

HOST RESPONSE TO THE
STREPTOCOCCUS MILLERI GROUP

CHARACTERIZATION OF THE HOST RESPONSE TO
CLINICAL ISOLATES BELONGING TO THE *STREPTOCOCCUS*
MILLERI GROUP

BY
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TITLE: Characterization of the host response to clinical isolates
belonging to the *Streptococcus* Milleri Group

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Abstract

The *Streptococcus* Milleri Group (SMG) asymptomatically colonize the gastrointestinal, female urogenital, and upper respiratory tract in the healthy population, and are therefore traditionally considered commensals. The SMG, however, are also pathogens that cause pyogenic and pulmonary infections. The factors that differentiate pathogenic from non-pathogenic isolates have proven difficult to identify, and consequently the determinants of SMG pathogenicity remain unknown. Characterization of the immune response to the SMG is important towards advancing the understanding of SMG pathogenicity, however there are limited studies that have done so.

Herein, we sought to investigate the cytokine profiles produced by human peripheral blood mononuclear cells in response to 35 clinical isolates of the SMG. Cytokine profiles varied across isolates resulting in a spectrum of responses that separated into three subgroups including a high, intermediate, and low response group. The responses were consistent across three individuals with the exception of several differences, which are discussed and warrant further studies on host susceptibility to SMG infections. The high and intermediate response groups were enriched with clinical isolates from invasive infections, which were found to induce significantly higher cytokine production than airway isolates. Cytokine induction was independent of TLR2 activation, suggesting that other pattern recognition receptors are involved in the recognition of and response to the SMG. Phenotypic characteristics, which are used in the clinical identification of the SMG, did not correlate with cytokine induction; therefore phenotypic tests are not sufficient to identify immunostimulatory isolates. The host response to the SMG characterized in this study provides foundational knowledge for future studies to investigate the mechanism of recognition as well as the function of downstream effector responses in the control of colonization and infection.

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Abbreviations and Symbols

| | |
|----------------|---|
| AP-1 | Activator protein-1 |
| ATCC | American Type Culture Collection |
| CF | Cystic fibrosis |
| CFU | Colony forming unit |
| CS | Chondroitin sulfatase |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNase | Deoxyribonuclease |
| FACS | Fluorescence-activated cell sorting |
| HA | Hyaluronidase |
| HEK293 | Human embryonic kidney cells 293 |
| IFN γ | Interferon gamma |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IL-1 β | Interleukin-1beta |
| LRT | Lower respiratory tract |
| Mbp | Million base pair |
| NF- κ B | Nuclear-factor kappa B |
| PAMP | Pathogen associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCoA | Principle coordinate analysis |
| PI3K/AKT | Phosphoinositide 3-kinase/Akt |
| RPM | Rotations per minute |
| SEAP | Secreted embryonic alkaline phosphotase |
| SMG | <i>Streptococcus</i> Milleri Group |
| Th | T helper |
| THY | Todd Hewitt Broth supplemented with yeast extract |

| | |
|-------|-----------------------------|
| TLR2 | Toll-like receptor 2 |
| TNF | Tumor necrosis factor |
| Tregs | Regulatory T cell |
| TSS | Toxic shock syndrome |
| URT | Upper respiratory tract |
| VGS | Viridans group streptococci |

Declaration of Academic Achievement

Julienne C. Kaiser contributed to the conception and design of experiments in this study, performed all experiments, analyzed and interpreted data, and performed statistical analysis.

Dr. Chris Verschoor prepared and ran cytokine samples on the Luminex xMAP platform, ran samples for flow cytometry on the BD LSR II and assisted with analysis in FlowJo software, and assisted in the interpretation of results and statistical analysis.

Drs. Michael Surette and Dawn Bowdish contributed to the conception and design of the experiments in this study and the interpretation of results.

All studies involving human samples were approved by the McMaster Research Ethics Board and written informed consent was obtained for all participants.

Contents

| | |
|--|----------|
| Abstract | iii |
| Acknowledgements | iv |
| Abbreviations and Symbols | v |
| Declaration of Academic Achievement | vii |
| 1 Introduction | 1 |
| 1.1 The airway microbiota in health and disease | 2 |
| 1.2 The Genus <i>Streptococcus</i> | 4 |
| 1.3 The <i>Streptococcus</i> Milleri Group | 7 |
| 1.3.1 Phenotypic and genotypic heterogeneity | 7 |
| 1.3.2 Virulence factors | 8 |
| 1.3.3 Host factors | 9 |
| 1.4 The role of cytokines in host defense | 10 |
| 1.4.1 The Th17 responses to <i>S. pneumoniae</i> colonization in the airway | 13 |

| | | |
|----------|---|-----------|
| 1.4.2 | Immunomodulation by <i>S. aureus</i> and its relation to commensalism and pathogenicity | 14 |
| 1.4.3 | Cytokine responses to other members of the airway microbiota | 16 |
| 1.5 | Experimental questions and hypothesis | 17 |
| 2 | Materials and Methods | 20 |
| 2.1 | Bacterial strains | 20 |
| 2.2 | Primary cell culture | 21 |
| 2.3 | PBMC stimulation | 21 |
| 2.4 | Analysis of cytokine data | 22 |
| 2.5 | Detection of pre-existing antibodies | 22 |
| 2.6 | TLR2 NF- κ B:SEAP Reporter Assay | 23 |
| 3 | Results | 24 |
| 3.1 | Optimization of variables for surveying the cytokine response to the SMG | 24 |
| 3.2 | <i>S. anginosus</i> , <i>S. constellatus</i> , and <i>S intermedius</i> elicit similar cytokine responses from human peripheral blood mononuclear cells . | 25 |
| 3.3 | Cytokine profiles define a spectrum of cytokine responses to the SMG | 26 |
| 3.3.1 | Isolate-specific differences | 26 |
| 3.3.2 | Donor-specific differences | 29 |
| 3.4 | Invasive isolates induce higher IL-1 β , TNF, IL-10, IL-23, and IL-12p70 production than airway isolates | 32 |
| 3.5 | Phenotypic traits of the SMG do not correlate with induction of cytokines | 33 |
| 3.6 | Cytokine production is independent of TLR2 activation | 34 |

| | | |
|----------|--|-----------|
| 4 | Discussion | 37 |
| 4.1 | Induction of pro- and anti-inflammatory cytokines by the SMG | 38 |
| 4.1.1 | Induction of the pro- and anti-inflammatory cytokines IL-1 β , TNF, IL-10 | 38 |
| 4.1.2 | Induction of the T cell polarizing cytokines IL-12p70, IL-23, and IL-4 | 40 |
| 4.2 | Cytokine responses to invasive and airway isolates | 41 |
| 4.2.1 | Invasive isolates | 42 |
| 4.2.2 | Airway isolates | 42 |
| 4.2.3 | Immune recognition of the SMG at other mucosal sites | 44 |
| 4.2.4 | Implications for CF | 45 |
| 4.3 | Donor variation in IL-17A induction | 46 |
| 4.4 | Future directions to investigate the dynamics of the cytokine spectrum | 47 |
| 5 | Conclusion | 50 |
| A | Appendix | 70 |
| A.1 | Methods | 70 |
| A.1.1 | Bacterial strains and culture conditions | 70 |
| A.1.2 | PBMC stimulation | 71 |
| A.1.3 | Capsule stain | 72 |
| A.2 | Results | 73 |

List of Tables

| | | |
|-----|--|----|
| 3.1 | Clinical source of SMG isolates used in this study. | 25 |
| 3.2 | Average and range of cytokine production from human PBMCs (n=3) in response to <i>S. anginosus</i> , <i>S. constellatus</i> , and <i>S. intermedius</i> isolates. | 26 |
| 3.3 | Slope and r^2 values for pairwise plots | 31 |

List of Figures

| | | |
|-----|---|----|
| 1.1 | Phylogenetic tree of the Genus <i>Streptococcus</i> | 5 |
| 1.2 | Summary of functions of cytokines produced by innate immune cells in response to bacteria. | 12 |
| 3.1 | Species differences in cytokine profiles | 27 |
| 3.2 | Heatmap and cluster analysis of cytokine profiles for 35 isolates of the SMG. | 28 |
| 3.3 | PBMC cytotoxicity and antibody responses. | 29 |
| 3.4 | Pairwise plots of pro- and anti-inflammatory cytokines IL-1 β , TNF, and IL-10. | 31 |
| 3.5 | Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by invasive and airway of the SMG. | 32 |
| 3.6 | Cytokine induction by isolates of varying phenotypic characteristics. . | 33 |
| 3.7 | Location of phenotypic biotypes of the SMG within the cytokine spec- trum. | 35 |
| 3.8 | TLR2 activation in response to the SMG using a reporter cell line . . | 36 |
| A.1 | Temporal induction of cytokines in response to <i>Streptococcus</i> spp. . . | 73 |
| A.2 | Intra-donor variability in response to C984. | 74 |
| A.3 | Dose response curves to the SMG | 77 |

| | | |
|------|---|----|
| A.4 | Cytokine responses to all SMG isolates from three Donors | 80 |
| A.5 | Spectrum of cytokine responses to 35 isolates of SMG in three individual donors | 83 |
| A.6 | PBMC cell death following stimulation with isolates of the SMG . . . | 84 |
| A.7 | Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by invasive isolates from different sources of infection. | 85 |
| A.8 | Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by CF airway isolates. | 86 |
| A.9 | Relative fold induction of TLR2 activation upon stimulation with the SMG | 87 |
| A.10 | Production of IL-17A by PBMCs stimulated with the SMG for 5 days | 88 |
| A.11 | Capsule stains of SMG and <i>S. pneumoniae</i> grown on solid media or in liquid broth | 89 |
| A.12 | Recipricol IL-17A and IL-12/IL-23 cytokine responses to SMG in five individuals | 90 |
| A.13 | Affect of lysozyme digestion on cytokine production and TLR2 signalling | 91 |

Chapter 1

Introduction

The *Streptococcus* Milleri Group (SMG) asymptotically colonize the gastrointestinal, female urogenital, and upper respiratory tract (URT) in the healthy population (Poole and Wilson, 1979), and are therefore traditionally considered commensals. The SMG, however, are also causative agents of acute pyogenic infections including abscesses and pleural empyema, and chronic polymicrobial infections in cystic fibrosis (CF) patients (Gossling, 1988; Shinzato and Saito, 1995; Sibley *et al.*, 2010a). Intra-species phenotypic and genetic heterogeneity complicates discerning the factors that differentiate pathogenic from non-pathogenic isolates, and although putative virulence factors have been identified, the determinants of SMG pathogenicity remain unclear. An alternative approach to studying the commensal-pathogenic spectrum of the SMG is to characterize the host response to isolates from along the spectrum. Differences in immune responses to such isolates might provide an explanation for the range of outcomes in the host, from asymptomatic colonization to symptomatic infection.

The Surette laboratory has a collection of clinical isolates of the SMG cultured

from a range of sources, including the sputum of CF patients during periods of stability and exacerbation, and acute invasive infections. This clinical collection provides the opportunity to study the host response to isolates along the commensal-pathogen spectrum. Surveying the spectrum of responses to such isolates will help address how the immune system distinguishes commensal and pathogenic bacteria and assist in identification of more pathogenic isolates.

1.1 The airway microbiota in health and disease

A diverse community of commensal microorganisms, as well as periodic, asymptomatic carriage of respiratory pathogens such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, comprise the URT microbiota (Human and Project, 2012; Gorwitz *et al.*, 2008; García-Rodríguez and Fresnadillo Martínez, 2002). The lower respiratory tract (LRT), in contrast, was thought to be sterile and now the current view based on culture-independent studies on explanted lung tissue or bronchiolar lavage fluid, is that a lung microbiome exists and resembles the URT microbiota but at a relatively reduced density (reviewed in (Dickson *et al.*, 2013)).

The immune system in the airway permits asymptomatic colonization of the microbiota by maintaining a primarily anti-inflammatory environment, while also retaining the ability to mount acute inflammatory responses to pathogenic bacteria (Holt *et al.*, 2008). This state of immunological homeostasis limits the potentially damaging effects of inflammation, which is of particular importance in the lungs, a vital organ for survival. Innate immune cells produce cytokines and chemokines in response to bacteria that regulate the homeostatic state and control the development of adaptive

immunity. This includes T helper (Th) 1 responses (cellular mediated immunity), Th2 responses (humoral immunity), and/or Th17 responses, which are gaining recent attention in the control of respiratory mucosal pathogens through the activation of anti-microbial defenses in the airway epithelium and neutrophil recruitment (Khader *et al.*, 2009).

Perturbations to the homeostatic state can have negative consequences on the health status of the host. For example, imbalances in the microbiota in the lungs of individuals with CF, asthma, and chronic obstructive pulmonary disease are thought to contribute to chronic inflammation and subsequently gradual lung deterioration (Dickson *et al.*, 2013). This suggests that the airway microbiota itself has the potential to act pathogenically. Alternatively, a disruption to homeostasis might occur if a particular strain of bacteria is pathogenic, or if the host's response to it is adverse, resulting in local infection or dissemination from the airways to cause serious invasive disease. For example, of the 90 serotypes of *S. pneumoniae*, approximately 15 are associated with a higher incidence of invasive disease. Recent acquisition of an invasive serotype in the nasopharynx is therefore a more important determinant of invasive disease than long-term carriage of a non-invasive serotype (Brueggemann *et al.*, 2003, 2004). In contrast, strain variation of *S. aureus* does not distinguish invasive isolates from colonizing isolates (van Belkum *et al.*, 2009). As a result, there have been efforts towards identifying host susceptibilities as determinants of carriage or infection. Such studies indicate that genetic polymorphisms in genes encoding IL-4 and C-reactive proteins are associated with the carriage state (Emonts *et al.*, 2008; Ruimy *et al.*, 2010). Although additional studies are required to ascertain the role of host genetics in susceptibility to *S. aureus* infection, this example illustrates the

importance of investigating the contribution of host variability to the commensal-pathogenic spectrum.

The transition from a healthy to disease state in the airway is therefore multifactorial and involves identifying more pathogenic strains or microbial communities as well as susceptible hosts. Studies that investigate the host-microbe interaction bridge these two approaches and contribute to the understanding of how the host regulates the state of colonization and infection. The innate and adaptive immune responses are well studied for clinically recognized respiratory pathogens, such as *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* and are described in detail in *Section 1.4*. The immune response to the other members of the airway microbiota that are perhaps less clinically recognized as pathogens, however, are poorly characterized. An understanding of the immune response to airway commensals will provide insight as to the events that lead to disruption of homeostasis and consequently, symptomatic disease.

1.2 The Genus *Streptococcus*

One of the predominant genera of the airway microbiota, and a focus of research in the Surette laboratory, is the genus *Streptococcus* (Human and Project, 2012). The genus *Streptococcus* consists of six major phylogenetic groups, Pyogenic, Mutans, Salivarius, Bovis, Mitis, and Anginosus/Milleri (**Fig. 1.1**) (Kawamura *et al.*, 1995). The Pyogenic streptococci are typically classified as pathogens and are identifiable as β -hemolytic on sheep's blood agar plates. Members of this group include *Streptococcus pyogenes* (Group A Streptococcus) and *Streptococcus agalactiae* (Group B Streptococcus). *S. pyogenes* causes rheumatic fever, invasive infections, and pharyngitis (strep

throat) and globally accounts for approximately 1.78 million infections and 500,000 deaths a year (Carapetis *et al.*, 2005). *S. agalactiae* is the leading cause of neonatal sepsis in developed countries (Stoll *et al.*, 2011), with an average fatality of 9.8% (Edmond *et al.*, 2012). The remaining five groups - Mutans, Salivarius, Bovis, Mitis, and Anginosus/Milleri - are collectively termed the viridans group streptococci (VGS). The VGS are typically classified as commensals of the human URT, however they are also implicated in a spectrum of invasive disease (Doern and Burnham, 2010).

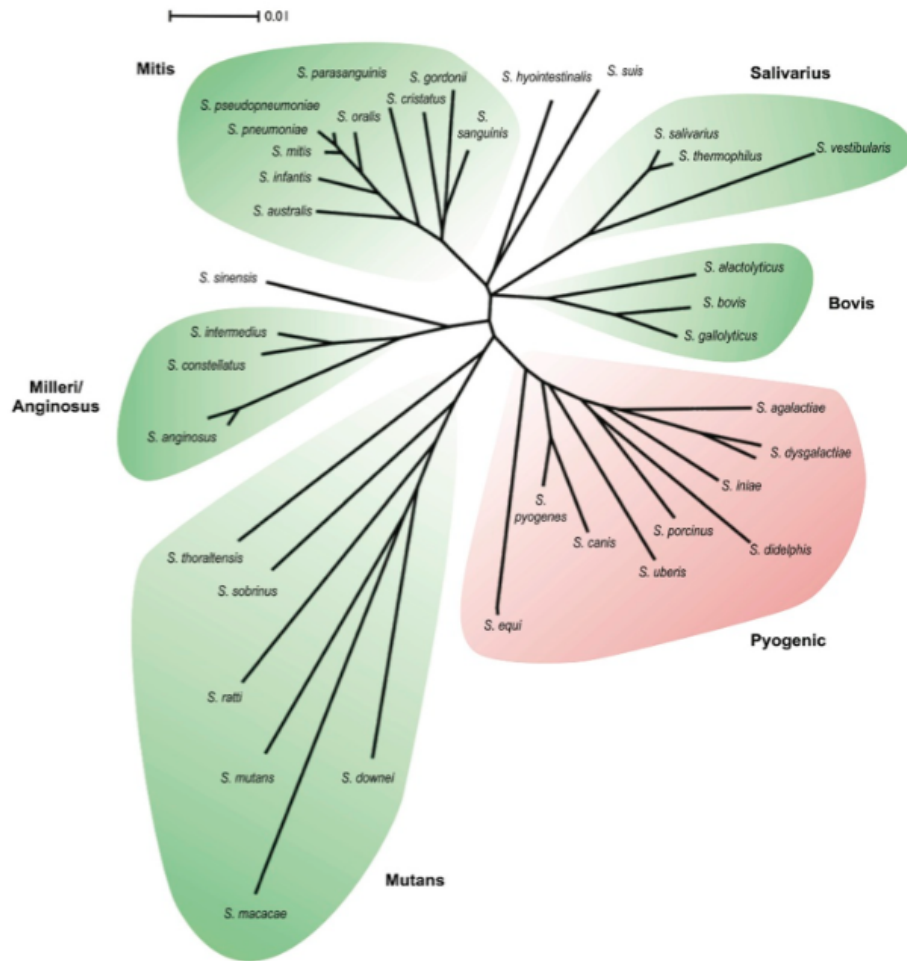


Figure 1.1: Phylogenetic tree of the Genus *Streptococcus*

Streptococcus mutans of the Mutans group is an important commensal in the maintenance of oral health in addition to being the primary causative agent of dental caries (Selwitz *et al.*, 2007). The Mitis group contains *S. pneumoniae*, which is responsible for 11% of all deaths in children under 5 years of age and is the leading cause of childhood bacterial pneumonia, meningitis, and sepsis worldwide (OBrien *et al.*, 2009). Asymptomatic colonization of *S. pneumoniae* in the URT precedes invasive disease, therefore strategies to reduce pneumococcal disease include preventing colonization (Bogaert *et al.*, 2004). Members of the *Streptococcus* Milleri group (SMG) are members of the microbiota of the intestinal tract, female urogenital tract, and URT in 15-30% of healthy individuals (Poole and Wilson, 1979; Gossling, 1988). The SMG are also causative agents of pyogenic infections, accounting for 28-40% of brain abscesses (Prasad *et al.*, 2006; Deutschmann *et al.*, 2013), 20-44% of liver abscesses (Alvarez Pérez *et al.*, 2001; Pang *et al.*, 2011; Kaplan *et al.*, 2004), 20% of head and neck abscesses (Lee and Kanagalingam, 2011), and 30-50% of pleural empyema (Ahmed *et al.*, 2006; Maskell *et al.*, 2005). Approximately 12-32% of invasive infections are complicated by bacteremia (Bert *et al.*, 1998). A retrospective survey conducted in the Calgary region observed that the SMG have the highest incidence of annual pyogenic streptococcal infections, more than all other streptococci combined (Laupland *et al.*, 2006). Our research has identified the SMG as under-recognized pathogens in CF. The SMG are the most abundant pathogen identified at the onset of pulmonary exacerbation in 40% of hospital admitted CF patients during a 6 month period and antibiotic therapy directed against the SMG relieves symptoms of exacerbation (Sibley *et al.*, 2008a; Parkins *et al.*, 2008).

