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Systematic Assessment of the Contribution of Superantigens to Nasopharyngeal Colonization in a Mouse Model of Streptococcal Infection

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Systematic Assessment of the Contribution of Superantigens to Nasopharyngeal Colonization in a Mouse Model of Streptococcal Infection

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Abstract

Streptococcus pyogenes is adapted for persistence in humans. It typically colonizes the tonsils and skin, and humans are the only known reservoir. *S. pyogenes* can cause a wide range of mild to serious infections. Most streptococci-related deaths are due to complications of rheumatic fever and invasive infections. *S. pyogenes* produces virulence factors that contribute to the pathogen's ability to colonize and cause disease, including streptococcal superantigens (SAgs), also known as streptococcal pyrogenic exotoxins (Spes). SAgs function by cross-linking T cells and antigen presenting cells (APC) which may cause a massive inflammatory response, and as such have been found to contribute to streptococcal toxic shock syndrome (STSS). The role of SAgs in adaptation of *S. pyogenes* to its target niche has not been investigated. *S. pyogenes* experimentally colonizes mouse complete nasal turbinates (cNT). Murine models of streptococcal infection are imperfect systems due to inherent host-specific tropism. In this thesis, the colonization potential of *S. pyogenes* MGAS8232, associated with rheumatic heart disease, and *S. pyogenes* MGAS5005, associated with invasive infections, were assessed to explore the contribution of SAgs to nasal colonization in a mouse model. Colonization was tested in multiple mouse strains expressing human or mouse major histocompatibility complex (MHC) class II. C57BL/6 (B6) mice expressing human leukocyte antigen (HLA) transgenes showed enhanced colonization, up to ~100,000-fold at 48 hours post-inoculation. Individual and combined SAg deletions were assessed for nasal colonization. *S. pyogenes* MGAS8232 deletion colonization in mice expressing HLA-DR4 and HLA-DQ8 (DR4/DQ8) determined that SpeA was the major contributing SAg to the establishment of colonization, with minor contributions from SpeL and streptococcal mitogenic exotoxin Z (SmeZ). Colonization of *S. pyogenes* MGAS5005 was assessed in FVB mice, which express murine MHC class II q ($H\text{-}2^q$), and SpeJ and SmeZ were the main contributing SAgs to the establishment of nasal colonization. SAgs contribute to 48-hour streptococcal recovery, but do not change the kinetics of bacterial clearance from cNT. These findings support the hypothesis that SAgs play an important role in niche adaptation and the establishment of colonization in mild or asymptomatic streptococcal infections in a murine model of nasal infection.

Keywords

Superantigen, *Streptococcus pyogenes*, streptococcal pyrogenic exotoxin, colonization, transgenic mice, mouse models, pharyngitis, group A *Streptococcus*

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List of Abbreviation

Ω	Ohms
°C	Degrees Celsius
μF	Microferridae
μg	Microgram
μl	Microliter
μM	Micromolar
× *g*	Times gravity
Δ	Deletion
ΔA	Deletion of *speA*
ΔAJZ	Deletion of *speA*, *speJ*, and *smeZ*
ΔC	Deletion of *speC*
ΔG	Deletion of *speG*
ΔJ	Deletion of *speJ*
ΔLM	Deletion of *speL* and *speM*
ΔSAg	Deletion of all streptococcal SAgs
ΔSAg + A	Deletion of all streptococcal SAgs complemented with *speA*
ΔZ	Deletion of *smeZ*
Amp	Ampicillin
APC	Antigen presenting cell
APS	Ammonium persulfate
ARF	Acute rheumatic fever
B	Brain
BHI	Brain heart infusion media
bp	Base pair
CBB	Coomassie brilliant blue
CD	Cluster of differentiation
cfu	Colony forming unit
cNT	Complete nasal turbinates

cRPMI	Complete media, supplemented Rosewell Park Memorial Institute tissue culture media
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DNase	Deoxynuclease
dNTP	Deoxyribonucleotide triphosphate
DQ8	Mice transgenically expressing MHC class II HLA-DQ8
DR4	Mice transgenically expressing MHC class II HLA-DR4
DR4/DQ8	Mice transgenically expressing MHC class II HLA-DR4 and HLA-DQ8
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
Erm	Erythromycin
ET	Ethmoid turbinate
FBS	Fetal bovine serum
FVB	Friend leukemia virus B-type mice
HBSS	Hanks balanced saline solution
HF	High-fidelity buffer, for Phusion polymerase
HLA	Human leukocytic antigen
HP	Human plasma
HPLC	High power liquid chromatography
HRP	Horseradish peroxidase
ICE	Integrative conjugative element
IFNγ	Interferon gamma
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase
kDa	Kilo dalton
L	Liter
LB	Luria-Bertani media
Mb	Mega base

MCP-1	Monocyte chemotactic protein-1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1 alpha
ml	Milliliter
mM	Millimolar
mm	Millimeters
NALT	Nasal-associated lymphoid tissue
NF	Necrotizing fasciitis
NK	Natural killer cells
No	Nose
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with tween-20
PCR	Polymerase chain reaction
pM	Picomolar
PVDF	Polyvinylidene fluoride
RF	Rheumatic fever
RHD	Rheumatic heart disease
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
rSAg	Recombinant superantigen
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	Staphylococcal enterotoxin A
SmeZ	Streptococcal mitogenic exotoxin Z
Spe	Streptococcal pyrogenic exotoxin
SSA	Streptococcal superantigen

STSS	Streptococcal toxic shock syndrome
TE	Tris-EDTA buffer
TAE	Tris-acetate EDTA buffer
TCA	Trichloroacetic acid
TCR	T cell receptor
TEMED	Tetramethylenediamine
TGF-β	Transforming growth factor beta
THY	Todd-Hewitt and yeast extract
TMB	3,3',5,5' tetramethylbenzidine, ELISA substrate reagent
TNFα	Tumor necrosis factor alpha
TSST-1	Toxic shock syndrome toxin-1
U	Units
V	Volts
Vβ	Variable beta chain
v/v	Volume per volume
w/v	Weight per volume

Chapter 1: Introduction

1.1. Group A *Streptococcus*

Streptococcus pyogenes are Gram-positive, β-haemolytic cocci that are aerotolerant anaerobes [1-2], and obligate human pathogens [3]. Although *S. pyogenes* is known to cause a wide range of active infections, they may also persist asymptomatically in the human population by colonizing the human oropharynx, specifically in the tonsils [4].

Streptococcal infections are contracted from direct, indirect or droplet contact with oral or wound secretions from an infected individual [5-7], and can be transmitted orally to cause streptococcal disease in atypical sites [8-9]. Casual contact rarely leads to disease [6]. The incubation period for streptococcal disease is one to three days post-exposure and the infection is communicable until seven days after the start of antibiotic treatment [6].

S. pyogenes produces infections of varying clinical severities which may be generally separated into diseases of two categories, namely severe streptococcal infections and mild or moderate infections. *S. pyogenes* is associated with significant morbidity and mortality globally and was ranked ninth among individual pathogens causing highest estimated deaths as reported by the World Health Organization in 2002 [10]. In high-income countries, the *S. pyogenes* diseases that are of the greatest significance are pharyngitis and invasive diseases, whereas in low-income countries rheumatic fever, rheumatic heart disease and glomerulonephritis are the most major *S. pyogenes* diseases with endemic incidences of impetigo [11]. The greatest burden of streptococcal diseases is encountered in low-income countries [11]. Streptococcal infections also result in a significant economic burden ranging in the hundreds of millions of dollars a year in the United States [12]. Genetically identical strains can cause both severe and non-severe disease, indicating an important role for host factors in determining the clinical outcome of streptococcal infections [13].

Serotypic classification of *S. pyogenes* has traditionally relied on variable surface antigens such as the M protein and the T antigen [14]. The M-protein is an anti-phagocytic surface protein and the T antigen is a pilus protein that binds fibronectin and collagen [15] and is resistant to trypsin digestion [16]. At any given time, there are multiple serotypic lineages of *S. pyogenes* circulating within the human population.

Differences in the distribution of *S. pyogenes* serotypes vary both by year and geographically [17]. There are molecular differences in the M type of strains of *S. pyogenes* that circulate in Africa and in the Pacific region compared to high-income countries [11]. Certain M serotypes of *S. pyogenes* have been linked to the prevalence of certain types of infections.

1.2. Infections by *Streptococcus pyogenes*

1.2.1. Infection versus colonization versus asymptomatic colonization

Strictly by definition within the context of bacteriology colonization means that there is a presence of bacteria in a given area. The implication of colonization may be that the bacteria will not cause disease. Asymptomatic colonization specifically means that the presence of the bacteria within the host results in no appreciable or detectable symptoms of infection and may additionally imply variable lengths of colonization, either transient or persistent infection. Finally, the definition of bacterial infection is the proliferation of a bacterium within a tissue which results in cellular injury and is symptomatic.

1.2.2. Severe streptococcal infections

Severe streptococcal disease includes necrotizing fasciitis (NF), streptococcal toxic shock syndrome (STSS) and chronic post-infection sequelae such as glomerulonephritis, streptococcal arthritis, and rheumatic fever (RF), which may ultimately lead to rheumatic heart disease (RHD). Severe streptococcal infections can be the result of a previous streptococcal infection by *S. pyogenes*, nosocomial infections, or in up to 50% of cases there is no obvious portal of entry for the streptococcal infection [18]. Severe streptococcal diseases can be further sub-classified as being either invasive disease or post-infection sequelae. Invasive diseases are defined as infections in which streptococci are isolated from an otherwise sterile site, whereas post-infection sequelae are defined as a severe consequence of a streptococcal infection. Monitoring of invasive streptococcal diseases over an 8-year period in Ontario, Canada revealed that 12% of invasive streptococcal cases were hospital-acquired, and that the common modes of transmission were from patient to patient or by a staff carrier [19]. The major risk factors

for severe and invasive streptococcal infections are pre-existing wounds, chicken pox, use of immunosuppressive drugs, pregnancy and underlying illnesses [6, 18, 20]. In developing countries, half of the patients with severe streptococcal diseases have an underlying disease or chronic illness such as human immunodeficiency virus infections, cancer, diabetes, alcohol abuse, or chicken pox [21].

It is estimated that there are approximately 18.1 million cases of severe streptococcal infections, including post-infection sequelae, and an additional 1.78 million new cases annually globally [10]. In the United States, there are 9,600-9,700 cases of invasive streptococcal diseases each year that result in 1,100-1,300 deaths [17]. In Halton Region in the province of Ontario, there were between 1 to 5 cases of severe streptococcal disease per 100,000 individuals between 1997 and 2009 [22-23].

Necrotizing fasciitis is an infection of the subcutaneous tissue, fascia, and fat that can lead to tissue necrosis, severe illness, streptococcal toxic shock syndrome (STSS) and mortality [24]. *S. pyogenes* is the most common cause of NF [25], and is diagnosed by a positive culture for *S. pyogenes* from an otherwise sterile site such as blood, connective tissue, or fascial tissue with either gross cutaneous necrosis or histopathology that demonstrates necrosis and polymorphonuclear cell infiltration in the fascia, reticular dermis or subcutaneous fat [19, 25]. NF most commonly presents with localized pain described to be disproportionate to the size of injury, chills, fever, sore throat, and respiratory symptoms, rash and vomiting [25]. The incidence of NF in children in Canada has been reported to be between 1.3 and 2.93 cases per million [25-27] and is associated with up to 20% mortality, which occurs in a shorter timeframe than any other severe streptococcal disease [25, 28] and is often preceded by a *Varicella* infection (chicken pox) [25-26].

Streptococcal toxic shock syndrome is a host immune-mediated systemic disease that is induced by superantigens (SAgs; described below) [29]. STSS is very severe in nature with a rapid onset that can result in hypotension and multiple organ failure [30]. Streptococcal SAgs have been detected in patients with STSS and a lack of anti-SAg antibodies has been associated with an increased risk of STSS [31-32]. STSS often occurs in conjunction with other invasive streptococcal diseases such as cellulitis, bacteremia and necrotizing fasciitis, and can be the result of a tegumentary breech [29,

32], or have no apparent source. The diagnostic criteria for STSS include hypotension, renal function impairment, disseminated coagulopathy, adult respiratory distress syndrome, erythematous macular rash, tissue necrosis and central nervous system disorders [33]. The early symptoms of STSS are flu-like with fever, swollen lymph nodes, irritated throat, vomiting, diarrhea, rash, and muscle and joint pain, which may be disproportionately severe [18, 32]. Resulting intravascular coagulation can result in circulatory impairment which may lead to gangrene and may require surgical intervention [18]. Mortality rates in patients with STSS have been estimated to be up to 81% [21].

Glomerulonephritis is the immune-mediated swelling of and subsequent damage to the kidneys which may ultimately result in kidney failure. This condition has been linked to preceding streptococcal infection, and typically occurs in children or young adults [34]. Symptoms of post-streptococcal glomerulonephritis are edema, hypertension, hematuria, urinary sediment abnormalities, fever and decreased serum complement levels [34]. Glomerulonephritis occurs after a latency period of an average of ten days following streptococcal infection for acute glomerulonephritis, and within three to six weeks for glomerulonephritis [34]. Nephrogenicity seems to be related to M protein serotypes, in particular with M49 serotype strains in instances of skin infection [35], but all strains within the same M serotype are not necessarily nephrogenic [34]. The incidence of skin infections and glomerulonephritis is seasonal, and peaks in the summer months in temperate climates, and is pharyngitis-associated acute glomerulonephritis in northern climates [34]. The median incidence of acute post-streptococcal glomerulonephritis in children in less developed countries was 24.3 cases per 100,000 [10]. It is estimated that there are 470,000 global cases annually with deaths in about 1% of all cases, mostly in adults [10]. The incidence of glomerulonephritis has continually declined globally, but there are still parts of the world that have a high incidence such as Africa, the Caribbean, South America, New Zealand, and Kuwait [34].

Rheumatic fever is a disease linked to poverty which is a post-streptococcal infection sequelae that may develop 2-5 weeks after the initial streptococcal infection [36-38]. Each year there are 470,000 estimated new cases of RF and 233,000 deaths attributable to RF or RHD [10]. A decrease in the incidence of RF in the developed world has been in part attributed to the lower incidence of streptococcal infection due to good nutrition,

good hygiene, and treatment with antibiotics [10, 39]. In developing countries *S. pyogenes* is the most common cause of invasive bacterial disease in young infants during the first two months of life [40]. This is due to both the greater incidence of any type of streptococcal infection, and the chances that infections are more likely to go untreated [38]. *S. pyogenes* are rarely recovered in RF cases because the preceding streptococcal infection occurs well before the first signs of RF . Clinical diagnosis of RF is made according to updated Jones criteria [41] (Table 1). The symptoms of RF are due to antibody cross-reactivity against shared epitopes from the streptococcal M-protein similar to epitopes found in cardiac myosin, synovial, and neuronal tissues, which ultimately results in autoimmunity [42-43]. Sera in patients with RF frequently have high concentrations of myosin-reactive antibodies [34]. This autoimmune reaction ultimately results in synovial inflammation (polyarthritis), neuronal tissue reactivity (Sydenham's chorea) and inflammation of the heart. Carditis is the most serious manifestation of RF with polyarticular arthritis being the most common manifestation. RF-associated carditis is associated with permanent valve damage [34]. *S. pyogenes* M-serotypes that are associated with the development of RF are found to have increased virulence, higher M protein expression and form mucoid colonies with upregulated capsule production to prevent phagocytosis [38].

RHD follows RF autoimmune reactivity to the heart resulting in lesions and inflammation in the heart valves that may lead to permanent and progressive cardiac damage [42]. The mitral valve is the most common valve affected, followed by the aortic, tricuspid, and occasionally the pulmonary valve [38]. With 15.6 million cases, RHD is the most common cause of heart disease in children worldwide and is the leading cause of cardiovascular death in the first five decades of life in developing countries [10, 36, 44]. RHD can sometime cause strokes; it is estimated that 3-7.5% of strokes in all developed countries are due to underlying RHD, which results in 108,000-269,000 deaths due to strokes per year in developing countries [10]. There are an estimated 2.39 million cases of RHD globally in children aged five to fourteen years, and it is estimated that one and a half percent of those cases will result in death each year [10]. Mortality due to RHD varies geographically with the total number of deaths being approximately 23,877 in more developed countries, and 468,164 in less developed countries [10]. The highest

Table 1. Jones criteria for the diagnosis of rheumatic fever. The diagnosis of RF requires the co-presence of either two major criteria, or one major criterion and two minor criteria, together with supporting evidence of a preceding infection with *S. pyogenes* [41].

Major Criteria	**Minor Criteria**
Migratory polyarthritis	Fever
Carditis	Elevated acute-phase reactants
Subcutaneous nodules	Arthralgia
Chorea	Prolonged PR interval on electrocardiography
Erythema marginatum	

prevalence of RHD is found in sub-Saharan Africa where there are 5.7 cases per 1,000, approximately five times higher compared to developed countries [10]. It is estimated that two to three percent of children in Cambodia and Mozambique have RHD which is often undiagnosed [39], and that 60% of RF patients will develop RHD [10], which is why prophylaxis is often used to prevent subsequent cardiac damage.

1.2.3. Mild-to-moderate streptococcal infections

Aside from serious, severe and invasive streptococcal diseases, *S. pyogenes* can also be the causative agent of less severe, mild-to-moderate infections such as scarlet fever, impetigo, and streptococcal pharyngitis. These infections are more common than severe streptococcal infections and are more likely to go unreported and untreated. Mild-to-moderate *S. pyogenes* infections are often considered to be inconsequential, despite the potential complications of developing severe streptococcal disease.

Scarlet fever is a SAg-mediated streptococcal disease [45] that is characterized by the swelling of the tongue that gives it a strawberry colour and appearance, a distinctive sandpaper rash on the trunk of the body, fever, and exudative pharyngitis [46]. The incubation period for scarlet fever is one to two days. The rash may persist for more than a week and as it fades may result in desquamation [46].

Impetigo is a topical skin infection, often caused by *S. pyogenes*, that results in skin abrasions which most often form around the mouth [47]. Estimates of the prevalence of impetigo in the global population vary based on the study. Studies done in Brazil, Ghana, and Mali found impetigo in 9.6-12.3% of the population; other studies have indicated that the prevalence of impetigo is between 0.2-35%; the range in prevalence may be due to differences in the definition of cases and bacteriological sampling techniques between groups [47]. *S. pyogenes* was isolated from approximately 95% of impetiginous lesions in studies in Brazil and Africa, which was consistent with previous American studies [47]. Impetigo is especially prevalent in children between the ages of 2 and six years old, especially those in developing countries. At any given time 111 million children in developing countries are estimated to have streptococcal pyoderma globally [10].

Streptococcal pharyngitis, otherwise known as ‘strep throat’, is a common illness in which a streptococcal infection of the pharynx results in swelling of the tonsular tissue and mucus production over the tonsils, and is often accompanied by fever. Large numbers of *S. pyogenes* are isolated from the saliva of convalescents [7] and droplets containing infected saliva can be passed by sneezing, coughing or talking [5].

Pharyngeal infections are some of the most common streptococcal infections with an estimated 616 million pharyngeal infections globally each year [10]. It is estimated that annually in developed countries, approximately 15% of school-aged children and four to ten percent of the population will suffer from a symptomatic episode of streptococcal pharyngitis [10], and that the average cost in the treatment (both medical and non-medical) of each case is approximately $200, which amounts to an estimated 224-539 million dollars per year cost for streptococcal pharyngitis in the United States alone [12]. The incidence of pharyngeal infection in less developed countries are estimated to be five to ten times greater than in the developed world [10]. Pharyngeal infections may resolve on their own or with aid of antibiotic treatment. Streptococcal pharyngitis may potentially precede more severe streptococcal infections or post-infection sequelae.

1.2.4. Asymptomatic colonization by *Streptococcus pyogenes*

Streptococcus pyogenes association with humans may also result in asymptomatic persistence of the bacteria within the human population. As this association is asymptomatic, there is no regular monitoring for the detection of *S. pyogenes* in healthy populations globally. The prevalence of *S. pyogenes* carriage varies by age. A general survey of the throat carriage of *S. pyogenes* in 2,626 asymptomatic individuals in Denmark revealed that 2.2% of the population was asymptomatically colonized. The incidence of colonization was inversely correlated with the age of the individual. The incidence of streptococcal colonization in individuals less than 14 years of age was between 10.9-12% [48-49], and the prevalence of streptococcal asymptomatic colonization in children under 5 was 24% [49]. The lowest incidence of streptococcal carriage was in individuals older than 45 years (0.6%) [48].

Asymptomatic carriers of *S. pyogenes* can be the cause of severe streptococcal infection in a healthcare environment [19]. There have been reported incidences of asymptomatic carriage of the same strains of *S. pyogenes* which were the cause of severe and invasive infections in an outbreak in healthcare and household contacts [21]. A study of 46 patients with invasive streptococcal infection in Ontario, Canada determined that the rate of streptococcal colonization in household contacts was 12% in throat cultures approximately 5-12 days after the onset of illness in the index patient. It was determined that household contacts were more likely to be colonized if they had 4 or more hours of contact daily with the index patient, and if they were younger in age [21]. The asymptomatic persistence of a streptococcal serotype within the population, regardless of the previous association with an infectious disease outbreak, likely plays a role in the overall incidence of streptococcal disease, either mild or severe.

1.2.5. Treatment of streptococcal infections

Effective resolution of non-severe *S. pyogenes* infections (such as pharyngitis or impetigo) can prevent the potential complications of severe streptococcal disease or the development of post-infection sequelae. Resolution of streptococcal infection is dependent on a fast and accurate diagnosis and effective treatment with antibiotics and, where necessary, supportive therapy [28]. *S. pyogenes* is most often treated with penicillin or amoxicillin, or alternatively in the case of penicillin allergy with clindamycin or macrolide antibiotics [25, 36, 50]. Prophylaxis is recommended in cases of colonization of healthcare workers carrying an outbreak strain of *S. pyogenes* [6].

Treatment of RF involves a two-pronged approach involving the treatment of the streptococcal infection with antibiotics and anti-inflammatory medications to treat the symptoms of RF. Intravenous immunoglobulin (IVIG) has also been used as a treatment for carditis [38]. The best prevention of RF is primary prophylaxis to prevent subsequent streptococcal infections. Long-term prophylaxis is recommended by the World Health Organization and the American Heart Association guidelines for people with a history of RF every three to four weeks until the age of 21 to prevent subsequent rheumatogenic incidents [10, 39]. When left untreated, episodes of RF resolve on their own within three months, but may ultimately result in cardiac damage. Sixty-five to seventy-five percent

of patients recover from the carditis without any further consequences; this recovery rate decreases if there are subsequent RF occurrences or if the carditis is severe [38].

There have been some reported cases of macrolide and multi-drug resistance in *S. pyogenes* including resistance to azithromycin, tetracycline, clindamycin, lincomycin, streptogramin, chloramphenicol and erythromycin [36, 51-52]. There are different incidences of antibiotic resistance reported in geographically distinct outbreaks that range from 1-48% [36], and antibiotic resistance has been considered to be a contributing factor to outbreaks of streptococcal disease [53]. Specifically, a recent report of a 2011 outbreak of scarlet fever in Hong Kong, China reported that the causative strain of *S. pyogenes* had acquired resistance to macrolide-lincomycin-streptogramin and tetracycline and encoded a MATE efflux pump, which is thought to be associated with antimicrobial resistances [53-54]. Nevertheless, despite the long-term use of β-lactam antibiotics to treat *S. pyogenes* infection, there has never been a documented case of penicillin resistance for this organism.

1.3. Genetic diversity in *Streptococcus pyogenes*

Streptococcal strains vary in genome size from 1.33 to 1.94 Mb in size. The variable size is due to the insertion of mobile genetic elements into the base streptococcal genome (NCBI genome sequence database). A large part of the genetic diversity in *S. pyogenes* is attributed to differences in the mobile genetic elements that are integrated into a base genome [55]. There is very little genetic change in the base genome between *S. pyogenes* serotypes [55]. There is limited variability in the clonal populations of *S. pyogenes* isolated in an outbreak. On average, there are 49 single nucleotide polymorphisms and 11 insertion and deletion events in the core genome of surveyed clonal *S. pyogenes* strains that were surveyed over the course of fifteen years [56]. Critically, the streptococcal population is not genetically static, but made of distinct sub clones that appear in each epidemic group [56].

Streptococci can acquire bacteriophage, transposons and integrative conjugative elements that often contain virulence factors and antibiotic resistance determinants [57]. The acquisition or loss of virulence factors and antimicrobial resistance encoding deoxyribonucleic acids (DNA) has been linked with changes in infectious properties and

outbreaks [53, 58]. Integrative conjugative elements (ICE) have been associated with the acquisition of antibiotic resistance in streptococcal populations, but do not encode SAgs [53]. ICE differ in their GC content more than bacteriophage, which may suggest that they originate from organisms that are not streptococci [57].

Bacteriophages are viral particles that may infect and introduce foreign DNA into a bacterial host. All sequenced strains of *S. pyogenes* are polylysogenic [55] and many of these bacteriophage elements encode virulence factors including SAgs [59]. Acquisition of a bacteriophage can change the infective properties of the streptococci due to the acquisition of new virulence factors. Often there is excision and reintegration of the bacteriophage DNA during normal growth (Kasper and McCormick, Unpublished data) and this movement may be a method regulating gene expression in *S. pyogenes* [60]. The phage-associated hypermutation is growth phase dependent and may allow the bacteria to better adapt to a new or changing environment [60].

Lysogenic bacteriophages found in *S. pyogenes* are mosaic in nature and may have pieces in common with other phages [61]. Bacteriophages that carry streptococcal SAgs can be transferred between different strains of *S. pyogenes*, and are not restricted by M serotypes. Transfer has been detected between group A, group C and group G streptococci, indicating a potentially common pool of bacteriophage [62-64]. This body of work also indicated that toxigenic conversion can happen by lysogenic transfer [61]. This lysogenic transfer, including the transfer of virulence factors, can provide plasticity that could be advantageous for adapting to a host challenge and may possibly result in increases in virulence. A bacteriophage can be induced to enter the lytic cycle in the presence of mammalian cells, including in a mouse nasopharyngeal model [55], which may indicate that phages can be transferred *in vivo* in the host. Toxin encoding lysogenic bacteriophages can be considered as early pathogenicity islands, which eventually undergo mutagenesis and selective pressure to alter sequences such that the island can no longer excise or enter the lytic cycle. This may be due to the selective advantage provided by the presence of the mobile genetic element and thus, pathogenicity islands and prophage can represent varying points of evolution, and depending on the stringency of the search criterion, may become indistinguishable from the surrounding genome [65].

The acquisition of bacteriophages that encode novel virulence factors may contribute to the increased fitness. The resurgence of the M1 strain of *S. pyogenes* that is widely circulating globally, is thought to be, in part, due to the acquisition of a bacteriophage encoding *speA* [2, 14]. The presence of the *speA* gene may also be a major contributing factor in the resurgence of severe and invasive streptococcal infections reported in the 1980s and 1990s [14]. It stands to reason that the introduction of a novel bacteriophage that encodes a SAg, or other phage-encoded virulence factor, could increase the efficiency of asymptomatic carriage, although this has yet to be shown experimentally.

1.4. Regulation of streptococcal virulence factors

Gene expression in *S. pyogenes* is dependent on two main types of regulatory proteins: stand-alone regulators and two component regulatory systems [66-67]. A two component regulatory system is comprised of a sensory histidine kinase that autophosphorylates in response to an environmental signal, and transfers the phosphate to a response regulator, which then affects gene transcription [68]. A stand-alone regulator is a regulatory protein that does not have an identified sensory kinase associated with it [66]. There are currently thirteen two-component systems described in *S. pyogenes* and multiple stand-alone regulators. The two best-studied virulence regulators in *S. pyogenes* are the stand-alone regulator *mga* operon and the *covRS* two-component system.

mga was the first positive acting gene regulator of a virulence network discovered in *S. pyogenes [66]*. All *S. pyogenes* have the *mga* operon which is required for the adaptation into new tissue sites and controls the expression of surface associated molecules such as the M protein, and secreted factors like C5a peptidase and the secreted inhibitor of complement, which are each necessary for immune evasion [66]. *The mga* operon controls genes in response to environmental conditions, such as the upregulation of C5a peptidase in response to elevated carbon dioxide levels, changes in osmolarity, temperature and iron restriction [66]. *mga* is encoded on what is believed to be an ancient pathogenicity island with the M-protein, whose integration into the streptococcal genome likely occurred prior to speciation [65]. Regulation by the *mga* regulon reaches its peak during the exponential phase of bacterial growth, which implies that *mga* regulated

proteins are necessary for adaptation of *S. pyogenes* to new tissue sites at early stages of infection [66].

Regulatory systems are very sensitive to the introduction of single nucleotide polymorphisms. It has been observed that there is an overabundance of single nucleotide polymorphisms in transcriptional regulators between streptococcal strains [56]. Modest polymorphisms in regulatory genes can result in considerable differences in the streptococcal transcriptome which can result in differences in pathogenicity [56].

The *covRS* two-component system influences the transcription of approximately 15% of genes in *S. pyogenes,* including genes involved in stress adaptation, gene regulation, pathogenesis, and suppresses surface and secreted proteins. Regulation by *covRS* is growth condition-dependent [69] and regulates many streptococcal virulence factors including the hyaluronic acid synthesis operon, streptokinase, cysteine protease, mitogenic factor, capsule and streptolysin S [70-74]. CovRS detects stressful conditions endogenous to the human body such as low iron, temperature of 39°C, pH, exogenous Mg^{2+}, and growth in the presence of saline [75-76], and is required for the adhesion to keratinocytes [70]. Single nucleotide polymorphisms in the *covS* gene have been linked to the ability of streptococcal strains to transition from causing pharyngeal infections to being more apt to cause invasive infections [77-79]. However, the reported mutations that are linked to invasive disease protein profile were reported to be present in both pharyngeal and invasive isolates; conversely, the pharyngeal *covS* mutations protein expression profile were also found in both pharyngeal and invasive isolates [80]. *S. pyogenes* isolated from a patient with scarlet fever from a 2011 outbreak in Hong Kong failed to switch to the *covRS* mutant SpeB-negative form 3 days post infection [53], as was reported to be characteristic. This may indicate that mutations in *covRS* may not be the key determinant of the severity of streptococcal infections in all strains.

Other regulatory systems have been identified and characterized to play a role in the regulation of virulence genes, often in response to external stimuli such as bacterial growth phase, temperature, gas concentrations, iron concentration and nutritional availability. One example is the regulator CodY which regulates the production of DNases, protease, and hyaluronidase, in response to growth phase and the abundance of

nutrients, which may contribute to the dissemination of *S. pyogenes* and the hydrolysis of host macromolecules [81].

There is surprisingly little known about the regulation of superantigens in *Streptococcus pyogenes*. There is evidence that streptococcal superantigens are upregulated early on in infection [82]. *In vitro* streptococcal superantigens are upregulated in late log phase or stationary phase [83]. The *rgg* (also known as *ropB*) regulon upregulates transcription is higher in pharyngeal infections, and in general is affected by growth phase, pH, NaCl concentration and carbohydrate-poor and peptide-rich nutrient environments. Recently, chromatin immunoprecipitation revealed that Rgg binds upstream of *speH* [84].

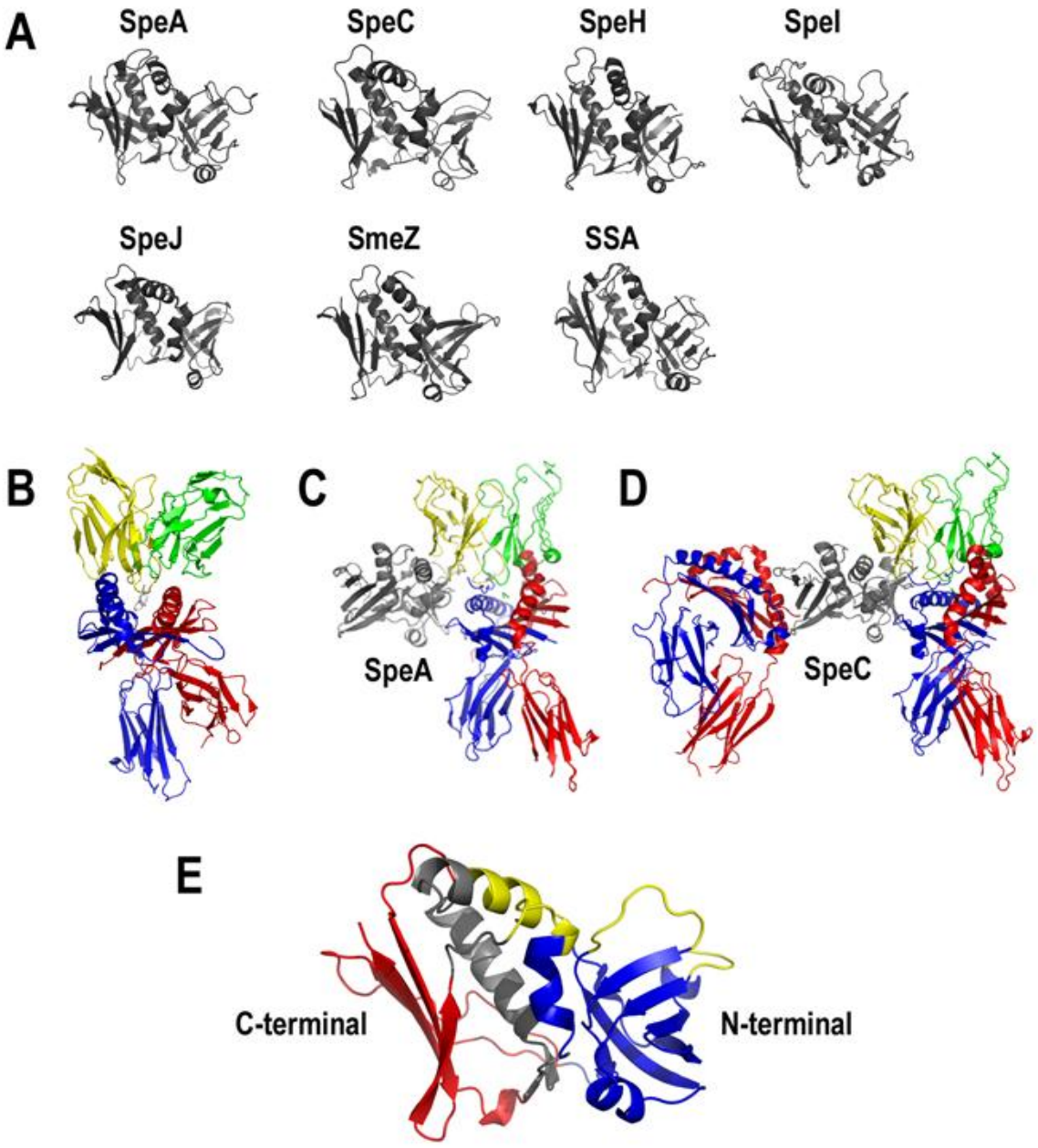
1.5. Superantigens

SAgs are a family of proteins that are produced by a limited number of bacterial, and viral species. Staphylococcal enterotoxin A (SEA) was the first SAg to be identified and it was purified and characterized in 1966 [85], although it was not known to be a SAg at the time. The term 'superantigen' was coined in 1989 when it was determined that the mitogenic capability of these toxins resulted in a massive expansion of T cells that occurred in a T cell receptor (TCR) variable β-chain (Vβ)-dependent manner [86]. SAgs stimulate both cluster of differentiation (CD) 4+ and CD8+ T cells polyclonally [87-88], and have been reported to stimulate contact-dependent T regulatory cells and invariant natural killer T cells in a concentration-dependent manner [87, 89]. Although they typically bind select TCR Vβ families and major histocompatibility complex (MHC) class II molecules (Figure 1C-D), their binding selectivity can be less stringent at higher SAg concentrations [87, 90].

1.5.1. Superantigen structure

Staphylococcal and streptococcal SAgs are between 22 and 31 kilodaltons (kDa) in size. Although the SAg family vary greatly in their amino acid similarity, between 15.5% to 90% amino acid sequence homology, they all share the same general ternary structure. Staphylococcal and streptococcal SAgs may have evolved from a single ancestral SAg [31] and a recent phylogenic tree has outlined the relatedness of all known

Figure 1. Streptococcal superantigen structures and immunoreceptor interactions. (A) Cartoon representations of all of the available streptococcal superantigen crystal structures including SpeA (PDB 1HA5) [91], SpeC (PDB 1KTK) [92], SpeH (PDB 1ET9) [93], SpeI (PDB 2ICI) [94], SpeJ (PDB 1TY0) [95], SmeZ (PDB 1EU3) [93], and SSA (PDB 1BXT) [96]. (B) Cartoon representation of conventional antigen presentation [97] in which the TCR Vα (green) and Vβ (yellow) chains recognize the specific peptide antigen presented by MHC class II (PDB IJ8W [98]. (C) Cartoon representation of a streptococcal superantigen which only has a low-affinity MHC class II binding site, such as SpeA and SSA. The superantigen (grey) interacts with the TCR Vβ chain (yellow) and the MHC class II α-chain (blue) in a manner that is less-specific than conventional antigen presentation. The Vα chain of the TCR makes contact with the β chain of MHC class II (red). This model was constructed by superimposition of SEB-MHC class II (PDB 1D6E [99]) with SEB-TCR β chain (PDB 1SBB [100]), a mouse TCR was used to align the Vβ chain of the TCR (PDB 1U3H) [101]). (D) The majority of the streptococcal superantigens have a zinc-mediated high-affinity MHC class II binding site in addition to a low-affinity MHC class II binding site. These superantigens bind the Vβ chain of the TCR and the α-chain of the MHC class II in a manner similar to Panel C, and have an additional zinc-mediated high-affinity MHC class II binding site which binds to the β-chain of the MHC class II binding site (red). The model was constructed as detailed in [102] by superimposition of the SpeC-TCR structure (PDB 1KTK [92]) with the SEC3-MHC class II (low-affinity) structure (PDB 1JWM [103]), and the SpeC-MHC class II (high-affinity) structure (PDB 1HQR [104]), and with TCR-MHC class II structure (PDB 1FYT [97]) to align the α-chain of the TCR. (E) Despite significant differences in their primary amino acid sequences, all SAgs share a conserved ternary structure. In red at the C-terminal end of the superantigen is a β-grasp domain which is the site of the zinc-mediated high-affinity MHC class II binding site in SAgs. In blue, at the N-terminal of the protein is a β-barrel domain which is the site of the low-affinity MHC class II binding site. In yellow is the T cell receptor binding site. In the center of the superantigen structure in grey is an α-helix that connects to two domains.


A
SpeA
SpeC
SpeH
SpeI
SpeJ
SmeZ
SSA
B
C
SpeA
D
SpeC
E
C-terminal
N-terminal

bacterial SAgs [105]. Each SAg contains a C-terminal β-grasp domain, and an N-terminal β-barrel domain, arranged around a long central α-helix (Figure 1A, 1E). SAgs bind directly to the Vβ-chain of the TCR [27, 81], and it has been demonstrated that the TCR α-chain plays an important role in the stabilization of the ternary complex between some of the TCR-SAg-MHC class II complexes [106-107].

The human MHC class II locus is a collection of genes known as the human leukocytic antigen (HLA) complex [108]. MHC class II molecules are heterodimeric glycoproteins with α- and β-chains that are expressed on the surface of macrophages, dendritic cells and B cells, and present peptides for recognition by CD4+ T cells. MHC class II molecules associate with antigens before being expressed on the surface of the cell. Human MHC class II has three loci, HLA-DR, -DQ, and –DP, and each individual has a maternal and paternal copy of each allele; 90% of all individuals are heterozygous for HLAs at each locus [108]. There are multiple allotypic forms of HLA-DR, HLA-DQ, and HLA-DP [109-110]. HLA haplotypes are expressed on the cell surface of APCs in different proportions, in the following general pattern: DR>DQ>DP [111].

