyogenes* colonies on blood agar plates is due to the oxygen-stable toxin SLS [69]. Encoded in a nine-gene operon (*sagA* - *sagI*) [70], SLS is typically expressed and secreted in stationary phase; however, with mutations in the *covR/S* locus, SLS is secreted during earlier stages [50]. SLS functions to form pores in membranes of lymphocytes, neutrophils, platelets, and red blood cells causing osmotic cell lysis, and aiding in virulence [68, 69].

1.3.6 Superantigens

Superantigens are non-enzymatic proteins that are secreted and range in size from ~22-29 kDa [71]. Named due to their "peculiarly strong and specific reactivity", superantigens are immunostimulatory toxins produced by some bacterial species [72]. They generally function by contacting the β-chain variable (Vβ) region of the T cell receptor (TCR) on T lymphocytes and the peptide-presenting major histocompatibility complex (MHC) class II molecules of antigen presenting cells (APC) causing large-scale immune activation (Figure 1) [71, 73]. These toxins are present in a number of bacterial species including *Mycoplasma arthritidis*, *Yersinia pseudotuburculosis*, coagulase negative staphylococci, and group G and C streptococci; however, the most notorious superantigens are from *Staphylococcus aureus* and *Streptococcus pyogenes*. Although superantigens are mainly known for their ability to activate and expand T lymphocyte populations, they are also known for their pyrogenic (fever-causing) and/or emetic (vomit-inducing) activity [74].

1.3.6.1 Streptococcal superantigens

Currently, there are 14 superantigens expressed by streptococcal species: streptococcal pyrogenic exotoxin (Spe) A, SpeC, SpeG-SpeP, streptococcal mitogenic exotoxin Z

A

B

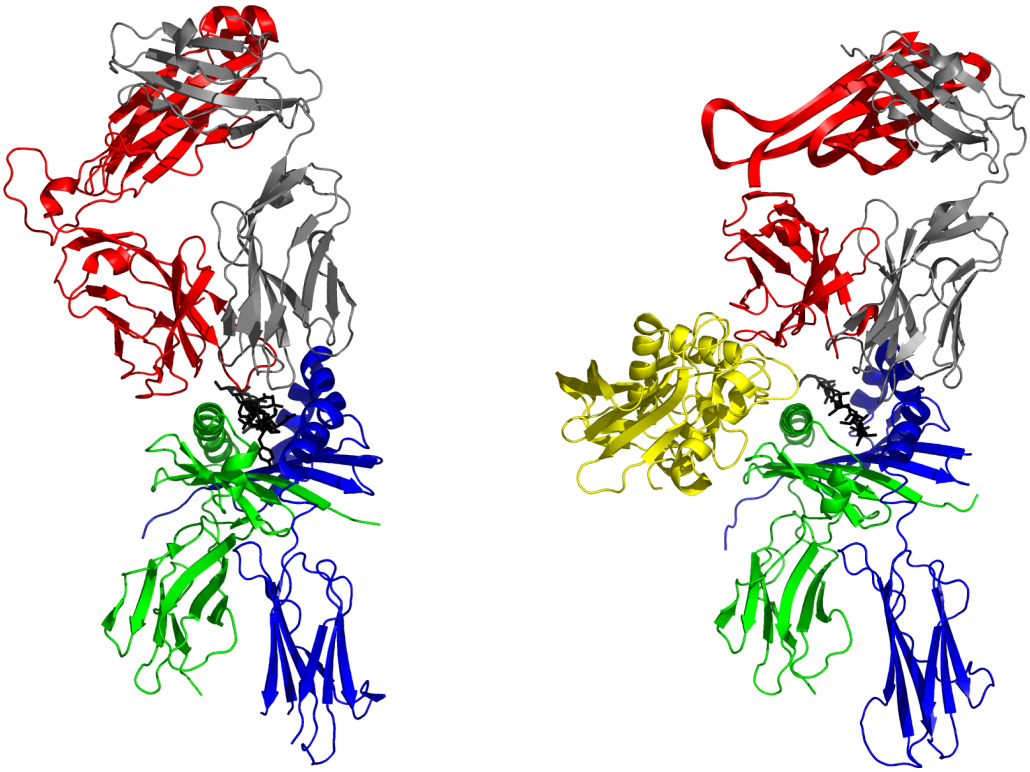


Figure 1. Antigen presentation complexes with and without superantigen interactions. (A) Conventional antigen presentation complex with TCR α-chain (grey), TCR β-chain (red), MHC class II α-chain (green), MHC class II β-chain (blue), and the antigen peptide (black). (B) Streptococcal pyrogenic exotoxin A (SpeA; yellow) interacting with the TCR β-chain (red) and MHC class II α-chain (green) of the antigen presentation complex. Also depicted are the TCR α-chain (grey), MHC class II β-chain (blue), and antigen peptide (black).

(SmeZ), and streptococcal superantigen A (SSA) [75]. However, only 11 of these – SpeA, SpeC, SpeG-SpeM, SmeZ and SSA – are found in *S. pyogenes*; the others are found in group C and group G streptococcal species [75]. Research groups have used the presence or absence of superantigen genes to "profile" different *S. pyogenes* strains in order to distinguish epidemiological distribution patterns [76, 77].

Superantigens target and expand T lymphocyte populations expressing specific Vβ regions. For example, SpeA targets human T lymphocytes expressing Vβ9, -12, and -14 [78], while SpeJ targets Vβ2, -3, -12, -14, and -17 [79]. SmeZ, which is an extremely potent superantigen, is able to activate human peripheral blood mononuclear cells (PBMC) at concentrations as low as 1 fg/mL [80], and targets T lymphocytes expressing Vβ8 [81-83]. Superantigen contribution to streptococcal disease is discussed below.

Although named with superantigen nomenclature, SpeB does not have superantigen activity nor sequence similarity to superantigens [41]. Original purifications of the enzyme were crude and likely contained SmeZ as an unknown contaminant. Interestingly, SpeB can degrade SmeZ, as well as SpeA and SpeG but to a lesser extent, while SpeJ is completely resistant to SpeB cleavage [84]. Similarly, SpeF was also mislabeled, and is a streptodoronase (streptococcal DNAse) originally named mitogenic factor (MF), but is now more commonly known as DNAse B [85]. Although DNAse B is heat stable like superantigens, it has no mitogenic activity [85, 86].

1.3.6.2 Superantigens from other species

M. arthritidis secretes a T lymphocyte mitogen (MAM) that targets murine Vβ6- and Vβ8-expressing lymphocytes, as well as human T lymphocytes expressing Vβ17 [87]. Interestingly, MAM has direct interactions with both TCR Vα and Vβ chains [88].

Y. pseudotuberculosis also expresses a T lymphocyte mitogen, *Y. pseudotuberculosis* mitogen (YPM), targeting human TCR Vβ3, Vβ9, Vβ13.1 and Vβ13.2 [71]. The YPM protein toxins are smaller in size at 14.5 kDa [71]. The superantigens from both *M. arthritidis* and *Y. pseudotuberculosis* have been found to exacerbate *in vivo* infections [89].

S. aureus produces several superantigens that contribute to numerous staphylococcal diseases including (but not limited to) staphylococcal food poisoning and staphylococcal TSS [73, 90]. They can be classified as toxic shock syndrome toxin 1 (TSST-1), the staphylococcal enterotoxins (SE) A-E, G, H, I, R, and T, as well as the SE-like (SEl) proteins SElJ-Q, S, U, V, and X [90]. The SEs each demonstrate emetic (vomit-inducing) activity and have been linked to staphylococcal foodborne illness [91], though this is attributed to a cysteine loop structure separate from the T lymphocyte stimulating activity [92]. Conversely, the SEl toxins do not have emetic activity, or have yet to formally demonstrate emetic activity [90]. SEH instead activates and expands T lymphocytes expressing Vα10, -14, and -17 TCR [93].

The only viral superantigens currently known are from the rabies virus and mouse mammary tumour viruses (MMTV). The nucleocapsid N protein of the rabies virus functions as a superantigen expanding (and causing eventual deletion of) T lymphocytes bearing human Vβ8 [94]. One of the recently proposed models for the mechanism by MMTV superantigens includes the protein binding to two MHC class II molecules and the TCR, bringing the TCR CDR3 into contact with the MHC class II α-chain [95]. MMTV superantigens are not thought to play a role in virus tumour formation, but instead are thought to be important for maintaining cell division and infection among T and B lymphocytes [96].

1.3.6.3 Superantigen genetics, expression, and structure

Since prophage elements can encode up to 10% of the *S. pyogenes* genome, many of the genetic differences seen between strains can be attributed to the presence or absence of these and other MGE [97-99]. Several *S. pyogenes* superantigens are encoded on prophage elements including SpeA, C, H, I, K, L, M, and SSA, while SpeG, J, and SmeZ are each encoded on the core chromosome [7, 97, 99, 100]. All strains of *S. pyogenes* annotated in the NCBI genomes database contain at least one superantigen, most with between two and seven superantigens with a median of four [77].

Superantigens contain an N-terminal signal sequence for secretion [90, 101] and are secreted in late stationary phase [102]. Once expressed, superantigens are generally very

hardy proteins, with most being unusually resistant to heat, and some are resistant to proteolysis, acidity, and desiccation [103].

Most virulence factors in *S. pyogenes* are regulated by Mga or CovR/S as previously discussed; however, regulation of superantigens is much less clear. Although expression has been linked to late stationary phase of the bacteria, a number of environmental factors including temperature, osmolarity, pH, and nutrient conditions could influence superantigen regulation. As some superantigen genes reside in active prophage elements, induction of the bacteriophage due to internal or external environmental signals (chemicals or UV light) may affect superantigen expression [97]. For example, SpeC expression and its bacteriophage production were both detected upon co-culture of *S. pyogenes* with the human pharyngeal cell line Detroit 562 cells [104]. SpeA expression can be induced by human transferrin and lactoferrin, two iron-binding proteins important for iron transport and sequestration in the host; although induction of SpeA expression was dependent on the level of iron-saturation, indicating that SpeA expression may be triggered as a response to low iron in the host [105]. Interestingly, strains with mutations in either or both of the *covR* and *covS* genes have increased *speA* transcript production [51]. Regulation of superantigens *in vivo* is a complex – and likely multifactorial – process that has yet to be fully elucidated.

Structures for several staphylococcal and streptococcal superantigens reveal similar tertiary structure, but fewer sequence similarities. The common N-terminal mixed β-barrel domain composed of antiparallel β-sheets, connected to a C-terminal β-grasp motif (an α-helix against a β-sheet) [106] indicates evolutionary conservation of the ability to bind both the TCR and MHC class II molecule [107]. Upon grouping superantigens (from all bacterial species) based on amino acid sequences, five distinct evolutionary groups are formed [90]. The sole member of group I is TSST-1 secreted by *S. aureus*, which upon binding the TCR and peptide-MHC (pMHC) (HLA-DR1) synapse contacts the polymorphic regions of the MHC class II α- and β-chain as well as peptide in the binding groove, indicating TSST-1 interaction with MHC class II molecules may be dependent on both the antigen presented and the specific MHC class II [108]. TSST-1 largely targets T lymphocytes bearing $V\beta2^+$ TCR [78]. Notable superantigens of group II include

staphylococcal SEB and SEC, along with streptococcal superantigens SpeA and SSA. Group II interactions with the pMHC are through a low-affinity N-terminal site and seem to be peptide independent [109], while TCR interactions are largely conformation-dependent [110]. Group III superantigens are all staphylococcal, including SEA, where the C-terminal β-grasp domain interacts with pMHC in a zinc-dependent manner [111]. They also contain both high-affinity and low-affinity MHC class II binding sites, allowing them to crosslink MHC class II molecules on the cell surface for optimum activity [112]. Both group II and group III superantigens contain a "cysteine loop" structure associated with staphylococcal foodborne illness [92]. Group IV consists of only streptococcal superantigens (SpeC, G, H, J, L, M, and SmeZ) that contain a high-affinity, zinc-dependent binding site [113]. SpeC, specifically, contacts specific residues of Vβ2.1 CDR1 and CDR2 in an energetically favourable interaction [114, 115]. Superantigens in group IV also contain a conserved "hydrophobic ridge" between the two β-sheets of the β-barrel domain and a shorter "cysteine loop", both of which are important for MHC class II binding [116]. Group V contains staphylococcal superantigens, and the streptococcal superantigen SpeI. These toxins require the zinc-dependent binding site [117], and also contain a unique "α3-β8" loop domain between the third α-helix and eighth β-sheet important for T lymphocyte activation [118]. Despite tertiary structural similarities between superantigens, each protein, even within groups, can have different interactions with MHC class II molecules and TCR. Not surprisingly, superantigens from *Y. pseudotuberculosis* and *M. arthritidis* cluster independent from the five main evolutionary groups of superantigens upon alignment [90].

1.3.6.4 Activation of immune cells by superantigens

To understand how superantigens interact with and activate the host immune system, we must first understand canonical antigen presentation and T lymphocyte activation. When the human immune system encounters conventional protein antigens, the foreign proteins are processed and presented by APC in the context of MHC class II molecules to T lymphocytes bearing complementary TCR. Recognition of the peptide antigen is based on specific contacts and conformational changes by the TCR complementarity determining region (CDR) 3 [119]. The CDR1 and CDR2 loops of the TCR α- and β-

chains form stabilizing interactions with the α-helices of the MHC class II molecules α- and β-chains of the pMHC class II complex [119, 120]. Conversely, superantigens contact both the MHC class II molecule and the TCR and bridge the antigen presentation complex, a process that can be irrespective of the specific antigen presented in the context of the MHC class II molecule [115, 121]. Superantigens bind both the MHC class II α-chain and the TCR outside of the antigen-binding groove, contacting the TCR in a less-specific manner that is critically dependent on the germline encoded CDR2 variable region of the β-chain (Vβ) [122], giving theses toxins their hallmark Vβ specificity [78]. The exception to this rule is SEH, which is the only known superantigen to activate T lymphocytes in a Vα-specific manner [93]. While conventional antigen presentation results in the activation of ~0.01% of naïve T lymphocytes [107], superantigens are able to activate entire Vβ subsets of T lymphocytes comprising up to 20% of the T lymphocyte population [73]. Of note, superantigens can activate both CD4+ and CD8+ T lymphocytes [88], in addition to γδ T lymphocytes [123].

Although there are multiple proposed models of molecular architecture detailing how TCR-triggering works (TCR aggregation, conformational change of parts in the TCR complex, or impediment of inhibitory molecules), TCR:pMHC recognition of conventional antigens leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) on the cytoplasmic tails of CD3 molecules by CD4- and CD8-coreceptor-associated protein tyrosine kinases Lck and Fyn [124]. Phosphorylated ITAM on CD3 cytoplasmic tails allows subsequent recruitment of the kinase ZAP70 [125]. A phosphorylation cascade and a non-linear framework of signaling follows, including the phosphorylation of adaptor proteins linker for the activation of T lymphocytes (LAT) and Src-homology 2 domain-containing linker protein of 76 kDa (SLP-76) which serve as docking sites for further signaling. Phospholipase C (PLCγ1), recruited to dock on phosphorylated LAT, is phosphorylated to produce its active form and cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol triphosphate (IP_3) and diacylglycerol (DAG) [125, 126].

IP_3 is an important messenger in calcium (Ca^{2+}) signaling, activating Ca^{2+}-dependent channels on the endoplasmic reticulum and leading to intracellular Ca^{2+} release. This, in

turn, leads to the activation of Ca^{2+} and calmodulin-dependent transcription factors. For example, intracellular Ca^{2+}, along with signals through the Ras pathway, activate calcineurin, which in turn dephosphorylates the nuclear factor of activated T lymphocyte (NFAT) protein, allowing its translocation to the nucleus. NFAT in complex with activator protein 1 (AP-1) ensures transcription of genes necessary for T lymphocyte activation such as interleukin-2 (IL-2) [125]. Alternatively, NFAT activity lacking AP-1 results in the transcription of genes promoting a state of T lymphocyte unresponsiveness [127]. Alternatively, DAG induces a pathway involving the guanine-nucleotide exchange factor Ras, which through a phosphorylation cascade of mitogen-associated protein kinases (MAPK), eventually leads to AP-1 transcription complex activation. DAG also binds to and activates the protein kinase C θ (PKC-θ), which has a role in integrin-based signaling in addition to regulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. PKC-θ phosphorylates caspase recruitment domain (CARD)-containing and membrane-associated guanylate kinase (MAGUK)-containing scaffolding protein (Carma1), which then associates with Bcl-10 and mucosa-associated lymphoid tissue lymphoma translocation gene 1 protein (MALT1) [128]. This complex then polyubiquitinates the inhibitor of NF-κB (IκB) kinase (IKK) complex regulatory subunit (IKKγ), sending it for degradation and allowing access to IκB [129]. The catalytic subunits of the IKK complex then phosphorylate IκB, facilitating its degradation and liberating NF-κB, which is capable of translocating into the nucleus to enable transcription of genes indicative of T lymphocyte activation. In summary, antigen recognition by the T lymphocyte leads to downstream events including cytoskeletal rearrangement, integrin signaling, and intracellular Ca^{2+} release, and downstream expression of genes indicative of cell activation [125, 126]. Costimulation, by recognition of the T lymphocyte CD28 molecule by CD80 or CD86 on APC, serves to enhance each of the activating signals produced; alternatively, TCR engagement without costimulation leads to anergy [130].

In contrast, T lymphocyte activation by some superantigens including staphylococcal enterotoxins (SE) A, SEB, SEC_1, SEE, and TSST-1, as well as streptococcal superantigens SpeC, SpeI, SpeJ, and SmeZ, can proceed in a CD4-independent manner [131]. These toxins do not require CD4-dependent Src-family kinase (Lck, Fyn)-

mediated signaling (including ZAP-70, LAT, and PLCγ1) [132]. Instead, they can initiate an alternative signaling pathway dependent on G protein Gα11, the activation of PLCβ, subsequent Ca^{2+} release, translocation of NFAT and NF-κB, and expression of IL-2 [132]. Although the alternative pathway and canonical Lck-dependent pathways do share crosstalk and converge at the level of ERK-1/2 in the MAPK cascade, and superantigens are capable of activating T lymphocytes through the Lck-dependent pathway, activation of the alternative pathway is sufficient to activate T lymphocytes [132]. Superantigen-induced T lymphocyte activation is an APC-dependent process, and costimulation with CD28 serves to increase the strength of the stimulated T lymphocyte response [133]. Although the canonical T lymphocyte marker of activation is IL-2, superantigens have also been known to induce the expression of TNF-β, and interferon-γ from T lymphocytes, in addition to TNF-α, and IL-1 from APC [106, 134, 135].

Post-superantigen activation, T lymphocyte populations can expand, remain unresponsive to normal antigenic and superantigen stimulation (anergy), or be deleted via apoptosis. Upon encountering superantigens *in vivo*, T lymphocytes bearing appropriate TCR will be activated and proliferate for up to four days, peaking around 24 hours [136]. In the following days, T lymphocytes may be rendered unresponsive to further superantigen stimulation [137]. This unresponsive state can last for several weeks, although recovery is possible [138]. It is also pertinent to remember that *in vitro* studies may not always reflect the *in vivo* state, as Heeg *et al.* (1995) determined that while anergy was detected *in vitro*, cells were still responsive *in vivo* [139].

Just as TCR Vβ chains are important for superantigen binding, so too are MHC class II molecules. Humans express MHC class II molecules from the highly polymorphic HLA locus – DP, DQ, and DR [140]. The MHC expressed, and polymorphisms within it (haplotype), affect the binding ability of the superantigen, the magnitude of response evoked [141], and potentially even the clinical outcome of the patient [142]. Specifically, expression of the DRB1*14/DQB1*0503 haplotype were associated with higher risk of developing invasive streptococcal disease, while the DRB1*1501/DQB1*0602 haplotype conferred a protective effect in human severe streptococcal infections [142]. Nooh and colleagues (2006) also determined that a lower cytokine response was elicited when

superantigens activated APC expressing DQ6 alleles (DQB1*06) than when APC expressed DR4/DQ8 alleles (DRB1*0402/DQB1*0302), and that HLA-DQ6-expressing mice survived longer in a mouse model of streptococcal sepsis [143]. It has been suggested that MHC class II molecules may play a role in modulating human superantigen responses, and thus may affect disease severity [143].

1.4 *S. pyogenes* infections

S. pyogenes uses its numerous virulence factors to survive in their hosts. In a relationship between organisms, classification of 'commensal' is applicable when one organism benefits without affecting the other, while a 'pathogenic' organism causes detriment to its host. Humans infected with *S. pyogenes* can be symptomatic or asymptomatic carriers of the bacterium. Meta-analysis of 209 studies concluded that approximately 12% of children between the ages of 5 and 18 years were found to be pharyngeal carriers for *S. pyogenes*, while the carriage rates for children under 5 years of age are much lower (3.8%) [144]. Asymptomatic carriage of *S. pyogenes* can last for up to a year [145], and has been implicated in transmission of *S. pyogenes* [146, 147]. Though globally there are *S. pyogenes* strains resulting in cases of documented resistance to antibiotics including tetracycline, erythromycin as well as other macrolides, there has yet to be a case where recorded resistance to penicillin G has been observed [7, 148-153]. As such, penicillin G remains the most effective treatment for *S. pyogenes* infections [151]. *S. pyogenes* is an extremely successful human pathogen that uses its arsenal of virulence factors to cause a number of different infections with varying clinical manifestations. *S. pyogenes*-associated diseases can be classified as direct infections, immune-mediated diseases, and toxin mediated diseases.

1.4.1 Direct infections

1.4.1.1 Pharyngitis

Perhaps the most common and well-known infection caused by *S. pyogenes* is pharyngitis or "strep throat". In 2005, it was estimated that there are more than 616 million incident cases of *S. pyogenes* pharyngitis per year, worldwide [4]. Additionally, it is estimated that the cost of *S. pyogenes* pharyngitis cases among children is more than $224 million

annually in the United States alone [154]. *S. pyogenes* usually establishes its niche in the pharynx and the crypts of the palatine tonsils and the infection proceeds with a sore throat and fever [155]. Other symptoms may include headache, abdominal pain, nausea, and vomiting, while the diagnosis is confirmed with a rapid antigen test and a *S. pyogenes*-positive throat culture [154]. Spread of infection is thought to be by droplet contact from infected or asymptomatically colonized individuals or by fomites [156, 157]. Pharyngeal infections are treated with penicillin V (oral), amoxicillin (oral), or benzathine penicillin G (intramuscular; IM); however, incidence of penicillin allergy dictates other treatment options including cephalexin (oral), cefadroxil (oral), clindamycin (oral), azithromycin (oral), and clarithromycin (oral) [158]. Prophylaxis with benzathine penicillin G, or erythromycin for those with a penicillin allergy, showed a 5-fold decrease in *S. pyogenes* pharyngeal infections when compared to those not given the prophylaxis [159]. Preventing *S. pyogenes* pharyngitis, or timely antibiotic treatment of infection, also serve to protect those from post-streptococcal infection sequelae (discussed below). There have not yet been any superantigens definitively associated with human pharyngitis infections.