Despite the pathogenic potential of the VGS, they are often overlooked as pathogens

when isolated from infections, particularly in the URT, because of their frequent isolation from healthy airways. Overlapping biochemical properties (Facklam, 1977; Doern and Burnham, 2010) and shared virulence genes in pathogenic and non-pathogenic species (Donati *et al.*, 2010) impose a clinical challenge in determining whether the VGS isolated from a respiratory or invasive infection are pathogenic.

1.3 The *Streptococcus* Milleri Group

1.3.1 Phenotypic and genotypic heterogeneity

The SMG reflect the complications of identifying pathogenic traits in the VGS. The SMG are a phenotypic and genotypic heterogeneous group of three closely related species; *S. anginosus*, *S. intermedius*, and *S. constellatus* (Whiley and Hardie, 1989; Whiley and Beighton, 1991; Bartie *et al.*, 2000). Strains vary in Lancefield grouping (A,C,G,F, non-groupable) (Clarridge *et al.*, 1999), hemolysis on sheep's blood agar (α , β , γ) (Clarridge *et al.*, 1999), fermentation of carbohydrates (Whiley *et al.*, 1990), and production of hydrolytic enzymes (hyaluronidase (HA), chondroitin sulfatase (CS), DNase, proteases) (Ruoff and Ferraro, 1987; Grinwis *et al.*, 2010a). This phenotypic diversity complicates clinical identification of the SMG and likely contributes to an underestimation of the number of infections they cause.

The SMG are naturally competent and able to acquire genetic material from the environment (Havarstein *et al.*, 1997). This likely contributes to the genetic heterogeneity that has been observed in the genomes of the SMG. A comparative genomics study of seven clinical isolates from the Surette laboratory collection, including two *S. anginosus*, three *S. constellatus*, and two *S. intermedius*, revealed that genomes ranged from 1.91 to 2.23 Mbp in size, with over 10% originating from mobile genetic

elements (Olsen 2013, submitted). Comparison of genomes to a virulence database containing 189 known virulence factors in other streptococci found that the number of genes present in the SMG varied across strains, with *S. anginosus* strains carrying 30-34 genes, *S. constellatus* strains carrying 30-35 genes, and *S. intermedius* carrying 27-36 genes (Olsen 2013, submitted). This study provides essential information to identifying genetic determinants of SMG pathogenesis, however this approach is still in its infancy. A previous study that questioned whether an association exists between genotypes and pathogenicity of SMG in the CF population found that genotypes of the SMG in the CF lung were patient-specific and strains of variable genotypes were associated with exacerbation (Sibley *et al.*, 2010b). This study supports that SMG infections are not the result of clonal expansion of a hypervirulent strain. It therefore remains unknown whether all genotypes of the SMG are potential pathogens and are able to cause infection under opportune circumstances (i.e. a susceptible CF patient) or whether some strains are more pathogenic than others.

1.3.2 Virulence factors

The virulence factors that are thought to contribute to the pathogenicity of the SMG include hydrolytic enzymes, toxins, and capsule production. Production of extracellular hydrolytic enzymes is associated with degradation of host tissue and evasion of host immune defenses by the SMG and other pathogenic streptococci (Starr and Engleberg, 2006; Shain *et al.*, 1996; Buchanan *et al.*, 2006). The human-erythrocyte toxin intermedilysin, only found in *S. intermedius*, is expressed 6 and 10 fold higher in isolates from brain and liver abscesses respectively compared to non-pathogenic isolates (Nagamune *et al.*, 2000), and is cytotoxic to human liver cells *in*

vitro and blocking intermedilysin reduces adhesion and invasion of these cells (Sukeno *et al.*, 2005). Encapsulated isolates of the SMG cause abscesses in mice whereas unencapsulated isolates cause abscesses at a lower frequency (Kanamori *et al.*, 2004). Formation of abscesses might be the result of inefficient killing by polymorphonuclear cells [(Wanahita *et al.*, 2002), which is inhibited by the presence of a capsule (Kanamori *et al.*, 2004). Despite the identification of these putative virulence factors, the presence or absence of such traits in clinical isolates of the SMG are not correlated with pathogenicity (Grinwis *et al.*, 2010a; Jacobs and Stobberingh, 1995).

Another feature of SMG pathogenicity is their synergistic virulence with anaerobic bacteria (Robertson and Smith, 2009). The mortality of mice from pulmonary infections or orofacial abscesses caused by *S. constellatus* is synergistically increased in the presence of *Prevotella intermedia* and *Fusobacterium nucleatum* respectively (Shinzato and Saito, 1994; Kuriyama *et al.*, 2000). Clinically, the SMG are often isolated from polymicrobial infections (Clarridge *et al.*, 2001; Sibley *et al.*, 2012; Belko *et al.*, 2002), although the importance of synergism in humans is not known.

1.3.3 Host factors

The SMG are unique from the other subgroups of the VGS in that they are capable of causing invasive disease in otherwise healthy hosts (Doern and Burnham, 2010). It is estimated that only 18% of patients with invasive SMG infections have risk factors of infection, such as diabetes, trauma, or are immunocompromised (Belko *et al.*, 2002). Retrospective studies on SMG infections report that the frequency of infections is 2 fold higher in males than females (Salavert *et al.*, 1996; Bert *et al.*, 1998) and although carriage rates of the SMG increase with age (Poole and Wilson,

1979), no studies have characterized chronic colonization in the healthy population or investigated age-associated SMG infections. The incidence of SMG carriage in the CF airways during a 6 month period is 40%, however not all carriers of the SMG experience SMG-associated exacerbation (Sibley *et al.*, 2010a). The factors that contribute to host susceptibility to SMG infections remain elusive, consequently complicating the identification of persons at risk.

The determinants of SMG infections are not easily identifiable, complicating the differentiation of pathogenic and non-pathogenic strains. To date, the pathogenicity of the SMG has mainly been characterized in relation to bacterial factors, however the interaction between the SMG and the host immune system is poorly understood. Characterization of the immune response to the SMG is important for improving the understanding of SMG infections and investigating host susceptibility.

1.4 The role of cytokines in host defense

Innate immune cells produce cytokines upon the recognition of pathogen-associated molecular patterns (PAMPs) that activate the inflammatory process and direct the effector response of adaptive immunity. Activated macrophages are the primary producers of TNF, a pro-inflammatory cytokine that activates the vascular endothelium, epithelial cells, dendritic cells and macrophages to promote a localized inflammatory response (Waters *et al.*, 2013). Blood monocytes, tissue macrophages and dendritic cells are the primary sources of IL-1 β . IL-1 β induces chemokine and cytokine production, contributes to the development of the Th17 response, and promotes infiltration of leukocytes and inflammatory mediators to the localized site of

inflammation by activating the expression of adhesion molecules on the vascular endothelia (Dinarello, 2009; Sahoo *et al.*, 2011). T cells, including Th2 cells, regulatory T cells (Tregs), and Th17 cells, as well as monocytes, macrophages, and dendritic cells are producers of IL-10 (Mosser and Zhang, 2008). IL-10 is an anti-inflammatory cytokine that exerts its effects by down-regulating antigen presentation on macrophages and dendritic cells to suppress activation of T cells, as well as directly inhibiting pro-inflammatory cytokine responses from macrophages and dendritic cells (Mosser and Zhang, 2008).

IL-12p70, IL-4 and IL-17A are produced primarily by activated macrophages and dendritic cells and polarize activated naive CD4⁺ T cells towards a specific Th subset, which vary in effector function and cytokine production. IL-12 polarizes CD4⁺ T cells to an IFN- γ -producing Th1 cell phenotype, which control infections with intracellular pathogens by activating macrophage phagocytosis (Szabo *et al.*, 2003). IL-4 polarizes CD4⁺ T cells towards an IL-4- and IL-5-producing Th2 cell phenotype, leading to the production of antibodies directed against extracellular parasites (Paul and Zhu, 2010). IL-23 is implicated in polarization of CD4⁺ T cells towards a Th17 phenotype, as well as the development of the Th1 response, and induction of pro-inflammatory cytokines from macrophages and dendritic cells (Iwakura and Ishigame, 2006; Szabo *et al.*, 2003). The Th17 response is a recently discovered branch of adaptive immunity that drives inflammation in models of chronic inflammatory disorders, including multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (Maddur *et al.*, 2012), and promotes a pro-inflammatory response to control bacteria at mucosal sites (Khader *et al.*, 2009). Th17 cells produce IL-17A, which induces cytokine and chemokine production from a variety of immune cells and recruits and activates

neutrophils and monocytes, as well as IL-22, which activates anti-microbial defenses in the mucosal epithelium (Korn *et al.*, 2009).

IFN γ and IL-4 suppress the development of Th2 and Th1 cells respectively, and both suppress Th17 development (Iwakura and Ishigame, 2006). The effector response initiated upon PAMP recognition is therefore directed by the specific Th cell lineage activated. A simplified summary of the functions of these cytokines is depicted in **Figure 1.2**. Current research on *S. pneumoniae* and *S. aureus* exemplifies the importance of these cytokines in the recognition of respiratory pathogens during colonization and infection.

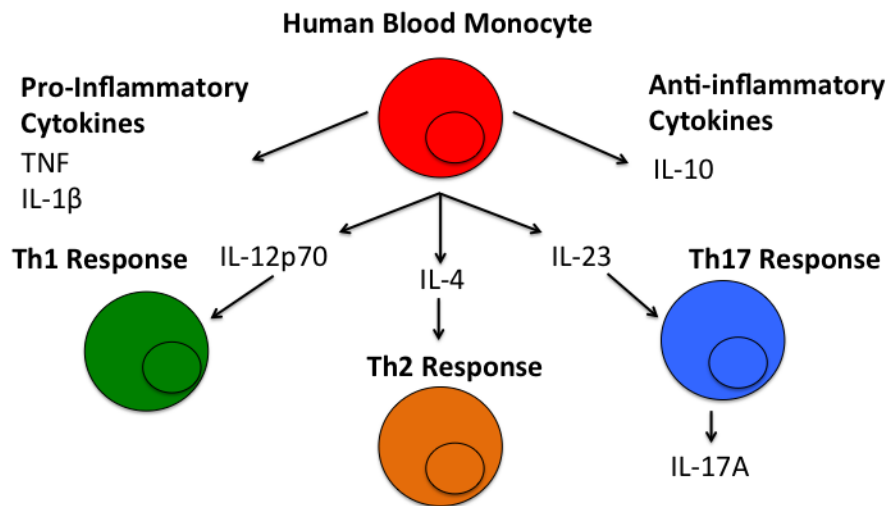


Figure 1.2: Summary of functions of cytokines produced by innate immune cells in response to bacteria.

Innate immune cells produce cytokines that initiate pro- and anti-inflammatory immune responses and polarize T cells towards effector phenotypes.

1.4.1 The Th17 responses to *S. pneumoniae* colonization in the airway

S. pneumoniae establishes asymptomatic colonization in the naso-oropharynx in early childhood, reaching a peak prevalence of 50% in children 3 years of age and decreasing to <10% in children over the age of 10 (Bogaert *et al.*, 2004). Colonization predisposes carriers to invasive infections, including otitis media, meningitis, pneumonia, or sepsis (Bogaert *et al.*, 2004). Resident macrophages in the nasopharynx of mice are the primary effector cells that phagocytose *S. pneumoniae* upon primary colonization (Dorrington *et al.*, 2013; Zhang *et al.*, 2009). Recruitment of additional monocytes and macrophages is required for complete clearance, correlating with a decrease in CFUs of *S. pneumoniae* from days 7-21 post-inoculation (Dorrington *et al.*, 2013; Zhang *et al.*, 2009). This recruitment is delayed in mice lacking TLR2 signalling or IL-17A-producing CD4⁺ T cells (Zhang *et al.*, 2009), suggesting that IL-17A plays a chemotactic role in monocyte/macrophage recruitment during colonization. Secondary exposure to *S. pneumoniae* is followed by a more rapid effector response. Memory Th17 cells secrete IL-17A, promoting neutrophil infiltration and activation leading to the phagocytosis of *S. pneumoniae* and clearance by day 4 (Zhang *et al.*, 2009; Lu *et al.*, 2008). Mice deficient in IL-23, a cytokine required for development of Th17 cells, are more susceptible to dissemination of *S. pneumoniae* from the lungs, which is attributed to impairment of both Th1 and Th17 responses (Kim *et al.*, 2013). *S. pneumoniae* colonization also triggers the humoral immune response, resulting in production of antibodies (Cohen *et al.*, 2011). Antibodies provide greater protection against pneumococcal bacteremia than Th17 cells by facilitating opsonic phagocytosis, demonstrating that multiple effector responses are important to respond to pneumococcal colonization and infection.

URT mucosal lymphoid tissue from humans produces IFN γ , IL-17, and IL-22 following stimulation with pneumococcal antigen (Lu *et al.*, 2008; Pido-Lopez *et al.*, 2011), suggesting that localized memory Th17 cells similarly exist in the nasopharynx of humans and might be involved in clearance. A localized population of Tregs also respond to pneumococcal antigen and suppress the production of the pro-inflammatory cytokines IL-17A, IFN- γ , and TNF by CD4⁺ T cells in response to pneumococcal antigen through the production of IL-10 and IL-5 (Zhang *et al.*, 2011; Pido-Lopez *et al.*, 2011). The consequence of Tregs and suppression of the pro-inflammatory response on the outcome of colonization, however, is not known.

1.4.2 Immunomodulation by *S. aureus* and its relation to commensalism and pathogenicity

S. aureus is a resident commensal in the anterior nares of the nose in a third of the population, yet also causes soft tissue infections, sepsis, and toxic shock syndrome (TSS) (Wertheim *et al.*, 2005). Approximately 50% of *S. aureus* isolates produce superantigens; potent toxins that activate up to 20% of T cells, resulting in systemic inflammation and shock, clinically referred to as TSS (Schmitz *et al.*, 1997). Given that the carriage rate of *S. aureus* is relatively high, it is puzzling that the occurrence of TSS is a relatively rare event (average incidence of 0.52/100,000 persons at risk) (DeVries *et al.*, 2011). *S. aureus* has been found to suppress widespread T cell activation in the presence of superantigens through induction of IL-10 from monocytes and macrophages, an immunomodulatory strategy that is thought to permit colonization in the URT without the symptoms of TSS (Mele and Madrenas, 2010; Chau *et al.*, 2009).

An unidentified component of *S. aureus* peptidoglycan is recognized by TLR2 on monocytes and macrophages and induces production of the anti-inflammatory cytokine IL-10 in an NF- κ B and PI3K/Akt signalling dependent manner (Chau *et al.*, 2009; Frodermann *et al.*, 2011). Macrophages comprise 90% of cells in the airways, leading to the hypothesis that this regulatory mechanism prevents massive T cell responses and symptomatic infection in the ecological niche of *S. aureus* (Mele and Madrenas, 2010). Dendritic cells, in contrast, are thought to initiate the appropriate inflammatory response to control *S. aureus* infections outside of the lung since TLR2 signalling in these cells induces minimal IL-10 production and high production of IL-12 and IL-23, which promote the Th1/Th17 response (Frodermann *et al.*, 2011).

Production of IL-10 by monocytes regulates the outcome of staphylococcal TSS and bacteremia. Positive blood cultures of *S. aureus* occur in less than 5% of staphylococcus TSS cases and a negative blood culture is actually included in the case definition (Lappin and Ferguson, 2009). In contrast, positive blood cultures are required for diagnosis of staphylococcal sepsis in which disease pathogenesis is distinct from TSS (McAdow *et al.*, 2011). This epidemiological data corresponds to the molecular data; that is if superantigens gain access to the blood in the absence of *S. aureus*, widespread T cell activation will occur, whereas if bacteremia occurs simultaneously, T cell activation is down-regulated by IL-10 produced by blood monocytes, therefore the symptoms of TSS are not observed. Indeed, the mortality of mice from TSS is reduced when administered purified staphylococcal peptidoglycan (Chau *et al.*, 2009). The IL-10 response during bacteremia, however, may act as a double-edged sword if while suppressing widespread T cell activation, it also suppresses development of a

proper antigen-specific inflammatory response to clear *S. aureus* bacteremia. Clinically, serum cytokine levels of IL-10 in patients with *S. aureus* bacteremia correlate to a higher incidence of mortality, whereas higher levels of the pro-inflammatory cytokine IL-1 β correlates to shorter duration of bacteremia (Rose *et al.*, 2012), indicating that IL-10 may play a negative role in preventing development of an immune response required to clear infection.

1.4.3 Cytokine responses to other members of the airway microbiota

A recent study characterized the production of IL-23, IL-12p70 and IL-10 from dendritic cells in response to pathogenic and non-pathogenic species from the airway (Larsen *et al.*, 2012). The cytokine profiles segregated into three subgroups. One subgroup consisted of the pathogenic species *M. catarrhalis* and *H. influenzae* and was characterized by production of high IL-23 (11-14 ng/mL), high IL-12p70 (4-5 ng/mL), and high IL-10 (12-14 ng/mL). An intermediate group consisted of non-pathogenic *Prevotella* spp. and was characterized by intermediate IL-23 (3-5 ng/mL), intermediate IL-12p70 (1-2 ng/mL) and high IL-10 (6-10 ng/mL). A low subgroup consisted of the non-pathogenic species *Veillonella dispar* and *Actinomyces* spp. and was characterized by low levels of IL-23 and IL-12p70 comparable to the unstimulated control, and low levels of IL-10 (1-2 ng/mL) (Larsen *et al.*, 2012). Although the downstream effector responses were not investigated, IL-10 appears to be an important cytokine produced in response to bacteria from the airway, and ratios of pro- and anti-inflammatory cytokines in this study suggest that species-specific responses regulate whether a bacteria is recognized as a commensal or pathogen.

Based on the studies of *S. pneumoniae* and *S. aureus* it is evident that the host

employs multiple strategies to limit adverse inflammation during colonization in the URT, yet also promote effective clearance during infection. This includes development of divergent branches of adaptive immunity (i.e. Th17/Tregs to control pneumococcal colonization and humoral responses to protect against bacteremia) and compartmentalization of pro- and anti-inflammatory cytokine production (i.e. induction of IL-10 from monocytes/macrophages and IL-12/IL-23 from dendritic cells in response to *S. aureus*). IL-10 is also produced in response to other airway commensals, suggesting that this cytokine might be important in regulating asymptomatic colonization in the airway.

1.5 Experimental questions and hypothesis

Given the importance of cytokines in the initiation and regulation of immune responses to URT bacteria, and the lack of knowledge of interactions between the SMG and the immune system, we therefore sought to investigate production of the cytokines IL-1 β , TNF, IL-10, IL-12p70, IL-23, IL-17A, and IL-4 from innate immune cells in response to the SMG. The Surette laboratory has a collection of 176 clinical isolates of the SMG, including three ATCC typed strains, that are phenotypically characterized (Grinwis *et al.*, 2010a,b). The pathogenic spectrum of the clinical isolates ranges from acute infections, including hip abscesses (1), brain abscesses (5), liver abscesses (4), empyema (11), blood (15), and unknown invasive infections (12), to chronic infections, including isolates from the CF airways cultured during periods of stability and exacerbation (isolated at an abundance of $\geq 10^7$ CFU/mL) (128). This diversity provides the opportunity to address both immunological and microbiological questions in order to better understand the commensal-pathogenic spectrum

of the SMG.

