

Superantigen Interactions in Streptococcal Tonsillitis

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Abstract

Streptococcus pyogenes is the commonest cause of bacterial throat infections, and asymptomatic throat carriage acts as an important reservoir for invasive soft tissue infections. Despite the high rates of infection little is known about the immunology of streptococcal throat infections. Superantigens have been identified as one of the bacterial virulence factors associated with the establishment of streptococcal throat infections. This PhD investigates superantigen expression both *in vitro* and in clinical disease, and the immune effects of superantigens in human tonsils.

Results:

The production of superantigens was assessed using quantitative RT-PCR and biological assays both directly from patient samples and from the corresponding strain *in vitro*. The presence of superantigens was demonstrated in tissues which cultured live bacteria. *In vitro* production of the superantigen SMEZ from throat strains was higher than from invasive strains.

Human tonsils were cultured as cell suspensions or solid block histocultures and stimulated with recombinant superantigens. There was a marked clonal T cell expansion and pro-inflammatory cytokine release in the presence of superantigens. There was apoptosis of tonsil B cells during co-culture with superantigens, with inhibition of IgG, IgA and IgM production. This corresponded with an alteration of the tonsil T cell phenotype from CXCR5 expressing T follicular helper cells to proliferating cells expressing high levels of the TNF receptor superfamily members OX40 and ICOS and the immune synapse signalling molecule SLAM.

Conclusions:

Superantigens are actively produced during clinical infection with *S. pyogenes*. They profoundly alter the functions of tonsil B and T cells *in vitro*, resulting in impaired immunoglobulin production.

Hypothesis and Aims

Experimental hypothesis:

Streptococcal superantigens are important in the pathogenesis of pharyngeal infections, by altering the adaptive immune responses in the pharynx and tonsils.

Experimental aims:

- 1) To assess streptococcal superantigen production *in vitro* and in patients with clinical disease
- 2) To investigate the immunological effects of *S. pyogenes* superantigens on human tonsils
- 3) To establish the effects of superantigen exposure on the generation of immunological memory
in vivo

Declaration of Originality

I confirm that the data, ideas and concepts presented in this document are either my own or have been fully acknowledged and referenced.

Frances Davies.

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List of Abbreviations

AID/AICDA = activation induced cytidine deaminase

ASOT = Anti-streptolysin O titres

AV = Annexin V

Bcl6 = B cell lymphoma 6

Blimp-1 = B cell inducible maturation protein 1 (also known as PDRM1)

BSA = Bovine serum albumin

cAMP = Cyclic adenosine monophosphate

CAS = CRISPR associated proteins

CBA = Columbia blood agar

CD = Cluster of differentiation

CFU = Colony forming units

cMAF = Musculoaponeurotic fibrosarcoma oncogene

ConA = Concanavalin A

CovRS = Control of virulence RS

Cpm = Counts per minute (proliferation)

CPA = Clinical pathology accreditation

CRISPR = Clustered regularly interfaced short palindromic repeats

CXCL = Chemokine C-X-C motif

CXCR = C-X-C chemokine receptor

ddH₂O = Double-distilled deionised water

DMSO = Dimethyl sulphoxide

DNA = Deoxyribonucleic acid

EDTA = Ethylenediaminetetraacetic acid

ELISA = Enzyme linked immunosorbent assay

ELISpot = Enzyme linked immunosorbent spot assay

Emm/M = *S. pyogenes* M protein, and the gene encoding it

EndoS = *S. pyogenes* endoglycosidase

ETOH = Ethanol
 FCS = Foetal calf serum
 FITC = Fluorescein isothiocyanate
 GAPDH = Glyceraldehyde-3-phosphate dehydrogenase
 GC = Germinal centre
GyrA = gene encoding *S. pyogenes* Gyrase A subunit
 H&E = Haematoxylin and Eosin
 HIV = Human immunodeficiency virus
 HLA (DP/DQ/DR) = Human leucocyte antigen (DP/DQ/DR)
 ICOS = Inducible co-stimulator
 IdeS = Immunoglobulin G degrading enzyme of *S. pyogenes*
 IgA/G/M = Immunoglobulin A/G/M
 IGHG = Immunoglobulin heavy chain gene
 IL = interleukin
 INF = interferon
 IVIG = Intravenous immunoglobulin
 L = Ligand
 LB = Lysogeny broth
 Lck = Lymphocyte-specific protein tyrosine kinase
 LCMV = Lymphocytic choriomeningitis virus
 LS = Lymphoepithelial symbiosis
 LT_i = Lymphoid tissue inducer
 MF = Multiplication factor
 MFI = Median fluorescent intensity
 Mga = Master gene regulator
 MHC = Major histocompatibility complex
 NALT = Nasal associated lymphoid tissue
 NETs = Neutrophil extracellular traps
 NF κ B = Nuclear factor kappa B
 NK = Natural killer cell
 NLRp3 = Nod-like receptor family pyrin domain-containing 3

Nod = Nucleotide-binding oligomerisation domain

OB = Oligosaccharide/nucleotide binding

PAM = Plasminogen binding M-like protein

PANDAS = Paediatric autoimmune disorders associated with streptococcal infections

PAX5 = Paired box protein 5

PBMC = Peripheral blood mononuclear cell

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

PD-1 = Programmed cell death 1

PE = Phycoerythrin

PI = Propidium iodide

ProS = Prolyl-tRNA synthetase

qRT-PCR = Quantitative real time reverse transcription polymerase chain reaction

RNA = Ribonucleic acid

RPMI1640 = Roswell park memorial institute media 1640

SAP = SLAM associated protein

ScpA = Alternative name for *S. pyogenes* C5a peptidase

S.D. = Standard deviation

SE (B, C) = Staphylococcal enterotoxin (B, C)

SEI = Staphylococcal enterotoxin-like

SIC = Streptococcal inhibitor of complement

SLAM = Signalling lymphocyte activation molecule

SLO = Streptolysin O

SLS = Streptolysin S

SMEZ = Streptococcal mitogenic exotoxin Z

SPE (A-K) = Streptococcal pyrogenic exotoxin (A-K)

SpyCEP = Streptococcus pyogenes cell envelope proteinase

SSA = Streptococcal superantigen

TCR = T cell receptor

TCRV β = T cell receptor variable beta chain

T_{FH} = T follicular helper cell

TGF = Transforming growth factor

T_H = T helper subset

TLR = Toll like receptor

TMB = 3,3',5 5'-Tetramethylbenzidine

TNF = Tumour necrosis factor

TRAF = TNF receptor associated factor

TReg = Regulatory T cell

TSST-1 = Toxic shock syndrome toxin-1

XPB1 = X box binding protein 1

ZAP70 = Zeta-chain associated protein kinase 70

1 Introduction

Streptococcus pyogenes is responsible for an estimated 600 million cases of tonsillitis worldwide each year, and as such carries a significant morbidity and economic burden of disease. Although *S. pyogenes* is susceptible to penicillin, treatment failures in tonsillitis are common, with many patients having repeated and persistent infections (Podbielski, Beckert, Schattke et al. 2003). In countries where penicillin is not readily available or treatment courses inadequate, there are the added post-streptococcal sequelae of immunological diseases, particularly rheumatic fever, and there are an estimated 15 million people worldwide living with rheumatic heart disease (Carapetis, Steer, Mulholland et al. 2005). In addition the pharynx represents the main site of carriage of *S. pyogenes*, and can act as a reservoir for other invasive streptococcal diseases, including skin infections, bone and joint infections, puerperal fever, necrotising fasciitis and toxic shock syndrome (Cunningham 2000).

Although there has been considerable enhancement of understanding about bacterial virulence factors, the human immunological response to *S. pyogenes* pharyngeal infection remains poorly understood. Human models of disease have so far been limited to *in vitro* blood work (Hopkins, Fraser, Pridmore et al. 2005), or investigation of samples taken from patients with clinical disease (Norrby-Teglund, Thulin, Gan et al. 2001). Animal models have been used for assessment of *in vivo* responses to infections, but due to differences in the immune systems between different mammals and their susceptibility to streptococcal infections, these have been most successful using higher primates (Shea, Virtaneva, Kupko, III et al. 2010) or when analysing the response of a specific component of the immune system using “humanised” transgenic animal models (Sanderson-Smith, Dinkla, Cole et al. 2008).

This project set out to expand the understanding of the host-pathogen interactions in the pharynx by developing an *ex-vivo* model of tonsillitis, to study immune responses at the primary site of *S. pyogenes* disease with particular reference to the streptococcal superantigens. Superantigen production in patients with invasive disease and pharyngitis was measured, and the impact of

superantigens on the development of immunological memory in superantigen sensitive HLADQ8 transgenic mice assessed.

1.1 Clinical diseases associated with *S. pyogenes*

Since the development of modern society, bacterial pathogens have evolved with humans to develop a niche of communicable diseases. Among the most notorious of these is *Streptococcus pyogenes* (Group A Streptococcus), which throughout history has been responsible for epidemics of scarlet fever, puerperal sepsis (child-bed fever), rheumatic fever and “the flesh-eating disease” necrotising fasciitis. Epidemics of scarlet fever in 19th century England and Wales occurred every 5 to 6 years, and carried a high mortality, particularly in children under 5 years of age (though the mean age of infection was 13 years). These epidemics were closely linked with crop failures in the previous year, a general state of malnutrition and overcrowding in the cities. They were linked to outbreaks of other contagious diseases such as smallpox and measles, and mortality was particularly high in the offspring of women who had been pregnant during the periods of malnutrition (Duncan, Duncan, & Scott 1996; Duncan, Scott, & Duncan 2000). Health and nutrition started to improve after 1880, and the regular epidemics of scarlet fever began to fade away, long before the discovery of penicillin in 1928 and introduction of antibiotic use in 1936 (Bentley 2009). Cases of *S. pyogenes* infection peaked again in the 1918 influenza pandemic, though it was not until 1933 that the current streptococcal classification system was described by Rebecca Lancefield (Lancefield 1933) and *S. pyogenes* definitively linked to diseases such as rheumatic fever.

Today it is unusual for people from developed countries to die from scarlet fever or streptococcal tonsillitis, but the incidence of rheumatic heart disease worldwide is still high, particularly in sub-Saharan Africa, indigenous populations of Australia and New Zealand and south/central Asia (Carapetis et al. 2005). The global case fatality rate from invasive disease is nearly 25%. In 2003/4 in the UK, 20% of patients with invasive streptococcal disease died within 30 days of the infection, with the highest mortality in patients aged over 45 or with significant co-morbidity (Lamagni, Neal, Keshishian et al. 2009). The nature of the illness was a contributing factor, with necrotising fasciitis or gastrointestinal symptoms resulting in a two-fold higher risk of death within the first 7 days of presentation, and cellulitis accounting for 30% of the total deaths. Overall deaths attributable to *S.*

pyogenes place it among the top 10 infectious disease causes of death worldwide each year (Carapetis et al. 2005).

It is difficult to accurately establish the rates of asymptomatic carriage, but there are estimates that 1:3 of household contacts of a case of paediatric streptococcal pharyngitis will go on to develop symptoms themselves (Danchin, Rogers, Kelpie et al. 2007), demonstrating the highly contagious nature of *S. pyogenes* infections.

1.1.1 Clinical presentation

There is a wide spectrum of clinical diseases and syndromes attributed to *S. pyogenes* infection, which are listed in Table 1. In severe infections the development of systemic collapse is referred to as streptococcal toxic shock syndrome, where two or more of the following criteria are met: hypotension, renal failure, coagulopathy, liver failure, acute respiratory distress, a generalised erythematous rash that may desquamate, necrotising fasciitis or gangrene and the laboratory identification of *S. pyogenes* from a sterile site (or non-sterile site with no other cause for the symptoms) (Anon 2011a). Among the post-infective immunological sequelae, rheumatic fever and post-streptococcal glomerulonephritis have definitely been linked to *S. pyogenes*. Kawasaki Disease and PANDAS (Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections) have been linked to *S. pyogenes* infections but the aetiology has not been proven (Cunningham 2000).

Non-life threatening disease	Invasive disease	Post-infections disease	Disease possibly linked to <i>S. pyogenes</i>
Pharyngitis Tonsillitis Localised skin infection Asymptomatic carriage	Scarlet fever Cellulitis Necrotising fasciitis Bacteraemia Septic arthritis Endocarditis Meningitis Necrotising pneumonia Puerperal sepsis Toxic Shock syndrome	Rheumatic fever Glomerulonephritis	Kawasaki Disease Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS)

Table 1: Spectrum of disease attributable to *S. pyogenes*

Compiled from various resources, including Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases (Bisno and Stevens 2005) and Pathogenesis of Group A streptococcal infections (Cunningham 2000).

1.1.2 Laboratory diagnosis of *S. pyogenes*

In diagnostic laboratories, *S. pyogenes* is identified as a beta-haemolytic colony on a blood agar plate (Figure 1A), and throat or wound swabs are plated onto media which specifically aid the identification of this organism from amongst the normal upper respiratory tract flora. In common with other streptococci *S. pyogenes* stains as chains of Gram positive cocci (Figure 1C), has a negative catalase reaction, and can be distinguished from other beta-haemolytic streptococci by agglutination with the group A carbohydrate (Bisno & Stevens 2005). Occasional mis-identification of streptococci from the milleri group (comprising *S. anginosus*, *S. intermedius* and *S. constellatus*), which can also cross-react with carbohydrate group A, can have significant consequences for patients as the disease associations are very different. In this case confirmatory testing in different growth conditions (*S. pyogenes* will grow in aerobic, microaerophilic or anaerobic environments where *S. milleri* group bacteria are not aero tolerant) or biochemical testing will reliably distinguish *S. pyogenes* from all other streptococci. Some strains of *S. pyogenes* are highly mucoid in appearance (Figure 1B), due to increased production

of a hyaluronic acid capsule. In this case the haemolysis can be difficult to see and can delay laboratory identification.