Superantigens associate with MHC class II and TCRs simultaneously which can result in a massive immune response. Streptococcal SAgs have been reported to, in general, bind to HLA-DQ better than HLA-DR and –DP [32], and isotypic and allotypic variations can influence the immune response and the response to SAgs [88] in *in vitro* binding and proliferation assays [112], and can result in different clinical outcomes in streptococcal infection [112-114]. The MHC class II polymorphisms have been demonstrated to contribute to the magnitude and quality of the T cell response to SAgs [112], and protective MHC class II haplotypes are associated with higher IL-10 production and lower levels of pro-inflammatory cytokines [115].

Streptococcal SAgs bind to MHC class II through their two MHC class II binding sites. Streptococcal SAgs can either bind to MHC class II through a low-affinity MHC class II α-chain binding site (as is the case for SpeA and SSA) [116] (Figure 1C), or through both the low-affinity interaction, and an additional zinc-mediated high-affinity MHC class II β-chain binding site [117] (Kasper and McCormick, unpublished) (Figure 1D). Streptococcal SAgs have a higher affinity for human MHC class II than for mouse

MHC class II, explaining why they are more potent in activating human T cells as compared to mouse T cells [31].

1.5.2. Staphylococcal Superantigens

Staphylococcal enterotoxins (SEs) were initially characterized based on their emetic ability at concentrations less than 1 μg, which causes diarrhea and vomiting in staphylococcal food poisoning [31]. However, many of the staphylococcal SAgs do not possess emetic activity, or have not been tested, and these are referred to as the SAg-like toxins (SE-like or SEl). Staphylococcal SAgs are associated with illnesses such as staphylococcal food poisoning, toxic shock syndrome (both menstrual and non-menstrual) and are potentially also linked to diseases in which SAgs may play a contributing role such as Kawasaki disease, chronic rhinosinusitis, atopic dermatitis and guttate psoriasis [105]. The toxic shock syndrome toxin-1 (TSST-1) SAg is associated with menstrual and non-menstrual toxic shock syndrome and is fairly unique among the superantigen family. TSST-1 is both evolutionarily distinct [118-119], and engages both TCR Vβ and MHC class II with a unique architecture by making peptide-contacts [120-121]. Staphylococcal enterotoxin B (SEB), and sometimes SEC, are also historically associated with the non-menstrual form of toxic shock syndrome [122]. The role of staphylococcal SAgs in colonization is unclear [105]. The work presented in this study focuses exclusively on streptococcal SAgs, and further information on the staphylococcal SAgs can be found in a recent review by Xu and McCormick [105].

1.5.3. Streptococcal Superantigens

There are currently eleven known SAgs that have been characterized from *S. pyogenes*: streptococcal pyrogenic exotoxin SpeA, SpeC, SpeG-M, streptococcal mitogenic exotoxin Z (SmeZ), and streptococcal SAg (SSA) [116, 123-125]. Streptococcal SAgs that have been crystallized are shown in Figure 1 [91-93, 95-96]. SpeB and SpeF were previously labelled as SAgs, but have since been found to encode a cysteine protease and deoxyribonuclease (DNase), respectively. All sequenced strains of *S. pyogenes* encodes multiple SAgs within their genomes, and distinct streptococcal strains express different compliments of SAgs [126]. Although, there is no common SAg

profile linked to a particular strain or to invasive diseases such as STSS [2, 127], SpeA has historically been associated with STSS [128-129], and has been detected in the serum of mice infected with an invasive streptococcal infections at microgram (μg) levels [130], and in the serum of acute phase patients at pictogram levels [130-132].

Although SAgs have been associated with severe diseases like STSS, many healthy adults who have not previously developed severe SAg-mediated diseases, produce antibodies against these toxins, suggesting that SAgs are also made during mild or asymptomatic streptococcal infections [133-134]. Streptococcal superantigens are upregulated in pharyngeal infections in a macaque model of pharyngeal infection [82], which also indicates that streptococcal superantigens may play a role in mild infections or asymptomatic colonization by *Streptococcus pyogenes*.

Certain streptococcal superantigens have been found to preferentially bind HLA-DR or –DQ such as SpeA, which preferentially bind HLA-DQ [59], but that SpeA did not react differently to allotypic variations between HLA-DQ6 and –DQ8 [88]. SpeC was better-presented by HLA-DR than –DQ alone [135]. Streptococcal superantigens also have the ability to stimulate different immune system responses depending on the concentration of superantigens [87]. At high concentrations superantigens stimulate inflammatory responses and low stimulating concentrations of superantigens at the picogram and femtogram levels stimulate the production of IL-10 which can stimulate the suppression of co-stimulatory ligands on APCs such as activated B cells and monocytes [87].

The true evolutionary function of SAgs within *S. pyogenes* is not understood, however it is likely that these toxins have evolved in the context of asymptomatic persistence of *S. pyogenes* within the human population.

1.6. Streptococcal strains

There are many serotypes of *S. pyogenes* circulating in the human population at any given time. The specialization of *S. pyogenes* as an obligate human pathogen is thought to be due to a lack of biosynthetic genes, and the expression of a large number of scavenging systems which are thought to be typical of human specialization [136]. The efficiency of *S. pyogenes* to persist within a human host is largely thought to be due to the

inherent virulence factors produced by the bacteria, in combination with the sensitivity of the host immune system to streptococcal infections. Closely related streptococci, such as *Streptococcus canis* and *Streptococcus dysgalactiae*, lack host specific adaptation which indicates that the ancestor to *S. pyogenes* was unlikely to be human-specific [137]. This further suggests that a primary feature in the evolution of *S. pyogenes* was the stringent adaptation to humans.

1.6.1. *Streptococcus pyogenes* MGAS5005

Streptococcus pyogenes MGAS5005 serotype was isolated from the cerebrospinal fluid in 1996 from a patient in Toronto, Ontario, Canada, and is an M1 serotype which has been indicated to be the most widely-circulating cause of invasive streptococcal infections in North America, and Western Europe [138]. This specific strain was originally isolated from a patient with an invasive case of *S. pyogenes* in Ontario, Canada [138]. The total size of the *S. pyogenes* MGAS5005 genome is 1.838554 megabases (Mb). The strain contains 4 mobile genetic elements including three bacteriophage elements and one ICE and encodes four SAgs. The gene for *speA* is encoded on the bacteriophage 5005.1. This lineage of *S. pyogenes* has persisted globally for more than 25 years and has been isolated from both severe and mild cases [14]. Genetically identical M1 isolates can cause both severe and non-severe disease [13].

1.6.2. *Streptococcus pyogenes* MGAS8232

Streptococcus pyogenes MGAS8232 is an M18 serotype of *S. pyogenes* and this strain was isolated from a patient with ARF in 1987 [136]. The M18 serotype strains are often associated with incidences of ARF in the USA. The genome is 1.895017 Mb, and contains five bacteriophages and no ICE. Of the six SAgs encoded in the MGAS8232 genome, four are encoded on bacteriophage. The 8232.1 bacteriophage encodes *speA1*; *speC* is encoded on 8232.2; and *speL* and *speM* are encoded close together on 8232.3 [136]. *Streptococcus pyogenes* MGAS8232 has a common genetic backbone to other streptococcal strains with some large regions of insertion but no major rearrangements [136].

1.7. Murine strains as experimental tools in investigating *Streptococcus pyogenes*

In order to study the role of virulence factors in the context of colonization, mild infections and disease, it is important to use a model system that is sensitive to the virulence factors produced by the pathogen being assessed. It is also important to reiterate that most mouse MHC class II molecules do not interact efficiently with streptococcal SAgs [139], and thus many experiments have utilized exogenous or chemical agents, such as the liver damaging agent D-galactosamine to sensitize mice to SAg-mediated responses [140]. These agents can also potentially mask or exaggerate significant pathogenic effects of the bacterial infection on the animal [140].

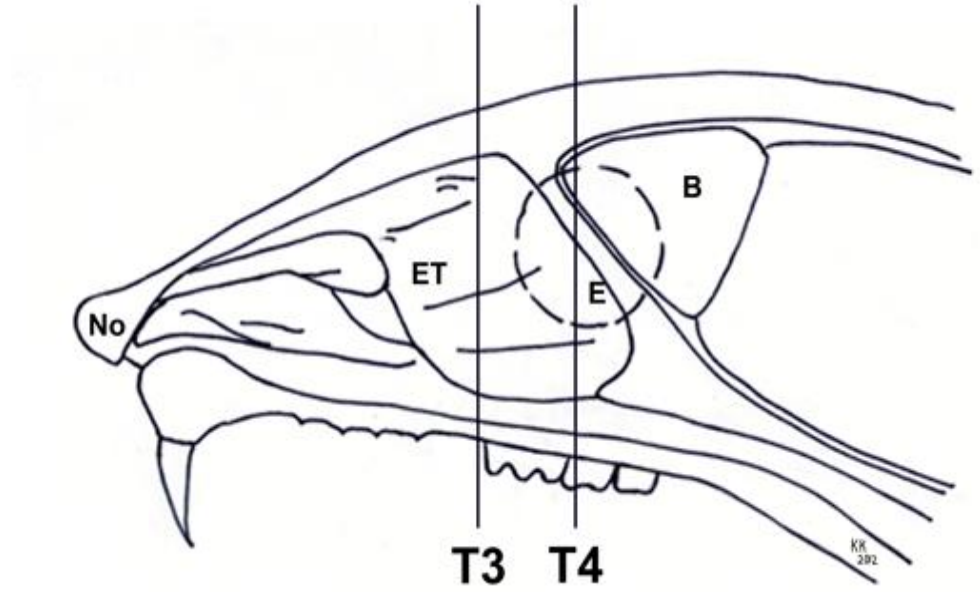
Mice expressing transgenic human MHC class II molecules have been described to have a greater sensitivity to SAg activation and are considered to be better models for the study of SAg-mediated pathogenesis [112-113, 141]. These transgenic mice have been used to determine the impact that various human MHC class II molecules could potentially have on the clinical outcomes of severe and invasive infections [112-113, 141].

1.8. Pathophysiological structure of the murine nose and oropharynx

In mice, as in all mammals, the nose is both a portal of entry into the respiratory system and the sensory organ for olfaction. Nasally inspired air is passed through the nasal passage which extends, bi-laterally divided by the septum, from the nostrils to the nasopharynx [142] (Figure 2). The complex folding of the nasal structure warms and conditions the air before it enters the lungs [143]. The nasal passage is lined by ciliated nasal mucosa, which beat to move nasal mucus, produced by goblet cells, distally to the oropharynx where it is swallowed [142].

There are five types of epithelia in the nose: neuroepithelium, respiratory epithelium, squamous epithelium, lymphoepithelium, and transitional epithelium. Squamous epithelium is located in the nasal vestibule just inside the nostrils and is similar to the epidermis in the skin. The translational epithelium is situated between the squamous epithelium and the respiratory epithelium. Respiratory epithelium is more distal from the nares and makes up the majority of the non-olfactory epithelium.

Figure 2. The physiological location of the ethmoid turbinates, nasal-associated lymphoid tissue and the maxillary sinuses. A cross-section drawing of the head of a mouse indicates where standard sections are taken for histological analysis at T3 and T4. The nose (No), and brain (B) are identified for orientation. The T3 section is located anterior of the T4 section and slices through the ethmoid turbinate (ET) ahead of both the molar teeth and the eye (E). The NALT (N) is located bilaterally at the base of the ethmoid turbinates. Lateral to the NALT in the T3 section is the maxillary sinus (MS). The T4 section cuts through both the brain and the eye. The ethmoid turbinate is better-separated and the NALT is located bilaterally at the base of the ethmoid turbinates.


B
ET
E
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T3
T4
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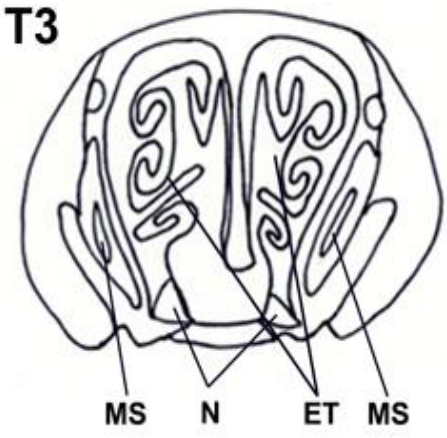
292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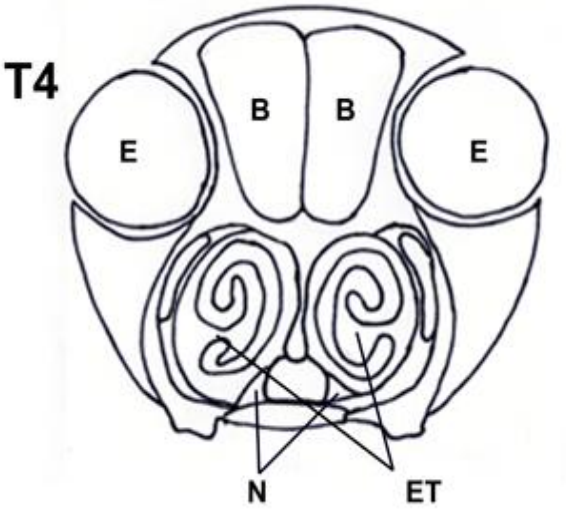
T4
E
B
B
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ET

Respiratory epithelium and neuroepithelium are both ciliated, although the cilia beat more slowly to locomote the mucus distally over the neuroepithelium than the respiratory epithelium. The fifth type of nasal epithelium is the lymphoepithelium; this epithelium covers the discrete foci of nasal-associated lymphoid tissue (NALT) that run bilaterally on the ventral face of the nasopharyngeal meatus, which is otherwise lined with respiratory epithelium.

1.8.1. Pathophysiological comparison of nasal tissue between mice and humans

Although there are many similarities in the function and structure of the nasal epithelia between mice and humans, there are also some notable differences [143]. The nasal passage in both mice and humans is separated by a septum and the nasal passage extends from the nostrils to the nasopharynx. Unlike humans and other primates who are able to breathe orally and nasally, mice are obligate nose breathers [142]. Physiologically, mice lack hair in the vestibule just inside the nasal opening [142] and have more complicated nasal turbinates in their folding and branching patterns [143]. In particular, the extremely complex folded shape of the ethmoid turbinates which are lined with olfactory neuroepithelium, and respiratory epithelium in mice [143] are thought to be designed for acute, more sensitive olfaction [142].

1.8.2. Nasal-associated lymphoid tissue

Human tonsils, otherwise known as Waldeyer's ring, are composed of the human adenoid, bilateral tubule, palatine, and lingual tonsils [142]. Similar analogous structures have been reported in monkeys, horses, cattle, rats, and mice [144-145]. The tonsular surface is highly invaginated which increases the surface area of the tonsils and aids in the capture of foreign materials for immune surveillance [146]. The human tonsils are the primary site of streptococcal colonization. Tonsils that were excised from healthy individuals due to recurrent pharyngotonsillitis had a carrier rate for *S. pyogenes* of 25%, and in healthy individuals with hypertrophic tonsils, this carrier rate for *S. pyogenes* was 7%, which suggests that the tonsils are an important reservoir for asymptomatic carriage of *S. pyogenes*, which may seek refuge intracellularly [147]. The NALT in mice has been reported to correlate functionally to Waldeyer's ring in humans [142]. The location of the

NALT within the nasal passage is strategic in that all antigenic-laden inspired air must pass across the area [142].

The NALT is overlaid by the lymphoepithelium, which includes cuboidal cells with luminal microvilli called membranous cells (M cells) that are involved in the uptake and translocation of inhaled antigens to the underlying lymphoid structures [142-143]. The NALT and the nasal mucosa drains directly to the cervical lymph nodes and is a major mucosal inductive site [148]. It is an organized tissue with T lymphocyte- and B lymphocyte-enriched zones [149]. NALT is an immune inductive and tolerogenic site which contains T cell precursors which are thought to downregulate T cell activation. It has been suggested that NALT may be capable of both mucosal and systemic immune response [142, 150].

1.8.3. Streptococcal colonization and the murine nasal infection model

Streptococcal colonization of the nasal passage in mice is thought to be analogous to tonsular asymptomatic colonization or mild pharyngeal infection and has been previously described in papers from the Cleary laboratory [149, 151-153]. It has been determined that BALB/c mice infected with 5×10^8 colony forming units (cfu) of *S. pyogenes* remained infected with detectable concentrations of bacteria for seven days in the nasal cavity, and that the greatest concentration of streptococci was located in the NALT at 48 hours post-inoculation [154]. Kinetically, it was observed that after an initial clearance of the bacteria, as imaged at four and a half hours, the streptococcal burden recovered at 24 hours and reached the maximum concentration at 48 hours [154].

Streptococci are indicated to survive intracellularly in NALT infection models as indicated by antibiotic protection assays of infected NALT tissue with *S. pyogenes* resulted in about 1-10% bacterial recovery [154]. Similar studies done with excised human tonsils also observed *S. pyogenes* existing intracellularly [147]. The uptake of *S. pyogenes* into the NALT was downregulated by pre-treatment with UEA-1 lectin which indicates that the portal of entry into the NALT is via the M cells [149]. Streptococcal induction of TGF-β upregulates the expression of α5β1 integrins and fibronectin in tonsular fibroblasts which are necessary for S. pyogenes internalization into the host cells [155], additionally it also increases Th17 and T regulatory cells which may dampen

cellular responses to antigens and increases the infiltration of neutrophils and macrophages into infected host tissues [155]. Similar Th17 response was observed in both mouse nasal and tonsular tissue [155].

1.9. Rationale and hypothesis

Scientific and clinical studies in the literature are largely focused on the examination of severe and invasive streptococcal infections. There are relatively fewer clinical papers, however, that examine mild streptococcal infections or asymptomatic persistence of *S. pyogenes* within the human population [49, 156-158]. It can be argued that although severe streptococcal diseases are dramatic and important, mild or asymptomatic infections occur much more frequently and are thus of greater significance for streptococcal persistence within the host, and within the human population as a whole, as well as for the mechanisms of evolution of these bacteria. Streptococcal superantigens have been shown to be upregulated in a pharyngeal infection model in *Cynomolgus macaques*, which implies that streptococcal superantigens play a role in mild streptococcal infection or streptococcal colonization [82], however the relationship and contribution of streptococcal superantigens to the establishment of mild infection and colonization has not been systematically assessed. As a result, the study of the mechanisms that *S. pyogenes* will use to persist within the population are extremely important. Although the role of streptococcal SAgs in severe disease such as STSS is clear, the natural niche of *S. pyogenes* is in a state of colonization or mild infection, and as such we hypothesize that these toxins have likely evolved within the context of mild infection or colonization. Therefore, the hypothesis of this thesis is that SAgs play an important role in the course of colonization of *S. pyogenes* in an appropriate SAg-sensitive nasal mouse model.

Chapter 2: Materials and Methods

2.1. Bacterial growth conditions

2.1.1. *Escherichia coli* growth conditions

Escherichia coli strains used in this study are listed in Table 2. All *E. coli* were grown aerobically at 37°C in either Difco Luria-Bertani (LB) broth (Becton, Dickinson and Company, Sparks, MD, USA) or Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) or on solid LB or BHI media supplemented with 1.5% agar (BD, Franklin Lakes, NJ, USA). The media were prepared to contain antibiotics appropriate to the plasmids within those strains (Table 3). Ampicillin (Amp) (EMD, Darmstadt, Germany) was used at a concentration of 150 μg/ml, and kanamycin (Kan) (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 50 μg/ml. Erythromycin (Erm) (Acros Organics, NJ, USA) was used at a concentration of 150 μg/ml in. All antibiotics were filter sterilized through a 0.2 μm syringe filter (Pall Life Sciences, Ann Arbor, MI, USA). Viable stocks of *E. coli* were maintained and stored in LB or BHI containing the appropriate antibiotics and 20% (v/v) glycerol at -80°C.

2.1.2. *Streptococcus pyogenes* growth conditions

S. pyogenes strains used in this study are listed in Table 2. All *S. pyogenes* cultures were grown without aeration at either 30°C, 37°C, or 40°C in Todd Hewitt media (Becton, Dickinson, and Company, Sparks, MD, USA) supplemented with 1% (w/v) with yeast extract (EMD Chemicals Inc., Darmstadt, Germany) (THY). Media was prepared to contain 1 μg/ml Erm for growth with plasmids as necessary (Table 3). After the SAg genes were inactivated (described below), of all of the *S. pyogenes* were grown in THY media supplemented with 10% human plasma (HP) to restore the streptococcal capsule [159]. Human plasma was collected after being diluted 1:1 in Roswell Park Memorial Institute medium (RPMI) 1640 and reserved from peripheral blood mononuclear cells

Table 2. Bacterial strains used in this study

Strain	**Description**	**Source**
Escherichia coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 supE44 relA1 lac* [F'*proABlacI*^{q}*ZΔM15 Tn10* (Tet^{r})]	Novagen Stratagene
Escherichia coli BL21 (DE3)	F- *ompT*[*lon*] *hsdSB* with λDE3 prophage carrying the T7 RNA polymerase gene	Novagen
Streptococcal strains		
Streptococcus pyogenes MGAS5005 wild type	M1 streptococcal strain isolated from an invasive case in Ontario	Sumby *et al*. [138]
Streptococcus pyogenes MGAS5005 Δ*speA*	*S. pyogenes* MGAS5005 with *speA* disrupted	This study
Streptococcus pyogenes MGAS5005 Δ*speG*	*S. pyogenes* MGAS5005 with *speG* disrupted	This study
Streptococcus pyogenes MGAS5005 Δ*speJ*	*S. pyogenes* MGAS5005 with *speJ* disrupted	This study
Streptococcus pyogenes MGAS5005 Δ*smeZ*	*S. pyogenes* MGAS5005 with *smeZ* disrupted	This study
Streptococcus pyogenes MGAS5005 Δ*speA*/*speJ*/*smeZ*	*S. pyogenes* MGAS5005 with *speA*, *speJ*, and *smeZ* disrupted	This study
Streptococcus pyogenes MGAS8232 wild type	M18 streptococcal serotype associated with acute rheumatic fever outbreaks in USA. Isolated from a patient with acute rheumatic fever.	Smoot *et al*. [136]
Streptococcus pyogenes MGAS8232 Δ*speA*	*S. pyogenes* MGAS8232 with *speA* disrupted	This study
Streptococcus pyogenes MGAS8232 Δ*speC*	*S. pyogenes* MGAS8232 with *speC* knocked out	This study
Streptococcus pyogenes MGAS8232 Δ*speG*	*S. pyogenes* MGAS8232 with *speG* disrupted	This study
Streptococcus pyogenes MGAS8232 Δ*speL*/*speM*	*S. pyogenes* MGAS8232 with *speL* and *speM* disrupted	This study
Streptococcus pyogenes MGAS8232 Δ*smeZ*	*S. pyogenes* MGAS8232 with *smeZ* disrupted	This study

Streptococcus pyogenes MGAS8232 ΔSAg	*S. pyogenes* MGAS8232 with *speA*, *speC*, *speG*, *speL*, *speM*, and *smeZ* disrupted	This study
Streptococcus pyogenes MGAS8232 ΔSAg + *speA*	*S. pyogenes* MGAS8232 ΔSAg complemented with *speA*	This study
Superantigen expression strains		
Escherichia coli BL21 with SpeA	pET28a::TEV::*speA* wild type	[160]
Escherichia coli BL21 with SpeC	pET41a::TEV::*speC* wild type	[161]
Escherichia coli BL21 with SpeG	pET28::TEV::*speG* wild type	[160]
Escherichia coli BL21 with SpeJ	pET28::TEV::*speJ* wild type	[160]
Escherichia coli BL21 with SpeL	pET28::TEV::*speL* wild type	Shipa Gupta, unpublished
Escherichia coli BL21 with SpeM	pET28::TEV::*speM* wild type	Shipa Gupta, unpublished
Escherichia coli BL21 with SmeZ	pET41a::TEV::*smeZ* wild type	Christine Herfst, unpublished

Table 3. Plasmids used in this study

Plasmid	**Description**	**Source**
pG+host5	Erythromycin resistance, Gram-negative origin of replication, Gram-positive temperature-sensitive origin of replication	Appligene, [162]
pG+host5::I-*Sce*I	pG+host5 base plasmid with I-*Sce*I restriction enzyme cut site	This study
pG+host5::I-*Sce*I::MGAS8232 Δ*speA*	Plasmid to knock out *speA* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS8232 Δ*speC*	Plasmid to knock out *speC* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS8232 Δ*speG*	Plasmid to knock out *speG* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS8232 Δ*speL/M*	Plasmid to knock out *speL* and *speM* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS8232 Δ*smeZ*	Plasmid to knock out *smeZ* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS5005 Δ*speA*	Plasmid to knock out *speA* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS5005 Δ*speG*	Plasmid to knock out *speG* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS5005 Δ*speJ*	Plasmid to knock out *speJ* in *S. pyogenes* MGAS8232	This study
pG+host5::I-*Sce*I::MGAS5005 Δ*smeZ*	Plasmid to knock out *smeZ* in *S. pyogenes* MGAS5005	This study
pG+host5::I-*Sce*I::Int MGAS5005	pG+host5::I-*Sce*I containing sequence for homologous recombination between *tsf* and *pepO* of *S. pyogenes* MGAS5005 with multiple cloning site	This study
pG+host5::I-*Sce*I::Int MGAS8232	pG+host5::I-*Sce*I containing sequence for homologous recombination between *tsf* and *pepO* of *S. pyogenes* MGAS8232 with multiple cloning site	This study
pG+host5::I-*Sce*I::Int MGAS8232 *speA* Comp	Plasmid for complementation of *speA* between *tsf* and *pepO* of *S. pyogenes* MGAS8232	This study

(PBMC) isolation. THY media with 10% HP was filtered in 0.2 μm filters (Pall Life Sciences, Ann Arbor, MI, USA) into sterile bottles. Viable stocks of *S. pyogenes* were maintained and stored in THY with 20% (v/v) glycerol at -80°C.

2.2. Genetic isolation

2.2.1. Plasmid isolation from *Escherichia coli*

Plasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN Sciences, MD, USA) as per manufacturer instructions. Plasmid DNA was eluted in distilled water and was stored at -20°C.

2.2.2. Isolation of genomic deoxyribonucleic acid from S*treptococcus pyogenes*

Genomic DNA was isolated by pelleting 1-2 ml of an overnight culture of *S. pyogenes*. The bacterial pellet was washed in 1 ml of 0.2 M sodium acetate (Sigma Chemical Company, St. Louis, MO, USA) and resuspended in 350 μl of *S. pyogenes* lysis buffer [50 mM ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Company, St. Louis, MO, USA), 0.2% sodium dodecyl sulfate (SDS; Sigma Chemical Company, St. Louis, MO, USA)] and incubated at 75°C for 30-40 minutes. Lysed streptococci were treated 100 μl with 5 M potassium acetate (EMD Chemicals Inc., Darmstadt, Germany) and placed in the -20°C for 1 hour. The frozen lysate was spun at 21,130 × *g* for 10 minutes. Supernatant was drawn off and added to 1 ml of -20°C 100% ethanol (Commercial Alcohols, Brampton, Canada) and placed in the -20°C freezer for 30-40 minutes. DNA was spun at 21,130 × *g* for 10 minutes. Ethanol is poured off and 1 ml of -20°C 70% ethanol was added and spun at 21,130 × *g* for 1 minute. The ethanol was poured off and the pellet was dried. To isolate total genetic material (DNA and RNA) the pellet was resuspended in distilled water. To isolate DNA alone the pellet was resuspended in tris-EDTA buffer (TE) 10 mM Tris, 1 mM EDTA with 10 μg/ml ribonuclease A (RNaseA) (Sigma-Aldrich Company, St. Louis, MO, USA), pH 8.0).

2.3. Deoxyribonucleic acid visualization

2.3.1. Agarose gel electrophoresis

DNA was visualized on ethidium bromide-stained tris-acetate EDTA buffer (TAE) (40 mM Tris-acetate, 1 mM EDTA) agarose gels. DNA loading dye (5% glycerol, 0.04% (w/v) bromophenol blue (International Biotechnologies, New Haven, CT, USA), 0.04% xylene cyanol) was mixed with the samples prior to loading the gel. A 1 kilobase (Kb) standard DNA molecular weight ladder (Invitrogen, Burlington, ON, Canada) was included in the gels as a size standard. Agarose (Invitrogen, Burlington, ON, Canada) gels were prepared at concentrations from 0.8% to 2% depending on the desired resolution of a given product. Gels were stained in 0.05% (w/v) ethidium bromide (EMD, Darmstadt, Germany) in 1 × TAE for 10-20 minutes before being rinsed in water and visualized and photographed under ultraviolet light.

2.3.2. Deoxyribonucleic acid agarose gel extraction

DNA fragments were purified from agarose gels by excising the DNA band of the appropriate size from the gel with a fresh scalpel blade. The gel fragment was placed in a microfuge tube and frozen at -20°C for at least an hour or -80°C for at least 20 minutes. The gel fragment was centrifuged at 21,130 × *g* for 10 minutes. The supernatant was drawn off and retained and the agarose pellet was spun a second time. The pooled supernatant was purified by a QIAquick PCR purification kit (QIAGEN Sciences, MD, USA) as specified by manufacturers instructions for DNA purification. Samples were eluted in 20-50 μl of distilled water.

2.4. Polymerase chain reaction

2.4.1. Standard polymerase chain reaction methods

Polymerase chain reaction (PCR) products were amplified in a Peltier Thermocycler PTC-200 (MJ Research, Waterdown, MA, USA). All oligonucleotide primers were obtained from Sigma-Genosys and are described in Table 4. Reactions were prepared in 100 μl reaction volumes or master mixes containing 1 × high fidelity (HF) buffer (New England Biolabs, Ipswich, MA, USA), 100 mM deoxyribonucleotide triphosphate (dNTP) mixture (Roche Diagnostics, Mannheim, Germany), 2 mM $MgSO_4$ (Sigma Chemical Company, St. Louis, MO, USA), 200 pM of each of the forward or reverse primers, 1 μl of template DNA and 1 Unit of Phusion polymerase (New England

Table 4. Primers used in this study

Primer	**Sequence (5'-3')[a]**	**Purpose**
Sequencing and screening primers		
M13 For	GTAAAACGACGGCCAGTGAG	Sequencing and screening
M13 Rev	CAGGAAACAGCTATGACCATG	Sequencing and screening
Int/Screen *speA* 5005 For	GCAAGCTACGGTTACGAAATTG	Integration, screening and sequencing
Int/Screen *speA* 5005 Rev	CAGGTTTGGGATAAGGACACAC	Integration, screening and sequencing
speA Screen II 8232 Rev	CCATCCTTTTTTGATTCTCC	Integration, screening and sequencing
speC Int II For	GCAAAGCACTGGTTCGATGT	Integration, screening and sequencing
speC Int II Rev	GGATAACCTTAACCGCGCTAC	Integration, screening and sequencing
speG 5005 Int/Screen II For	GAAGCCGGCGTCAAACTG	Integration, screening and sequencing
speG 5005 Int/Screen II Rev	CAGCTTCTGTTACTTGGCCTTG	Integration, screening and sequencing
speG int II For	GCCCTCGTCAGAATGACTGT	Integration, screening and sequencing
speG Screen II Rev	CCGATACCGATCACCACAAG	Integration, screening and sequencing
Int/Screen *speJ* 5005 For	GACGCTAGCCGGTACTGTC	Integration, screening and sequencing
Int/Screen *speJ* 5005 Rev	CCTTATGGTACCCAACATAC	Integration, screening and sequencing
speL/M Int II For	GGCTTGAGTTATGTGTCTTTA	Integration, screening and sequencing
speL/M Screen II Rev	GTTTGGTGGTATAGATGTAGCAAGG	Integration, screening and sequencing
smeZ Int II For	CATGCCTGCTCAAACAAGATT	Integration, screening and sequencing
smeZ Screen II Rev	ATACGACTCCATCTCATTATAGC	Integration, screening and sequencing
Plasmid construction		

I-*Sce*I OH 1	CTAGTTAGGGATAACAGGGTAATT	Construction of pG+host5::I-*Sce*I
I-*Sce*I OH 2	CTAGAATTACCCTGTTATCCCTAA	Construction of pG+host5::I-*Sce*I
*Kpn*I-*tsf* For	GGGGGGGGTACCACTTGCTCAATTG AACCACG	Constructing pG+host5::Int complementation plasmid
*Eco*RI-MCS-*tsf* Rev	TTTTTTGAATTCGATATCAAGCTTAT CGATACCGTCGACCTCGAGGGGGGG CCCTCCGTTTGACACAACAAAAAGA	Constructing pG+host5::Int complementation plasmid
*Eco*RI-MCS-*pepO* For	GGGGGGGAATTCCTGCAGCCCGGG GGATCCACTAGTTCTAGACACCAAT AAGGAAGCAAAAA	Constructing pG+host5::Int complementation plasmid
*Not*I-*pepO* Rev	GGGGGGGCGGCCGCAGCCTAAATG ATTGGTGGA	Constructing pG+host5::Int complementation plasmid
*Bam*HI-upstream *speA*5005 For	CCCGGATCCTTATCTAGCACACAGG CTGATGTG	Superantigen deletion construct
*Kpn*I-downstream *speA* 5005Rev	CCCGGTACCGGACGTGGGTTCGACT CCC	Superantigen deletion construct
*Bam*HI-upstream *speA* MGAS8232 For	CCCGGATCCGCCAAGTTATGCCATT ACTGTGTTG	Superantigen deletion construct
*Pst*I-upstream *speA* MGAS8232 Rev	CCCCTGCAGGAAGTCTACCTAACAA CCAAGTAA	Superantigen deletion construct
*Pst*I-downstream *speA* MGAS8232 For	CCCCTGCAGCAATACTTTTTTATTGT TTTCCATTAATAT	Superantigen deletion construct
*Kpn*I-downstream *speA* MGAS8232 Rev	CCCGGTACCGCAAATGACAAATCGC TATATCAATAA	Superantigen deletion construct
*Spe*I-*speA* Comp For	GGGGGGACTAGTATTTTATAATAAA ATTATTAATATAAGTTAA	Superantigen complementation construct
*Sal*I-*speA* Comp Rev	GGGGGGGTCGACAAAACCGCTCATC AAATGA	Superantigen complementation construct
*Pst*I-downstream *speC* MGAS8232 For	CCCCTGCAGTTTGATGATGTTAATCT TTTTCAT	Superantigen deletion construct
*Kpn*I-downstream *speC* MGAS8232Rev	CCCGGTACCCGGATATCAATTTTGT GGATTAAACG	Superantigen deletion construct

*Bam*HI-upstream *speC* MGAS8232 For	CCCGGATCCCGGTTACACCATGCGCACTGTCG	Superantigen deletion construct
*Pst*I-upstream *speC* MGAS8232 Rev	CCCCTGCAGTTCGATATTTATCTTGAAAAATAATTCATC	Superantigen deletion construct
*Bam*HI-upstream *speG* MGAS5005 For	CCCGGATCCGAATCGGTATTAGAAGGTTTGCTAGTG	Superantigen deletion construct
*Pst*I-upstream *speG* MGAS5005 Rev	CCCCTGCAGAATTGTCAAAATGTTTGTTTTCATTTAAAA	Superantigen deletion construct
*Kpn*I-downstream *speG* MGAS5005 Rev	CCCGGTACCCATGTTGTCCCGCAAATGAC	Superantigen deletion construct
*Bam*HI-upstream *speG* MGAS8232 For	CCCGGATCCGCCCAGCTTCAAGACGTTGGTTAA	Superantigen deletion construct
*Pst*I-upstream *speG* MGAS8232 Rev	CCCCTGCAGAAATTCTAAAATGAACTTAGCCAC	Superantigen deletion construct
*Pst*I-downstream *speG* For	CCCCTGCAGGATATCTACTTAAAAACGCACTAG	Superantigen deletion construct
*Kpn*I-downstream *speG* MGAS8232 Rev	CCCGGTACCGGAAGATCAAGCCAACCCAAGAAA	Superantigen deletion construct
*Bam*HI - downstream *speJ* MGAS5005 For	CCCGGATCCGATGGAAGAGAGACTCTTAGAGAAGC	Superantigen deletion construct
*Pst*I-downstream *speJ* MGAS5005 Rev	CCCCTGCAGTACCTTTGGACTAAATAAGATAGGAGC	Superantigen deletion construct
*Pst*I-upstream *speJ* MGAS5005-For	CCCCTGCAGTTTTATTATTCTTTTCATACCTCTCCTCG	Superantigen deletion construct
*Kpn*I-upstream *speJ* MGAS5005-Rev	CCCGGTACCGAGTCACTTTGACATTCAGAGATGAAG	Superantigen deletion construct
*Bam*HI-upstream *speL/M* MGAS8232 For	CCCGGATCCCCTATGGACGTAGACAAATATGTTG	Superantigen deletion construct
*Pst*I-upstream	CCCCTGCAGTTATTCACAAACAAAG	Superantigen deletion construct

speL/M MGAS8232 Rev	AAAATTAATTAGTA	
*Pst*I-downstream *speL/M* MGAS8232 For	CCCCTGCAGCAAAGTCAAGGTATTTTTTTTCAT	Superantigen deletion construct
*Kpn*I-downstream *speL/M* MGAS8232 Rev	CGCGGTACCCGGTGTATTGACATTGATGTATTC	Superantigen deletion construct
*Bam*HI-upstream *smeZ* MGAS8232 For	GGGGGATCCGGGGAATTATGCCAATTGTCTCTA	Superantigen deletion construct
*Pst*I-upstream *smeZ* MGAS8232 Rev	CCCCTGCAGAAAAATAAGTTTTGTTTTTTTCATAAATAG	Superantigen deletion construct
*Pst*I-downstream *smeZ* MGAS8232 For	CCCCTGCAGTTAGATATAGAAATTGACTCCTAATTC	Superantigen deletion construct
*Kpn*I-downstream *smeZ* MGAS8232 Rev	GGGGGTACCGGGCAATTGTTTAACTGGTTAATTAG	Superantigen deletion construct
Superantigen internal screening primers		
speA RT For	AAAGTTGCCATCTCTTGGTTC	Internal screening primer
speA RT Rev	CAAGAGGTATTTGCTCAACAAGAC	Internal screening primer
speC RT For	TTTGAGCAGGCGTAATTCCT	Internal screening primer
speC RT Rev	TTCAACGACACACACATTAAACA	Internal screening primer
speG RT For	ACCCCATGCGATTATGAAAA	Internal screening primer
speG RT Rev	GGGAGACCAAAAACATCGAC	Internal screening primer
speJ RT For	GCTCTCGACCTCAGAATCAA	Internal screening primer
speJ RT Rev	CTTTCATGGGTACGGAAGTG	Internal screening primer
speL RT For	ATAAGTCAGCACCTTCCTCTTTC	Internal screening primer
speL RT Rev	AAATCTCCCGTTACCTTCCA	Internal screening primer
speM RT For	AACTTCTTCTTCCTTAAAGCGTCT	Internal screening primer
speM RT Rev	TGCTGTGTTGGTTAATAGCGA	Internal screening primer
smeZ RT For	TTTCTCGTCCTGTGATTGGA	Internal screening primer
smeZ RT Rev	AATGGGACGGAGAACATAGC	Internal screening primer
DR4/DQ8 genotyping primers		
DQA (1027) For	GAAGACATTGTGGCTGACCATGTTG	Mouse genotype

	CC	
DQA (1029) Rev	AGCACAGCGATGTTTGTCAGTGCAA ATTGCGG	Mouse genotype
DQ8b (vA) For1	AGGATTTGGTGTACCAGTTTAAGGG CAT	Mouse genotype
DQ8b (vT) For 2	AGGATTTGGTGTTCCAGTTTAAGGG CAT	Mouse genotype
DQ8b (vii) Rev	TGCAAGGTCGTGCGGAGCTCCAA	Mouse genotype
DR4A (1101) For	GGAGATAGTGGAACTTGCGG	Mouse genotype
DR4A (1104) Rev	CCGATCACCAATGTACCTCC	Mouse genotype
DR4B (1098) For	GTTTCTTGGAGCAGGTTAAACA	Mouse genotype
DR4B (1099) Rev	CTGCACTGTGAAGCTCTCAC	Mouse genotype

[a] Underline in primer sequences indicates restriction endonuclease sites used for cloning purposes.