1. The isolates from the CF airways allow investigation of the immune response to members of the airway microbiota, especially those implicated in chronic inflammatory disorders. This is of particular importance in the field of CF research, since it is currently unknown whether the pathogenicity of the SMG is through a direct or indirect mechanism (Parkins *et al.*, 2008). SMG pathogenicity in the CF airways is dependent on reaching a threshold of bacterial abundance $\geq 10^7$ CFU/mL (Sibley *et al.*, 2010a). Whether this correlates with an increase in immune activation can be explored by testing the immune response to isolates from stable periods or exacerbation, while also providing general knowledge on how the immune system recognizes mucosal-associated bacteria.

2. Given that there are no clear host determinants of SMG invasive infections, attention to human variability in the immune response to the SMG will aid in identification of differences to focus on in future studies. This might include ratios of pro- and anti-inflammatory cytokines, since human variability in ratios of pro- and anti-inflammatory cytokine production during sepsis or bacteremia correlates with clinical morbidity and mortality (Van Dissel *et al.*, 1998; Rose *et al.*, 2012), or differences in development of adaptive immune responses.

3. Considering the phenotypic (biochemical and exoenzyme) and genetic heterogeneity of the SMG, it can be investigated whether this diversity is reflected in their

immunostimulatory properties. The cytokine profiles of the SMG can be used to define a spectrum of responses that can then be related to pathogenicity to investigate whether cytokine profiles distinguish more pathogenic isolates.

These questions were explored in this study by characterizing the cytokine profiles produced by human peripheral blood mononuclear cells (PBMCs) to 35 clinical isolates of the SMG that originated from a variety of invasive infections and the CF airways. We hypothesized that the commensal-pathogen spectrum of the SMG would be related to their immunostimulatory properties, since isolates from invasive infections and the airway have divergent outcomes on the host's health. Specifically, we hypothesized that the airway isolates would induce a higher amount of the anti-inflammatory cytokine IL-10 relative to the invasive isolates.

Chapter 2

Materials and Methods

2.1 Bacterial strains

The 35 isolates of SMG used in this study included three ATCC reference strains (*S. anginosus* strain ATCC33397, *S. constellatus* strain ATCC27823, and *S. intermedius* strain ATCC27335) and 32 clinical isolates. Isolates from invasive infections (hip abscess, brain abscess, empyema, blood, or unknown source) were obtained from the Calgary Laboratory Services. Airway isolates were cultured from the sputum of cystic fibrosis patients during periods of stability or exacerbation (Sibley *et al.*, 2010b; Grinwis *et al.*, 2010a). The airway isolates from exacerbation were cultured at an abundance of ($\geq 10^8$ CFU/mL). The SMG were cultured in Todd Hewitt broth supplemented with 5 g/L yeast (THY) at 37 °C, 5% CO₂. Twenty-four hour cultures were centrifuged at 13,000 rpm for 5 minutes, washed 1X in sterile phosphate buffered saline (PBS) and resuspended in sterile PBS. Colony forming units (CFUs) were enumerated by plating serial dilutions on THY agar followed by incubation at 37 °C, 5% CO₂ for 24 h. Cell suspensions were heat-killed for 10 minutes at 65 °C.

2.2 Primary cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood of healthy human donors (n=3, age range 30-50 years, male:female 2:1) using Ficoll-Paque (GE Healthcare) and Leucosep tubes (Greiner Bio-One). Cells were cultured in X-VIVO 10 media (Lonza) containing gentamicin and 1% autologous serum. Cells were seeded at a density of 5×10^5 cells/well in a 96-well round bottom plate (BD Bioscience) in a final volume of 200 μ L. These studies were approved by the McMaster Research Ethics Board and written informed consent was obtained for all participants.

2.3 PBMC stimulation

Heat-killed SMG were added to freshly isolated PBMCs in triplicate at a ratio of 5:1 (PBMCs:Bacteria) in a final volume of 210 μ L. After 24 h of stimulation, PBMCs were centrifuged at 1,500 rpm for 5 minutes and supernatants were collected and stored at -20°C until analysis. Levels of IL-1 β , TNF, IL-10, IL-12p70, IL-23, IL-17A, and IL-4 were measured in cell supernatants using the MILLIPLEX Map Human Th17 Magnetic Bead Panel (EMB Millipore) following the manufacturer's recommendations and run on the Luminex[®] xMAP[®] platform. PBMCs from a single isolation from donor C were used to survey all 35 isolates. PBMCs isolated on three separate occasions were used to survey the 35 isolates in donors A and B. Isolations occurred within 4 weeks of each other. The isolate C984 was included in each experiment to control for intra-donor variability across isolations. Release of lactate dehydrogenase (LDH) from PBMCs was used to assess PBMC viability using the CytoTox96

® non-radioactive cytotoxicity assay kit (Promega) following the manufacturer's instructions. The formula $(\text{OD of treated} - \text{OD of untreated}) / (\text{OD of complete lysis control} - \text{OD of untreated}) \times 100$ was used to calculate percentage of dead cells.

2.4 Analysis of cytokine data

The mean cytokine value of replicates was scaled to the highest value for each donor tested to generate the heatmap and dendrogram for cluster analysis and principle coordinate analysis. Cluster analysis was performed by calculating the distance matrix using the Euclidean method followed by complete linkage hierarchical clustering using the `gplots` package in R. Principle coordinate analysis was performed using the Bray-Curtis method in the `vegan` package in R. The slopes and r^2 values for pairwise plots of IL-1 β vs IL-10, IL-1 β vs TNF, and IL-10 vs TNF were calculated in R. For all other cytokine analysis, the mean cytokine value across all three donors was used. Statistical analysis was performed using ANOVA with Tukey HSD post-hoc comparisons in R.

2.5 Detection of pre-existing antibodies

Heat-killed bacteria were incubated with donor plasma for 1 hour at room temperature in a v-bottom 96-well plate (Costar). Cells were washed 2X with PBS and incubated with the Alexa Fluor ® 488-conjugated AffiniPure Donkey Anti-Human IgG detection antibody (Jackson ImmunoResearch) diluted 1/800 in FACS wash buffer (0.2 mM EDTA, 0.5% fetal bovine serum in PBS) for 30 minutes at room temperature. Cells were washed and resuspended in FACS wash buffer and run on the BD LSR II. FlowJo

software was used to analyze results.

2.6 TLR2 NF- κ B:SEAP Reporter Assay

The HEK-Blue-2 cell line (InvivoGen Plasmotest) stably expresses TLR2 and a secreted embryonic alkaline phosphatase (SEAP) reporter gene inducible by the transcription factors NF- κ B and AP-1. Cells were cultured in complete DMEM (2 mM L-glutamine, 10% fetal bovine serum, 50 U/mL penicillin, 50 μ g/mL streptomycin) with the addition of 350 μ g/mL of hygromycin, 5 μ g/mL blasticidin, and 25 μ g/mL zeocin. Upon reaching 80% confluency, cells were seeded at a density of 2×10^4 cells/well in complete DMEM in a 96-well flat bottom plate (BD Biosciences). After 24 h media was replaced with HEK-Blue Detection media (InvivoGen) and cells were treated with 10^6 heat-killed cells of SMG or dilutions of Pam₃Csk₄ (InvivoGen) (highest final concentration of 0.1 μ g/mL) as a positive control. Each sample was performed in triplicate. Absorbance was read at 630 nm at 24 on the BioTek Synergy H1 plate reader. Fold change was calculated relative to the unstimulated control.

Chapter 3

Results

3.1 Optimization of variables for surveying the cytokine response to the SMG

Thirty-five isolates of SMG were selected from the Surette laboratory collection of characterized clinical isolates (Grinwis *et al.*, 2010a,b), including 12 *S. anginosus*, 12 *S. intermedius*, and 11 *S. constellatus*. Isolates varied in phenotypic traits and clinical source to represent the heterogeneity of the SMG (**Table 3.1**). PBMCs were stimulated with heat-killed SMG for 24 h, as this was determined to be the optimal time point for stimulation (**Fig. A.1**). Levels of the anti-inflammatory cytokine IL-10, and pro-inflammatory cytokines TNF, IL-1 β , IL-12p70, IL-17A, IL-23, and IL-4 were measured in cell supernatant by multiplex assaying. All 35 isolates were surveyed with PBMCs from a single isolation for one donor (donor C), and over three isolations for two donors (donors A and B). The response to isolate C984 served as an internal control across experiments for donors A and B and did not differ significantly with each isolation (**Fig. A.2**). Dose curves for all 35 isolates were performed in donor

C (**Fig. A.3**) and based on this a dose of 10^5 cells were added to 5×10^5 PBMCs for experiments with donors A and B. Both conserved responses and divergent donor responses to the 35 isolates of SMG were observed (**Fig. A.4**).

Table 3.1: Clinical source of SMG isolates used in this study.

| Species | Clinical source | No. of isolates |
|------------------------|-----------------------|-----------------|
| <i>S. anginosus</i> | ATCC Typed | 1 |
| | Airway | 7 |
| | Blood | 1 |
| | Empyema | 1 |
| | Exacerbation | 1 |
| | Invasive ^a | 1 |
| <i>S. constellatus</i> | ATCC Typed | 1 |
| | Airway | 3 |
| | Blood | 2 |
| | Brain | 1 |
| | Empyema | 2 |
| | Exacerbation | 1 |
| | Invasive ^a | 1 |
| <i>S. intermedius</i> | ATCC Typed | 1 |
| | Airway | 3 |
| | Blood | 4 |
| | Brain | 1 |
| | Exacerbation | 1 |
| | Hip Abscess | 1 |
| | Invasive ^a | 1 |
| Total | | 35 |

^a Source of infection unknown.

3.2 *S. anginosus*, *S. constellatus*, and *S. intermedius* elicit similar cytokine responses from human peripheral blood mononuclear cells

The SMG induced all cytokines tested to varying levels, including high levels of IL-1 β , TNF, IL-10, and IL-23 (average of 5113 pg/ml, 5406 pg/ml, 902 pg/ml, 330 pg/ml respectively) and lower levels of IL-12p70, IL-17A and IL-4 (average of 28 pg/ml,

8 pg/ml, 5 pg/ml respectively). Within a species, isolates varied in their ability to induce cytokine production from immune cells, resulting in a range of cytokine production for each cytokine measured (**Table 3.2**). The mean value of each cytokine produced was comparable across the three species except for IL-17A (**Fig. 3.1A**). *S. intermedius* induced significantly higher production IL-17A compared to *S. constellatus* ($P < 0.001$) and *S. anginosus* ($P = 0.031$). Aside from this species-specific difference, the cytokine response was generally conserved across the three species. Principle coordinate analysis (PCoA) confirmed that the variability in cytokine production was not species-specific except for IL-17A (**Fig. 3.1B**).

Table 3.2: Average and range of cytokine production from human PBMCs (n=3) in response to *S. anginosus*, *S. constellatus*, and *S. intermedius* isolates.

| Cytokine | Control pg/mL | <i>S. anginosus</i> Average (Range) pg/mL | <i>S. constellatus</i> Average (Range) pg/mL | <i>S. intermedius</i> Average (Range) pg/mL |
|--------------|------------------|---|--|---|
| IL-1 β | 31.3 | 4993.4 (26-22731) | 4067.5 (78-15555) | 6795.9 (218-22834) |
| TNF | 58.2 | 6031.9 (71-19541) | 5864.5 (92-17245) | 4955.4 (63-17929) |
| IL-10 | 9.4 | 852.5 (9-2632) | 938.6 (22-5736) | 1086.7 (13-4580) |
| IL-23 | 0 | 345.6 (0-1666) | 425.3 (0-2603) | 243.9 (0-2523) |
| IL-12p70 | 0.7 | 37.8 (0-211) | 27.2 (0-162) | 21.7 (0-189) |
| IL-17A | 0 | 7.4 (0-44) | 3.6 (0-28) | 12.8 (0-78) |
| IL-4 | 0.4 | 5.8 (0-17) | 5.5 (0-13) | 5.9 (0-17) |

3.3 Cytokine profiles define a spectrum of cytokine responses to the SMG

3.3.1 Isolate-specific differences

A heatmap of cytokine profiles for all 35 isolates was generated and clustered by hierarchical clustering to visualize isolate-specific and donor-specific responses. The cluster

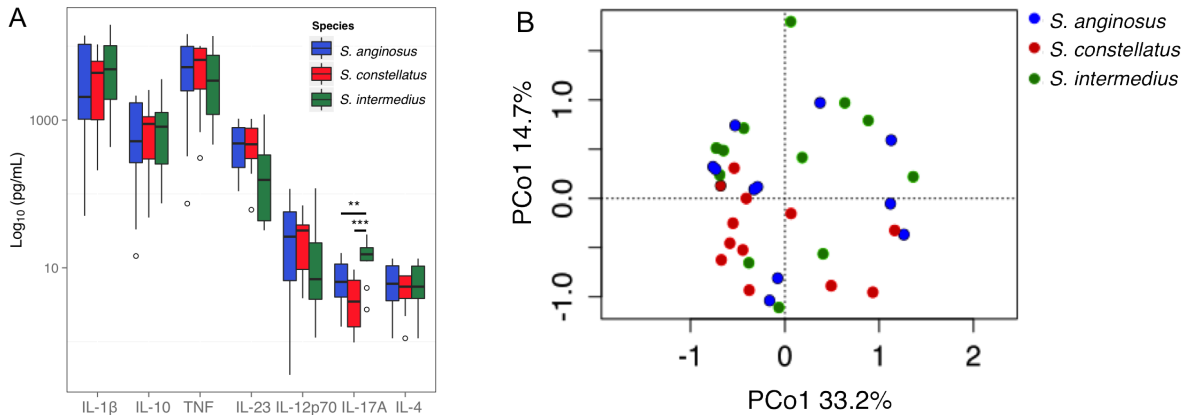


Figure 3.1: Species differences in cytokine profiles

PBMCs from three donors were stimulated with 35 heat-killed isolates of the SMG (12 *S. anginosus*, 11 *S. constellatus*, and 12 *S. intermedius*) at a ratio of 5:1 (PBMCs:Bacteria). Levels of IL-1 β , TNF, IL-10, IL-12p70, IL-23, IL-17A and IL-4 were measured in cell supernatant after 24 h by multiplex assaying. A. Cytokine values were averaged for all three donors. The mean cytokine production for each species was not significantly different for IL-1 β , TNF, IL-10, IL-12p70, IL-23 and IL-4. *S. intermedius* induced significantly higher production of IL-17A in comparison to *S. constellatus* and *S. anginosus*. Statistical significance was determined by ANOVA with Tukey HSD post-hoc comparisons. *** $P < 0.001$, ** $P < 0.01$. B. PCoA of cytokine profiles for 35 isolates of the SMG. Cytokine values were scaled to the highest value for each individual donor. Data was analyzed by PCoA. Isolates are coloured by species.

analysis revealed three subgroups of cytokine profiles: high, intermediate, and low responses (**Fig. 3.2**). Isolates did not cluster by species (High: 50% *S. intermedius*, 40% *S. anginosus*, 10% *S. constellatus*, Intermediate: 70% *S. constellatus*, 30% *S. anginosus*, Low: 47% *S. intermedius*, 33% *S. anginosus*, 20% *S. constellatus*), corresponding with the PCoA analysis. Rather, the isolates appeared to separate based on whether they originated from invasive infections or the airway. In both the high and intermediate response groups, 80% of isolates originated from invasive infections, compared to 20% in the low response group in which the majority originated from the airways. Cytokine profiles did not group invasive isolates from the same source of infection together in the spectrum. To eliminate the possibility that the lack of cytokine production was due to cytotoxicity, the amount of lactate dehydrogenase

(LDH) released by PBMCs was measured to assess viability. The SMG induced a range of cell death (1-20% cytotoxicity) (**Fig. A.6**), however cytotoxicity did not correlate with cytokine responses (**Fig. 3.3A**).

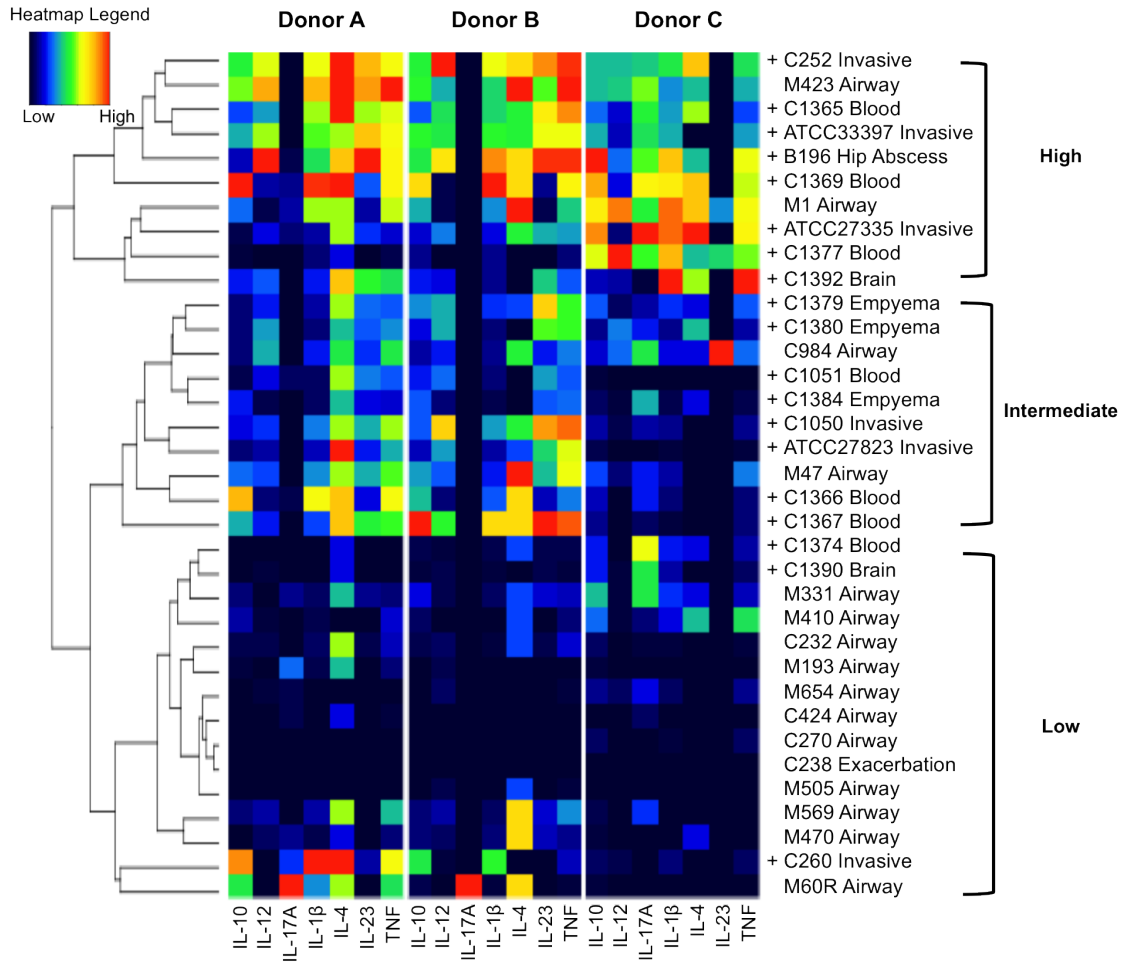


Figure 3.2: Heatmap and cluster analysis of cytokine profiles for 35 isolates of the SMG. Cytokine values were scaled to the highest value for each individual donor. A heatmap of scaled data was generated and analyzed by hierarchal clustering. Cytokine profiles separate into three major groups of responses; high, intermediate, and low. Isolates are labelled with the isolate ID and clinical source. The + symbol indicates isolates from invasive infections.