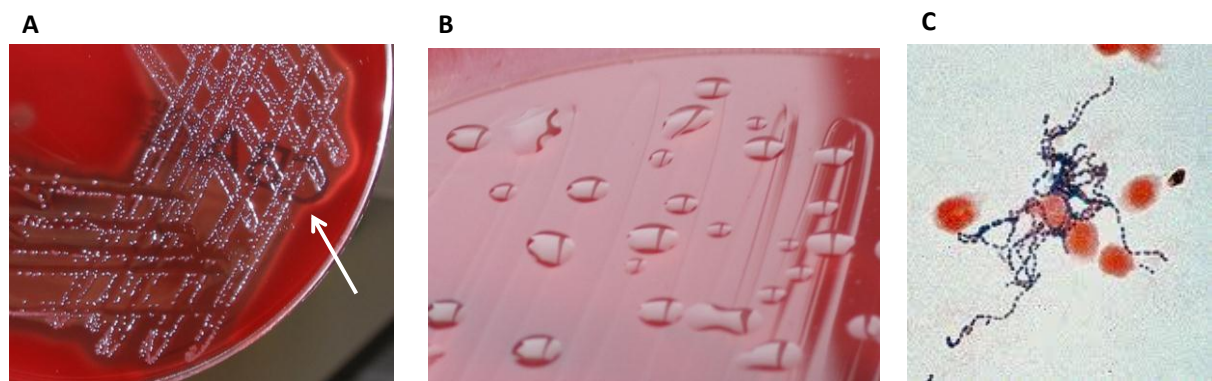


Figure 1: Laboratory appearance of *S. pyogenes*

A/ Typical appearance of *S. pyogenes* on a Columbia blood agar (CBA) plate. Colonies are small, round, white and glossy. There is a large area of beta-haemolysis seen around the colonies (white arrow). B/ Appearance of a mucooid strain of *S. pyogenes* with an oil-drop appearance. C/ Typical Gram stain appearance of *S. pyogenes*, reproduced from Todar's online textbook of microbiology (Anon 2011d).

Further classification of *S. pyogenes* has traditionally relied on M typing or T typing. The M protein is carried on the surface of all *S. pyogenes* and differences in the length and composition of this protein correlate well with different disease associations. M typing can be performed by serotyping or *emm* gene sequencing, and over a hundred different *emm*/M types have now been identified (Cunningham 2000). The T antigen has now been discovered to represent the streptococcal pilus (Mora, Bensi, Capo et al. 2005), with at least 20 T types being identified.

Rapid testing by PCR or direct grouping tests on swabs have been developed, though the sensitivity and specificity of these tests can vary widely and they have failed to replace traditional culture and identification methods in routine use to date (Clerc and Greub 2010). Serology to detect antibodies to streptolysin O (ASO titres) and DNase (anti-DNase B titres) are useful in helping to achieve a retrospective diagnosis of *S. pyogenes* infection (especially with the post-infectious immunological sequelae) or monitoring patients with recurrent disease. These antibodies take about 6 weeks to develop after an infection, and are detectable in the serum for up to a year (Johnson, Kurlan, Leckman

et al. 2010), so serial testing across several weeks/months should be performed to monitor changes in titres and to help establish the timing of the preceding infection.

1.1.3 Management and prevention of clinical infections due to *S. pyogenes*

S. pyogenes is still susceptible to penicillin, and this remains the treatment of choice in many situations. The standard treatment course for a confirmed case of pharyngitis/tonsillitis is 10 days, with increased rates of disease recurrence or immune sequelae if the course is not completed (Bisno & Stevens 2005). Macrolide (erythromycin, clarythromycin or azithromycin) resistance has been reported with varying rates between countries. In Germany there is currently an 8.2% macrolide resistance rate, with 31% being due to acquisition of the *mef* genes (which confers resistance just to macrolides by a drug efflux pump) and 48.3% due to the *erm* genes, which confer class resistance to macrolides and lincosamides (clindamycin) by modification of the ribosomal binding site of the drugs (Bley, van der Linden, & Reinert 2011). This presents a particular challenge in the treatment of patients with penicillin allergy, but also lincosamide resistance can adversely affect the outcome of patients with toxic shock syndrome, where the protein synthesis inhibitor role of lincosamides is key to management (Lappin and Ferguson 2009).

Early surgery is of utmost importance in the management of necrotising fasciitis due to *S. pyogenes* where the disease is characterised by the rapid spreading of bacteria along fascial planes. Antimicrobial therapy fails to contain such severe infections, and surgery may need to occur on several occasions to ensure adequate removal of necrotic tissue and live bacteria (Stevens 2000). The release of virulence factors, such as superantigens, in invasive disease is thought to contribute to the degree of systemic shock which is seen to accompany the local infections, and for this reason the use of IVIG has been advocated by some authors in the adjuvant management of the streptococcal toxic shock syndrome. A clinical trial looking at the use of IVIG failed to recruit sufficient patients to

achieve significance in the primary endpoint, but a decrease in mortality and organ failure was noted (Darenberg, Ihendyane, Sjolín et al. 2003).

There is still no vaccine available for *S. pyogenes*. M protein vaccine trials have not provided the desired coverage, due to the variation of *emm* types seen in different communities (Lynskey, Lawrenson, & Sriskandan 2011) and there are safety concerns that M protein is important in the aetiology of rheumatic fever and that such vaccines may have adverse side effects relating to this (Ellis, Kurahara, Vohra et al. 2010). A variety of other non-M protein vaccine candidates have been suggested, including SpyCEP (a cell surface protease) and C5a peptidase (Fritzer, Senn, Minh et al. 2010; Steer, Batzloff, Mulholland et al. 2009).

With the increased risk of disease developing in close contacts of patients with invasive *S. pyogenes* disease, UK guidelines have been drafted recommending the use of antibiotic prophylaxis for high risk contacts (Smith, Lamagni, Oliver et al. 2005).

1.1.4 Current *S. pyogenes* epidemiology

M/*emm* typing of *S. pyogenes* is useful both for tracking a local cluster of cases of infections and for monitoring the regional or global trends in streptococcal diseases. Certain M/*emm* types have been linked to different diseases, such as M28 strains with puerperal sepsis and M1 or M3 strains with particularly severe invasive disease and toxic shock syndrome. In 2003-4 a large study collecting strains from 11 countries across Europe compared clinical disease to M/*emm* type, and found considerable variation in the M types from different countries. More concerning is that the *emm*/M types differ considerably between different regions of the world, and many of the common strains in the poorest countries of the world are not those covered by the 26 valent vaccine which has been trialled (Steer, Law, Matatolu et al. 2009).

1.2 Virulence factors produced by *Streptococcus pyogenes* and their interaction with the immune system

S. pyogenes is renowned for producing a large number of different virulence factors, which are thought to contribute to the high morbidity and mortality of clinical *S. pyogenes* disease and to the species specificity of *S. pyogenes* infections. The majority of these virulence factors have been found to target specific components of the innate or adaptive human immune systems and promote survival in the hostile environment of the human pharynx.

Whole genome sequencing projects and microarray technology have revealed numerous strategies employed by *S. pyogenes*, to tackle these human defence systems (Olsen, Shelburne, & Musser 2008), with increased expression of numerous genes identified during the establishment of infection (Virtaneva, Porcella, Graham et al. 2005; Virtaneva, Graham, Porcella et al. 2003), and also to promote bacterial survival in inter-epidemic periods (Beres, Richter, Nagiec et al. 2006).

1.2.1 Interactions with the innate immune system

The first defence mechanisms in the oropharynx are mucus and saliva. Saliva contains lysozyme and antimicrobial peptides, which have specific targeted action against the bacterial cell wall (De and Contreras 2005; Janeway 2008; Schneider, Unholzer, Schaller et al. 2005), as well as being a nutrient poor environment already colonised by other bacteria. Mucus acts as a physical barrier to prevent the adhesion of *S. pyogenes* to epithelial cells. Other key components of the innate immune system which are encountered by *S. pyogenes* during attempts to colonise a human include complement and the innate immune cells; neutrophils, monocytes/macrophages and dendritic cells. Secretory IgA is also present in oropharyngeal secretions, to bind and opsonise bacteria for immune system recognition. With such an advanced array of defences to overcome before *S. pyogenes* can colonise a host, let

alone proliferate and cause disease, it is not surprising that so many of the virulence factors are directed against these specific targets. There are several transcription regulators described in *S. pyogenes* which control the expression of virulence factors, of which two have been extensively studied: *Mga* (master gene regulator), with control of M protein expression and *CovRS* (Control of virulence), a two-component system regulating capsule production and several other virulence factors (Bisno & Stevens 2005). A pictorial representation of some of the bacterial virulence factors interacting with the innate immune system is shown in Figure 2, and specific virulence factors discussed in the text below.

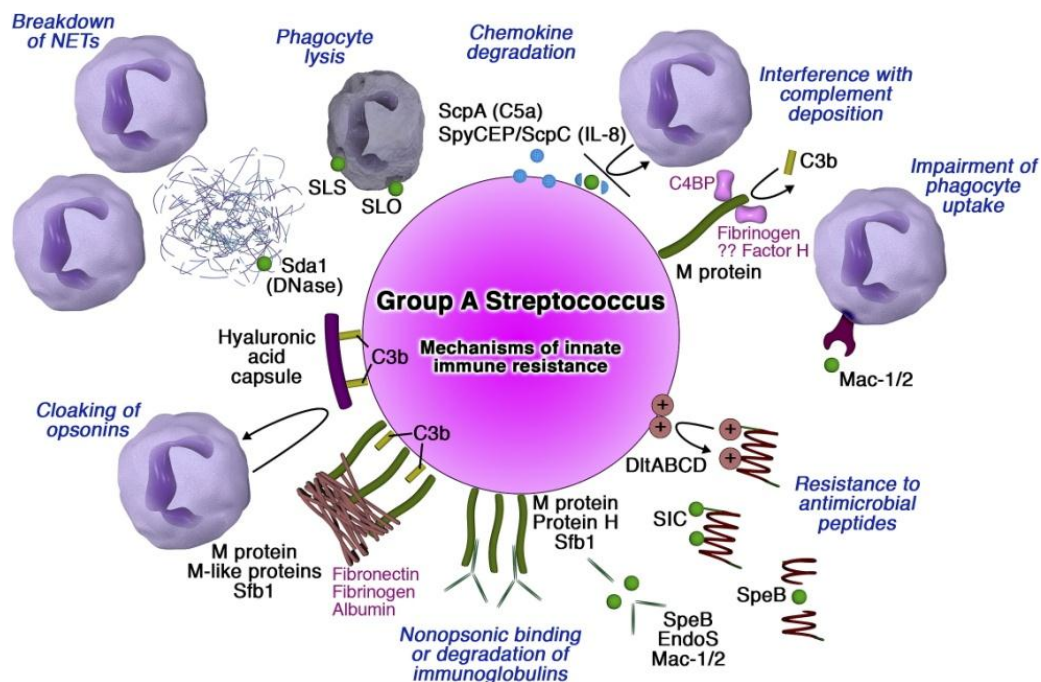


Figure 2: Interaction of *S. pyogenes* and the innate immune system.

Mechanisms by which the bacterial pathogen Group A Streptococcus subverts host innate immune defence. Phagocyte recruitment is reduced by peptidases ScpA and SpyCEP/ScpC that degrade C5a and IL-8, respectively. Complement deposition is limited by M protein binding of host counter regulatory factors C4bBP and Factor H. Phagocytic uptake is reduced by Mac-1/2 binding of phagocyte Fc receptors. Resistance to cationic antimicrobial peptides is afforded by D-alanyl modification of cell wall teichoic acids, cysteine protease SpeB-mediated degradation, and binding/inactivation by protein streptococcal inhibitor of complement (SIC). M protein and protein H bind Fc domains of Ig's in a non-opsonic manner, and proteolytic inactivation of Ig is a property of SpeB, Mac-1/2, and endopeptidase S. DNase production facilitates escape from NETs, and the pore-forming cytolysins streptolysin S (SLS) and streptolysin O (SLO) exhibit lytic activity against host neutrophils and macrophages. Figure and legend reproduced from (Nizet 2007).

1.2.1.1 Antimicrobial peptides

S. pyogenes has been found to produce a protein called SIC (Streptococcal inhibitor of complement, so named because of its ability to interfere with the complement membrane attack complex), which is able to bind to and inhibit the key Gram positive antimicrobial peptides: human alpha defensins, beta defensins 1, 2 and 3 and cathelicidin LL37 (Fernie-King, Seilly, & Lachmann 2004; Frick, Akesson, Rasmussen et al. 2003; Shelburne, III, Granville, Tokuyama et al. 2005). The cysteine protease SPEB has been shown to be associated with LL37 production at the site of infection in humans (Johansson, Thulin, Sendi et al. 2008) and has a role in the degradation of LL37 (Schmidtchen, Frick, Andersson et al. 2002). In response to antimicrobial peptides, *S. pyogenes* also increases production of its capsule (Gryllos, Tran-Winkler, Cheng et al. 2008). Human tonsils removed from patients with recurrent tonsillitis have been shown to have decreased levels of beta defensins 1 and 3 and LL37, compared to patients whose tonsils were removed for sleep disorders (Ball, Siou, Wilson et al. 2007), suggesting the importance of these molecules in the pathology of throat infections.

1.2.1.2 Saliva

Studies on bacterial growth in saliva and macaque monkey models of pharyngitis have shown that some strains of *S. pyogenes* are capable of producing phospholipase A2, which promotes entry to host cells and causes a pronounced inflammatory reaction (Sitkiewicz, Stockbauer, & Musser 2007). Other colonising bacteria are present in the pharynx, and *S. pyogenes* has to compete with them for carbohydrates and gain a survival advantage, in the nutrient-poor environment of saliva. Microarray technology has revealed that *S. pyogenes* is able to metabolise several carbohydrates in the oropharynx, especially maltose and mannose, and that transcription of the metabolic genes is up-regulated during the early phase of colonisation (Shelburne, III, Keith, Horstmann et al. 2008).

1.2.1.3 Mucus

Mucus acts to bind bacteria and prevent adherence to the epithelia, before forcibly expelling the bacteria via the mucociliary escalator system. *S. pyogenes* have been shown to produce pili (also

known as T antigens), which help in adherence of the bacteria to epithelia in early colonisation and biofilm formation (Abbot, Smith, Siou et al. 2007; Abbott and Hayes 1984; Manetti, Zingaretti, Falugi et al. 2007), by attaching to the ridged sections of tonsil epithelia (Lilja, Silvola, Bye et al. 1999). Other mechanisms which *S. pyogenes* has available to promote binding to epithelium, and hence colonisation, include Fibronectin binding protein F1 (Hyland, Wang, & Cleary 2007), serum opacity factor (Gillen, Courtney, Schulze et al. 2008), lipoteichoic acid and M protein (Cunningham 2000). The binding of M protein to pharyngeal epithelium via membrane co-factor protein CD46 and $\beta 1$ integrin has been shown to create an inflammatory response, with release of IL1, IL6 and Prostaglandin E2 (Cunningham 2000), yet also aid the internalisation and colonisation by *S. pyogenes*, possibly by actin cytoskeleton rearrangement (Rezcallah, Boyle, & Sledjeski 2004). *S. pyogenes* is coated with a hyaluronic acid capsule, with expression controlled by the genes *has A*, *B* and *C*. Capsule production varies between strains, and strains which are less capsular have attenuated virulence. Along with M protein, capsule has an anti-phagocytic activity (Cunningham 2000). In addition, binding of plasminogen both on the bacterial cell surface (by enolase and GAPDH) and extracellularly (Streptokinase) helps to prevent bacterial invasion by preventing matrix metallo-proteinase production (Cunningham 2000).