Biolabs, Ipswich, MA, USA). General cycle conditions were as follows: 98°C for 5 minutes; 98°C for 30 seconds; primer-specific annealing temperature which was in general Tm + 4°C for 30 seconds; 72°C for a time appropriate for the gene being amplified at 15 seconds per 1 Kb; steps 2 to 4 were repeated 35 times; 72°C for 5 minutes; 4°C indefinitely. PCR products were purified using QIAquick PCR purification kit (QIAGEN Sciences, MD, USA) as per manufacturer's instructions and were eluted in 20-50 μl of distilled water or 10 mM Tris-Cl, pH 8.5.

2.4.2. *Escherichia coli* colony screening by polymerase chain reaction

To screen for recombinant plasmids in *E. coli*, colony-screening PCR was performed on ligation-transformed *E. coli* colonies. A standard master mix for PCR was prepared with the exception of added template and aliquoted into 10-20 μl across the number of reaction tubes. Single colonies were picked from the plates and dipped into the aliquoted master mix before being placed in a snap cap tube with appropriate media and antibiotics. Resulting PCR products were analyzed by agarose gel electrophoresis.

2.4.3. Deoxyribonucleic acid sequencing

All DNA sequencing was performed at the John P. Robarts Research Institute Sequencing Facility at Western University in London, Ontario, Canada. Sequencing was performed with appropriate primers (Table 4).

2.5. Molecular cloning

2.5.1. Restriction digestions

Restriction digestions were performed in a 90 μl reaction volume containing 30-70 U of each restriction enzyme in 1 × the appropriate reaction buffer supplemented with 1 μl of bovine serum albumin as indicated, and incubated following the manufacturer's recommended temperature. Enzymes were purchased from Invitrogen (Burlington, ON, Canada), New England Biolabs (Ipswich, MA, USA) or Roche Diagnostics (Mannheim, Germany). Once the DNA was cut to completion it was purified using the QIAquick PCR purification kit (QIAGEN Sciences, MD, USA). Antarctic phosphatase (New

England Biolabs, Ipswich, MA, USA) was used in stock plasmid digestions to reduce the incidence of non-specific empty vector ligation.

2.5.2. Deoxyribonucleic acid ligation

All ligations were performed in 25 µl reaction volumes containing 40 U of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and an excess of insert to vector. Reactions were incubated at either room temperature for 1 hour or overnight at 17°C before transformation.

2.5.3. Deoxyribonucleic acid ethanol precipitation

Plasmid DNA, previously isolated from a QIAprep spin miniprep kit (QIAGEN Sciences, MD, USA), was mixed with 2-3 volumes of -20°C 99% ethanol supplemented with 0.1 volumes of 3 M Sodium Acetate (Sigma, St. Louis, MO, USA) and placed at -20°C for 1 hour or more. The precipitated DNA was spun at 21,130 × *g* for 10 minutes before the supernatant was poured off. The pellet was washed with 1 ml of -20°C 70% ethanol without mixing, and spun for 1 minute at 21,130 × *g* and the supernatant was poured off. The pellet was dried and resuspended in HPLC water (Fisher Scientific, Fair Lawn, NJ, USA).

2.5.4. Preparation of rubidium chloride competent *Escherichia coli*

Escherichia coli XL1-Blue and *E. coli* BL21 (DE3) competent cells were prepared by growing 100 ml culture in PSI broth (5 g/L Bacto yeast extract, 20 g/L Bacto tryptone, 5 g/L magnesium sulphate, pH 7.6 with KOH) to an OD_{600} of 0.4. Cells were placed on ice for 15 minutes and were then centrifuged at 3,000 × *g* at 4°C for 5 minutes. The cell pellet was resuspended in 40 ml of TfbI buffer [30 mM KAc (EMD Chemicals Inc., Darmstadt, Germany), 100 mM $RbCl_2$ (Alfa Aesar, Ward Hill, MA, USA), 10 mM $CaCl_2$ (Sigma Chemical Co., St Louis, MO, USA), 50 mM $MnCl_2$ (Sigma-Aldrich Co., St. Louis, MO. USA), 15% glycerol, pH 5.8] and put on ice for 15 minutes. Cells were again centrifuged at 3,000 × *g* at 4°C resuspended in 4 ml of TfbII buffer [10 mM MOPS (Sigma Chemical Co., St. Louis, MO, USA), 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% glycerol,

pH 6.5]. Cells were aliquoted on ice into 100 µl volumes and flash frozen in an ethanol bath and stored at -80°C.

2.5.5. *Escherichia coli* transformations

Escherichia coli XL1-Blue and *E. coli* BL21 rubidium chloride competent cells were thawed on ice for approximately 10 minutes. Plasmid DNA or ligation mixtures were added to the thawed *E. coli* cells and were left on ice for up to 30 minutes. The cells were heat shocked at 42°C for 45 seconds and then placed on ice for 2 minutes and 900 µl of LB broth was subsequently added. The cells were then incubated at 37°C for 1 hour with shaking. The transformation was plated on the appropriate media plates with antibiotic and grown overnight at 37°C.

2.5.6. Preparation of electrocompetent *Streptococcus pyogenes*

Electrocompetent *S. pyogenes* was prepared by subculturing 1 ml an overnight culture into 100 ml of pre-warmed THY + 0.6% glycine (Fisher Scientific, Fair Lawn, NJ, USA) which was then grown at 37°C without shaking. After 1.5 hours, 8 mg of hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) was added and the culture was returned to the 37°C incubator until the OD_{600} reached 0.21. The culture was pelleted at 6,000 × *g* for 10 minutes. The cells were washed twice in 10-20 ml of ice cold 15% sterile glycerol. Cells were resuspended after a final wash in 4 ml ice cold 15% glycerol and aliquoted in 220 µl aliquots. Aliquots were flash-frozen in a dry-ice ethanol bath and stored at -80°C.

2.5.7. Electroporation of *Streptococcus pyogenes*

Plasmids to be electroporated into *S. pyogenes* were ethanol precipitated and resuspended in HPLC water (Fisher Scientific, Fair Lawn, NJ, USA). DNA (1-2 µg) was added to ice-thawed electrocompetent *S. pyogenes*. Cells were electroporated at 2500 volts (V), 600 ohms (Ω), and 25 microfarads (µF) in 2 mm electroporation cuvettes (VWR, Westchester, PA, USA) in a Biorad Gene Pulser XCell electroporator. After electroporation, cells were immediately transferred to 10 ml THY broth at room

temperature and placed in a 30°C incubator to recover for 1 hour before a sub-inhibitory concentration of erythromycin (0.01 μg/ml) was added. Cells were returned to 30°C for an additional four hours before being pelleted and resuspended in 1 ml THY broth plated on THY plates with the appropriate antibiotics for the plasmids. Cultures were grown for up to 3 days at 30°C.

2.5.8. Streptococcal genome mutagenesis

In-frame deletions were made of all of the streptococcal SAgs in *S. pyogenes* MGAS5005 (Figure 3) and *S. pyogenes* MGAS8232 (Figure 4). The construction of the streptococcal SAg mutants was made in a manner similar to the procedure reviewed in Cho *et al*. [163]. Briefly, *S. pyogenes* were electroporated with a pG+host5-based plasmid (Table 3) [162] including the mutation flanked by the contextual upstream and downstream homologous regions. The bacterial transformants that grew on THY + 1 μg/ml erythromycin (THY Erm_1) at 30°C were picked and grown in THY Erm_1 broth at 30°C. A total nucleic acid preparation was made of this culture and the presence of the plasmid was confirmed by PCR. Transformed bacteria were plated on THY Erm_1 plates and incubated at 40°C. Resulting colonies were picked and grown in THY Erm_1 broth at 40°C. DNA was isolated and the integration of the construct was confirmed by PCR. Bacteria with confirmed integrations were subcultured into THY broth without antibiotics and grown at 30°C with daily subculturing at 1%. Cultures were screened for the loss of the pG+host5-based plasmid by replicate plating onto THY, THY Erm_1, and TSAII plates that are supplemented with 5% defibrinated sheep blood [Becton, Dickinson, and Company, Franklin Lakes, NJ USA; 1.45% pancreatic digest of casein (w/v), 0.5% papaic digest of soybean meal (w/v), 0.5% NaCl (w/v), 1.4% agar (w/v), 0.15% (w/v) growth factors, 5% (v/v) sheep blood, defibrinated]. Beta-haemolytic cultures that were sensitive to erythromycin were grown in THY broth culture and were screened by total nucleic acid isolation and PCR for excision or insertion of the targeted gene of interest. PCR primers used for *S. pyogenes* mutagenesis are listed in Table 4 and the mutagenesis strategy is depicted in Figure 5. All strains of *S. pyogenes* were sub-cultured daily at 1% in filter-sterilized THY + 10% HP for 5 days before glycerol stocks were made and stored at

Figure 3. *Streptococcus pyogenes* MGAS5005 superantigens in genomic context. (A) The genome of *S. pyogenes* MGAS5005 contains three bacteriophage like-elements (triangles), and four SAgs within its 1.839 Mb genome. (B) *S. pyogenes* MGAS5005-encoded SAgs in their immediate genetic context. The *speA* gene is flanked upstream by a hypothetical phage protein spy_0995 and downstream by phage proteins spy_0997 and spy_0998 that are oriented in the opposite direction of the *speA2* gene. The *speG* gene is flanked upstream by the spy_0180 gene, which is in reverse orientation and is an S-layer-like domain containing protein. Directly upstream of *speG* in the same orientation is spy_0181 which is a hypothetical cytoplasmic protein. Downstream of *speG* are three genes that are in the same orientation as the *speG* gene, spy_0183, which is a hypothetical protein, spy_0184, which is a predicted cytoplasmic proteins and glucose-6-phosphate isomerase. *speJ* is flanked by two hypothetical proteins. The upstream protein is in the same orientation as *speJ* and the downstream protein is in reverse orientation. The *smeZ* gene is flanked upstream by two genes in the same orientation. spy_1700 encodes an acetyltransferase and spy_1701 encodes a topology modifying protein. Immediately downstream of *smeZ* there is a gene in reverse orientation that is a hypothetical cytoplasmic protein and beyond that there is a gene in the same orientation that is a dipeptide binding protein.

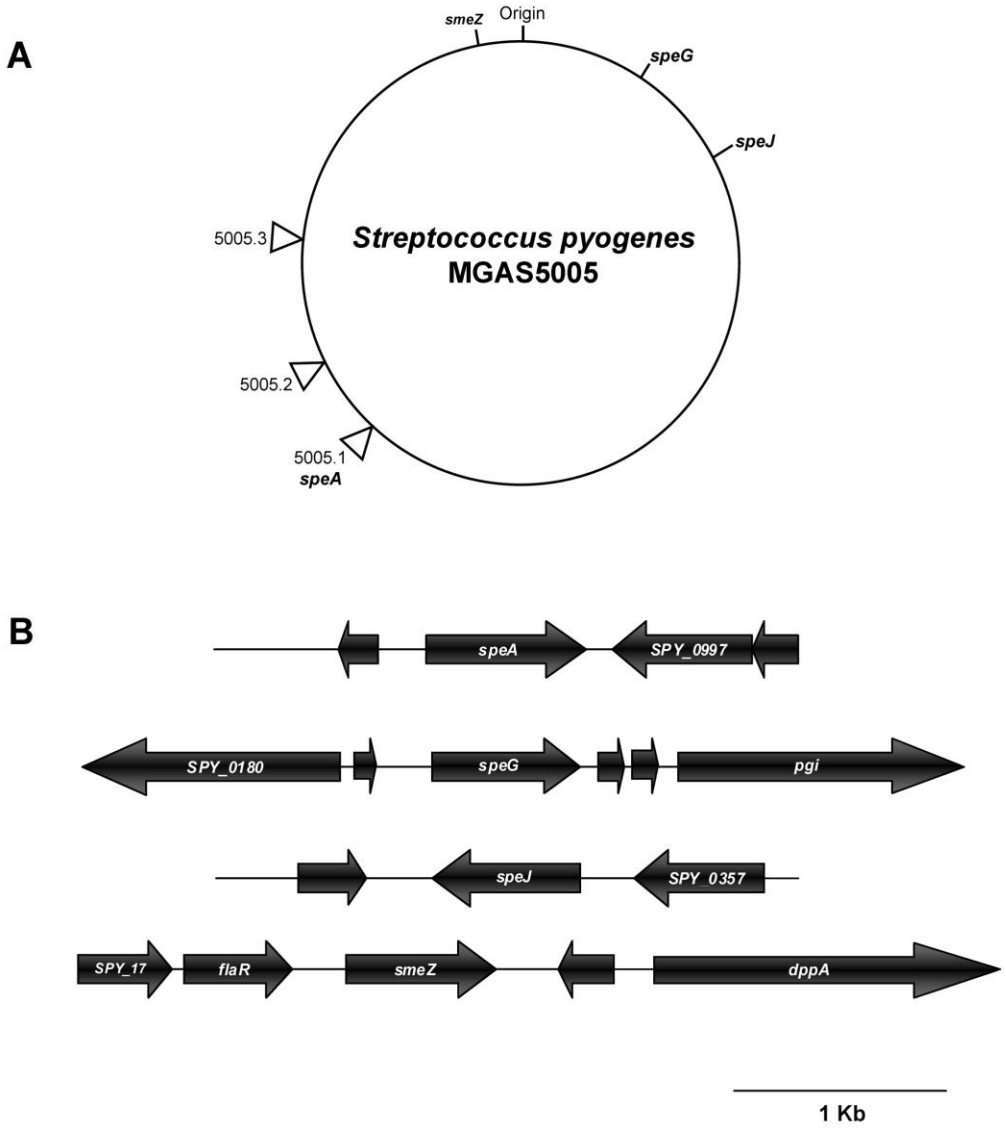

A
smeZ
Origin
speG
speJ
5005.3
Streptococcus pyogenes
MGAS5005
5005.2
5005.1
speA
B
speA
SPY_0997
SPY_0180
speG
pgi
speJ
SPY_0357
SPY_17
flaR
smeZ
dppA
1 Kb

Figure 4. *Streptococcus pyogenes* MGAS8232 superantigens in genomic context. (A) The 1.895 MB genome of *S. pyogenes* MGAS8232 encodes five bacteriophage-like elements (triangles). Four of the six SAgs encoded in the chromosome of *S. pyogenes* MGAS8232 are bacteriophage-encoded including *speA1* encoded on 8232.1, *speC* on 8232.2, and *speL* and *speM* are encoded on 8232.3. (B) Within the immediate genetic context *speA* is flanked upstream by two genes that are in the opposite orientation to *speA* and encode hypothetical proteins, and downstream by spyM18_0391 which encodes N-acetylmuramoyl-L-alanine amidase which is also in reverse orientation. The *speC* gene is flanked upstream in the same orientation by *mf2* and downstream by a hypothetical protein in reverse orientation. The *speG* gene is flanked upstream by spyM18_0199 which is a transposase that is in the reverse orientation. Downstream of *speG* are two genes in the same orientation, a hypothetical protein and glucose-6-phosphate isomerase. The *speL* and *speM* genes are encoded beside one another in the same orientation. The genes directly flanking *speL* and *speM* are both hypothetical proteins oriented in the same orientation. The *smeZ* gene is flanked by hypothetical proteins in the opposite direction. Further upstream of *smeZ* is a topology modulation protein, and further downstream is *dppA* which is a surface lipoprotein.

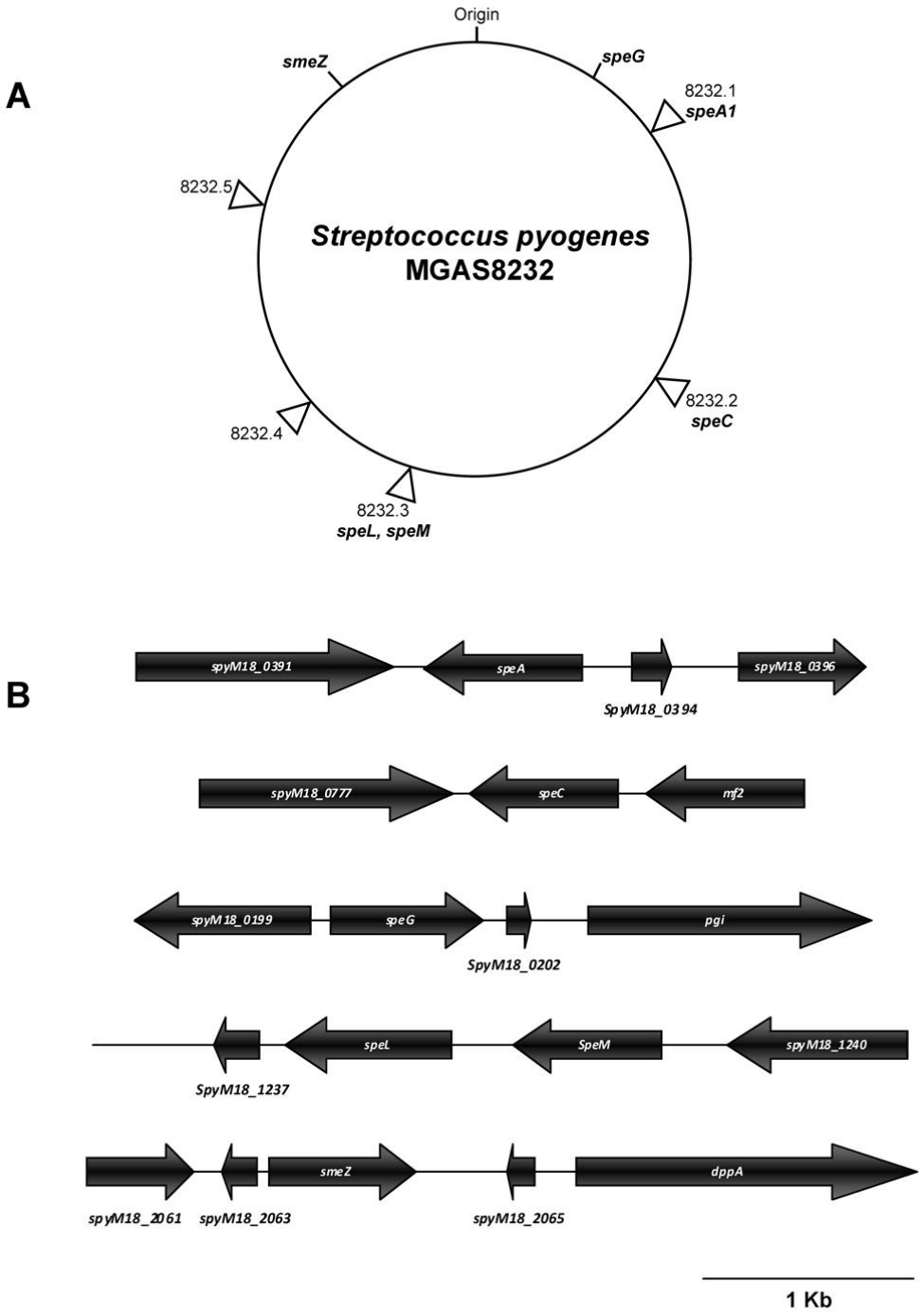
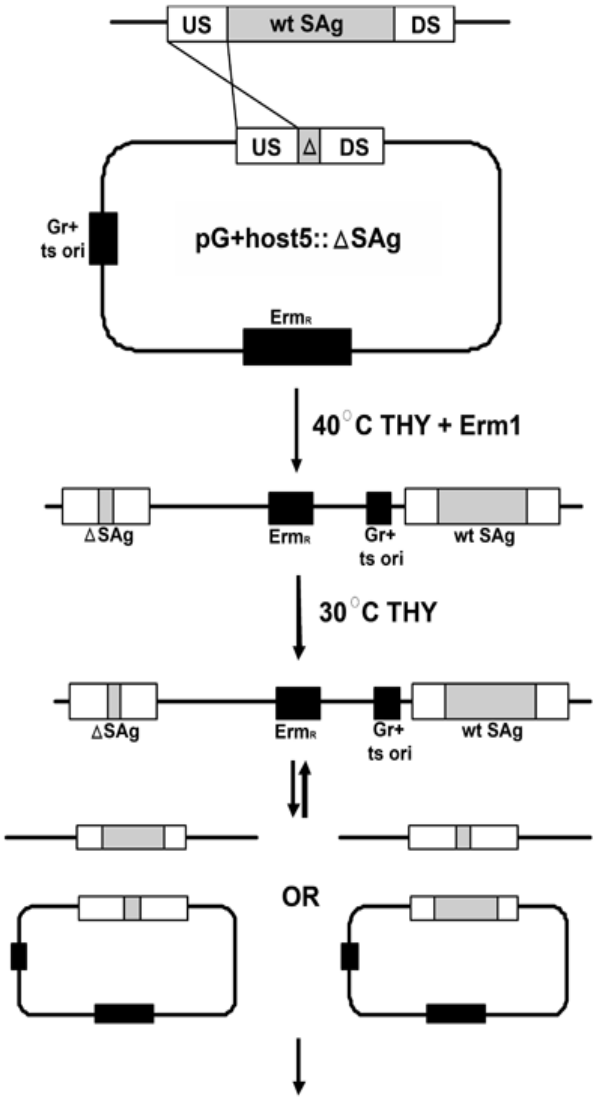

A
Origin
smeZ
speG
8232.1
speA1
8232.5
Streptococcus pyogenes
MGAS8232
8232.2
speC
8232.4
8232.3
speL, speM
B
spyM18_0391
speA
spyM18_0396
SpyM18_0394
spyM18_0777
speC
mf2
spyM18_0199
speG
pgi
SpyM18_0202
speL
SpeM
spyM18_1240
SpyM18_1237
smeZ
dppA
spyM18_2061
spyM18_2063
spyM18_2065
1 Kb

Figure 5. Markerless mutagenesis in *Streptococcus pyogenes*. To create precise markerless deletions of each superantigen, all constructs were generated in pG+host5::I-*Sce*I (Table 3) by cloning PCR products containing ~500 bp homologous regions both upstream and downstream of the superantigen gene, but leaving the first and last three codons of the superantigen intact. *S. pyogenes* containing the integration plasmids were grown at 40°C with erythromycin to select for a single cross-over integration of the plasmid within the streptococcal genome. The integration was confirmed by PCR and bacterial growth was shifted to the permissive temperature of 30°C without antibiotics to allow for the second cross-over event and eventual clearance of the plasmid. Erythromycin-sensitive *S. pyogenes* were screened for the superantigen deletion and clearance of the plasmid with specific primers by PCR.


US
wt SAg
DS
US
Δ
DS
Gr+
ts ori
pG+host5::ΔSAg
Erm$_R$
40°C THY + Erm1
ΔSAg
Erm$_R$
Gr+
ts ori
wt SAg
30°C THY
ΔSAg
Erm$_R$
Gr+
ts ori
wt SAg
OR
Clones that have lost the plasmid were erythromycin-sensitive
and the presence of superantigens was assessed by PCR

minus 80°C. All stocks at -80°C were tested for growth defects by doing a computer-automated optical density of 600 nm (OD_{600}) growth curve using a Bioscreen C MBR (Piscataway, NJ, USA).

2.5.9. Complementation of *speA* into the superantigen deletion strain of *Streptococcus pyogenes* MGAS8232

Streptococcus pyogenes MGAS8232 complete superantigen deletion strain was complemented with the *speA* gene with its native promoter as determined by promoter mapping by PPP software (http://bioinformatics.biol.rug.nl/websoftware/ppp) [164]. The complementation cassette was created in pG+host5 using homologous recombination sites for insertion between *pepO* and *tsf*. The process by which speA was introduced was similar to the superantigen deletion protocols outlined in Figure 5 in that it used the Gram-positive temperature-sensitive origin of replication to ensure integration of the plasmid into the genome and relied on antibiotic sensitivity to indicate the loss of the plasmid and PCR screening to confirm each step.

2.6. Protein visualization

2.6.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

All proteins were visualized by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separating gels were made with 12% polyacrylamide [0.1% (w/v) SDS (Fisher Scientific, Fair Lawn, NJ, USA), 0.25% (v/v), 1.5 M Tris-HCl (EMD Chemicals Inc., Darmstadt, Germany), pH 8.8, 12% (v/v) acrylamide (EMD Chemicals Inc., Darmstadt, Germany), 0.1% (w/v) ammonium persulfate (APS) (Sigma Chemical Co., St. Louis, MO, USA) and 0.15% (v/v) tetramethylenediamine (TEMED) (EMD Chemicals Inc., Darmstadt, Germany)] with 5% acrylamide stacking gels [1.3% (w/v) SDS, 25% (v/v), 0.5 M Tris-HCl, pH 6.8, 5% (v/v) acrylamide, 0.1% (w/v) APS and 0.2% (v/v) TEMED], using a BioRad Mini-PROTEAN 3 system. Laemmli buffer [125 mM Tris pH 6.8, 50% glycerol, 4% SDS, 5% β-mercaptoethanol [Omni Pur, Calbiochem, EMD Chemicals Inc., Gibbstown, NJ, USA], and 0.1% (w/v) bromophenol blue] was added to each protein sample before being boiled for five minutes. Broad-Range molecular weight marker (New England BioLabs, Ipswich, MA, USA) was used.

Gels were run in tris-glycine electrophoresis buffer (25 mM Tris, 190 mM glycine, 1% SDS) for 30 minutes at 85 V and then at 150 V for an additional 90 minutes. Gels were stained in coomassie [45% methanol, 10% glacial acetic acid (Caledon, Georgetown, ON, Canada), 0.1% (w/v) coomassie brilliant blue stain R-250) for at least 1 hour and then destained for visualization (45% methanol (Caledon, Georgetown, ON, Canada), 10% acetic acid (Caledon, Georgetown, ON, Canada)].

2.6.2. Western blots

Proteins for Western blots were run on 12% SDS-PAGE gels and were then transferred to a polyvinylidene difluoride (PVDF) Amersham HybondP membranes (GE Healthcare, Little Chalfont, Buckingshire, UK) using a BioRad Trans-Blot system. A piece of PVDF membrane was immersed in 100% methanol for 10 minutes and then equilibriated in transfer buffer (182 mM glycine, 34 mM Tris, 20% methanol) for 10 minutes. SDS-PAGE gels were also equilibrated in transfer buffer. The PVDF membrane was placed on top of the protein gel and sandwiched between 6 pieces of filter paper and was placed in the transfer apparatus. Proteins were transferred for one hour at 100 V at 4°C or overnight at 25 V at 4°C. Membranes were subsequently blocked with 5% (w/v) skim milk (Equality) diluted in 1 × phosphate buffered saline (PBS) [125 mM NaCl (BDH, Westchester, PA, USA), 1.5 mM KH_2PO_4 (Sigma Aldrich Co., St. Louis, MO, USA), 8 mM Na_2HPO_4 (EMD Chemicals Inc., Darmstadt, Germany), 2.5 mM KCl (Sigma Aldrich Co., St. Louis, MO, USA), pH 7.6] for 1 hour at room temperature. The blocking buffer was replaced with the appropriate dilution of primary antibody diluted in 1% (w/v) skim milk in PBS incubated at room temperature for 1 hour. The membranes were washed three times for 5 minutes in PBS with 0.01% (v/v) tween20 (PBST) and then once for 15 minutes in PBS. The secondary antibody was incubated with the membrane in 1% (w/v) skim milk in PBS for an hour. The membrane was washed as before. All Western blots were visualized on LI-COR Odyssey (LI-COR Biosciences, Lincoln, NB, USA).

2.7. Protein expression and purification

2.7.1. Histidine-tagged superantigen purification

Histidine-tagged SAgs were expressed from pET-based expression plasmids in *E. coli* BL21 (DE3). Expression strains of *E. coli* were grown in LB supplemented with Kanamycin at 50 μg/ml (Kan_{50}). Overnight cultures of bacteria were subcultured (0.1%) into 1 L of LB Kan_{50} and grown at 37°C with shaking for 3 hours before being induced with 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was returned to 37°C incubator with shaking overnight. Cultures were pelleted at 3,500 × *g* (Avanti J-25 centrifuge, Beckman Coulter) for 10 minutes. The pellet was resuspended in 10 ml of 1 × hepes buffer [10 mM Hepes (BDH, VWR, West Chester, PA, USA), 150 mM NaCl (BDH, VWR, West Chester, PA, USA), pH 7.4] with 10 μl DNaseI and 50 μl of 10 mg/ml lysozyme in a shaking ice bath for 1 hour. Bacteria were sonicated in 3 rounds of 50 pulses of 40% duty cycle, output 4 on Branson Sonifier (Danbury, CT, USA) and allowed to rest on ice between each round of sonication. Cellular debris was pelleted at 10,000 × *g* at 4°C for 10 minutes and the supernatant was passed over a Ni NTA His Bind Resin nickel affinity chromatography column (Novagen, San Diego, CA, USA) and histidine-tagged SAgs were eluted in either the 60 mM or 200 mM imidazole in 1 × hepes buffer elution fractions. The proteins were dialyzed 3 times against 1 L of 1 × hepes buffer for 1 hour. The SAgs were cleaved with either tobacco etch virus protease (McCormick lab) or thrombin for 24-72 hours at 4°C. Cleaved proteins were passed again over a nickel affinity chromatography column, and eluted from the column in the 15 mM or 30 mM imidazole fractions in 1 × hepes buffer. The proteins were dialyzed 3 times against 1 L of 1 × hepes buffer, 0.9% saline or distilled water for 1 hour. The purity of the SAgs was assessed by SDS PAGE and the concentration of the SAgs was assessed by BCA assay (Thermo Scientific, Rockland, IL, USA). Proteins were diluted to the correct concentration in either 1 × hepes buffer or 0.9% saline, aliquotted and stored in -20°C. For antibody production proteins dialyzed into water were lyophilized.

2.7.2. Trichloroacetic acid streptococcal supernatant protein precipitation

Streptococcal cultures were subcultured into 100 ml pre-warmed THY broth and grown to late exponential phase. The bacteria were pelleted and 90 ml of culture supernatant was removed and 50% trichloroacetic acid (TCA) was added to reach a final

concentration of 6%. The supernatant was chilled on ice for 30 minutes. Precipitated supernatants were pelleted at 10,000 × *g* for 10 minutes at 4°C. The supernatant was removed and the pellet was washed once in 10 ml of -20°C acetone and spun at 10,000 × *g* for 10 minutes. The supernatant was removed and the pellet was dried and resuspended in 900 µl of 8M urea for a final concentration of 10:1.

2.8. Antibody generation

2.8.1. Antibody production

Purified and lyophilized SpeA, SpeC, SpeG, SpeL, SpeM, and SmeZ were sent to ProSci Incorporated (Poway, California, U.S.A.). SAgs were resuspended in 0.9% saline and were injected at a concentration of 200 µg in complete Freund's adjuvant at week 0. Rabbits were boosted with 100 µg of SAg in incomplete Freund's adjuvant at weeks 2, 4 and 6. Serum samples were collected at week 0, 5 and 7 with a terminal bleed taken at week 8. Serum samples were assessed for antibody titres, aliquoted and were stored at minus 80°C.

2.8.2. Antibody titres

Antibody titres were determined by enzyme-linked immunosorbant assay (ELISA). SAgs were diluted to a concentration of 10 µg/ml in 50 mM carbonate buffer (Na_2CO_3 0.0015 M; $NaHCO_3$ 0.0349 M; NaN_3 0.00307 M; pH to 9.5) in Costar ELISA 96-well plates (Costar, Corning, NY, USA). Coated plates were incubated at room temperature wrapped in aluminum foil. Plates were washed twice with PBST wash buffer and then blocked with blocking buffer [1% BSA (Sigma Aldrich Co., St. Louis, MO, USA), 0.02% tween20 (Fisher Scientific, Fair Lawn, NJ, USA) in 1 x PBS] for 2 hours in the dark at room temperature. Dilutions of serum were prepared in dilution buffer (0.1% BSA, 0.02% tween20 in 1 × PBS). Plates were washed twice after blocking with PBST and flipped on Whatman paper to dry between washes. One hundred microliters of each diluted sample was added to the plates in triplicate. Samples were incubated for 2 hours at room temperature in the dark. Plates were washed three times with PBST, and three times with distilled water. Goat anti-rabbit antibody conjugated with horseradish peroxidise (HRP) (Rockland, Gilbertsville, PA, USA) at a dilution of 1:10,000 in 1 × PBS

with 1% BSA, 0.02% Tween-20 was incubated in the plates for 2 hours at room temperature in the dark. Plates were washed with PBST five times, followed by 5 washes with distilled water. One hundred microliters of BD EIA 3,3',5,5' tetramethyl benzidine (TMB) substrate reagent (Becton, Dickinson, Company, Sparks, MD, USA) developed the reaction for 10 minutes at room temperature in the dark before being stopped by adding 50 μl of 1 N H_2SO_4. Plates were immediately read at OD_{450} with a reference of OD_{570}

2.9. Mouse housing and breeding

2.9.1. Housing

Mice were housed in an inclusion/exclusion facility during experimentation in micro-isolator cages. Transgenic DR4, DQ8, and DR4/DQ8 breeding pairs were housed and cared for at the West Valley Barrier Facility at Western University, Canada in positive pressure microisolator system Micro-VENT System cages (model # MD75 Ju14OMVS PCD3, Allentown Inc., Allentown, New Jersey, USA).

2.9.2. Mice

A list of all mice used in this study can be found in Table 5. Human MHC class II transgenic mice were bred from McCormick Laboratory colonies specifically for this study and were in a C57BL/6 (B6) background. Transgenic H-2 null, MHC class II human leukocytic antigen-DR4 (DR4) mice originated from a colony housed at Western University, West Valley Barrier Facility and were a kind gift from Dr. Ewa Cairns. Expression of the transgenic HLA-DR4 immunoreceptor was confirmed by flow cytometry at regular intervals (data not shown). H-2 null, HLA-DR4/DQ8 transgenic mice were a kind gift from Dr. M. Kotb at the University of Cincinnati, Cincinnati, OH, U.S.A. HLA-DQA1*0301 and DQB1*0302 (DQ8) mice were originally developed by the C.S. David group to examine the contribution of HLA-DQ type to the development of human polyarthritis in a collagen-induced arthritis model [165]. The DQ8 alleles were introduced into a mouse that was mouse class II-deficient H-2Ab^0 by cosmid injection into mouse embryos [165]. Mice were also developed to express DRB1*0402 in addition to DQ8 are classified as DR4/DQ8 mice [166]. Mice were isolated in quarantine and

Table 5. Mouse strains used in this study

Strain Name	Characteristics	MHC class II	Source	Stock Number
C57BL/6	Inbred black mouse	H-2^b	The Jackson Laboratory	000664
BALB/cJ	Inbred white mouse	H-2^d	The Jackson Laboratory	000651
FVB/NJ	Inbred white mouse	H-2^q	The Jackson Laboratory	001800
A/J	Inbred white mouse	H-2^a	The Jackson Laboratory	000646
DR4	Inbred homozygous transgenic black mouse	H-2 null, Human HLA-DR4	Gift from Dr. E. Cairns [167]	McCormick Lab Breeding colony
DQ8	Bred from a heterozygous DR4/DQ8 mouse	H-2 null, Human HLA-DQ8	Bred from DR4/DQ8 heterozygous mice	McCormick Lab Breeding colony
DR4/DQ8	Expressed human MHC class II HLA-DR4 and HLA-DQ8	H-2 null, Human HLA-DR4 and HLA-DQ8	Gift from Dr. M. Kotb, [113, 166]	McCormick Lab Breeding colony

were assessed for enteric pathogens before being transferred to the West Valley Barrier Facility to be bred specifically for this study. All other mice were purchased from The Jackson Laboratories. Mice were used between nine and thirteen weeks old for nasal infection and both male and female mice were used in the study.

2.9.3. Genotyping

Transgenic HLA-DR4/DQ8 mice were genotyped to verify the presence of the transgenes. The breeding colony was confirmed to be homozygous for the HLA-DQ8 allele but heterozygous for the HLA-DR4 allele. As such, all mice used were genotyped. Unsuccessful attempts were made to establish a completely homozygous breeding colony. Mice (10-14 days old) were ear punched to identify individuals and the ear punches were used to isolate DNA from the tissue using the QIAGEN DNeasy blood and tissue kit (QIAGEN Sciences, MD, USA) using the manufacturer's directions. Resulting DNA preparations were used with primers specific to the alpha and beta chains of the HLA-DR4 and HLA-DQ8 alleles (Table 4).

2.10. Streptococcal colonization and persistence in a mouse model

2.10.1. Bacterial preparation

Streptococcus pyogenes cultures that had been grown in serial culture in un-autoclaved, 0.2 μm-filtered THY media supplemented with 10% human serum for five consecutive days before storage in -80°C were started in 10 ml THY media in a 15 ml falcon tube. Cultures were subcultured at 1:100 in THY broth daily for two days. On the third day the culture of *S. pyogenes* was subcultured at 5% into a pre-warmed 150 ml bottle of 100 ml of THY and grown for 3-4 hours at 37°C, which allowed the cultures to reach logarithmic phase which was considered to be when the OD_{600} was between 0.150 and 0.350. Optical density was measured at 600 nm and was used to calculate the number of bacteria in a millilitre of culture. The volume of bacteria necessary to prepare the inoculation was calculated, measured and spun at 3,000 RPM (Allegra 6 centrifuge; Beckman Coulter) for 10 minutes. Supernatant was gently poured off without disturbing the pellet. The pellet was transferred to a microfuge tube and the culture was spun at 21,130 × *g* for one minute and the supernatant was drawn off and the pellet was washed

in 1 ml of 1 × Hanks Balanced Saline Solution without phenol red (HBSS; Hyclone Laboratories, South Logan, UT, USA), *S. pyogenes* were re-pelleted and again the supernatant was removed. The culture was prepared so that it was resuspended in 15 µl of HBSS per dose of bacteria at room temperature.

2.10.2. Mouse nasal inoculation

Mice between 9 and 13 weeks of age were anesthetized with isoflurane (Baxter Corp., Mississauga, ON, Canada) and were monitored in accordance with the Western University animal care and veterinary services post-operative/post-anesthetic care protocol for rodents (standard operating procedure # 330-03). Mice were scruffed and 7.5 µl of the bacterial preparation was used to inoculate each nostril of the mouse very slowly so that there was no accumulation of inoculum on the nares of the mouse while the dose was being given. The mouse was allowed to recover and was monitored for the duration of the experiment.

2.10.3. Endpoint surgery

Mice were sacrificed at the end of the experiment as per the animal protocol approved by the Western University Animal Ethics Board. Endpoint timing was determined by desired readout. To analyze the establishment of colonization data all mice were sacrificed at 48 hours. To examine the persistence of colonization, mice were sacrificed at 24, 48, 72, 96, and 144 hours post-infection.