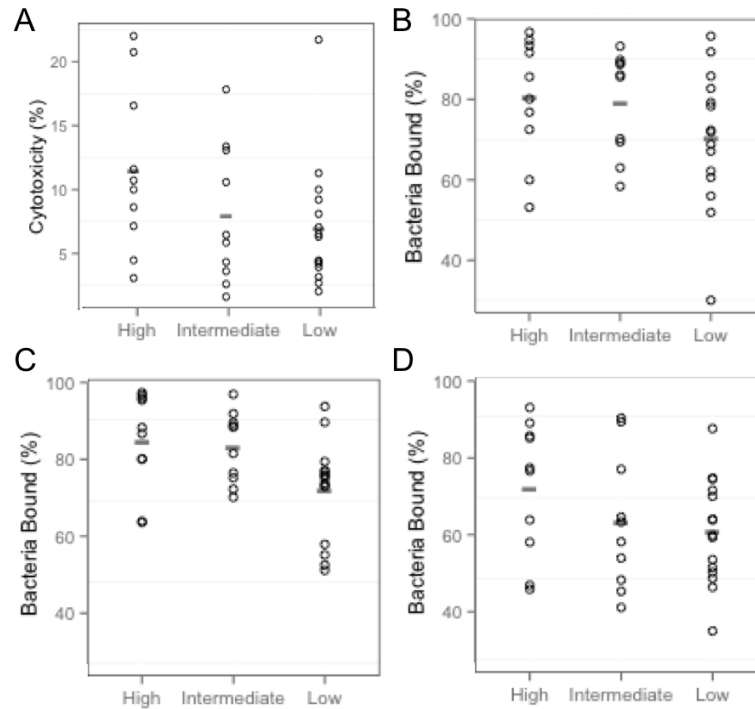


Figure 3.3: PBMC cytotoxicity and antibody responses.

A. PBMC viability was assessed by detection of LDH release by PBMCs stimulated with heat-killed SMG for 24 h. Viability was expressed as percent dead cells relative to a complete lysis control. B-D. IgG was detected in plasma of donors A, B and C by flow cytometry. Bound antibody was calculated as a percentage of fluorescently positive cells compared to cells not bound by IgG (no plasma negative control). Data was plotted based on grouping of cytokine responses (High = 10 isolates, intermediate = 10 isolates, low = 15 isolates). Neither cytotoxicity nor antibody responses were significantly different across cytokine response groups. Statistical analysis was performed using ANOVA with Tukey HSD post-hoc comparisons.

3.3.2 Donor-specific differences

The cytokine spectrums were fairly consistent across the three donors (**Fig. A.5**), although donor-specific responses to individual isolates were observed. ATCC27335 and C1377 induced low cytokine responses in donors A and B, but induced a high

cytokine response in donor C. Conversely, C260 and M60R induced low cytokine responses in donor C, but high cytokine responses in donors A and B (**Fig. 3.2**). It was investigated whether these donor differences were related to pre-existing antibodies to the SMG. The presence of SMG-specific IgG in donor plasma was measured by flow cytometry. All three donors possessed antibodies to all 35 isolates, although the percentage of bacterial cells bound varied across isolates. The variation did not correlate with cytokine responses (**Fig. 3.3B-D**). Previous evidence suggests that the IgG response to the SMG is isolate-specific (Surette, personal communication), therefore it is questionable whether the positive results were due to antibody cross-reactivity or false positives.

In addition to donor-specific responses to certain isolates, overall differences in the quality of the immune response to the SMG from the three donors was observed. In general, donors A and B responded more similarly to the SMG than donor C. One contributing factor was that donors A and B elicited an IL-23 response to isolates but IL-17A was absent, whereas donor C elicited an IL-17A response to isolates but IL-23 was absent (**Fig. 3.2**). The isolate M60R, however, induced an IL-17A response in donors A and B (3.8 and 71.5 pg/mL respectively), suggesting that the IL-17A response is inducible in these donors. Similarly, donor C produced IL-23 in response to isolates M1, C1377, and C984 (73, 97, and 222 pg/mL respectively) suggesting the IL-23 response is intact in this donor. Another difference noted between donor C and donors A and B was the ratios of pro- and anti-inflammatory cytokines induced. IL-1 β , TNF, and IL-10 were positively correlated for all three donors (**Fig. 3.4**) however the slope of the line for the pairwise plot of IL-1 β vs IL-10 and IL-1 β vs TNF in donor C varied from those of donors A and B (*Table 3.3*). The slope of the pairwise plot

of IL-10 and TNF did not vary across the three donors.

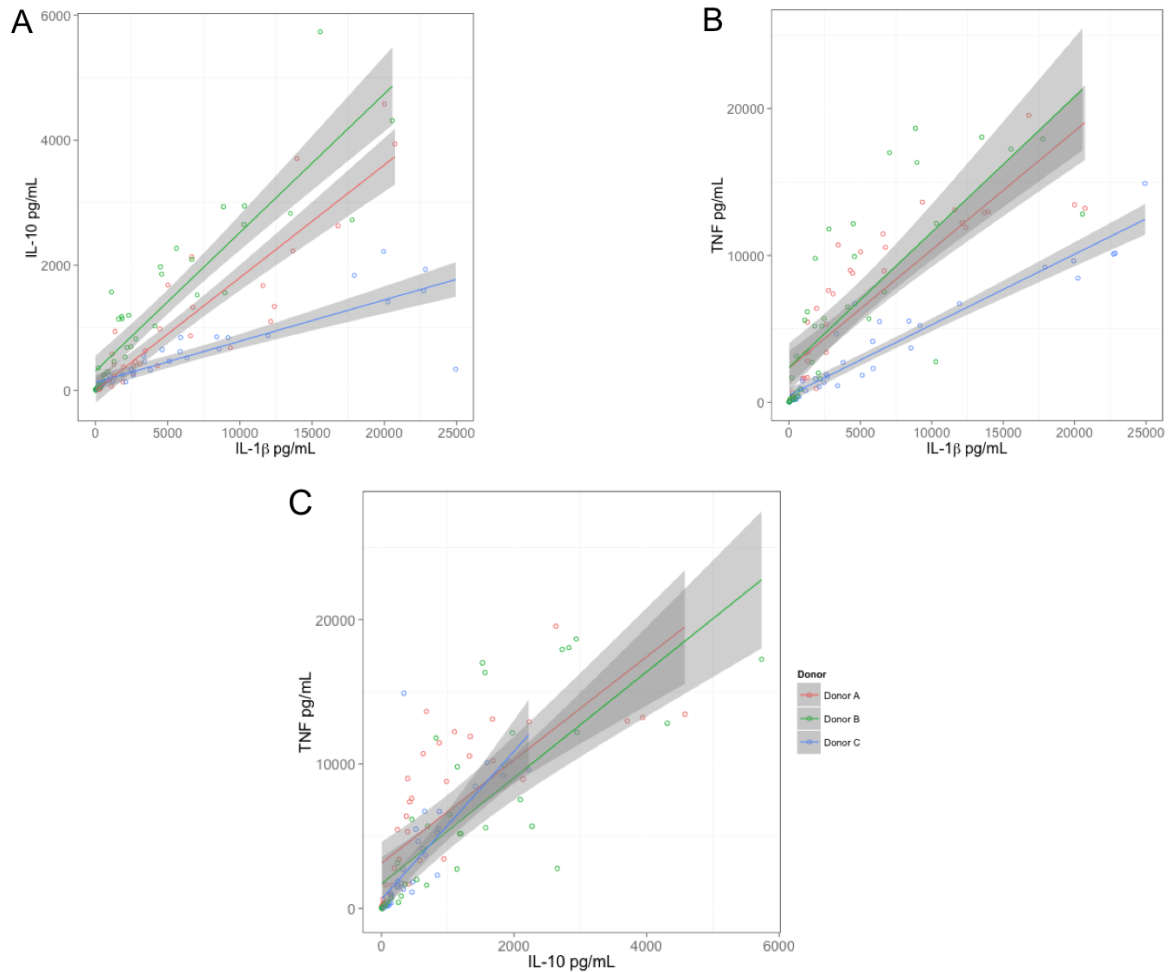


Figure 3.4: Pairwise plots of pro- and anti-inflammatory cytokines IL-1 β , TNF, and IL-10. Pairwise plots of IL-1 β , TNF, and IL-10 and the linear regression line were generated for donors A, B, and C. The grey shading represents the standard deviation.

Table 3.3: Slope and r^2 values for pairwise plots

| Cytokines | Donor A | | Donor B | | Donor C | |
|-----------------------|---------|-------|---------|-------|---------|-------|
| | Slope | r^2 | Slope | r^2 | Slope | r^2 |
| IL-1 β vs IL-10 | 0.18 | 0.951 | 0.22 | 0.9 | 0.066 | 0.853 |
| IL-1 β vs TNF | 0.81 | 0.871 | 0.92 | 0.789 | 0.48 | 0.953 |
| IL-10 vs TNF | 3.57 | 0.76 | 3.67 | 0.774 | 5.12 | 0.786 |

3.4 Invasive isolates induce higher IL-1 β , TNF, IL-10, IL-23, and IL-12p70 production than airway isolates

The variability in cytokine responses was next compared to the source of isolation. Isolates from acute invasive infections (blood, brain, empyema, hip abscess) were compared to isolates from chronically infected CF airway during periods of stability and exacerbation. Invasive isolates induced significantly higher IL-1 β , TNF, IL-10, IL-23, and IL-12p70 production than airway isolates (**Fig. 3.5**). There was no significant difference in cytokines induced by invasive isolates from different sources of infection (**Fig. A.7**) or airway isolates cultured during periods of stability or exacerbation (**Fig. A.8**).

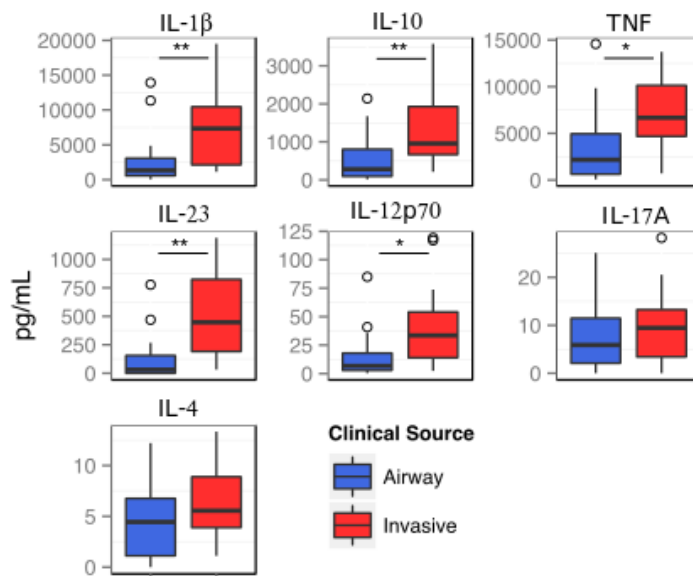


Figure 3.5: Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by invasive and airway of the SMG.

Invasive isolates include all isolates from blood, brain abscesses, empyema, hip abscess, and invasive infections of an unknown source. Airway isolates include airway isolates from periods of stability and exacerbation. Statistical significance between invasive and airway isolates was analyzed by ANOVA. ** $P < 0.01$, * $P < 0.05$.

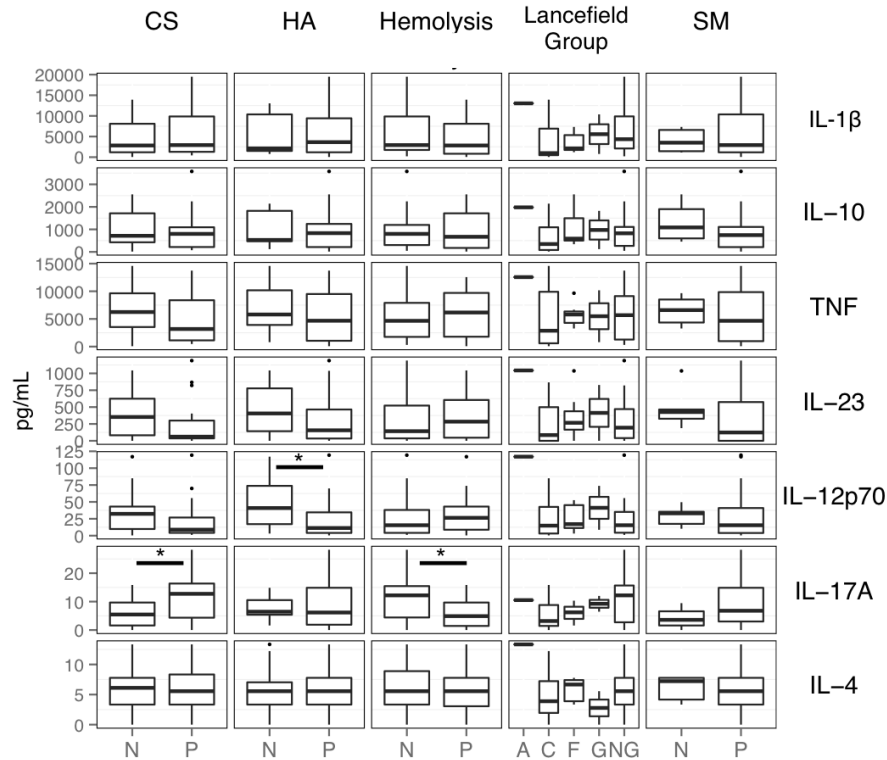


Figure 3.6: Cytokine induction by isolates of varying phenotypic characteristics. Cytokine production by each isolate was compared to phenotypic characteristics, including putative virulence factors. Phenotypic traits: CS = Chondroitin sulfatase, HA = Hyaluronidase, SM = Proteolytic activity on skin milk agar, Hemolysis = β -hemolysis on sheeps blood agar, Lancefield = Lancefield Group. X-axis: N = Negative test, P = Positive test. Statistical significance was analyzed by ANOVA. * $P < 0.05$.

3.5 Phenotypic traits of the SMG do not correlate with induction of cytokines

Phenotypic traits, including Lancefield grouping, β -hemolytic activity on sheep's blood agar, and production of proteases, DNases, HA, and CS, are used clinically to differentiate isolates of SMG (Clarridge *et al.*, 2001; Jacobs and Stobberingh, 1995; Whiley *et al.*, 1990). Comparison of phenotypic traits of isolates, as previously characterized by Grinwis *et al.*, (Grinwis *et al.*, 2010a), to cytokine production revealed

no association between phenotypic traits and induction of TNF, IL-1 β , IL-10, IL-23, or IL-4, with the exception of HA positive isolates, which induced significantly lower amounts IL-12p70 than HA negative isolates ($P = 0.04$), CS positive isolates, which induced significantly lower amounts of IL-17A than negative isolates ($P = 0.012$), and β -hemolytic positive isolates, which induced significantly lower amounts of IL-17A than β -hemolytic negative isolates ($P = 0.033$) (**Fig. 3.6**). Grinwis *et al.*, also identified phenotypic biotypes within the *S. anginosus* species (phenotypically active, phenotypically non-active, and HA positive) and *S. constellatus* species (Non-groupable/Lancefield group F non-active, Lancefield group F active, and Lancefield group C and β -hemolytic) based on 18 biochemical properties, including the aforementioned phenotypes (Grinwis *et al.*, 2010a). *S. anginosus* isolates of the active biotype and *S. constellatus* isolates of the Lancefield Group F active biotype were present in only the intermediate response group, whereas the other four biotypes were present in two or more response groups (**Fig. 3.7**). The phenotypic traits therefore do not provide diagnostic value to identifying more inflammatory isolates.

3.6 Cytokine production is independent of TLR2 activation

The observation that the SMG induce production of IL-10 from PBMCs was intriguing due to recent evidence that an unidentified ligand embedded in *S. aureus* peptidoglycan induces a TLR2-, PI3K/Akt-dependent IL-10 response from human monocytes and macrophages (Frodermann *et al.*, 2011; Chau *et al.*, 2009). We examined whether the SMG signal through a similar mechanism using a HEK293 NF- κ B:SEAP reporter cell line expressing TLR2. The SMG induced a range of TLR2 activation (1-13-fold increase compared to the unstimulated control) (**Fig. A.9**). Isolates from the low

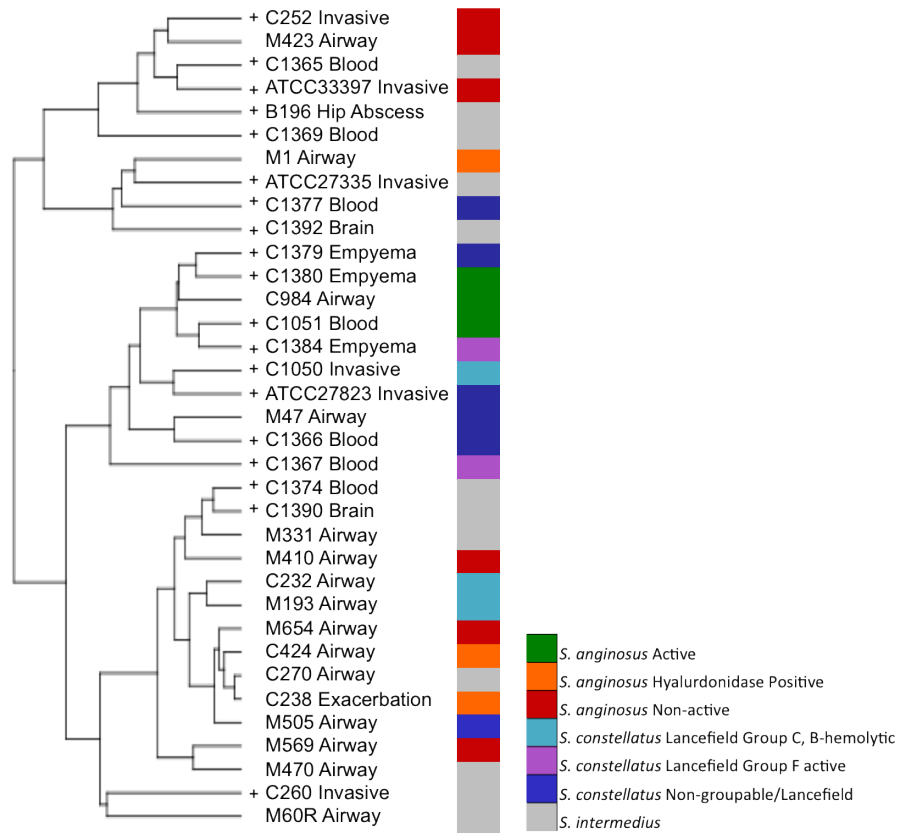


Figure 3.7: Location of phenotypic biotypes of the SMG within the cytokine spectrum. *S. anginosus* and *S. constellatus* biotypes were aligned to the dendrogram from the cytokine spectrum. Isolates are coloured according to biotypes. The + symbol indicates isolates from invasive infections.

cytokine response group were able to activate TLR2 signalling (**Fig. 3.8**). This suggests that the mechanism of recognition of the SMG differs from that of *S. aureus* since TLR2 recognition of *S. aureus* is positively correlated with cytokine induction (Frodermann *et al.*, 2011). The activation of TLR2 by isolates in the low response group also suggests that the lack of a cytokine response from PBMCs might involve active down-regulation of the response.