Oral fluids contain high levels of CXCL9 (monokine induced interferon γ , MIG), which are higher in patients with acute streptococcal pharyngitis, and which has direct antibacterial properties (Egsten, Eliasson, Johansson et al. 2007). Again *S. pyogenes* attempts to overcome this host immune mechanism, with neutralisation of CXCL9 occurring in the presence of SIC (Egsten et al. 2007).

1.2.1.4 Complement

Complement is found in most body fluids, and is involved in three methods of defence against bacteria – inflammatory cell recruitment, opsonisation of bacteria for phagocytosis and direct bacterial killing via the membrane attack complex. The key binding pathway for complement against *S. pyogenes* is the alternative pathway (Yuste, Ali, Sriskandan et al. 2006), and *S. pyogenes* has developed several mechanisms to overcome the specific actions of C3b, including inhibition by Factor

H binding (Cunningham 2000) and inactivation and cleavage by SPEB (Kuo, Lin, Chuang et al. 2008). Activation of the classical complement pathway via C1q is also important in *S. pyogenes* disease (Yuste et al. 2006) and maybe particularly relevant in pharyngitis, as production of C1q in tonsils is largely controlled by immature dendritic cells and is reduced as they mature in response to antigen detection (Castellano, Woltman, Nauta et al. 2004). Additionally, some strains of *S. pyogenes* have been found to interfere with C4b binding protein (Suvilehto, Jarva, Seppänen et al. 2008). *S. pyogenes* has been shown to produce a specific C5a peptidase (Chen and Cleary 1989), affecting phagocyte recruitment, and as mentioned above *S. pyogenes* is also able to produce SIC, which interferes with the complement membrane attack complex (Shelburne, III et al. 2005).

1.2.1.5 Innate immune cells

The primary effector cells of the innate immune system are phagocytic neutrophils and monocytes in the blood, macrophages and dendritic cells in the lymphoid organs, including tonsils. These cells phagocytose invading bacteria, processing their cellular components for antigen presentation, and stimulating the inflammatory response by releasing prostaglandins, leucotrienes, platelet activating factors, TNF α and cytokines. This results in reduced blood flow around the site of inflammation and endothelial changes to attract circulating immune cells. Mechanisms are being discovered by which *S. pyogenes* is able to inhibit the recruitment of innate immune cells, most importantly by the production of the IL-8 cleaving enzyme SpyCEP, which prevents neutrophil recruitment to the site of infection (Edwards, Taylor, Ferguson et al. 2005). In addition MAC protein (also known as IdeS) secreted by *S. pyogenes* has homology to human Mac-1 protein, and so can bind to human PMNs to inhibit opsonophagocytosis and bacterial killing, as well as preventing IgG from binding to CD16 and having a specific IgG proteinase activity (Lei, Liu, Meyers et al. 2003). Streptolysins O and S (both haemolysins) have been demonstrated to have a directly toxic effect on human phagocytes, including disruption of the cell membrane (Bisno & Stevens 2005).

Regrettably the importance of neutrophils in the pharyngeal immune responses is yet to be established, as the level of recruitment from the systemic circulation to acutely inflamed tonsils can only accurately be assessed by removing tonsils during acute infection – which is contraindicated as it

increases the risk of haemorrhage and surgical site infection. Monocytes and macrophages, which are known to be present in tonsils, express toll like receptors (TLR) on their surface, which control cell signalling and the release of inflammatory cytokines in response to antigenic stimulation. Superantigens produced by *S. pyogenes* have been shown to up-regulate the expression of TLR4 (Hopkins et al. 2005), and there is altered expression of TLRs in patients with recurrently infected tonsils compared to patients with tonsillar hypertrophy due to other causes (Mansson, Adner, & Cardell 2006). Production of cytokines can also influence antigen presentation by macrophages, as TGFβ1 reduces MHC II expression, contrasting with INFγ which enhances it, in response to streptococcal M protein (Delvig, Lee, Chrzanowska-Lightowlers et al. 2002).

Dendritic cells have been shown to prevent the dissemination of *S. pyogenes* in murine models of infection, due to the production of IL12 (Loof, Rohde, Chhatwal et al. 2007). As these cells are present in large numbers in tonsils, it is likely that they play a significant role in the pharyngeal defence against *S. pyogenes*, which is yet to be fully established. Certainly they are the major antigen presenting cell type in tonsils, and can bind and activate both B and T cells (Plzak, Holikova, Smetana, Jr. et al. 2003). *In vivo* work with a staphylococcal superantigen SEB has shown that superantigens can induce the maturation and migration of dendritic cells (Muraille, De, Pajak et al. 2002), but *in vitro* whole *S. pyogenes* has been shown to inhibit the maturation of monocyte derived macrophages by capsule binding and direct streptolysin O toxicity (Cortes and Wessels 2009).

1.2.2 Interactions with the adaptive immune system

The adaptive immune cells in the human pharynx are located predominantly in the lymphoid follicles of Waldeyer's ring, and comprise predominantly T cells and B cells (see section 1.5). To date, the most pronounced interaction of *S. pyogenes* virulence factors with T cells has been in the discovery of superantigens, which cause profound T cell proliferation and cytokine release (see section 1.3). However, given the array of virulence factors produced by *S. pyogenes* against factors of the innate

immune system, it is likely that further mechanisms directed against T cells or the cytokines they produce will be identified in the future. The balance between pro-inflammatory responses to *S. pyogenes* infection and controlling mechanisms through regulatory T cells (TRegs) has also to be established, though there is evidence that regulatory T cells also increase in number in response to superantigens (Taylor and Llewelyn 2010).

B cells are known to produce specific antibodies in response to streptococcal infection, particularly anti-M protein antibodies, anti-DNAse B antibodies and anti-Streptolysin O (ASO) antibodies. As mentioned in section 1.1.2, anti-DNAse B and ASO titres are used in diagnostic microbiology laboratories to help in the diagnosis of recent streptococcal infections, particularly in the context of the post-streptococcal immune complex disorders glomerulonephritis and rheumatic fever (Johnson et al. 2010). Research into the harnessing of antibody reactions to *S. pyogenes* is being actively pursued in the hope of developing an effective vaccine against *S. pyogenes*, though to date no successful vaccines have been produced which do not result in harm to the host (Cunningham 2000). Immunoglobulins also play a role in mucosal defences, as secretory IgA is found in mucosal secretions. Both IgA and IgG have been shown to bind to *S. pyogenes* (Lilja et al. 1999), and again *S. pyogenes* virulence factors have been shown to interfere with their mechanism of action (Lei et al. 2003). EndoS, IdeS (MAC) and SPEB have all been shown to directly cleave human immunoglobulin, as part of the *S. pyogenes* array of virulence factors, with the end results that opsonisation of the bacteria is compromised (Collin, Svensson, Sjöholm et al. 2002).

A summary of these complex interactions between *S. pyogenes* and both the innate and adaptive immune system is shown in Table 2. Although some of the key mechanisms so far identified are outlined here, these are specific to the pharyngeal immune response, and *S. pyogenes* is known to produce a number of other pathogenic factors, which have not been mentioned. Further research into these is necessary to establish their role in tonsillo-pharyngitis.

Human immune System	Activity against <i>S. pyogenes</i>	<i>S. pyogenes</i> virulence factors
Saliva	Lysozyme Antimicrobial peptides Competing bacteria	SIC, phospholipase A2, increased carbohydrate scavenging
Mucus	Mechanical prevention of adhesion High levels of cytokines and chemokines	Pili M protein Lipoteichoic acid Capsule Fibronectin binding protein Serum opacity factor
Complement	Classical pathway Alternative pathway Membrane attack complex	C4b binding proteins SPEB Factor H binding protein C5a Peptidase SIC
Innate immune Cells	Neutrophils Monocytes/macrophages Dendritic cells	SpyCEP MAC/IdeS Superantigens
Adaptive immune cells	T cells B cells	Superantigens C5a peptidase, SPEB, MAC and EndoS cleaving IgG
Other	Plasminogen Direct toxicity	Plasminogen binding M-like protein (PAM), streptokinase, enolase, GAPDH Streptolysin

Table 2: *S. pyogenes* virulence factors target the human immune system

Summary of the key components of the pharyngeal immune system and the virulence factors produced by *S. pyogenes* which have been demonstrated to act against them. This table was compiled from information published in numerous reviews (Cunningham 2000; Nizet 2007; Olsen and Musser 2010) and specific publications which are detailed in the accompanying text (section 1.2).

1.2.3 Immune recognition of *S. pyogenes*

The production of *S. pyogenes* specific antibodies to opsonise bacteria and enhance phagocytosis is one of the main mechanisms of host defence against *S. pyogenes* infection. Antibody production has been demonstrated against numerous cell surface and extracellular *S. pyogenes* proteins, with M protein being the most prominent of these (Cunningham 2000). Other virulence factors which induce the production of opsonising antibodies include those being studied for potential vaccine development, such as C5a peptidase.

Among the first phagocytic cells *S. pyogenes* encounters in the pharynx are dendritic cells. Recognition of bacterial components by dendritic cells has been found to be multifactorial and through several TLRs simultaneously in order to activate and elicit strong dendritic cell responses to *S. pyogenes* infection, including expression of CD40, CD80 and CD86 and cytokine production (Loof, Goldmann, & Medina 2008). As antigen presenting cells, dendritic cells also present *S. pyogenes* antigens to T cells for the formation of cognate immune responses (Loof et al. 2007). T cell recognition of *S. pyogenes* M protein was found to be determined by the nature of MHC Class II presentation of M protein fragments by antigen presenting cells, either by formation of new MHC Class II complexes or by MHC Class II complex recycling (Delvig and Robinson 1998).

In macrophages, it has been recently shown that as well as TLR signalling, the production of an IL1 β response to streptolysin O is possible in a TLR independent fashion, via the NLRp3 (Nod (nucleotide-binding oligomerisation domain)-like receptor family pyrin domain-containing 3) inflammasome and the caspase-1 pathway (Harder, Franchi, Munoz-Planillo et al. 2009).

1.2.4 Bacterial defence systems

Recently systems which act as a primitive bacterial immune system have been identified in a number of bacteria, including *S. pyogenes*. The CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-associated proteins (Cas) can protect the bacteria from potentially harmful plasmids and phage's (Deltcheva, Chylinski, Sharma et al. 2011; Nozawa, Furukawa, Aikawa et al. 2011). They work by integrating foreign genetic material into a specific CRISPR locus, producing short guiding CRISPR RNAs and then controlling the fate of the foreign genetic material, though the precise mechanisms by which this happens are yet to be confirmed.

1.3 Bacterial Superantigens

Among all the virulence factors produced by *S. pyogenes*, superantigens stand out as the main factors with activity against the adaptive immune system. They have also been shown to be among those expressed early in monkey models of pharyngitis, and so are likely to play a role in pharyngeal colonisation by *S. pyogenes* (Virtaneva et al. 2005).

Superantigens have been identified in *S. pyogenes*, *Staphylococcus aureus* and various other bacteria including *Mycoplasma arthritidis*, *Yersinia sp.* and several viruses including HIV and Rabies. Most work has so far been done on *S. aureus* superantigens (staphylococcal enterotoxins (SE)A-E, SEG-I and staphylococcal enterotoxin-like superantigens (SEI)J-R and U, and Toxic shock syndrome toxin (TSST)-1) and streptococcal superantigens (Streptococcal pyrogenic exotoxins (SPE) A, C and G-M, Streptococcal superantigen (SSA) and streptococcal mitogenic exotoxin (SME)Z-1/2) (Proft and Fraser 2003). In addition specific B cell superantigens have been identified in *S. aureus*, which cause B cell clustering and proliferation, but so far these have not been identified in *S. pyogenes*.

Different M types of *S. pyogenes* carry different superantigen genes in their chromosome, and this correlates to different mitogenic capacity (Turner, Namnyak, McGregor et al. 2011). Superantigens vary in their potency, with SMEZ being the most powerful streptococcal superantigen so far described: SMEZ reaches half maximal proliferation at a concentration of 100 pg/ml, in contrast to SPEA, at a concentration of 1.8ng/ml (Muller-Alouf, Proft, Zollner et al. 2001).

1.3.1 Mechanism of action

Streptococcal pyrogenic exotoxin A (SPEA) was among the first identified streptococcal superantigens (Watson 1960). In common with the other streptococcal and staphylococcal superantigens, it was later shown that T cells were being driven to proliferate and produce large quantities of pro-inflammatory cytokines after stimulation with SPEA. This was due to abnormal binding of the superantigen to both the variable beta subunit of the T Cell Receptor (TCRV β) and the MHC Class II molecule of antigen presenting cells. The normal process of antigen presentation occurs in a carefully regulated manner, with antigen presenting cells processing material, before presenting it on the cell surface via MHC molecules. These are then identified by the T cell receptor, and downstream signalling events occur. Superantigens bypass this usual antigen presentation process, by binding ectopically to the MHC class II molecules of antigen presenting cells, and the T cell receptor (Figure 3). This interferes with MHC class II and T cell receptor interactions, effectively driving a wedge or bridge between the molecules (Brosnahan and Schlievert 2011), and prevents normal signalling from occurring. Not only does this cause clonal expansion of the TCRV β subset(s) of T cells to which an individual superantigen binds, but also the activation of these cells (Bueno, Criado, McCormick et al. 2007). The release of pro-inflammatory cytokines in response to superantigens causes profound shock and circulatory collapse in the affected host, leading to Toxic Shock Syndrome in the most severe cases (Llewelyn and Cohen 2001).

There are two major structural domains in all superantigens: the amino terminal oligosaccharide/nucleotide binding (OB) fold and the carboxy terminal β -grasp domain. Similarities in amino acid sequences and structures allow superantigens to be classified into 5 groups, which are listed in Table 3. The structural differences alter the ability of superantigens to act as a wedge (as in the case of SPEA) or a bridge (as seen with SPEC) between the MHC class II molecule and the T cell receptor (Brosnahan & Schlievert 2011). Some superantigens have an additional zinc binding domain, including SMEZ and SPEJ, and some have the ability to form homodimers at high concentrations (including SPEJ) (Proft, Arcus, Handley et al. 2001; Proft & Fraser 2003).