1.4.1.2 Skin infections

Erysipelas and cellulitis, in addition to impetigo and ecthyma (pyoderma) are all skin infections caused by *S. pyogenes*. Erysipelas and cellulitis, while not as common as pyoderma, are no less severe. Both conditions have symptoms of inflammation (erythema) and pain, sometimes accompanied by fever, high leukocyte counts, inflammation of the lymphatic channels, and enlarged lymph nodes [160]. Erysipelas is characterized by a distinct raised area of affected and inflamed skin. The majority of cases are caused by *S. pyogenes*, though other types of streptococci may be the causative agent [160]. In cellulitis, the distinction between affected and unaffected skin is less clear. While *S. pyogenes* is one cause of cellulitis, it should be noted that a number of other bacterial species, including *Staphylococcus aureus*, can also cause this condition [160]. In the instance of pyoderma, there are more than 111 million cases due to *S. pyogenes* annually [4]. Pyoderma presents as contagious pustules that rupture, forming honey-coloured scabs and is treated by topical or oral antibiotics [160, 161]. Expression of specific collagen-binding and fibronectin-binding proteins contributes to the ability of

S. pyogenes to cause skin infections [2]. *S. pyogenes* skin infections are also associated with the development of post-streptococcal immune sequelae (discussed below).

1.4.1.3 Invasive infections

It is estimated that there are over 600,000 new invasive infections and 163,000 deaths from *S. pyogenes* invasive infections each year [4]. Invasive infections are defined as the isolation of *S. pyogenes* from an otherwise sterile site. *S. pyogenes* can cause bacteraemia, sepsis, puerperal sepsis, and necrotizing fasciitis. Streptococcal bacteraemia, defined as bacteria in the blood, can lead to the life-threatening condition of severe sepsis. Streptococcal severe sepsis is characterized by organ impairment as well as coagulation abnormalities, and can then lead to septic shock (discussed below as streptococcal toxic shock syndrome) [162]. Puerperal sepsis refers to sepsis following childbirth and is a particularly dangerous condition as the disease progresses rapidly and can become critical within hours to days [163]. Necrotizing fasciitis is discussed below.

1.4.2 Immune-mediated diseases

Immune-mediated post-streptococcal immune sequelae can result following untreated pharyngitis or other *S. pyogenes* infections. These diseases include acute post-streptococcal glomerulonephritis (APSGN), paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS), and ARF.

Acute post-streptococcal glomerulonephritis (APSGN) is a disorder of the kidneys as a result of immune complex deposition in the glomeruli. Symptoms of APSGN, which include proteinuria, edema, hypertension, and low levels of complement from the serum, usually appear 10 days post-pharyngeal infection and 14-21 days post-pyoderma infection [164]. As with other *S. pyogenes* diseases, there are specific *emm*-types associated with APSGN, deemed “nephritogenic” strains [6]. It is estimated that there are over 470,000 cases of APSGN annually, with approximately 5,000 deaths [4].

There have been proposed links between *S. pyogenes* infections and prepubescent neuropsychiatric manifestations including obsessive-compulsive disorder (OCD) and Tourette’s syndrome, which are cumulatively referred to as PANDAS [165]. Symptoms

are proposed to originate due to anti-neuronal antibodies; however, these links, and even the existence of PANDAS are highly controversial [6].

There are high rates of ARF in developing countries, as well as indigenous populations in developed countries. ARF is the leading cause of heart disease in children in the developing world [166]. In Australia, high prevalence is attributed to social determinants such as poverty, low employment, poor nutrition and education, overcrowded living conditions, and decreased access to medical care [166]. ARF is diagnosed according to the Jones criteria: carditis, migratory polyarthritis, chorea, along with subcutaneous nodules and erythema marginatum [26]. ARF is an autoimmune response to the heart, joints, subcutaneous tissues, brain, and skin that can develop following an upper respiratory tract *S. pyogenes* infection [166]. Molecular mimicry of *S. pyogenes* antigens M protein and hyaluronic acid capsule leads to the development of antibodies to epitopes on or within *S. pyogenes* that cross-react with similar epitopes in human tissues [27]. ARF can lead to rheumatic heart disease (RHD) development and long-term heart damage, where the affected individual may eventually need a heart valve replacement. Ralph *et al.* have developed a continuous quality improvement strategy for prevention and management of ARF and RHD, using prophylactic intramuscular (IM) injections of benzathine penicillin G [167]. This is a common strategy following *S. pyogenes* infections [166, 168, 169].

1.4.3 Toxin-mediated diseases

Necrotizing fasciitis is classified as the progressive breakdown of fat, subcutaneous tissue, fascia, and muscle, which may or may not include the destruction of the skin itself [170, 171]. A key symptom for necrotizing fasciitis is severe pain [172]. Production of proteases, phospholipases, and other enzymes cause disintegration of the affected tissue [6]. Necrotizing fasciitis has also been associated with a lack of neutrophils at the site of infection [172], a condition in part caused by SpyCEP [65], as well as the production of superantigens causing widespread immune system activation and contributing to local tissue damage [6].

Scarlet fever cases usually present with fever, pharyngitis, and rash with sandpaper

texture, with or without strawberry tongue (glossitis with hyperplastic fungiform papillae) and circumoral pallor [152]. Children between 2 and 10 years of age are the most commonly affected population, typically with a median age of 4 years of age [152, 153, 173]. Scarlet fever remains a relevant disease as outbreaks were reported in China (Beijing, Shanghai, and Hong Kong) in 2011 [152, 174, 175] and increasing numbers of cases were reported in the United Kingdom in early 2014 [173]. The superantigens SSA, SpeA, and SpeC have also been associated with scarlet fever isolates of *S. pyogenes* [153].

Superantigens are known mediators for toxic shock syndrome (TSS), a condition in which symptoms include disseminated intravascular coagulation, vasodilation, myocardial suppression, acute respiratory distress syndrome, multiple organ failure, and potentially death [73]. Superantigen-induced activation of numerous immune cells causes a 'cytokine storm', and further activation of the immune system [73, 90]. These immune-reactive toxins are most commonly produced by *S. aureus* or *S. pyogenes* in TSS. Clinically, TSS has been associated with TSST-1 production during *S. aureus* infections [73], but streptococcal TSS (STSS) has been linked to the superantigen SpeA, as well as SpeC [176], SpeJ, and streptococcal mitogenic exotoxin Z (SmeZ) [177]. Interestingly, lack of neutralizing anti-superantigen antibodies are considered a risk-factor for TSS [177], and more than 60% of patients with STSS have bacteraemia [73].

1.5 Superantigen redundancy

Multiple alleles of some superantigens exist in nature. As of 2014, 91 unique streptococcal superantigen gene sequences had been annotated [75]. SmeZ is likely the superantigen with the highest number of variant forms; interestingly, most forms remain equally potent, regardless of their antigenic differences [82]. Some forms are inactive due to a single base pair deletion that truncates the protein [82, 178].

Additionally, most *S. pyogenes* strains contain more than one superantigen [77, 179]. Considering many superantigens are encoded in prophage elements [7, 97], and MGE enable transfer of genes between strains, *S. pyogenes* strains can naturally acquire extra genes including superantigens [7, 180]. Each strain usually contains a different

superantigen repertoire and although this feature has been harnessed for epidemiological purposes by searching for superantigen presence or absence in the genomes of clinical isolates [76, 77], the question of why each strain maintains a unique superantigen profile remains unanswered.

1.6 Superantigens in murine models

Certain host factors, including CD44 and human plasminogen, enhance *S. pyogenes* nasopharyngeal infection in murine models [18, 181]. Additionally, different mice have different susceptibilities to infection, as emphasized by Aziz *et al.* [182], indicating that MHC class II molecules are important for infection. In addition, mice expressing human MHC class II molecules serve as better models of *S. pyogenes* disease as they are more sensitive to superantigens [80, 183-185].

Previous nasopharyngeal murine models have demonstrated that *S. pyogenes* resides in the tonsil homologue nasal-associated lymphoid tissues (NALT) [186]. It has been proposed that *S. pyogenes* enters the NALT via membranous cell (M cell) transfer across the epithelium, similar to Peyer's patches in the intestines [186]. However, our current mouse model demonstrated that *S. pyogenes* cells localize to the murine nasal turbinates (but not the NALT) following nasal inoculation. Using this model, we established that human MHC class II molecules are necessary for acute nasopharyngeal infection by *S. pyogenes* strain MGAS8232 and that the establishment of infection by that strain was also dependent on expression of the superantigen SpeA [183].

1.7 Rationale and Hypothesis

With continued research, the role for superantigens in invasive streptococcal diseases has become clearer in the past few years. Alternatively, the role of superantigens in less severe infections has remained undefined, though we know they are being expressed *in vivo* during nasopharyngeal infections [183] (McCormick Lab, unpublished data). Also unclear is the reason why strains frequently carry more than one superantigen; however, one proposed theory is that different superantigens allow for infection of humans expressing different MHC class II molecules [183]. Thus, I hypothesize that encoding

different superantigens allows *S. pyogenes* to infect a wider host range. I also hypothesize that different superantigens, in addition to SpeA, can promote infection.

1.8 Specific Aims

The specific aims for this project were to i) generate isogenic superantigen-complemented strains to determine if a single superantigen could modify a host's susceptibility to infection and ii) generate a series of isogenic superantigen-knockout and -complemented strains to determine if superantigens other than SpeA are functionally redundant and if so, iii) determine which superantigens are important for establishment of a non-severe nasopharyngeal infection by *S. pyogenes*.

Chapter 2 : Materials and Methods

2 Materials and Methods

2.1 Bacterial growth conditions

2.1.1 *Escherichia coli* growth conditions

Escherichia coli XL1-Blue cultures were grown aerobically with shaking in Luria Bertani (LB; EMD Millipore; Etobicoke, ON, Canada) or Brain Heart Infusion (BHI) broth at 37°C. Solid media was created by adding 1.5% (w/v) agar (Thermo Fisher Scientific; Ottawa, ON, Canada). For cultures possessing pG^{+}host5, media was supplemented with 150 µg/mL Erythromycin (Erm; Thermo Fisher Scientific). Supplementation of 150 µg/mL Ampicillin (Amp; EMD Millipore) was required for pBluescript containing clones. A complete list of plasmids used in this study can be found in Table 1. Stock cultures of *E. coli* were frozen in LB or BHI liquid media supplemented with 25% glycerol. A complete list of bacterial strains used in this study can be found in Table 2.

2.1.2 *S. pyogenes* growth conditions

Streptococcus pyogenes liquid cultures were grown in stationary Todd Hewitt Broth (Becton Dickinson [BD] Biosciences; Mississauga, ON, Canada) supplemented with 1% (w/v) yeast extract (BD Biosciences) (THY) at 37°C. For solid media, 1.5% (w/v) agar (Thermo Fisher Scientific) was added. Cultures containing the pG^{+}host5 plasmid were grown in media supplemented with 1 µg/mL Erm. Stock cultures of *S. pyogenes* were frozen in THY supplemented with 25% glycerol.

2.1.3 *S. pyogenes* growth curves

Prior to growth evaluation, cultures were grown from -80°C freezer stocks overnight and subcultured twice (1%) in THY at 37°C. For automated growth curves, cultures were adjusted to an optical density at 600 nm (OD_{600}) value of 0.02 prior to placement in a 100-well Bioscreen plate. The OD_{600} value for each well was measured every 30 minutes by a Bioscreen C MBR (Piscataway, NJ, USA). For manual growth curves, 1 mL of culture with an OD_{600} value of 0.9 was added to a pre-warmed 100 mL bottle of THY.

Table 1. Plasmids used in this study.

Plasmid	Description[a]	Source
pBluescript SK+	*E. coli* expression vector, expression is under the Lac promoter, multiple cloning site (MCS) is in the *lacZ* gene; Amp^r	Stratagene (Aligent Technologies, Santa Clara, CA, USA)
pBluescript::*smeZ*	pBluescript with the *smeZ* gene from MGAS5005 inserted in the MCS; Amp^r	This study
pG^+host5	Gram-negative origin of replication, and temperature-sensitive Gram-positive origin of replication; Erm^r	[187]
pG^+host5::*I-Sce*I::IntMGAS8232	pG^+host5 with the *I-Sce*I restriction enzyme cut site inserted; 1 kb of homologous DNA to the *tsf* and *pepO* region of MGAS8232 with its own MCS is inserted in the *I-Sce*I site; Erm^r	[183]
pG^+host5::*I-Sce*I::IntMGAS8232::*speJ*	Plasmid for complementation of *speJ* from MGAS5005 between *tsf* and *pepO* of MGAS8232; Erm^r	This study
pG^+host5::*I-Sce*I::MGAS5005Δ*smeZ*	Plasmid for the deletion of *smeZ* in MGAS5005; Erm^r	This study
pG^+host5::*I-Sce*I::IntMGAS5005	pG^+host5 with the *I-Sce*I restriction enzyme cut site inserted; 1 kb of homologous DNA to the *tsf* and *pepO* region of MGAS5005 with its own MCS is inserted in the *I-Sce*I site; Erm^r	[188]
pG^+host5::*I-Sce*I::IntMGAS5005::*speA*	Plasmid for complementation of *speA* between *tsf* and *pepO* of MGAS5005; Erm^r	This study
pG^+host5::*I-Sce*I::IntMGAS5005::*speJ*	Plasmid for complementation of *speJ* between *tsf* and *pepO* of MGAS5005; Erm^r	This study
pG^+host5::*I-Sce*I::IntMGAS5005::*smeZ*	Plasmid for complementation of *smeZ* between *tsf* and *pepO* of MGAS8232; Erm^r	This study

[a]Abbreviations: Amp^r – Ampicillin resistance, Erm^r – Erythromycin resistance

Table 2. Bacterial strains used in this study.

Strain	Description	Source
E. coli XL1 Blue	Cloning strain	Novagen, Stratagene
S. pyogenes MGAS5005	*emm1* (M1) invasive isolate from Ontario, Canada	[189]
S. pyogenes MGAS5005Δ*speA*	MGAS5005 with *speA* deletion	[188]
S. pyogenes MGAS5005Δ*speG*	MGAS5005 with *speG* deletion	[188]
S. pyogenes MGAS5005Δ*speJ*	MGAS5005 with *speJ* deletion	[188]
S. pyogenes MGAS5005Δ*smeZ*	MGAS5005 with *smeZ* deletion	[188]
S. pyogenes MGAS5005Δ*speA/smeZ*	MGAS5005 with *speA* and *smeZ* deletions	[188]
S. pyogenes MGAS5005Δ*speA/speJ/smeZ*	MGAS5005 with *speA*, *speJ*, and *smeZ* deletions	[188]
S. pyogenes MGAS5005Δ*speA/speJ/smeZ::speJ*	MGAS5005Δ*speA/speJ/smeZ* with *speJ* complement	This study
S. pyogenes MGAS5005Δ*smeZ::smeZ*	MGAS5005Δ*smeZ* with *smeZ* complement	This study
S. pyogenes MGAS5005Δ*speA/smeZ::speA*	MGAS5005Δ*speA/smeZ* with *speA* complement	This study
S. pyogenes MGAS5005Δ*speA/smeZ::smeZ*	MGAS5005Δ*speA/smeZ* with *smeZ* complement	This study
S. pyogenes MGAS5005Δ*smeZ* (new)	MGAS5005 with *smeZ* deletion	This study
S. pyogenes MGAS5005Δ*speA/smeZ* (new)	MGAS5005 with *speA* and *smeZ* deletions	This study
S. pyogenes MGAS5005Δ*smeZ::smeZ* (new)	MGAS5005Δ*smeZ* with *smeZ* complement	This study
S. pyogenes MGAS5005Δ*speA/smeZ::speA* (new)	MGAS5005Δ*speA/smeZ* with *speA* complement	This study
S. pyogenes MGAS5005Δ*speA/smeZ::smeZ* (new)	MGAS5005Δ*speA/smeZ* with *smeZ* complement	This study
S. pyogenes MGAS8232	*emm18* (M18) rheumatic fever isolate from an outbreak in USA	[190]

S. pyogenes MGAS8232ΔSAg	MGAS8232 with *speA*, *speC*, *speG*, *speL*, *speM*, and *smeZ* deletions	[183]
S. pyogenes MGAS8232ΔSAg::*speJ*	MGAS8232ΔSAg with *speJ* complement	This study

OD_{600} values were measured every hour in a spectrophotometer (DU® 530 Life Science UV/Vis Spectrophotometer, Beckman Coulter Canada LP; Mississauga, ON, Canada).

2.2 Deoxyribonucleic acid isolation

2.2.1 Plasmid isolation from *E. coli*

Plasmids were extracted from overnight cultures of *E. coli* using either the Spin Miniprep Kit (Qiagen; Toronto, ON, Canada), or E. Z. N. A. kit (Omega Bio-Tek; Norcross, GA, USA). Plasmid concentrations were measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific).

2.2.2 Genomic DNA isolation from *S. pyogenes*

S. pyogenes genomic DNA was isolated by pelleting 2 mL of overnight culture. The pellet was resuspended and washed twice with 0.2 M sodium acetate (Thermo Fisher Scientific), followed by resuspension in Tris Ethylene Glucose buffer (100mM Tris (Amresco; Solon, OH, USA), 1 mM ethylenediaminetetraacetic acid (EDTA; Bioshop Canada Inc., Burlington, ON, Canada), 25% glucose (Sigma Aldrich; Oakville, ON, Canada)). Mutanolysin (Sigma Aldrich) and lysozyme (Sigma Aldrich) were added, and samples were left at 37°C for one hour. Samples were then centrifuged and the supernatants were poured off. Pellets were resuspended in lysis buffer (0.2% sodium dodecyl sulphate (SDS; EMD Millipore) and 50mM EDTA), followed by the addition of ribonuclease (RNAse) A (Sigma Aldrich) and Proteinase K (MP Biomedicals, LLC; Solon, OH, USA). Samples were then left at 65°C for two hours. Upon cooling, potassium acetate (EMD Millipore) was added to a concentration of 0.6M and samples were centrifuged at 20,800 × *g* at 4°C for 10 minutes. Supernatants were removed, added to 500 µL ice cold 95% ethanol, and then centrifuged for 10 minutes at 4°C at 20,800 × *g*. Supernatants were drained and pellets were washed with 70% ethanol, prior to drying. DNA was resuspended in autoclaved MilliQ water. Plasmid concentrations were measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific).

2.3 Amplification of DNA

2.3.1 Standard polymerase chain reaction protocol

All primers used in this study (Table 3; Sigma Genosys; Oakville, ON, Canada) were resuspended in Tris-EDTA buffer (MilliQ water, 10 mM Tris, 1 mM EDTA, pH 7.5-8) or MilliQ water to a concentration of 100 μM. Primers were used at 100 nM for Polymerase Chain Reactions (PCR). DNA was amplified using either Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) or Pfu polymerase. Reactions were run in a Peltier Thermocycler PTC-200 (MJ Research, Waterdown, MA, USA).

2.3.1.1 Reactions with Phusion polymerase

For Phusion reactions, 2 mM magnesium chloride ($MgCl_2$; Thermo Fisher Scientific), 3 mM deoxyribonucleotide triphosphate (dNTP) mixture (Hoffmann-La Roche; Mississauga, ON, Canada), 10 pM each of the forward and reverse primers, 0.6 μL of template DNA, and Phusion polymerase were mixed into 1 × high fidelity (HF) buffer (Thermo Fisher Scientific). Cycling for Phusion reactions proceeded as follows: 98°C for 10 minutes, 98°C for 30 seconds, primer-specific annealing temperature for 30 seconds, 72°C for 15 seconds/kb of expected gene product for 36 cycles, 72°C for previous time plus one minute (final extension), 4° until reactions were removed.

2.3.1.2 Reactions with Pfu polymerase

Reactions to amplify DNA with Pfu polymerase contained 2 mM magnesium chloride ($MgSO_4$; Sigma Aldrich), 3 mM dNTP mixture, 10 pM each of the forward and reverse primers, 0.6 μL of template DNA, and Pfu polymerase were mixed into 1 × Pfu buffer containing 200 mM Tris-hydrochloric acid (HCl; Caledon Laboratories Ltd.; Georgetown, ON, Canada), 100 mM potassium chloride (Sigma Aldrich), 1% triton X-100 (Bio-Rad Laboratories Ltd.; Mississauga, ON, Canada), and 1mg/mL bovine serum albumin (BSA; Sigma Aldrich). Cycling for Pfu reactions proceeded as follows: 95°C for 10 minutes, 95°C for 30 seconds, primer-specific annealing temperature for 30 seconds, 74°C for 1 minute/kb of expected gene product for 36 cycles, 74°C for previous time plus two minutes (final extension), 4°C until reactions were removed.

Table 3. Primers used in this study

Primer	Sequence (5'-3')[a]	Purpose
*Bam*HI-Upstream *smeZ*	GGGGGATCCGGGGAATTATGCCAATTGTC TCTA	Generation of knockout construct
Erm For	TTACTTATTAAATAATTTATAGCTATTGAA AAGAGA	Screening for loss of plasmid
Erm Rev	ATGAACGAGAAAAATATAAAACACAGTC	Screening for loss of plasmid
*Kpn*I-Downstream *smeZ*	GGGGGTACCGGGCAATTGTTTAACTGGTT AATTAG	Generation of knockout construct
M13 For	GTAAAACGACGGCCAGTGAG	Sequencing and screening
M13 Rev	CAGGAAACAGCTATGACCATG	Sequencing and screening
pepO Seq Rev	CCAGCCCACTTAGTCAAT	Sequencing
pepO RT Rev	CGAAGAAGGCAACGAAAAAG	Sequencing and screening
*Pst*I-Upstream *smeZ*	CCCCTGCAGAAAAATAAGTTTTGTTTTTTT CATAAATAG	Generation of knockout construct
*Pst*I-Downstream *smeZ*	CCCCTGCAGTTAGATATAGAAATTGACTC CTAATTC	Generation of knockout construct
smeZ Int II For	CATGCCTGCTCAAACAAGATT	Sequencing and screening
smeZ Screen II Rev	ATACGACTCCATCTCATTATAGC	Sequencing and screening
smeZ RT For	TTTCTCGTCCTGTGATTGGA	Sequencing and screening
smeZ RT Rev	AATGGGACGGAGAACATAGC	Sequencing and screening
speA RT For	AAAGTTGCCATCTCTTGGTTC	Sequencing and screening
speA RT Rev	CAAGAGGTATTTGCTCAACAAGAC	Sequencing and screening
speJ RT For	GCTCTCGACCTCAGAATCAA	Sequencing and screening
speJ RT Rev	CTTTCATGGGTACGGAAGTG	Sequencing and screening
tsf RT For	GGCGTTATGGACGCTAAAAA	Sequencing and screening

[a] Underlined nucleotides indicate restriction enzyme recognition site

2.4 Sequencing of DNA

DNA Sequencing was completed by the Sequencing Facility at the John P. Robarts Research Institute in London, Ontario, Canada. Primers used for sequencing can be found in Table 3.