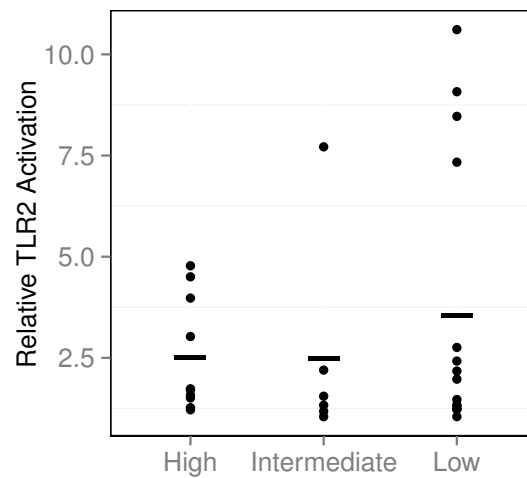


Figure 3.8: TLR2 activation in response to the SMG using a reporter cell line

HEK293 cells expressing TLR2 and an NF- κ B:SEAP reporter were stimulated with heat-killed SMG at a ratio of 500:1 Bacteria:HEK293 cells. Serial dilutions of the TLR2 ligand Pam₃Csk₄ were included as a positive control. Detection of SEAP expression was determined by reading absorbance at 630 nm at 24 h. Data are mean of three replicates from a single experiment. Fold activation is plotted based on cytokine response groups.

Chapter 4

Discussion

The search for factors that distinguish pathogenic from non-pathogenic isolates of the SMG in the past has proven unsuccessful. Most studies approach this problem from a purely microbial perspective, and although putative virulence factors have been identified, the equal presence of such factors in both pathogenic and non-pathogenic isolates supports the idea that all isolates of the SMG are equally pathogenic (Grinwis *et al.*, 2010a; Jacobs and Stobberingh, 1995). This is the first study to characterize the cytokines produced by innate immune cells in response to a range of clinical isolates of the SMG and relate this to pathogenicity, aside from unpublished data from additional collaborative studies between the laboratories of Drs. Michael Surette and Dawn Bowdish. The SMG induced a broad range of pro- and anti-inflammatory cytokines that varied across 35 clinical isolates in both magnitude and proportion. The cytokine profiles of the SMG resembled those induced by other Gram-positive pathogens in the airway (Frodermann *et al.*, 2011; Martner *et al.*, 2009; Niers *et al.*, 2005), and as such, future studies can compare the mechanisms of recognition and signalling. The most notable difference in cytokine profiles across the SMG was that

invasive isolates induced significantly higher cytokine production than airway isolates. The separation of invasive from airway isolates by innate stimulatory capacity provides a basis for future studies to compare the variables that might affect this response and are therefore involved in pathogenicity. This might include a comparative genomic strategy to identify the presence or absence of genes encoding cell wall proteins or capsular synthesis genes. Characterization of the host response to the SMG provides a basis for future studies to investigate the downstream effector responses as well as identifying microbial differences that result in the variability of responses. Furthermore, the donor-specific differences observed in this study provide rationale for future studies to focus on the Th17 branch of adaptive immunity and the consequences of this response in terms of host susceptibility.

4.1 Induction of pro- and anti-inflammatory cytokines by the SMG

4.1.1 Induction of the pro- and anti-inflammatory cytokines IL-1 β , TNF, IL-10

There are limited studies that use *in vivo* mouse models to study SMG infections (Kuriyama *et al.*, 2000; Takahashi *et al.*, 2011; Kanamori *et al.*, 2004; Shinzato and Saito, 1994, 1995) and of those, even fewer have characterized the immune response to infection (Salam *et al.*, 2006). Collaboration between the Bowdish and Surette labs has resulted in the development of a mouse model of SMG sub-cutaneous abscesses. Experimental data suggests that SMG abscesses involve an influx of neutrophils and that this response is strain specific (Jayanth and Pinto, unpublished data). It might

prove interesting to examine the role of IL-1 β and TNF in this model, since these cytokines are shown to be required for the formation and clearance of *S. aureus* induced cutaneous infections and brain abscesses in mice (Kielian, 2004; Cho *et al.*, 2012).

The SMG also induced the anti-inflammatory cytokine IL-10, an intriguing observation since recently *S. aureus* was also documented to induce IL-10 from monocytes and macrophages in a TLR2-dependent manner (Frodermann *et al.*, 2011; Chau *et al.*, 2009). The SMG isolates in this study were found to signal through TLR2, however the degree of activation did not reflect the location of the isolate in the cytokine spectrum, suggesting a linear relationship between TLR2 activation and cytokine induction does not exist as it does for *S. aureus*. This does not rule out the importance of TLR2, but suggests that additional pattern recognition receptors and signalling pathways are implicated in the recognition of and response to the SMG. Preliminary data from the Bowdish and Surette labs suggest that the SMG induce the NF- κ B signalling pathway as well as the PI3K/Akt signalling pathway in human macrophages (Pelka and Stearns, unpublished data). The PI3K/Akt signalling pathway plays an important role in regulating ratios of IL-10 and IL-12 production (Fukao and Koyasu, 2003; Weichhart and Saemann, 2008), therefore this pathway would make an interesting focus of future studies that elucidate the mechanisms involved in IL-10 induction.

The anti-inflammatory capacity of IL-10 is favourable to the host if it prevents damaging inflammation, however if it impairs a proper pro-inflammatory immune response it can also be detrimental. The pairwise plots of IL-1 β , TNF, and IL-10 from donors A, B, and C in this study illustrate the human variability in the proportions of

pro- and anti-inflammatory cytokines induced in response to bacteria. Since 12-32% of abscess infections with SMG are complicated by bacteremia (Bert *et al.*, 1998), I predict that human variability in ratios of pro- and anti-inflammatory cytokines regulate the outcome of SMG infections and clinically, might provide prognostic value, particularly during systemic infections.

4.1.2 Induction of the T cell polarizing cytokines IL-12p70, IL-23, and IL-4

We included IL-23, IL-12p70, and IL-4 in the cytokine panel because of their role in polarization of CD4⁺ cells towards specific Th cell lineages. The SMG induced higher levels of IL-23 than IL-12p70 and IL-4 (average of 12 fold induction of IL-23 relative to IL-12 and 66 fold induction relative to IL-4). These levels are also higher than the response reported to other Gram-positive bacteria (Martner *et al.*, 2009; Niers *et al.*, 2005). Furthermore, donor C was observed to elicit an IL-17A response to some isolates in this study (26/35 isolates, range of 3-78 pg/ml). Preliminary data suggests that donor A also develops a Th17 cell response with prolonged stimulation. PBMCs stimulated for 5 days with a selection SMG isolates from the high, intermediate, and low response groups produced IL-17A, which corresponded with the location of the isolate in the cytokine spectrum, except for C1392 (**Fig. A.10**). The Th17 response is gaining increasing attention in the control of respiratory pathogens at mucosal surfaces, including *S. pneumoniae* (Zhang *et al.*, 2009; Lu *et al.*, 2008), *Klebsiella pneumoniae* (Aujla *et al.*, 2008; Ye *et al.*, 2001) and *Candida albicans* (Conti *et al.*, 2009; Hernández-Santos *et al.*, 2012; Pandiyan *et al.*, 2011). Based on the preliminary evidence and the literature supporting a role for Th17 cells in the airway, I predict that

this response is involved in controlling colonization of the SMG. Immunostimulatory isolates that induce an IL-17 response would effectively be cleared in the airway, which is why immunostimulatory isolates from the airway did not appear in this study. This could be tested by characterizing the Th cell cytokines IFN γ , IL-17A, and IL-10 from human PBMCs stimulated with the SMG *in vitro* assays or using a mouse model of colonization in the airway.

It is important to consider that the cytokine profiles in this study originate from healthy PBMCs, and that these responses should not be generalized across all innate immune cells. Additional cell types, such as macrophages, dendritic cells, and neutrophils, might respond differently to the SMG. As with *S. aureus*, compartmentalization of pro- and anti-inflammatory responses to specific cell types might be important in the control of SMG infections.

4.2 Cytokine responses to invasive and airway isolates

The variability in the cytokine response to the SMG corresponded to the clinical source of the isolate and not the phenotypic properties. These results were expected, given that there is no clear association between SMG phenotypes and pathogenicity, and because we hypothesized that pathogenicity is more likely linked to the immune response to an isolate. We originally hypothesized that isolate-specific ratios of pro- and anti-inflammatory cytokines would distinguish isolates from acute invasive infections and chronic infections. This was in part due to the evidence previously discussed on the importance of cytokine ratios for determining whether *S. aureus* is recognized as a commensal or pathogen (i.e. high IL-10 production from monocytes/macrophages is speculated to be involved in suppression of immune responses during colonization,

whereas dendritic cells induce pro-inflammatory cytokines, speculated to be involved in activation of the immune response during infection (Frodermann *et al.*, 2011; Mele and Madrenas, 2010)). The differences we observed in cytokine profiles to invasive and airway isolates, however, were strictly a matter of magnitude.

4.2.1 Invasive isolates

It is not an unexpected result that isolates from invasive infections induce pro-inflammatory cytokine production. Recognition of PAMPs by innate immune cells initiates the inflammatory process to clear infection but if the inflammatory response is sustained and in parallel causes host tissue damage, inflammatory mediators accumulate and tissue healing is delayed resulting in the physiological state of inflammation (Nathan, 2002). Discerning the exact mechanisms of immune activation, and particularly whether the role of IL-10 is favourable to the host or the pathogen is an interesting question to pursue in future studies. I predict that the resultant effects of IL-10 are dependent on the host, strain, and location.

4.2.2 Airway isolates

The absence of cytokine production to airway isolates was an interesting observation that we did not originally hypothesize, however this result can be used to formulate new hypotheses about the recognition of the SMG at mucosal sites. Given the importance of maintaining an anti-inflammatory environment at mucosal sites, the absence of a cytokine response to airway isolates is not unexplainable. The data suggest that the immune system in the airway selects for non-immunostimulatory isolates at the mucosal surface, which therefore suggests that in the healthy state,

the immune system in the airway is effective at clearing immunostimulatory isolates of the SMG. Some of the airway isolates in this study were located in the high and intermediate response group, suggesting that there may be periodic carriage of immunostimulatory isolates, however it should be considered that the airway isolates in this study originate from the CF airways, a chronic inflammatory airway disorder with impaired bacterial clearance (Cohen and Prince, 2012). It would prove interesting to test the immune response to airway isolates cultured from the URT of healthy individuals with the expectation that isolates would fall into the low response group. There would likely be outliers, as seen in this study, that induce high cytokine production if perhaps a particular host is unable to clear an isolate, or if polymicrobial interactions in the airway alter the recognition of SMG. For example, dendritic cells produce IL-12p70 in response to *H. influenzae* but this response is suppressed in the presence of *Prevotella* spp. (Larsen *et al.*, 2012). I would predict that carriage of an immunostimulatory isolate of SMG might predispose an individual to infection upon breach of the mucosal barrier.

Currently it is unknown whether the lack of a response to the SMG is active suppression or simply ignorance. The TLR2 data suggests that active suppression might be used as an immunomodulatory strategy by some isolates. Alternatively, ignorance might be a strategy used by the SMG and might be influenced by whether a strain is encapsulated, since capsules of some streptococci species shield against phagocytosis (Meijerink *et al.*, 2012). The genomes of the SMG encode capsular synthesis genes (Olsen 2013, submitted) and preliminary data suggests that the SMG are able to produce a capsule as detected by microscopy (**Fig. A.11**). The presence of capsule is therefore an important phenotype to characterize in future studies.

It should be noted that several isolates in the low response group induced cytokine production at an increased dose in donor C (10^6 bacteria per 5×10^5 PBMCs) (**Fig. A.3**). This did not, however, correlate with isolates from CF exacerbations. The isolates C232, C238, M505, M569, and M470 did not induce cytokine production at higher doses, isolates M410, M193, M654, C424, C270, C260, and M60R induced higher amounts of primarily IL- 1β and TNF, and isolates C1374, C1390, and M331 induced higher production of three or more cytokines at the high dose. A dose of 10^5 bacteria per 5×10^5 PBMCs dose was originally selected because it provided the most variable responses across isolates, but it is important to recognize the effect of the dose on cytokine production.

4.2.3 Immune recognition of the SMG at other mucosal sites

The SMG are members of the normal microbiota in the gastrointestinal tract (GI) and female urogenital tract in addition to being URT commensals. Studies that have phenotypically characterized isolates of SMG from different mucosal tissues have found that *S. anginosus* is overrepresented in the urogenital tract compared to the two other species (Clarridge *et al.*, 2001; Whiley *et al.*, 1992) and one study suggests a distinct *S. anginosus* biotype is commonly found in the urogenital tract (Clarridge *et al.*, 1999). Research in the Surette lab has also identified the presence of *S. anginosus* in stool samples originating from healthy individuals and irritable bowel syndrome patients (Lau, unpublished data). The GI, similarly to the airway, is a primarily anti-inflammatory environment to prevent adverse responses to the plethora of non-pathogenic antigen it is exposed to, but distinct differences exist between the mucosal-associated lymphoid tissue (Kunisawa *et al.*, 2008). The isolates from other

mucosal sites are likely adapted to colonize those anatomical locations, and since each mucosal site utilizes different mechanisms to maintain the state of immunological homeostasis, I predict that they would form separate subgroups in the cytokine spectrum. Studies that characterize the cytokine profiles to these commensals will help understand immune recognition of commensals at other mucosal sites.

4.2.4 Implications for CF

The airway isolates cultured from CF patients at the time of exacerbation ($\geq 10^8$ CFU/mL) did not induce significantly different cytokine profiles than isolates cultured during periods of stability. It is possible that the role of SMG in CF exacerbations is indirect pathogenesis through synergistic interactions with other infecting pathogens, such as *Pseudomonas aeruginosa* (Parkins *et al.*, 2008). Our research has demonstrated that commensal streptococci synergistically increase *P. aeruginosa* virulence in experimental models of infection by modulating *P. aeruginosa* virulence gene expression and anti-microbial gene-expression in the host (Duan *et al.*, 2003; Sibley *et al.*, 2008b). Alternatively, it is possible that SMG pathogenesis occurs through direct activation of the immune response if a CF patient elicits an adverse response to the isolate they are infected with. Macrophages from CF patients elicit an increased immune response to the pathogen *Burkholderia cenocepacia* compared to healthy controls (Kopp *et al.*, 2012) and overall CF patients are impaired in a variety of immune defense mechanisms (Cohen and Prince, 2012). I predict that the cytokine profiles induced by innate immune cells from CF patients would be more pro-inflammatory than the cytokine profiles from healthy individuals, possibly contributing to persistent immune activation in the airways of these individuals.

4.3 Donor variation in IL-17A induction

Donors A and B in this study elicited more similar responses to each other than to donor C. We noticed that one of the major differences was that donors A and B responded to isolates with production of IL-23 and IL-12p70, but little IL-17A, whereas donor C produced IL-17A but little or no IL-23 and IL-12p70. These divergent responses were also observed in two additional donors that were used in preliminary experiments (**Fig. A.12**). IL-17A is produced by Th17 cells, suggesting that in the span of 24 h, some individuals develop a Th17 response to the SMG, however this occurs in the absence of IL-23, a cytokine required for Th17 cell development, and occurs earlier than the regularly reported time point of 72 h (Lu *et al.*, 2008). A study recently demonstrated that IL-2, IL-15, IL-18, and IL-21 are also able to polarize CD4⁺ T cells towards IL-17A-producing Th17 cells and that the IL-23-induced IL-17A is observed as early as 24 h after stimulation (Hoeve *et al.*, 2006). Interestingly, this study also demonstrated that IL-12p70 inhibits IL-17A production from T cells, which might explain the absence of IL-17A production in donors A and B. IL-12p70, as well as IL-23 to a lesser degree, are required for Th1 cell development (Murphy *et al.*, 2003; Hoeve *et al.*, 2006). The different cytokine responses therefore might translate into divergent adaptive immune responses (i.e. Th1 vs Th17), which could have implications in outcome of SMG infections.

Th1 and Th17 cells have divergent roles in autoimmune disease, where the Th1 response is important for controlling disease pathology and the Th17 response exacerbates it (Murphy *et al.*, 2003). Functions of Th17 cells during mucosal infections range from protective, to exacerbating disease, to no effect at all (Khader *et al.*, 2009). This spectrum of functions is thought to reflect the importance of regulation of the Th17

branch of immunity. It is hypothesized that production of IL-23 by macrophages and dendritic cells at sites of acute infection trigger rapid and localized IL-17 production from resident $\alpha\beta/\gamma\delta$ T cells or natural killer (NK) or NKT cells (McKenzie *et al.*, 2006). This rapid response initiates the inflammatory process and provides time for development of the Th1 adaptive branch to clear infection. If however, the infection is not controlled, sustained IL-17 production could lead to chronic inflammation (McKenzie *et al.*, 2006). I predict that these donor differences are involved in host susceptibility to infection. An interesting observation in SMG pathogenicity is that the incidence of infection is 2X higher in males compared to females (Salavert *et al.*, 1996; Bert *et al.*, 1998). The two individuals in this study that produced the IL-17A response were both male, whereas the other three were a mix of male and female. A gender bias is observed in the Th cell type generated during the pro-inflammatory response, where females are skewed towards Th1 cell development and males are skewed towards Th17 cell development (Zhang *et al.*, 2012). These differences in adaptive immunity might contribute to host susceptibility to infection.

4.4 Future directions to investigate the dynamics of the cytokine spectrum

The donor differences observed in this study demonstrate the dynamics of the spectrum across individuals. It might prove interesting to investigate additional variables that affect the dynamics of the spectrum. On the host side, previously discussed variables include using PBMCs from healthy vs. SMG-infected individuals, or testing different immune cell types. On the microbial side, it might prove interesting to test isolates prepared differently or grown in different conditions.

A preliminary experiment was conducted to test whether lysozyme digestion of isolates releases immunostimulatory components of the bacterial cell, particularly for the low response isolates. The data revealed that production of IL-1 β is significantly reduced in response to lysozyme digested cells (**Fig. A.13A**). The lysozyme digested cells retained the ability to activate TLR2 signalling, and activated signalling to a significantly higher extent than whole cells (**Fig. A.13B**). It would therefore be interesting to separate and purify the cell wall components, including capsule and peptidoglycan, to test the stimulatory capacities of these components. The recognition of the SMG might also be altered if live cells were used for stimulation. Live cells of *S. pneumoniae* stimulate monocytes to produce IL-12p70 leading to development of IFN γ -producing Th1 cell, whereas dead cells skew towards the Th17 phenotype (Olliver *et al.*, 2011). Additionally, the growth conditions of the SMG could also be varied to include bacteria grown on THY agar, since preliminary evidence suggests that SMG grown on agar plates for 72 h express capsular polysaccharide (CPS). The CPS of a particularly pathogenic serotype of *Streptococcus suis* modulates the ratios of pro- and anti-inflammatory cytokine production from dendritic cells (Meijerink *et al.*, 2012) and it would be interesting to investigate whether there is a relationship between capsule production, cytokine profiles, and pathogenicity for the SMG.

As mentioned in Section 1.3, SMG virulence is synergistically increased in mouse models of infection by anaerobic bacteria, an observation that our laboratory has also made (Jayanth and Pinto, unpublished data). Studies have demonstrated that this effect is mediated by the supernatant of anaerobes (Kuriyama *et al.*, 2000; Shinzato and Saito, 1994). These studies suggest that the anaerobic bacteria supernatant promotes the growth of SMG, but it is also observed to prevent phagocytic killing

of the SMG. I predict that the cytokine profiles induced to the SMG would change during co-infection with anaerobes or following growth in the presence of anaerobic bacteria supernatant, resulting in suppression of the pro-inflammatory response either directly or indirectly, resulting in a delayed or impaired pro-inflammatory response required to control infection *in vivo*.

Chapter 5

Conclusion

In conclusion, this is the first study to characterize the cytokine profiles produced by innate immune cells in response to a range of clinical isolates of the SMG. The work presented here contributes to the field of SMG pathogenesis by identifying differences in immune recognition of pathogenic and non-pathogenic isolates, and adds to the knowledge of how commensal bacteria are recognized in the airway. The spectrum of cytokine responses provides a foundation for future studies to investigate the mechanism of recognition, as well as the global consequences of cytokine profiles during colonization and infection. The isolate-specific and donor-specific differences illustrate the importance of examining both microbial variability and host variability when studying the host-microbe interaction. In the case of the SMG, it should be recognized that responses to individual isolates should not be generalized across the entire group or across hosts. This approach is necessary for identifying the isolates with increased virulence as well as hosts susceptible to infection.