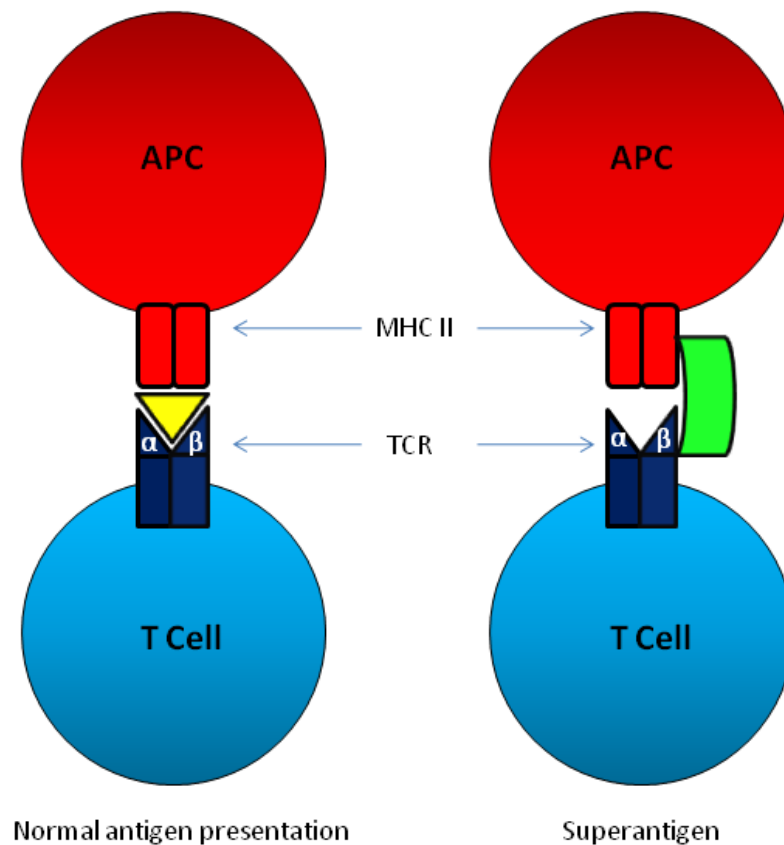


Figure 3: Superantigen binding

Schematic representation of the abnormal binding of superantigens to the T cell receptor variable β chain and MHC Class II molecules (right, green) in contrast to normal antigen presentation (left, yellow).

Group	Superantigens	MHCII Binding	Structural features
I	TSST-1	Low affinity α -chain site	Unique amino acid sequence, no cysteine loop
II	SEB, SEC, SPEA , SSA	Low affinity α -chain site	Variable length cysteine loop
III	SEA/D/E/J/H	Low and high affinity α - and β -chain sites	Nine amino acid length cysteine loop
IV	SPEC, SPEJ , SMEZ-2	Low and high affinity α - and β -chain sites	No cysteine loop
V	SEI, SPEH,	Low and high affinity α - and β -chain sites	No cysteine loop, 15 amino acid insert

Table 3: Superantigen group classification

Classification of staphylococcal and streptococcal superantigens as defined by amino acid sequence and structural features. Examples of each group of superantigen are listed; superantigens of importance in this project are highlighted in bold. Adapted from (Brosnahan & Schlievert 2011).

1.3.2 T cell receptor binding

Superantigens have a unique ability to stimulate variable β chain subunit specific T cells, from their baseline levels to as much as 20% of the total T cell population. Despite the cross-linking occurring with MHC Class II molecules, this T cell expansion is not limited to CD4 cells, but also involves CD8 cells. For some superantigens binding to a single $V\beta$ subunit has been identified (for example SPEJ and $V\beta 2$), whereas others, such as SPEA bind to a variety of $V\beta$ subunits, depending on the exposure concentration (Llewelyn, Sriskandan, Terrazzini et al. 2006; Proft & Fraser 2003).

The T cell effects of superantigen binding result in an early burst of pro-inflammatory cytokines (1-2 hours) followed by a later sustained release of both T_H1 and T_H2 cytokines (Faulkner, Cooper, Fantino et al. 2005; Muller-Alouf, Capron, Alouf et al. 1997). Normal T cell signalling involves Lck tyrosine kinase activation, associated with CD4/CD8 molecules, with subsequent ZAP-70 activation, downstream cell signalling events, and the formation of the complex immunological synapse between T cells and APCs. It was initially assumed that this normal signalling cascade was employed by superantigens, but more recent work has shown that superantigens are also able to activate T cells via a Lck-independent pathway, through $G\alpha_{11}$ -dependent phospholipase C- β activation (Bueno, Lemke, Criado et al. 2006). The importance of each signalling method *in vivo* has yet to be investigated, and it remains to be seen whether the different binding modalities of individual superantigens can influence the resultant T cell signalling, resulting in different outcomes.

Chronic exposure to streptococcal superantigens in mouse models has been shown to cause T cell anergy and clonal T cell depletion (McCormack, Callahan, Kappler et al. 1993). Similar experiments with SEB have shown that after repeated exposure there was increased production of the anti-inflammatory cytokine IL-10, and an induction of CD4⁺ TRegs and polyclonal B cell expansion (Florquin, Amraoui, Abramowicz et al. 1994; Noel, Florquin, Goldman et al. 2001). The long term influence of this on the host, the ability to generate anti-superantigen and anti-streptococcal antibodies, and any influence on recurrence of infection is unknown.

1.3.3 MHC Class II binding

Superantigens have low (K_d $10^{-5}M$) and high affinity (K_d $10^{-7}M$) binding sites which attach independently to MHC Class II molecules. Low affinity binding occurs between the superantigen OB folding domain and the MHC class II α chain. High affinity binding has only been characterised for a few superantigens, but appears to involve contact between the superantigen β -grasp domain and the MHC Class II β chain (Bueno et al. 2007). Some superantigens, such as SPE-C/J, are able to form a zinc-mediated homodimer, between the low affinity binding sites (Proft & Fraser 2003). Different superantigens bind with preference to different MHC Class II molecules, for example staphylococcal enterotoxin B (SEB) binds preferentially HLA-DR>DQ>DP (Llewelyn, Sriskandan, Peakman et al. 2004). To date, the down-stream effects of superantigen binding on the antigen presenting cells themselves has not been fully explored, but there is evidence that there is alteration of cell signalling (Hopkins et al. 2005), and it is likely that this influences the degree of T cell activation and apoptosis (Lavoie, McGrath, Shoukry et al. 2001). Certainly T cell binding to superantigens in the absence of MHC Class II binding does not result in T cell activation (Tiedemann and Fraser 1996).

Although it is still unclear the precise role that superantigens play on MHC II signalling, there is evidence that binding of superantigens does not trigger normal downstream signalling events to occur: although there is an increase in cAMP production, there is no increase in intracellular calcium, inositol phosphates or phosphatidic acid. In B cells these would usually be increased upon stimulation of either the B cell receptor or MHC Class II (Abram and Lowell 2007).

1.3.4 SPEA

Streptococcal pyrogenic exotoxin A (SPEA) is one of the superantigens produced *S. pyogenes* which is studied in this project. Four distinct alleles have been recognised (Nelson, Schlievert, Selander et al. 1991), and it has been shown to be active in concentrations of 1 ng/ml and above, causing proliferation of human TCRV β 2, 12, 14 and 15 (Proft & Fraser 2003). Dose response experiments have shown that V β 14 is the predominant subset of human T cells recruited, with other subsets only being activated at higher concentrations of SPEA exposure (Llewelyn et al. 2006). In mice, expansion of mV β 2 and 8 occurs. The crystallographic structure of SPEA and its binding to the human and murine receptors is shown in Figure 4.

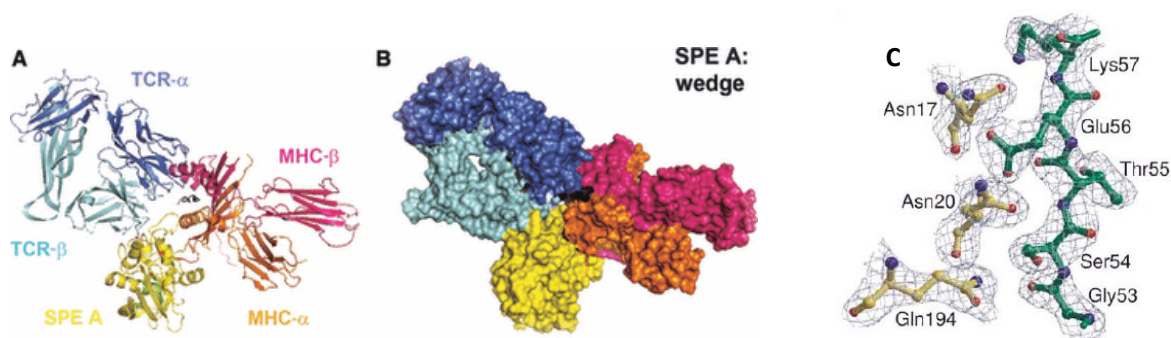


Figure 4: Structure of SPEA

A and B: representation of the binding of SPEA to the human T cell receptor and MHC Class II molecules as a wedge, reproduced from (Brosnahan & Schlievert 2011), SPEA yellow, MHC class II molecule pink (β) and orange (α), T cell receptor blue (α) and cyan (β). C: Structural representation of the SPEA-murine V β 2.1 complex: Superantigen carbon atoms yellow, TCRV β carbon atoms green, Nitrogen atoms blue, Oxygen atoms red, reproduced from (Sundberg, Li, Llera et al. 2002).

Animal models of necrotising fasciitis have shown that SPEA can be detected in kidneys and liver of mice infected with live bacteria, with independent systemic spread from the original site of infection (Sriskandan, Moyes, Buttery et al. 1996). The creation of an isogenic *speA*- mutant M1 *S. pyogenes* strain did not attenuate the virulence *in vivo*, with the *speA*- strain resulting in a higher rate of

bacteraemia and more intense local bacterial infection (Sriskandan, Unnikrishnan, Krausz et al. 1999; Unnikrishnan, Cohen, & Sriskandan 2001), though later models of disease in HLA transgenic mice showed increased inflammation with SPEA (Sriskandan, Unnikrishnan, Krausz et al. 2001). The importance and true role of SPEA in the course of *S. pyogenes* infection has still to be fully explained.

1.3.5 SMEZ

Streptococcal mitogenic exotoxin Z (SMEZ) is the most potent streptococcal superantigen so far described, with proliferation occurring at concentrations of less than 1 pg/ml (Unnikrishnan, Altmann, Proft et al. 2002). It has multiple alleles, resulting in the production of two main crystallographic structures; SMEZ-1 and SMEZ-2 (Proft, Moffatt, Weller et al. 2000), the structure of SMEZ-2 being shown in Figure 5. SMEZ binds predominantly to TCRV β 8 in humans and TCRV β 11 in mice. Interestingly, SMEZ lacks the low-affinity MHC class II binding site, but does have the high affinity binding site and zinc binding domain, potentially allowing homodimer formation to occur.

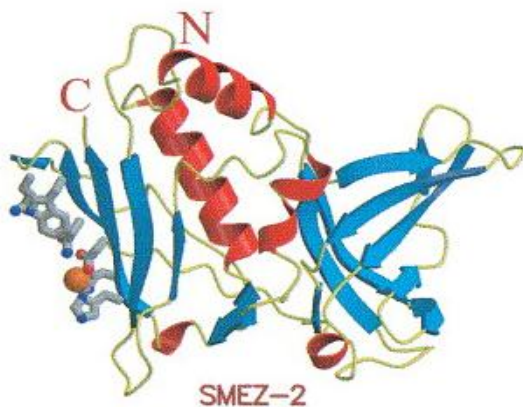


Figure 5: Crystal structure of SMEZ-2

Structure of SMEZ-2 showing the peptide folding and binding sites. Bound zinc is represented by the orange sphere. Conserved residues on the MHCII binding face shown in grey. The shape is roughly trapezoidal, with the concave faces of the β sheets (blue) being potential binding faces. Reproduced from (Arcus, Proft, Sigrell et al. 2000).

There is a mutation in the M3 *S. pyogenes* gene of SMEZ, which renders the gene inactive, and results in reduced proliferation *in vitro* (Turner et al. 2011). However, M3 strains of *S. pyogenes* frequently cause aggressive disease and some of the more severe cases of streptococcal toxic shock syndrome (Lamagni, Darenberg, Luca-Harari et al. 2008). Similarly, there was no difference in survival from infection in a mouse model of streptococcal infection, comparing a wild type M89 strain of *S. pyogenes* and the isogenic mutant *smeZ*- strain (Unnikrishnan et al. 2002). There is also evidence that SMEZ is vulnerable to cleavage by another virulence factor secreted by *S. pyogenes*, the cysteine protease SPEB (Nooh, Aziz, Kotb et al. 2006).

This brings into question the role of SMEZ in invasive disease, despite its strong mitogenic power and the fact that SMEZ can be identified in the serum of patients with toxic shock syndrome. Anti-SMEZ neutralising antibodies develop during convalescence, and specific antibodies against SMEZ-1 can be found in the serum of healthy volunteers, which are capable of fully neutralising the mitogenic effects in 85% of people tested (Proft, Sriskandan, Yang et al. 2003). As with other superantigens, the true role of SMEZ in *S. pyogenes* disease has yet to be established.

1.3.6 SPEJ

Streptococcal pyrogenic exotoxin J is one of the newer superantigens to be discovered on whole genome sequencing. It has mitogenic ability in a concentration range similar to that of SMEZ, and similarly binds to the MHC Class II β chain, though it is structurally more similar to SPEC with its ability to form homodimers at high concentrations. It has been found to expand human TCRV β 2 cells almost exclusively (Proft et al. 2001), but has not yet been tested in mouse models of disease. Along with SMEZ, it has been detected in the serum of patients with active streptococcal infections (Proft et al. 2003), and so is likely to play an important role in disease.