2.5 Molecular Cloning

Standard cloning techniques, with the following modifications, were used to generate superantigen deletion and complement strains.

2.5.1 Plasmid vector generation

Standard cloning techniques were used to generate cloning vectors, using restriction enzymes purchased from New England Biolabs Ltd. (Whitby, ON, Canada). Blue/white screening with pBluescript was used as an intermediate step in cloning the *smeZ* complementation vector. The *smeZ* PCR product was bluntly ligated into *Eco*RV-cut pBluescript to form pBluescript::*smeZ* and transformed into $RbCl_2$-competent *E. coli* XL1 Blue (see below). The pG^+host5::*I-Sce*I::IntMGAS5005 and pG^+host5::*I-Sce*I::IntMGAS8232 vectors for SAg complementation had previously been engineered to contain 500 bp each upstream and downstream of the *tsf/pepO* region of the indicated strain, with a new MCS (including *Sal*I or *Spe*I restriction enzyme sites) in between. Each SAg gene was amplified from the original chromosome by PCR using primers containing either a *Sal*I or *Spe*I restriction enzyme site, and inserted into this new MCS. T4 DNA ligase was used to seal the plasmid prior to transformation. Superantigen knockout vectors were generated by amplifying 500 bp regions of DNA homologous to the upstream and downstream areas of the target gene. These fragments were each cut using a *Pst*I restriction enzyme site and ligated together using T4 DNA ligase (New England Biolabs Ltd.). The resulting fragment and the pG^+host5-based vector backbone were then cleaved at *Kpn*I and *Bam*HI sites and ligated together prior to transformation into *E. coli*.

2.5.2 Rubidium chloride competent *E. coli*

E. coli XL1-Blue cells were grown in 100 mL Psi broth (5 g/L Bacto yeast extract (BD Biosciences), 20 g/L Bacto Tryptone (BD Biosciences), 5 g/L magnesium sulfate, pH

7.6) at 37°C to an OD_{550} of 0.48. After sitting on ice for 15 minutes, the culture underwent centrifugation at 4°C for 5 minutes at 5000 × *g*. The pellet was resuspended in 40 mL TfbI (30 mM potassium acetate, 100 mM rubidium chloride (Amresco), 10 mM calcium chloride (Sigma Aldrich), 50 mM manganese chloride (Sigma Aldrich), 15% glycerol, pH 5.8) and placed on ice for 15 minutes. Following centrifugation at 4°C for 5 minutes at 5000 × *g*, the pellet was resuspended in TfbII (10 mM 3-(N-morpholino) propanesulfonic acid (Sigma Aldrich), 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol, pH 6.5) and aliquots were frozen at -80°C until use.

2.5.3 Transformation into rubidium chloride-competent *E. coli*

Ligated vectors were transformed into $RbCl_2$-competent *E. coli* using a heat-shock method. Cells were thawed on ice and 5 µL of the plasmid or ligation to be transformed was added and incubated on ice for 30 minutes. Cells with DNA were heat-shocked for 45 seconds, followed by incubation on ice for two minutes. The transformation was relocated to a tube with 800 µL of LB broth and left shaking at 37°C for one hour. Cells were plated on LB or BHI agar with the appropriate antibiotic and left to grow overnight at 37°C. For blue/white screening (pBluescript plasmid derivative transformations), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Gold Biotechnology Inc.; St. Louis, MO, USA) dissolved in N,N-dimethylformamide (DMF; EMD Millipore), was mixed with isopropyl β-D-1-thiogalactopyranoside (IPTG) and liquid LB. This mixture was applied to LB plates and allowed to dry prior to transformation plating.

2.5.4 Generation of Electrocompetent *S. pyogenes*

A bottle of pre-warmed THY with 0.6% glycine (Thermo Fisher Scientific) was inoculated 2% (v/v) with an overnight culture of *S. pyogenes* and left in the 37°C incubator. After two hours, 5 mg of hyaluronidase (Sigma Aldrich) was added, and the culture was left to grow for another three hours. Cells were spun at 7000 × *g* for 15 minutes at 20°C. The supernatant was removed and the pellet was washed once with 15% (v/v) glycerol and spun at 7000 × *g* for 15 minutes at 20°C. Upon resuspension in 15% glycerol, aliquots were stored at -80°C.

2.5.5 Transformation of *S. pyogenes* by electroporation

Electrocompetent cells were thawed on ice, prior to the addition of 2 μg of vector DNA All plasmids utilized in this experiment were based on the pG$^+$host5 vector and were used with Erm. Upon relocation to a 2 mm cuvette (VWR; Mississauga, ON, Canada), the transformation was electroporated in a Bio-Rad Gene Pulser XCell machine (Bio-Rad Laboratories Ltd.) with the following settings: 2500 V, 25 μF, and 600 Ω. Cells were immediately transferred to THY broth for 1 hour at 30°C. A sub-inhibitory concentration of Erm (0.01 μg/mL) was added and the cells were returned to 30°C for four hours. Cells were then plated on THY plates with Erm (1 μg/mL). Plates were placed in the 30°C incubator for up to three days.

2.5.6 *S. pyogenes* temperature shift knockout and complement protocol

Double homologous recombination using the temperature-sensitive origin of replication of the pG$^+$host5-based vectors was used to either complement a superantigen gene (with its natural promoter) into, or to knock out the *smeZ* gene from, the *S. pyogenes* genome. A schematic of the complementation protocol can be found in Figure 2, while a schematic of the knockout protocol can be found in Figure 3. Each protocol followed the following similar general steps. Following electroporation of the plasmid of interest into the *S. pyogenes* target strain, colonies recovered the next day were started in THY Erm (1 μg/mL) liquid cultures and were left at 30°C overnight. Cultures were then streaked on THY plates with Erm (1 μg/mL) left to grow at 40°C overnight. Liquid THY Erm (1 μg/mL) cultures were started from the 40°C colonies, and were left at 40°C overnight. Again, cultures were streaked on THY plates with Erm (1 μg/mL) left to grow at 30°C overnight. Colonies were used to start liquid THY Erm (1 μg/mL) cultures and were left at 30°C overnight. Liquid 30°C cultures were subcultured 0.1% (v/v) into fresh THY for 5 days at 37°C to cure the remaining plasmid. Cultures were then streaked on THY plates and patched onto both THY and THY plates with Erm (1 μg/mL). Cured cultures (susceptible to Erm) were then grown in liquid THY culture for DNA isolation and to screen by PCR for both loss of the Erm gene (Erm For and Erm Rev primers), and integration (using *tsf* For and *pepO* Rev primers which lie outside of the homologous recombination region) or knockout (using primers up- or downstream of the deletion

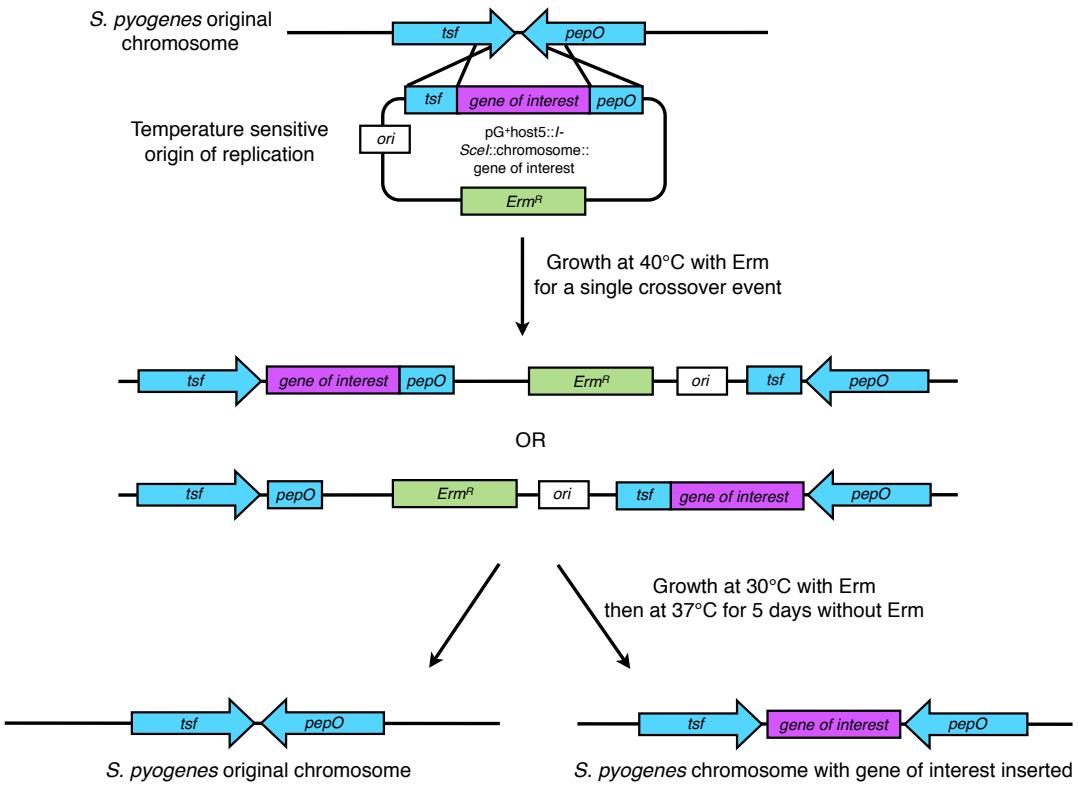


Figure 2. Schematic of cloning strategy for double-crossover insertion of a superantigen gene of interest into the *S. pyogenes* chromosome. The gene of interest, flanked by *tsf* and *pepO* (500 bp of each gene) was cloned into the vector pG$^+$host5 with a temperature sensitive origin of replication; the resulting plasmid was named using the nomenclature of pG$^+$host5::*I-Sce*I::chromosome::gene of interest. The plasmid was then electroporated into the target strain of *S. pyogenes* and single-crossover colonies (with the gene of interest integrated) were selected for by growth at the non-permissive temperature of 40°C. Double-crossover events were obtained by growth at 30°C, followed by subculturing at 37°C for 5 days without erythromycin.

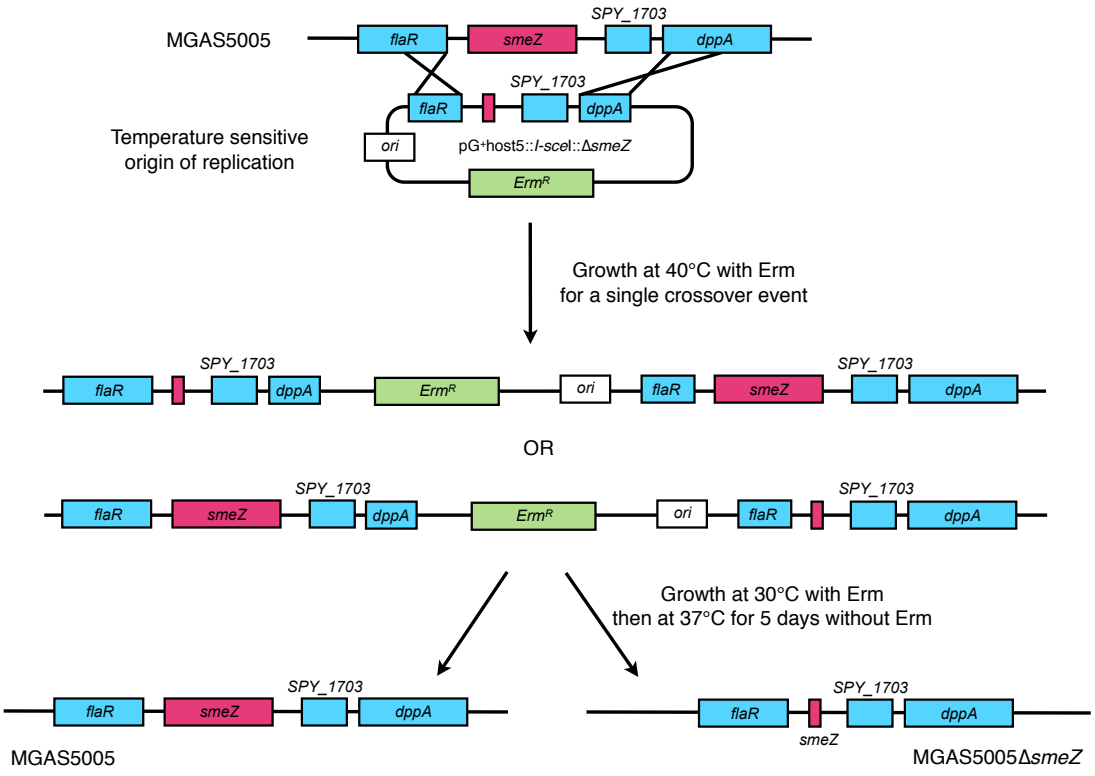


Figure 3. Schematic of cloning strategy for double-crossover deletion of *smeZ* from the MGAS5005 or MGAS5005Δ*speA* chromosome. A truncated version of the *smeZ* gene from *S. pyogenes* MGAS5005, with flanking regions of MGAS5005 *flaR* and *Spy_1703* with *dppA* (500 bp up and downstream of *smeZ*) were cloned into the temperature sensitive backbone of the vector pG^{+}host5; the resulting plasmid was named pG^{+}host5::*I-sce*I::Δ*smeZ*. The plasmid was electroporated into MGAS5005 and MGAS5005Δ*speA* and single-crossover colonies (with the *smeZ* deletion construct integrated) were selected for by growth at the non-permissive temperature of 40°C. Double-crossover events were obtained by growth at 30°C, followed by subculturing at 37°C for 5 days without erythromycin.

region) of the superantigen gene of interest. At each stage of liquid culture, aliquots were used to isolate the *S. pyogenes* genome to screen with PCR for the correct intermediate step. Following successful cloning (ensured by sequencing analysis as mentioned above), clones were passaged through five days of growth in 0.2 μm-filtered THY + 10% human plasma (HP) at 37°C prior to freezing in glycerol stocks.

2.6 Isolation of streptococcal supernatant proteins

2.6.1 Trichloroacetic acid (TCA) precipitation of streptococcal supernatant proteins

Cultures were subcultured into 100 mL bottles of THY and grown for 12 hours (until late exponential phase). Once the bacteria were pelleted, supernatants were separated and incubated for 30 minutes with 50% TCA (Sigma Aldrich) to reach a final concentration of 6%. Precipitated supernatants were then centrifuged for 15 minutes at 10,000 × *g* at 4°C. Pellets were washed with ice-cold acetone (Thermo Fisher Scientific), centrifuged for 15 minutes at 10,000 × *g* at 4°C, and left to dry. Precipitated proteins were resuspended in 8M urea (BioShop Canada Inc.).

2.7 Protein visualization

2.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 12% or 15% (v/v) acrylamide gels as indicated. Proteins were mixed with Laemmli buffer (125 mM Tris pH 6.8, 50% glycerol, 4% SDS, 5% β-mercaptoethanol (EMD Millipore), and 0.1% (w/v) bromophenol blue) and boiled for 5 minutes prior to loading. Gels were run for one hour at 200 V in electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) and stained with coomassie stain (0.1% (w/v) coomassie brilliant blue R-250 (Bio-Rad Laboratories Ltd.) in 45% (v/v) methanol (EMD Millipore) and 10% (v/v) acetic acid), and destained for visualization (45% (v/v) methanol and 10% (v/v) acetic acid).

2.7.2 Western blot

When necessary, proteins were transferred onto a polyvinylidine difluoride (PVDF) membrane (EMD Millipore) for Western blot. The membrane was first incubated in

methanol for 5 minutes, followed by immersion in Western blot transfer buffer (39 mM glycine, 48 mM Tris, 0.037% (w/v) SDS, 20% (v/v) methanol) for 5 minutes. The protein gel was placed on the membrane and the pair were placed between pieces of filter paper. This set was placed between two sponges in the transfer apparatus. Proteins were transferred overnight at 10V with a cold block. Equilibration of the PVDF membrane in transfer buffer for 30 minutes at room temperature was followed by blocking with 5% (w/v) skim milk (Equality) in 1 × tris buffered saline (TBS; 100 mM Tris, 1.5 M sodium chloride, pH 7.5) for 30 minutes. The primary antibody was always an anti-superantigen antibody (Table 4; ProSci Incorporated; Poway, CA, USA), and was diluted 1:8,000 in 1% skim milk in TBS, followed by one hour incubation. PVDF membranes were subjected to three five-minute washes with TBS with 0.01% (v/v) tween-20 (TBS-T). IRDye800-conjugated goat anti-rabbit (Rockland Inc.; Limerick, PA, USA) was used as a secondary, and was diluted 1:10,000 in 1% skim milk in TBS and incubated for one hour at room temperature. Membranes were imaged on a Li-Cor Odyssey (Li-Cor Biosciences; Lincoln, NB, USA).

2.8 *In vivo* experiments

2.8.1 Mice

Mice were housed in the West Valley Barrier Facility at the University of Western Ontario, Canada under specific pathogen-free conditions and the care of Animal Care and Veterinary Services staff. All experiments involving animals were completed in accordance with the Canadian Council on Animal Care Guide to Care and Use of Experimental Animals, and was approved by the Animal Use Subcommittee at the University of Western Ontario (Appendix A).

A list of the types of mice used in this study may be found in Table 5. Humanized mice transgenic for HLA-DR4 and HLA-DQ8 (HLA-DR4/DQ8 mice) were a generous gift from Dr. Malak Kotb [143] and are bred in-house. Mice transgenic for human HLA-DQ8 were bred from the HLA-DR4/DQ8 heterozygous breeding. FVB mice were purchased specifically for each experiment.

Table 4. Antibodies used in this study

Antibody	Target	Purpose	Source
Rabbit anti-SpeA	Polyclonal SpeA	Passive immunization	ProSci Inc., [188]
Rabbit anti-SpeC[A]	Polyclonal SpeC	Passive immunization, Western blot	ProSci Inc., [188]
Rabbit anti-SmeZ	Polyclonal SmeZ	Passive immunization	ProSci Inc., [188]
IRDye800 goat anti-rabbit	Polyclonal rabbit IgG	Western blot	Rockland Inc. (Limerick, PA, USA)
Goat anti-mouse HRP[B]	Polyclonal murine IgG	Antibody quantification	Clone A3673, Sigma Aldrich

[A] Anti-SpeC antibody cross-react with SpeJ [79], and therefore was used to detect SpeJ
[B] HRP – horseradish peroxidase

Table 5. Mice used in this study

Strain Name	Characteristics	MHC Class II	Source
C57Bl/6	Inbred black mouse	$H2^b$	Jackson Laboratories (Bar Harbor, ME, USA)
HLA-DR4/DQ8	Bred from heterozygous HLA-DR4/DQ8 × HLA-DR4/DQ8 cross	Human HLA-DR4 and HLA-DQ8	Gift from Dr. M. Kotb [143]; McCormick lab breeding colony
HLA-DQ8	Bred from heterozygous HLA-DR4/DQ8 × HLA-DR4/DQ8 cross, and also inbred from homozygous HLA-DQ8 × HLA-DQ8 cross	Human HLA-DQ8	McCormick lab breeding colony
FVB	Inbred white mouse	$H2^q$	Jackson Labs/Charles River

2.8.2 *In vivo* murine model and complete nasal turbinates removal

S. pyogenes strains were grown from freezer stock and subcultured twice in THY prior to growth to early exponential phase (OD_{600} between 0.2 and 0.3). Cells were then washed and resuspended in Hank's balanced saline solution (HBSS) (Hyclone; Logan, UT, USA) with ~1.5×10^8 colony forming units (CFU) per 15 µL dose. Two days prior to inoculation, mice were provided with water containing 2 mg/mL neomycin (Neo) to drink *ad libitum*. On the day of inoculation, mice were anesthetized under 4% isofluorane and administered ~1.5×10^8 CFU (total) by pipetting 7.5 µL of bacterial suspension dropwise onto each nare, allowing natural inhalation of *S. pyogenes*. Mice were monitored during recovery, and placed back in their cages and monitored daily. Forty-eight hours post-inoculation, mice were again anesthetized under 4% isofluorane. With the mouse on its back, its nose still receiving anaesthesia, and its limbs secured under T-shaped pins (not punctured), a midline vertical incision was made through the fur, dermis, and peritoneum, extending from the bottom of the rib cage and diaphragm down to the genitalia. Lateral incisions from the original midline incision were also made (dorsally), ensuring not to damage other tissues. Internal organs (intestines and fat pads) were moved aside to reveal the inferior vena cava, from which the mouse was exsanguinated using a 25-gauge heparinized needle. The mouse was then decapitated and the lower jaws and upper cheeks were removed using straight blade operating scissors. T-shaped pins were used to secure the upper part of the head prior to removal of the upper incisors and nose tip. The palate was peeled away and disposed of. Both sets of upper molars were removed by inserting opened curved iris scissors around each side of the maxilla, and rolling outward away from the head. The complete nasal turbinates (cNT) are off-white, mucoid tissue which lay bilateral to the septum and are approximately the size of a grain of rice [188]. This tissue was removed using curved tweezers and a scooping motion and placed in 500 µL of ice-cold, sterile HBSS until further processing. The cNT tissue was manually homogenized using 1 mL glass homogenizers, serially diluted (10-fold) in sterile HBSS, and plated on Trypticase Soy Agar plus 5% sheep blood plates (TSAII + 5% SB plates; BD Biosciences). Blood samples were diluted 1:10 with sterile HBSS and 100 µL of each sample was spread plated on TSAII + 5% SB plates. Plates were left for 48 hours at 37°C. Counts less than 3 CFU/10 µL were considered below the detectable

limit.