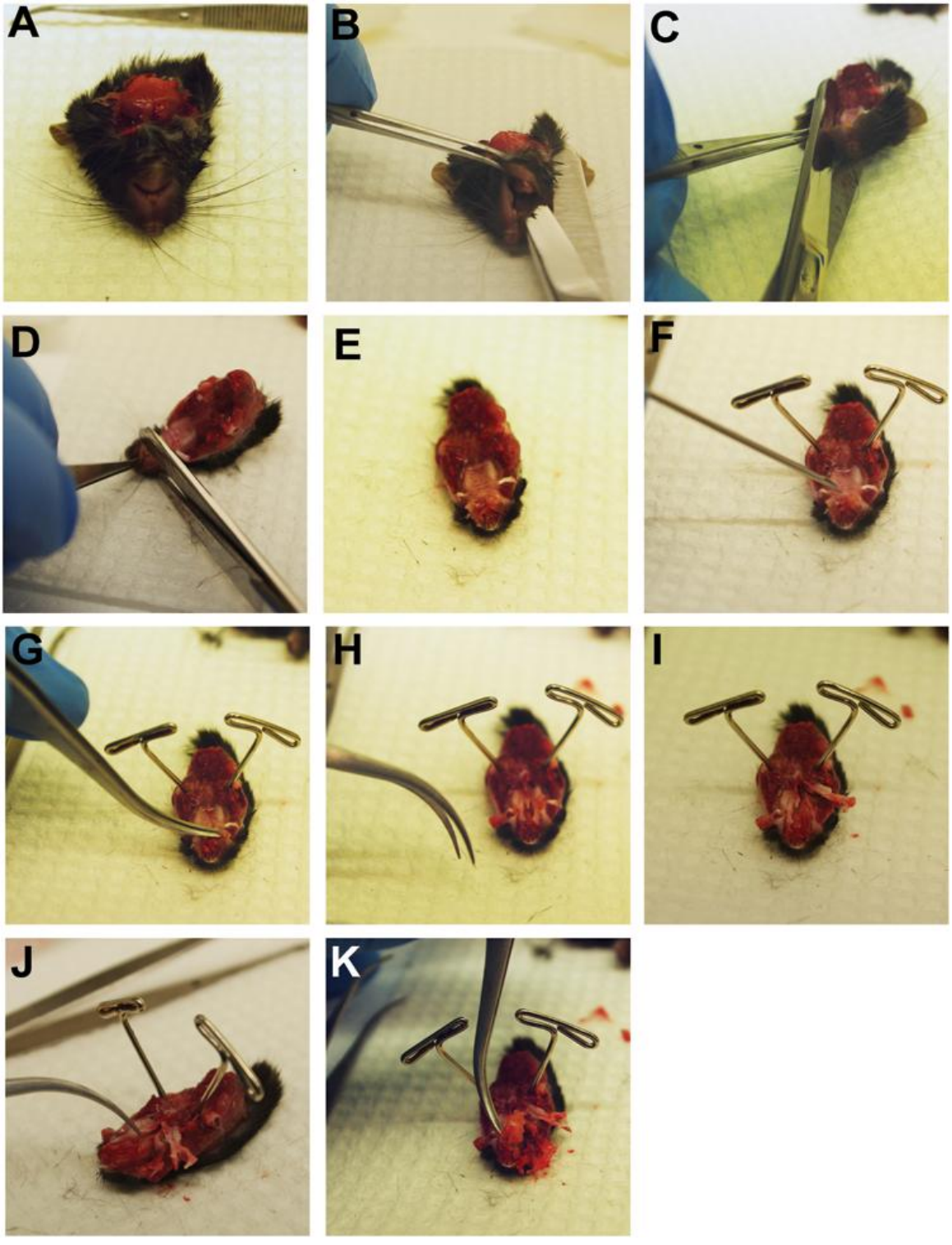
At the desired endpoint mice were anesthetized with isoflurane until they were not responsive to pain and anesthesia was maintained on a nose cone for the duration of the surgery. Mice were opened down the ventral midline with small straight sterile scissors. Organs were moved to the animal's left exposing the dorsal abdominal wall. Blood was drawn from the portal vein into a 1 ml syringe (Becton, Dickinson, and Co., Sparks, MD, USA) with a 25 gauge needle coated with Heplean heparin (Organon Canada Ltd., Toronto, Ontario, Canada). After the blood draw, the animal was sacrificed by breaking the diaphragm and decapitation with scissors. The spleen, kidneys, liver (with gallbladder removed), lungs and heart were harvested. Organs were surgically removed into tubes containing 4 ml HBSS. Organs and blood samples were placed on wet ice until

homogenization. To remove the complete nasal turbines including the NALT and the maxillary sinuses, the head was positioned so that the snout of the head was facing the surgeon upside down on the surgical platform. The head was stabilized using tweezers to hold the lower jaw, gripping the tongue to the roof of the mouth. Sturdy large scissors were inserted pointed end into the mouth all the way to the back and held so that they were parallel to the soft palate and used to sever the lower jaw from the upper portion of the head. Curved tweezers were then used to grip the cheek with the curve of the tweezers pointing away from head. Large scissors were used to cut between the tweezers and the head to cut away and remove excess cheek tissue from both cheeks, without cutting the skull bones, all the way to the ears. Tweezers were subsequently used to grip the tip of the nose and the sturdy scissors were used to cut behind the front teeth to remove the nose and the front teeth. With the nose of the head still pointing directly at the surgeon and the head positioned so that the soft palate is facing up, and cross-pinned the head behind the palate. A fresh needle was used as a micro-scalpel to slice a very shallow cut midline through the soft palate. The soft palate was peeled off using very fine curved tweezers. Small curved scissors were inserted pointed side up shallowly into one side of the curved jaw bone back to the back teeth. The NALT and maxillary sinuses are attached to the ventral surface of the ethmoid turbinate, so it is important that the cut of the curved part of the jaw bone is very shallow as to not disturb the ethmoid turbinates. The bone was cut and the facial bone with remaining tissue was gently and slowly rolled/pressed away from the septum. The entire nasal turbines including the NALT and the maxillary sinus were removed without disturbing the septum by breaking and removing the cartilage at the front of the tissue and breaking the ethmoid turbinates free from the back. This harvested tissue will henceforth be referred to as the ‘complete nasal turbinates’ (cNT). The other side cNT was removed in the same manner. The cNT were collected in a microfuge tube with 500 µl HBSS and stored on ice until processed (Figure 6).

2.10.4. Tissue processing and plating

Isolated tissues were processed by homogenization for determination of colony forming units (cfu). Spleen, kidneys, liver, lung, and heart were processed with a

Figure 6. Surgical removal of the murine complete nasal turbines. The head (A) was prepared by the removal of the lower jaw (B) and cheek muscles on either side of the head (C). The nostrils were removed by cutting posterior to the incisors (D). The head was pinned down just ahead of the ear canals and a needle was used as a microsurgical tool to make a very shallow cut in the centre of the palate (F) and the palate was peeled off with fine smooth curved tweezers (G) to expose the top of the premaxilla on either side of the septum (H). Very fine curved scissors are inserted very shallowly under the top edge of the premaxilla all the way back to the back of the molars, the premaxilla was cut and moved away from the tissue beneath it (I) which exposed the nasal turbines and the ethmoid turbinate which were posterior to the more bony maxilloturbinate and nasoturbinate (I, J). The maxilloturbinate and nasoturbinate can be distinguished from the ethmoid turbinate as they appear to be redder in appearance and feel bony to the tweezers. The maxilloturbinates and nasoturbinates were removed with the fine, smooth curved tweezers. The tweezers were then placed posterior to the ethmoid turbinate and then scooped out of the head without disturbing the septum (K). The other ethmoid turbinate was then removed in the same manner.


A
B
C
D
E
F
G
H
I
J
K

PRO200 electronic homogenizer (PRO Scientific Inc., Monroe, CT, USA). Between samples, the homogenizer was cleaned by rinsing twice in 4 ml of water or HBSS, being wiped with a tissue wipe, rinsed with 10% bleach, 70% ethanol, and finally rinsed twice more in each of 4 ml sterile distilled water and 4 ml sterile HBSS. Samples were homogenized until they appeared homogeneous. The cNT were homogenized in a 1 ml glass homogenizer. Between samples, the glass homogenizer was cleaned three times with 70% ethanol and rinsed twice with both sterile distilled water and sterile HBSS. Tissues were plated both 100 μl neat and serially diluted on TSAII plates containing 5% sheep blood agar (Becton, Dickinson, and Co., Sparks, MD, USA). Bacterial growth was observed after growth at 37°C. Total bacterial growth and β-haemolytic *S. pyogenes* were assessed.

2.11. Histology

2.11.1. Organ isolation

Mice were fully anesthetized with isoflurane and the mouse was perfused through the heart with sterile PBS containing heparin to flush the blood from the mouse using a Gilson Minipuls 3 peristaltic pump (Middletown, WI, U.S.A) at a constant flow rate. Mice were then perfused with 10% neutral buffered formalin (BDH, VWR, West Chester, PA, USA) through the peristaltic pump at a constant flow rate until organs were fully perfused with formalin. Organs were surgically removed without tool marks and were placed in 10-volumes of fresh formalin. The head was prepared for histology by removing the lower jaw and tongue and the tip of the nose which was removed by cutting behind the front teeth and then placed 10-volumes of formalin.

2.11.2. Histology tissue processing

Formalin-perfused soft tissues (spleen, kidneys, liver, lung and heart) were further fixed in formalin for 48 hours at 4°C, changed daily. Organs were rinsed in 1 × PBS before being resuspended in 10-volumes of 1 × PBS twice a day for three days, and washed in 10-volumes of 70% ethanol twice and stored in 70% ethanol until processed. To process bony tissues, such as the head, the formalin-perfused mouse tissues were soaked in 10 volumes of formalin for 24 hours and then the formalin was removed and

the tissue was re-suspended in Shandon TBD-2 Decalcifier (TBD; Thermo Scientific, Kalamazoo, MI, USA) for 96 hours. The TBD-treated tissue was tested for decalcification by cutting the back of the skull with a scalpel to determine whether or not the bone could be sectioned smoothly after further processing. TBD-decalcified heads were placed in formalin for 48 hours and washed with 1 × PBS, and resuspended in 10-volumes of 1 × PBS twice daily for 4 days and washed in 70% ethanol twice and stored in 10-volumes of 70% ethanol until cassetted and processed.

2.11.3. Cassetting, embedding and sectioning fixed tissues

Fixed tissues were placed in 4 mm Fisherbrand TRU-Flow tissue cassettes. The head was sectioned between the first and second molar and the front section of the head was placed in one cassette and the back portion in a second for processing. Formalin-fixed cassetted tissues in 70% ethanol were sent to The Robarts Research Institute Molecular Pathology Core Facility for processing in preparation for embedding in wax. Cassettes were processed in Leica ASP300 fully enclosed paraffin wax tissue processor overnight in the bone program. Cassettes were transferred into a warm wax bath and embedded in paraffin wax. Embedded tissues were stored at room temperature until sectioning.

Tissues were sectioned on a microtome HM335E Microtome Leica in the Robarts Research Institute Molecular Pathology Core Facility using MB35 Premier Microtome blades (Thermo Scientific) into 5 micron sections. Serial sections were collected for head sections, and representative sections were cut for the spleen. Sections were mounted on Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Fair Lawn, NJ, USA) and were dried at 45°C for 48 hours prior to storage/staining.

2.11.4. Haematoxylin and eosin staining of processed tissue

Tissues were stained with haematoxylin and eosin in a Leica Autostainer XL. Slides were allowed to dry and Fisher Finest Premium Cover Glass (Fisher Scientific, Fair Lawn, NJ, USA) cover slips were affixed to the slides using Cytoseal 60 low viscosity mounting medium (Richard-Allen Scientific, Kalamazoo, MI, USA). Cover slipped slides were dried for at least 24 hours horizontally before vertical storage.

2.11.5. Histology evaluation

Slides were blindly evaluated in collaboration with a pathologist. After all slides were observed evaluation criteria were determined for each tissue type. The relative amount of mucus present covering the epithelia, the presence of red blood cells, and the presence of nucleated cellular debris on the surface of the epithelia was assessed. The presence and severity of these findings were used to assign a score of zero to two points to each of two sections per mouse. The scores were averaged to determine differences in histological pathology in the mice.

2.12. Flow cytometry

2.12.1. Tissue isolation and processing

Mice were anesthetized as previously described. The cervical lymph nodes were surgically removed and collected and suspended in RPMI 1640 (Gibco, Burlington, Ontario, Canada) and the mouse was decapitated. The spleen was surgically removed and placed into 5 ml of complete RPMI (cRPMI). The remaining lymph nodes were collected into the lymph node collection tube. The cNT were removed as described above and placed in a tube with 5 ml RPMI 1640. Cells were isolated by passing the organs through 40 μm nylon cell strainers (BD Falcon, San Jose, CA, U.S.A) with the end of a syringe. Cells were spun at 1,200 RPM (Beckman Coulter Allegra 6) for 5 minutes and the pellet was resuspended into ACK lysis buffer (15 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM EDTA) for five minutes with inversions every 30 seconds. ACK lysis buffer was neutralized with 10 ml cRPMI and the cells were pelleted as before. Cells were loosened by racking and resuspended into filtered PBS with 5% fetal bovine serum (FBS). Cells were enumerated and their viability was assessed by trypan blue (Gibco, Burlington, Ontario, Canada).

2.12.2. Flow cytometry

Isolated cells were aliquoted at 500,000 cells per 5 ml tube. Cells were pelleted at 1,200 RPM (Allegra 6 centrifuge, Beckman Coulter) for 5 minutes and the supernatant was carefully poured off. The pellet was gently racked to resuspend the cells. Cells were

pre-treated with Fc block from hybridoma clone 2.4G2 cell culture supernatant (produced by the Haeryfar lab) for 10 minutes prior to cell staining. A master mix of the antibodies (Table 6) were used to stain the cells for each panel (Table 7) were added, the cells were mixed and incubated on ice in the dark for 30 minutes. Cells were washed twice with 1 × PBS + 5% FBS, pelleting the cells at 1,200 RPM for 5 minutes between washes. Cells were finally resuspended in 500 μl of 1 × PBS + 5% FBS. Stained cells were run on a BD FACS Canto II flow cytometer, with capture of 100,000 events per sample. Standard compensations were used for each tissue using FACSdiva software.

2.13. Cytokine analysis

2.13.1. Human peripheral blood mononuclear cell activation assay

Human blood was collected in heparinized vacuum tubes, diluted 1:1 with RPMI, layered over pre-warmed ficoll and spun at 2,500 RPM for one hour. Peripheral blood mononuclear cells (PBMC) were removed by pipetting and washed in 40 ml of warm RPMI, pelleted for 5 minutes at 2,500 RPM, and resuspended in cRPMI. Cells were assessed for viability by trypan blue and plated at 2×10^5 PBMCs per well in a 96-well plate. Cells were stimulated with streptococcal culture supernatant so that the final volume per well was 200 μl. The potential mitogens were incubated at 37°C with 5% CO_2 for 18 hours. Cell culture supernatant was removed and activation was assessed by measuring IL-2 by ELISA.

2.13.2. Mouse splenocyte activation assay

The spleen was surgically removed from the mouse and was suspended in cRPMI. The spleen was passed through a 40 μm nylon cell strainer, spun at 1,200 RPM (Beckman Coulter Allegra 6) for 5 minutes, and resuspended in ACK lysis buffer for five minutes with inversions every 30 seconds before being neutralized with 10 ml cRPMI. Pelleted cells were loosened by racking and resuspended into cRPMI. Cells were enumerated and their viability was assessed by trypan blue. Cells were plated at 2×10^5 splenocytes per well in a 96-well plate and were stimulated with either recombinant SAg (rSAg) or streptococcal culture supernatant so that the final volume per well was 200 μl. The potential mitogens were incubated at 37°C with 5% CO_2 for 18 hours. Cell culture

Table 6. Antibodies for flow cytometry

Target	Fluorochrome	Identifies	Order Number	Source
Mouse CD3	APC	Mature T cells	17-0031-82	eBioscience
Mouse CD3	PE Cy7	Mature T cells	25-0051	eBioscience
Mouse CD4	APC Cy7	CD4+ T cells	100414	Biolegend
Mouse CD8	PE Cy7	CD8+ T cells	25-0081	eBioscience
Mouse CD25	PE	Active and mature T cells and B cells and regulatory T cells	12-0251-82	eBioscience
Mouse CD45	Alexafluor 700	Leukocytes	560510	BD Pharmigen
Mouse CD19	FITC	B cells	11-0191-81	eBioscience
Mouse NK1.1	PE	Natural killer and natural killer T cells	12-5941-82	eBioscience
Mouse CD1d tetramer, Unloaded	APC	Control for iNKT cell staining		NIH, Dr. M. Haeryfar
Mouse CD1d tetramer, PBS-57 Loaded	APC	iNKT cells		NIH, Dr. M. Haeryfar
Mouse CD11c	APC Cy7	Dendritic cells	17-0114-82	eBioscience
Mouse F4/80	FITC	Macrophages	11-4801	eBioscience
Mouse GR1	FITC	Neutrophils	53-5931	eBioscience
Anti 7AAD		Dead cells	51-68981E	BD Pharmigen

Table 7. Flow cytometry staining panels

Panel	**Tissues stained**	**Antibodies**
Panel 1	Lymph nodes Spleen Complete nasal turbinates	7AAD CD45 Alexaflour700 (cNT only) CD19 FITC (cNT only) CD3 APC CD4 APC Cy7 CD8 PE Cy7 CD25 PE
Panel 2	Lymph nodes Spleen	7AAD CD1d APC (loaded and unloaded) CD3 PE Cy7 CD19 FITC NK 1.1 PE
Panel 3	Lymph nodes Spleen	7AAD F4/80 FITC CD11c APC Cy7
Panel 6	Lymph nodes Spleen Complete nasal turbinates	7AAD CD45 Alexaflour700 (cNT only) CD11c APC F4/80 FITC NK 1.1 PE
Panel 7	Lymph nodes Spleen Complete nasal turbinates	7AAD CD45 Alexaflour700 (cNT only) GR1 FITC CD11b PE

supernatant was removed and activation was assessed by measuring mouse IL-2 by ELISA.

2.13.3. Enzyme-linked immunosorbant assay

Human and mouse IL-2 ELISA kits were purchased from eBioscience, and were used to assess primary cell culture supernatants stimulated for 18 hours. Kits were run as specified by the manufacturer in Costar ELISA plates. Plates were read at OD_{450} and at the reference OD_{570}.

2.13.4. Multiplex cytokine analysis

Cytokines were analyzed in cNT homogenates isolated from mice treated by either wild-type *S. pyogenes* MGAS8232 or *S. pyogenes* MGAS5005, isogenic SAg deletion strains or saline at either 24 or 48 hours in DR4/DQ8 and FVB mice respectively. The homogenates were analyzed in one of three way; by ELISA (eBioscience), Luminex was performed by the Screening Lab for Immune Disorders (Lawson Health Research Institute, London, Ontario, Canada) using the Bio-Rad BioPlex 200 to run a Millipore 12-plex cytokine array, or with an eBioscience Flow Cytomix mouse Th1/Th2 10-plex ready-to-use cytokine analysis kit (San Diego, CA, U.S.A) as per manufacturer instructions.

Chapter 3: Results

3.1. Establishment of a murine model of mild nasal infection by *Streptococcus pyogenes*

3.1.1. Surgical removal of the complete nasal turbinates of mice

Mice are obligate nose breathers, and hence all inspired air passes through the nasal passage, which is the first site of contact for inhaled infectious agents [143]. The lymphoid tissue that lines the basolateral surface of the nasopharyngeal meatus is known as the NALT, which is physiologically similar to the human tonsils [144]. The NALT has been established as a site of colonization of *S. pyogenes* in mouse nasal infection model at 24 hours post-inoculation [149].

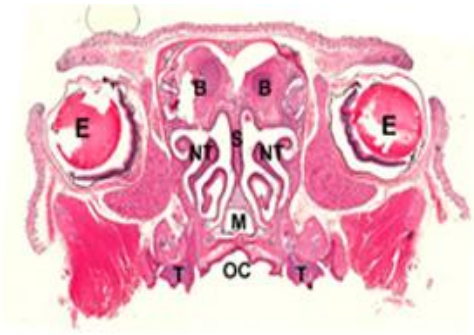
Although there is a good description of one method of NALT extraction and processing in which the palate is excised around the perimeter with a small scalpel and then carefully separated from the head to solely isolate the NALT which is attached to the posterior of the palate [144]. This process differs from the removal of the complete nasal turbinates (cNT) whose removal includes the NALT, the maxillary sinuses, and the ethmoid turbinates. There was no previous existing detailed diagrammatic protocol of surgical removal of the complete nasal turbinates before this work (Figure 6). The cNT isolation procedure differs in that the palate is removed without first incising the perimeter. The premaxilla was shallowly cut all the way to the molars and the cheeks were separated away to reveal the cNT which is separated from the septum using fine tweezers.

To illustrate the physiological location of the structures in the nasal passage relative to the rest of the head, histological sections were prepared from 10% formalin-perfused TBD decalcified mouse heads embedded in wax. Sequential cross-sections of the mouse head were prepared between the T3 and T4 sections to demonstrate the differences in the nasoturbinates throughout the head (Figure 7). Cross-sections of the mouse heads, stained with haematoxylin and eosin, were visualized by microscopy and have been used to indicate the location of the NALT in relation to the ethmoid nasal turbinates at the T4 cross-section (Figure 8).

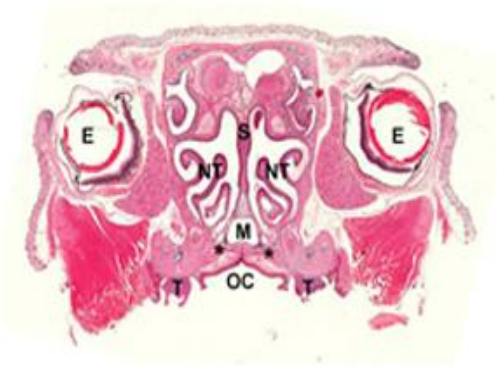
The surgical technique used for the isolation of the cNT in this work was learned in the laboratory of Dr. P. P. Cleary (University of Minnesota), an established expert in this model [149, 151-152, 168]. Despite the fact that in previous literature the primary site of colonization has been established to be highly localized in the NALT [149], the

Figure 7. Histological transition in the haematoxylin and eosin-stained murine head cross sections from T4 to T3. The prepared sections of the fixed mouse head were scanned in their entirety. The sections were taken at 200 μm intervals from the T4 cross-section to the T3 cross-section, a total of 1,400 μm (A-G). At the T4 section the eye (E) and the optical lobes of the brain (B) are visible. The nasal turbinates (NT) are distinct from the nasopharyngeal meatus (M) and are divided by the septum (S). The oral cavity (OC) is located at the base of the section and the molar teeth (T) are visible. Starting at 200 μm towards the anterior of the head (B) the NALT (*) is visible bilaterally throughout the rest of the sections (B-G). At 300 μm towards the anterior of the head the maxillary sinuses (MS) are visible bilaterally through the rest of the sections (C-G). At 800 μm (D), the nasopharyngeal meatus connects to the nasal turbinates and the teeth are no longer apparent.

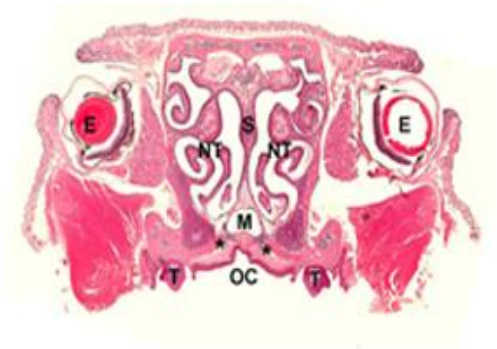
A



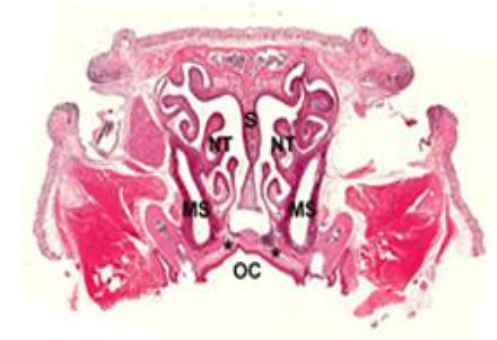
B



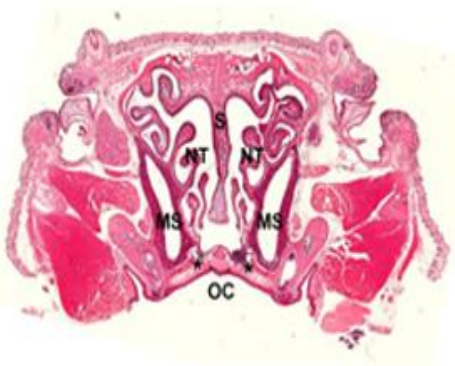
C



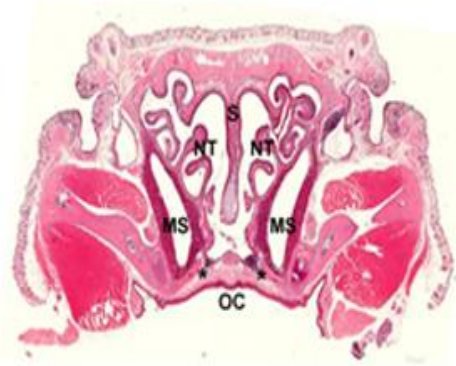
D



E



F



G

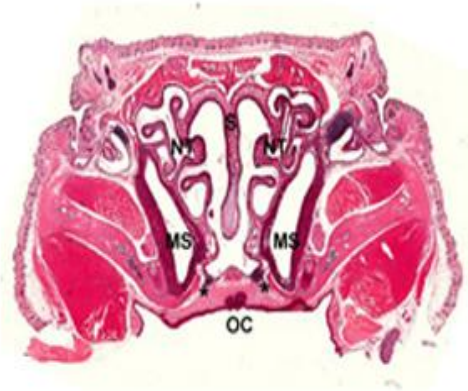
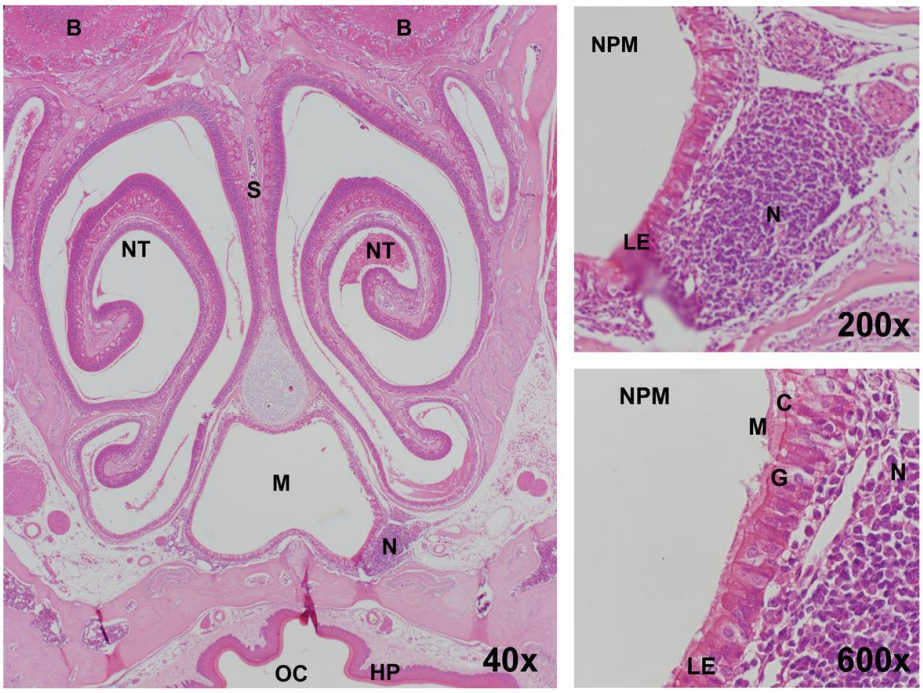


Figure 8. Haematoxylin and eosin-stained section of transected murine head indicating the position of the NALT from a wild type *S. pyogenes* MGAS8232-infected mouse. The 40× magnification orients where the NALT (N) is located in relation to the rest of the tissue in the head. The oral cavity (OC) and palate (HP) and optical lobe of the brain (B) serve to orient the diagram. The septum (S) divides the two ethmoid turbinates (NT) of the sinuses. The 200× magnification image shows the lymphoepithelium (LE) separating the NALT from the nasopharyngeal meatus. The 600× magnification image highlights the LE and identifies the ciliated (C) cells, goblet cells (G) and overlying mucus (M) which is located on the basolateral surface of the nasopharyngeal meatus (NPM).


B
B
S
NT
NT
M
N
OC
HP
40x
NPM
N
LE
200x
NPM
C
M
G
N
LE
600x

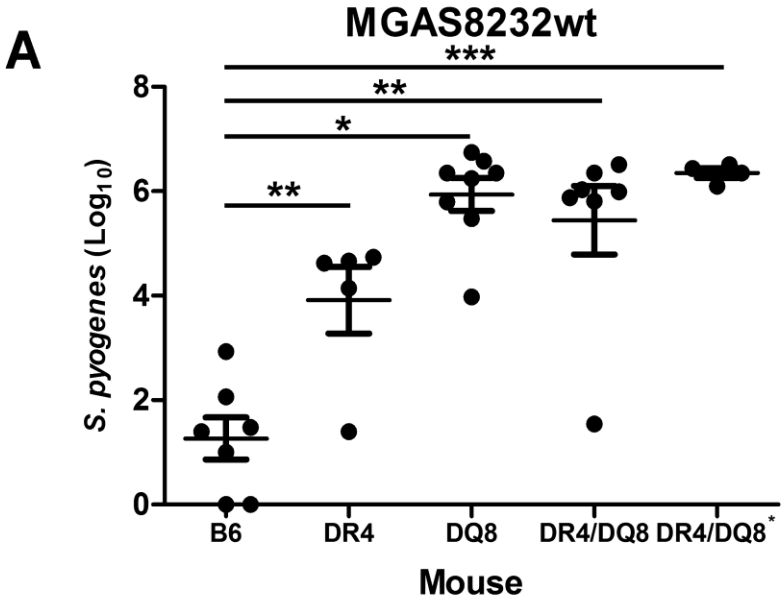
surgical technique learned from the Cleary lab varies slightly from the documented NALT surgical technique described in Asanuma *et al*. [144] in that it removes the cNT (Figure 6). All surgeries in this work recovered the cNT which includes the ethmoid turbinates along with the NALT and maxillary sinuses. This thesis has provided a detailed pictorial diagrammatic protocol of how to perform the removal of the cNT (Figure 6). Importantly, this technique resulted in reproducible streptococcal counts within the expected range (described below).

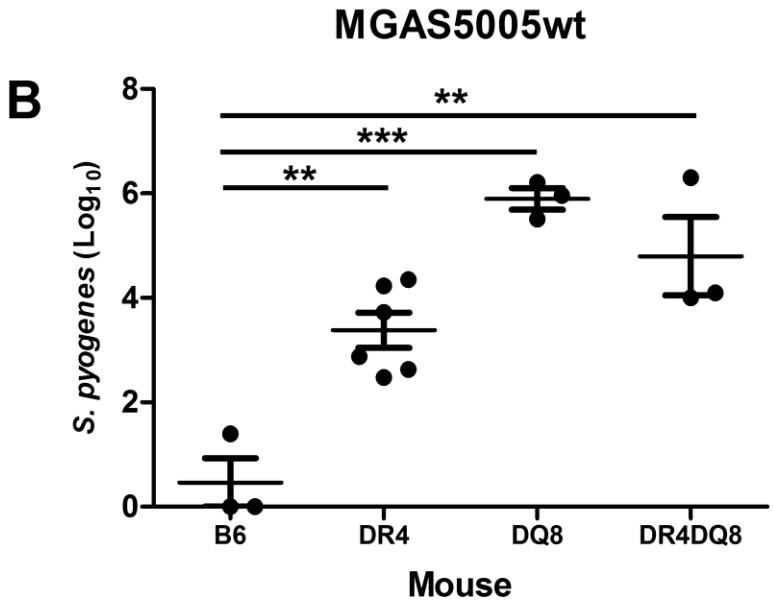
3.1.2. Bacterial dose determination for streptococcal colonization in mice

To examine streptococcal colonization potential, mice were sacrificed at 48 hours post-inoculation. The 48 hour time point was chosen as the time point to assess streptococcal colonization based on previous work by Park *et al*. [149], which described that in a BALB/c mouse model of streptococcal nasal infection with an M1 strain of *S. pyogenes* (strain 90-226), after an initial decrease in the streptococcal burden at four hours post-infection, the population of streptococci in the cNT recovers and peaks at 48 hours.

To determine the optimal dose of bacteria to use in a mouse model of nasal colonization, 10^7, 10^8 and 10^9 cfu of wild-type *S. pyogenes* MGAS8232 were prepared and tested in B6 mice, or B6 mice transgenic for HLA-DR4 (hereafter referred to as DR4). Mice were sacrificed at 48 hours and their blood, organs and cNT were assessed for the presence of live *S. pyogenes* MGAS8232. The streptococci colonized B6 mice very poorly at all dose concentrations tested; however, *S. pyogenes* MGAS8232 consistently colonized DR4 mice 2-3 logs higher compared with the B6 mice (Figure 9). Ultimately, due to the viscosity and technical difficulty in the preparation of the 10^9 cfu dose of *S. pyogenes* MGAS8232 (data not shown), it was decided to use a dose of 1×10^8 cfu for both *S. pyogenes* MGAS8232 and *S. pyogenes* MGAS5005 for colonization experiments in the mice.

Figure 9. Efficient nasal colonization in humanized mice by *S. pyogenes* is dependent on human MHC class II. Mice expressing either mouse or transgenic human MHC class II molecules were nasally inoculated with (A) *S. pyogenes* MGAS8232 (N=4-7) and (B) *S. pyogenes* MGAS5005 (N=3-6). At 48 hours post inoculation mice were sacrificed and the streptococcal burden in the cNT was assessed. DR4/DQ8* mice are F1 generation mice of a cross of DR4/DQ8 and DR4 mice from the Western University colony. Statistical differences were assessed by unpaired t-test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.


A
MGAS8232wt
***
**
*
**
S. pyogenes (Log10)
8
6
4
2
0
B6
DR4
DQ8
DR4/DQ8
DR4/DQ8*
Mouse


B
MGAS5005wt
**
***
**
S. pyogenes (Log10)
8
6
4
2
0
B6
DR4
DQ8
DR4DQ8
Mouse

3.1.3. Streptococcal nasal colonization varies depending on transgenic expression of human MHC class II

Different human MHC class II haplotypes have been previously established to alter susceptibility to streptococcal SAg-induced T cell proliferation and have been linked to clinical outcomes in patients with severe streptococcal infection [29, 59, 112-114]. Human MHC class II was also found to contribute to the severity of streptococcal sepsis in a transgenic mouse model [113]. To assess the sensitivity of mouse strain variation on streptococcal colonization, *S. pyogenes* MGAS5005 or *S. pyogenes* MGAS8232 were used to nasally inoculate B6 background mice which do not express mouse H-2 and express different MHC class II molecules with a 1×10^8 cfu/dose of bacteria. At 48 hours post-inoculation this resulted in strikingly different amounts of streptococci recovered. In particular it was noted that DR4/DQ8 or DQ8 mice were colonized more effectively than DR4 mice, and DR4 mice were colonized more effectively than the parental B6 mice, with both streptococcal strains (Figure 9). Specifically, mice expressing DQ8, with or without DR4, had counts that were ~ 4-5 orders of magnitude higher than B6 mice. DR4/DQ8-expressing mice are thought to be neutral in their susceptibility to severe streptococcal disease when compared to other human MHC class II and are responsive to some streptococcal SAgs [113]. These data indicate that human MHC class II molecules are critical host factors for the efficient colonization of cNT by *S. pyogenes*.

3.1.4. Streptococcal colonization is not dependant on the source of mice

The DR4/DQ8 mice are not available commercially and all transgenic mice were bred and genotyped on site by the researcher using MHC class II sequencing primers (Table 4) (data not shown). Because the DR4 and DR4/DQ8 mouse populations used in this study were from two different sources, the two strains were crossed and the F1 generation expressing both DR4 and DQ8 was used in colonization experiments. The DR4 × DR4/DQ8 mice had similar levels of streptococcal colonization at 48 hours post-inoculation with *S. pyogenes* MGAS8232 (Figure 9A., Right column denotes DR4/DQ8*). This data indicates that the source of the mouse is not likely a factor in the HLA-dependent colonization phenotype.

3.1.5. Nasal colonization by *S. pyogenes* in mice expressing different murine MHC class II is variable and *S. pyogenes* strain-dependant

Mice expressing different mouse MHC class II alleles (Table 5) were also assessed for their ability to be colonized by different strains of *S. pyogenes* (Figure 10). The Friend leukemia virus B-type (FVB) line of mice are albino inbred mice that were purchased from The Jackson Laboratory. They express the mouse MHC class II H2 haplotype q [169-170]. FVB mice were initially derived in 1966 from an outbred population of Swiss mice at the National Institutes of Health in the United States of America to have sensitivity to post-*pertussis* vaccination histamine challenge. These mice are reported to be susceptible to asthma-like airway responsiveness and have higher than average activity, anxiety and basal body temperature [169-170]. FVB mice infected with *S. pyogenes* MGAS5005 had the highest yield of streptococci of all mouse strains tested at 48 hours. Inoculation with MGAS8232 did not result in a similarly high yield of *S. pyogenes* in isolated cNT. Ultimately it was decided that HLA-DR4/DQ8 transgenic mice would be used to assess *S. pyogenes* MGAS8232 isogenic deletion strains, and FVB mice would be used to assess *S. pyogenes* MGAS5005 isogenic deletion strains.

3.1.6. Streptococcal superantigenic sensitivity varies with murine expression of MHC class II

It has been well-established that streptococcal SAgs have different activities depending on their interactions with MHC class II [29, 112-114, 171] and Vβ TCR [86, 172], whose specificities can vary with concentration [87]. In general, streptococcal SAgs do not interact with mouse MHC class II as efficiently as they do with human MHC class II [59], which often results in poor murine response to streptococcal SAg stimulation.

To assess the activation of murine lymphocytes by streptococcal SAgs, splenocyte activation was used as an *ex vivo* readout to predict *in vivo* sensitivity to SAgs (Figures 11 and 12) in strains of mice with different murine and human MHC class II molecules (Table 5). The activation of splenocytes was assessed by mIL-2 ELISA after 18 hours of stimulation by rSAg. The activation potential of each SAg encoded in the genomes of *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232 is summarized in Table 8. None of

Figure 10. *Streptococcus pyogenes* colonization variability in mice expressing murine MHC class II. Strains of mice expressing different mouse MHC class II were assessed for their ability to be colonized nasally by (A) *S. pyogenes* MGAS5005 wild type and (B) *S. pyogenes* MGAS8232 wild type. All mice were infected for 48 hours before the streptococcal burden was assessed in the cNT. FVB mice colonized with *S. pyogenes* MGAS5005 were colonized more efficiently than any mouse strain tested. Statistical differences were assessed by unpaired t-test. **, $P < 0.01$ (N=2-4).