1.4 *In vitro* and *in vivo* studies of *S. pyogenes* pathogenesis

The study of bacterial pathogenesis has seen many advances in recent decades, with the advent of high throughput bacterial genome sequencing, micro-arrays, proteomics and immunological techniques (Olsen, Shelburne, & Musser 2008). However, remarkably little is known about how *in vitro* research translates into the *in vivo* situation. This is mainly due to the inherent difficulties of obtaining and analysing clinical samples in a timely fashion and establishing accurately when the infection began. Although animal models of disease have been developed as a surrogate to measure the progress of an infection and host responses to this, in the case of *Streptococcus pyogenes*, the bacteria is so specifically adapted to a human host that only higher primate models are truly able to mimic human disease (Sumbly, Tart, & Musser 2008).

Bacteria are usually studied *in vitro* in very controlled and artificial environments: the bacteria are sub-cultured into broth or onto agar plates and encouraged to grow in a laboratory incubator which is set up to best mimic the conditions in the usual ecological niche for that organism. In the case of *S. pyogenes*, it prefers to grow in a 5% carbon dioxide or anaerobic atmosphere, as found in the human upper respiratory tract, and will only grow in a rich media such as Todd Hewitt broth or on an agar plate supplemented with blood (Bisno & Stevens 2005). However, these conditions were developed primarily to allow the bacteria to be cultured and isolated from clinical samples rather than to create a realistic laboratory environment in which to study their genetic or proteomic responses to stress. Certainly large differences in bacterial protein expression can be seen when these conditions are altered: for example, the surface expression of *S. pyogenes* M protein is altered during broth growth, and especially during repeated *in vitro* bacterial passage (MICKELSON 1964). Studies of the expression of key bacterial virulence factors and regulatory genes have shown that there is a difference in expression of virulence factors at different growth phases, with some being expressed preferentially during logarithmic growth phases, and others in stationary growth phase (Unnikrishnan, Cohen, & Sriskandan 1999). The addition of other factors to culture media, such as human saliva, has

allowed important information about bacterial nutrient scavenging to be discovered (Shelburne, III et al. 2008).

Although *in vitro* work allows for the study of changes to the bacterial transcription and protein production under specific influences, the environment is still artificial and does not adequately take into account the influence of human host factors in the development of clinical disease. Furthermore, it is unclear how *in vitro* growth phases relate to the clinical situation in disease. Streptococcal necrotising fasciitis frequently presents when the disease is well established and it is often unclear when or how the bacterial inoculation and infection began. The yield of bacteria from the site of infection into laboratory media can be altered by the sampling method and storage of the sample (Ross 1977). In clinical practice this has led to the development of rapid antigen testing and molecular diagnostics to increase the yield of bacterial culture results as well as the speed of diagnosis (Forward, Haldane, Webster et al. 2006; Wang, Kong, Yang et al. 2008). The potential low yield means that it is even more difficult to accurately assess the true *in vivo* nature of *S. pyogenes* during human infection.

To some extent this problem can be overcome by the use of animal models, with both mouse and macaque monkey models being well established in the investigation of streptococcal disease (Graham, Virtaneva, Porcella et al. 2006; Virtaneva et al. 2005; Virtaneva et al. 2003). However the problem remains that there are still significant differences between animals and humans, who are the only natural hosts of *S. pyogenes* disease. In mice, using Human HLA and other genetic manipulations can enhance the response to some bacterial antigens, including superantigens (Srisakandian et al. 2001). Although not natural hosts for *S. pyogenes*, both Baboons and Macaque monkeys have been used successfully to study the initiation and progress of streptococcal throat infections and the reciprocal mammalian responses to the infection (Shea et al. 2010). To study pharyngeal disease, a mouse nasal model of infection with *S. pyogenes* has been developed. Although the structure of mouse NALT (nasal associated lymphoid tissue) is very different from that of the human pharynx, consisting of lymphoid aggregates rather than defined lymphoid follicles, the model has been used successfully to examine immune responses to *S. pyogenes* (Costalonga, Cleary, Fischer et al. 2009; Hyland, Brennan, Olmsted et al. 2009).

Patient based studies are always reliant on good recruitment and samples processing, and it is difficult to account for other contributing patient factors that might influence results. A small amount of work has been done on biopsy samples from patients with necrotising fasciitis, and has shown that virulence factors can be detected at the site of infection (Johansson et al. 2008;Norrby-Teglund et al. 2001). Similarly one trial has shown that molecular analysis of *S. pyogenes* gene expression is possible directly from the throat swabs of patients with *S. pyogenes* pharyngitis (Virtaneva et al. 2003).

1.5 The Structure and function of human tonsils

The human pharynx has a unique immune system, which provides the first line of defence for both digestive and respiratory tracts. A ring of lymphoid tissue known as Waldeyer's Ring circles the pharynx, and comprises the palatine tonsils, lingual tonsil (also known as the pharyngeal tonsil or Adenoid) and lymphoid follicles on the surface of the tongue (Figure 6). The palatine tonsils are the largest of these lymphoid organs, and are situated between the anterior and posterior pharyngeal arches on both sides of the throat. Tonsils are separated from the pharynx by a thick mucosal capsule, which forms the dissection plane during tonsillectomy (Ellis 1992).

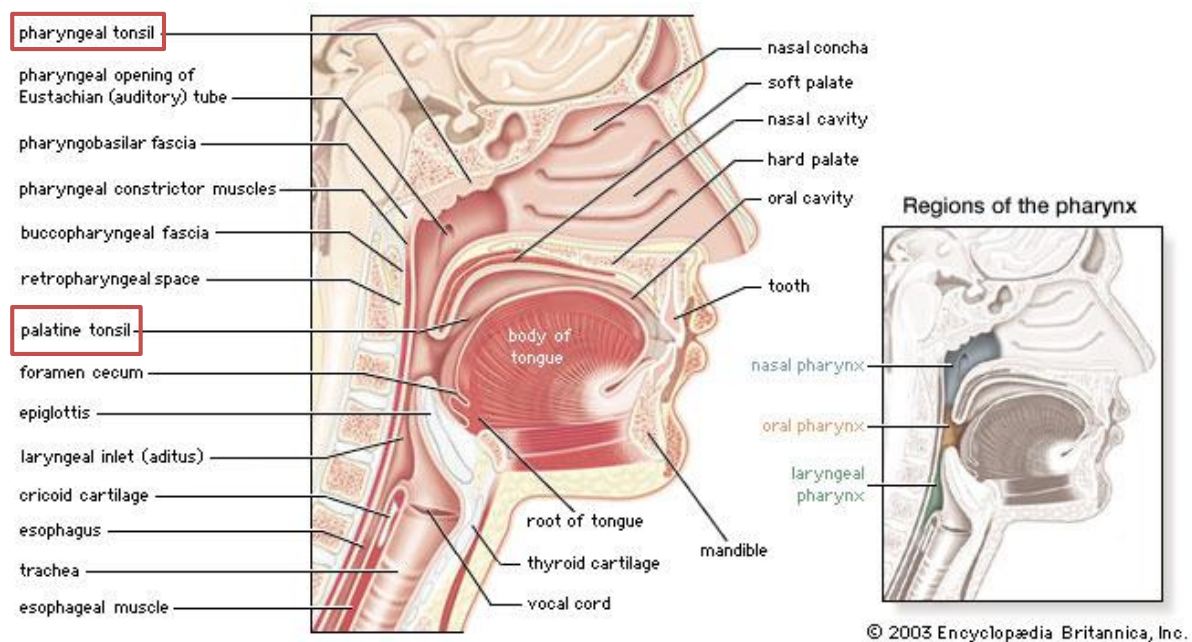


Figure 6: Structure of the human pharynx

A sagittal section of the human pharynx is shown. The location of the pharyngeal (adenoid) and palatine tonsils are highlighted in red boxes. Image reproduced from Encyclopædia Britannica 2003 (Anon 2011c).

The internal structure of tonsils is complex: tonsils are not a solid lymph node, but contain multiple fingers of tissue, each containing a structural fibrous lymphovascular core coated in lymphoid tissue and then covered in keratinised squamous epithelium (Isaacson 2008) as demonstrated in Figure 7 A-C. This structurally supporting conduit system provides lymph, nutrients and antigens to lymphoid tissue, and is surrounded by dendritic cells which sample antigens from the lymphoid channels (Roozendaal, Mebius, & Kraal 2008;Sixt, Kanazawa, Selg et al. 2005). Electron and fluorescent microscopy have demonstrated that the system in tonsils is similar to that in other lymph nodes, which are composed of a complex system of lymphatic drainage and blood vessels, connecting to the meshwork in the parafollicular matrix (Ohtani and Ohtani 2008). The lymphoid tissue is then formed into carefully arranged lymphoid follicles, with B cell germinal centres, marginal zones, and perifollicular T cell zones. Tonsils can enlarge both acutely and chronically, with differences in the number and size of follicles evident in patients with recurrent tonsillitis with or without hyperplasia (Alatas and Baba 2008;Dell'Aringa, Juarez, Melo et al. 2005).

The vascular supply to tonsils is via the tonsillar branch of the facial artery, and venous drainage is through the pharyngeal plexus to the paratonsillar vein. The tonsillar arteries branch into smaller arterioles and capillaries in both the follicles and parafollicular areas, ending in sinusoidal capillaries situated between the follicles and tonsil epithelium (Figure 7D). These then connect back to the tonsillar veins which are situated in the septa (Ohtani & Ohtani 2008). Lymphatic drainage starts in a complex meshwork of vessels which originate 200-300µm below the epithelium. Immune cells can travel to these vessels from the specialised epithelial lymphatic areas also referred to as the lympho-epithelial symbiosis (LS, Figure 7 E) (Ohtani & Ohtani 2008;Takahashi, Nishikawa, Sato et al. 2006). The peripheral lymphatic vessels in turn lead to perifollicular lymphatic sinuses, which drain into the lymphatic vessels in the septum, and ultimately the capsule (Ohtani & Ohtani 2008).The drainage is then to the jugulo-digastric or tonsillar lymph node which is situated at the angle of the jaw and the top of the deep cervical lymphoid chain; the “glands” which are frequently painful and enlarged during acute tonsillitis.

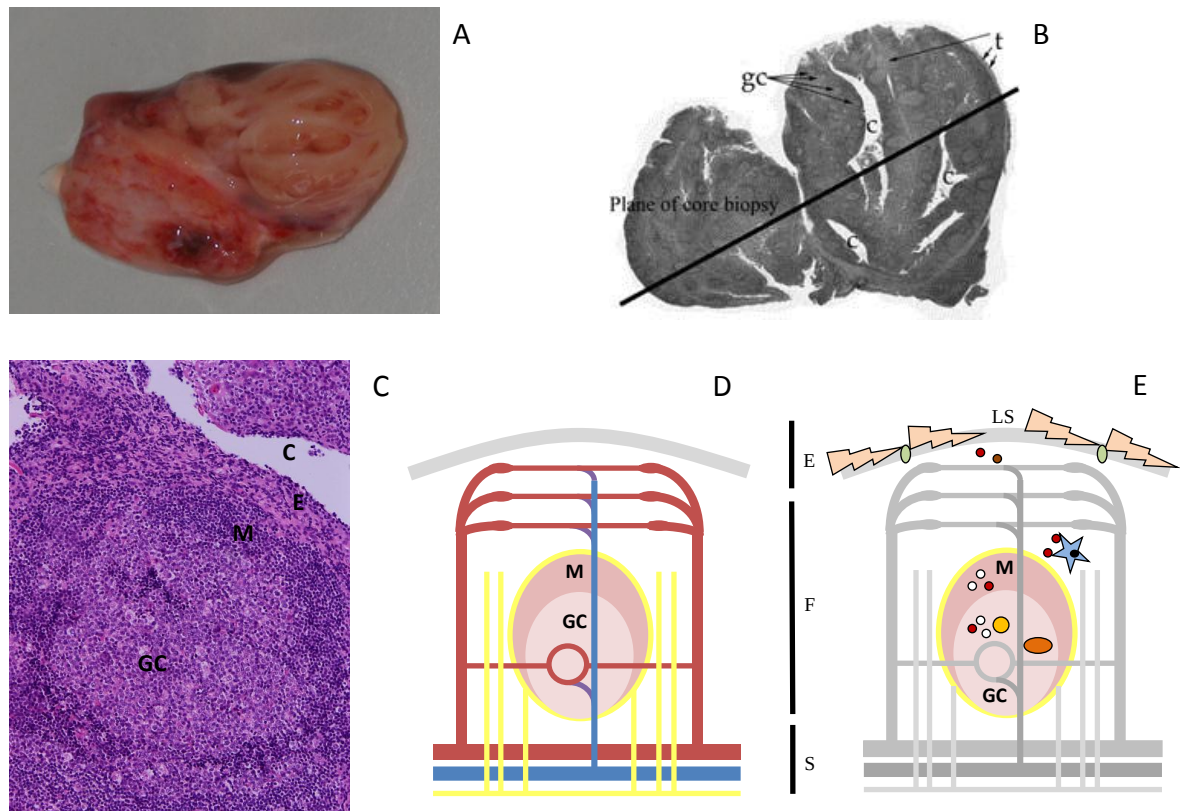


Figure 7: Structure of human tonsils

A/ appearance of the anterior section of a human palatine tonsil after tonsillectomy. B/ demonstration of the gross histopathology of a tonsil, and the usual plane of a core biopsy specimen, reproduced from (Isaacson 2008). C/ H&E stained section of a formalin fixed human tonsil, sectioned similarly to the biopsy plane shown in B, magnification x 100. D/ representation of the vascular and lymphatic supplies of a tonsil lymphoid follicle, red arterioles, blue venules and a mesh of capillaries reaching above the follicle, yellow lymphatics, adapted from (Ohtani & Ohtani 2008). E/ representation of the cell distribution in tonsils; blue = dendritic cell, red = T cell, white = B cell, yellow and orange = plasma cells, buff colour = epithelial cell, green = mucin producing cell. In all images, GC= germinal centre, M = mantle zone, E = epithelium, C = crypts, T = trabeculae, LS = lympho-epithelial symbiosis, F = follicle, S = septum.

Unlike other lymphoid nodes, tonsils are directly exposed to the mouth and are covered in a specialised keratinised epithelium, which covers the surface of the tonsil and also the surface of the crypts which extend deep into the tonsils (Ozbilgin, Kirmaz, Yuksel et al. 2006). This epithelium contains multiple mucin secreting cells, and so contributes to the muco-ciliary escalator system. Bacterial biofilms have been identified in the mucus overlying adenoid tonsils, though the

contribution this makes to disease and chronic hypertrophy is unknown (Winther, Gross, Hendley et al. 2009). Increased levels of antibacterial molecules lactoferrin and lysozyme have been detected in the mucus of patients with acute tonsillitis compared to healthy individuals, suggesting a significant antibacterial role (Stenfors, Bye, & Raisanen 2003). There are also lymphoid sinuses interspersed amongst the epithelium (the LS system, as above), containing Langerhan's-type immature dendritic cells, which serve a similar function to Payer's patches throughout the gut (Fujimura 2000;Plzak et al. 2003).