For experiments where the mice were passively immunized, 500 μL of the appropriate anti-superantigen antibody was injected into the intraperitoneal cavity (intraperitoneally; i.p.) at both 24 and two hours prior to inoculation. Anti-superantigen antibodies (ProSci Inc.) had been previously titred and used in the McCormick Laboratory [188]. Mice were monitored at regular intervals and the remainder of the experiment proceeded as detailed above.

2.9 Detection of *in vitro* superantigen production

2.9.1 Splenocyte activation assays

Mice were anesthetized under 4% isofluorane prior to spleen removal. Spleens were placed immediately into 5 mL ice-cold 1 × phosphate buffered saline (PBS; 137 mM sodium chloride (Amresco), 2.7 mM potassium chloride (Sigma Aldrich), 10 mM sodium phosphate (dibasic; EMD Millipore), 2mM potassium phosphate (monobasic; Thermo Fisher Scientific)). Spleens were processed into single-cell suspension with a glass homogenizer or pressed through a cell strainer, then treated with ammonium-chloride-potassium (ACK) lysis buffer (154.95 mM ammonium chloride, 9.99 mM potassium bicarbonate, 0.099 mM EDTA) until erythrocytes were lysed. 1 × PBS was added to dilute out the ACK buffer and cells were spun for 7 minutes at 447 × *g*. Splenocytes were washed with Roswell Park Memorial Institute medium (RPMI-1640; Life Technologies Inc.) and spun for 7 minutes at 447 × *g*). Cells were resuspended in complete RPMI (cRPMI; RPMI-1640 supplemented with 0.1 mM minimal essential medium non-essential amino acids (Life Technologies Inc.), 10% heat-inactivated fetal bovine serum (Sigma Aldrich), 100 units/mL penicillin (Life Technologies Inc.), 100 μg/mL streptomycin (Life Technologies Inc.), 1 mM sodium pyruvate (Life Technologies Inc.), and 2 mM L-glutamine (Life Technologies Inc.)), and seeded at 2 x 10^5 cells/well in a 96-well plate. Splenocytes were activated with varying 10-fold dilutions of either recombinant superantigens (rSAg), or supernatants from *S. pyogenes* cultures. T lymphocyte activating controls were also used: ionomycin (500 ng/mL; Sigma Aldrich) and phorbol 12-myristate 13-acetate (PMA; 10 ng/mL; Sigma Aldrich). Cells were

activated for 18 hours in a 37°C + 5% carbon dioxide (CO_2) incubator. Supernatants were removed analyzed for IL-2 production using ELISA.

2.9.2 Human peripheral blood mononuclear cell activation assays

Human peripheral blood mononuclear cells (PBMC) were isolated from blood acquired from healthy volunteers, with ethics approval (Appendix B). Whole blood was mixed 1:1 with pre-warmed RPMI-1640, and added to room temperature Ficoll (GE Healthcare; Fairfield, CT, USA) (2:1, Ficoll : diluted blood). Tubes were centrifuged for 30 minutes without the brake at 913 × *g*. The PBMC (buffy coat) layer were carefully moved to a new tube where they were then washed three times with RPMI with spins gradually decreased in intensity; the first at 514 × *g*, the second at 329 × *g*, and the third at 185 × *g*, each with the brake. Cells were resuspended in cRPMI and seeded at 2 x 10^5 cells/well in a 96-well plate. PBMC were stimulated with varying 10-fold dilutions of either rSAg or supernatants from *S. pyogenes* cultures. Multiple T lymphocyte activating controls were also used: 500 ng/mL ionomycin, 10 ng/mL PMA, and 500 ng/mL phytohaemagglutinin (PHA; Sigma Aldrich). Cells were activated for 18 hours in a 37°C + 5% carbon dioxide (CO_2) incubator. Supernatants were analyzed for IL-2 production using ELISA.

2.9.3 Enzyme-linked Immunosorbent Assays

Murine interleukin-2 (IL-2) in supernatants from splenocyte activation assays was measured using the mouse IL-2 ELISA Ready-Set-Go! kit (eBioscience Inc.; San Diego, CA, USA). Human IL-2 was measured using the Human IL-2 ELISA Set (BD Biosciences). Costar 96-well EIA/RIA plates (Thermo Fisher Scientific) were coated overnight using either the supplied capture buffer (murine IL-2 kit) or carbonate capture buffer (15 mM sodium carbonate, 35 mM sodium hydrogen carbonate, 3 mM sodium azide, pH 9.5) (human kit only) with the appropriate capture antibody. Kit instructions were followed in both cases with the following exceptions: the primary antibody-biotin and avidin-horseradish peroxidase (HRP) conjugates were incubated together for 30 minutes in assay diluent prior to adding to plates, and 3-3'-5-5'-tetramethylbenzidine (TMB; BD Biosciences) substrate was allowed to incubate for 30 minutes prior to stopping with 1M sulphuric acid (Caledon Laboratories Ltd.). All plates were read by a

Synergy H4 plate reader (BioTek Instruments Inc.; Winooski, VT, USA) at OD_{450} with a reference value of OD_{570}. The detection limit for the murine IL-2 ELISA was 3.125 ng/mL, while for the human IL-2 ELISA it was 7.8 ng/mL.

ELISA to measure murine serum antibody titre (following passive immunization) were conducted by adding 1 μg of rSAg per well in carbonate capture buffer in 96-well Costar EIA/RIA plates and allowing overnight incubation at room temperature. Plates were then washed twice with MilliQ water and patted dry on Whatman paper. To block, 200 μL of 1% (w/v) bovine serum albumin (BSA; Sigma Aldrich) and 0.02% (v/v) tween-20 in 1 × PBS was added to each well and left to incubate for two hours at room temperature. Plates were then washed twice with MilliQ water as above. The first well of each set of dilutions had 2 μL of serum (from terminal bleed) added to 200 μL of 0.1% BSA and 0.02% tween-20 in 1 × PBS to create a 1:100 dilution. Samples were serially diluted 1:1 in the plate, creating two-fold dilutions up to 1:204,800. Following two hours of incubation, serum samples were removed and plates were washed three times with 1 × PBS with 0.05% (v/v) tween-20 (PBS-T) then three times with MilliQ water. Plates were patted dry on Whatman paper prior to the addition of 100 μL of goat anti-mouse horseradish peroxidase conjugate (clone A3673; Sigma Aldrich) diluted 1:10,000 in 0.1% BSA and 0.02% tween-20 in 1 × PBS and incubation for two hours at room temperature. Five washes with PBS-T and five washes with MilliQ water were performed prior patting dry with Whatman paper and addition of 100 μL TMB for 15 minutes. The colorimetric reaction was stopped with the addition of 1M sulphuric acid. Plates were read by the Synergy H4 plate reader at OD_{450} with a reference value of OD_{570}.

2.10 Statistical Analyses

Data from *in vivo* experiments are displayed as mean ± standard error of the mean (SEM). Data were analyzed using unpaired students' t test and values of $p < 0.05$ were deemed significant. Analyses were performed in Prism v6.0 (Graphpad; La Jolla, CA, USA).

Chapter 3 : Results

3 Results

The *S. pyogenes* strain MGAS5005 was isolated from cerebrospinal fluid of a patient from Ontario, Canada in 1996 [51, 189]. This strain is a clonal variant of the globally disseminated M1T1 strain known for causing a number of invasive infections [29]. The MGAS5005 genome encodes four superantigens; SpeG, SpeJ, and SmeZ are all chromosomally encoded, while SpeA was acquired on a bacteriophage element (Figure 4) [191]. By contrast, *S. pyogenes* strain MGAS8232, a rheumatic fever (non-invasive) isolate, carries six superantigens: SpeA, SpeC, SpeL, and SpeM (bacteriophage-encoded), as well as chromosomally encoded SpeG and SmeZ (Figure 4) [190]. Prior work from our laboratory established that both SpeA and human MHC class II molecules (HLA-DR4/DQ8) were critical for establishing nasopharyngeal infection in mice [183]. In this thesis, I sought to evaluate the importance of superantigens in the nasopharyngeal infection model from the contemporary MGAS5005 strain, which encodes a different repertoire of superantigens compared to MGAS8232.

3.1 Evaluation of individual superantigen deletion MGAS5005 strains for nasopharyngeal infection in mice

Previously generated single superantigen deletion mutant strains of *S. pyogenes* MGAS5005 were evaluated in the nasopharyngeal infection model in HLA-DR4/DQ8 (Figure 5). Briefly, HLA-DR4/DQ8 mice were nasally inoculated with ~1.5×10^8 CFU of one of the different superantigen deletion strains from the MGAS5005 background. After 48 hours, the complete nasal turbinates (cNT) were removed, homogenized, and plated on TSAII + 5% SB plates. Individually, deletion of the *speA* or *speG* genes did not reduce the number of *S. pyogenes* recovered from the cNT of HLA-DR4/DQ8 mice, while MGAS5005Δ*speJ* and MGAS5005Δ*smeZ* both appeared to have reduced ability to infect HLADR4/DQ8 mice (Figure 5).

Previous research from our lab indicated that *S. pyogenes* are able to produce an efficient infection in the cNT of FVB mice similar to the infection seen in HLA-DR4/DQ8 mice [188]. However, this phenotype was dependent on the strain of *S. pyogenes* where

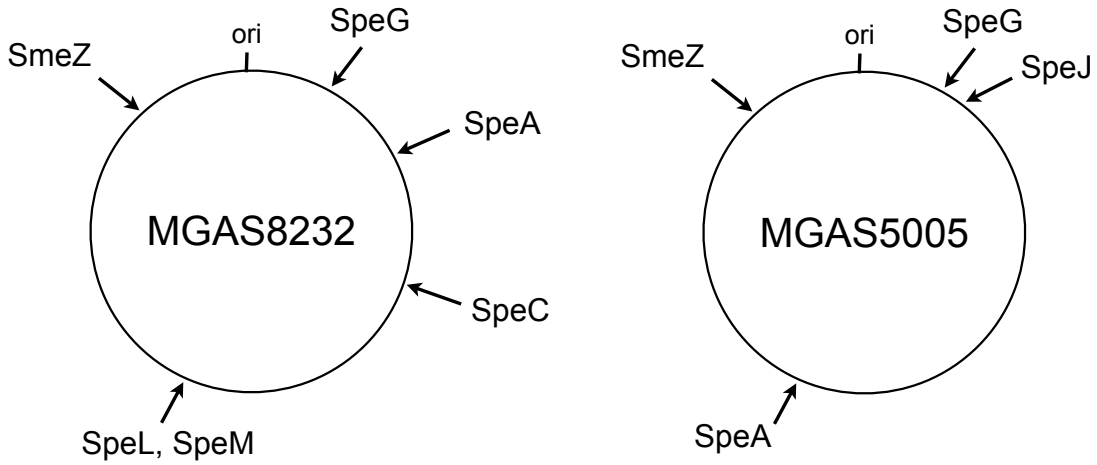


Figure 4. Schematics of the circular *S. pyogenes* strain core chromosome depicting superantigen location in reference to the origin of replication (ori). (A) Strain MGAS8232 encodes six superantigens: SpeA, C, G, L, M, and SmeZ (B) Strain MGAS5005 encodes four superantigens: SpeA, G, J, and SmeZ.

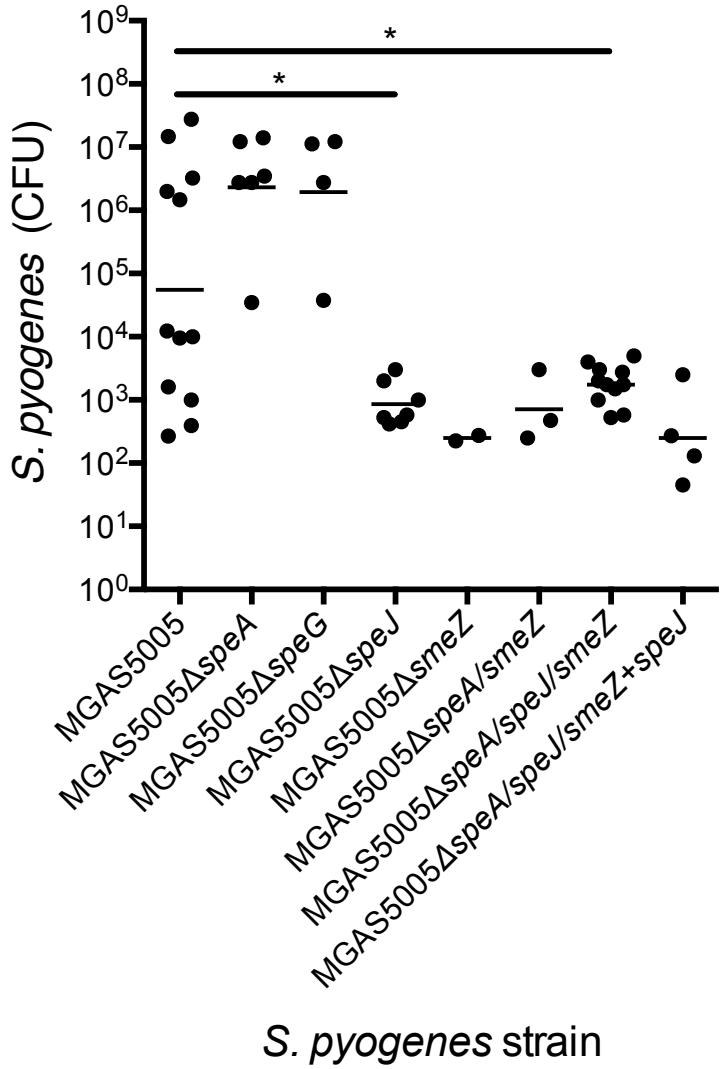


Figure 5. Evaluation of superantigen-knockout strains of MGAS5005 in HLA-DR4/DQ8 transgenic mice. Individual mice were nasally inoculated with ~1.5 × 10^8 bacterial CFU with the indicated strains and nasopharyngeal CFU were assessed at 48 hours. Each symbol represents an individual mouse; horizontal lines depict the mean of each group. * denotes $p < 0.05$ as determined by Student's *t* test.

MGAS8232 were not recovered in high numbers 48 hours post-inoculation, while *S. pyogenes* MGAS5005 were recovered from cNT of FVB mice in similar amounts to the HLA-DR4/DQ8 mice (Figure 6). FVB mice are inbred white mice of Swiss ancestry that were originally named for their susceptibility to Friend virus B [192]. They are traditionally used for the generation of transgenic mouse lines and possess murine MHC class II haplotype $H2^q$ [192]. As per our nasopharyngeal infection model, FVB mice were inoculated with 1.5×10^8 CFU of the different *S. pyogenes* strains. After 48 hours (Figure 6), high numbers of *S. pyogenes* were recovered from cNT of mice inoculated with *S. pyogenes* MGAS5005 wild-type strain and similar to the results in HLA-DR4/DQ8 mice, there was no impact on infection by the *speA* or *speG* individual deletion strains. Alternatively, MGAS5005 lacking *speJ* or *smeZ* were reduced in the ability to establish nasopharyngeal infection in FVB mice since (Figure 6). Two additional strains, containing a double deletion of *speA* and *smeZ*, and a triple deletion of *speA*, *speJ* and *smeZ* were also evaluated. Each strain poorly infected both HLA-DR4/DQ8 mice and FVB mice.

These collective data indicated that the superantigens SpeJ and SmeZ may each be individually important for establishment of infection by *S. pyogenes* MGAS5005 in the HLA-DR4/DQ8 and FVB mice. Conversely, SpeA and SpeG, individually, were not important. Considering previous research from our lab in which one single superantigen was critical for formation of a nasopharyngeal infection [183], we hypothesized that SpeJ or SmeZ could be important for MGAS5005 infection in the FVB mice.

3.2 Generation of *speJ*-complemented strains of *S. pyogenes*

To first evaluate the role of SpeJ for establishment of acute nasopharyngeal infection, *speJ* was complemented into MGAS5005Δ*speA/speJ/smeZ*. Double homologous recombination the pG^+host5 system was used to insert the *speJ* gene into the MGAS5005Δ*speA/speJ/smeZ* chromosome between the *tsf* and *pepO* genes. This region was chosen because it is a null region between two terminators, and additionally, the *speA* gene has been successfully complemented in this location [183]. Proper integration

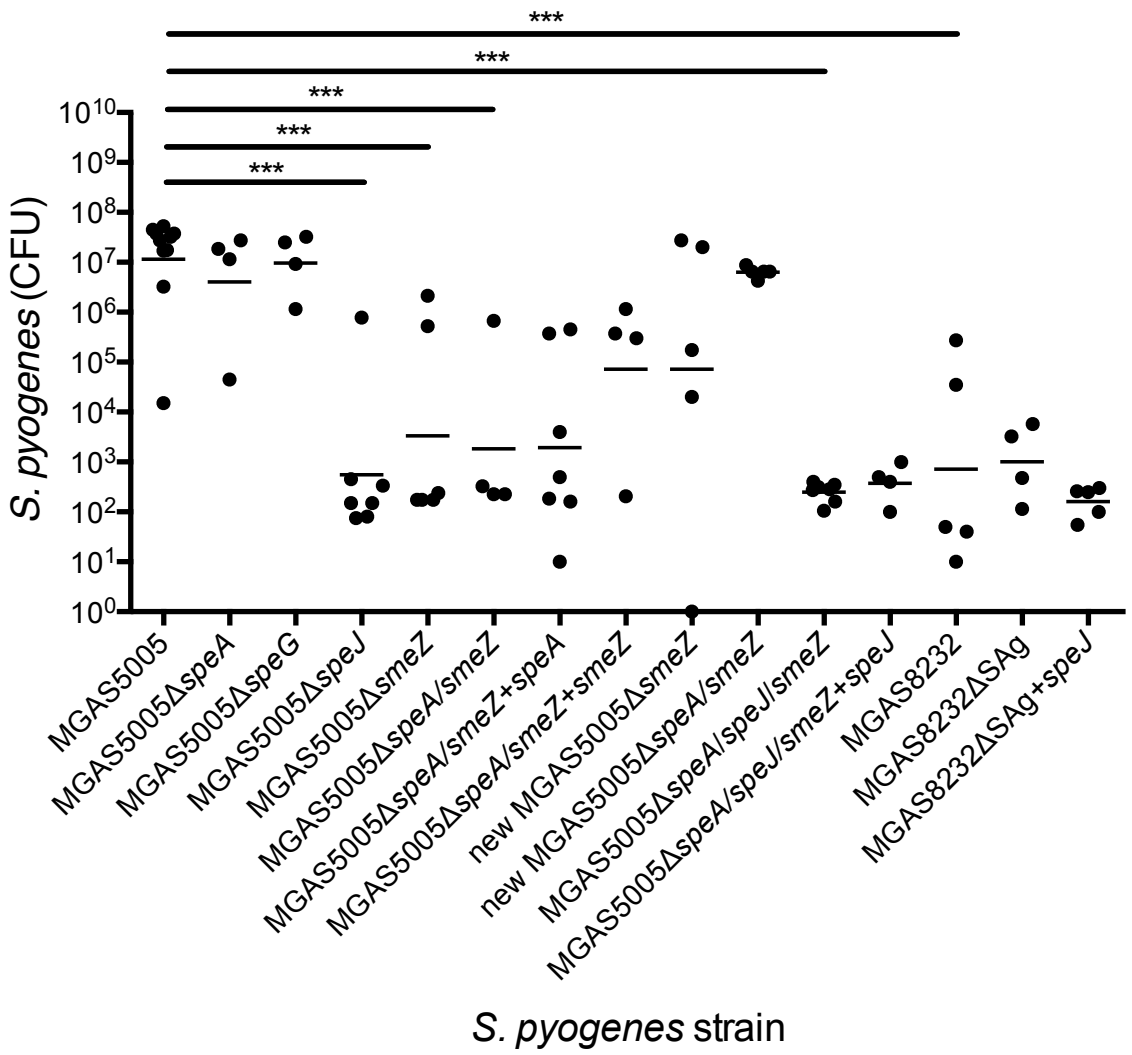


Figure 6. Evaluation of superantigen-knockout strains of MGAS5005 and MGAS8232 in FVB transgenic mice. Individual mice were nasally inoculated with ~1.5 × 10^8 bacterial CFU with the indicated strains and nasopharyngeal CFU were assessed at 48 hours. Each symbol represents an individual mouse; horizontal lines depict the mean of each group. *** denotes $p < 0.001$ as determined by Student's *t* test.

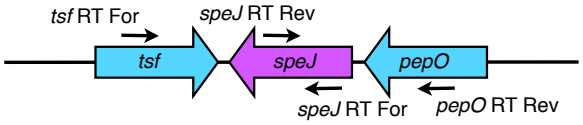
of *speJ* into the *tsf/pepO* region was demonstrated by PCR (Figure 7) and was confirmed with sequencing.

Concurrently, *speJ* from *S. pyogenes* MGAS5005 was also complemented into *S. pyogenes* MGAS8232ΔSAg, a strain mutated to lack all superantigens, and furthermore, did not originally encode *speJ*. Similar to the creation of MGAS5005Δ*speA/speJ/smeZ*+*speJ*, complementation of *speJ* in between *tsf* and *pepO* of MGAS8232ΔSAg was accomplished using double homologous recombination with a vector based on the pG^{+}host5 plasmid backbone. The successful *speJ* complements were confirmed by PCR (Figure 8) using primers internal and external to the homologous cloning region prior to further confirmation with sequencing.

Both *speJ*-complemented strains were subjected to *in vitro* testing to ensure the inherent characteristics of the strains were not altered during cloning. *In vitro* growth curves were conducted for each MGAS8232 strain and there were no notable differences distinguished (Figure 9). Manual growth curves of MGAS5005, MGAS5005Δ*speA*/*speJ*/*smeZ*, and MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* displayed some differences in the rate of growth rates with the MGAS5005 strain entering exponential phase marginally earlier than the other two strains (Figure 10). To evaluate SpeJ expression *in vitro*, supernatants from cultures in stationary phase were subjected to TCA precipitation prior to analysis using SDS-PAGE and Western blot analysis. Since anti-SpeC antibodies are cross-reactive with SpeJ [79, 177], polyclonal rabbit anti-SpeC antibody was used to detect SpeJ. Although recombinant SpeJ was detected by Western blot, SpeJ secreted by *S. pyogenes* was undetectable in culture supernatants from wild-type MGAS5005, or the *speJ*-complemented strains (Figure 11). In the MGAS8232 lane, the detected band is SpeC as it is one of the six naturally encoded superantigens of MGAS8232 [183].