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Appendix A

Appendix

A.1 Methods

A.1.1 Bacterial strains and culture conditions

The *S. pneumoniae* P1121 (serotype 23F) and P1547 (serotype 6A) were clinical isolates obtained from Dr. Dawn Bowdish (McMaster University). *S. agalactiae* P7G7 is a clinical isolate obtained from the sputum of an asthmatic patient. P1121 and P1547 were grown in tryptic soy broth (TSB) to mid-log phase ($OD_{600} = 0.5$), washed 1X in PBS, and enumerated on Columbia blood agar. P7G7 was grown in THY to mid-log phase, washed 1X in PBS, and enumerated on THY agar. Bacteria were heat-killed at 65 °C for 10 min.

A.1.2 PBMC stimulation

Time course

5×10^5 PBMCs from one donor were stimulated with isolates B196, P1547, and P7G7 at doses ranging from 10^2 - 10^6 heat-killed cells for 6, 24, and 48 h. Supernatants were collected and stored at -20°C until analysis. IL-8, IL-10, and TNF were analyzed by commercial ELISAs kits according to the manufacturer's instructions (eBioscience).

Extended stimulation

5×10^5 PBMCs from a single donor were incubated with heat-killed isolates of SMG including *S. intermedius* isolates C1365, C1369, C270, *S. constellatus* isolates C1379, C1384, and *S. anginosus* isolates M1 and M410 at a ratio of 2:1 bacteria:PBMCs. Cells were incubated for 5 days at 37°C , 5% CO_2 , after which supernatant was collected and stored at -20°C until analyzed. Levels of IL-17A were measured by ELISA (BioLegend).

Donor comparison of IL-17A, IL-12p70, and IL-23

5×10^5 PBMCs from five individuals (age range 30-50 years, male:female 4:1) were stimulated with heat-killed SMG at a ratio of 5:1 PBMCs:Bacteria. Supernatants were collected after 24 h stimulation and levels of IL-17A, IL-12p70 and IL-23 were measured using the MILLIPLEX Map Human Th17 Magnetic Bead Panel (EMB Millipore) following the manufacturer's recommendations and run on the Luminex®xMAP®platform.

Lysozyme digestion

Lysozyme digested-cells were prepared by addition of 50 μL of lysozyme (100 mg/mL in H_2O) was added to 1 mL of heat-killed cells suspended in PBS and incubated for 1.5 h at 37 °C. An equivalent of 10^5 heat-killed whole cells or lysozyme-digested cells were added to 5×10^5 PBMCs from a single donor for 24 h. Levels of IL-1 β were measured in cell supernatant using a commercial ELISA kit (eBioscience). Statistical significant between lysozyme-digested and whole cell treated samples was determined by a *t* test (two-tailed).

A.1.3 Capsule stain

The *S. intermedius* isolate B196 was either grown on THY agar for 72 h at 37 °C, 5% CO_2 , or in THY broth for 24 h at 37 °C, 5% CO_2 . Capsule stain was performed following the protocol for Anthony's capsule stain (Hughes and Smith, 2007).

A.2 Results

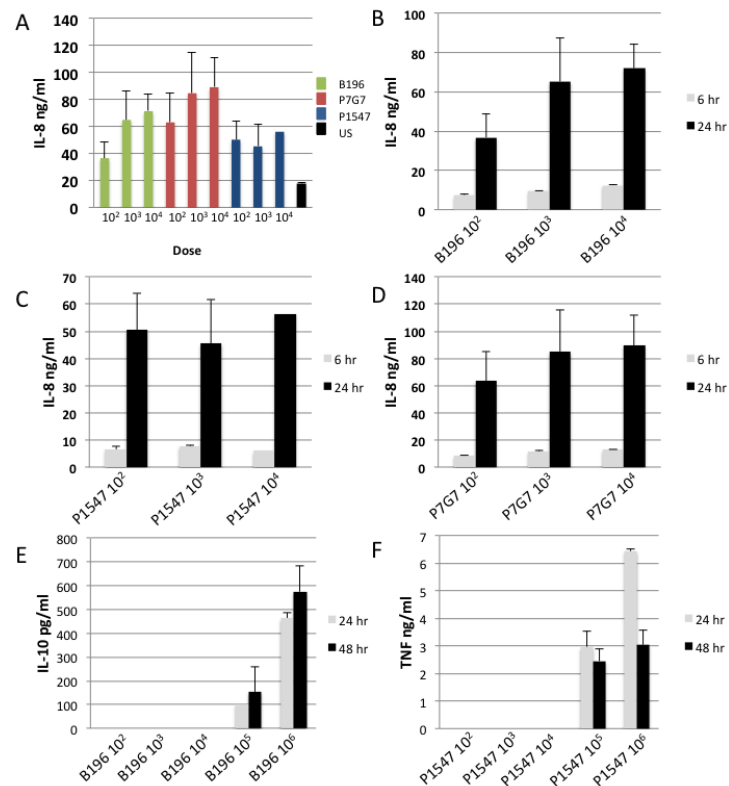


Figure A.1: Temporal induction of cytokines in response to *Streptococcus* spp. PBMCs from a single donor were stimulated with a dose range of 10^2 - 10^6 of heat-killed bacteria, including *S. intermedius* strain B196, *S. pneumoniae* strain P1547, and *S. agalactiae* strain P7G7. Supernatants were collected after 6, 24, and 48 h of stimulation. Cytokines were measured by ELISA. Data are mean \pm SD from a single experiment.

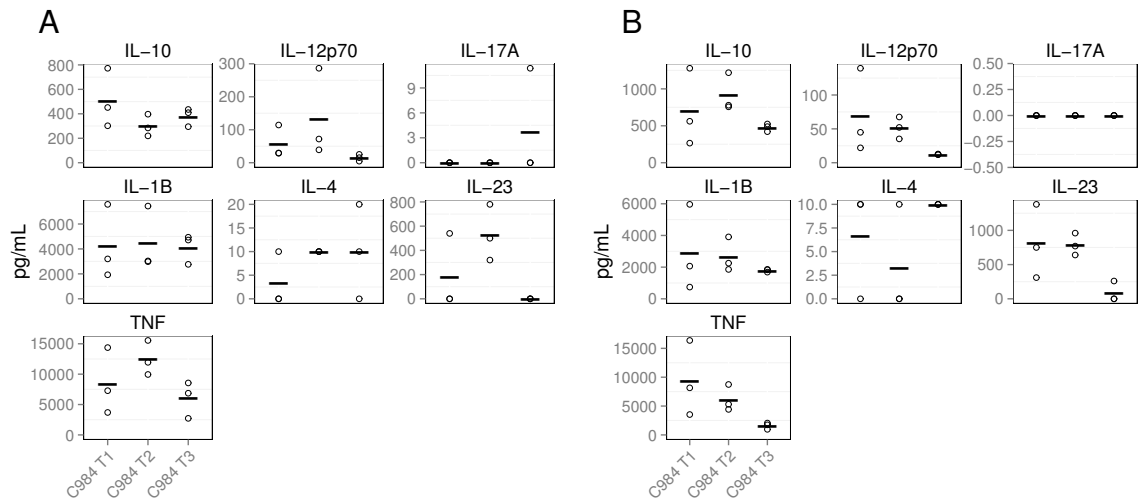
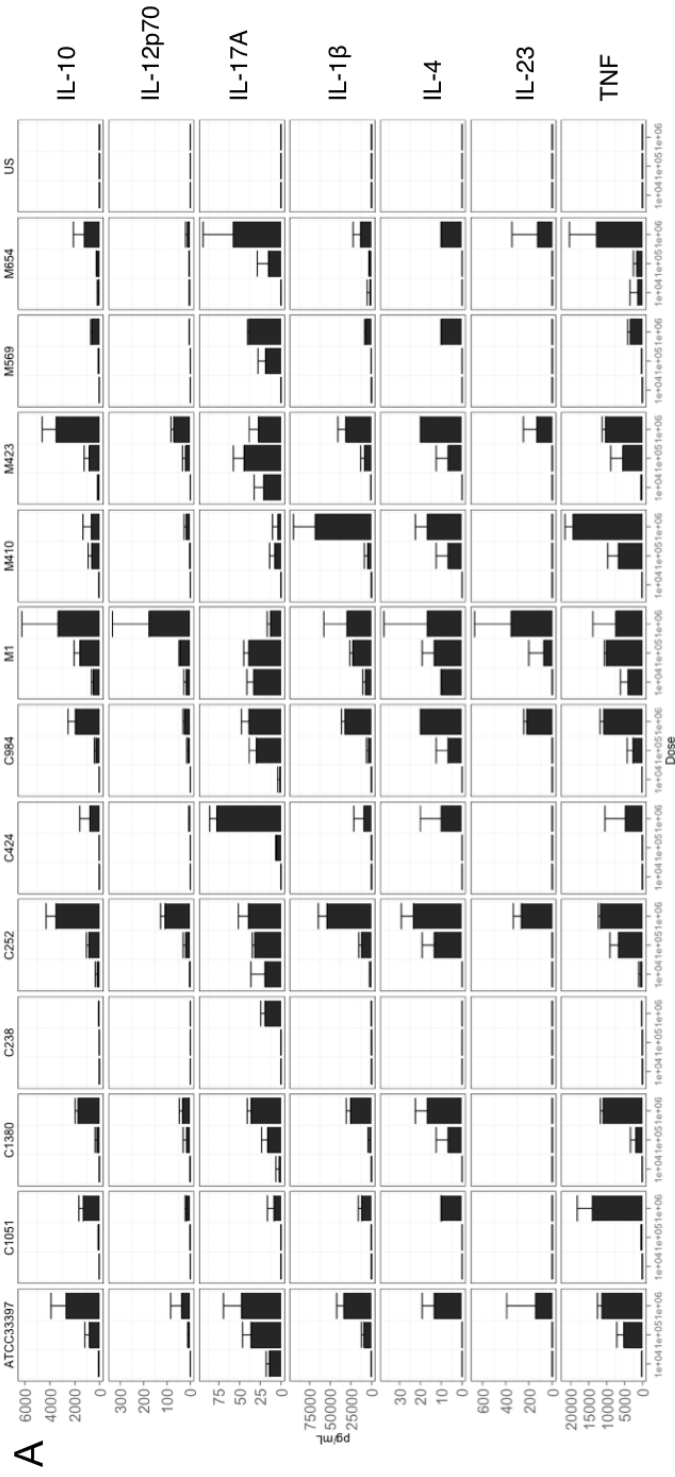
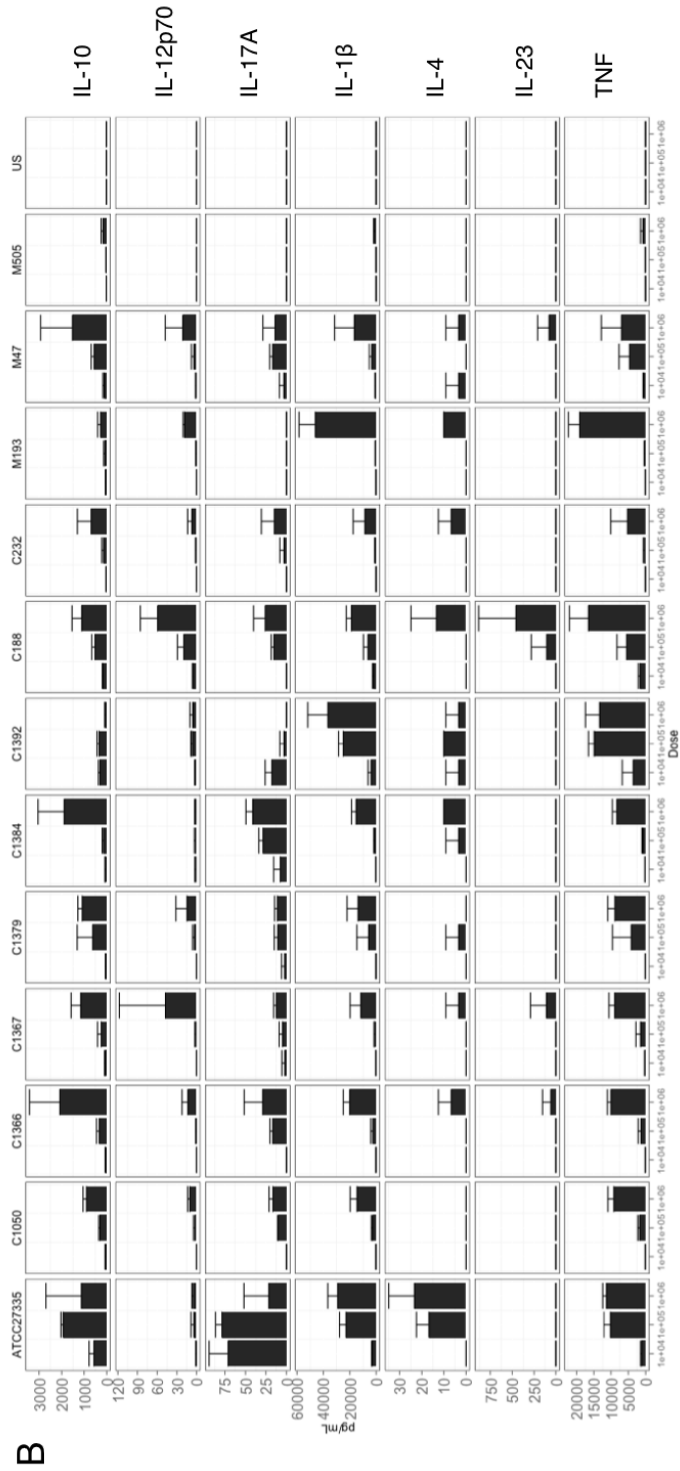


Figure A.2: Intra-donor variability in response to C984. PBMCs were isolated from Donors A and B on three separate occasions and stimulated with the *S. anginosus* isolate C984 at a ratio of 5:1 (PBMCs:Bacteria). IL-10, TNF, IL-1 β , IL-12p70, IL23, IL-17A, and IL-4 were measured in cell supernatant by multiple assaying. Data are mean \pm for each isolation for A. Donor A and B. Donor B. The response for each cytokine was not significantly different across isolations as determined by ANOVA with Tukey HSD post-hoc comparisons.





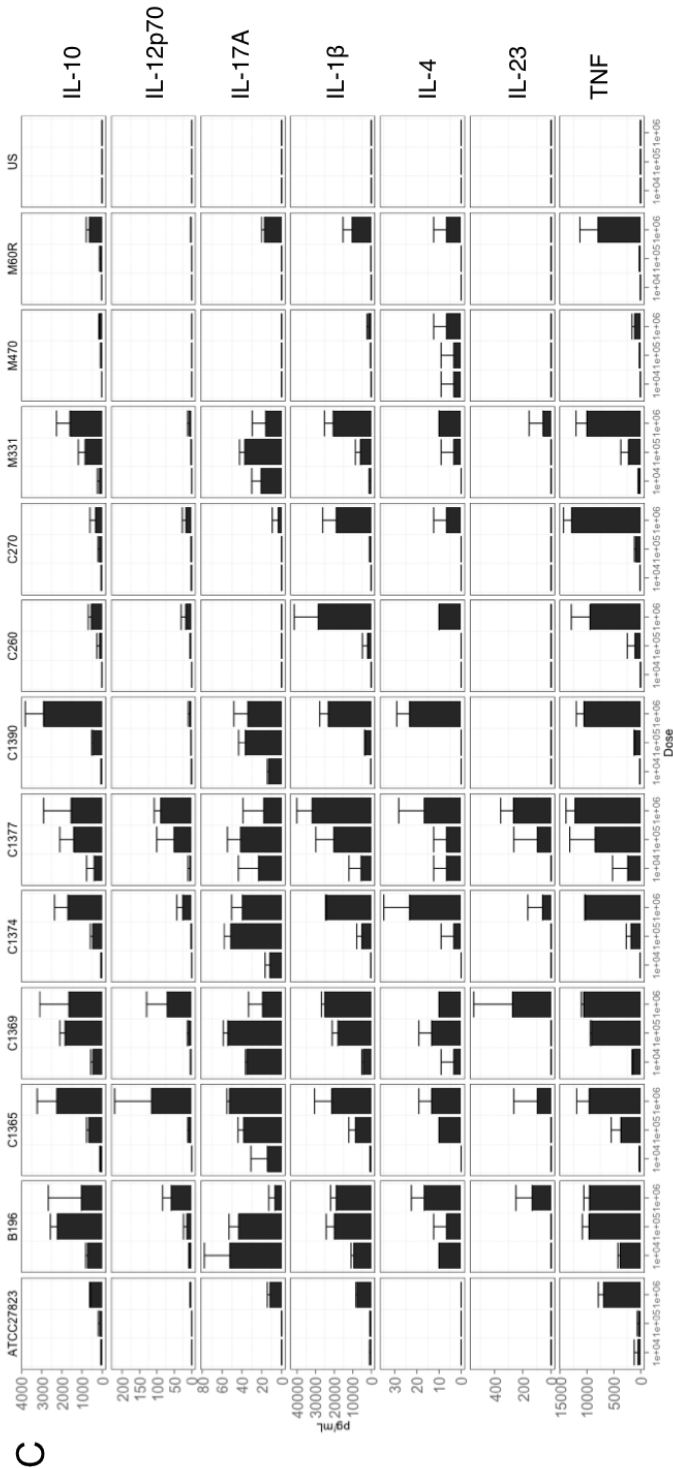
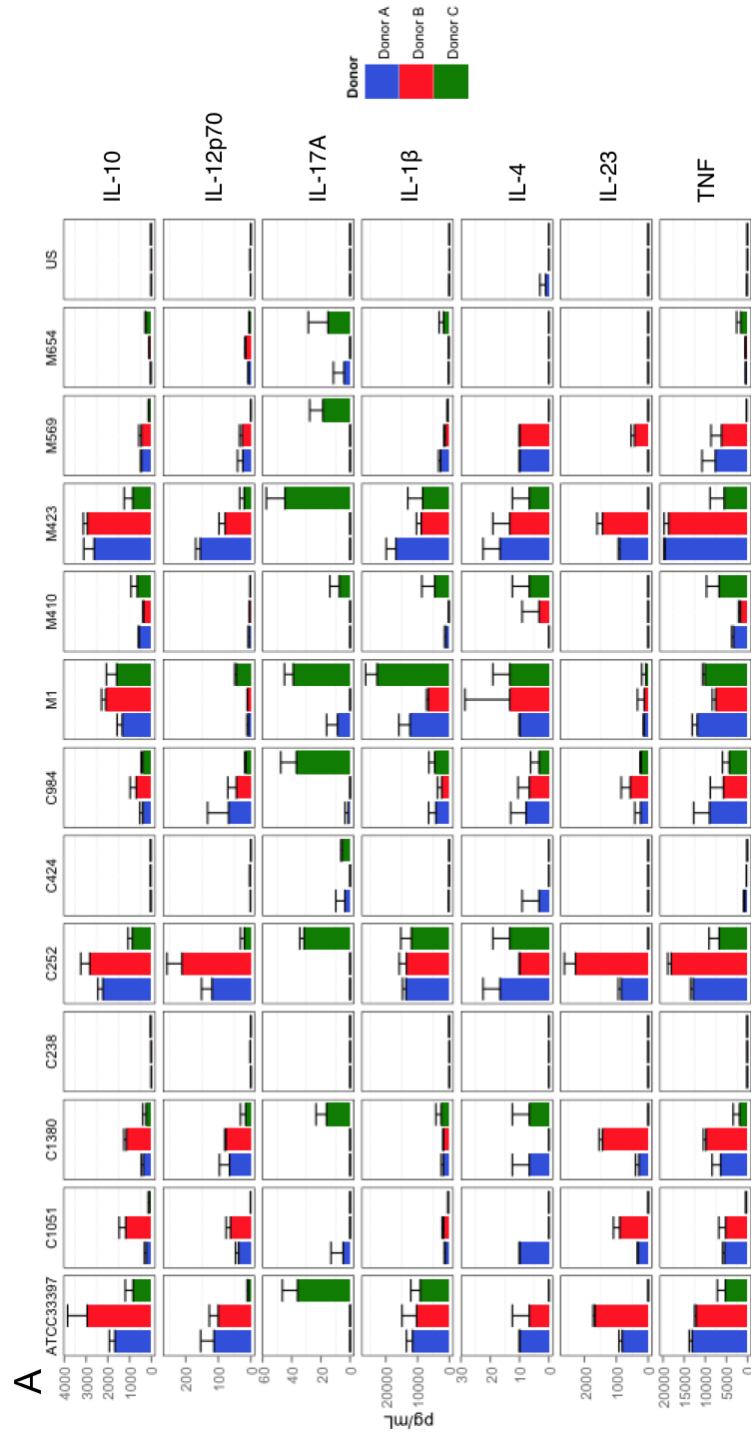
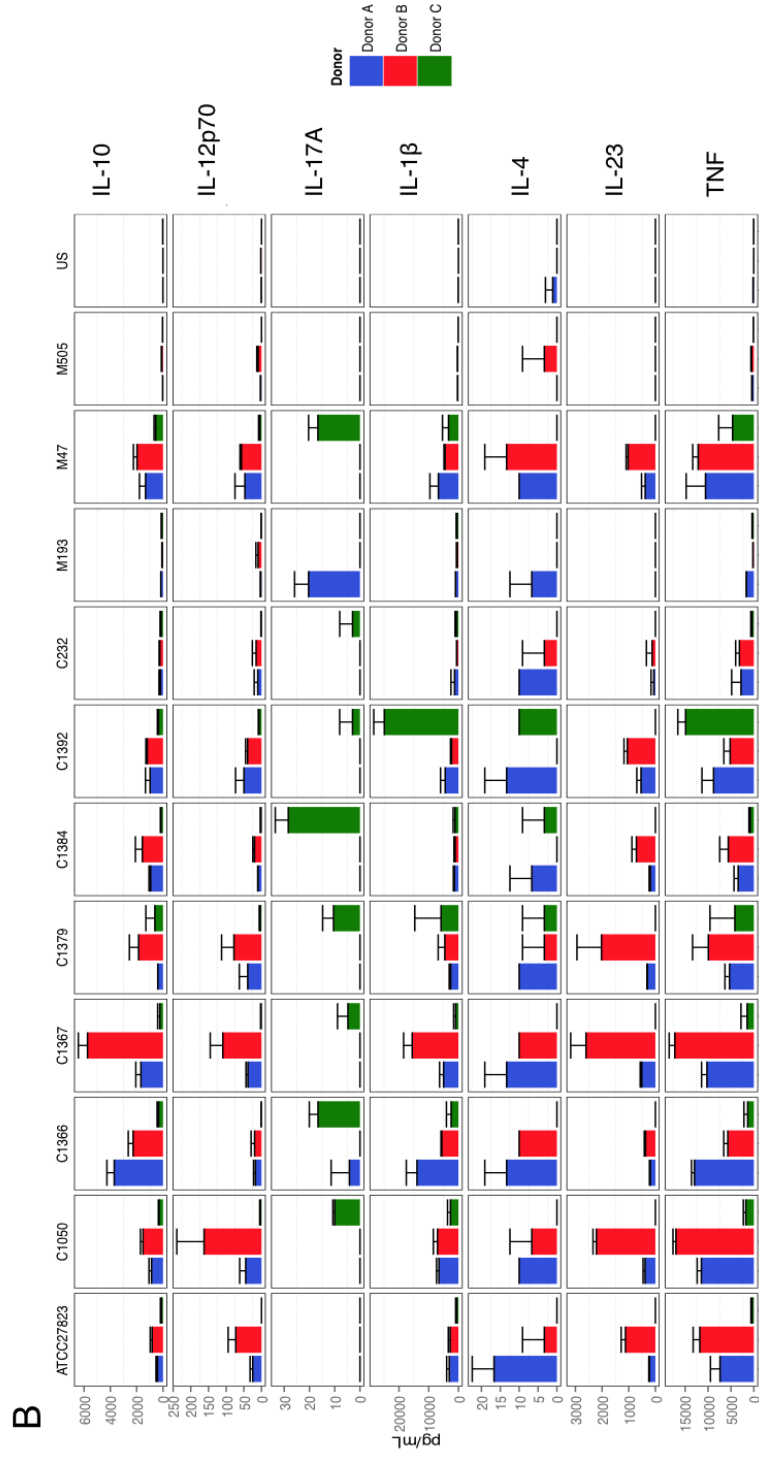