Secretion of IgG and IgA into the mucus and saliva is a further important role of the tonsil lymphocytes and epithelium, though it has been shown that the percentage of bacteria successfully coated in IgA during acute tonsillitis is reduced compared to viral or non-disease states (Lilja et al. 1999;Stenfors, Bye, & Raisanen 2001). It is unclear whether this is because the bacterial proliferation in disease outstrips the ability to produce immunoglobulin, or whether it is due to the production of bacterial immunoglobulin cleaving products such as SpeB and EndoS (Collin and Olsen 2001). Overall, the epithelium provides protection to the tonsils, but also allows for immune cells to pass easily from the tonsil to the surface and back, helping immune surveillance and antigen processing at the primary site of bacterial and viral exposure.

1.5.1 Cellular structure and functions of lymphoid follicles

As part of the human lymphoid network, it is thought that human tonsils form an integral part of both the innate and adaptive immune system. The exact cellular composition and interactions of tonsils and other lymphoid organs are still being explored, but it is known that they comprise a complex assortment of immune cells, at a wide range of stages of maturation. The dominant features are the germinal centres – these comprise mainly B cells (Brieva, Roldan, De la Sen et al. 1991), macrophages (Giger, Bonanomi, Odermatt et al. 2004) and follicular dendritic cells (Ozbilgin et al. 2006), all of which are capable of acting as antigen presenting cells. It has also been shown that the B

cells in tonsils are capable of maturing into long-lived plasma cells *in vitro* in response to stimulation (Arce, Luger, Muehlinghaus et al. 2004;Liu and Banchereau 1997;van Laar, Melchers, Teng et al. 2007) as well as having an antigen presentation role (Ozbilgin et al. 2006).

The follicles are surrounded by a mantle zone, comprising immature B cells (but not plasma cells) and dendritic cells (Giger et al. 2004). The areas between the follicles are the location for the majority of T cells (both CD4 and CD8 expressing cells), which interact with the dendritic cells and the fibroblastic reticular cells of the lymphoid vessels (Lammermann and Sixt 2008;Ozbilgin et al. 2006;Plzak et al. 2003). However, T cells have been identified throughout the tonsil, and have close relationships to the other cells which depend on their signalling for activation. A large proportion of T cells are of the T Follicular Helper (T_{FH}) phenotype, expressing CXCR5, ICOS (Inducible co-stimulator), PD-1 (programmed death-1) and IL21 (Haynes 2008;Withers, Fiorini, Fischer et al. 2007), and expression of these surface molecules can vary depending on where in the tonsils the cells are located (Bentebibel, Schmitt, Banchereau et al. 2011), with those in the extra-follicular regions often being referred to as pre- T_{FH} cells. T_{FH} cells have now been identified as a distinct subset of CD4+ T cells, under the control of the transcriptional regulator Bcl6 rather than Blimp-1, which is the main regulator in other T helper cell subsets. However they can share some of the characteristics of other T_H subsets in terms of the cytokines they produce (Crotty 2011;King, Tangye, & Mackay 2008).

A representation of these key tonsil cell types and their interactions is demonstrated in Figure 8 (Linterman and Vinuesa 2010). Although rather simplified, this does demonstrate the complexity of a lymphoid follicle, and how the different cells move around the lymph node and alter their phenotype depending on the signals they receive.

A large number of co-stimulatory molecules are involved in the communication between the different cells in tonsils, and help to determine the fate of the different cell types (Figure 8B), as well as the fundamental T cell receptor – MHC class II interaction. Among the key communication molecules expressed on T cells are ICOS, OX40, CD40L, CD30, CD27 (not shown) and CD28, which all interact with their ligands on B cells or dendritic cells. With the exception of CD28 these are all

members of the TNF receptor superfamily. Although CD30 has a more regulatory role, the remainder of these receptors have a T cell stimulatory/activation role, by intracellular signalling through TRAFs (TNF receptor associated factors), and numerous different downstream signalling cascades (Ha, Han, & Choi 2009).

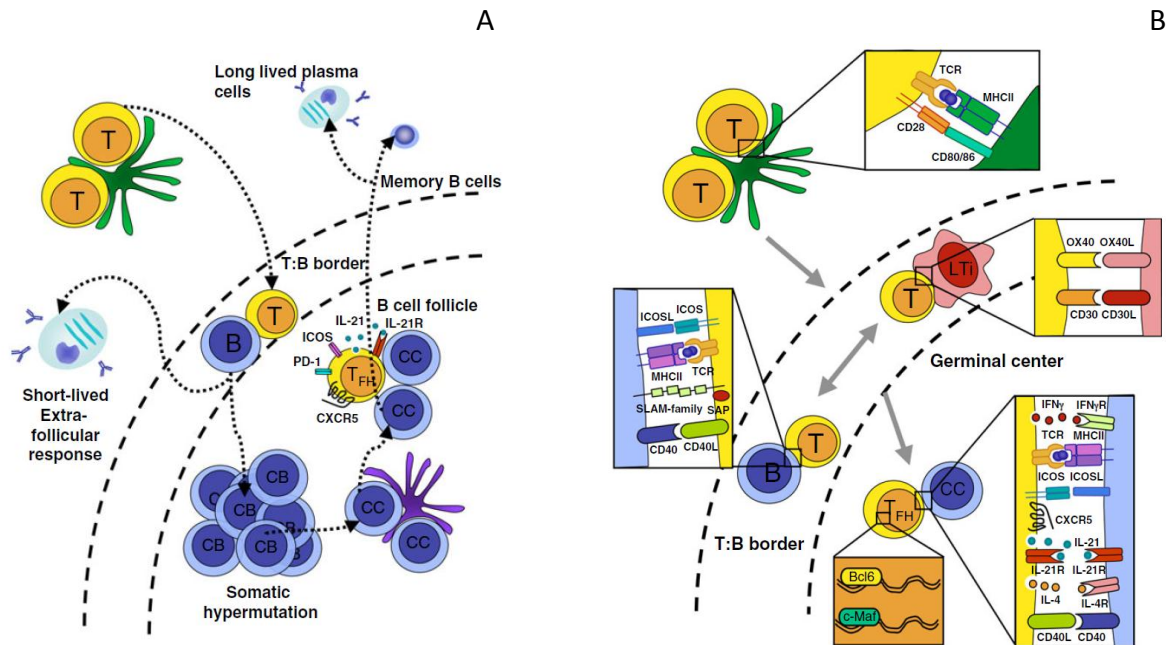


Figure 8: Cell types and intracellular communication in lymphoid follicles

A/ On receipt of foreign antigen dendritic cells (green) prime T cells (yellow) which then migrate to the T:B border (mantle zone) and interact with B cells (blue). B cells can then form short lived extra-follicular plasmablasts or migrate to the germinal centres to form centroblasts (CB), which proliferate and mature into centrocytes (CC). Mature centrocyte (germinal centre) B cells interact with follicular dendritic cells (purple) and germinal centre T follicular helper cells (T_{FH}). Depending on the signals received they can then mature into memory B cells or long lived plasma cells. All of these cell-cell communications require multiple signals across the immune synapse (figure B). LT_i = lymphoid tissue inducer cells, identified as CD4⁺ CD3⁻ cells (red) which promote CXCR5 expression on T cells, particularly through OX40/OX40L and CD30/CD30L interactions. SLAM and/or ICOS interactions between T and B cells are critical for T_{FH} development and functioning. Bcl6 and cMAF expression is characteristic of T_{FH} cells, and may be determined by the strength of these signals. Images reproduced from and text adapted from (Linterman & Vinuesa 2010).

Other lymphoid cells are present in smaller numbers, for example NK cells represent approximately 0.5% of the tonsil lymphoid cell population (Ferlazzo, Thomas, Lin et al. 2004), and their role in disease is still being established. Lymphoid tissue inducer (LT_i) cells are a specialised set of cells

which are thought to help boost the development of T_{FH} cells in marginal zones (Linterman & Vinuesa 2010). It is important to remember that in all lymph nodes, including tonsils, the cellular composition in each of the areas is dynamic, depending on local and systemic stresses, antigen presentation and release and recognition of different cell signals (Bronzetti, Artico, Pompili et al. 2006; Lammermann & Sixt 2008; Strowig, Brilot, Arrey et al. 2008). In particular the number of B cell follicles can increase in response to recurrent infections (Alatas & Baba 2008), which manifests as tonsillar hypertrophy, and together with recurrent infections forms the commonest indication for tonsillectomy.

1.5.2 Production of immunoglobulin by B cells

In humans, B cells are found throughout the bone marrow, peripheral blood and lymphoid organs, including tonsils. A spectrum of B cells at different stages of maturity and differentiation can be identified in most of these sites, from naive B cells to mature antibody-secreting plasma cells, and these subsets can be differentiated by size and the expression of surface markers. In lymph nodes, such as tonsils, immature B cells are not found, but three main sets of cells have been described: Marginal (mantle) zone B cells, which express high levels of surface IgM and CD21; Follicular B cells (B2 cells, centroblasts/centrocytes) which express high levels of IgD, moderate expression of CD21 and CD23 and IgM; and Plasma cells (immunoglobulin secreting cells), which lose CD19 and CD20 expression as they mature, and express CD38 and CD138 and intracellular Blimp-1. CD27 expression is a marker of memory B cells. B cell stimulation can be either T cell dependent (follicular B cells) or independent (marginal zone B cells), and T independent B cells can also help to stimulate T cells by presentation of antigen via MHC Class II (Fairfax, Kallies, Nutt et al. 2008; Pillai, Cariappa, & Moran 2004).

Once B cells receive signals to mature into plasma cells, for example via the B cell receptor and CD40 ligation, the class switch recombination system is irreversibly activated, which causes genetic editing of the immunoglobulin gene to occur via the actions of AICDA (activation induced cytidine deaminase), and the class of immunoglobulin to be produced is determined (Figure 9), (Stavnezer,

Guikema, & Schrader 2008). VDJ recombination occurs before B cells reach the tonsils. The constant (C) regions of the immunoglobulin heavy chain (H) gene are in a set order, expressing IgM and IgD before the class switch recombination is activated. AICDA causes double strand breaks in the DNA to occur at the appropriate switch (S) regions, which are then recombined by end-end joining. This process is under the control of numerous regulatory genes, particularly Blimp-1, Pax5 and Bcl6 (which are both counter-regulatory to Blimp-1), and Xbp1 (which is regulated by Blimp-1, and starts the process of class switch recombination). Expression of these determines the fate of the B cell depending on which stimulatory signals are received (e.g. class switch recombination, cell death or memory cell formation).

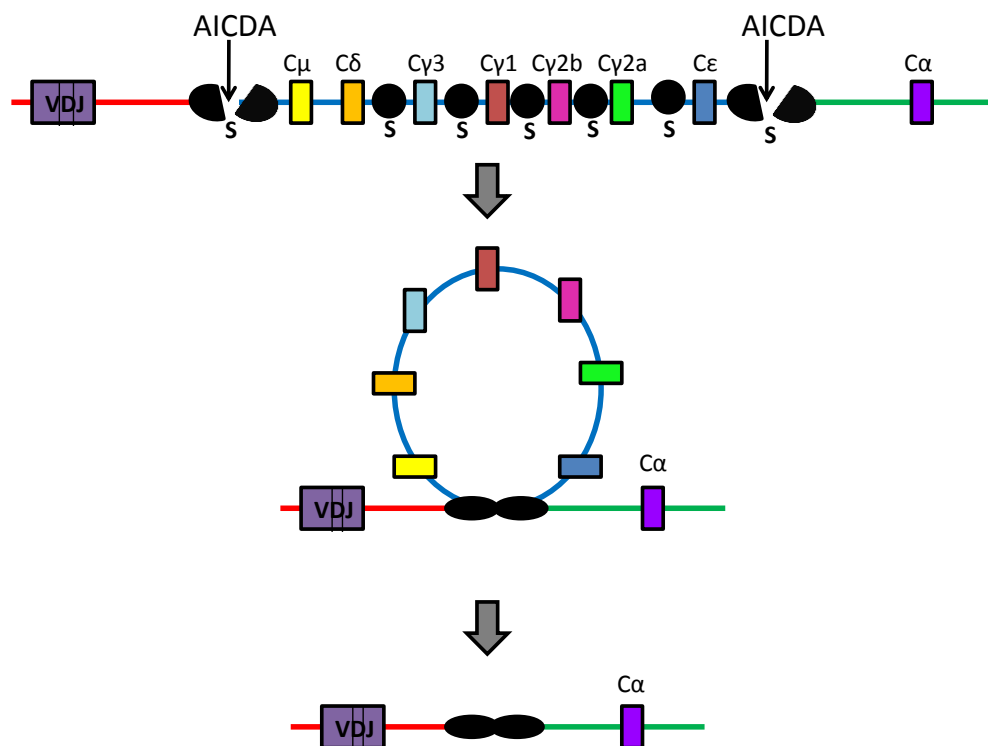


Figure 9: Mechanism of class switch recombination

Diagram of class switch recombination to IgA, adapted from (Staudt and Lenardo 1991; Stavnezer, Guikema, & Schrader 2008). The IgH locus in B cells expresses IgM and IgD, having previously undergone VDJ recombination. During class switch recombination, activation induced cytidine deaminase (AICDA) deaminates dC residues in the active switch (S) regions. This results in intra-chromosomal deletion of the unwanted sections of DNA – in this example the constant regions for IgM (Cμ), IgD (Cδ), IgG (subsets Cγ3, 1, 2b, 2a) and IgE (Cε) are removed, so the IgH locus is now in the correct formation to produce IgA (Cα).

1.5.3 T cell receptor structure and specificity

On the majority of T cells, T cell receptors are comprised of two chains, termed α and β chains. These have extracellular variable (V) and constant (C) regions, a hinge (H) region where the chains are linked by a disulfide bond, a transmembrane region and a cytoplasmic tail. A minority of T cells have structurally different receptors, with the chains termed γ and δ . Co-receptor molecules such as CD3 and CD4 or 8 are closely associated with the T cell receptor, and also play a role in the interaction of the T cell receptor with the peptide-presenting MHC molecules (Janeway 2008).