Figure 7. Complementation of *speJ* into MGAS5005Δ*speA/speJ/smeZ*. (A) Schematic of the gene organization and primer locations for the *speJ* complemented MGAS5005Δ*speA/speJ/smeZ* strain (MGAS5005Δ*speA/speJ/smeZ*+*speJ*). The *tsf* and *pepO* genes are labeled. The *tsf* RT For and *pepO* RT Rev primers fall outside of the 500 bp homologous region used for recombination. (Not to scale.) (B) Confirmation of integration of *speJ* from MGAS5005 into the *tsf/pepO* region of MGAS5005Δ*speA/speJ/smeZ*. PCR was performed on MGAS5005Δ*speA/speJ/smeZ* genomic DNA and MGAS5005Δ*speA/speJ/smeZ*+*speJ* genomic DNA with three sets of primers:, *speJ* RT Rev and *pepO* RT Rev (1), and *tsf* RT For and *speJ* RT For (2), and *tsf* RT For and *pepO* RT Rev (3). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Ladder band sizes are adjacent to the gel in base pairs.

A


tsf RT For
speJ RT Rev
tsf
speJ
pepO
speJ RT For
pepO RT Rev

B

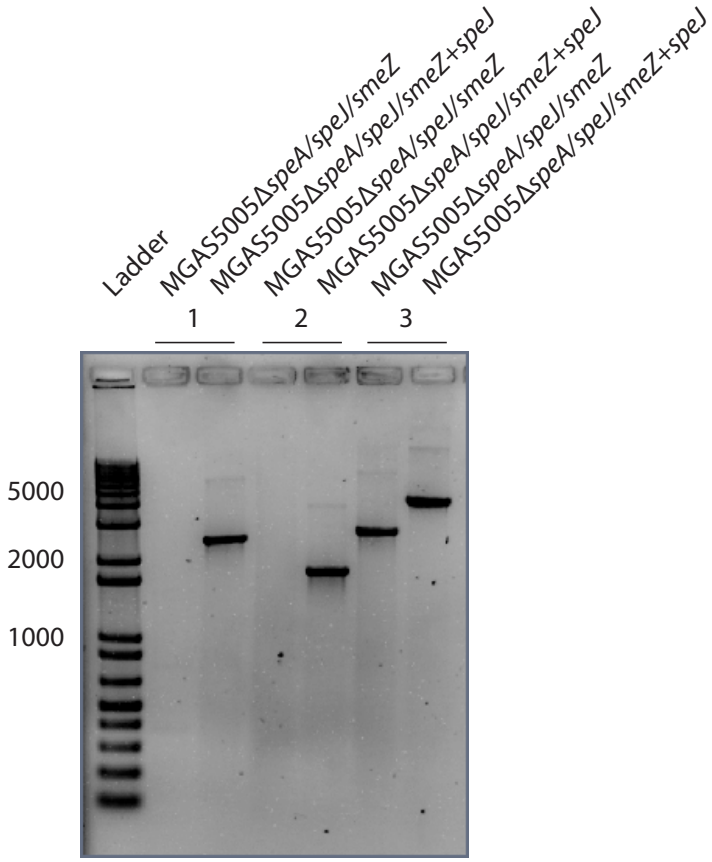
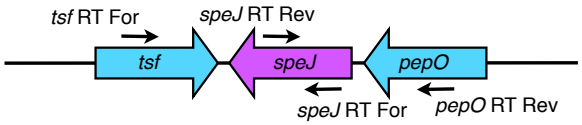
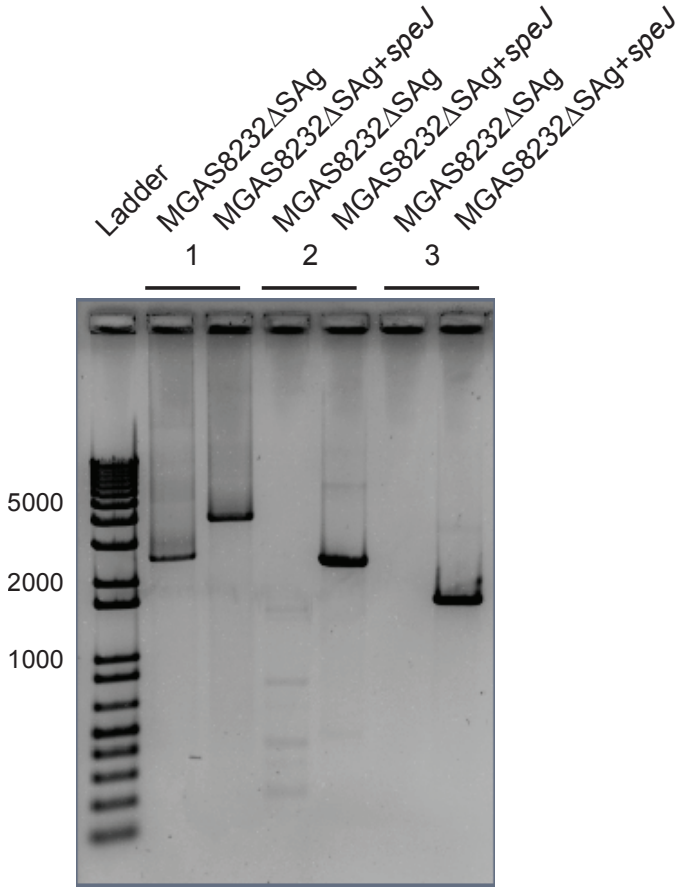

Ladder
MGAS5005ΔspeA/speJ/smeZ
MGAS5005ΔspeA/speJ/smeZ+speJ
MGAS5005ΔspeA/speJ/smeZ
MGAS5005ΔspeA/speJ/smeZ+speJ
MGAS5005ΔspeA/speJ/smeZ
MGAS5005ΔspeA/speJ/smeZ+speJ
1
2
3
5000
2000
1000

Figure 8. Complementation of *speJ* from *S. pyogenes* MGAS5005 into *S. pyogenes* MGAS8232ΔSAg. (A) Schematic of the gene organization and primer locations for the *speJ* complemented MGAS8232ΔSAg (MGAS8232ΔSAg+*speJ*). The *tsf* and *pepO* genes are labeled. The *tsf* RT For and *pepO* RT Rev primers fall outside of the 500 bp homologous region used for recombination. (Not to scale.) (B) Confirmation of integration of *speJ* into the *tsf/pepO* region of *S. pyogenes* MGAS8232ΔSAg. PCR was performed on *S. pyogenes* MGAS8232ΔSAg genomic DNA and MGAS8232ΔSAg+*speJ* genomic DNA with three sets of primers: *tsf* RT For and *pepO* RT Rev (1), *speJ* RT Rev and *pepO* RT Rev (2), and *tsf* RT For and *speJ* RT For (3). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Ladder band sizes are adjacent to the gel in base pairs.

A


tsf RT For
speJ RT Rev
tsf
speJ
pepO
speJ RT For
pepO RT Rev

B


Ladder
MGAS8232ΔSAg
MGAS8232ΔSAg+speJ
MGAS8232ΔSAg
MGAS8232ΔSAg+speJ
MGAS8232ΔSAg
MGAS8232ΔSAg+speJ
1
2
3
5000
2000
1000

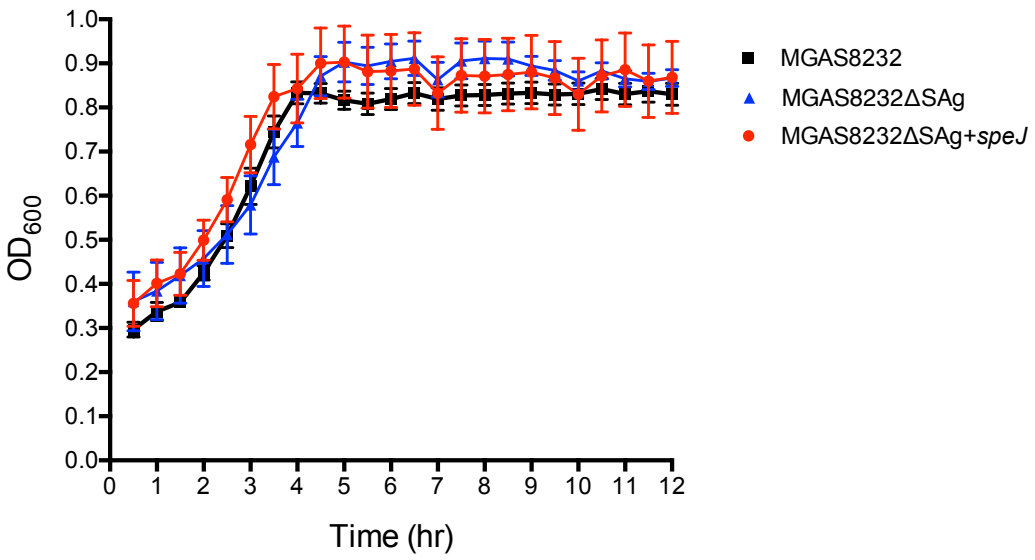


Figure 9. MGAS8232ΔSAg+*speJ* displays a similar growth rate to parent strains MGAS8232 and MGAS8232ΔSAg. Growth of strains was evaluated at 37°C where OD_{600} was measured in 30 minute intervals. Nine replicates of each culture were measured. Data represented as mean ± standard error of the mean (SEM).

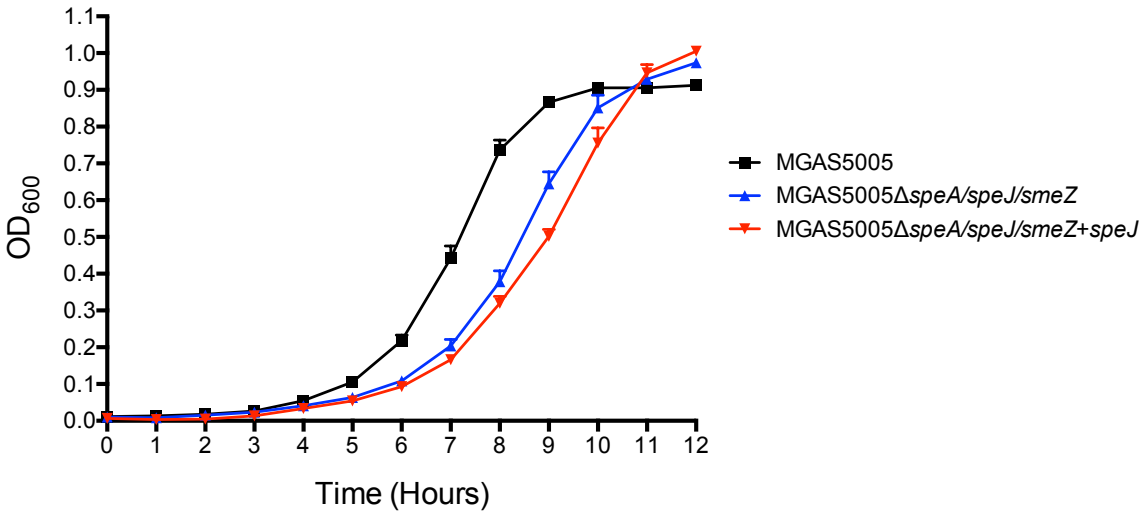


Figure 10. Manual growth curve of MGAS5005, MGAS5005Δ*speA*/*speJ*/*smeZ*, and MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* at 37°C. Optical density at 600 nm (OD_{600}) measurements of triplicate cultures were taken every hour.

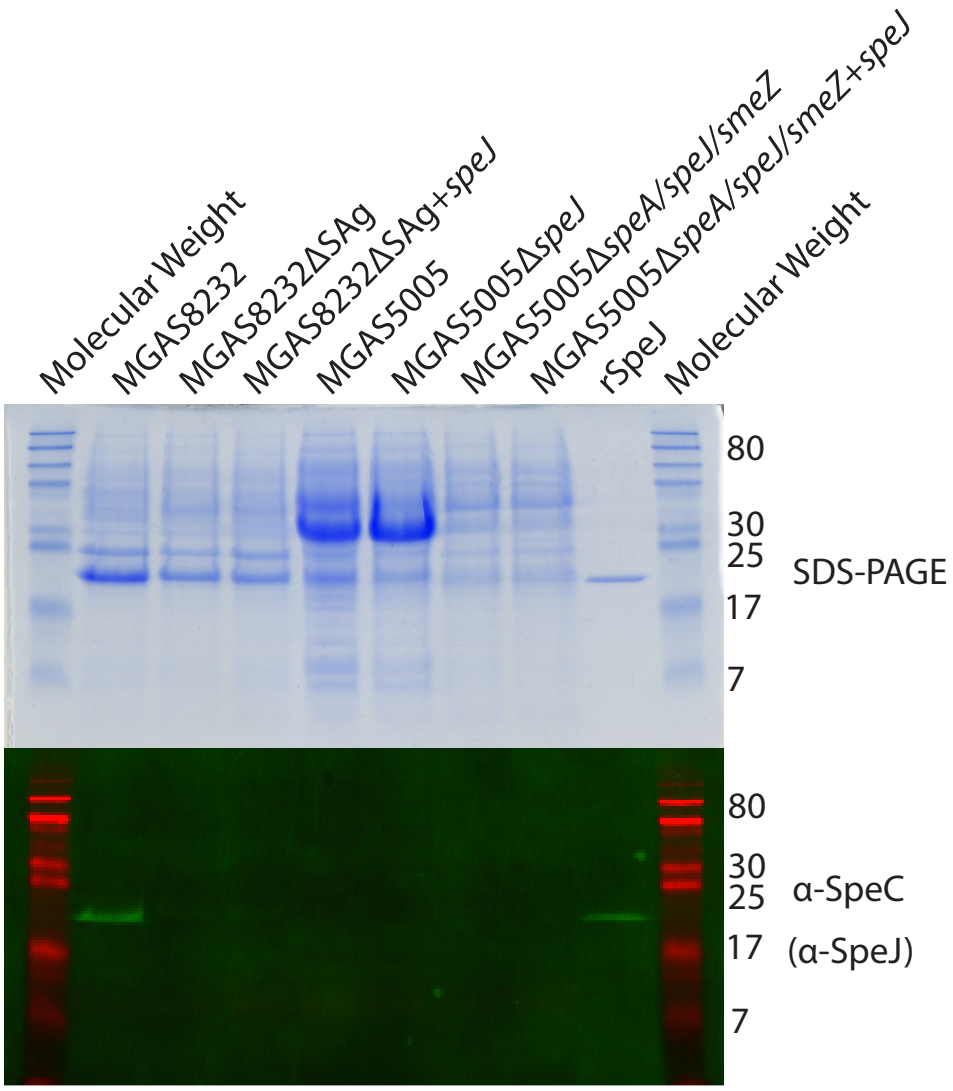


Figure 11. Expression of SpeJ by *S. pyogenes* is not detectable by Western Blot analysis from culture supernatants *in vitro*. Cultures of the indicated strains were grown for 12 hours prior to TCA precipitation. Precipitated proteins were resuspended in 8M urea, visualized alongside recombinant SpeJ on SDS-PAGE. Western blotting was performed with rabbit anti-SpeC antibody and detected with anti-rabbit IRDye. Molecular weight sizes are adjacent to the gel in kilodaltons.

3.3 Genetic SpeJ complementation does not enhance *S. pyogenes* acute infection in the cNT of HLA-DR4/DQ8 or FVB mice

Although *in vitro* expression of SpeJ was undetectable by Western blot analysis, the *speJ*-complemented strains were further evaluated in the nasopharyngeal infection model since *in vivo* expression patterns may differ. The number of bacteria recovered from cNT of HLA-DR4/DQ8 mice infected with MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* was not different than the number of bacteria recovered from the cNT of mice infected with MGAS5005Δ*speA*/*speJ*/*smeZ* (Figure 5), indicating that complementation of *speJ* did not provide an advantage to *S. pyogenes* for establishment of a nasopharyngeal infection in this transgenic mouse strain.

Strains complemented with *speJ* were also evaluated in FVB mice to assess importance the contribution of SpeJ to nasopharyngeal infection to this mouse strain. A similar number of *S. pyogenes* were recovered from FVB mice inoculated with MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* compared to mice inoculated with the base strain MGAS5005Δ*speA*/*speJ*/*smeZ* (Figure 6). In addition, the number of *S. pyogenes* recovered from cNT of FVB mice inoculated with MGAS8232ΔSAg+*speJ* was also not different from the number isolated from MGAS8232ΔSAg-inoculated mice. Thus, the addition of the *speJ* gene into the MGAS5005Δ*speA*/*speJ*/*smeZ* genome and the MGAS8232ΔSAg genome did not enhance the ability of the strain to infect FVB mice.

3.4 SpeJ is produced *in vitro* but does not stimulate murine splenocytes

After the unexpected results with the *in vivo* experiments in which the acquisition of *speJ* did not aid the *S. pyogenes* strains in establishment of infection in HLA-DR4/DQ8 or FVB mice, recombinant superantigens were evaluated in their ability to stimulate FVB splenocytes. Since superantigens are known to activate T lymphocytes, IL-2 secretion was used to measure levels of T lymphocyte activation. Curiously, each of the recombinant superantigens encoded by MGAS5005 produced only low levels of IL-2 from FVB splenocytes (Figure 12). Additionally, each strain was grown *in vitro* and bacterial supernatants were evaluated for their ability to activate FVB (Figure 13) and HLA-DR4/DQ8 splenocytes (Figure 14). As expected, neat (undiluted) supernatants induce secretion of low amounts of IL-2 (in comparison to other dilutions) due to the

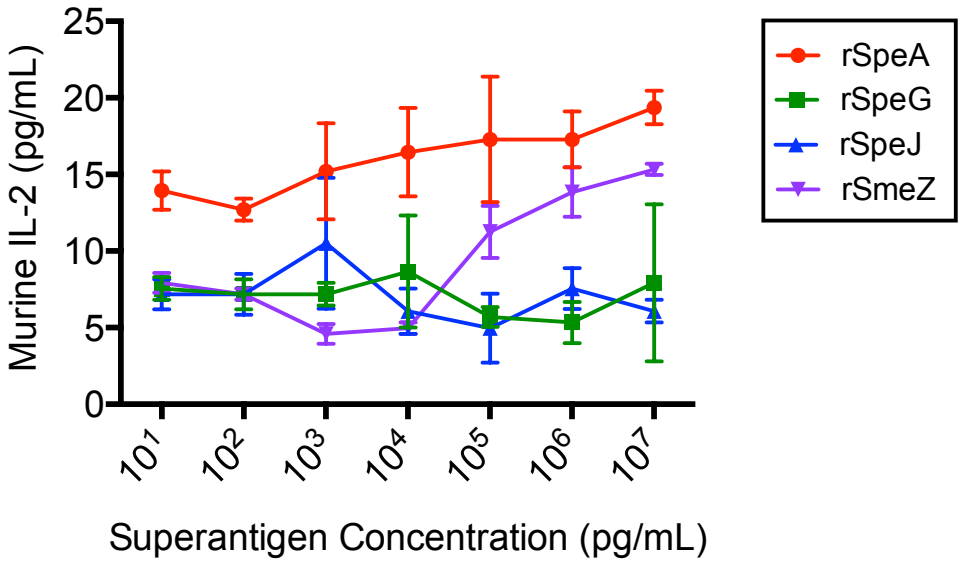


Figure 12. Recombinant superantigens do not activate FVB splenocytes. Splenocytes were harvested and stimulated with 10-fold dilutions of recombinant superantigen. Eighteen hours post-activation, supernatants were assayed for murine IL-2 production by ELISA.

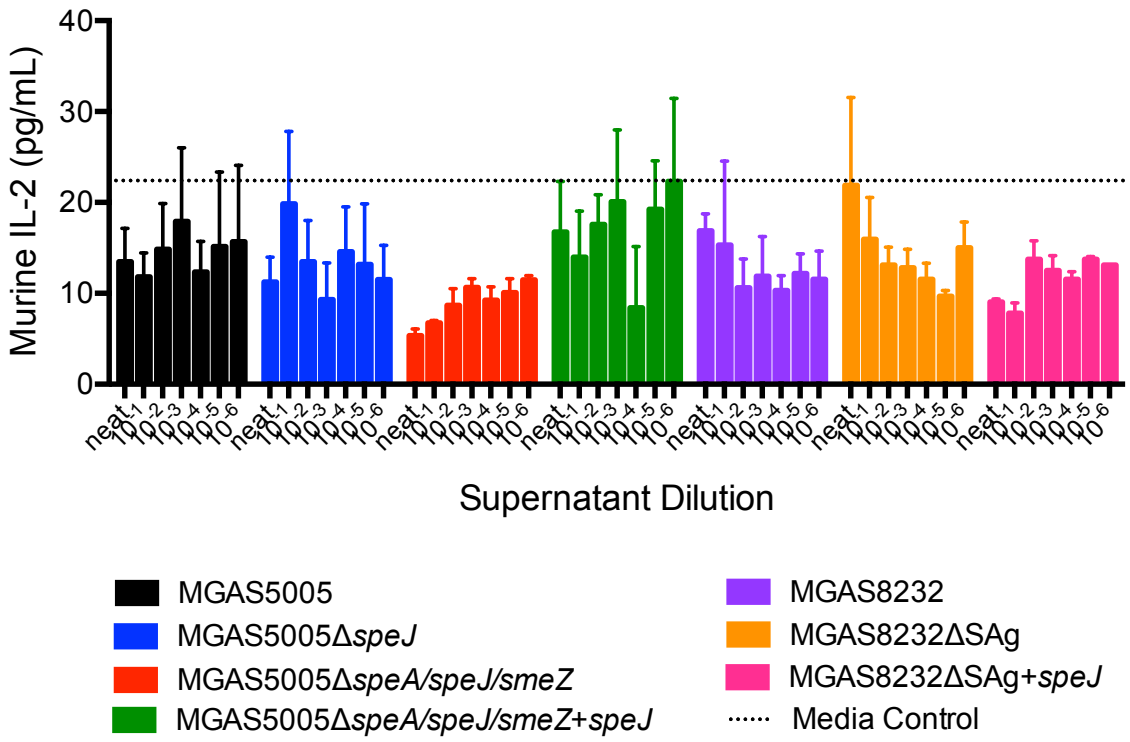


Figure 13. *S. pyogenes* strain supernatants do not activate FVB splenocytes. Splenocytes were harvested and stimulated with 10-fold dilutions of supernatants from stationary phase cultures. Eighteen hours post-activation, supernatants were assayed for murine IL-2 production by ELISA.