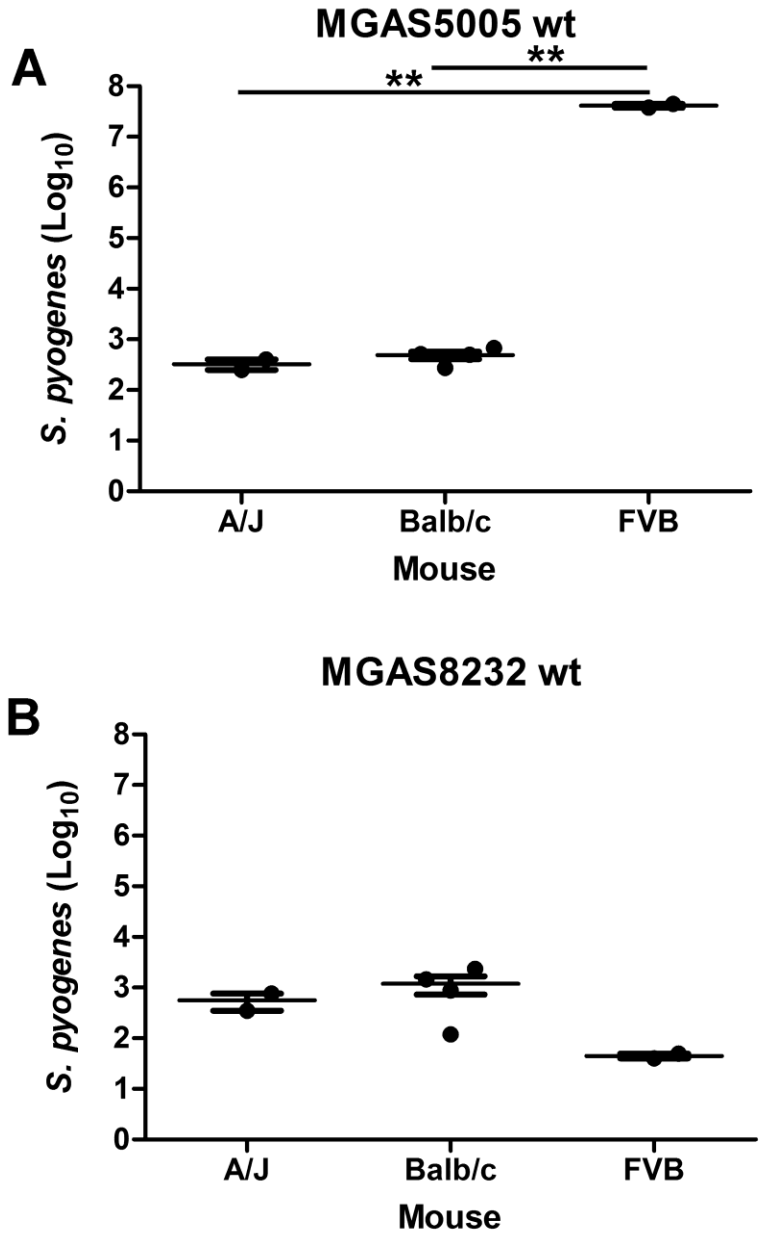

A
MGAS5005 wt
**
**
S. pyogenes (Log10)
0
1
2
3
4
5
6
7
8
A/J
Balb/c
FVB
Mouse
B
MGAS8232 wt
S. pyogenes (Log10)
0
1
2
3
4
5
6
7
8
A/J
Balb/c
FVB
Mouse

Table 8. Summary of mouse strain-specific superantigen sensitivity[A]

Superantigen	C57Bl/6	DR4	DR4/DQ8	BALB/c	FVB
SpeA	Neg	+	+ + +	Neg	Neg
SpeC	Neg	Neg	Neg[B]	Neg	Neg
SpeG	Neg	Neg	Neg	Neg	Neg
SpeJ	Neg	+	Neg	Neg	+
SpeL	Neg	Neg	Neg	Neg	Neg
SpeM	Neg	Neg	Neg	Neg	Neg
SmeZ	Neg	+	+ + + +	+ +	+ +

[A] Superantigen sensitivity was assessed by looking at the concentration of SAg in pg/ml neccessary to result in the production of mIL-2 at 18 hours.

[B] SpeC is considered a negative result because activation did not occur in concentrations above 100 ng/ml.

Figure 11. Splenocyte activation of B6 and transgenic HLA-mice by recombinant streptococcal superantigens. Splenocytes isolated from female B6, DR4, and DR4/DQ8 mice were treated with ten-fold serial dilutions of the pure recombinant streptococcal SAgs encoded in *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232 for 18 hours. Activation of the splenocytes was assessed by determining the amount of mIL-2 produced in the cell culture supernatants by ELISA. Results are representative of independent experiments in at least two mice.

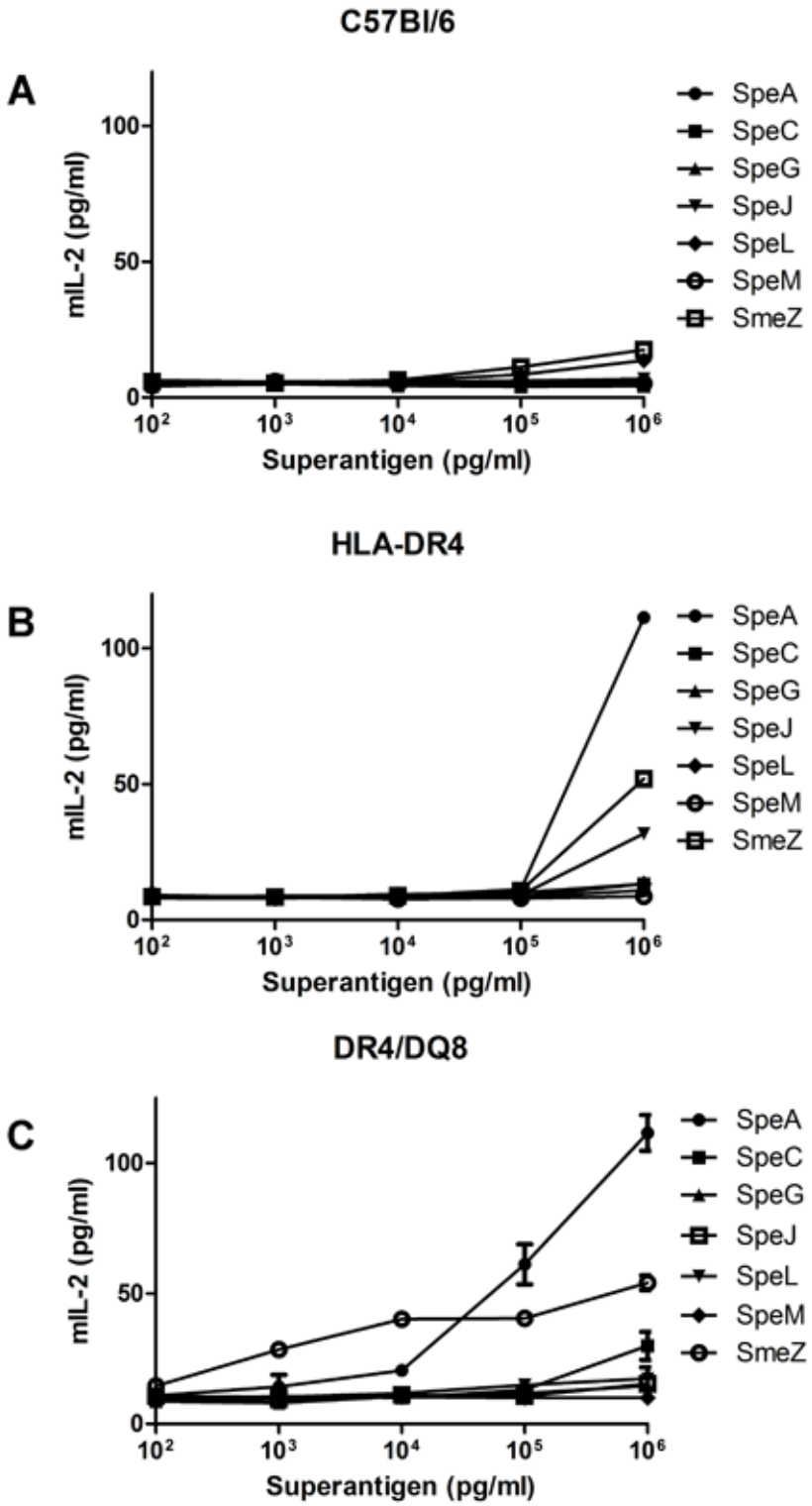
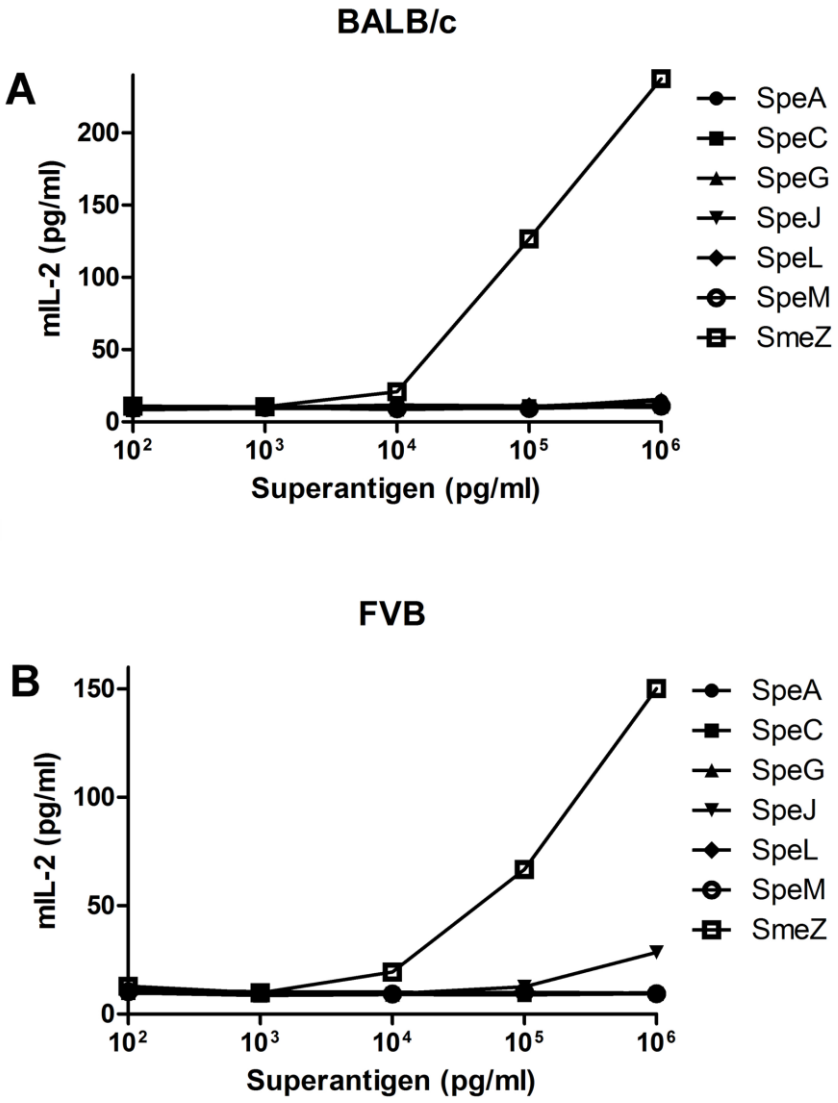

C57Bl/6
A
mIL-2 (pg/ml)
100
50
0
10^2
10^3
10^4
10^5
10^6
Superantigen (pg/ml)
SpeA
SpeC
SpeG
SpeJ
SpeL
SpeM
SmeZ
HLA-DR4
B
mIL-2 (pg/ml)
100
50
0
10^2
10^3
10^4
10^5
10^6
Superantigen (pg/ml)
SpeA
SpeC
SpeG
SpeJ
SpeL
SpeM
SmeZ
DR4/DQ8
C
mIL-2 (pg/ml)
100
50
0
10^2
10^3
10^4
10^5
10^6
Superantigen (pg/ml)
SpeA
SpeC
SpeG
SpeJ
SpeL
SpeM
SmeZ

Figure 12. Splenocyte activation of BALB/c and FVB mice by recombinant streptococcal SAgs. Splenocytes isolated from female (A) BALB/c and (B) FVB mice were treated with ten-fold serial dilutions of pure rSAg from *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232. Activation of the splenocytes was assessed by determining the concentration of mIL-2 in the cell culture supernatants after 18 hours of stimulation by ELISA.


BALB/c
A
mIL-2 (pg/ml)
200
150
100
50
0
10^2
10^3
10^4
10^5
10^6
Superantigen (pg/ml)
SpeA
SpeC
SpeG
SpeJ
SpeL
SpeM
SmeZ
FVB
B
mIL-2 (pg/ml)
150
100
50
0
10^2
10^3
10^4
10^5
10^6
Superantigen (pg/ml)
SpeA
SpeC
SpeG
SpeJ
SpeL
SpeM
SmeZ

the streptococcal rSAgs were found to efficiently stimulate B6 splenocytes at any concentration tested (Figure 11A) and streptococcal SAgs only activated DR4 splenocytes at the highest concentrations for SpeA, SmeZ, and SpeJ (Figure 11B). Splenocytes from DR4/DQ8 mice were stimulated by recombinant SpeA, SpeL and SmeZ (Figure 11C). White mice were also tested and were selected for their expression of different murine MHC class II haplotypes. BALB/c mice express H2 haplotype d and were stimulated by recombinant SmeZ and mildly stimulated by SpeA and SpeJ. FVB mice express H2 haplotype q and were stimulated by recombinant SmeZ and SpeJ (Figure 12).

3.2. *Streptococcus pyogenes* MGAS8232 and MGAS5005 superantigen isogenic deletion strain creation and characterization

3.2.1. Superantigen production by *Streptococcus pyogenes*

Streptococcal SAgs encoded in the genomes of *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232 were predicted by data mining their sequenced genomes which are annotated in the NCBI genome database for superantigens [136, 138] (Figures 3 and 4). The presence of four SAgs in *S. pyogenes* MGAS5005 and six SAgs in *S. pyogenes* MGAS8232 were initially confirmed by PCR analysis with primers for all eleven streptococcal SAgs (data not shown). Between the two strains of streptococci there are seven unique SAgs encoded: *speA*, *speC*, *speG*, *speJ*, *speL*, *speM*, and *smeZ*.

Anti-SAg antibodies were generated against six SAgs produced in MGAS5005 and MGAS8232, noting that SpeC and SpeJ have cross-reactive epitopes [173]. Recombinant SAgs were expressed by induction of the proteins from pET expression vectors in *E. coli* BL21 (DE3) (Table 2) and purified to apparent homogeneity as previously described (data not shown) [102]. The chromatography-purified SAgs were used as immunogens to generate polyclonal rabbit anti-SAg antibodies. The resulting rabbit polyclonal anti-SAg antibodies in sera were assessed for antibody titres by ELISA (data not shown) (Table 9), and tested for specificity against recombinant pure SAgs by Western blot (Figure 13). There was no cross-reactivity between any of rSAgs when probed with the polyclonal rabbit anti-SAg antibodies, which supports the notion that individual superantigens are immunologically distinct.

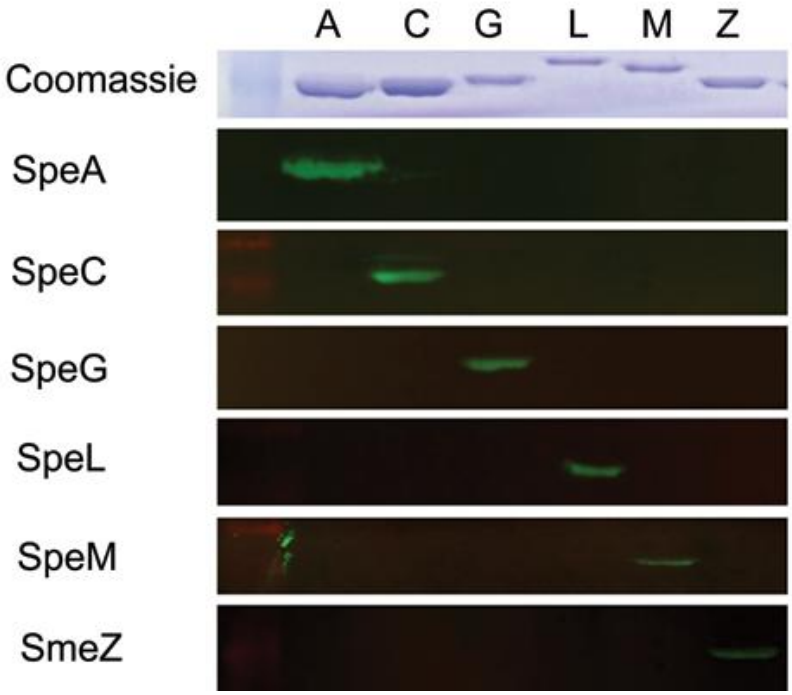
Table 9. Antibodies used for Western blot analysis

Antibody	**Target**	**Dilution[B]**	**Source**
Rabbit anti-SpeA	Polyclonal SpeA	1:32,000	Pro-Sci - This study
Rabbit anti-SpeC[A]	Polyclonal SpeC	1:32,000	Pro-Sci - This study
Rabbit anti-SpeG	Polyclonal SpeG	1:8,000	Pro-Sci - This study
Rabbit anti-SpeL	Polyclonal SpeL	1:32,000	Pro-Sci - This study
Rabbit anti-SpeM	Polyclonal SpeM	1:8,000	Pro-Sci - This study
Rabbit anti-SmeZ	Polyclonal SmeZ	1:8,000	Pro-Sci - This study
IRDye700× conjugated goat anti-human IgG	Human IgG	1:10,000	Rockland Inc. 609-130-123
IRDye800× conjugated donkey anti-rabbit IgG	Rabbit IgG	1:10,000	Rockland Inc. 611-732-127
goat anti-rabbit HRP	Rabbit IgG	1:10,000	Rockland Inc. 611-1302

[A] SpeC antibody cross-reacts with SpeJ [173] and was used for detection of SpeJ at 1:8,000

[B] The dilution was calculated by antibody titre ELISA and then tested by Western blot

Figure 13. Anti-streptococcal superantigen antibodies are specific for their target proteins. Polyclonal anti-streptococcal superantigen antibodies were tested by Western blot for specificity against streptococcal SAgs. Rabbit anti-SpeC cross-reacts with SpeJ, which is the only superantigen that is found in *S. pyogenes* MGAS5005 and not in *S. pyogenes* MGAS8232 (data not shown).


A
C
G
L
M
Z
Coomassie
SpeA
SpeC
SpeG
SpeL
SpeM
SmeZ

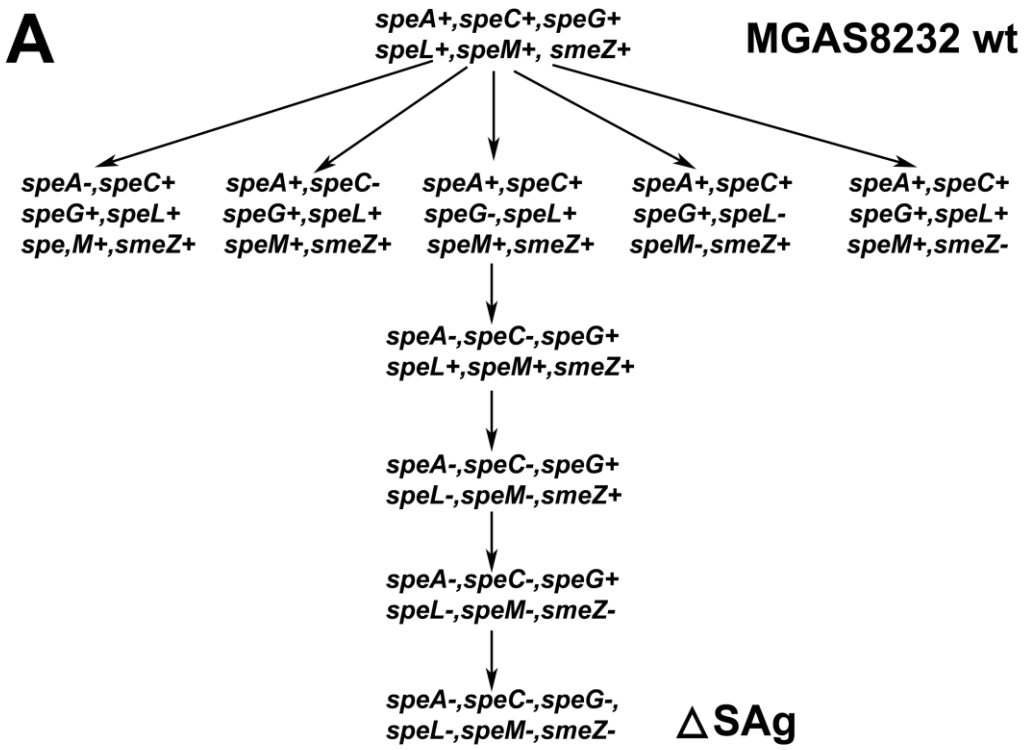
3.2.2. Construction of *Streptococcus pyogenes* MGAS5005 and *Streptococcus pyogenes* MGAS8232 superantigen deletion mutants

To assess the potential contribution of SAgs to the establishment and persistence of colonization by *S. pyogenes* in mouse nasal colonization, isogenic deletion strains of *S. pyogenes* were created with individual and complete SAg deletion profiles (Figure 14). To construct the isogenic deletion strains pG+host5-based plasmids were constructed with a truncated version of the SAg flanked by 500 bp homologous regions upstream and downstream of the SAg. The presence of the electroporated plasmid was confirmed by PCR before the clone was grown under antibiotic selection at a temperature that was not permissive for free-living plasmid replication (Figure 5). After shifting the growth temperature to the non-permissive plasmid replication temperature under antibiotic selection, the integration of the plasmid was confirmed by PCR in clones that grew at 40°C and were erythromycin-resistant (data not shown). Subculture without antibiotic selection at the permissive growth temperature encourages a second recombination event which excises the integrated plasmid from the genome. The plasmid was subsequently cured by growth at 30°C. Erythromycin sensitivity and growth at 30°C was an indicator that *S. pyogenes* transformants had lost the pG+host5 plasmid. Erythromycin-sensitive clones were further tested with primers that were outside of the homologous recombination areas flanking the SAg (Figure 15). The lack of SAg genes in the respective deletion strains was also confirmed using primers internal to the SAgs as listed in Table 4 (Figure 16).

3.2.3. Complementation of *speA* into the *S. pyogenes* MGAS8232 complete superantigen deletion strain.

S. pyogenes MGAS8232 ΔSAg was complemented by *speA* with its native promoter. The native promoter was determined from published data [174] and by scanning the upstream regions for putative (Lactococcal-like) SigmaA binding sites using PPP software (http://bioinformatics.biol.rug.nl/websoftware/ppp) [164]. This *speA* cassette was ‘knocked in’ to the non-coding transgenic region between genes encoding endopeptidase O (*pepO*) and EF-Ts (*tsf*; involved in translational protein elongation [175]). The intergenic region between *pepO* and *tsf* has been used previously for the

Figure 14. Streptococcal superantigen deletion strategy for individual and entire superantigen deletion strains. (A) *S. pyogenes* MGAS8232 encodes six SAgs. Each superantigen was disrupted to generate single deletion strains in *S. pyogenes* MGAS8232. The *speL* and *speM* genes were deleted in tandem, as they are encoded adjacent to one another on a bacteriophage. Each subsequent round deleted an additional superantigen until, after five rounds, a complete isogenic superantigen deletion strain was created. (B) *S. pyogenes* MGAS5005 encodes four SAgs. The SAgs are deleted in a manner similar to those in MGAS8232. The one notable difference was that the final round of superantigen disruption resulted in an isogenic knockout with *speA*, *speJ* and *smeZ* deleted.


A
speA+,speC+,speG+
speL+,speM+, smeZ+
MGAS8232 wt
speA-,speC+
speG+,speL+
spe,M+,smeZ+
speA+,speC-
speG+,speL+
speM+,smeZ+
speA+,speC+
speG-,speL+
speM+,smeZ+
speA+,speC+
speG+,speL-
speM-,smeZ+
speA+,speC+
speG+,speL+
speM+,smeZ-
speA-,speC-,speG+
speL+,speM+,smeZ+
speA-,speC-,speG+
speL-,speM-,smeZ+
speA-,speC-,speG+
speL-,speM-,smeZ-
speA-,speC-,speG-,
speL-,speM-,smeZ-
ΔSAg

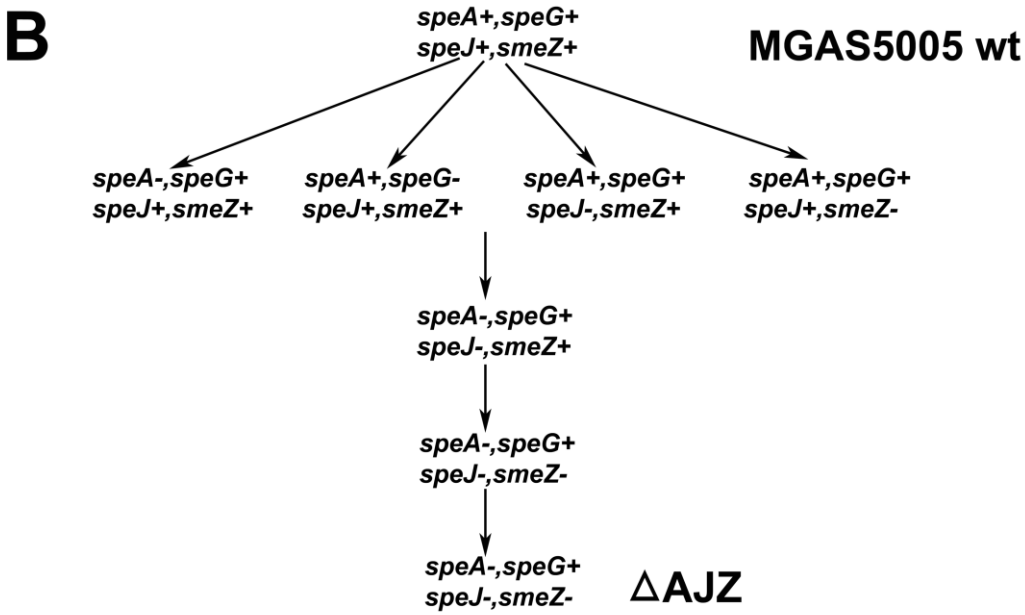

B
speA+,speG+
speJ+,smeZ+
MGAS5005 wt
speA-,speG+
speJ+,smeZ+
speA+,speG-
speJ+,smeZ+
speA+,speG+
speJ-,smeZ+
speA+,speG+
speJ+,smeZ-
speA-,speG+
speJ-,smeZ+
speA-,speG+
speJ-,smeZ-
speA-,speG+
speJ-,smeZ-
ΔAJZ

Figure 15. Strategy to evaluate the superantigen deletion strains. Primers were designed to create and screen for all of the integration and subsequent clearing stages of the pG+host5 superantigen deletion plasmids within the streptococcal genomes. Similarly located primers were designed for all of the superantigen deletion and complementation constructs. The primer sequences are listed in Table 4. All PCR templates were total DNA preparations isolated from *S. pyogenes* cultures. PCR products from primers 2 & 4, and 3 & 5 were cut, ligated together and cloned into pG+host5 to create the superantigen deletion and complementation construct plasmids. Primers 2 and 5 were used in PCR to confirm the presence of the superantigen deletion construct in electroporated clones of *S. pyogenes* by the presence of two different sized PCR products. Integration of the pG+host5 constructs was confirmed by PCR using primers 1, 6, 7, and 8. The final superantigen deletion was confirmed by PCR with primers 1 and 6 and by primers internal to the wild type SAgs. Internal superantigen primers 9 and 10 were used to screen for the presence of wild type SAgs in the isogenic mutant deletion strains.

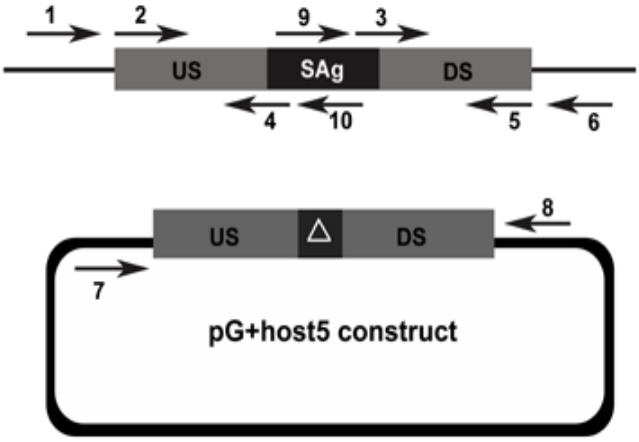
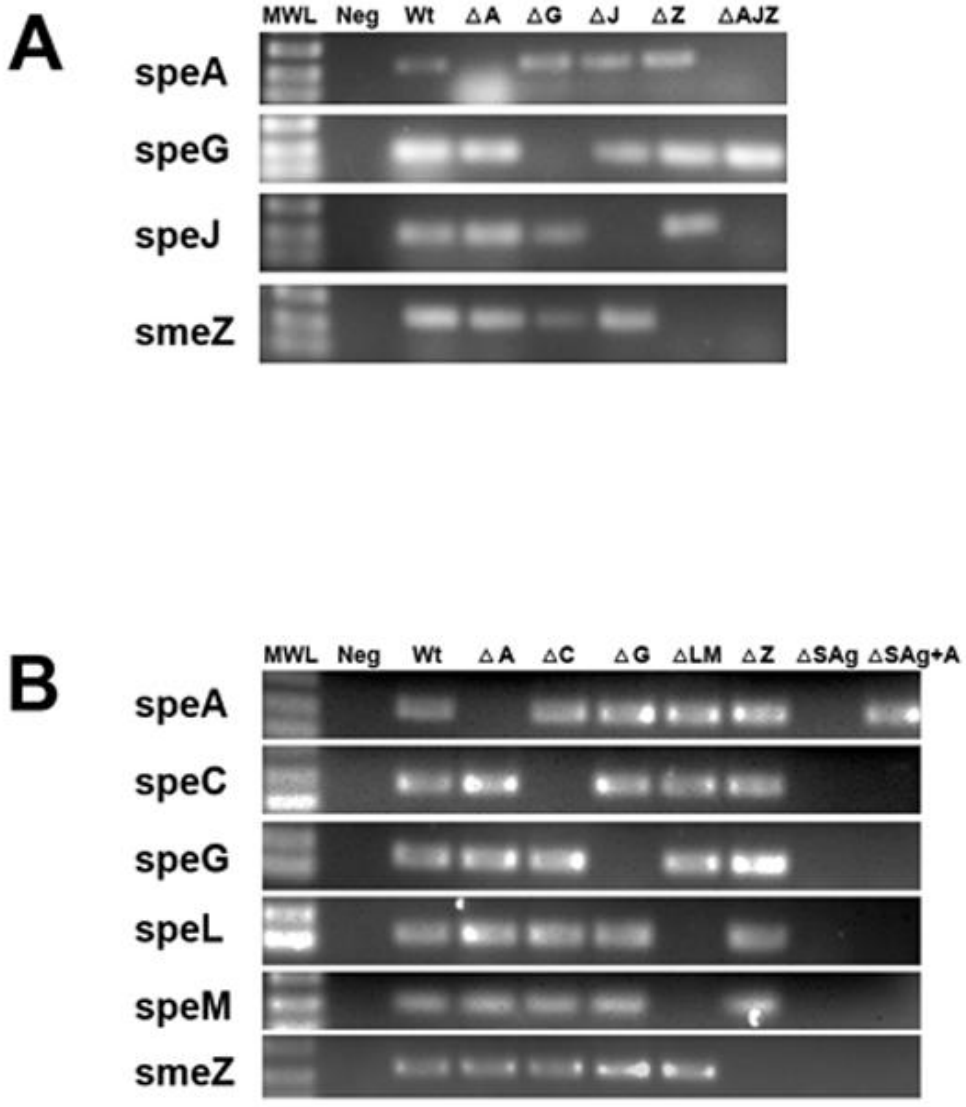

1
2
9
3
US
SAg
DS
4
10
5
6
US
DS
8
7
pG+host5 construct

Figure 16. Confirmation of superantigen deletions in mutant strains of *Streptococcus pyogenes*. Superantigen deletions were confirmed by PCR with primers internal to the superantigen genes for all (A) *S. pyogenes* MGAS5005 and (B) *S. pyogenes* MGAS8232 strains.


A
MWL
Neg
Wt
ΔA
ΔG
ΔJ
ΔZ
ΔAJZ
speA
speG
speJ
smeZ
B
MWL
Neg
Wt
ΔA
ΔC
ΔG
ΔLM
ΔZ
ΔSAg
ΔSAg+A
speA
speC
speG
speL
speM
smeZ

successful insertion of genes into streptococcal strains in our laboratory. This location was initially chosen because the *pepO* and *tsf* genes are oriented in opposite directions facing each other, and there are two hairpin structures present between the two genes which implies that there is no transcriptional read through in that area (Brent Armstrong, personal communication). The presence of the *speA* gene in the complemented clone was confirmed by PCR (Figure 16).

3.2.4. Superantigen deletion did not induce significant growth defects

Streptococcus pyogenes MGAS5005 and MGAS8232 SAg deletion mutants were assessed for growth both by manual (data not shown) and Bioscreen automated growth analysis (Figure 17). There were no gross differences in the *in vitro* growth between the parental wild type MGAS5005 and MGAS8232 and the individual and complete isogenic SAg deletion mutant strains of MGAS5005 and MGAS8232.

3.2.5. Superantigen protein production by *in vitro* cultured isogenic streptococcal deletion mutants confirms superantigen deletion

The production of SAgs from wild type *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232 and isogenic SAg deletion mutants grown *in vitro* was assessed. The precipitated culture supernatants were concentrated 10-fold by TCA precipitation and analyzed by SDS PAGE and Western blot analysis. Western blot using polyclonal rabbit anti-streptococcal SAg antibodies with rSAg used as a positive control (Figure 18). Of the four SAgs encoded in *S. pyogenes* MGAS5005 only SpeA was detectable by Western blot (Figure 18A). Of the six SAgs encoded within the genome of *S. pyogenes* MGAS8232, SpeC was produced in the greatest quantity, and SpeA and SpeL were also detectable by Western blot. SpeG, SpeM and SmeZ were not detectable (Figure 18B). Western blot analysis additionally confirmed that in the *S. pyogenes* deletion strains there was no production of the superantigens when the superantigens were otherwise produced and detected in the *in vitro* expression.

3.3. Role of superantigens in nasal colonization of *Streptococcus pyogenes* MGAS5005 in FVB mice.

Figure 17. Assessment of the growth of isogenic streptococcal superantigen deletion strains. (A) *S. pyogenes* MGAS5005 and (B) *S. pyogenes* MGAS8232 were assessed for growth defects potentially introduced by the superantigen deletion and complementation processes by growth in the Bioscreen assay.

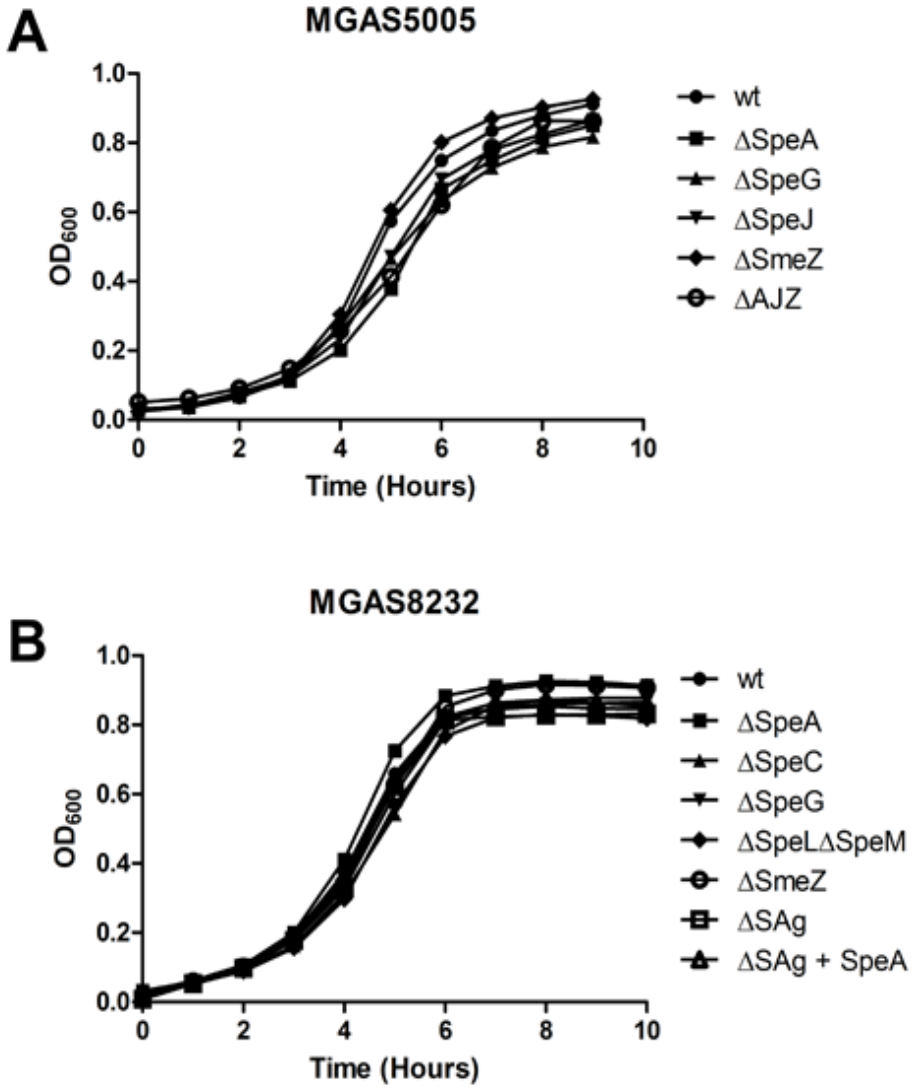
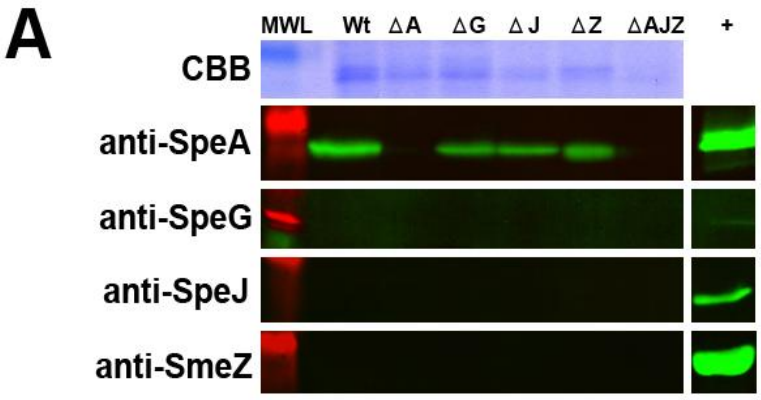
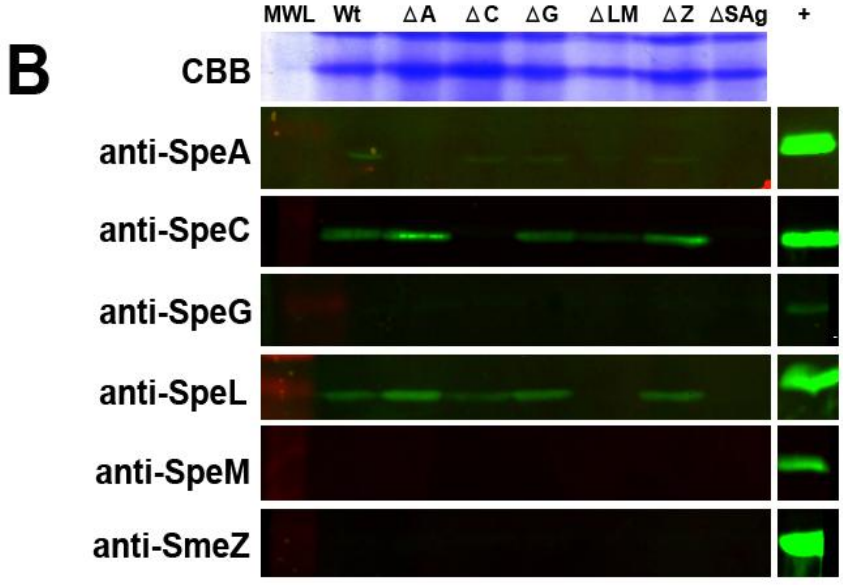

A
MGAS5005
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Time (Hours)
wt
ΔSpeA
ΔSpeG
ΔSpeJ
ΔSmeZ
ΔAJZ
B
MGAS8232
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Time (Hours)
wt
ΔSpeA
ΔSpeC
ΔSpeG
ΔSpeLΔSpeM
ΔSmeZ
ΔSAg
ΔSAg + SpeA

Figure 18. Expression analysis of streptococcal superantigens in the isogenic superantigen deletion strains. TCA protein precipitation of all streptococcal supernatants from cultures grown to late exponential phase were assessed for superantigen production by Western blot. (A) *S. pyogenes* MGAS5005 produced similar amounts of detectable SpeA in all strains with the exception of the deletion strains. (B) *S. pyogenes* MGAS8232 produced detectable SpeA, SpeC and SpeL that was of similar concentration in all isogenic strains unless the respective gene was deleted.