There is a wide variation in the structure of the variable chains, in the six complementarity-determining regions (CDR), allowing specificity to a vast array of antigens, in the order of 1×10^{13} potential different combinations after auto-reactive clones have been deleted in the thymus (Nikolich-Zugich, Slifka, & Messaoudi 2004). It is these CDRs which interact with the peptide-MHC complexes on antigen presenting cells. In similarity with immunoglobulin production, V(D)J recombination occurs to create the specificity of each T cell, though this occurs in the thymus for T cells as opposed to bone marrow. With the duplication of some cells, it is estimated that there are approximately 2.5×10^7 different T cell receptor variations present in a single human at any one time, allowing sufficient variety to recognise the immunodominant epitopes of a wide range of pathogens and also allowing for recognition of pathogen mutation (Nikolich-Zugich, Slifka, & Messaoudi 2004).

1.6 Human tonsils in disease

S. pyogenes is well recognised as a major cause of acute bacterial pharyngeal and tonsil infections, with infections often being recurrent despite prolonged antibiotic therapy. Many papers have looked at “tonsillar core biopsy” cultures from patients with recurrent tonsillitis, and established that the predominant bacteria living deep within the tonsil crypts are *Haemophilus influenzae* and *Staphylococcus aureus*, pushing *S. pyogenes* into third place (Lindroos 2000). Other potential pathogens which are frequently isolated include *Moraxella catarrhalis*, *Streptococcus pneumoniae*, anaerobes (including *Fusobacterium* species) and *Actinomyces* (Lindroos 2000). The contribution that these bacteria make to both acute disease and chronic tonsillar hypertrophy has yet to be established, though there is suggestion that when *S. aureus* are living intracellularly as opposed to just in the crypts, that they have an invasive phenotype (Zautner, Krause, Stropahl et al. 2010). Furthermore, *H. influenzae* can be isolated from blood samples taken during tonsillectomy, suggesting that they represent a potential source for invasive disease (Francois, Bingen, Lambert-Zechovsky et al. 1992). Tonsils removed from patients with IgA nephropathy appear to produce increased levels of IgA and chemokines in response to stimulation with *Haemophilus parainfluenzae*, suggesting a link between chronic tonsillar infection and renal disease with this bacterium (Kuki, Gotoh, Hayashi et al. 2004; Nozawa, Takahara, Yoshizaki et al. 2008). This has led to the thinking that although *S. pyogenes* is one of the more aggressive causes of acute disease and is easily cultured from surface swabs, the situation should be reassessed during recurrent and chronic tonsil infections, with the consideration that other bacteria may be playing a role in pathogenesis (Lindroos 2000).

1.7 Human tonsils in immunology studies

Human tonsils have been used for a number of years as a ready supply of cells for immunology research. As a primary lymphoid organ they have a structure which is broadly similar to that found in other lymphoid organs, consisting of follicles with germinal centres and mantle zones, which makes them amenable to the study of whole lymph node interactions during organ culture. They are also used as the primary source of B cells for numerous cell culture experiments, as they are present in far higher numbers than in peripheral blood, and once disrupted into a single cell suspension it is easy to select out the cell types of interest.

Disrupting the human tonsil structure to obtain a single cell suspension disturbs the structural architecture of the tonsils, which is essential for proper function of tonsils. Although responses can be gained when the architecture is disrupted, it is slower and the cell interactions are not necessarily the same. The alternative is to use tonsils for organ culture (histocultures), which allows preservation of the three-dimensional structure. The histoculture system consists of building an artificial nutrient matrix, which allows provision of nutrients to the blocks of tissues, whilst maintaining an air interface and the complex tissue architecture. For tonsils this system has been developed (Grivel and Margolis 2009) and used for the investigation of infection with viruses, particularly HIV (Audige, Schlaepfer, Bonanomi et al. 2004; Margolis, Glushakova, Grivel et al. 1998). In similarity with other organ culture models (Michelini, Rosellini, Simoncini et al. 2004) preservation of the 3 dimensional structure means that cytokine and immunoglobulin responses can be rapid (Margolis et al. 1998; van Laar et al. 2007). However, the model does have some significant drawbacks. A lack of the systemic blood supply does not take into account the contribution of and recruitment from the systemic circulation of other immune cells, particularly neutrophils which are found at low levels in healthy human tonsils. Also, there is a considerable degree of necrosis which sets in after 48-72 hrs of culture (Giger et al. 2004), dependent on several conditions including the type of tissue support and media used. This has knock on effects on the cell function and viability for prolonged analysis. Histoculture necrosis does not appear to affect the cellular functional properties though, as immunoglobulin secretion has been

shown to be stable over 14 days (van Laar et al. 2007). Cytokine production in histocultures varies widely between individuals, but only IL6 and IL8 production varies over time in unstimulated cultures, with an increase over the first 24 hours of culture before stabilising at a higher level (Bonanomi, Kojic, Giger et al. 2003).

There is an additional problem to be overcome when investigating streptococcal infection in tonsils: achieving live bacterial infection with *S. pyogenes* whilst suppressing indigenous bacterial populations. This is particularly the case as the commonest reason for tonsillectomy is recurrent tonsillitis, and many of these patients have tonsoliths in the crypts, hard foci of chronic inflammation comprising live bacteria and dead epithelial and immune cells. The location of tonsils in the upper respiratory tract means that cultures often become contaminated with endogenous bacteria, even when high strength antimicrobial agents are used in the culture media (Johnston, Sigurdardottir, & Ryon 2009). This has not been a problem for other authors recreating viral infections, as the presence of antibiotics in culture media does not significantly inhibit viral replication or cell function. However in this project, the presence of even trace amounts of antibiotics could have a significant effect on the growth of bacteria and success of achieving bacterial colonisation. To date investigations using live bacteria with human tonsils have been limited to less than 24 hours (Abbot et al. 2007).

1.8 Summary

Considering that both innate and adaptive components of the human immune system have developed to combat a wide range of pathogens, it is perhaps the ability of *S. pyogenes* to produce so many virulence factors which makes it one of the most versatile and aggressive human pathogens. It is unclear what the advantages of superantigens are to the bacterium, but there is evidence of up-regulation of superantigen gene expression in a monkey model of streptococcal pharyngitis (Virtaneva et al. 2003). Additionally there is superantigen up-regulation in inter-epidemic periods (Beres et al. 2006; Virtaneva et al. 2005). This has led to the theory that superantigens are perhaps useful in helping to establish pharyngeal infection by reducing normal streptococcal antigen processing, yet increasing inflammation to facilitate disease transmission.

Taking into account the unique structure and complex functions of human tonsils, the primary immune defence organ at the primary site of *S. pyogenes* infection, this project set out to investigate the effects of streptococcal superantigens on human tonsils.

2 Materials and Methods

2.1 Materials

Antibiotic Free Media (all Invitrogen, Paisley, UK)

- RPMI 1640
- 10% Foetal calf serum
- 100mM L-glutamine

Standard Culture Media (all Invitrogen)

- RPMI 1640
- 10% Foetal calf serum
- 100mM L-Glutamine
- 100iu/ml Penicillin
- 100µg/ml Streptomycin

Tonsil Media (based on Dissection HBSS, (Freshney 2005), all Invitrogen)

- RPMI 1640
- 10% Foetal calf serum
- 100mM L-Glutamine
- 250iu/ml Penicillin
- 250µg/ml Streptomycin
- 100µg/ml Kanamycin
- 2.5µg/ml Amphotericin B

ELISA Coating Buffer

20x (0.1M) concentrate, diluted in ddH₂O for use

- 5.3g Na₂CO₃
- 4.2g NaHCO₃

Made up to 1litre in ddH₂O, pH 9

Phosphate buffered saline (PBS)

- NaCl 137 mmol/L
- KCl 2.7 mmol/L
- Na₂HPO₄·2H₂O 10 mmol/L
- KH₂PO₄ 1.76 mmol/L

Adjusted to pH 7.4. For routine laboratory use made at 10x concentrate and diluted in ddH₂O for use.

For tissue culture purchased at 1x concentration from Invitrogen (Paisley UK).

Todd Hewitt Broth

Made from concentrate (36.4g/L ddH₂O autoclaved prior to use) powder by Oxoid (Basingstoke, UK), pH 7.8

- Infusion from 450g fat free minced meat 10g/L
- Tryptone 20g/L
- Glucose 2g/L
- Sodium bicarbonate 2g/L
- Sodium chloride 2g/L
- Disodium phosphate 0.2g/L

Lysogeny Broth (LB)

- Tryptone 10g
- Yeast extract 5g
- NaCl 10g

Made up to 1L in ddH₂O and autoclaved prior to use. To create LB Kanamycin agar plates 15g/L of agar and 50µg/ml Kanamycin was added.

SET Buffer

- NaCl 75mMol
- EDTA 25mMol
- Tris at pH 7.5 20mMol

Made up to 50 mls volume in ddH₂O

All chemicals were purchased from Sigma (Dorset, UK), Oxoid, VWR (Lutterworth, UK) or Invitrogen, unless stated otherwise.

2.2 Methods

2.2.1 Clinical studies, recruitment and sample collection

2.2.1.1 *Virulence factors in streptococcal tonsillitis*

Study participants were recruited from patients attending the paediatric ambulatory unit at the Hammersmith Hospital, Imperial College Healthcare NHS Trust, between January 2010 and March 2011. The inclusion criteria were children presenting with symptoms of tonsillitis or pharyngitis which clinically warranted antibiotic therapy, and the presence of at least one asymptomatic accompanying relative willing to participate in the study as a healthy volunteer. Exclusion criteria were any patients who had completed a course of antibiotics within the last 1 month, as it was felt that this could alter bacterial gene expression. The study protocol, consent forms and supporting documents are listed in Appendix 1.

For each study participant two throat swabs were collected – a plain swab into Stewart's media for routine processing in the diagnostic laboratory and a dual-headed swab for research. One head from the research swab was cut off into a 1.5ml tube, heated for 4 minutes at 95°C with 200 µl Max Bacterial Enhancement Reagent and then mixed into 1ml of Trizol Reagent (both Invitrogen Ltd, Paisley, UK). This was then stored at -80°C until RNA extraction and purification was completed, by the same protocol as detailed in section 2.2.3.5. For healthy volunteers, no routine swab was sent to the diagnostic laboratory; research samples were processed in the same manner.

DNA was also extracted from throat swabs after the aqueous phase had been removed for RNA extraction. DNA was precipitated from the interphase and organic phase by adding 300µl 100% ethanol and mixing. Samples were rested for 2 minutes at room temperature and the DNA precipitated by centrifugation at 2000g for 5 minutes at 4°C. The supernatant was discarded and the DNA pellet washed in 1ml of 0.1M sodium citrate in 10% ethanol solution. The sample was incubated at room temperature for 30 minutes, with periodic mixing before centrifuging at 2000g for 5 minutes, 4°C. After this step was repeated the DNA was washed in 1.5ml 75% ethanol for 10 minutes at room

temperature (occasional mixing) and centrifuged for 5 minutes at 2000g, 4°C. The pellet was air dried and then resuspended in 50µl of 8mM NaOH solution buffered with 1M HEPES as per manufacturers' recommendations. DNA samples were measured by spectrophotometry and stored at -20°C.

The other research swab head was first streaked onto a Columbia blood agar (CBA) plate (Oxoid) and then used in the Clearview Exact Strep A dipstick test (Inverness Medical, Alere, Stockport, UK). The CBA plate was incubated at 37°C in a 5% CO₂ atmosphere and examined at 24 and 48 hours incubation for the presence of beta haemolytic streptococci. The identity of any possible *S. pyogenes* colonies was confirmed by Gram staining (Gram positive cocci), negative catalase reaction (emulsification of a single colony on a glass slide in the presence of 0.03% hydrogen peroxide and observation of the presence of gas bubble formation under a glass cover slip) and latex agglutination with the Lancefield Group A carbohydrate (PathoDX Strep grouping kit, Oxoid, Basingstoke, UK). Culture was performed even if the rapid test was negative, as poor sampling can reduce the sensitivity of the rapid diagnostic test (Clerc & Greub 2010).

The Clearview Exact Strep A test was performed in the clinic and the result relayed to the clinician and the patient/volunteer. For all volunteers, an information sheet was provided with the result of their rapid test as rates of subsequent disease are higher in relatives of patients with confirmed *S. pyogenes* pharyngitis (Danchin et al. 2007) this was to allow them faster access to treatment should they become unwell after the clinic visit. Quality assurance was confirmed by noting a 100% concordance with the results from the diagnostic laboratory. The Clearview Exact Strep A Dipstick (Inverness medical) test was chosen as it can be used on swabs which have previously been streaked onto blood agar. According to manufacturer's instructions, 3 drops of reagent 1 and 3 drops of reagent 2 were placed in the supplied extraction tube, and checked for formation of a yellow colour. The swab was placed in the tube and rotated/agitated for 1 minute. The swab was removed, the dipstick inserted into the extraction fluid and incubated for up to 5 minutes at room temperature. The presence of a control band was confirmed and the dipstick checked for formation of a positive or negative test band.

2.2.1.2 Virulence factor expression in invasive *S. pyogenes* disease

Patients with necrotising fasciitis due to *S. pyogenes* were identified and notified to the Gram Positive Pathogenesis Group from clinical microbiology at Imperial College Healthcare NHS or the plastic surgery team at Chelsea and Westminster NHS Foundation Trust.

All available tissues were collected from samples sent to the diagnostic laboratory from surgery. The tissues were stored at 4°C until culture had been completed, and then transferred to the research laboratory. There was a delay of between 2 and 5 days between the time of operation and the arrival of isolates at the research laboratory for all samples. On arrival tissues were cultured on CBA to establish the presence or absence of antibiotic effect (growth of bacteria only where they have been streaked across the plate away from the initial inoculum indicates the presence of high concentrations on antibiotics in the tissues). Any fluid exudates surrounding the tissues or liquid samples were also cultured before being stored at -80°C until further use. Tissues were then dissected into blocks weighing approximately 300mg with a scalpel, and were processed in the following ways:

- 1) Heated at 95°C for 4 minutes in 200 µl Max Bacterial Enhancement Reagent and then homogenised into 1 ml of Trizol Reagent (Invitrogen).
- 2) Blocks homogenised into 1ml PBS and stored at -80°C until use.