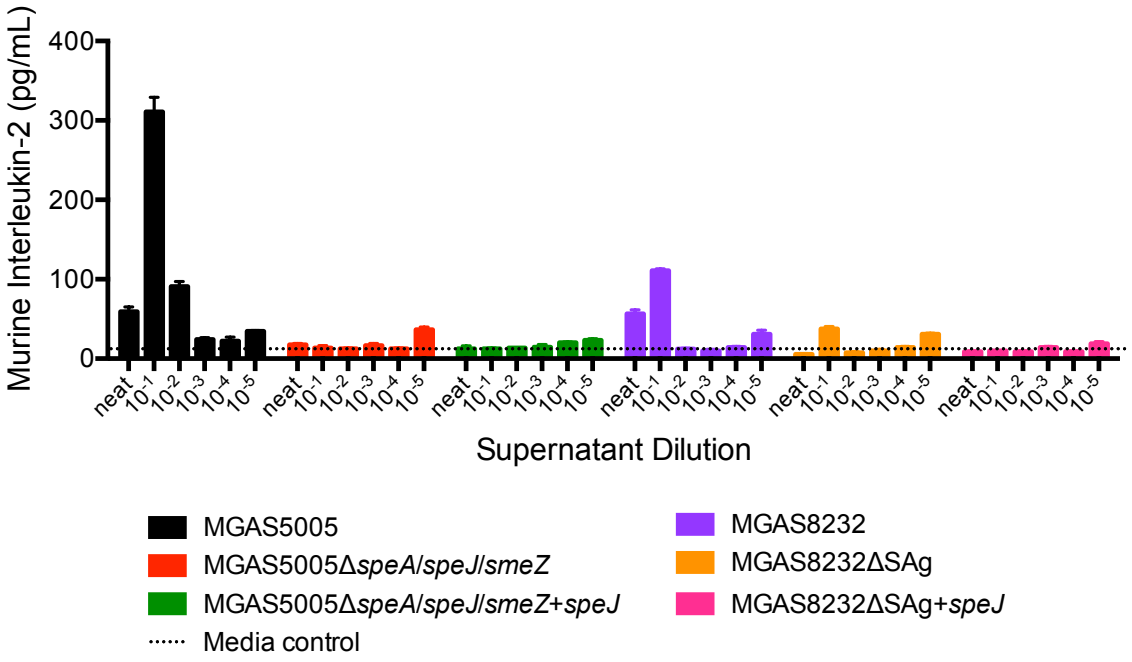


Figure 14. Supernatants of *speJ*-complemented *S. pyogenes* strains do not activate HLA-DR4/DQ8 splenocytes. Splenocytes were harvested and stimulated with 10-fold dilutions of supernatants from stationary phase cultures. Eighteen hours post-activation, supernatants were assayed for murine IL-2 production by ELISA.

presence of cytolysins which decrease splenocyte viability. There were no differences between supernatants from MGAS5005, MGAS8232, or any of the superantigen knockout or complement strains in the level of FVB splenoctye activation as measured by murine IL-2 secretion. These results were not altogether unexpected as *S. pyogenes* is a human specific pathogen and murine cells are typically less sensitive to superantigens than human T lymphocytes [184, 185]. The HLA-DR4/DQ8 splenocytes were stimulated by supernatants from wild-type MGAS5005, and less so by supernatants from MGAS8232, but were not stimulated by supernatants of any other strain evaluated, including the *speJ*-complemented strains.

Although SpeJ protein and the *speJ*-complemented strains did not activate the murine splenocytes, human lymphocytes are reactive to SpeJ [79]. In order to determine whether the genetically complemented strains were actually producing SpeJ protein, the same supernatants were incubated with human PBMC to evaluate level of T lymphocyte activation by measuring human IL-2 secretion (Figure 15). Neat (undiluted) supernatants induce secretion of low amounts of IL-2 (in comparison to other dilutions) due to the presence of cytolysins which decrease PBMC viability. As expected, supernatants from wild-type strains MGAS5005 and MGAS8232 activated the PBMC producing levels of IL-2 similar to the positive control PHA. This activation was decreased upon deletion of *speJ* from MGAS5005, and was effectively abrogated upon further deletion of *speA* and *smeZ* from MGAS5005, and all superantigens from MGAS8232. Interestingly, complementation of *speJ* was able to restore T lymphocyte activation to levels comparable to approximately half of the amount of IL-2 secreted with wild-type strains, indicating that SpeJ is indeed being produced by these strains *in vitro*. Thus, the MGAS5005Δ*speJ* strain was independently rebuilt from wild-type MGAS5005 strain and evaluated *in vivo.* This new strain produced similar results of decreased CFU recovered from cNT of mice, compared to the wild-type parent strain (data not shown). Given these findings, and since the phenotype could not be functionally restored *in vivo* by genetic complementation, we concluded that deletion of *speJ* caused a polar effect, altering the regulation of one or more genes up- or downstream of *speJ*, thus hindering the bacteria from establishing an infection. Further work in this thesis will focus on other superantigens and will exclude SpeJ.

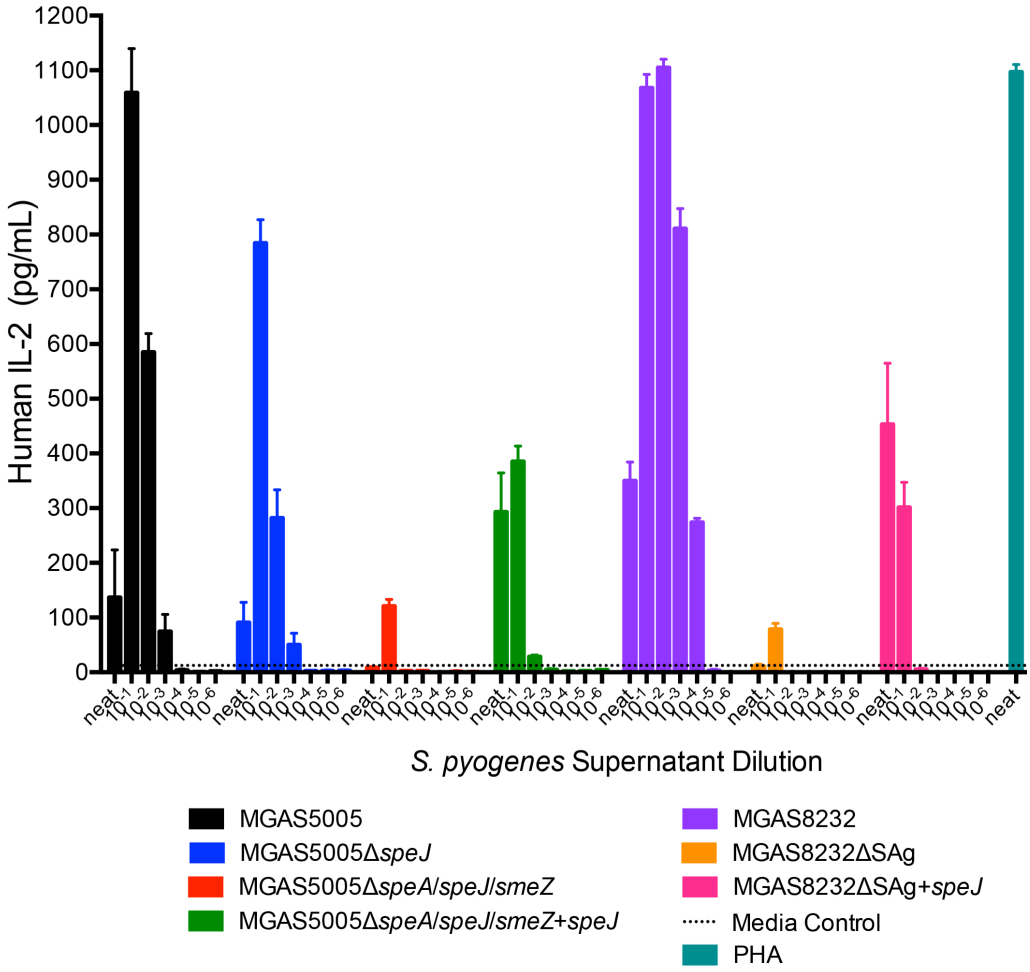


Figure 15. SpeJ is produced by both *S. pyogenes* MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* and *S. pyogenes* MGAS8232ΔSAg+*speJ in vitro*. Human PBMC were isolated from whole blood, and stimulated with 10-fold dilutions of supernatants from stationary phase cultures. Eighteen hours post-activation, supernatants were removed and human IL-2 production was measured by ELISA.

3.5 Evaluation of other superantigens in acute nasopharyngeal infection

Each of the *S. pyogenes* MGAS5005 superantigen deletion strains was evaluated for its ability to activate human PBMC (Figure 16). Interestingly, each single superantigen deletion strain, and the strain with both *speA* and *smeZ* deleted, demonstrated similar activity to the wild-type *S. pyogenes* MGAS5005 strain. This is presumably because while one superantigen is deleted, the strain still carries three other superantigens, each of which is active with human cells. There was however, a noted reduction in activity of the *S. pyogenes* MGAS5005 supernatants when *speA*, *speJ*, and *smeZ* all were deleted.

Each of the single superantigen deletion strains was evaluated in the HLA-DR4/DQ8 mice (Figure 5) and the strain lacking the gene for SmeZ, *S. pyogenes* MGAS5005Δ*smeZ*, displayed a reduction in ability to cause an infection indicating that SmeZ may be important for HLA-DR4/DQ8 infection. This phenotype was also seen in FVB mice (Figure 6). In order to determine whether the effects of the loss of *smeZ* could be restored, the *smeZ* gene was complemented back into the *S. pyogenes* MGAS5005Δ*smeZ* and *S. pyogenes* MGAS5005Δ*speA*/*smeZ* genomes to generate strains *S. pyogenes* MGAS5005Δ*smeZ*+*smeZ* and MGAS5005Δ*speA/smeZ*+*smeZ*, respectively. Similar to the *speJ* complements, *smeZ* was complemented into the null region between *tsf* and *pepO*, using the temperature sensitive vector system described earlier. Successfully integrated clones were confirmed with PCR and sequencing (Figure 17). Simultaneously but separately, the MGAS5005Δ*speA/smeZ* strain was complemented with the *speA* gene in the same *tsf* and *pepO* region as the other complements to generate MGAS5005Δ*speA/smeZ*+*speA*. These clones were evaluated in the FVB nasopharyngeal infection model (Figure 6), where complementation of *smeZ* into MGAS5005Δ*speA/smeZ*, although not statistically significant, showed a trend towards restoration of the number of *S. pyogenes* recovered from the cNT closer to wild-type levels. The MGAS5005Δ*speA/smeZ*+*speA* strain was also used in FVB nasopharyngeal infection and the number of *S. pyogenes* recovered was comparable to the number obtained without *speA* complemented. This was an expected result as the main murine target for SpeA, $V\beta8^{+}$-T lymphocytes [193], are missing in FVB mice. The MGAS5005Δ*smeZ*+*smeZ* clone was not tested in the FVB mice because concurrently in

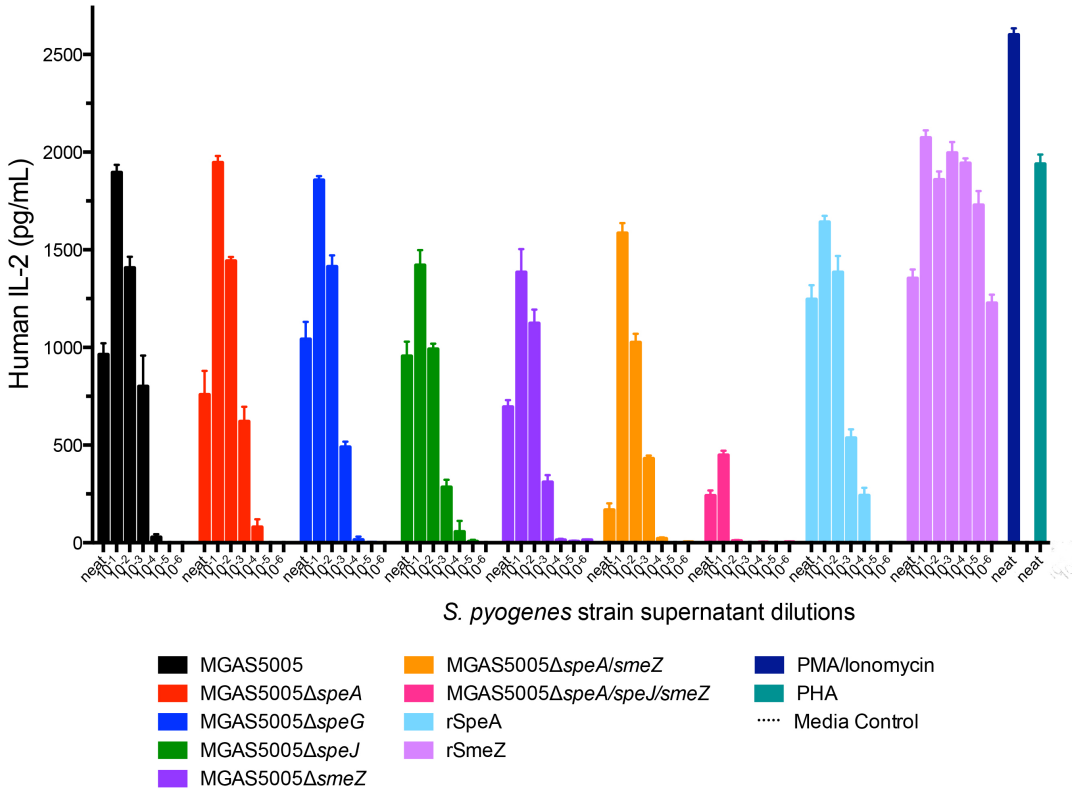


Figure 16. Human PBMC are activated by supernatants from *S. pyogenes* MGAS5005 and single-superantigen mutant strains. PBMC were isolated from whole blood, and stimulated with 10-fold dilutions of supernatants from stationary phase cultures. Eighteen hours post-activation, supernatants were assayed for human IL-2 production by ELISA.

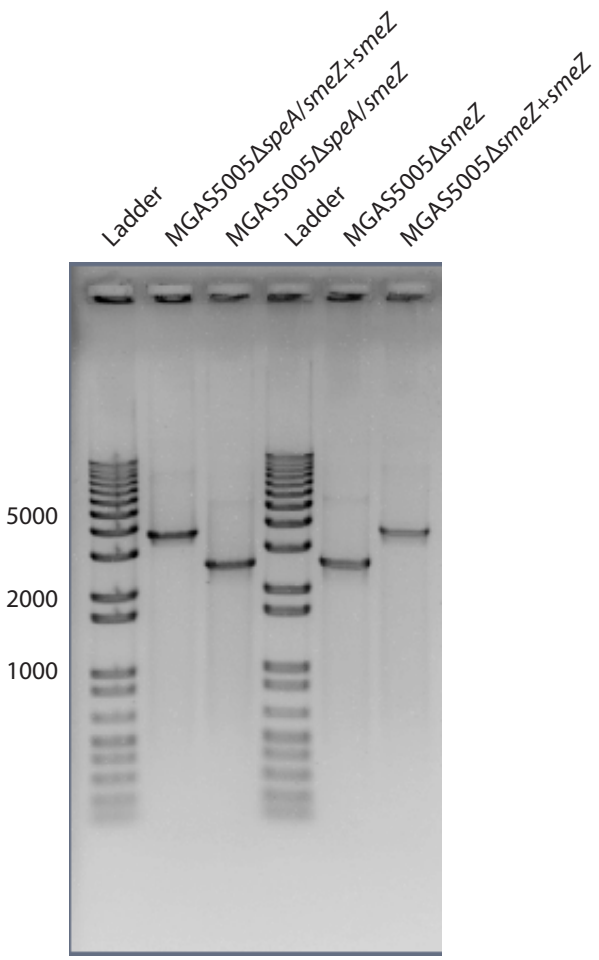


Figure 17. Generation of original *smeZ*-complemented strains. Confirmation of the integration of *smeZ* from MGAS5005 into the *tsf/pepO* region of both MGAS5005Δ*smeZ* and MGAS5005Δ*speA*/*smeZ.* Polymerase chain reaction (PCR) was performed on MGAS5005Δ*smeZ,* MGAS5005Δ*smeZ*::*smeZ,* MGAS5005Δ*speA/smeZ*, and MGAS5005Δ*speA/smeZ*+*smeZ* genomic DNA with primers *tsf* RT For and *pepO* RT Rev (outside of the integration site). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Ladder band sizes are adjacent to the gel in base pairs.

the lab, the MGAS5005Δ*speA*/*speJ*/*smeZ* strain had been sent to sequencing and results determined that there were ten single nucleotide polymorphisms (SNP) in the area surrounding the *smeZ* gene deletion, thus rendering the strains containing the *smeZ* deletion non-isogenic. Consequently, the deletion vector was re-created to be specific to the upstream and downstream regions of *smeZ* from MGAS5005, and strains containing a deleted *smeZ* gene were re-derived. MGAS5005Δ*smeZ* strain was regenerated on the MGAS5005 background, while MGAS5005Δ*speA/smeZ* was regenerated on the MGAS5005Δ*speA* base strain. Each newly created deletion was confirmed by PCR and sequencing (Figure 18). When evaluating these new strains in the FVB mice (strains labeled "new"), there were drastically different results for the new MGAS5005Δ*speA/smeZ*, where the strain's ability to cause an acute nasopharyngeal infection was not hindered (Figure 6). Infection with the new MGAS5005Δ*smeZ* strain produced high numbers of CFU recovered from cNT, though the results were more variable. Cloning was also underway to regenerate the *speA*- and *smeZ*-complemented strains, which was completed successfully and confirmed with sequencing (Figure 19).

While generating the new deletion and complement strains, the single mutant strains were evaluated in HLA-DQ8 mice (Figure 20), as previous research indicated that MGAS5005 can also infect these mice [188]. Deletion of *speA* and *speG*, each individually, did not have an effect on the ability of *S. pyogenes* to establish a nasopharyngeal infection. The result with SpeA was interesting, considering recombinant SpeA clearly activates HLA-DQ8 splenocytes (Figure 21). The new isogenic MGAS5005 *smeZ* deletion strain showed variance with high numbers of *S. pyogenes* recovered from two mice, and a low number recovered from one mouse. The most surprising results were the high and varying numbers of *S. pyogenes* recovered from HLA-DQ8 mice that had been infected with the new MGAS5005Δ*speA/smeZ* strain. This was an unexpected result as previous deletion of more than one superantigen in tandem in other murine models resulted in a poor establishment of infection by the bacteria [183]. Previously (Figures 5 and 6), the original double deletion strain was not effective at causing infections; however, consistently high numbers of *S. pyogenes*, similar to wild type counts, were recovered from the cNT of mice inoculated with the new MGAS5005Δ*speA/smeZ* strain.

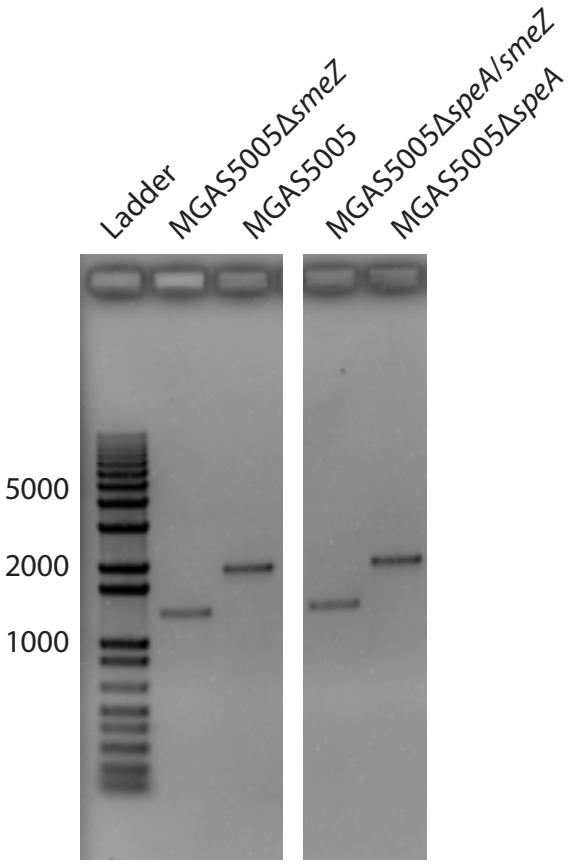


Figure 18. Generation of new *smeZ* deletion strains. Genomic DNA from MGAS5005Δ*smeZ* and MGAS5005Δ*speA*/*smeZ*, as well as parent strains MGAS5005 and MGAS5005Δ*speA* were amplified by polymerase chain reaction (PCR) with primers upstream and downstream of the *smeZ* gene. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Ladder band sizes are adjacent to the gel in base pairs.

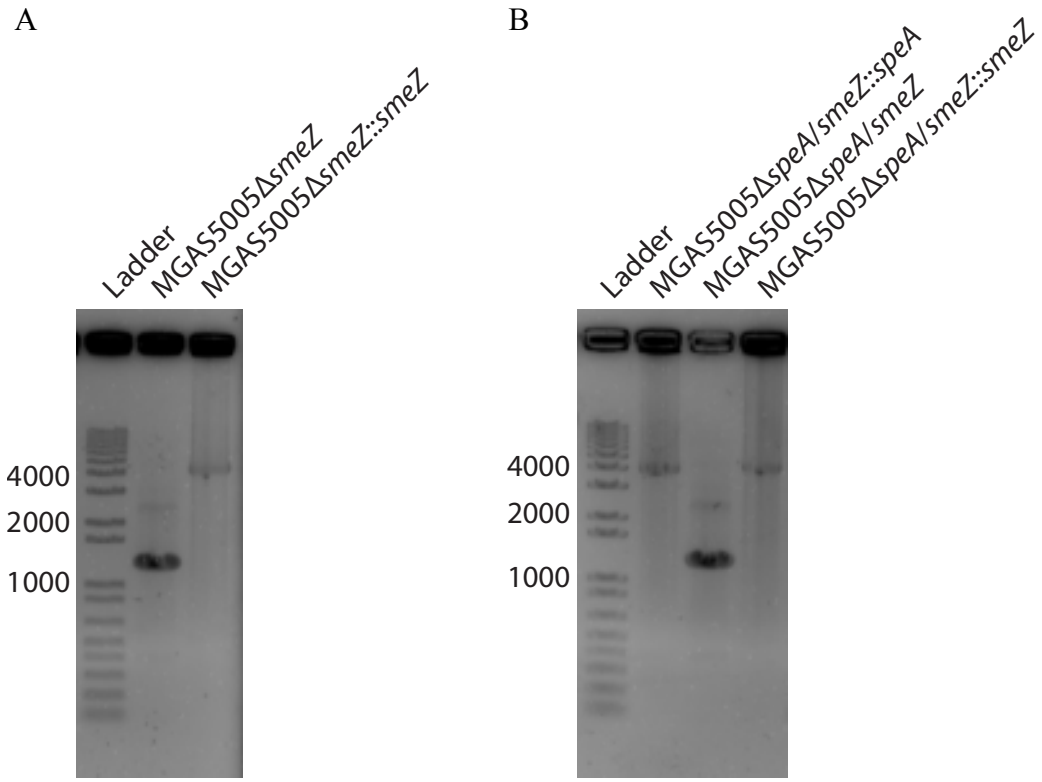


Figure 19. Generation of new *smeZ*- and *speA*-complemented strains. Confirmation of the integration of (A) *smeZ* from MGAS5005 into the *tsf/pepO* region of MGAS5005Δ*smeZ* and (B) *speA* or *smeZ* from MGAS5005 into the *tsf/pepO* region of MGAS5005Δ*speA*/*smeZ*. Polymerase chain reaction (PCR) was performed on MGAS5005Δ*smeZ,* MGAS5005Δ*smeZ*+*smeZ,* MGAS5005Δ*speA/smeZ*, MGAS5005Δ*speA/smeZ*+*speA*, and MGAS5005Δ*speA/smeZ*+*smeZ* genomic DNA with primers *tsf* RT For and *pepO* RT Rev (outside of the integration site). PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Ladder band sizes are adjacent to the gel in base pairs.