A
MWL
Wt
ΔA
ΔG
ΔJ
ΔZ
ΔAJZ
+
CBB
anti-SpeA
anti-SpeG
anti-SpeJ
anti-SmeZ


B
MWL
Wt
ΔA
ΔC
ΔG
ΔLM
ΔZ
ΔSAg
+
CBB
anti-SpeA
anti-SpeC
anti-SpeG
anti-SpeL
anti-SpeM
anti-SmeZ

3.3.1. Streptococcal superantigens influence the colonization of *Streptococcus pyogenes* MGAS5005 in the nasal passage of FVB mice

Wild type *S. pyogenes* MGAS5005 efficiently colonized FVB mice with ~8 logs of streptococci recovered from the cNT of the mice at 48 hours post-inoculation. FVB mice that were inoculated with the MGAS5005 Δ*speA* and MGAS5005 Δ*speG* isogenic deletion strains had no significant decrease in streptococcal burden recovered from the cNT of mice sacrificed 48 hours post-inoculation. Inoculation of FVB mice with *S. pyogenes* MGAS5005 Δ*speJ*, Δ*smeZ* and Δ*speA*/*speJ*/*smeZ* resulted in significantly fewer streptococci recovered from the cNT (Figure 19). There was also a striking difference in the amount of piloerection, which is indicative of stress in mice, observed in wild type MGAS5005-treated mice at 48 hours, which was notably absent in the saline-treated, and MGAS5005 Δ*speA*/*speJ*/*smeZ*-treated mice (Figure 20), and there were no significant differences in the amount of *S. pyogenes* recovered from the other organs (Figure 21). Mice inoculated with MGAS5005 Δ*speA* and Δ*speG* were piloerected similar to wild-type-treated FVB mice, and MGAS5005 Δ*speJ*-, and Δ*smeZ*-treated mice were not piloerected (data not shown), which corresponds to the streptococcal load assessed in the mice at 48 hour post-inoculation.

3.4. Role of superantigens in nasal colonization of *Streptococcus pyogenes* MGAS8232 in DR4/DQ8 mice

3.4.1. Streptococcal superantigens produced by *S. pyogenes* MGAS8232 contributed to the colonization of the nasal passage at 48 hours post-inoculation

Wild type MGAS8232 colonized the cNT of DR4/DQ8 mice at ~6 logs at 48 hours. Although most of the individual SAg deletions did not impact the colonization potential of MGAS8232, MGAS8232 Δ*speA* and MGAS8232 ΔSAg both had dramatic reductions in the ability to colonize nasal tissue. Although there was some variation in the number of streptococci recovered from the other isogenic mutant streptococci, there were no statistically significant differences between those groups and the wild-type MGAS8232-treated mice (Figure 22).

Streptococcus pyogenes MGAS8232 ΔSAg was complemented with a copy of speA integrated into the streptococcal genome between the *pepO* and *tsf* genes to create *S.*

Figure 19. Specific superantigens are critical for successful colonization of cNT by *Streptococcus pyogenes* MGAS5005 in FVB mice. Nine week-old FVB mice were inoculated with wild type MGAS5005 and isogenic superantigen deletion mutants as indicated. The cNT samples were homogenized and the bacterial burden of *S. pyogenes* strains was assessed at 48 hours. Shown are the data points from individual mice, with the average (± SEM) log10 cfu/cNT. (*, $P < 0.05$, and ***, $P < 0.0005$ as assessed by unpaired t-test) (N=4-8).

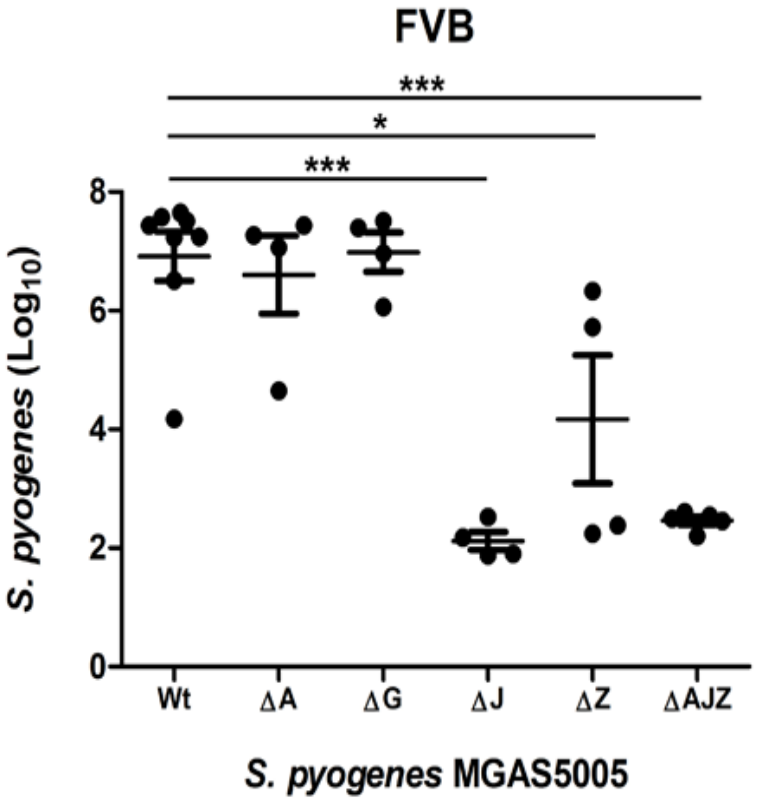

FVB
***
*
***
8
6
4
2
0
S. pyogenes (Log10)
Wt
ΔA
ΔG
ΔJ
ΔZ
ΔAJZ
S. pyogenes MGAS5005

Figure 20. Piloerection of FVB mice in *Streptococcus pyogenes* MGAS5005-treated mice. Nine week old FVB mice were inoculated nasally with 1×10^8 *S. pyogenes* MGAS5005. Mice were photographed under anesthesia at 48 hours post-infection, immediately prior to sacrifice. Photographs are representative of three mice per group.

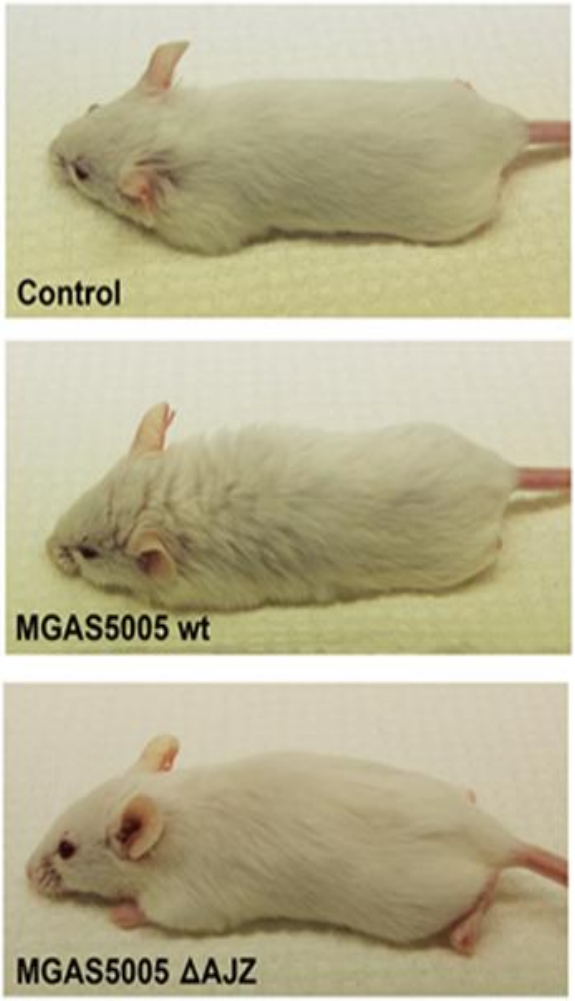

Control
MGAS5005 wt
MGAS5005 ΔAJZ

Figure 21. *Streptococcus pyogenes* MGAS5005 recovered from the organs of nasally-treated FVB mice. There were no streptococci recovered from the *S. pyogenes* MGAS5005-deletion mutants the blood, the kidney or the spleen. There were no significant difference in the number of streptococci recovered from the liver, and heart. There were significantly fewer streptococci recovered from the lungs of *S. pyogenes* MGAS5005 ΔSpeJ and ΔSpeA/SpeJ/SmeZ-treated mice. Statistics were assessed by unpaired t test. *, $P < 0.05$, and **, $P < 0.005$ (N=4-8).

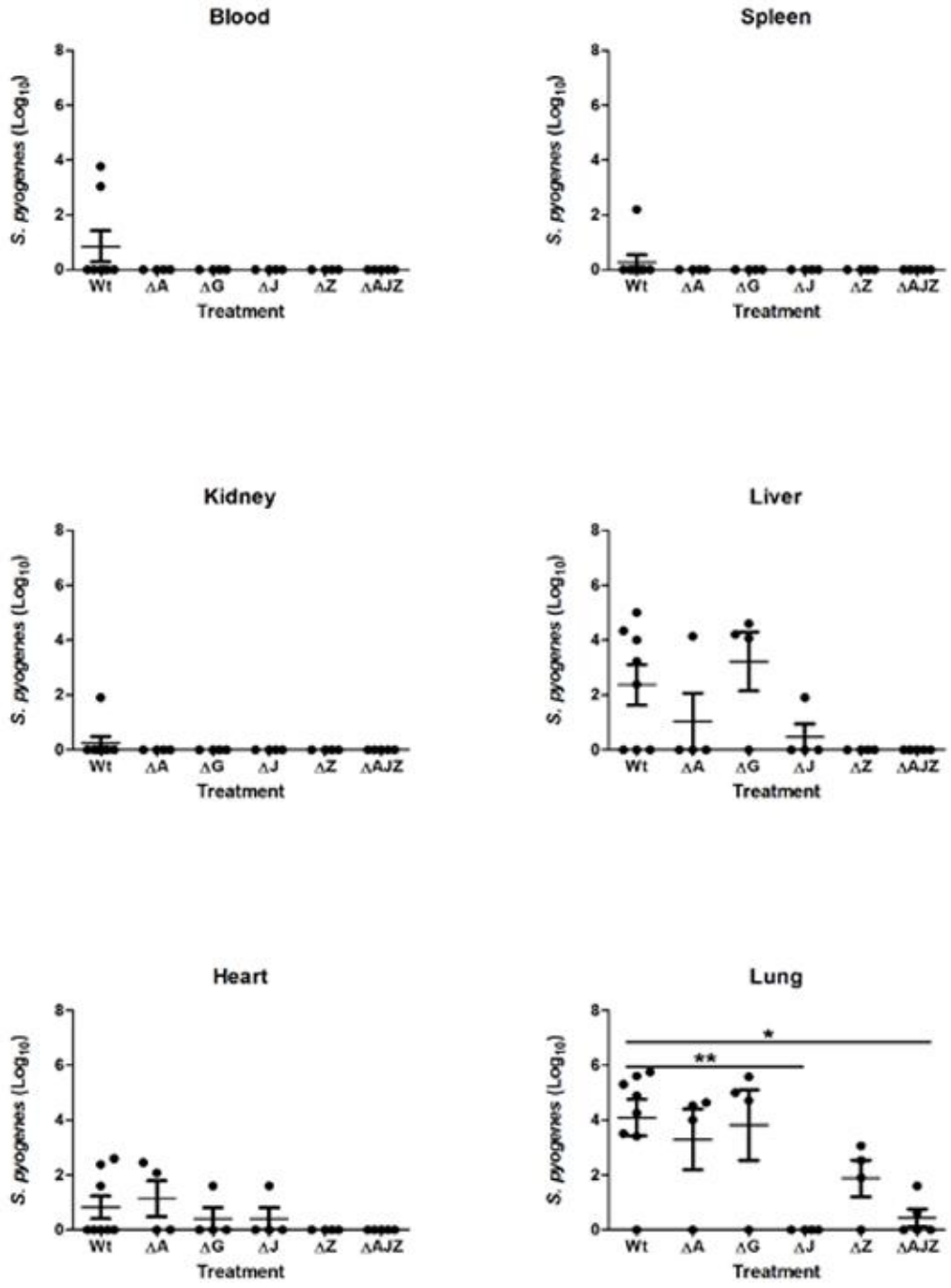
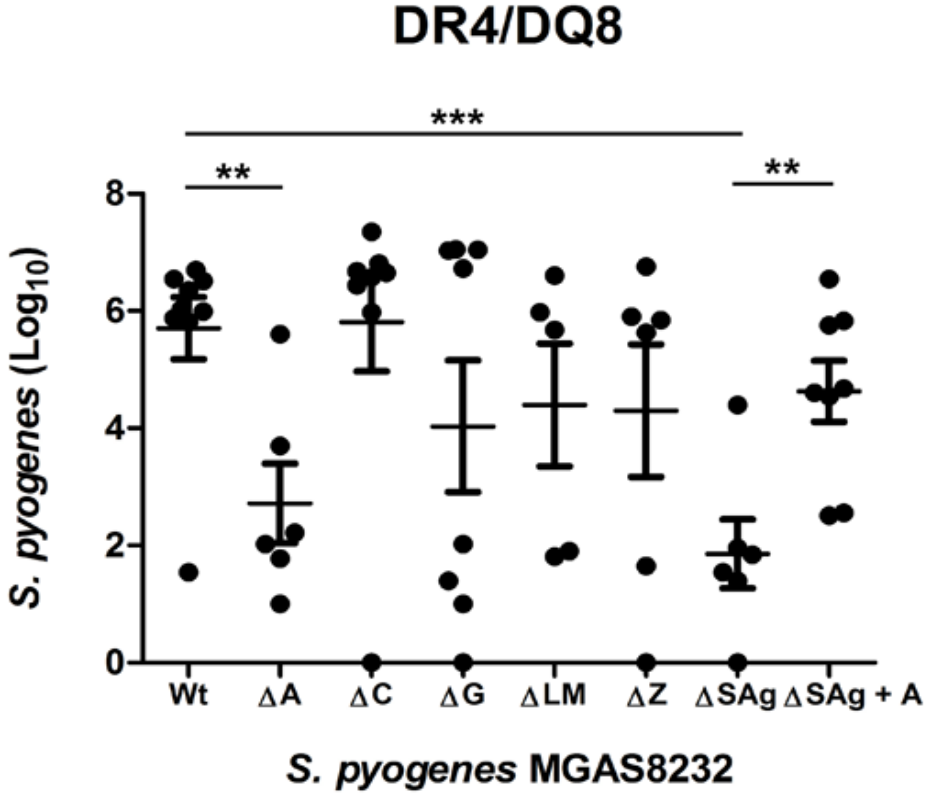

Blood
Spleen
Kidney
Liver
Heart
Lung
S. pyogenes (Log10)
Wt
ΔA
ΔG
ΔJ
ΔZ
ΔAJZ
Treatment
*
**

Figure 22. Nasal colonization by isogenic superantigen deletion strains of *Streptococcus pyogenes* MGAS8232. DR4/DQ8 transgenic mice were treated nasally with 1×10^8 cfu *S. pyogenes* MGAS8232 wild type and isogenic superantigen deletion mutant and were sacrificed at 48 hours post-infection. Recovered cNT were homogenized and the concentration of *S. pyogenes* was assessed as the number of colonies that were β-haemolytic on TSAII sheep blood agar plates. There was a statistically significant decrease in recoverable *S. pyogenes* in the Δ*speA* and the complete superantigen deletion-treated mice. *speA* complementation in the genome resulted in a statistically significant increase in recoverable *S. pyogenes*. Shown are the data points from individual mice, with the average (± SE) log10 cfu/cNT. (**, $P < 0.005$, and ***, $P < 0.0005$ as assessed by unpaired t-test) (N=5-8).


DR4/DQ8
***
**
**
S. pyogenes (Log10)
8
6
4
2
0
Wt
ΔA
ΔC
ΔG
ΔLM
ΔZ
ΔSAg
ΔSAg + A
S. pyogenes MGAS8232

pyogenes MGAS8232 ΔSAg + SpeA. Nasal infection with the complementation strain of *S. pyogenes* MGAS8232 ΔSAg + SpeA resulted in a significantly greater recovery of streptococci at 48 hours post-inoculation as compared to the *S. pyogenes* MGAS8232 ΔSAg, and it was not statistically significantly different in the recovery from the *S. pyogenes* MGAS8232 wild type.

3.4.2. Streptococcal burden in the organs of *Streptococcus pyogenes* MGAS8232-infected transgenic DR4/DQ8 mice

There were fewer streptococci recovered from the organs of *S. pyogenes* MGAS8232 mutant-infected DR4/DQ8 mice as compared to *S. pyogenes* MGAS5005 mutant-infected FVB mice (Figure 23). Streptococci were detected in the lungs and livers and hearts of some mice. However, there were no statistically significant difference in the amounts of streptococci recovered from the organs of mice inoculated with the wild type and mutant *S. pyogenes* MGAS8232.

3.4.3. Superantigen deletion impacts streptococcal burden at 48 hours, but not the persistence of *S. pyogenes* in DR4/DQ8 nasal passage

The kinetics of *S. pyogenes* MGAS8232 infections in transgenic DR4/DQ8 mice were addressed by nasally infecting mice and assessing the streptococcal burden in the cNT at one, two, three, four, and six day time points. Both the wild type and complete SAg deletion strains of *S. pyogenes* MGAS8232, at 24 hours, were cleared to a level that was lower than the inoculating dose and there was no significant difference in the streptococcal burden recovered from the cNT at this time point. At 48 hours, MGAS8232 ΔSAg colonization was dramatically decreased in comparison to wild type MGAS8232. However, despite the inability of the mutant to recover to wild-type levels at 48 hours it still persisted in the cNT of the transgenic DR4/DQ8 mice for the same length of time as the wild-type *S. pyogenes* MGAS8232, and was not recovered in statistically significantly different amounts between groups between days three and six (Figure 24). This suggests that streptococcal SAgs play a role in the initial establishment of colonization in this model, but not in the kinetics of clearance.

Figure 23. *Streptococcus pyogenes* MGAS8232 recovered from the organs of nasally-treated DR4/DQ8 mice. There were no detectable streptococci in the blood, spleen, kidney and heart of DR4/DQ8 mice nasally treated with *S. pyogenes* MGAS8232. Some streptococci were detected in liver and lung homogenates. Shown are the data points from individual mice, with the average (± SEM) log10 cfu/organ. There was no statistical significance between groups as assessed by unpaired t test.

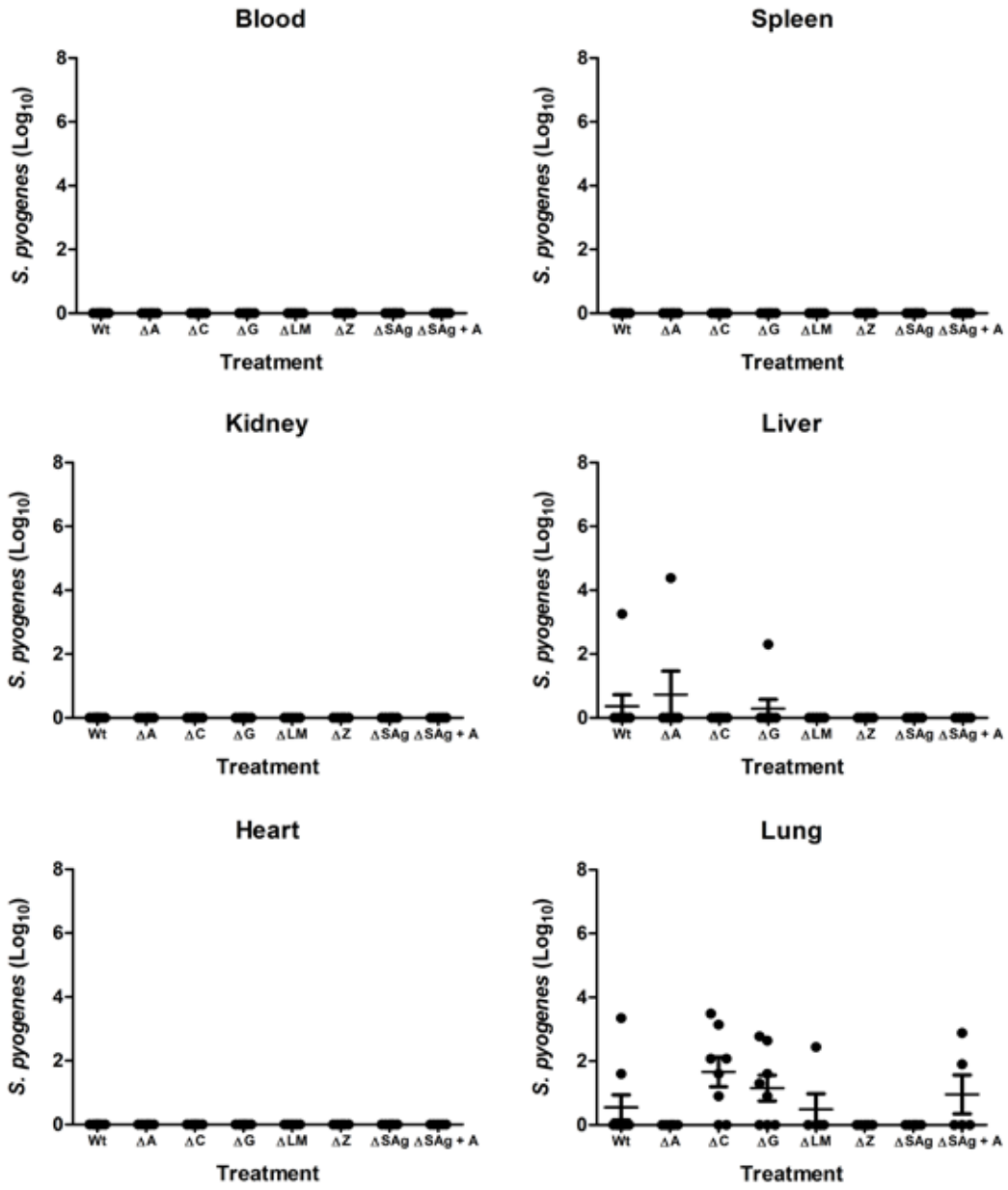
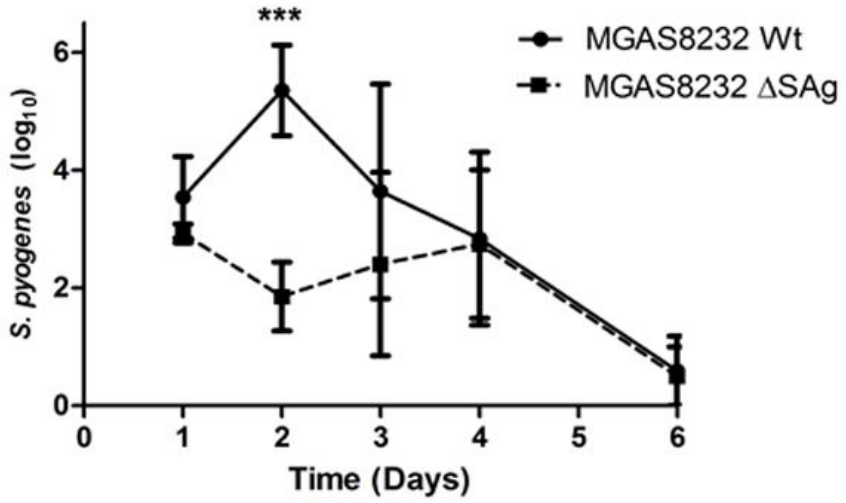

Blood
Spleen
Kidney
Liver
Heart
Lung
S. pyogenes (Log10)
0
2
4
6
8
Wt
ΔA
ΔC
ΔG
ΔLM
ΔZ
ΔSAg
ΔSAg + A
Treatment

Figure 24. Kinetics of nasal colonization by *Streptococcus pyogenes* MGAS8232 wild-type and ΔSAg strains in DR4/DQ8 mice. DR4/DQ8 mice were inoculated with *S. pyogenes* MGAS8232 wild type and ΔSAg and were sacrificed at day 1, 2, 3, 4, and 6 and the streptococcal burden in the cNT was assessed. Statistically different amounts of *S. pyogenes* MGAS8232 wild type and *S. pyogenes* MGAS8232 ΔSAg were recovered at day 2. There were no statistically significant differences in the number of streptococci recovered at any other time point assessed. Shown is the average of individual mouse data points, with the average (± SEM) log10 cfu/cNT. (***, $P < 0.0005$ as assessed by unpaired t-test) (N=4-8).


***
MGAS8232 Wt
MGAS8232 ΔSAg
S. pyogenes (log10)
0
2
4
6
0
1
2
3
4
5
6
Time (Days)

3.4.4. Infection with a superantigen-competent strain of *S. pyogenes* MGAS8232 results in greater mucus production and cellular debris in the nasopharyngeal meatus

Two representative haematoxylin and eosin-stained histological sections of the heads of three mice in each treatment group were examined along the entire length of the respiratory epithelial lining in the nasopharyngeal meatus, including the lymphoepithelium covering the NALT and over the entire length of the neuroepithelium in the ethmoid turbinates for the presence of mucus, cellular debris, tissue damage and other abnormalities by two independent researchers in a blinded fashion. Scores between zero to two points were assigned to each sample depending on the presence and amount of mucus, red blood cells and nucleated cellular debris along the respiratory (including lymphoepithelium) and the neuroepithelium in the nasal turbinates. Scores were averaged and assessed statistically by unpaired t test. There were no statistically significant differences between the treatment groups in the neuroepithelium (Figure 25B). In the respiratory epithelial lining of the nasopharyngeal meatus there was a greater incidence of red blood cells and nucleated cells in the mucus covering the respiratory epithelium in the nasopharyngeal meatus in wild type *S. pyogenes* MGAS8232-treated mice as compared to the saline- or *S. pyogenes* MGAS8232 ΔSAg-treated mice (Figure 25A). There was no visible damage or break in the continuity of the either the neuroepithelium or the respiratory epithelium observed in any of the sections examined, hence we were unable to attribute the cellular debris to a specific epithelial damage.

3.4.5. Immunophenotyping of spleen, lymph node, and nasal cells in *S. pyogenes* MGAS8232-treated mice

Transgenic mice expressing HLA DR4/DQ8 were inoculated with either saline, wild type *S. pyogenes* MGAS8232, or *S. pyogenes* MGAS8232 ΔSAg were sacrificed at 48 hours and the spleen, pooled lymph nodes and cNT were collected. The cells were stained for differential markers for immune cells and analyzed by flow cytometry. Gating strategies for flow cytometry are detailed in Figures 26, 28, and 30.

One hundred thousand events were captured for each treatment. Both the spleen and lymph nodes were gated on live cells based on 7AAD staining and were assessed for the presence of different cellular types based on staining. Cellular concentrations were

assessed as the proportion of cells that were live. Although there were subtle changes in the cell populations detected in the spleen, there were no statistically significant differences in the immune cell populations between treatment groups (Figure 27). The pooled lymph nodes included the cervical, the axillary, iliac and the popliteal lymph nodes. There was a statistically significant increase in the F4/80+ macrophage population between the *S. pyogenes* MGAS8232 wild type and isogenic deletion-treated mice (Figure 29).

One hundred thousand events were captured for each sample. The samples were gated on the live cell populations based on 7AAD staining. The live cells were further gated on the CD45+ cell population. Live CD45+ cell populations in the cNT were assessed by flow cytometry for various immune cell populations. There was a significant decrease in the CD11c+ DC cell populations in the cNT with *S. pyogenes* MGAS8232 wild type-treated mice as compared with the *S. pyogenes* MGAS8232 ΔSAg-treated mice, and lower than the saline-treated mice (Figure 31). The *S. pyogenes* MGAS8232 ΔSAg-treated mouse cNT CD11c+ cell population was not significantly different from the saline-treated mice. There were no other significant differences in any of the other cell populations assessed in the cNT.

Figure 25. Histological assessment of nasal tissue sections. Difference in the pathological severity in mice inoculated with saline, *S. pyogenes* MGAS8232 wild type and ΔSAg-treated mice sacrificed at 48 hours post-infection were assessed by examination of haematoxylin and eosin stained sections. Sections were assessed for the presence and severity of mucus, red blood cells, and nucleated cellular debris on the surface of the (A) respiratory epithelium and the (B) neuroepithelium. There was a significantly greater amount of mucus and cellular debris observed in the *S. pyogenes* MGAS8232 wild type-treated mice as compared to the saline- and ΔSAg-treated mice. Shown is the average of individual mouse data points, with the average (± SEM). (*, $P < 0.005$ as assessed by unpaired t-test).

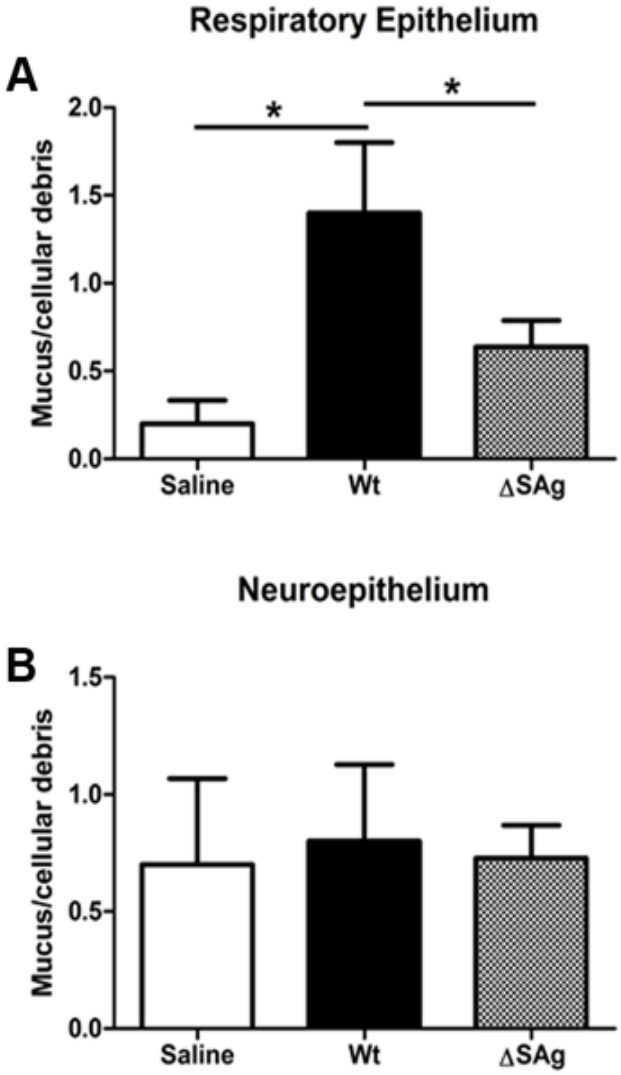
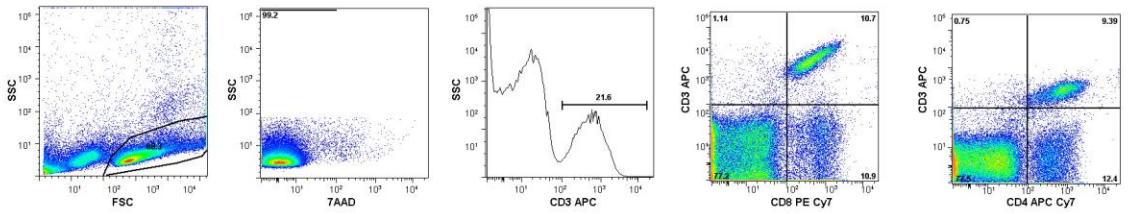
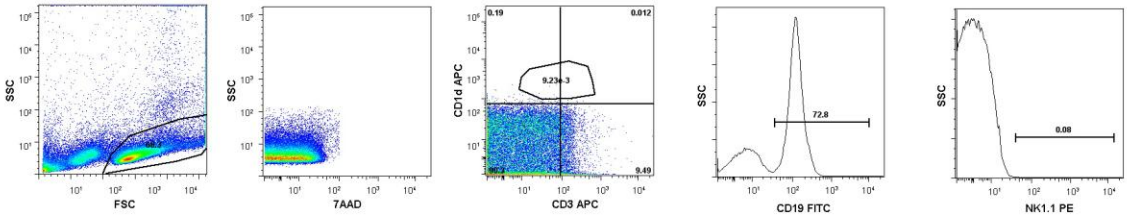

Respiratory Epithelium
A
Mucus/cellular debris
2.0
1.5
1.0
0.5
0.0
*
*
Saline
Wt
ΔSAg
Neuroepithelium
B
Mucus/cellular debris
1.5
1.0
0.5
0.0
Saline
Wt
ΔSAg

Figure 26. Splenocyte gating strategies to assess the proportions of immune cells in mice treated with *S. pyogenes*. Splenocytes were isolated from mice treated with HBSS, *S. pyogenes* MGAS8232 wild type or *S. pyogenes* MGAS8232 ΔSAg. Representative gating strategies are shown for all panels used to determine proportions of immune cells in the spleen. Splenocytes in all panels were first separated by forward and side scatter and gated on the lymphocyte population, and then further gated on the live cell populations based on 7AAD staining. Immune cells were assessed based on immunoreceptor staining. One hundred thousand events were captured for each sample.

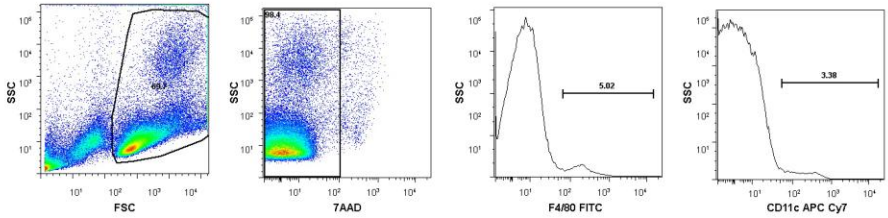
Panel 1



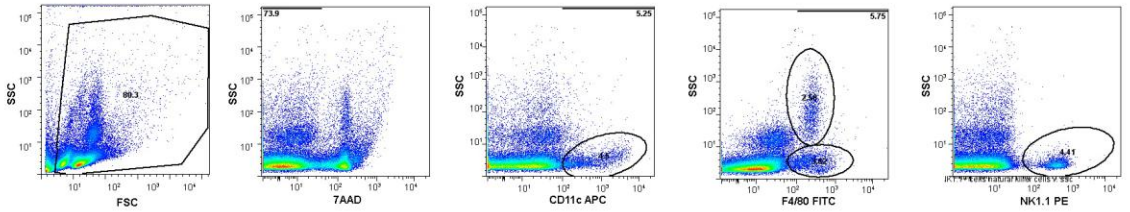
Panel 2



Panel 3



Panel 6



Panel 7

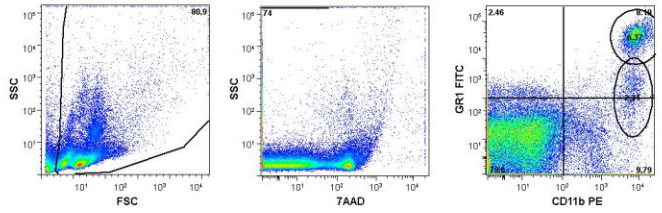


Figure 27. Nasal infection with wild type and superantigen deletion strains of *Streptococcus pyogenes* MGAS8232 does not result in changes in the lymphocyte population in the spleen. Mice were inoculated with HBSS (empty bar), 1×10^8 cfu *S. pyogenes* MGAS8232 wild type (black bar) or *S. pyogenes* MGAS8232 ΔSAg (grey bar). Mice were sacrificed at 48 hours post-infection. The spleen was harvested and cells were stained to identify immune cell sub-populations by flow cytometry. There were no statistically significant differences in (A) any of the immune cells assessed (N=6) including the (B) GR1+ and GR1+CD11b+ populations (N=2). Shown is the average of individual mouse data points, with the average (± SE).

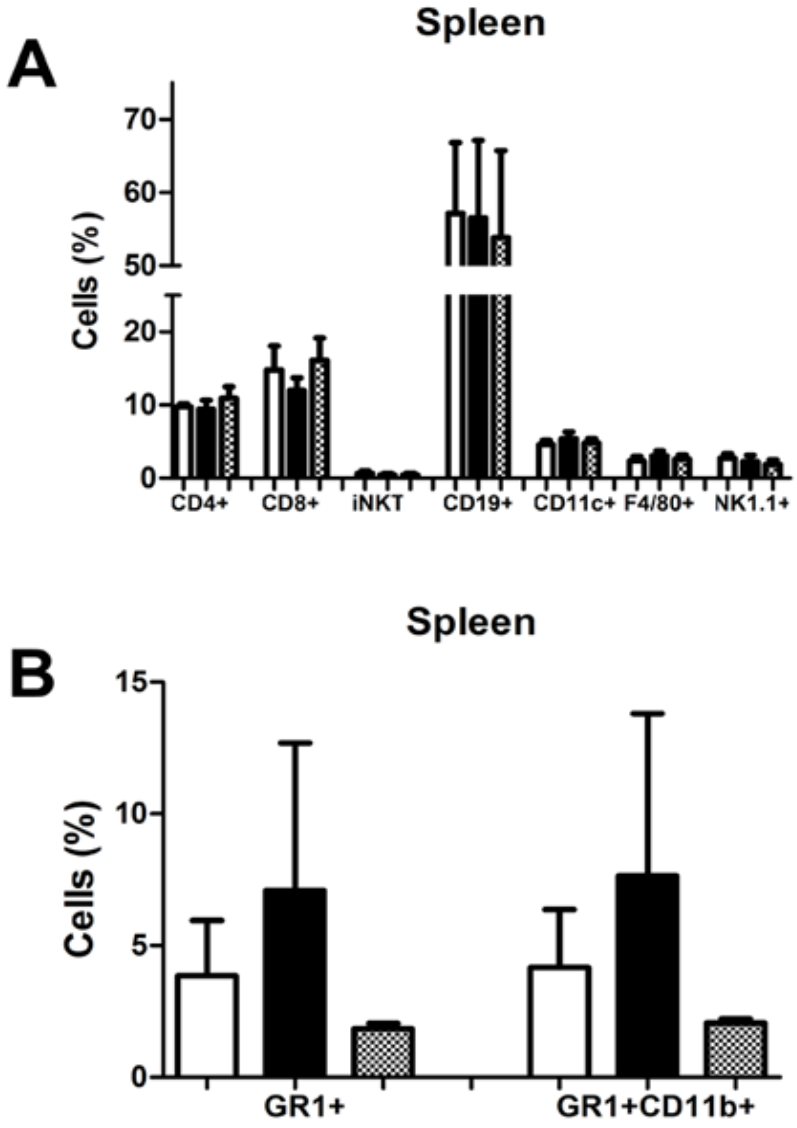
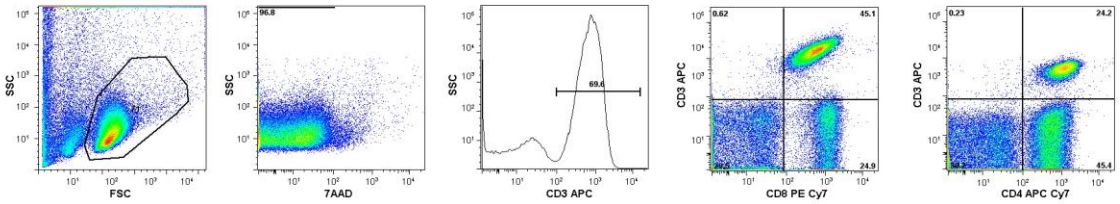
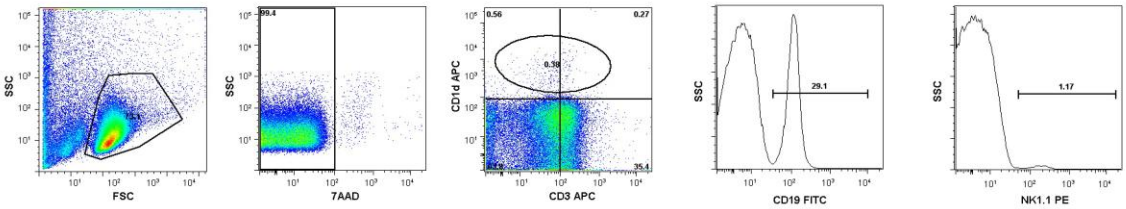

A
Spleen
Cells (%)
70
60
50
20
10
0
CD4+
CD8+
iNKT
CD19+
CD11c+
F4/80+
NK1.1+
B
Spleen
Cells (%)
15
10
5
0
GR1+
GR1+CD11b+

Figure 28. Lymph node gating strategies to assess the proportions of immune cells in mice treated with *S. pyogenes*. Total pooled lymph nodes were isolated from mice treated with HBSS, *S. pyogenes* MGAS8232 wild type or *S. pyogenes* MGAS8232 ΔSAg. Representative gating strategies are shown for all panels used to determine proportions of immune cells in the lymph nodes. Cells in all panels were first separated by forward and side scatter and gated on the lymphocyte population, and then further gated on the live cell populations based on 7AAD staining. Immune cells were assessed based on immunoreceptor staining. One hundred thousand total events were captured for each sample.