Figure A.3: Dose response curves to the SMG

10⁵ PBMCs from Donor C were stimulated with doses of 10⁴, 10⁵, and 10⁶ heat-killed cells of SMG for 24 h. IL-10, TNF, IL-1β, IL-12p70, IL23, IL-17A, and IL-4 were measured in cell supernatant by multiplex assaying. Data are mean ± SD from a single experiment. A. *S. anginosus* B. *S. constellatus* and C. *S. intermedius* isolates.





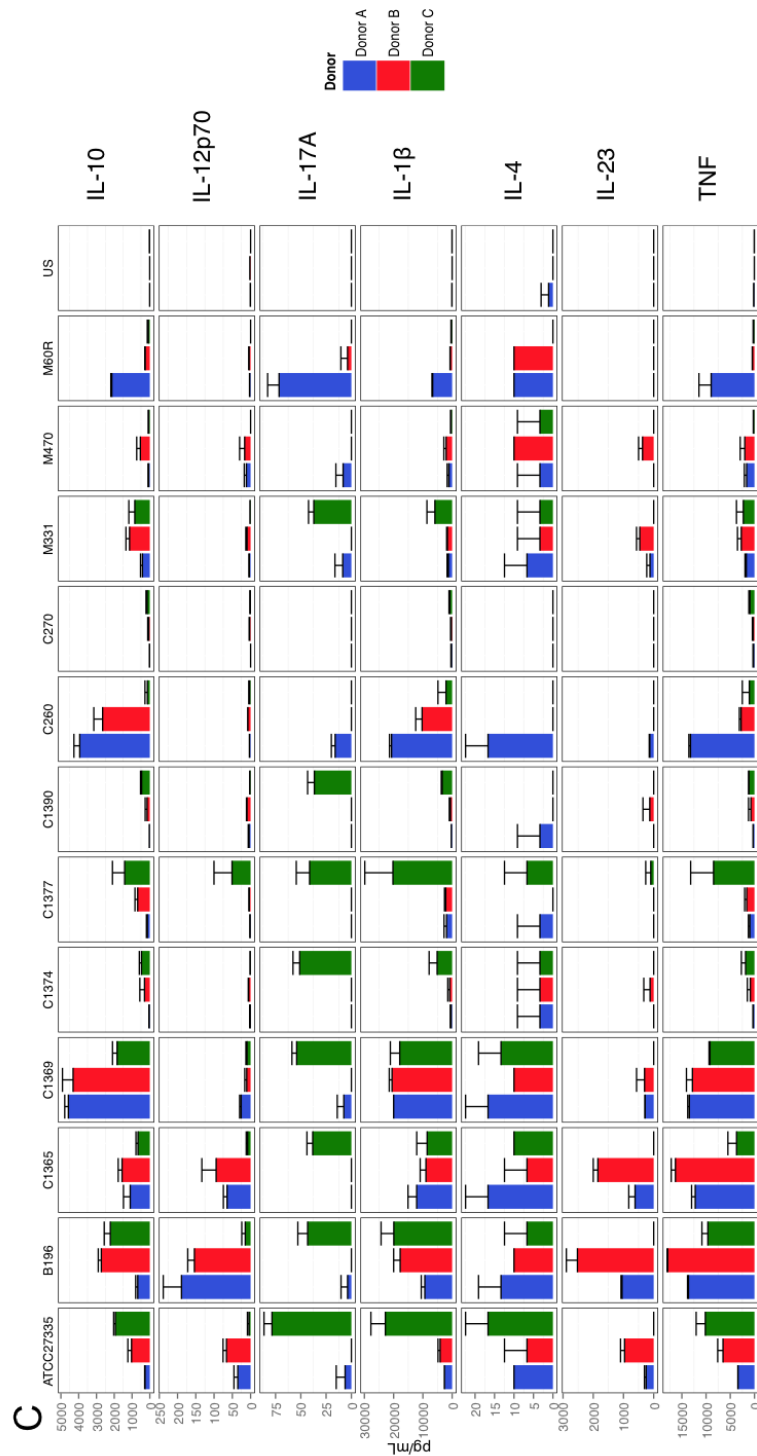
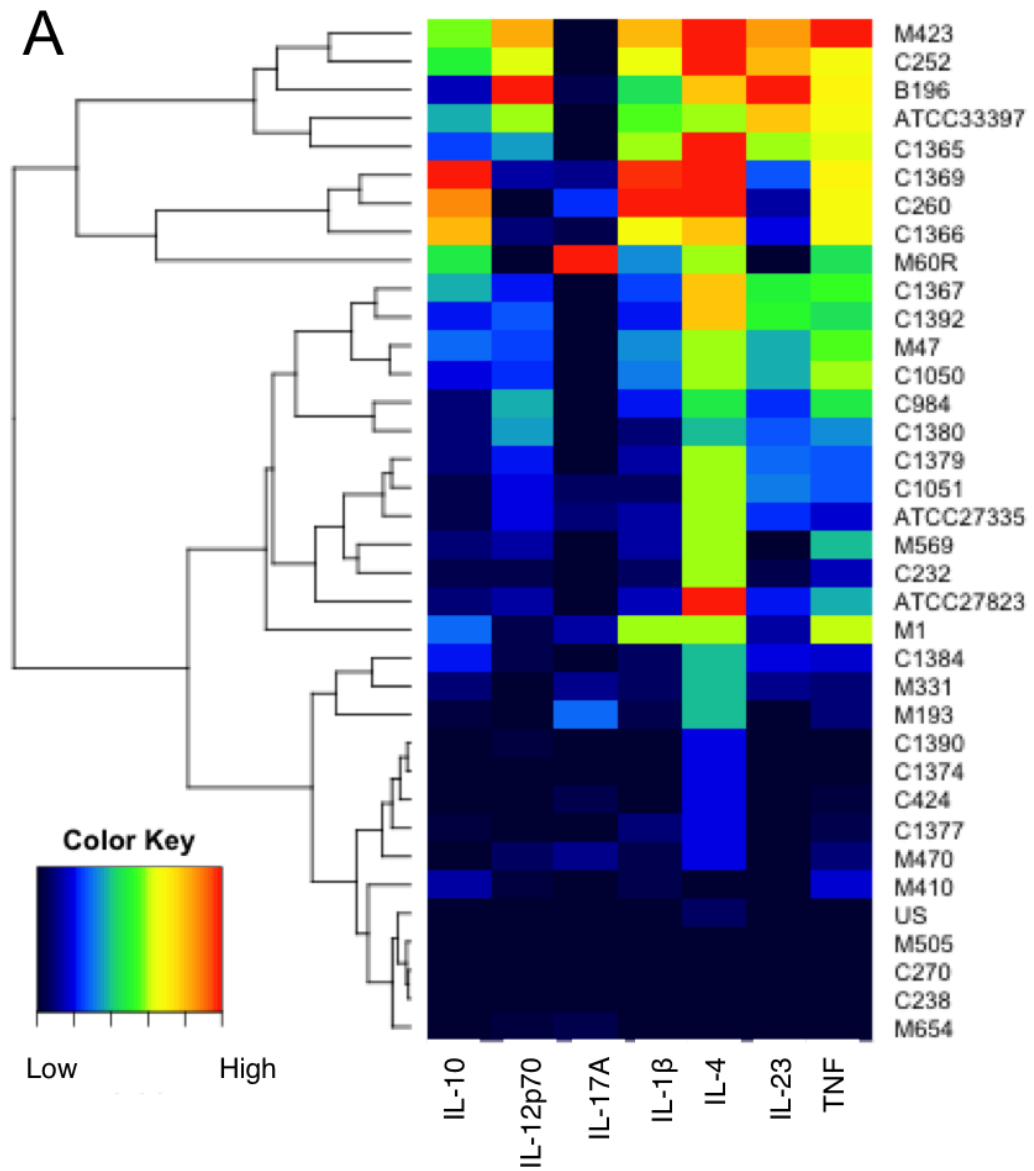
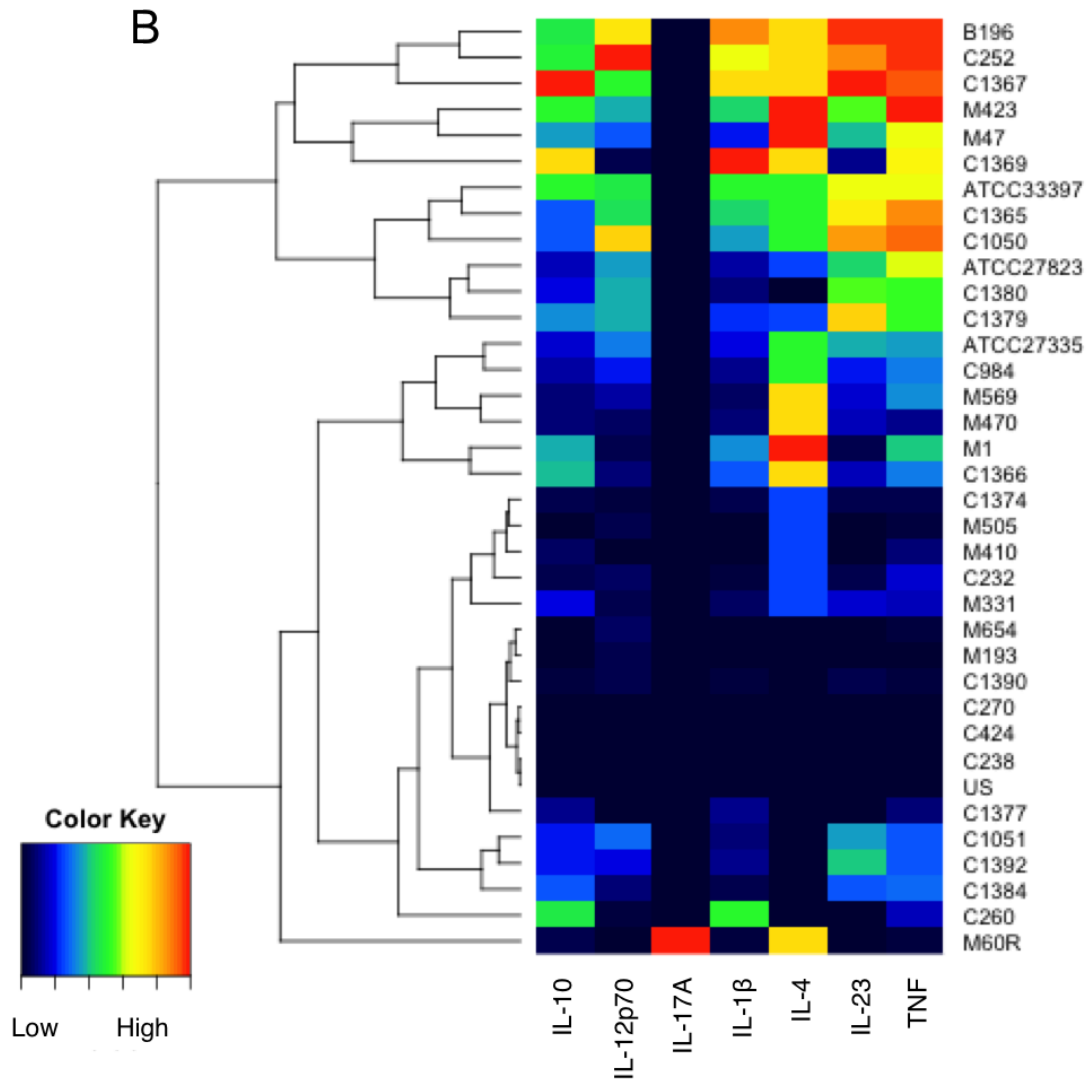


Figure A.4: Cytokine responses to all SMG isolates from three Donors

PBMCs from Donors A, B and C were stimulated with heat-killed cells of SMG at a ratio of 5:1 PBMCs:Bacteria for 24 h. IL-10, TNF, IL-1 β , IL-12p70, IL-23, IL-17A, and IL-4 were measured in cell supernatant by multiplex assaying. Data are mean \pm SD. A. *S. anginosus* B. *S. constellatus* and C. *S. intermedius* isolates.





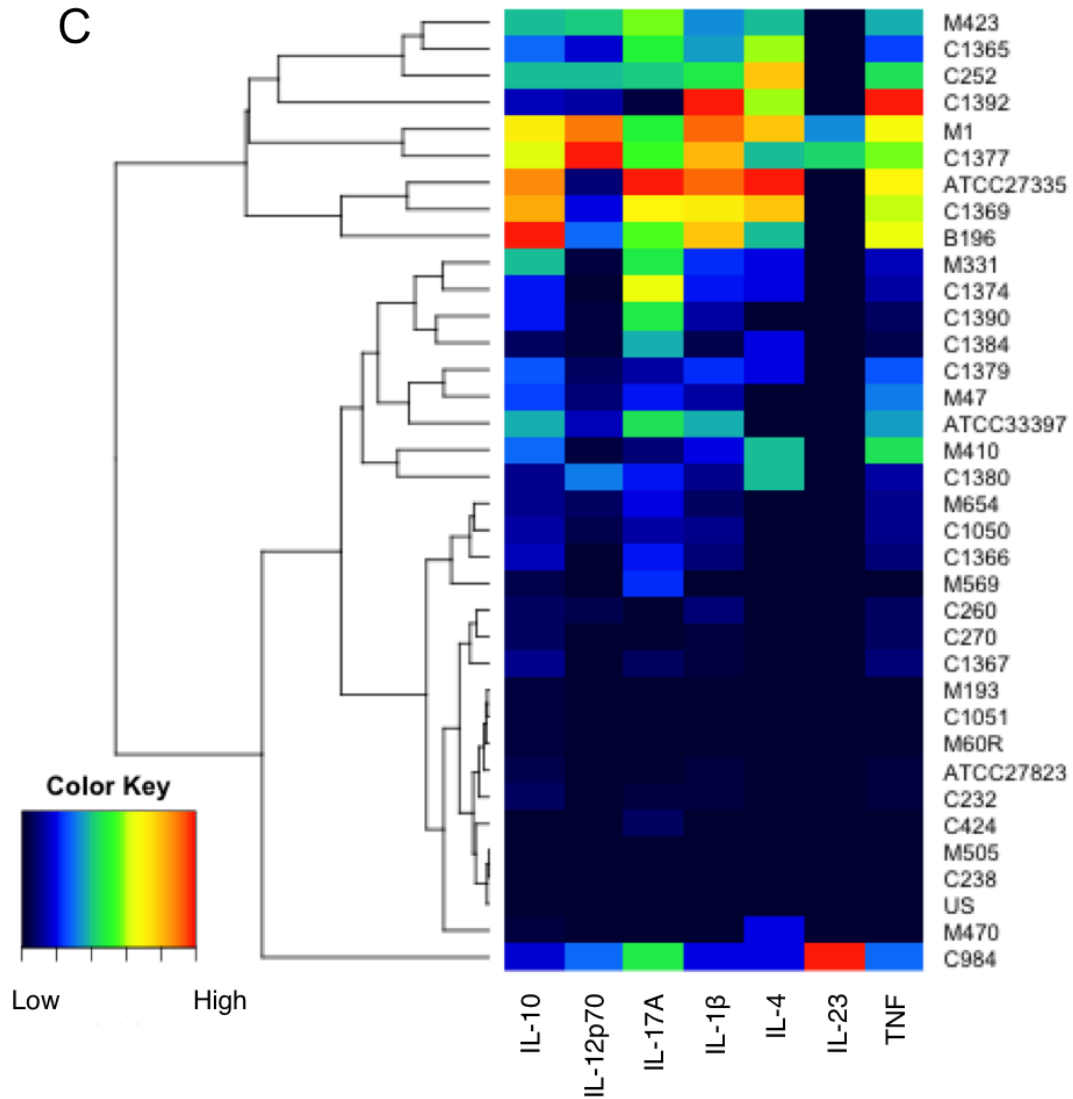


Figure A.5: Spectrum of cytokine responses to 35 isolates of SMG in three individual donors

Cytokine values for donors A, B, and C were scaled to the highest value for each donor and analyzed by a heatmap followed by hierarchical clustering individually. A. Donor A, B. donor B, C. donor C.