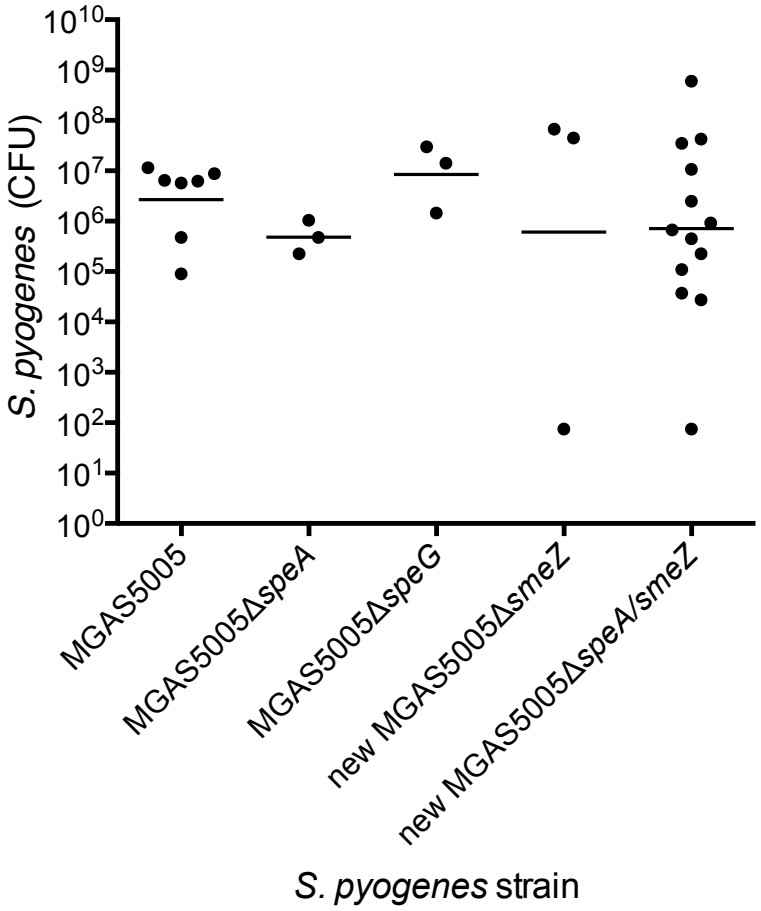


Figure 20. Evaluation of superantigen-knockout strains of MGAS5005 in HLA-DQ8 transgenic mice. Individual mice were nasally inoculated with ~1.5×10^8 bacterial CFU with the indicated strains in HLA-DQ8 mice. Nasopharyngeal CFU were assessed at 48 hours. Each symbol represents an individual mouse; horizontal lines depict the mean of each group.

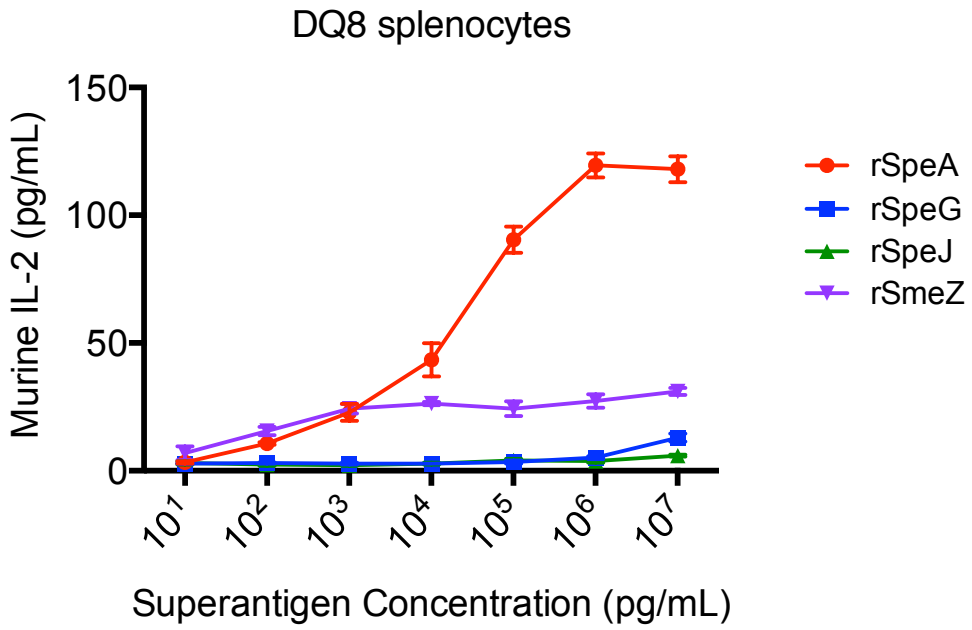


Figure 21. Recombinant SpeA and SmeZ activate HLA-DQ8 splenocytes. Splenocytes were harvested and stimulated with 10-fold dilutions of rSAg. Eighteen hours post-activation, supernatants were assayed for murine IL-2 production by ELISA.

3.6 MGAS5005Δ*speA*/*smeZ* phenotype was not MHC class II-dependent

There were high and variable numbers of *S. pyogenes* MGAS5005Δ*speA/smeZ* recovered from the cNT of both FVB and HLA-DQ8 mice (Figures 6 and 20). Since MGAS5005 does not efficiently infect C57Bl/6 mice using the nasopharyngeal cNT model (due to the lack of human MHC class II molecules), each of the MGAS5005 superantigen deletion strains were evaluated in C57Bl/6 mice to confirm that the phenotype seen in the HLA-DQ8 mice with MGAS5005Δ*speA/smeZ* was dependent on MHC class II molecules. Surprisingly, MGAS5005Δ*speA/smeZ* was recovered at higher levels compared to the wild-type MGAS5005 strain (Figure 22), indicating that the phenotype seen in the HLA-DQ8 mice was not dependent on MHC class II molecules, and therefore not superantigen-dependent. Further investigation with the single superantigen mutant strains determined that the MGAS5005Δ*speA* strain also had a significant increase in the number of *S. pyogenes* recovered when compared to MGAS5005, indicating that this phenotype was also not superantigen-dependent. The number of *S. pyogenes* recovered from the MGAS5005Δ*smeZ* infection, however, was not different than the number recovered from MGAS5005 infection.

3.7 SpeA and SmeZ are both important for MGAS5005 infection

Since the previous experiments were all dependent on genetic manipulation of *S. pyogenes* strain, an alternate, non-genetic experiment was proposed to determine the superantigen requirements for MGAS5005 nasopharyngeal infection of HLA-DQ8 mice. Mice were passively immunized with rabbit serum containing anti-superantigen polyclonal antibodies prior to inoculation with *S. pyogenes* MGAS5005 using the standard infection protocol (Figure 23). Four different treatment groups of anti-superantigen serum were utilized: anti-SpeA and anti-SmeZ combination to determine if both SpeA and SmeZ together are important for infection establishment; anti-SpeC as a negative control (MGAS5005 does not express SpeC); anti-SpeA/anti-SpeC combination as a control for anti-SpeA serum; and anti-SmeZ/anti-SpeC combination as a control for anti-SmeZ serum. The combinatorial controls were utilized instead of single antibody treatments (eg. anti-SpeA serum only) to maintain consistency of volume of treatments, as well as to ensure prospective effects were not due to double the amount of targeted

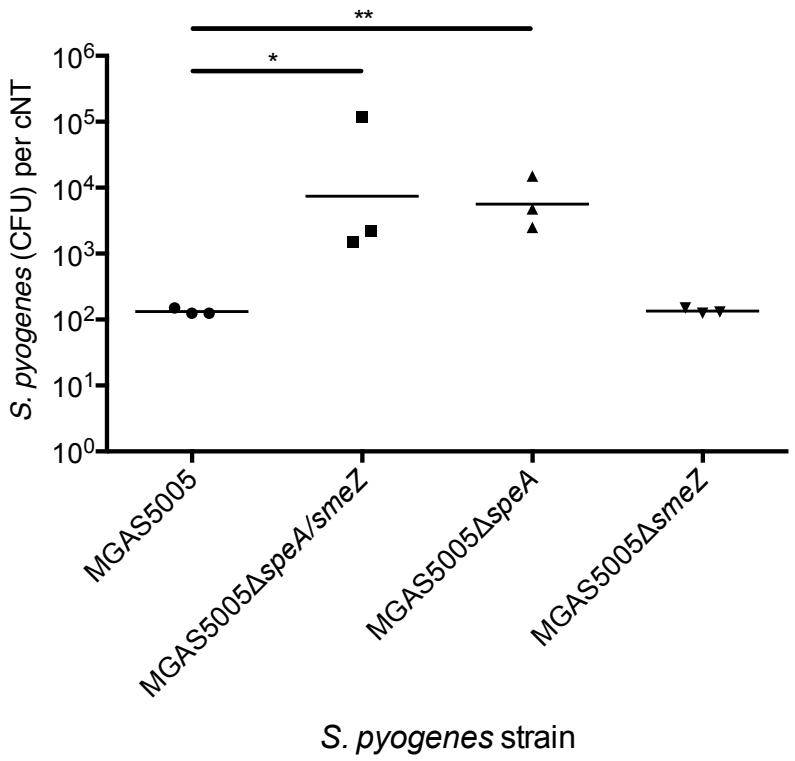


Figure 22. MGAS5005Δ*speA/smeZ* and MGAS5005Δ*speA* infect C57Bl/6 mice in an MHC class II-independent manner. Individual mice were nasally inoculated with ~1.5 × 10^8 bacterial CFU with the indicated strains in C57Bl/6 mice. Nasopharyngeal CFU were assessed at 48 hours. Each symbol represents an individual mouse; horizontal lines depict the mean of each group. * denotes $p < 0.05$, ** denotes $p < 0.01$ as determined by Student's *t* test.

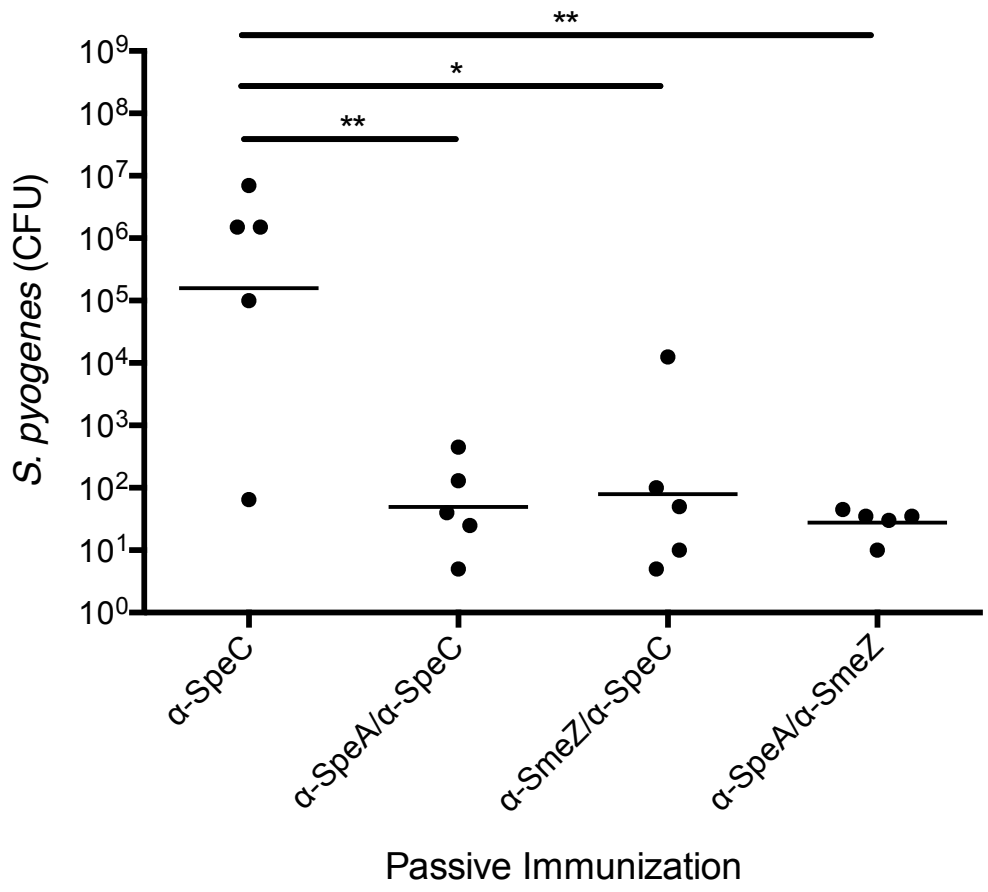


Figure 23. Both SpeA and SmeZ are important for establishment of acute infection of HLA-DQ8 mice with *S. pyogenes* MGAS5005. Mice were passively immunized at 24 and two hours pre-inoculation with 500 μL (i.p.) of one of four antibody combinations: anti-SpeC only, anti-SpeA and anti-SpeC, anti-SmeZ and anti-SpeC, or anti-SpeA and anti-SmeZ. Infection with ~1.5×10^8 bacterial CFU of MGAS5005 then proceeded, with sacrifice at 48 hours post-inoculation. Nasopharyngeal CFU were assessed at 48 hours. Each symbol represents an individual mouse; horizontal lines depict the mean of each group. * denotes $p < 0.05$, ** denotes $p < 0.01$ as determined by Student's *t* test.

superantigen-specific antibodies. As expected, *S. pyogenes* were able to establish an infection in mice that received passive immunization with antibodies to SpeC, a superantigen not expressed by MGAS5005. Passive immunization with the anti-SpeA and anti-SmeZ combination inhibited the infection by *S. pyogenes* in the cNT. Perhaps the most interesting result was that mice that were passively immunized with either an anti-SpeA and anti-SpeC combination or an anti-SmeZ and anti-SpeC combination had significantly less bacteria recovered from their cNT tissue, indicating that both SpeA and SmeZ superantigens are likely required for establishment of infection by *S. pyogenes* MGAS5005 in HLA-DQ8 mice. Antibody titres at time of sacrifice were determined by performing ELISA on serum acquired during the terminal bleed (Figure 24). Serum from each mouse contained antibodies that were administered via passive immunization, confirming proper administration of antibodies. The cNT data from this experiment implies that more than one superantigen could be required for *S. pyogenes* pharyngeal infection establishment.

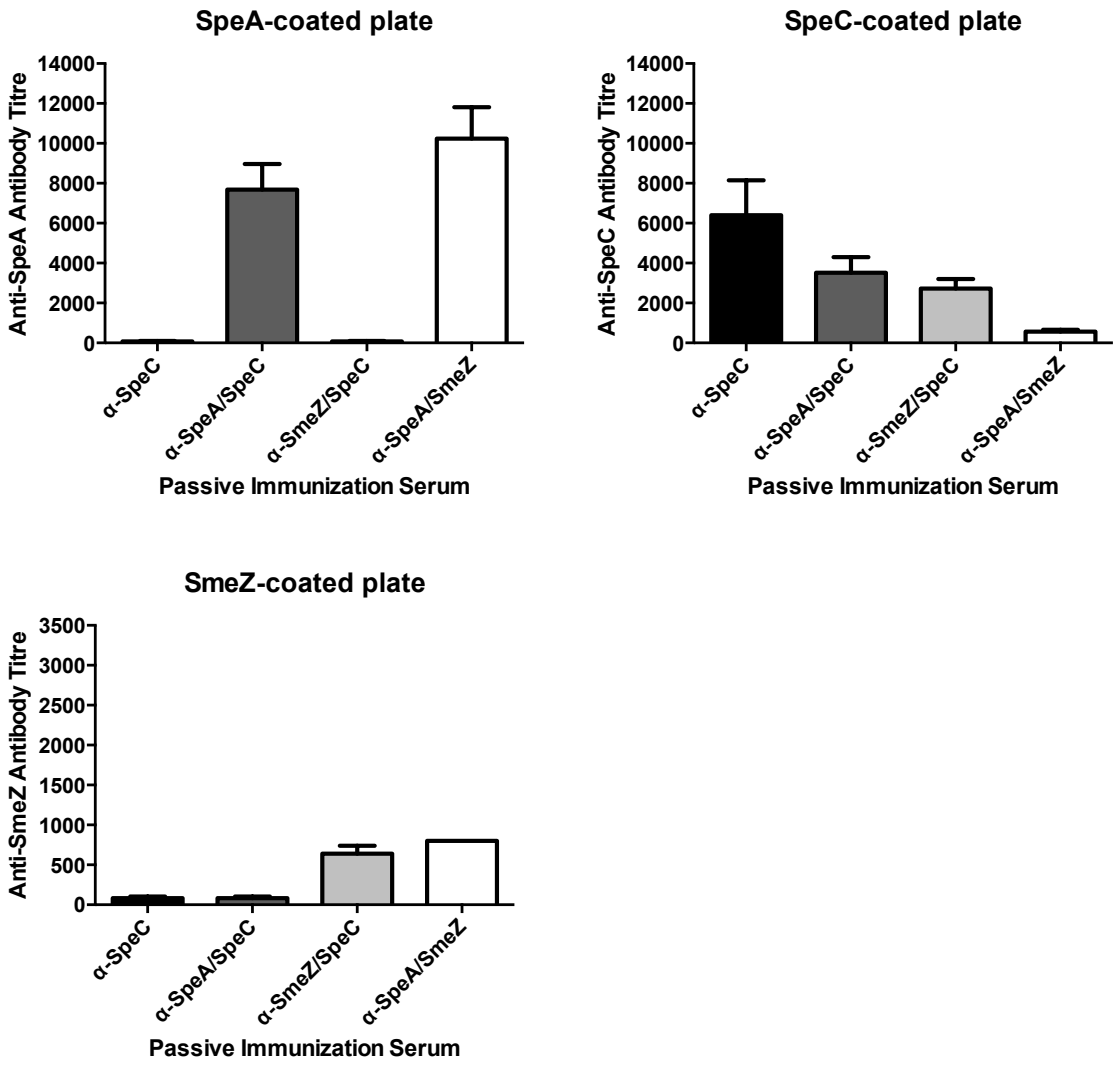


Figure 24. Terminal bleed antibody titres from passively-immunized HLA-DQ8 mice. Mice were passively immunized 24 and 2 hours prior to inoculation with MGAS5005. Forty-eight hours post-infection, mice were sacrificed and serum was assayed by ELISA to determine anti-superantigen antibody titers.

Chapter 4 : Discussion

4 Discussion

S. pyogenes has evolved to be an effective human-specific pathogen, employing several virulence factors to establish infections and maintain its survival. Although most virulence factors function to inactivate or avoid the innate immune system, superantigens deliberately activate the adaptive immune system. These toxins are extremely potent and can activate T lymphocytes in picogram quantities. Both MHC class II molecules, and a single superantigen, SpeA, were found to be critical for effective nasopharyngeal infection by *S. pyogenes* MGAS8232 [183]. A different strain of *S. pyogenes*, MGAS5005, was able to cause efficient nasopharyngeal infection in FVB mice [188]; however, since MGAS8232 was unable to cause a nasopharyngeal infection in these mice, and both strains have a different superantigen profile, we postulated that the different superantigens expressed by each strain may have contributed to this effect. Although superantigen research in previous years has determined various superantigen structures, functions, and roles in diseases such as STSS, the questions of why all *S. pyogenes* strains encode multiple, genetically distinct superantigens, if they are functionally redundant, and whether the bacteria use superantigens to aid in the establishment of infections within a wider range of the human population (based on MHC class II expression), still remain unanswered. In this study, the contribution of each of the four superantigens encoded in the *S. pyogenes* strain MGAS5005 to the establishment of non-severe infection was assessed in a model of murine acute nasopharyngeal infection.

S. pyogenes MGAS5005 encodes for four independent superantigens: SpeA, SpeG, SpeJ and SmeZ [191]. Although most streptococcal superantigens are variable traits in *S. pyogenes*, the *speG* gene appears to be chromosomally encoded and is found in nearly all strains of *S. pyogenes*. Recombinant SpeG has been shown to be functionally active with human PBMC [81], although recombinant SpeG did not activate HLA-DR4/DQ8 [183] or FVB (Figure 12) splenocytes. The *S. pyogenes* strain with *speG* as the only remaining superantigen encoded caused very limited activation of human PBMC (Figure 16) and deletion of *speG* did not alter the infection phenotype in all three murine models tested

(FVB, HLA-DR4/DQ8, and HLA-DQ8 mice). These experiments indicate that although SpeG is functional and expressed by MGAS5005, this superantigen does not appear to be a functionally dominant superantigen in MGAS5005. Although a very similar toxin can be identified in group G streptococci [194] and SpeG is not thought to play a critical role in pathogenesis of human infections [195], this superantigen may still be a contributor to establishment of human infections, albeit in a more limited capacity.