Panel 1



Panel 2



Panel 3

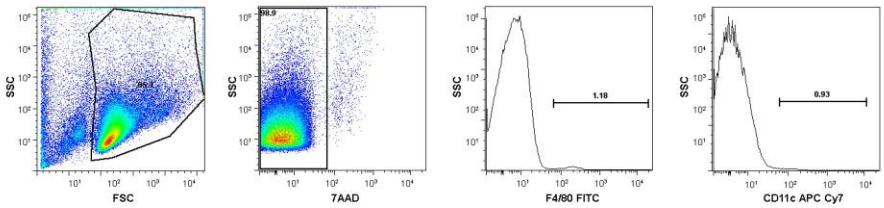


Figure 29. Nasal infection with wild type and superantigen deletion strains of *Streptococcus pyogenes* MGAS8232 results in significantly more macrophages in the pooled lymph nodes. Mice were inoculated with HBSS (empty bars), 1×10^8 *S. pyogenes* MGAS8232 wild type (black bars) or *S. pyogenes* MGAS8232 ΔSAg (grey bars). The mice were sacrificed at 48 hours post-infection. Cells were stained to identify immune cell sub-populations by flow cytometry. A statistically significant difference was detected in the F4/80+ macrophage population between *S. pyogenes* MGAS8232 and isogenic knockout-treated mice (P=0.0357) as assessed by unpaired t-test (N=4). Shown is the average of individual mouse data points, with the average (± SE). (*, P = 0.05 as assessed by unpaired t-test).

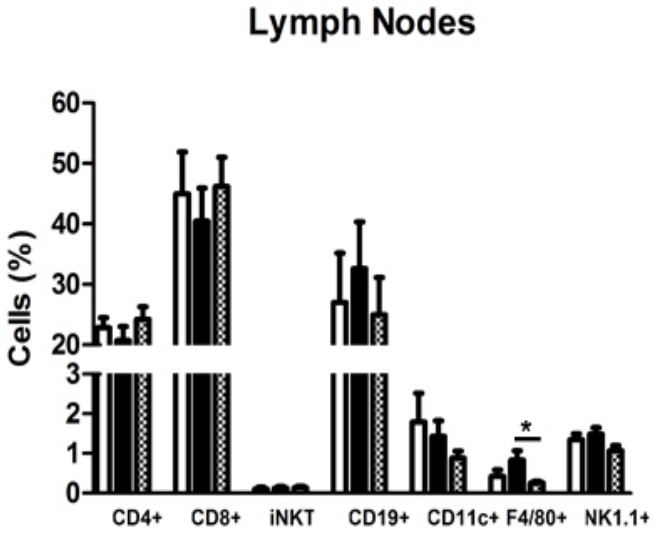
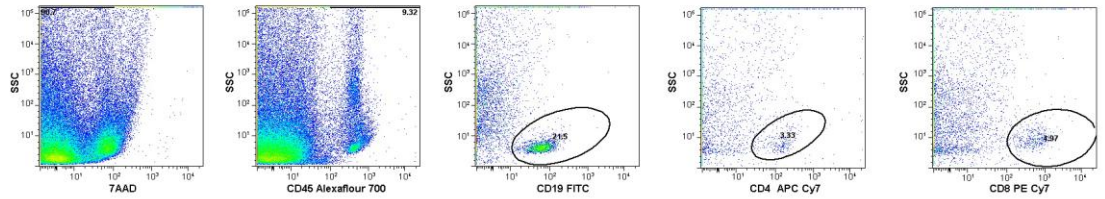
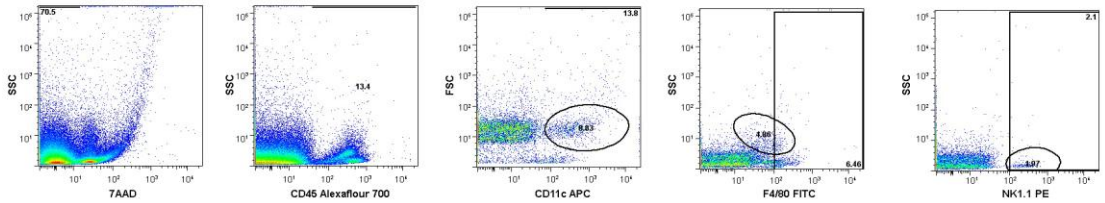

Lymph Nodes
Cells (%)
60
50
40
30
20
3
2
1
0
*
CD4+
CD8+
iNKT
CD19+
CD11c+
F4/80+
NK1.1+

Figure 30. Gating strategies to assess the proportions of immune cells in mice treated with *S. pyogenes* in the complete nasal turbinates. The complete nasal turbinates were isolated from mice treated with HBSS, *S. pyogenes* MGAS8232 wild type or *S. pyogenes* MGAS8232 ΔSAg at 48 hours post-infection. Representative gating strategies are shown for all panels used to determine proportions of immune cells in the complete nasal turbinates. The complete nasal turbinates cells were first separated based on 7AAD staining and gated on live cells only. The live cells were further gated on CD45 positive population before being assessed for the proportion of immune cells. One hundred thousand total events were captured for each sample.

Panel 1



Panel 6



Panel 7

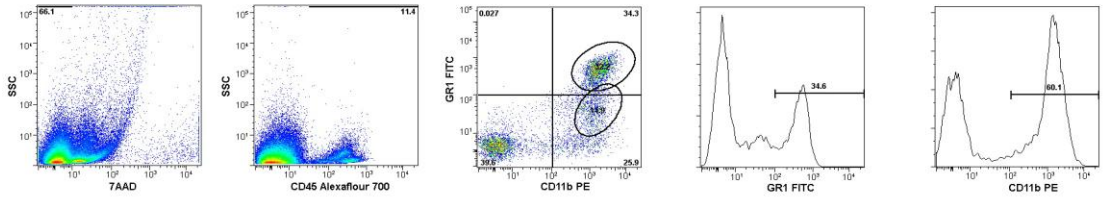
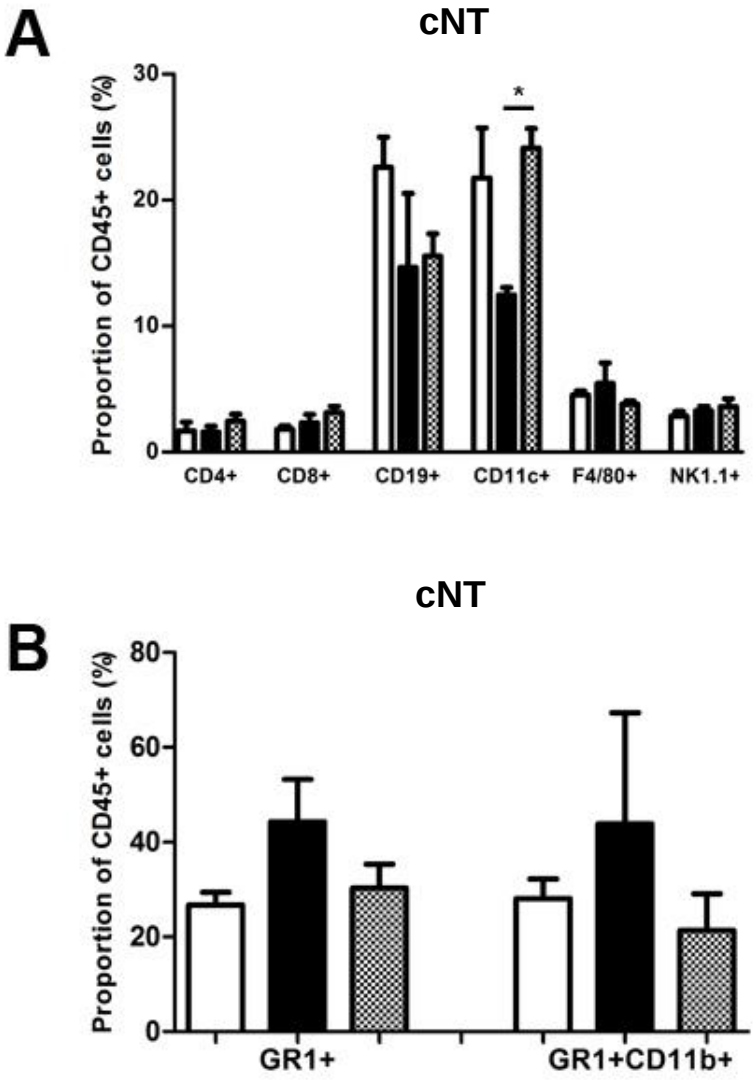


Figure 31. Nasal infection with wild type deletion strains of *Streptococcus pyogenes* MGAS8232 results in a decrease in CD11c+ cells in the cNT. Mice were inoculated with HBSS (clear bars), 1×10^8 cfu *S. pyogenes* MGAS8232 wild type (black bars) or *S. pyogenes* MGAS8232 ΔSAg (grey bars). Mice were sacrificed at 48 hours post-infection and the cNT including NALT were harvested and stained to identify immune cell sub-populations in the various tissues by flow cytometry. All samples were gated to exclude 7AAD positive cells (dead cells). Additionally, cells from the cNT were gated on all CD45-positive cells to indicate the lymphocytic population. Immune cell populations assessed in (A) had an N of ≥ 4. (B) The CD11b+ cell marker marks myeloid progenitor cells (N=2). A statistically significant difference in the CD11c+ DC cell populations in the cNT was observed between the wild-type and isogenic knockout-treated (P=0.0304). Shown is the average of individual mouse data points, with the average (± SEM). (*, $P < 0.05$ as assessed by unpaired t-test).


A
cNT
Proportion of CD45+ cells (%)
30
20
10
0
*
CD4+
CD8+
CD19+
CD11c+
F4/80+
NK1.1+
B
cNT
Proportion of CD45+ cells (%)
80
60
40
20
0
GR1+
GR1+CD11b+

Chapter 4: Discussion

The bacterial SAg toxins have evolved to target two critical receptors of adaptive immunity, and although the role of these toxins in severe disease is well established, little is known about why so many different SAgs are encoded within the *S. pyogenes* genome, and why ultimately these genes are maintained in the genome as the bacteria evolve. The natural lifecycle of *S. pyogenes* is a state of colonization in the pharynx, where most carriers do not develop symptomatic pharyngitis. In fact, ~20% of school children in the winter and spring may be asymptomatic chronic carriers of *S. pyogenes* [176]. Thus, we believe that SAgs have evolved in the context of "colonization", rather than "virulence", and therefore their true evolutionary role is likely important for the state.

There are eleven identified streptococcal SAgs [116, 123-125] and to date, every sequenced strain of *S. pyogenes* encodes multiple SAgs within its genome [35, 57-58, 126, 136, 138, 177-184]. Despite the impressive body of work focused on streptococcal SAgs as potent virulence factors, there have been no efforts made to understand the physiological function of streptococcal SAgs in the transmission, establishment of colonization, and persistence of *S. pyogenes* within the human population. In addition, there are very few studies that have examined the role of streptococcal SAgs using defined genetic deletion strains [2, 59, 88, 112-113], and even fewer that are coupled with live *in vivo* infections in appropriate animal models [185], and none have examined the role of SAgs in colonization and mild infection in an appropriate mouse model. Superantigens have been shown, indirectly, to play a role in the early stages of mild pharyngeal infection as examined by transcriptome analysis of *Streptococcus*-infected macaques [82]. Although there is a lack of mechanistic studies to address these questions, a role for SAgs in the persistence of *S. pyogenes* in humans with mild infection or colonization is supported by the existence of many healthy adults who have not had severe or invasive streptococcal infections, and yet have antibodies against streptococcal SAgs [133-134].

Group A streptococci are obligate human pathogens [3], and their primary site of colonization is the human tonsils and *S. pyogenes* is often found in surgically excised tonsils [147]. Human tonsils, or Waldeyer's ring, are composed of the adenoid, bilateral tubular, palatine, and lingual tonsils [142]. Physiologically similar structures have been

identified in other mammals [145], and in mice the functionally analogous tissues are the NALT, which are located bilaterally along the base of the nasal passage in mice [143].

The streptococcal colonization model of nasal infection has been previously established as a model to mimic human colonization or pharyngeal infection [149]. The surgical technique used in this work to isolate cNT was learned directly from the laboratory of Dr. P. Patrick Cleary at the University of Minnesota (Figure 6). Use of this technique deviates from other NALT extraction techniques published in the literature [144]. This technique resulted in consistent and highly reproducible streptococcal and bacterial counts from *S. pyogenes* colonized mice (Figures 9 and 10), and when assessed for immune cells by flow cytometry, gave consistent proportions of subsets of live CD45+ immune cells (Figure 31). Together, these results validate the surgical method as an alternate method for the removal of cNT which includes the NALT along with the ethmoid turbinates and maxillary sinuses. The details of this model and the surgical method, as described in this thesis, which represents a significant contribution to this field of research.

The genetic makeup, innate defenses, cytokine levels and the potential presence of underlying health conditions which may alter the immune responsiveness of the host may influence the course of a streptococcal infection [186]. Also, the ability of the MHC class II to determine the clinical outcome of severe or invasive streptococcal infection has been previously established [112-115, 135]. We found that murine lineages expressing different murine or human MHC class II molecules respond differently to nasal challenge by different *S. pyogenes* strains, as assessed at 48 hours post-infection (Figures 9 and 10). Mouse lineages that expressed human MHC class II colonized much more effectively than the parental B6 lineage (Figure 9); B6 mice did not colonize well with either strain of *S. pyogenes* tested. Mice expressing MHC class II HLA-DR4 colonized better than B6 mice but not as well as the mice expressing HLA-DQ8 or HLA-DR4/DQ8 (Figure 9). These experiments demonstrate clearly that host genetics can have an enormous influence on the ability of *S. pyogenes* to colonize the nasopharynx.

To assess whether the colonization phenotypes were due to divergent evolutionary differences in the DR4 and the DR4/DQ8 lineages of mice, DR4/DQ8 mice were back-crossed with the DR4 mice and infection of the F1 generation (DR4/DQ8*) resulted in

similar levels of colonization to the DR4/DQ8 mice when tested with the *S. pyogenes* MGAS8232 serotype (Figure 9). This experiment supports our findings that the human MHC class II molecules are responsible for the colonization differences.

Mice expressing different murine MHC class II (Table 5) were also assessed for their potential colonization with two strains of *S. pyogenes* (Figure 10). In general, the mice were found to have low levels of *S. pyogenes* recovered after 48 hours of colonization, with the exception of FVB mice. Strikingly, FVB mice infected with *S. pyogenes* MGAS5005 had the highest recovery of *S. pyogenes* of any group of mice tested with any strain of *S. pyogenes*, yet these mice were only poorly colonized by *S. pyogenes* MGAS8232, the main difference between the superantigen complement encoded in these strains being the presence of speJ in *S. pyogenes* MGAS5005, which is a streptococcal superantigen that is immunostimulatory in FVB mice but not in transgenic DR4/DQ8 mice. These experiments strongly suggest that the strain of *S. pyogenes* can also have a major influence on the ability to colonize, and that this is also linked with host genetics, specifically MHC class II.

To study infectious organisms, it is important to use a model system that is sensitive to the virulence factors produced by the pathogen being assessed. SAg research in mice has commonly utilized external sensitizing agents, such as D-galactosamine: However, this agent could mask or exaggerate significant pathogenic effects of the bacterial infection on the animal [140]. Theoretically, a naturally sensitive model is critical to the proper understanding of the course of pathogenesis and the interactions between pathogen and host. Streptococcal SAgs interact with human MHC class II with higher affinity than to murine MHC class II molecules [139]. The demonstrable sensitivity of a mouse to streptococcal SAgs, either with transgenic human MHC class II expression or sensitive mouse H-2 for the SAgs in question is important in the study of any streptococcal infection.

In order to understand the role of MHC class II in the nasal colonization model, recombinant SAgs were evaluated on the different mouse splenocyte cells *ex vivo*. Mice that expressed different MHC class II molecules were found to have different sensitivities to rSAg as judged by the ability of their splenocytes to secrete IL-2 in response to various SAgs (Figures 11 and 12). Mice expressing transgenic human MHC class II were more

sensitive to rSAg than B6 mouse splenocytes which demonstrated little to no activation by any of the toxins. Splenocytes from mice expressing DR4/DQ8 were more sensitive than splenocytes from mice expressing only DR4 (Figure 11). It is important to note that DR4/DQ8 mice were stimulated by SpeA and SmeZ, with SmeZ being 100-fold more potent than SpeA (Figure 11). This supports previous findings that SpeA is more responsive in DQ-expressing mice [59, 113]. Overall the reactivity to SmeZ in all mice with the exception of B6 was not unexpected as SmeZ has been reported to be, in general, at least 10 times more potent in its T cell stimulating ability [187]. FVB mouse splenocytes were also stimulated by both SpeJ and SmeZ (Figure 12). Given the universally poor superantigenic activity of streptococcal SAgs in B6 mice it may be concluded that B6 mice are not an appropriate murine model with which to study streptococcal SAgs. Additionally, B6 mice may not be appropriate murine models with which to study streptococcal pathogenesis, despite the availability of many interesting deletion strains in mice, as they are not reactive to streptococcal SAgs, which are considered to be major streptococcal virulence factors.

Streptococcus pyogenes MGAS5005 encodes four SAgs [79], and *S. pyogenes* MGAS8232 encodes six SAgs [136]. To evaluate the level of SAg expression from these strains, recombinant SAgs were used to generate SAg-specific polyclonal rabbit antibodies against the streptococcal SAgs encoded in both serotypes of *S. pyogenes* used in this study (Figure 13). The amount of SAgs produced by *S. pyogenes* MGAS8232 and *S. pyogenes* MGAS5005 *in vitro* were assessed by Western blot using the rabbit anti-SAg antibodies (Figure 18). *S. pyogenes* MGAS5005 produced detectable amounts of SpeA, and *S. pyogenes* MGAS8232 produced detectable amounts of SpeA, SpeC and SpeL. However, it is important to note that *S. pyogenes* may have distinctively different protein expression profiles *in vitro* depending on the medium or condition that they are grown in and the transcriptome also varies based on growth phase [188]. There is evidence that SAgs are produced *in vivo* at different points in the infection [82] and that the production of SAgs *in vivo* has been linked to the selective pressure exerted by the innate immune system [79, 189]. Previously, *S. pyogenes* MGAS5005 was grown in human blood and the transcriptome was analyzed at various time points. It was found that the SAgs SpeA, SpeJ, SpeG, and SmeZ were all upregulated within the first thirty minutes of growth in

blood [186]. In a macaque model of pharyngitis that was used to evaluate changes in the streptococcal transcriptome it was determined that the temporal upregulation of SAgs coincided with a successful infection and inflammatory reaction and that SpeA, SpeJ and SmeZ were expressed during distinct disease phases and in general were associated with the colonization phase [82]. This suggests that there may be changes in SAg protein expression profiles during nasal infection of the mouse which could affect virulence and colonization potential of the streptococci. Thus, the SAgs produced *in vitro* may not necessarily reflect the production of SAgs *in vivo*, but even if the SAg is produced, if the mouse is not sensitive to SAg stimulation then the SAg may not necessarily have an impact. Based on that premise it was surmised that the streptococci that will successfully infect a mouse nasally will express SAgs that are immunostimulatory to that mouse, and that it will not be able to effectively infect mice that are not stimulated by the SAgs expressed in that strain. As a result of both the initial nasal colonization experiments and the splenocyte sensitivity experiments it was ultimately decided to use the DR4/DQ8 transgenic mice to assess the colonization of the *S. pyogenes* MGAS8232 isogenic SAg deletion strains and FVB mice to assess the nasal colonization potential of *S. pyogenes* MGAS5005 isogenic SAg deletion strains.

Isogenic SAg deletions were created for *S. pyogenes* MGAS5005 and *S. pyogenes* MGAS8232 (Figure 14) to address the role that streptococcal SAgs play in colonization in a mouse model of nasal infection. The isogenic SAg deletion mutants were made as in-frame markerless deletions using Gram-positive temperature-sensitive plasmids (Figure 6). In-frame, markerless deletions have the advantage that they are stable and likely non-polar, and result in the deletion of the central portion of the gene, which maintains the genetic reading frame [163] (Figure 5). The resulting isogenic SAg deletion mutants were confirmed by PCR using primers that were located internally to the SAg gene (Figure 16), Western blot for deletion of protein production (Figure 18), and by growth curve to ensure that there were no serious growth defects introduced from the genetic manipulations (Figure 17). A similar scheme was used to complement wild type *speA* into the genome of the complete SAg deletion strain of *S. pyogenes* MGAS8232 between the *pepO* and *tsf* genes.

Streptococcus pyogenes MGAS5005 encodes 4 SAgs that were disrupted to make four single SAg isogenic deletion strains and one strain with deletions of three of four SAgs, Δ*speA*/*speJ*/*smeZ* (Figure 14A). Nasal inoculation of FVB mice with wild-type *S. pyogenes* MGAS5005 resulted in approximately $\log_{10}$ 7 recovery of *S. pyogenes* at 48 hours post-infection. The *S. pyogenes* MGAS5005 Δ*speA* and Δ*speG* strains resulted in *S. pyogenes* recovery that was not significantly different from that of wild type *S. pyogenes* MGAS5005. There was a significantly lower recovery from the single isogenic *S. pyogenes* MGAS5005 Δ*speJ* and Δ*smeZ* deletions (Figure 19), which corresponds to the superantigens that are immunostimulatory in the FVB mice (Figure 12B). Similarly, FVB splenocytes stimulated with *in vitro S. pyogenes* MGAS5005 isogenic SAg deletion culture supernatants and assessed for splenocyte activation showed a profile that indicated that the disruption of *speA*/*speJ*/*smeZ* resulted in an absence of splenocyte activation (Appendix 4B). FVB mice inoculated with *S. pyogenes* MGAS5005 wild type became piloerected by 48 hours, the *S. pyogenes* MGAS5005 Δ*speA*/*speJ*/*smeZ* deletion-treated mice did not show any piloerection at 48 hours, similar to the saline-treated mice (Figure 20), as did the Δ*speJ* and Δ*smeZ* deletion-treated mice (data not shown). Taken together this data indicates that in *S. pyogenes* MGAS5005 SpeJ and SmeZ play a significant role in the establishment of colonization at 48 hours post-inoculation in FVB mice.

There were no significant differences in the number of streptococci recovered from the blood, spleen, kidneys, liver or heart in any of the *S. pyogenes* MGAS5005 SAg deletion mutants in FVB mice sacrificed at 48 hours post-inoculation (Figure 21). There were statistically fewer streptococci recovered from the lungs of mice infected nasally with *S. pyogenes* MGAS5005 Δ*speJ* and Δ*speA/speJ*/*smeZ*-infected mice as compared to the wild type-infected mice, which may reflect the fact that there were fewer streptococci present in the nasal cavity of those mice, which resulted in fewer streptococci being inspired into the lungs of the mice at this time point.

Transgenic DR4/DQ8 mice infected nasally with wild type *S. pyogenes* MGAS8232 resulted in approximately $\log_{10}$ 6 recovery of *S. pyogenes* at 48 hours (Figure 22). There were significantly fewer *S. pyogenes* MGAS8232 Δ*speA* and *S. pyogenes* MGAS8232 ΔSAg isogenic deletion mutants recovered from the cNT. When *speA* was complemented into the genome of the *S. pyogenes* MGAS8232 ΔSAg strain there was a

significant recovery in the colonization potential (Figure 22). DR4/DQ8 splenocytes stimulated with *in vitro S. pyogenes* MGAS5005 isogenic SAg deletion culture supernatants assessed for splenocyte activation showed that the deletion of *speL/M*, and *speA* resulted in a decrease of mIL-2 produced, and that the complete deletion of all of the SAgs in *S. pyogenes* MGAS8232 resulted in an absence of splenocyte activation (Appendix 4C), which is in accordance with the superantigens produced by this bacterium *in vitro* (Figure 18B) and the superantigens that are immunostimulatory in the DR4/DQ8 mice (Figure 11C). This was a critical experiment, as it established that the phenotypes observed for both the MGAS8232 Δ*speA* and ΔSAg strains are not due to secondary mutations introduced through the mutagenesis process. There were no statistically significant differences in the numbers of streptococci recovered from the organs of infected mice (Figure 23). This indicates that the superantigen deletions in *S. pyogenes* MGAS8232 did not affect the invasiveness of *S. pyogenes* in the nasal infection of DR4/DQ8 mice.

A time-course study was performed in which wild type and the isogenic ΔSAg deletion strains of *S. pyogenes* MGAS8232 were inoculated at the same initial concentration of 10^8 cfu per animal, and streptococcal burden was assessed over the course of a week (Figure 24). There was an initial drop in streptococcal recovery 24 hours after inoculation. Although there was a significant difference in the recovery of wild type and ΔSAg *S. pyogenes* MGAS8232 at 48 hours, both strains ultimately took the same time to clear from the nasal passages of the mice. Both the *S. pyogenes* MGAS8232 wild type and *S. pyogenes* MGAS8232 ΔSAg were not recovered at significantly different concentrations after the 48 hour time point up to when the streptococci were cleared from the cNT after day six. The results of colonization kinetics with wild type *S. pyogenes* in DR4/DQ8 mice were similar to what was previously documented in streptococcal nasal infections in BALB/c mice inoculated with five-times the streptococcal burden with S. pyogenes 90-226 [149]. In the *S. pyogenes* 90-226-infected BALB/c mice, the infection initially appeared to clear but then recovered to reach peak colonization at 48 hours post-infection and was detected in the nasal cavity of the mice for greater than 72 hours and up to seven days [149].

Haematoxylin and eosin stained histological sections of nasal turbinates from mice nasally treated with saline, *S. pyogenes* MGAS8232 wild type, and *S. pyogenes* MGAS8232 ΔSAg were assessed along the respiratory epithelium and neuroepithelium for production of mucus, nucleated cellular debris, and red blood cells along the epithelium. There was a significant increase in the pathological severity in the *S. pyogenes* MGAS8232 wild type-treated mice as compared to the saline and the *S. pyogenes* MGAS8232 ΔSAg-treated mice (Figure 25A). There were no significant differences in the pathophysiology along the neuroepithelium between groups (Figure 25B). This indicates that the histological severity of the infection at the level of the T4 cross-section (Figure 2) was significantly different along the respiratory epithelium in the nasopharyngeal meatus. This may be due to the fact that mice are quadrupedal and that inspired antigens, mucus, and bacteria gravitationally settle in the nasopharyngeal meatus where they are subsequently cleared by cilia [143]. This may be the practical physiological reason for the location of the NALT being located on the basolateral surface of the nasopharyngeal meatus in mice.

Investigation into how SAgs alter host immunity during the establishment of colonization revealed that there were no significant differences in the number of T cell or B cell populations amongst various treatment groups in the splenocyte, pooled lymph nodes and cNT cell populations (Figures 27, 29, and 31). Flow cytometric analysis revealed that there was an increase in the number of F4/80+ cells in the lymph nodes of mice treated with wild type *S. pyogenes* MGAS8232 (Appendix 2), which indicated that there was an increase in the macrophage population in mice infected with wild type *S. pyogenes* MGAS8232 compared to the *S. pyogenes* MGAS8232 ΔSAg treated mice. Macrophages are able to rapidly recognize and internalize pathogens and can further recruit immune cells by producing chemokines and can also serve as APCs. Macrophages can play a critical role in streptococcal infections as previous work found that the depletion of macrophages within the first 24 hours post-infection rendered resistant mice susceptible to streptococcal intraperitoneal infection with higher bacterial loads and mortality [190]. The presence of increased numbers of macrophages in the lymph nodes of wild-type infected mice may indicate an active immune response to *S. pyogenes*. As there were significantly fewer macrophages in the lymph nodes of mice infected with *S.*

pyogenes MGAS8232 ΔSAg at 48 hours, this may indicate the SAg activity has altered this response. Further study on the kinetics of the macrophage response to *Streptococcus pyogenes* infection with and without superantigens focusing mainly on the cervical lymph nodes will allow us to draw further conclusions about the role of superantigens in macrophage response.

Conventional DCs circulate within the peripheral tissues, take up antigens, and become activated upon contact with pathogens after which they become potent stimulators of T cell responses in the peripheral lymphoid organs [191]. As such, DCs play a major role in the identification and immune response to pathogens, and can be detected with the use of anti-CD11c antibodies. There was a significant decrease in the proportion of CD11c+ cells in the CD45+ live population in the cNT of wild type *S. pyogenes* MGAS8232-treated mice (Appendix 3) indicating fewer dendritic cells present in the cNT of these mice. In addition to antigen presentation and T cell activation DC cells can also produce IL-6 and TGF-β, which in the abscence of IL-12, IL-4 and IFN-γ stimulate the differentiation of Th17 cells [191]. It has previously been reported in the literature that there were fewer CD45+ live cells that stained with anti-CD11c antibody in mice infected with *S. pyogenes*, than mice inoculated with *Lactobacillus murinus*, a commensal bacterium in mice [153]. Although it was suggested that the presence of *S. pyogenes* induced a migration of DC cells from the NALT to the lymph nodes or blood [153], we did not observe such an increase in the DC cell population in the lymph nodes. It is important to note that in *S. pyogenes* MGAS8232 wild type-treated mice, there was a statistically insignificant, but potential trend, for an increase in the GR1+CD11b+ cell population in the CD45+ live cells in the cNT of two mice (Appendix 3). GR1 and CD11b together are markers of myeloid progenitor cells, and potentially myeloid-derived suppressor cells (MDSC) in mice [192]. MDSCs are a heterogeneous cell population of myeloid origin, are in an immature state and have the potential to suppress T lymphocyte and adaptive immune responses [192]. The expansion of MDSCs is triggered by prostaglandin, macrophage colony stimulating factor, IL-6, granulocyte-macrophage colony stimulating factor and capsular endothelial growth factor [192]. It has been suggested that some diseases can block or partially block the differentiation of immature myeloid cells into granulocytes, macrophages or dendritic cells [192]. MDSCs are

considered part of the regulatory arm of the immune system, and may induce the expansion of the T regulatory cells [192]. Further study is needed to better characterize the cellular immune response to *S. pyogenes*, and the SAg deletion strain, to better understand the contribution of SAgs to the cellular immune response.

Cytokines can be classified as regulatory, anti-inflammatory or pro-inflammatory and are often produced in a balance that is important for a controlled functional immune response [108]. Streptococcal superantigens have been shown to elicit different cytokine expressions depending on the concentration of superantigens used [87]. *In vitro* SAgs have been reported to induce a Th1 proinflammatory response that results in the release of IFN-γ and TNF [83]. Similarly, mice with severe streptococcal infections had high serum levels of inflammatory cytokines including IFN- γ, IL-12 and IL-1β, which strongly indicates a Th1 response [193-194]. Cytokine profiles were assessed by Luminex and Multiplex on homogenized cNT supernatants from FVB mice inoculated with either saline, *S. pyogenes* MGAS5005 or *S. pyogenes* MGAS5005 Δ*speA*/*speJ*/*smeZ* at 48 hours (Appendix 1) and by Luminex on cNT homogenates from transgenic DR4/DQ8 mice infected with saline, *S. pyogenes* MGAS8232 or *S. pyogenes* MGAS8232 ΔSAg at 24 and 48 hours (Appendices 5 and 6). At 24 hours there were no statistically significant differences in the cytokines between the saline-, *S. pyogenes* MGAS8232 wild type-, and *S. pyogenes* MGAS8232 ΔSAg-treated samples in the DR4/DQ8 mice. At 48 hours post-infection in both FVB mice treated with *S. pyogenes* MGAS5005 and DR4/DQ8 mice treated with *S. pyogenes* MGAS8232 there were notable differences in the amount of IL-1, IL-6, IL-17, MCP-1, MIP-1α in the samples from both mice. Additionally, FVB mice also had significantly more IL-10, IFN-γ, and TNF-α produced in the *S. pyogenes* MGAS5005 wild type-treated mice. The luminex cytokine analysis of the cNT tissue homogenate supernatant, and particularly the FVB experiments which produced more inflammatory cytokines, suggests that the mice could have potentially been mounting a Th17 response [191] in the FVB mice treated with *S. pyogenes* MGAS5005 wild type. The mice treated with the wild type streptococci had a stronger cytokine response than the saline-, *S. pyogenes* MGAS5005 Δ*speA*/*speJ*/*smeZ*-, and *S. pyogenes* MGAS8232 ΔSAg-treated mice indicating that this response is likely SAg driven and likely contributes to the enhance survival of *S. pyogenes*.

One potential immunomodulatory route that has been suggested to be induced in streptococcal nasal infection is the development of Th17 T cells by TGF-β1 assisted generation [195]. IL-17-producing T cells have been reported to enhance the infiltration of neutrophils and macrophages in infected tissue which aids in pathogen clearance [196]. In the literature, the frequency of NALT cells with a Th17 phenotype is similar to the number of CD4+IL-17+ cells that have been characterized in human tonsils [197]. The Th17 population of T cells (CD4+IL-17+) was not directly examined in the cNT of *S. pyogenes*-infected mice by flow cytometry in this study; however, we did detect a low but statistically significant increase in IL-17 in DR4/DQ8 mice that were inoculated with wild-type *S. pyogenes* MGAS8232 as compared to saline-treated mice. There was a lower trend, but not statistically significant decrease in the amount of IL-17 between the wild-type- and SAg deletion-treated mice. In the *S. pyogenes* MGAS5005-treated FVB mice there was a significantly higher amount of IL-17 produced in the wild-type-treated mice as compared to the saline- and Δ*speA*/*speJ*/*smeZ*-treated mice (Appendix 1). Future experiments to examine cells producing IL-17, by flow cytometry doing ICS staining for IL-17 or RORγT at different time points, are needed to better characterize the significance of *S. pyogenes* SAgs to the Th17 response in the mouse model of cNT colonization.

In all instances we observed a striking increase in the amount of IL-6 in all wild-type streptococci-treated mice compared to the other two treatment groups. IL-6 is necessary, along with TGF-β1, which was not assessed in this study, TNF-α and IL-1β to contribute to Th17 cellular expansion in mice [198]. In the *S. pyogenes* MGAS5005-treated FVB mice there was an increase in both IL-1β and TNF-α that was not observed in either the saline or SAg deletion-treated mice. This indirectly suggests that there is an up regulation of the Th17 population of cells in the mice that are infected with SAg-competent *S. pyogenes* that is absent in mice treated with SAg-disrupted *S. pyogenes*.

It is tempting to draw broad sweeping conclusions about the contributory role of streptococcal SAgs in the immune response by comparing the immunological results of *S. pyogenes* MGAS8232-treated DR4/DQ8 mice (Figures 27, 29, 31, and Appendix 2 and 3) and *S. pyogenes* MGAS5005-treated FVB mouse data (Appendix 1). Both data sets assess the contributory role of SAgs in nasal colonization of mice at the 48 hour time point, in different strains of *S. pyogenes* that produce different virulence factors which

may play a contributory role in the overall colonization potential in their different, and respective, mouse models, and despite this both models yielded similar results indicating that superantigens played a contributory role in the establishment of murine nasal colonization. Further studies examining the cell populations and cytokines within the cNT at various time points in one strain of mouse infected with one serotype of *S. pyogenes* is necessary to elucidate a clear mechanism by which SAgs contribute to mediating the establishment of colonization in the murine nasal passage.

There are multiple strains of *S. pyogenes* circulating globally at any given time, and these strains vary in prevalence region-to-region [11], and different strains are associated with the different incidences of *S. pyogenes*-related diseases in a regional manner [11]. Different subsets of SAgs are produced by different strains of *S. pyogenes*, which may affect the persistence of the strain within a population of people who encode MHC class II that optimally binds the superantigens in the particular strain of *S. pyogenes*. Future studies examining the immune response to *S. pyogenes* with different SAg complements naturally within the human population, will help us better understand the differences in sensitivity to superantigens within the human population. Specific immune cell responses may be examined by cellular depletion, and cytokine neutralization in a murine model of colonization to help further elucidate the mechanism by which superantigens subvert the immune system to allow *S. pyogenes* to establish colonization.

To apply Koch's postulates to microbial pathogenesis, Stanley Falkow's "molecular Koch's postulates' suggests that the (1) phenotype or factor under investigation should be associated with pathogenic organisms, (2) the virulence genes should be identified, and isolated (3) an inactivation of the virulence factor should result in a decrease in pathogenicity and (4) the re-introduction of the wild type gene should restore pathogenicity [199]. Based on this bacterial pathogenesis application of Koch's postulates, the role of streptococcal SAgs in severe and invasive disease has been well-documented [18]. Secondly, SAg genes have been identified in the genomes of all *S. pyogenes* strains sequenced [57, 116, 123-125], and these streptococcal SAgs have been isolated and recombinantly produced and mutagenized to characterize their immunoreceptor interfaces [160-161]. Thirdly, in this work streptococcal SAgs were

completely inactivated by markerless in-frame deletions of all of the streptococcal SAgs in *S. pyogenes* MGAS8232, and three of four SAgs in *S. pyogenes* MGAS5005, the resulting SAg deletion strains revealed decreased ability to establish colonization in a nasopharyngeal-associated lymphoid tissue murine model of infection in strains with the SAgs that were immunostimulatory to the mouse model employed in the colonization. Finally, the re-introduction of the wild type *speA* into an alternate location within the genome of *S. pyogenes* MGAS8232 ΔSAg restored the pathogenicity of the strain by recovering its ability to establish colonization in a similar manner to wild type *S. pyogenes* MGAS8232. Therefore having satisfied Koch's postulates, as they can be applied to microbial pathogenesis, it can be said that in regards to the hypothesis that the results indicate that streptococcal SAgs play a role in the establishment in the colonization of a nasopharyngeal-associated lymphoid tissue model of murine streptococcal infection.

In summary, this work has provided important evidence that the true evolutionary function of the bacterial SAgs, at least those produced by streptococci, is to aid in the establishment of nasopharyngeal infection in SAg-sensitive mice, and potentially the persistence of *S. pyogenes* within human populations. The role of SAgs in the life-cycle of bacterial pathogens has remained an enigma, and this work will contribute to a more complete understanding of this interesting and important family bacterial toxins.