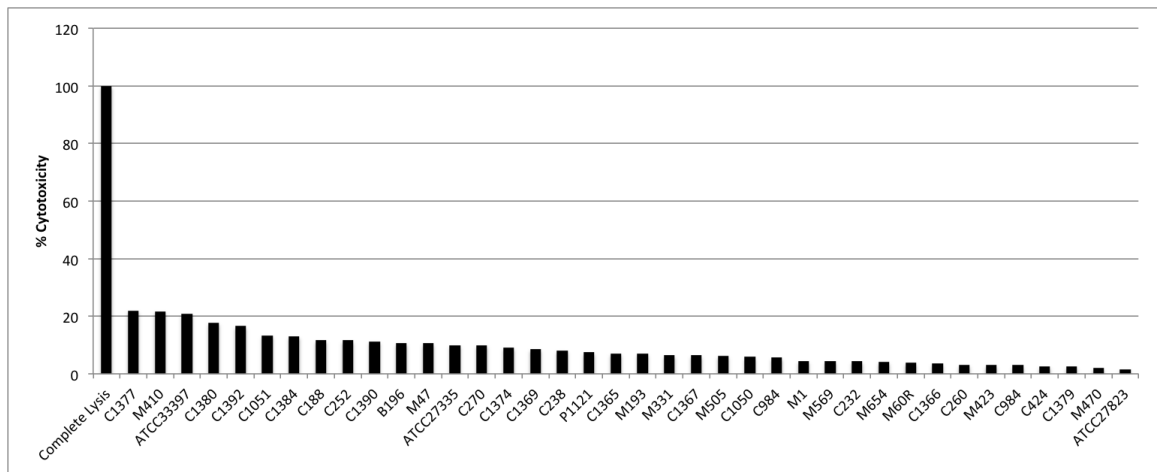


Figure A.6: PBMC cell death following stimulation with isolates of the SMG

. PBMC viability was assessed by detection of LDH release by PBMCs stimulated with heat-killed SMG for 24 h. A complete lysis positive control and untreated negative control were used to calculate percent cytotoxicity.

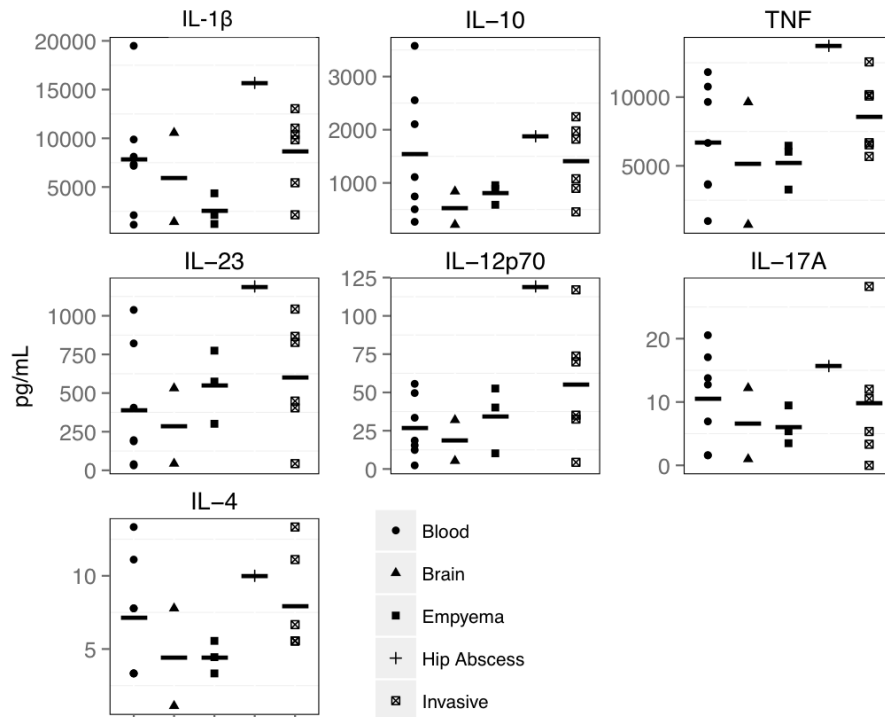


Figure A.7: Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by invasive isolates from different sources of infection.

Isolates from different sources of invasive infection did not induce significantly different cytokine production as determined by ANOVA with Tukey HSD post-hoc comparisons

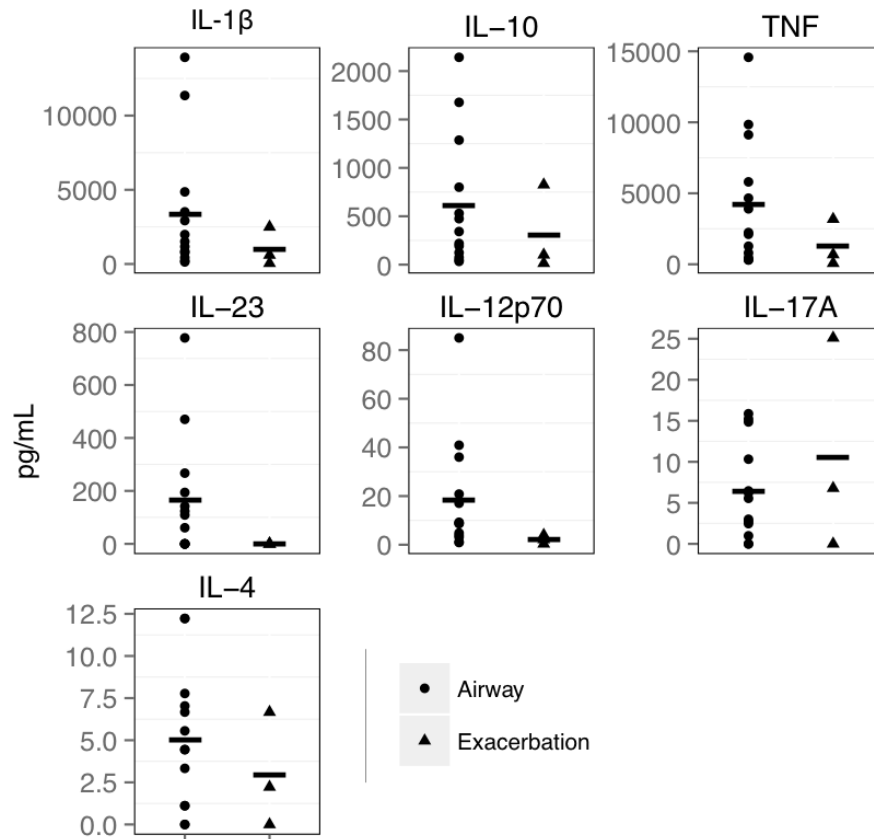


Figure A.8: Induction of IL-1 β , IL-10, TNF, IL-23, IL-12p70, IL-17A, and IL-4 by CF airway isolates.

Airway isolates cultured from sputum during from periods of stability did not induce significantly different cytokine production from isolates associated with exacerbation. Statistical significance was determined by ANOVA.

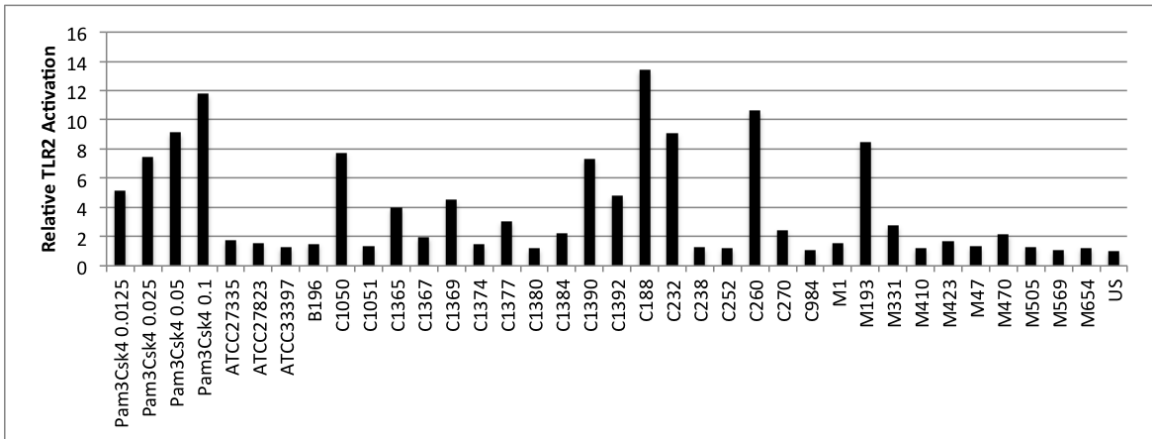


Figure A.9: Relative fold induction of TLR2 activation upon stimulation with the SMG HEK293 cells expressing TLR2 and an NF- κ B reporter expressing were stimulated with heat-killed SMG at a ratio of 500:1 bacteria:TLR2 cells. Serial dilutions of the TLR2 ligand Pam₃Csk₄ were included as a positive control. Detection of SEAP expression was determined by reading absorbance at 630 nm at 24. Data are mean of three replicates from a single experiment. Fold increase in SEAP expression compared to the unstimulated control. The SMG induced a range of TLR2 activation up to 13 fold increase.

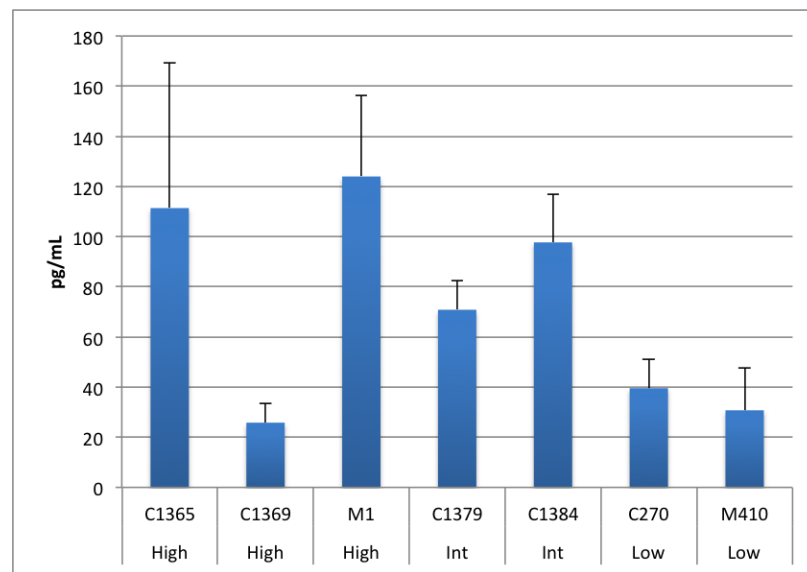


Figure A.10: Production of IL-17A by PBMCs stimulated with the SMG for 5 days
PBMCs from Donor B were incubated with heat-killed *S. intermedius* isolates C1365, C1369, C270, *S. constellatus* isolates C1379, C1384, and *S. anginosus* isolates M1 and M410 at a ratio of 2:1 bacteria:PBMCs. IL-17A was measured in cell supernatant after 5 days of stimulation by ELISA. Data are mean \pm SD from a single experiment. Strains are labelled based on cytokine response groups. Int = Intermediate.

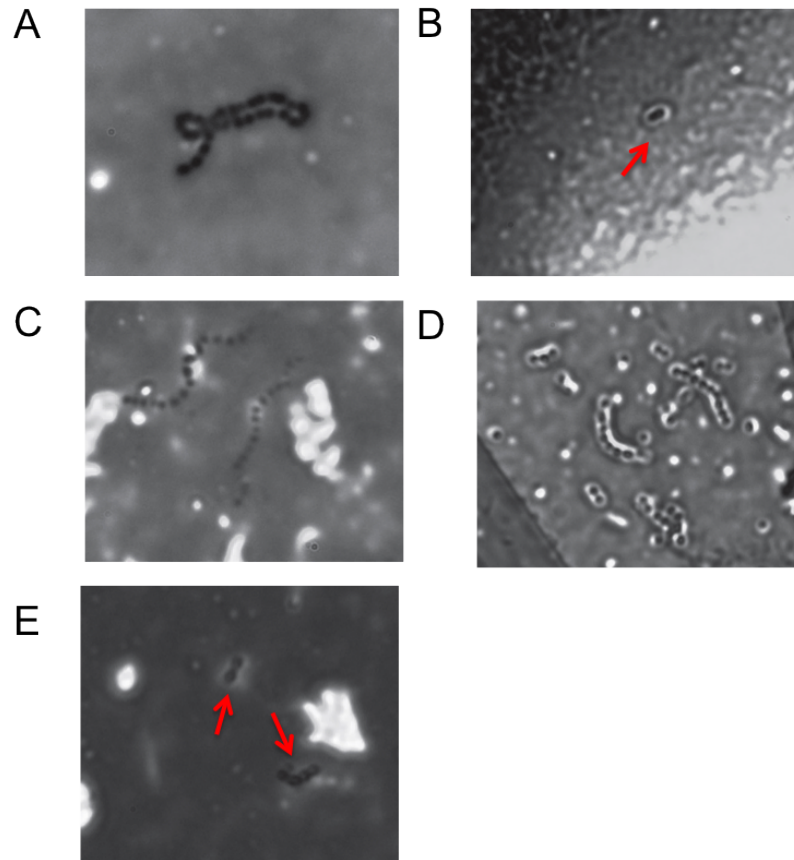


Figure A.11: Capsule stains of SMG and *S. pneumoniae* grown on solid media or in liquid broth

Streptococcus spp. were either grown in liquid broth for 24 hr or grown on solid media for 72 h prior to capsule staining. *S. intermedius* isolates B196 and C1365 did not produce capsule when grown in liquid broth (A, C), but did appear to produce capsule following growth on solid media (B, D). The encapsulated *S. pneumoniae* isolate P1121 expressed capsule following grown in in liquid broth (E).

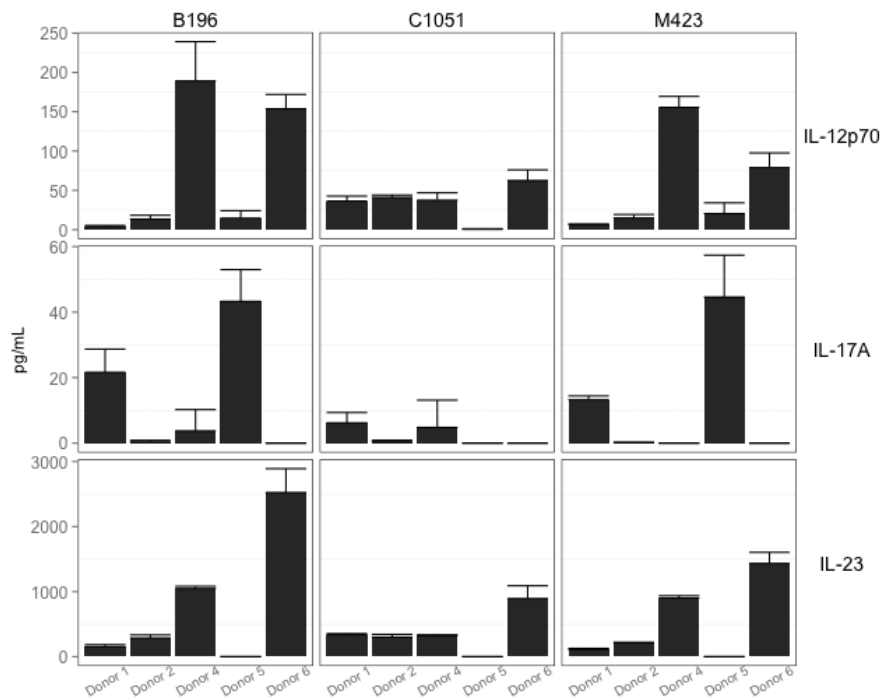


Figure A.12: Recipricol IL-17A and IL-12/IL-23 cytokine responses to SMG in five individuals

PBMCs from 5 individuals were stimulated heat-killed strains of SMG including *S. intermedius* isolate B196 and *S. anginosus* isolates C1051 and M423 at a ratio of 5:1 (PBMCs:Bacteria). PBMC supernatants were collected after 24 h and levels of IL-17A were measured by multiplex assaying. Data are mean \pm SD.

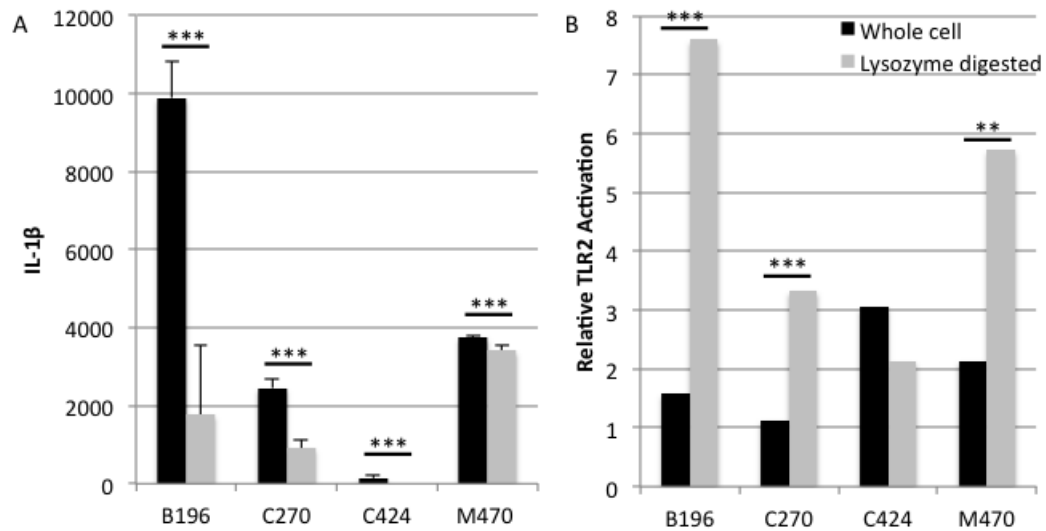


Figure A.13: Affect of lysozyme digestion on cytokine production and TLR2 signalling
 A. 5×10^5 PBMCs were stimulated with an equivalent of 10^5 heat-killed whole cells or lysozyme-digested cells of *S. intermedius* isolates B196, C270 and M470, and *S. anginosus* isolate C424. Supernatants were collected after 24 h and levels of IL-1 β were measured by ELISA. Data are mean \pm SD from a single experiment. B. Heat-killed whole cells or lysozyme digested cells were added to a TLR2 reporter cell line at a ratio of 50:1 bacteria:reporter cells. Alkaline phosphatase activity was measured after 24 h. Data are mean \pm SD from a single experiment. Significance between heat-killed and lysozyme-digested samples was determined by *t* test (two-tailed). ** $p < 0.01$, *** $p < 0.001$.