Initial data with the MGAS5005Δ*speJ* strain had indicated that this superantigen may be important for establishment of infection in FVB mice by *S. pyogenes* MGAS5005 (Figure 6). Additionally, *S. pyogenes* strain MGAS8232, a strain that lacks the *speJ* gene, was unable to establish a nasopharyngeal infection in FVB mice, and thus, we hypothesized that transferring the *speJ* gene of MGAS5005 into *S. pyogenes* MGAS8232 would provide MGAS8232 the ability to cause an infection in FVB mice. Thus, isogenic *speJ*-deletion and *speJ*-complemented strains were generated and utilized to evaluate the importance of SpeJ in acute nasopharyngeal infection establishment. Although genetic manipulation was successful, complementation of *speJ* into *S. pyogenes* MGAS8232ΔSAg, a strain lacking all encoded superantigens, did not increase the number of bacteria recovered from the cNT of FVB mice compared to mice that were infected with *S. pyogenes* MGAS8232 (Figure 6). In addition, complementing *speJ* into a strain in which three superantigen genes (including *speJ*) had previously been deleted did not restore the ability of *S. pyogenes* to establish an acute nasopharyngeal infection in any of the mouse strains tested (Figures 5 and 6). Recombinant SpeJ was not capable of activating HLA-DQ8 or FVB splenocytes (Figures 21 and 12), nor were supernatants from *speJ*-complemented strains able to activate murine splenocytes (Figure 13). Interestingly, it was confirmed that SpeJ was expressed *in vitro* by both MGAS5005Δ*speA*/*speJ*/*smeZ*+*speJ* and MGAS8232ΔSAg+*speJ* strains, as supernatant from culture growth caused activation of human PBMC (Figure 15). Real-time quantitative PCR was performed on post-infection murine cNT samples in order to quantify the number of *speJ* transcript messenger ribonucleic acids (mRNA), though results were inconclusive (data not shown). Attempts to quantify SpeJ protein from the post-infection cNT by capture ELISA were also inconclusive (data not shown). Each of these findings may have been the result of low numbers of bacteria in the cNT of mice

inoculated with *speJ*-complemented strains (Figure 6). The *speJ* deletion strain was generated multiple times and revealed similar results *in vivo*. Although in these experiments the genes were complemented *in trans* in an attempt to prevent polar effects, we now conclude that the deletion of *speJ* likely introduced a polar effect that was not corrected upon complementation. Since a polar effect is suspected, quantifying gene or RNA expression using microarray or RNA-seq could reveal expression changes between the *S. pyogenes* MGAS5005 wild-type strain, deletion, and complemented strains. Alternatively, the *speJ* gene could be replaced by a mutated, non-functional version of the gene. Structure models could be utilized to predict which amino acids are important for MHC class II or TCR binding. We have previously generated a toxoid gene for SpeA, $SpeA_{Y100A}$ (containing a tyrosine to alanine mutation at position 100) that demonstrated reduced activity on murine splenocytes when compared to wild-type SpeA, and was successfully complemented into the *S. pyogenes* genome (albeit at a different location than the original *speA* gene) [183]. Replacing the native *speJ* with a non- or less-functional gene may eliminate the polar effects experienced with the *speJ* deletion strain. Results from the experiments in this thesis suggest that SpeJ is not the important superantigen for MGAS5005 nasopharyngeal infection establishment in these models.

Interestingly, experiments with an 86-day pharyngeal model in cynomolgous macaques using the same strain of *S. pyogenes*, MGAS5005, revealed that temporally, SpeJ is the first superantigen expressed, followed by SmeZ and SpeA [196]. Early expression of SpeJ also correlated with low CFU counts [196], indicating that it is perhaps important in initial establishment of pharyngeal infection in the macaque model. The failure of mouse T cells to respond to SpeJ may be due to the lack of murine equivalent TCR Vβ chains. SpeJ targets human T lymphocytes expressing Vβ2, -3, -12, -14, and -17 [79], and as a consequence, mouse T lymphocytes may not have been reactive to SpeJ, a proposed situation similar to what occurs with SpeC, that targets human Vβ2 [114, 197] but does not interact with the murine TCR and is incapable of activating mouse T lymphocytes [198]. Alternatively, SpeJ may not bind HLA-DR4 or HLA-DQ8.

Deletion of *speA* from *S. pyogenes* MGAS5005 did not impact the ability of MGAS5005 to establish an infection in FVB mice. This was a predicted result in the FVB mouse

model, as these mice are missing the TCR gene for murine Vβ8 [199], one of the main targets of SpeA [193]. Based on previous evidence of how important SpeA of *S. pyogenes* MGAS8232 is for establishing a nasopharyngeal infection in HLA-DR4/DQ8 mice [183], and considering the fact that the *speA* allele from *S. pyogenes* MGAS5005 only differs by one nucleotide, and thus one amino acid (Gly in MGAS8232 vs. Ser in MGAS5005 at position 110) as annotated in the NCBI genomes database, it was expected that SpeA would be important for MGAS5005 infection in HLA-DR4/DQ8 mice. In HLA-DR4/DQ8 mice and HLA-DQ8 mice, the number of *S. pyogenes* recovered from infections in which *speA* was deleted was not different from the number recovered from wild-type MGAS5005 infections (Figures 5 and 19, respectively). However, upon evaluating the strain in the C57Bl/6 mice, there was a significant increase in the number of bacteria recovered from the murine cNT (Figure 22). This effect was also seen with the double *speA* and *smeZ* deletion strain, which indicated that the previous high numbers of *S. pyogenes* recovered from cNT were likely not MHC class II-dependent, and thus not superantigen-dependent. The molecular basis of this finding, although very interesting, is currently not clear. Consequently, a new MGAS5005Δ*speA* strain will need to be generated, in addition to a new *speA*/*smeZ* double deletion strain generated on the *S. pyogenes* MGAS5005Δ*smeZ* background. It is only logical to assume that the effects seen in the C57Bl/6 model by the *speA*/*smeZ* double deletion strain were due to whatever change happened in the *speA* deletion strain, as that is the parent strain of MGAS5005Δ*speA*/*smeZ.*

The *smeZ* deletion strains were recreated as the original strains contained multiple SNPs in the regions surrounding the deletion. New strains were tested in the FVB and HLA-DQ8 model, yet, as previously mentioned, high numbers of the double deletion strain were recovered from cNT. Since there were difficulties encountered with the genetic manipulations in this study, an experiment using the wild-type MGAS5005 strain and neutralizing antibodies to different superantigens was accomplished as a non-genetic-based experiment. An experiment in which both SpeA and SmeZ were neutralized, both individually and in tandem, revealed that in HLA-DQ8 mice, both superantigens were required for *S. pyogenes* MGAS5005 to establish the nasopharyngeal infection phenotype (Figure 23), thereby nullifying our hypothesis that the SpeA and SmeZ are functionally

redundant. The use of multiple superantigens simultaneously is interesting and helps to provide new understanding as to why many strains encode multiple superantigens [77]. Future experiments could examine temporal expression since *S. pyogenes* gene expression can be phase-dependent, for example shifting from a $SpeB^{+}/SpeA^{-}$ phenotype to a $SpeB^{-}/SpeA^{+}$ phenotype in murine tissue [43].

Evidently, a superantigen-independent change occurred in regulation of *S. pyogenes* MGAS5005Δ*speA*/*smeZ* and MGAS5005Δ*speA* genes to allow for better bacterial recovery 48 hours post infection from the C57Bl/6 mice. There are a number of different candidates for this including changes in capsule expression, SpeB, or other genes altogether. *S. pyogenes* produces an anti-phagocytic capsule composted of hyaluronic acid. It should be noted that capsule expression is quickly reduced following culture in artificial media, on fomites, and during convalescent throat carriage [200]. Flores *et al.* [34] suggest that down-regulation of capsule synthesis contributes to an asymptomatic carriage state for *S. pyogenes*, while increased capsule expression has been associated with more severe infections [37]. Though India ink assays were briefly attempted to visualize differences in hyaluronic acid capsule production among the superantigen-deletion strains, results were inconclusive (data not shown). Quantitative real-time PCR focused on the *hasA*, *hasB*, and *hasC* genes is recommended for an accurate evaluation of whether capsule expression differences are responsible for the 'gain of function' mouse infection phenotypes. Alternatively, ELISA for capsule expression has been previously used successfully for quantification [69] and is an option for future consideration. Following cloning techniques, strains were cultured for five days in THY + 10% HP to simulate animal passage. It is possible, however, that a mutation was selected for in the MGAS5005Δ*speA* strain during this process. Potentially, a mutation in one of the CovR/S two-component system genes could affect bacterial fitness. Longevity of nasopharyngeal infection was shorter for mice inoculated with strains with a mutated CovR/S system than those with a wild-type allele, yet an increase in the number of viable bacteria recovered from the ipsilateral lymph node, signifying invasive infection [201]. Similar to the *speJ*-complemented strains, whole genome sequencing of the MGAS5005Δ*speA*/*smeZ* and MGAS5005Δ*speA* clones will determine where changes in sequence occurred. Though this will be useful, it is expected that there will be changes

after several rounds of replication during the genetic deletion/complementation experiments, and thus, determining exactly which SNPs or mutations are responsible for the demonstrated phenotypic change may be challenging. RNA-seq or microarray experiments are recommended to quantify gene expression levels of the *speA* and *speA*/*smeZ* double deletion strain. These experiments would also provide information on a number of other genes and expression patterns as well. When combined with the genomic sequences, these data may provide insight into how these strains were able to better infect in the C57Bl/6 murine model compared to the wild-type MGAS5005 parent strain.

One of the limitations of this study was the mouse model itself. Simply stated, this is merely a model of what could potentially be happening in humans. There are challenges to this model, including the human MHC class II requirement, and lack of other known human-specific host factors such as plasminogen [18] and human T cell receptors. Future work with the acute nasopharyngeal infection model includes extending the infection timeline. Currently, we know that six days post-inoculation, there are very low levels of *S. pyogenes* in the cNT [183]. Other murine nasopharyngeal models have demonstrated bacterial shedding and persistence in the nasopharynx 15 to 20 days post-infection [201]. *S. pyogenes* also has been known to have differential expression of genes *in vivo* as the infection progresses [196], and thus, examining the contributions of superantigens to *S. pyogenes* persistence and carrier status, perhaps using non-human primates, would contribute to understanding of how and why superantigens are utilized. Alternatively, different mouse models could suffice as well. Immunodeficient NOD.Cg-Prkdc^{scid} Il2rgt^{m1Wjl}/SzJ (NSG) mice, if engrafted with a human immune system – such as in the case of BLT mice which are engrafted with human hematopoietic stem cells, liver, and thymus [202] – would be an interesting model as the MHC class II molecules and T cell repertoire would be human, and all of the streptococcal superantigens would be active.

Despite the fact that our experiments did not show that one superantigen is capable of modifying a host's susceptibility to infection, we are still in favour of the theory that superantigens help determine host range of each *S. pyogenes* strain. Superantigens, when they bind and activate T lymphocytes, do not need to be processed and presented in the

context of MHC class II molecules like traditional antigens to trigger immune activation; however, it is possible to produce anti-superantigen neutralizing antibodies [203]. Considering the fact that neutralizing antibodies can be effective at preventing infection establishment (Figure 23) and may be a beneficial adjunct therapy for some superantigen-associated diseases [204, 205], we continue to postulate that *S. pyogenes* carries multiple superantigens to maximize its host range within humans. While the number and frequency of Vβ targets in a host typically does not change, each strain possesses multiple superantigens, or potentially antigenically distinct alleles in the case of SmeZ [82]. The benefits of antigenic variation are obvious; however, expression of previously unencountered superantigens would provide the same effect – evasion of the host humoral immune system. Alternatively, the MHC class II expressed by the host may contribute to a host's susceptibility to infection and may affect patient outcomes [142]. Since superantigens bind MHC class II molecules to function [73], and binding affinities (and therefore, downstream activation effects) are dependent on the MHC class II polymorphisms [141], it is possible that having a different repertoire of superantigens to target different MHC class II polymorphisms could increase the host range of *S. pyogenes* within humans.

The versatility of *S. pyogenes* to face and overcome almost all aspects of the human immune system [206] including complement evasion and degradation, chemokine destruction, resistance to antimicrobial peptides, direct phagocyte killing, and binding of immunoglobulins are testament to the extreme host specificity and targeted evolution of the organism. *S. pyogenes* also has the ability to induce changes in human gene expression, as within 24 hours of nasopharyngeal infection in cynomolgus macaques, expression of host genes for cytokine biosynthesis and inflammatory response had decreased [39]. Interestingly, the number of differentially expressed genes correlated directly with the number of CFU and pharyngitis score assigned to each host [39].

The various infections and disease manifestations caused by *S. pyogenes* indicates that the bacterium may exhibit different lifestyles. Although this organism can cause very severe infections, including death in some cases, death of the host is not an ideal circumstance for the bacteria. Instead, we continue to believe that host colonization

(either in the form of a non-severe or an asymptomatic infection) is the preferred lifestyle, and host immune over-activation is a consequence of attempting to achieve this desired state, albeit in a “the best defense is a good offense” manner.

Superantigen expression is likely a dynamic and multi-factorial process that is dependent on a variety of host genetic and environmental elements. As these exotoxins are secreted in both severe and non-severe infections, they remain an excellent therapeutic target for future consideration and research.

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Appendices

Appendix A. Ethics approval for *in vivo* mouse experiments in this study.

From: **eSiriusWebServer**
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2009-038::5
Date: May 6, 2014 at 3:05 PM
To:
Cc: ,

Western

2009-038::5:

AUP Number: 2009-038
AUP Title: Bacterial SAgs Play a Key Role in the Pathogenesis of Streptococcus pyogenes

Yearly Renewal Date: 04/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-038 has been approved, and will be approved for one year following the above review date.

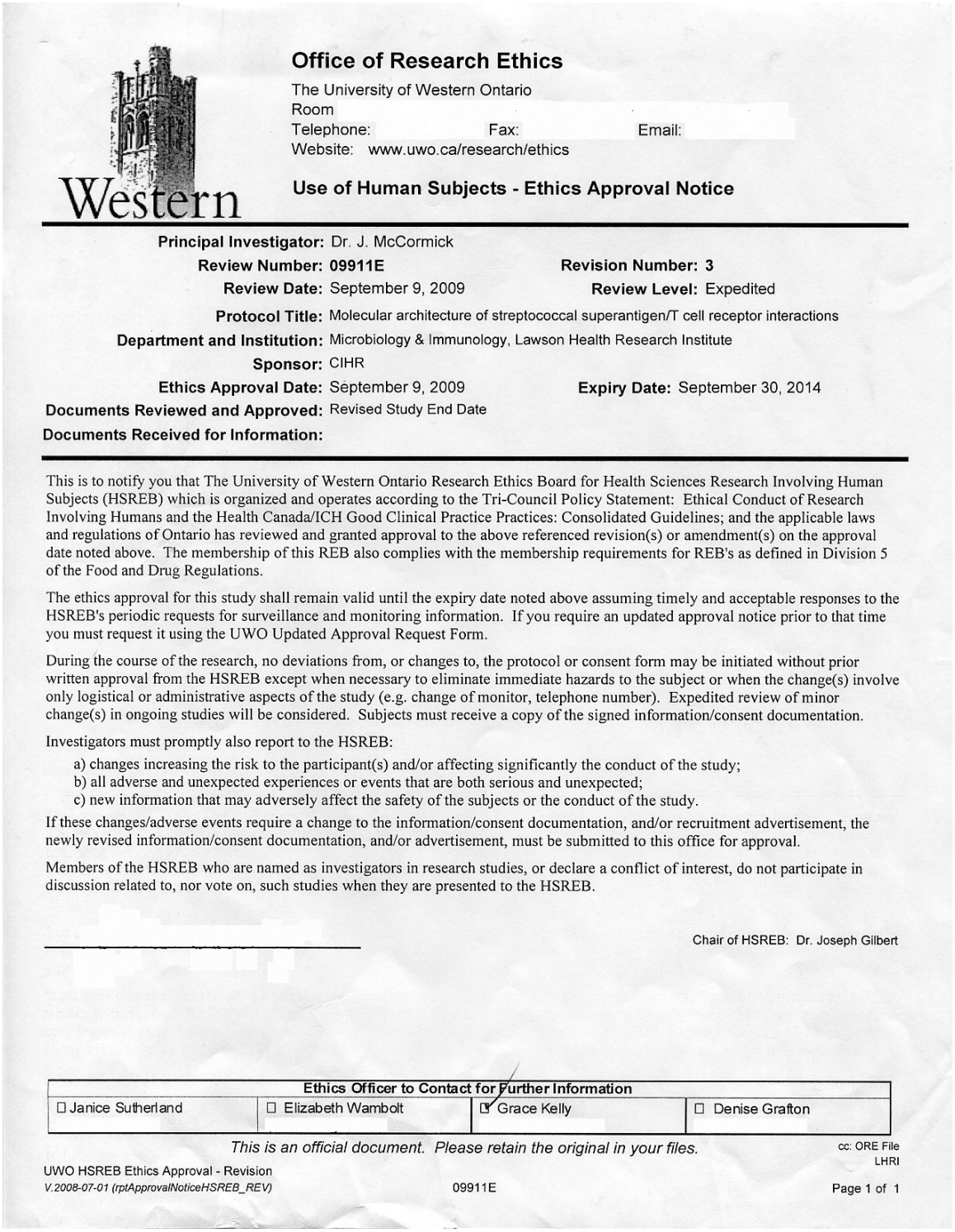
1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Appendix B. Ethics approval for collection of blood from healthy donors for PBMC experiments in this study.



Western

Office of Research Ethics

The University of Western Ontario
Room
Telephone: Fax: Email:
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. J. McCormick
Review Number: 09911E **Revision Number:** 3
Review Date: September 9, 2009 **Review Level:** Expedited
Protocol Title: Molecular architecture of streptococcal superantigen/T cell receptor interactions
Department and Institution: Microbiology & Immunology, Lawson Health Research Institute
Sponsor: CIHR
Ethics Approval Date: September 9, 2009 **Expiry Date:** September 30, 2014
Documents Reviewed and Approved: Revised Study End Date
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information			
☐ Janice Sutherland	☐ Elizabeth Wambolt	☑ Grace Kelly	☐ Denise Grafton

This is an official document. Please retain the original in your files.

cc: ORE File
LHRI

UWO HSREB Ethics Approval - Revision
V.2008-07-01 (rptApprovalNoticeHSREB_REV)

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Page 1 of 1

Curriculum Vitae

Adrienne Tamiko Wakabayashi

Post-Secondary Education and Degrees

2007 –2012	**Bachelor of Medical Science** (B.M.Sc.) University of Western Ontario, London, Ontario, Canada
2012 – present	**Master of Science** (M.Sc.) University of Western Ontario, London, Ontario, Canada

Honours and Awards

2012 – 2014	**Western Graduate Research Scholarship** ($9000) Schulich School of Medicine and Dentistry, University of Western Ontario (London, Canada)
2012	**Dean's Honor Roll** Faculty of Science, University of Western Ontario (London, Canada)
2007	**Western Scholarship of Distinction** ($1000) University of Western Ontario (London, Canada)

Publications

* denotes equal authorship contribution

1. K.J. Kasper, J.J. Zeppa, **A.T. Wakabayashi**, S.X. Xu, D.M. Mazzuca, I. Welch, M.L. Baroja, M. Kotb, E. Cairns, P.P. Cleary, S.M.M. Haeryfar, and J. K. McCormick. 2014. Bacterial superantigens promote acute nasopharyngeal infection by *Streptococcus pyogenes* in a human MHC class II-dependent manner. *PLoS Pathogens*. 10(5):e1004155

2. J. Delport, **A.T. Wakabayashi***, R.V. Anantha*, R. Lannigan, M. John, and J.K. McCormick. 2014. Case Report: *Cellulosimicrobium cellulans* isolated from a patient with acute renal failure. *J Med Micro Case Reports*. 1(2): 0.000976

Poster Presentations

* denotes presenter

1. J.J. Zeppa*, K.J. Kasper, **A.T. Wakabayashi**, D.M. Mazzuca, S.M.M. Haeryfar, and J.K. McCormick. Vaccine approaches targeting colonization by *Streptococcus pyogenes*. Presented at the International Union of Microbiology Societies Congress 2014. Montreal, Quebec, Canada.

2. J.J. Zeppa*, K.J. Kasper, **A.T. Wakabayashi**, D.M. Mazzuca, S.M.M. Haeryfar, and J.K. McCormick. Vaccine approaches targeting colonization by *Streptococcus pyogenes*. Presented at the American Society of Microbiology's General Meeting 2014. Boston, Massachusetts, United States of America.

3. **A.T. Wakabayashi***, K.J. Kasper, and J.K. McCormick. *Streptococcus pyogenes* requires superantigen expression to promote acute nasopharyngeal infection. Presented at London Health Research Day 2014. London, Ontario, Canada.

4. **A.T. Wakabayashi***, K.G. Patterson, M.L. Baroja, J.D. Dikeakos, S.M.M. Haeryfar, and J.K. McCormick. Molecular basis of human invariant Natural Killer T cell antigen receptor targeting by the bacterial superantigen staphylococcal enterotoxin B. Presented at London Health Research Day 2013. London, Ontario, Canada.

Related Work and Teaching Experience

2012 – present	**Masters Candidate** (Supervisor: Dr. John K. McCormick) University of Western Ontario (London, Canada) Thesis: *Streptococcus pyogenes* superantigens: Studies into host specificity and functional redundancy
2012 – 2014	**Let's Talk Science Classroom Volunteer** Tweedsmuir Public School and Matthew's Hall (London, Canada)
2013	**Teaching Assistant** (Dr. Steven Kerfoot, Dr. Rodney DeKoter, Dr. Bryan Heit) Microbiology and Immunology 3300: Immunology University of Western Ontario (London, Canada)
2012 – 2013	**Tutor** (Dr. Kelly Summers and Dr. David Colby) Microbiology and Immunology 3810: Microbiology for Nursing Students University of Western Ontario (London, Canada)
2012	**Research Assistant** (Supervisor: Dr. John K. McCormick) The University of Western Ontario (London, Canada) Research topic: Determining superantigen specificity to a T cell receptor
2011 – 2012	**4th Year Honors Thesis Student** (Supervisor: Dr. John K. McCormick) University of Western Ontario (London, Canada) Thesis: Targeting the human invariant Natural Killer T cell antigen receptor with the bacterial superantigen staphylococcal enterotoxin B

Volunteer Work

2012 – 2013	**Chair** Microbiology and Immunology Department Social Committee University of Western Ontario (London, Canada)
2012 – 2014	**Co-chair and Volunteer** Outreach Committee, Department of Microbiology and Immunology University of Western Ontario (London, Canada)
2012 – 2013	**Vice President, Administration** Infection and Immunity Research Forum Organizing Committee University of Western Ontario (London, Canada)