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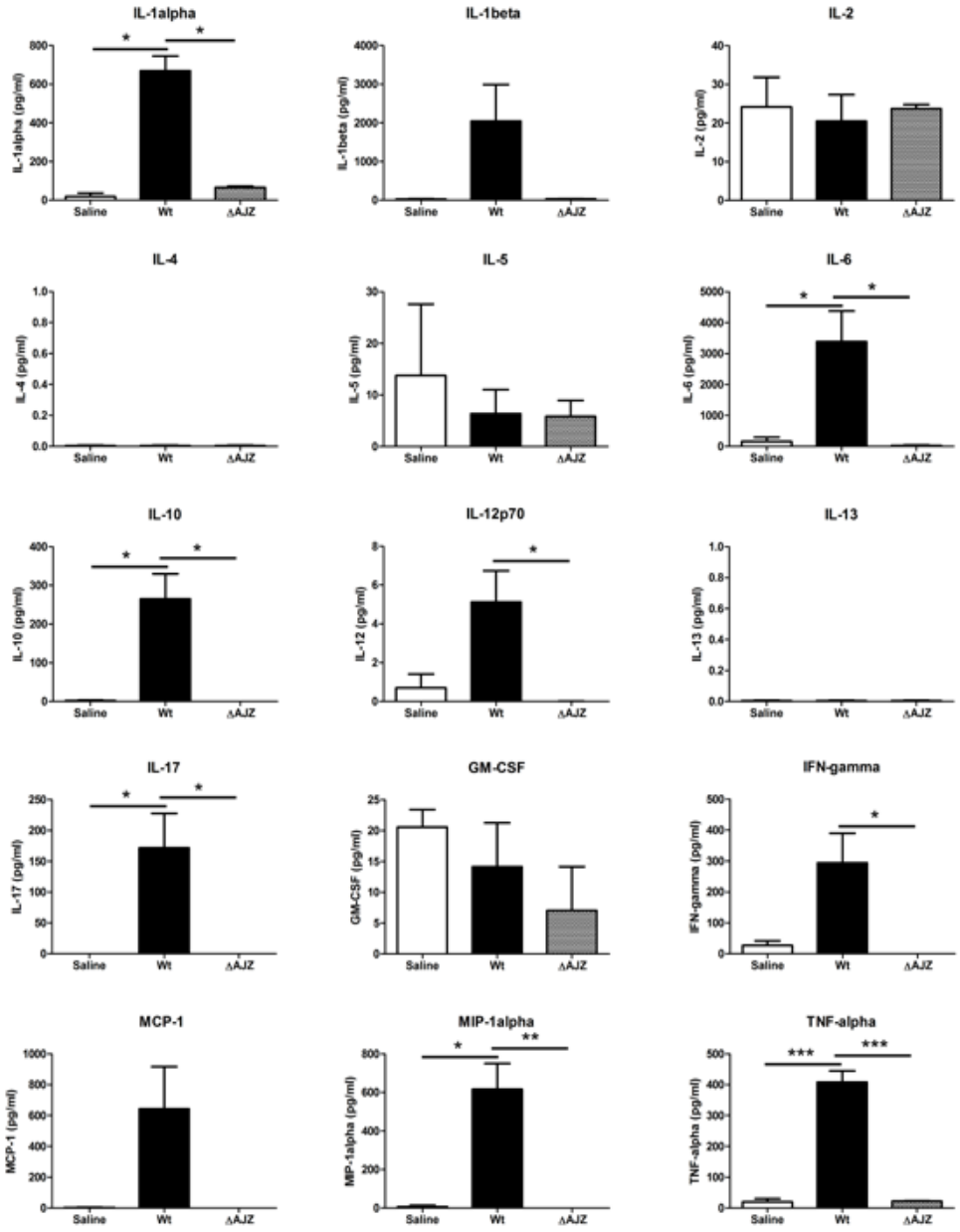
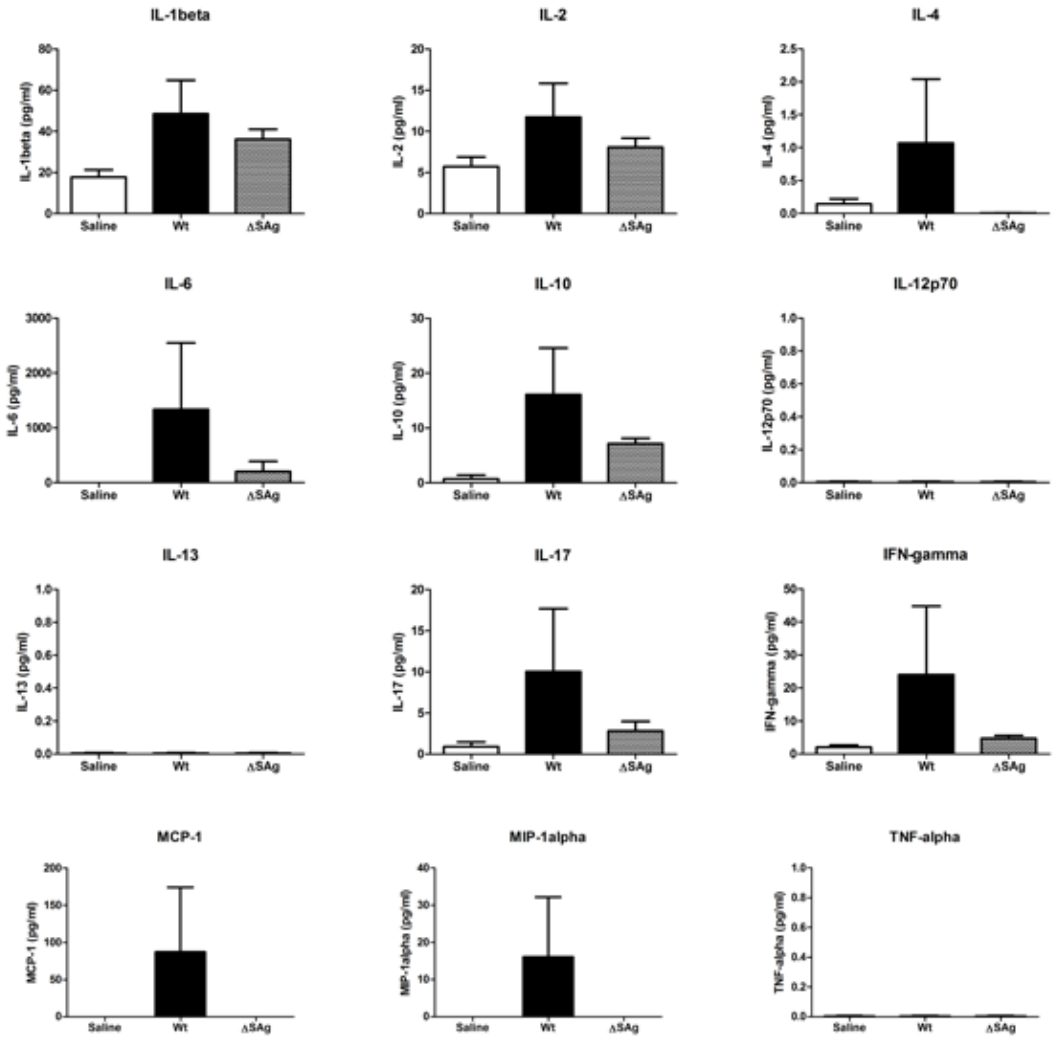
Appendices

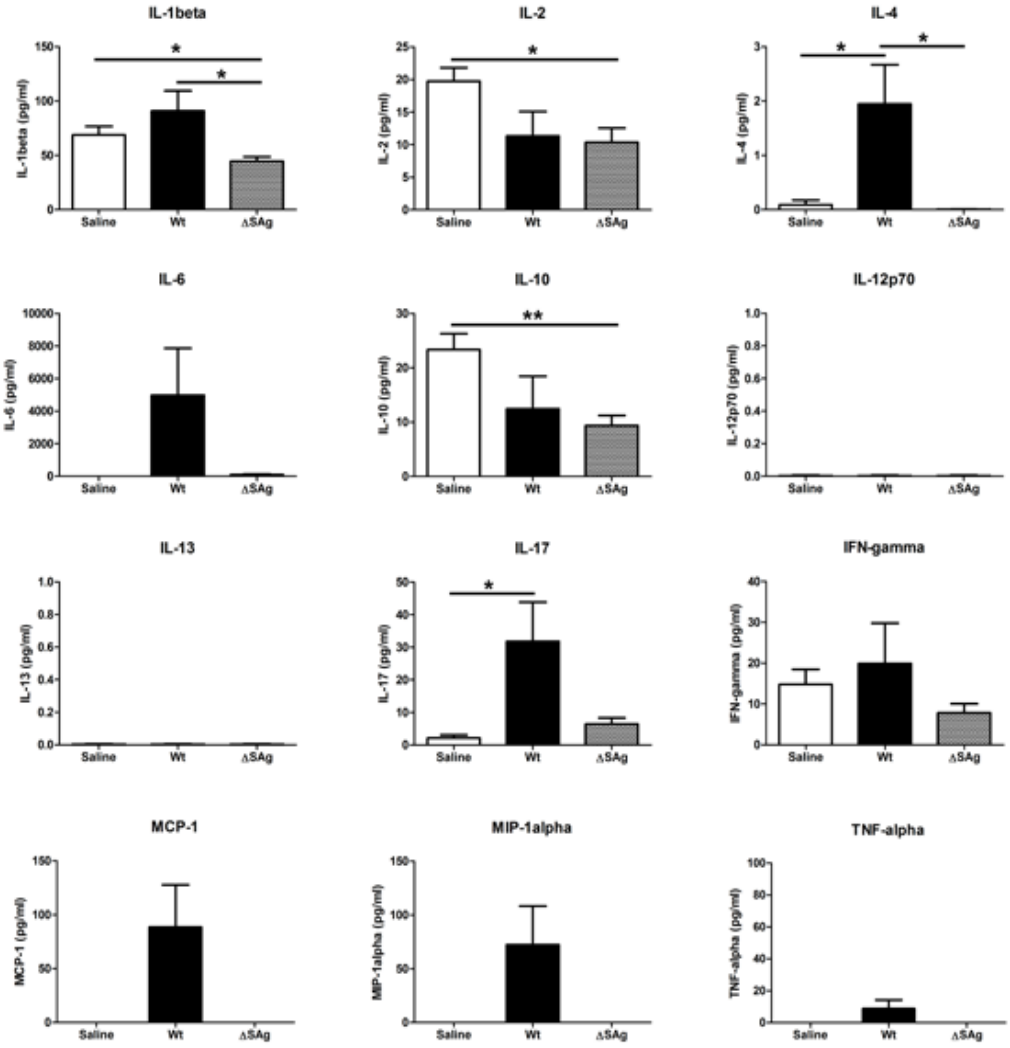
Appendix 1. Cytokine analysis of complete nasal turbinate tissue homogenate supernatants from FVB mice inoculated with *Streptococcus pyogenes* MGAS5005 at 48 hours. The cNT homogenates were assessed for cytokine production by luminex and multiplex analysis (N=3). Shown is the average of individual mouse data points, with the average (± SE). (*, $P < 0.05$, and ***, $P < 0.0005$ as assessed by unpaired t-test).


IL-1alpha
IL-1alpha (pg/ml)
IL-1beta
IL-1beta (pg/ml)
IL-2
IL-2 (pg/ml)
IL-4
IL-4 (pg/ml)
IL-5
IL-5 (pg/ml)
IL-6
IL-6 (pg/ml)
IL-10
IL-10 (pg/ml)
IL-12p70
IL-12 (pg/ml)
IL-13
IL-13 (pg/ml)
IL-17
IL-17 (pg/ml)
GM-CSF
GM-CSF (pg/ml)
IFN-gamma
IFN-gamma (pg/ml)
MCP-1
MCP-1 (pg/ml)
MIP-1alpha
MIP-1alpha (pg/ml)
TNF-alpha
TNF-alpha (pg/ml)
Saline
Wt
ΔAJZ

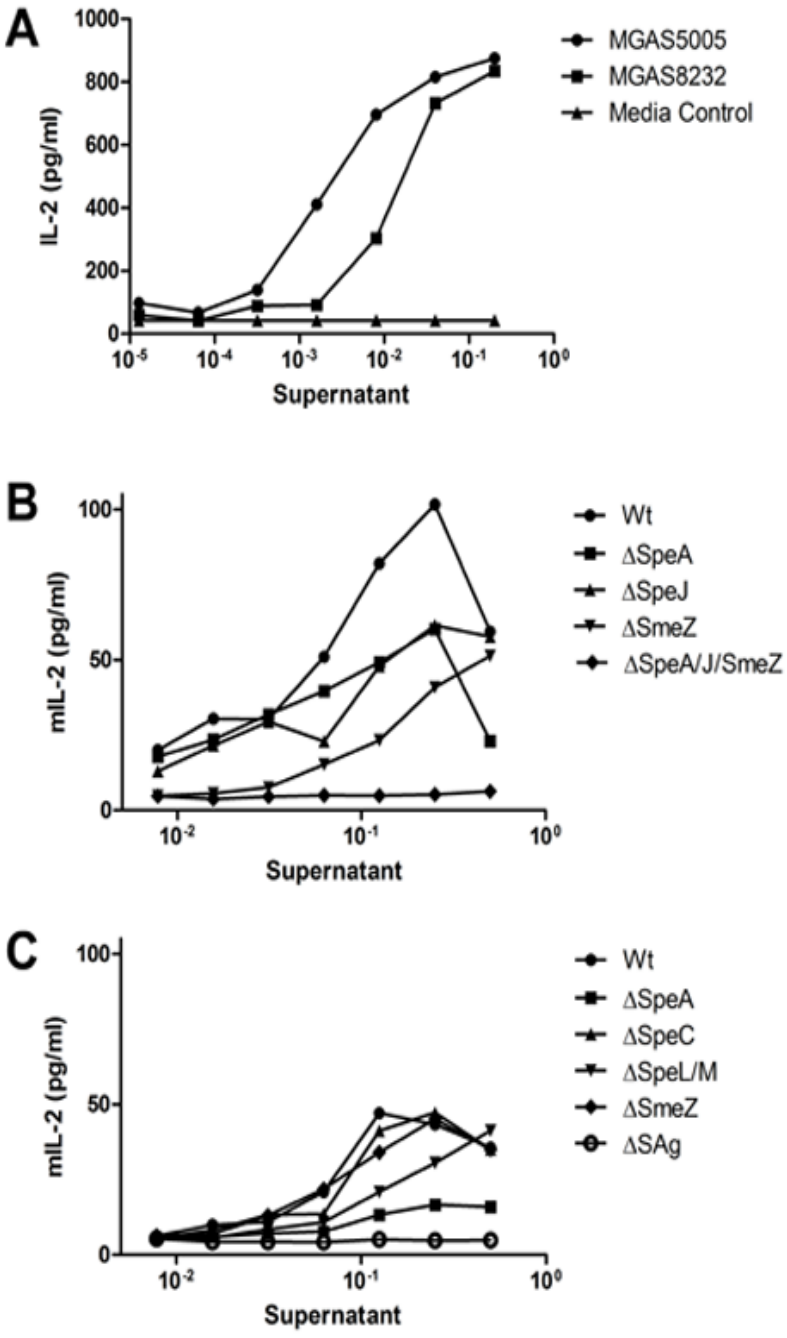
Appendix 2. Cytokine analysis of complete nasal turbinate tissue homogenate supernatants from DR4/DQ8 mice infected with *Streptococcus pyogenes* MGAS8232 at 24 hours. The homogenates were assessed for cytokine production by luminex. There were no statistically significant differences between groups (N=4). Shown is the average of individual mouse data points, with the average (± SE). Assessed by unpaired t-test.


IL-1beta
IL-1beta (pg/ml)
0
20
40
60
80
Saline
Wt
ΔSAg
IL-2
IL-2 (pg/ml)
0
5
10
15
20
Saline
Wt
ΔSAg
IL-4
IL-4 (pg/ml)
0.0
0.5
1.0
1.5
2.0
2.5
Saline
Wt
ΔSAg
IL-6
IL-6 (pg/ml)
0
1000
2000
3000
Saline
Wt
ΔSAg
IL-10
IL-10 (pg/ml)
0
10
20
30
Saline
Wt
ΔSAg
IL-12p70
IL-12p70 (pg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
Saline
Wt
ΔSAg
IL-13
IL-13 (pg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
Saline
Wt
ΔSAg
IL-17
IL-17 (pg/ml)
0
5
10
15
20
Saline
Wt
ΔSAg
IFN-gamma
IFN-gamma (pg/ml)
0
10
20
30
40
50
Saline
Wt
ΔSAg
MCP-1
MCP-1 (pg/ml)
0
50
100
150
200
Saline
Wt
ΔSAg
MIP-1alpha
MIP-1alpha (pg/ml)
0
10
20
30
40
Saline
Wt
ΔSAg
TNF-alpha
TNF-alpha (pg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
Saline
Wt
ΔSAg

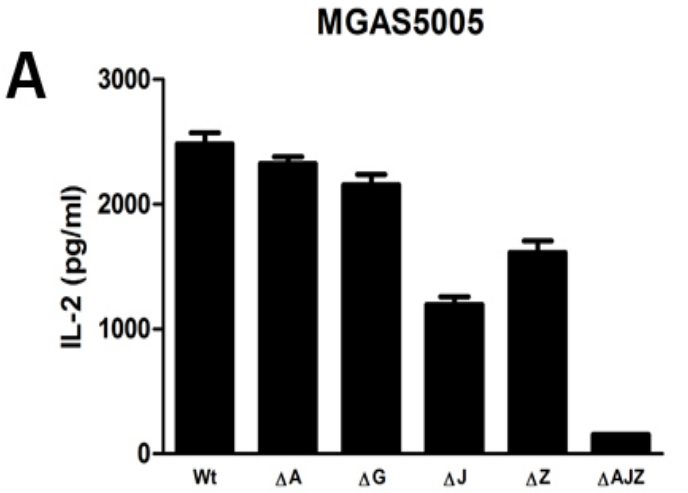
Appendix 3. Cytokine analysis of complete nasal turbinate tissue homogenate supernatants from DR4/DQ8 mice inoculated with *Streptococcus pyogenes* MGAS8232 taken at 48 hours. The cNT homogenates were assessed for cytokine production by luminex analysis (N=3). Shown is the average of individual data points, with the average (± SEM). (*, $P < 0.05$, and **, $P < 0.005$ as assessed by unpaired t-test).

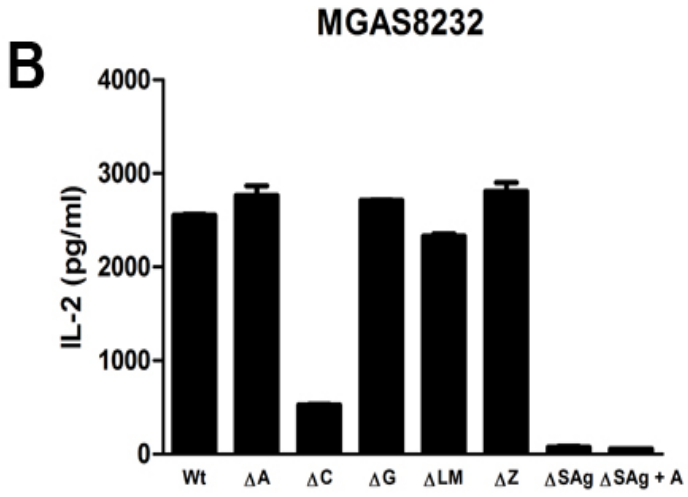

IL-1beta
IL-1beta (pg/ml)
150
100
50
0
*
*
Saline
Wt
ΔSAg
IL-2
IL-2 (pg/ml)
25
20
15
10
5
0
*
Saline
Wt
ΔSAg
IL-4
IL-4 (pg/ml)
3
2
1
0
*
*
Saline
Wt
ΔSAg
IL-6
IL-6 (pg/ml)
10000
8000
6000
4000
2000
0
Saline
Wt
ΔSAg
IL-10
IL-10 (pg/ml)
30
20
10
0
**
Saline
Wt
ΔSAg
IL-12p70
IL-12p70 (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Saline
Wt
ΔSAg
IL-13
IL-13 (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Saline
Wt
ΔSAg
IL-17
IL-17 (pg/ml)
50
40
30
20
10
0
*
Saline
Wt
ΔSAg
IFN-gamma
IFN-gamma (pg/ml)
40
30
20
10
0
Saline
Wt
ΔSAg
MCP-1
MCP-1 (pg/ml)
150
100
50
0
Saline
Wt
ΔSAg
MIP-1alpha
MIP-1alpha (pg/ml)
150
100
50
0
Saline
Wt
ΔSAg
TNF-alpha
TNF-alpha (pg/ml)
100
80
60
40
20
0
Saline
Wt
ΔSAg

Appendix 4. Activation of human PBMCs and murine splenocytes by *Streptococcus pyogenes* wild-type and isogenic mutant bacterial culture supernatants. (A) Human PBMCs were treated with *S. pyogenes* MGAS5005 and MGAS8232 bacterial supernatants. Mouse splenocytes were treated with (B) *S. pyogenes* MGAS5005 and (C) MGAS8232 bacterial supernatants. Cells were treated with serial dilutions of bacterial culture supernatants for 18 hours. The amount of IL-2 detected in human PBMC cultures stimulated with wild type *S. pyogenes* serotype supernatants was in general higher than mIL-2 produced by murine splenocytes stimulated with the same wild type streptococcal supernatants. The superantigen complete knockout strain in *S. pyogenes* MGAS8232 and the triple deletion mutant in *S. pyogenes* MGAS5005 supernatant-treated splenocytes did not produce mIL-2. Mouse and human IL-2 cytokines was assessed by ELISA.


A
1000
800
600
400
200
0
IL-2 (pg/ml)
10-5
10-4
10-3
10-2
10-1
100
Supernatant
MGAS5005
MGAS8232
Media Control
B
100
50
0
mIL-2 (pg/ml)
10-2
10-1
100
Supernatant
Wt
ΔSpeA
ΔSpeJ
ΔSmeZ
ΔSpeA/J/SmeZ
C
100
50
0
mIL-2 (pg/ml)
10-2
10-1
100
Supernatant
Wt
ΔSpeA
ΔSpeC
ΔSpeL/M
ΔSmeZ
ΔSAg

Appendix 5. Human PBMC stimulated with isogenic *Streptococcus pyogenes* mutant supernatants. Human PBMCs were treated with (A) *S. pyogenes* MGAS5005 and (B) *S. pyogenes* MGAS8232 bacterial culture supernatants for 18 hours. Human IL-2 was assessed by ELISA. There was a significantly lower amount of IL-2 produced between the wild-type *S. pyogenes* MGAS5005 and the Δ*speJ*, Δ*smeZ* and the Δ*speA*/*speJ*/*smeZ* isogenic knockout supernatant-treated PBMCs. There was statistically significant lower amount of IL-2 produced between the *S. pyogenes* MGAS8232 wild-type and the Δ*speC*, ΔSAg and the ΔSAg + *speA*-treated PBMCs. Shown is the average of individual data points, with the average (± SE). (*, P <0.05, and **, P <0.005, and ***, P <0.0005 as assessed by unpaired t-test).


MGAS5005
A
IL-2 (pg/ml)
3000
2000
1000
0
Wt
ΔA
ΔG
ΔJ
ΔZ
ΔAJZ


MGAS8232
B
IL-2 (pg/ml)
4000
3000
2000
1000
0
Wt
ΔA
ΔC
ΔG
ΔLM
ΔZ
ΔSAg
ΔSAg + A

Appendix 6. Animal protocol ethics approval.

Western

April 27, 2009

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2013

Dear Dr. McCormick:

Your Animal Use Protocol form entitled:
Bacterial SAgs Play a Key Role in the Pathogenesis of Streptococcus pyogenes
Funding Agency CIHR – Grant #RDAF-107845

has been approved by the University Council on Animal Care. This approval is valid from **April 27, 2009 to April 30, 2010.** The protocol number for this project is **2009-038**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

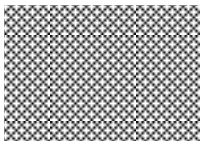
ANIMALS APPROVED FOR 4 Years

SPECIES & SECT D.5.1 GROUP ID#	STRAIN &/or OTHER SPECIES DETAIL For Rodents, Also Provide Vendor Stock #	AGE or WEIGHT & SEX	4-YEAR TOTAL ANIMAL NUMBER
Mouse ID# 2,5,8,B1	C57Bl/6 HLA-DR4	8-11 week old/Male	72+4 breeding mate
Mouse ID# 3,6,9,B2	C57Bl/6 HLA-DR4/DQ8	8-11 week old/Male	72+4 breeding mate
Mouse ID# 11	C57Bl/6 HLA-DQ6	8-11 week old/Male	24+4 breeding mate
Mouse ID# 1,4,7,10	C57Bl/6	8-11 week old/Male	96
Mouse ID# 2,5,8,B1	C57Bl/6 HLA-DR4	8-11 week old/Female	72+4 breeding mate
Mouse ID# 3,6,9,B2	C57Bl/6 HLA-DR4/DQ8	8-11 week old/Female	72+4 breeding mate
Mouse ID# 11	C57Bl/6 HLA-DQ6	8-11 week old/Female	24+4 breeding mate
Mouse ID# 1,4,7,10	C57Bl/6	8-11 week old/Female	96

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.

c.c. Approved Protocol - J. McCormick, D. Mazzuca Siroen, W. Lagerwerf
Approval Letter - J. McCormick, D. Mazzuca Siroen, W. Lagerwerf



Appendix 7. Human ethics approval.

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
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.

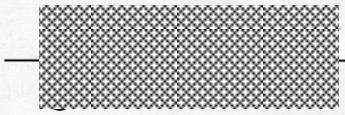
During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects 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 (jsutherl@uwo.ca)	☐ Elizabeth Wambolt (ewambolt@uwo.ca)	☑ Grace Kelly (grace.kelly@uwo.ca)	☐ Denise Grafton (dgrafton@uwo.ca)

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

cc: ORE File
LHRI

Curriculum Vitae

KATHERINE JESSICA KASPER

Education:

2007-2013	Ph.D. Candidate (Microbiology and Immunology), The University of Western Ontario, London, Ontario.
2004-2007	M.Sc. (Microbiology and Immunology) The University of Western Ontario, London, Ontario.
1998-2004	B.MSc. Honors (Microbiology and Immunology, Minor Biochemistry) The University of Western Ontario, London, Ontario.

Employment Experience:

May 2007-Jan. 2013	**Ph.D. candidate** The University of Western Ontario, London, Ontario Successfully completed Ph.D. candidacy examination in March 2008.
May 2004-Apr. 2007	**Masters Student** The University of Western Ontario, London, Ontario.
Sept. 2003-May 2004	**4th Year Honors Project Student** The University of Western Ontario, London, Ontario.
Nov. 2000-May 2004	**Work Study Student** Department of Biochemistry, The University of Western Ontario, London, Ontario.
Sept. 2002-Sept. 2003	**Internship Student, Quality Control Manager** Labatt Breweries, Packaging Q.C. Dept., London, Ontario.
May 2002-Aug. 2002	**Summer Student (Summer Studentship)** Samuel Lunenfeld Institute, Casper Lab, Toronto, Ontario.

Teaching Experience:

Sept. 2007-Dec. 2010	**Laboratory Teaching Assistant, Microbiology 2100a** The University of Western Ontario, London, Ontario -Nominated for "Graduate Student Teaching Award" by students in all three years.
Sept. 2005-Dec. 2005	**Laboratory Teaching Assistant, Microbiology 2100a** The University of Western Ontario, London, Ontario -Microbiology and Immunology introductory lab course. Nominated for "Graduate Student Teaching Award"
Sept. 1995-Dec. 1997	**Continuing Education Instructor and Coordinator** Waterloo County Board of Education, Kitchener, Ontario. -Ran and coordinated the "Toy Box Theatre" program.

Awards:

Jan. 2013	**John A. Thomas Award** ($1000) The Department of Microbiology and Immunology, Western University
May 2011-Apr. 2012	**Ontario Graduate Scholarship** ($15,000)
May 2011	**Drs Charles & Madge Macklin Award for Teaching and Research** ($3000) Schulich School of Medicine and Dentistry
May 2008-Apr. 2011	**Doctoral Research Award** ($63,000) Canadian Institute for Health Research.
May 2005-Apr. 2011	**Western Graduate Research Scholarship** ($28,000) The University of Western Ontario, London, Ontario.
May 2009	**Drs Charles & Madge Macklin Award for Teaching and Research** ($3000) Schulich School of Medicine and Dentistry
Sept. 2007-May 2008	**Schulich Graduate Enrichment Scholarship Research Award** ($12,000)

Schulich School of Medicine and Dentistry

Oct. 2008 **Travel Award** ($500)
Gram-positive Pathogens Conference in Omaha, Nebraska, U.S.A.

Oct. 2006 **Travel Award** ($1,000)
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

Oct. 2006 **Travel Award** ($500)
Gram-positive Pathogens Conference in Omaha, Nebraska, U.S.A.

Summer 2002 **Summer Studentship** ($5,000)
The Samuel Lunenfeld Institute, Mount Sinai Hospital, Toronto, Ontario

Publications:

1. Rahman, A. K. M. N., D. A. Bonsor, C. A. Herfst, F. Pollard, M. Peirce, A. W. Wyatt, D. M. Mazzuca, **K. J. Kasper**, J. Madrenas, E. J. Sundberg, and J. K. McCormick. 2011. The T cell receptor β-chain second complimentarity determining region loop (CDR2β) governs T cell activation and Vβ-specificity by bacterial SAgs. *Journal of Biological Chemistry* 286(6):4871-81.

2. Hayworth J. L.*, **Kasper, K. J.***, Leon-Ponte, M., Herfst, C. A., Yue, D., Brintnell, W. C., Mazzuca, D. M., Heinrichs, D. E., Madrenas, J., Hoskin, D. W., McCormick, J. K., and S. M. Haeryfar. 2009. Attenuation of massive cytokine response to the staphylococcal enterotoxin B superantigen by the innate immunomodulatory protein lactoferrin. *Clinical Experimental Immunology*. 157(1):60-70. *Equal contribution.

3. Chau, T. A., McCully, M. L., Brintnell, W., An, G., **Kasper, K. J.**, Vines, E. D., Kubes, P., Haeryfar, S. M., McCormick, J. K., Cairns, E., Heinrichs, D. E., and J. Madrenas. 2009. Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nature Medicine*. 15(6):641-8.

4. **Kasper, K. J.**, W. Xi, A. K. M. N. Rahman, M. Kotb, J. Madrenas, E. J. Sundberg, and J. K. McCormick. 2008. Molecular Requirements for MHC Class II α-Chain Engagement by the Group IV Bacterial Superantigen SpeC. *Journal of Immunology*. 181(5):3384-92.

5. Günther, S., Varma, A. K., Moza. B., **Kasper, K. J.,** Wyatt, A. W., Zhu, P., Rahman, A. K. M. N., Li, Y., Mariuzza, R.A., McCormick, J.K. and E.J. Sundberg. 2007. A novel loop domain in SAgs extends their T cell receptor recognition site. *Journal of Molecular Biology*. 371(1):210-21.

6. **K.J. Kasper**. 2007. Functional Characterization of Streptococcal Superantigen Low-Affinity MHC Class II Binding Interfaces. The University of Western Ontario. (Master's thesis)

Oral Presentations At Professional Meetings:

January 2013 "Superantigens dictate host-specific nasopharyngeal infection by Streptococcus pyogenes". PhD public lecture. Western University, London, Ontario.

November 2007 "Functional Mapping of the Low-Affinity MHC Class II Site of the Streptococcal Superantigen SpeC". Infection and Immunity Research Forum. The University of Western Ontario, London, Ontario.

Poster Presentations At Professional Meetings:

1. **Kasper, K. J.**, Xu, S. X., Mazzuca, D. M. Baroja, Land McCormick, J. K. Development of a model to assess the role of streptococcal virulence factors in nasal-associated lymphoid tissue colonization in mice. London Health Research Day. London, Ontario. March, 2012.

2. **Kasper, K. J.**, Xu, S. X., Mazzuca, D.M. and McCormick, J. K. Development of a model to assess the role of streptococcal virulence factors in nasal-associated lymphoid tissue colonization in mice. Canadian Society of Microbiology Conference. St. Johns, Newfoundland. June 2011

3. **Kasper, K. J.**, Mazzuca, D. M., and J. K. McCormick. Development of a model to assess the role of streptococcal SAgs in nasal-associated lymphoid tissue colonization in mice. Margaret Moffat Research Day, London, Ontario. March, 2011.

4. **Kasper, K. J.**, Mazzuca, D. M., and J. K. McCormick. Development of a model to assess the role of streptococcal SAgs in nasal-associated lymphoid tissue colonization in mice. Banff Conference on Infectious Diseases, Banff, Alberta. May, 2010

5. Patterson K. G., Dixon, J. L., Bastedo, P. S., Gupta, S. S., **Kasper K. J.**, and J. K. McCormick. Development of superantigen-derived anti-cancer immunotherapeutics. Infection and Immunity Research Forum. London, Ontario. November 2009.

6. Kwon, T., **Kasper, K. J.**, Pollard, F., and J. K. McCormick. *Allium sativum* as a potential antimicrobial against group A streptococcal infections. The Infection and Immunity Research Forum. London, Ontario, Canada. November 2009.

7. Gupta, S., Herfst, C. A., **Kasper, K. J.**, and J. K. McCormick. Inhibition of streptococcal SAgs by IVIG. The Third Annual Infection and Immunity Research Forum. London, Ontario, November 2008.

8. Patterson K.G., Dixon, J.L., Bastedo, P.S., Gupta, S.S., **Kasper K.J.**, and J.K. McCormick. Development of superantigen-derived anti-cancer immunotherapeutics. Infection and Immunity Research Forum. London, Ontario. November 2008.

9. Gupta, S., Herfst, C. A., **Kasper, K. J.**, and J. K. McCormick. Inhibition of streptococcal SAgs by IVIG. The Third Annual Canadian Society for Life Sciences Research Conference. Toronto, Ontario, November 2008.

10. Gupta, S., Herfst, C. A., **Kasper, K. J.**, and J. K. McCormick. Inhibition of streptococcal SAgs by IVIG. The National Conference for Gram-positive Pathogens. Omaha, Nebraska, U.S.A. October 2008.

11. Patterson K. G., Dixon, J. L., Bastedo, P. S., Gupta, S. S., **Kasper K. J.**, and J. K. McCormick. Development of superantigen-derived anti-cancer immunotherapeutics. Canadian Society of Microbiology Conference. Calgary, Alberta. June 2008.

12. **Kasper, K. J.**, A. K. M. N. Rahman, and J. K. McCormick. Low-affinity MHC class II binding of streptococcal SAgs. The National Conference for Gram-positive pathogens. Omaha, Nebraska, USA. October 2006.

13. **Kasper, K. J.**, A. K. M. N. Rahman, and J. K. McCormick. Low-affinity MHC class II binding of streptococcal SAgs. Canadian Society of Microbiologists Annual Meeting Student Poster Competition. London, Ontario. June 2006.

14. **Kasper, K. J.**, A. K. M. N. Rahman, and J. K. McCormick. Low-affinity MHC class II binding of streptococcal SAgs. Margaret P. Moffatt Graduate Research Day. University of Western Ontario, London, Ontario. May 2006.

15. **Kasper, K. J.**, A. K. M. N. Rahman, and J. K. McCormick. Low-affinity MHC class II binding of streptococcal SAgs. Lawson Health Research Day. London, Ontario. March 2006.

16. McCormick, J. K., C. A. Herfst, J. M. Laughton, J. L. Dixon, J. N. W. Herbert, A. K. M. N. Rahman, C. M. Kerr, **K. J. Kasper**, I. Gryski, N. Brouillard, and S. A. Cuozzo. Molecular biology of Gram-positive bacteria: toxins, islands, communication, and *in vivo* expression technology. The Canadian Institutes of Health Institute for Infection and Immunity New Investigator Forum. Toronto, Ontario. April 2005.

17. **Kasper, K.**, and J. K. McCormick. Evolution of pathogenicity islands in *Streptococcus pyogenes*. University of Toronto Microbiology and Infectious Diseases Research Day. Toronto, Ontario. July 2004.

18. **Kasper, K.**, and J. K. McCormick. Evolution of pathogenicity islands in *Streptococcus pyogenes*. 15th Annual Sister Mary Doyle Research Day. London, Ontario. March 2004.

19. **Kasper, K.**, Detmar, J., and R. Casper. The role of Bax in apoptosis in mouse placenta and the creation of an inducible mouse model for preeclampsia. Samuel Lunenfeld Institute Summer Studentship Poster Competition. August 2002.

Scholarly and Professional Activities:

Student Mentorship and Supervision

Feb. 2008 –Jan. 2013 **Actua Student Mentor**
Actua Science Mentorship Program, CIHR.

Sept. 2010-May 2011 **Direct Supervisor and Mentor to 4th year Honors Thesis Student**
Supervised and mentored a 4th year honors thesis student in day-to-day lab work.

Nov. 2007-Aug. 2010 **Supervisor and Mentor High School Student**
Mentored a high school student (Tony Kwon) who worked on science fair projects. He placed first in the local science fair for three years, which

allowed him to participate in Canada-Wide Science Fair. In 2009 he placed first at The National Science Fair and was invited to present his work at an international science fair in Tunisia in the summer of 2009. In 2010 was named one of the top 20 under 20.

Roles in Conferences

Nov. 2012 **Volunteer**
Infection and Immunity Research Forum Organizing Committee.
The Department of Microbiology and Immunology, Western University London, Ontario.

Nov. 2009-Jan 2011 **VP of Donor/Sponsor Relations**
Infection and Immunity Research Forum Organizing Committee.
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

Nov. 2009-Jan. 2010 **Past Chair**
Infection and Immunity Research Forum Organizing Committee.
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

Nov. 2008-Jan. 2009 **Chair**
Infection and Immunity Research Forum Organizing Committee.
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

May 2007–2008 **VP Advertising and Event Promotion**
Infection and Immunity Research Forum Organizing Committee
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

June 2006 **Student Volunteer**
The Canadian Society of Microbiologists Annual Meeting

Memberships in Professional Societies

2009-Present Society for Industrial Microbiology and Biotechnology

2007-Present American Society for Microbiology

2006-Present Canadian Society of Microbiologists

University Committees

May 2007-Sept. 2010 **Student Representative**
Infection and Immunity Seminar Series Committee Student Representative. The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

Sept. 2009-Sept. 2010 **Graduate Student Representative**
Student Liaison to the Faculty Committee
The Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario.

Sept. 2004-Sept. 2008 **Social Committee Member**
Department of Microbiology and Immunology Social Committee
The University of Western Ontario, London, Ontario.

Outreach and Volunteer Work

March 2012 **Science Fair Judge**
Orchard Park Public School

Oct. 2010 **Representative of Schulich School of Medicine and Dentistry**
Represented The Schulich School of Medicine and Dentistry at a graduate training recruitment event at The University of Toronto at Mississauga Campus.

March 2010 **Science Fair Judge**
London District Science and Technology Fair, London, Ontario.

March 2010 **Representative at Faculty of Science March Break Open House**
The University of Western Ontario, London, Ontario.

March 2009 **Science Fair Judge and Assistant Division Chair**
London District Science and Technology Fair, London, Ontario.

March 2009	**Representative at Faculty of Science March Break Open House** The University of Western Ontario, London, Ontario.
April 2008	**Science Fair Judge** London District Science and Technology Fair, London, Ontario.
March 2007	**Representative at Faculty of Science March Break Open House** The University of Western Ontario, London, Ontario.

Professional Development

2012	Alternative Curriculum Delivery (Schulich School of Medicine and Dentistry)
2012	Rethinking Multiple Choice (Schulich School of Medicine and Dentistry)
2011	Microsoft Access 2010 Level 1 (Schulich School of Medicine and Dentistry)
2011	Microsoft Excel 2010 Level 2 (Schulich School of Medicine and Dentistry)
2011	Microsoft Powerpoint Level 2 (Schulich School of Medicine and Dentistry)
2011	Teamboard Instruction (Schulich School of Medicine and Dentistry)
2010	WHMIS (The University of Western Ontario
2010	Radiation Safety (The University of Western Ontario)
2009	Gas anesthesia training (UWO ACVS)
2009	Rodent Handling (UWO ACVS)
2009	Basic animal care and use (UWO ACVS)