2.2.1.3 Human tonsil collection

Human tonsils were collected from 62 different adults undergoing routine tonsillectomy (all surgery performed by Mr Shula, ENT surgeon Charing Cross Hospital) by the Imperial College Hospitals NHS Trust Tissue bank, according to Ethics reference 07/Q0407/38. The details of the tonsil donors can be found in Appendix 2. Tonsils were collected immediately after surgery, and were bisected by the tissue bank staff. The posterior portion of each tonsil was snap frozen at -80°C for future research by users of the tissue bank, and H&E staining and reporting of sections was prepared from this portion for the first 22 donors. Samples for this research study were stored at 4°C either plain in a sterile container or soaking in Tonsil media until collection within 6 hours of surgery. The experimental protocols for tonsils are detailed in section 2.2.4.

2.2.2 Bacteriology

2.2.2.1 Bacterial strains

The following bacterial strains (Table 4) were previously collected and characterised by the Gram Positive Pathogenesis group and isogenic mutant strains with alterations in superantigen (*speA* and *smeZ*) expression created (Russell and Sriskandan 2008). Wild Type strain H305 is the M1 Scarlet Fever reference strain NCTC 8198, and was originally supplied by the Health Protection Agency (Colindale, London). Wild Type parent M89 strain H293 was originally from a patient with necrotising fasciitis (1995).

Serotype	Strain	Designation in text	Toxin Genotype		
Isogenic M1 Strains	H305	M1 WT	<i>speA</i>⁺	<i>smeZ</i>⁺	<i>speC</i> ⁻ <i>speG</i> ⁺ <i>speJ</i> ⁺ <i>ssa</i> ⁻
	H326	M1 WTΔ <i>speA</i>	<i>speA</i> ⁻	<i>smeZ</i> ⁺	
	H361	M1 WTΔ <i>speA</i> comp	<i>speA</i> ⁺⁺	<i>smeZ</i> ⁺	
Isogenic M89 strains	H293	M89 WT	<i>speA</i>⁻	<i>smeZ</i>⁺	<i>speC</i> ⁻ <i>speI</i> ⁻ <i>speG</i> ⁺ <i>speH</i> ⁺ <i>speJ</i> ⁺ <i>ssa</i> ⁻
	H362	M89 WT <i>speA</i> ⁺	<i>speA</i> ⁺⁺	<i>smeZ</i> ⁺	
	H377	M89 WTΔ <i>smeZ</i>	<i>speA</i> ⁻	<i>smeZ</i> ⁻	
	H432	M89 WTΔ <i>smeZ</i> comp	<i>speA</i> ⁻	<i>smeZ</i> ⁺⁺	
	H435	M89 WTΔ <i>smeZ</i> <i>speA</i> ⁺	<i>speA</i> ⁺⁺	<i>smeZ</i> ⁻	

Table 4: Bacterial strains

Isogenic mutant bacterial strains as previously created by the Gram Positive Pathogenesis group. Wild type strains in bold. For toxin genotype, +/- represents gene present or absent. ++ represents gene complementation.

All new bacterial strains from the clinical studies, confirmed as *S. pyogenes*, were grown overnight in 10 mls Todd-Hewitt Broth (Oxoid, Basingstoke, UK), allocated a unique identifier and stored in 20% glycerol stocks indefinitely at -80°C in the Gram Positive Pathogenesis isolate collection.

2.2.2.2 Bacterial strains from clinical studies

All cultured bacteria which were collected as a result of the two clinical studies and confirmed as *S. pyogenes*, were allocated a unique identifier and stored in 20% glycerol stocks indefinitely at -80°C in the Gram Positive Pathogenesis isolate collection. Bacterial DNA was extracted from overnight culture pellets and *emm* and superantigen typing performed by PCR (see section 2.2.3). 16S ribosomal RNA PCR was also performed on DNA extracted directly from all throat swabs to confirm that there were no further cases of *S. pyogenes* infection that had been missed on Clearview testing or conventional plate culture, and confirmed by PCR for the housekeeping gene *proS*, which is highly specific for *S. pyogenes* (Virtaneva et al. 2003), the primers for which are listed in Table 6.

2.2.2.3 Superantigen preparations

Recombinant SPEA was obtained from Toxin Technologies (Florida, USA). Recombinant SMEZ and SPEJ were provided by Thomas Proft (Auckland, NZ), produced as previously described (Proft, Moffatt, Berkahn et al. 1999). As controls, recombinant staphylococcal enterotoxins (SE) B and C and toxic-shock syndrome toxin 1 (TSST-1) were also purchased from Toxin Technologies.

To create superantigen-containing bacterial supernatants, 1-4 well isolated bacterial colonies of the appropriate strain were taken from a fresh purity plate, emulsified in 20 mls Antibiotic Free Media and cultured overnight at 37°C in 5% CO₂. Bacterial counts were quantified as colony forming units/ml (CFU/ml) and supernatants were prepared as follows: bacteria were spun at 800g for 10 minutes, and supernatant removed with a 20ml syringe. The supernatant was then filter sterilised through a 0.2µm syringe filter into a clean collecting tube. Supernatants were aliquoted and stored at -20°C until needed.

2.2.2.4 Bacterial culture preparation

Bacteria for live tonsil co-culture experiments were cultured overnight in Antibiotic Free Media, as described above. Cultures were then diluted 1:10 and incubated for a further 4 hours, to reach Log phase of growth prior to infecting tonsil cultures. Cultures were quantified before use (CFU/ml). A typical inoculum of 5µl of bacterial suspension was applied to each block, containing approximately 1×10^6 CFU bacteria.

Bacteria for mouse experiments were cultured in a similar manner overnight in volumes of 50mls in Antibiotic Free media. Bacterial counts were quantified (CFU/ml) and the culture supernatant removed and filter sterilised as above. Bacterial pellets were washed twice in sterile PBS and re-suspended in 1ml PBS, before heat killing in 1.5ml tubes incubated at 80°C for 45 minutes in a water bath, with frequent inversion. Bacteria were then centrifuged and the pellet re-suspended in 1 ml of filter sterilised culture supernatant. Complete bacterial killing was confirmed for each culture. RPMI 1640 with 10% FCS was used as a control. Injections typically contained $1-5 \times 10^8$ CFU heat killed bacteria per mouse.

For live bacterial infections, mice were injected into the right thigh with 50µl of live bacterial suspension from overnight culture prepared as above, which was washed twice in sterile PBS and re-suspended to an optical density A_{600} of 10, which yielded 7.45×10^7 CFU per mouse.

2.2.3 Molecular methods

2.2.3.1 Bacterial DNA extraction

Bacterial DNA was extracted from culture pellets of 10 ml overnight growth in Todd Hewitt broth, by centrifuging cells at 800g for 10 minutes. Supernatant was discarded and the pellet re-suspended in 1ml PBS. 20µl mutanolysin and 20µl lysozyme (Sigma) were added and incubated for 10 minutes at 37°C and then centrifuged at 200g for 10 minutes. Supernatant was removed and the pellet resuspended in 500µl SET buffer with 50µl 10% SDS solution and 5µl proteinase K (at 50mg/ml) for

2 hours at 55°C (with occasional mixing). 175µl 5M NaCl and 500µl chloroform was added and incubated at room temperature for 30 minutes. Solutions were centrifuged at 15,000g for 15 minutes and the aqueous layer removed into 500µl isopropanol and mixed. This was centrifuged at 15,000g for 5 minutes and the supernatant discarded. The DNA pellet was washed in 1ml 70% ethanol and then centrifuged again at 15,000g for 5 minutes. The ethanol was removed and the pellet air dried before re-suspending in DEPC treated H₂O.

2.2.3.2 *Emm* typing

Emm typing was performed according to the CDC recommended protocol (Anon 2011b). A 100µl reaction was created using GoTaq (Promega, Madison, USA): 20µl of 5x colourless GoTaq reaction buffer, 10µl 1mM dNTP, 1µ Forward primer (TATTCGCTTAGAAAATTAA), 1µl reverse primer (GCAAGTTCTTCAGCTTGTTT), 0.5µl GoTaq Polymerase (5u/µl), 2µl DNA template and 66.5µl H₂O. The PCR reaction was run in a thermal cycler (Biorad, Hemel Hempstead, UK) according to the following protocol: 94°C 1 min, 10 cycles of 94°C (15s), 46.5°C (30s) 72°C (1 min 15s), 20 cycles of 94°C (15s), 46.5°C (30s), 72°C (1 min 15s with a 10 sec increment for each of the subsequent 19 cycles), 72°C for 10 min, then 4°C storage. PCR products were checked by electrophoresis on a 2% agarose gel. PCR product was purified using Qiagen PCR purification kit (according to manufacturers' instructions, Qiagen, Crawley, UK) and sequenced by the MRC sequencing centre (Imperial College, London), using the primer TATTCGCTTAGAAAATTAAAAACAGG. Sequences were submitted to the CDC for analysis and *emm* type confirmation.

2.2.3.3 *Superantigen* typing

Superantigen typing was performed by PCR using the method described by Lintges (Lintges, Arlt, Uciechowski et al. 2007), carried out separately for each gene. The primer pairs used are listed in Table 5. PCR reactions were performed using Megamix Blue (Microzone, Hayward's Heath, UK) (20.5µl Megamix Blue, 1.25µl each primer and 2µ DNA template) and the PCR cycle: 95°C for 15 mins, 35 cycles of 94°C (30s), 57°C (90s), 72°C (90s), and final extension of 72°C for 10 mins. Samples were analysed for band size by electrophoresis on a 2% agarose gel. PCR for *speA* and *smeZ*

was confirmed using the primers and PCR protocol used to create the qRT-PCR plasmid (section 2.2.3.7).

16S ribosomal RNA PCR was also performed on DNA extracted directly from all throat swabs to confirm that there were no further cases of *S. pyogenes* infection that had been missed on Clearview testing or conventional plate culture.

Gene	Primer sequence
16S 1	F: GTGAGTAACGCGTAGGTAACCTACCTCATAG R: CCCAGGCGGAGTGCTTAATG
16S 2	F: GGTGAGTAACGCGTAGGTAACCTACC R: GCCCAACTTAATGATGGCAACTAACA
<i>speA</i> 1-4	F: CAAGAAGTATTTGCTCAACAAGACCCCA R: TTAGATGGTCCATTAGTATATAGTTGCTTGTTATC
<i>speA</i> 1-3,5	F: GGTATTTGCTCAACAAGACCCCGAT R: TGTGTTTGAGTCAAGCGTTTCATTATCT
<i>speC</i>	F: GGTAAATTTTCAACGACACACACATTAAA R: TGTTGAGATTCTCCCGAAATAAATAGAT
<i>speG</i>	F: GCTATGGAAGTCAATTAGCTTATGCAGAT R: TTATGCGAACAGCCTCAGAGG
<i>speH</i>	F: TCTATCTGCACAAGAGGTTTGTGAATGTCCA R: GCATGCTATTAAAGTCTCCATTGCCAAAA
<i>speI</i>	F: AAGGAAAAATAAATGAAGGTCCGCCAT R: TCGCTTAAAGTAATACCTCCATATGAATTCTTT
<i>speJ</i>	F: CAATTAAATTACGCATACGAAATCATACCAGTA R: ACGAGTAAATATGTACGGAAGACCAAAAATA
<i>speK</i>	F: TATCGCTTGCTCTATACACTACTGAGAGT R: CCAAAGTGTAGTATTTTCATCCGTATTAAA
<i>speL</i>	F: GGACGCAAGTTATTATGGATGCTCA R: TTAAATAAGTCAGCACCTTCCTCTTTCTC
<i>speM</i>	F: GCTTTAAGGAGGAGGAGGTTGATATTTATGCTCTA R: CAAAGTGACTTACTTTACTCATATCAATCGTTTC
<i>Ssa</i>	F: AATTATTATCGATTAGTGTTTTTGCAAGTA R: AGCCTGTCTCGTACGGAGAATTATTGAACTC
<i>smeZ</i>	F: CAATAATTTCTCGTCCTGTGTTTGGAT R: GATAAGGCGTCATTCCACCATAG

Table 5: Superantigen typing primer sequences

Primer sequences for superantigen typing were taken from Lintges et al (Lintges et al. 2007). F= forward primer sequence, R=reverse primer sequence.

2.2.3.4 Bacterial RNA extraction

Bacteria were cultured A/ overnight in 20mls Todd-Hewitt broth, B/ to late log (optical density at A600 of 0.7-0.9) growth in 20 mls Todd Hewitt broth and C/ on a CBA plate. For broth growth, bacteria were centrifuged at 800g for 10 mins and the supernatant discarded. The pellets were resuspended, or bacterial colonies from a 1/3 plate of confluent growth (C), were emulsified in 0.5mls sodium acetate buffer (0.1% SDS, 30mM sodium acetate at pH5.5). This was added to 0.5mls Hot Acid Phenol (Qbiogene, MP lifesciences, UK) and heated at 70°C for 10 minutes with frequent inverting. After centrifugation at 12,000g for 10 minutes the aqueous layer was removed into an equal volume of Phenol: Chloroform: Isoamyl alcohol (Sigma). This was centrifuged for 5 minutes at 12,000g and the aqueous layer removed and precipitated in 500µl isopropanol and 500µl 3M sodium acetate pH 7.0 for several hours at -20°C. Samples were centrifuged at 12,000g for 30 minutes at 4°C and the supernatant discarded. RNA pellets were washed in 500µl ice cold 80% ethanol and centrifuged at 12,000g for 5 minutes at 4°C. Pellets were air dried and the RNA dissolved in 50µl DEPC treated H₂O. RNA concentrations were measured on a spectrophotometer (Picodrop) and samples stored at -80°C.

2.2.3.5 RNA extraction from tissues and swabs

To ensure extraction of both human and bacterial RNA from tissue samples or swabs, samples were placed into a 1.5ml tube, and heated for 4 minutes at 95°C with 200 µl Max Bacterial Enhancement Reagent (Invitrogen) and then mechanically disrupted using a disposable sterile pestle (VWR). 1ml of Trizol Reagent (Invitrogen) was added. RNA extraction was then either completed immediately or samples stored at -80°C until needed.

Samples were rested at room temperature for 5 minutes to allow complete cell disruption. 200µl of chloroform was added and the samples shaken vigorously for 15 seconds. Tubes were incubated at room temperature for 2 minutes and then centrifuged at 12,000g for 15 minutes, 4°C. The aqueous phase was removed to a separate tube and precipitated in 500µl isopropanol. Samples were incubated for 10 minutes at room temperature, or for up to 2 hours at -20°C. Samples were then centrifuged at 12,000g for 10 minutes at 4°C and the supernatant discarded. The RNA pellet was washed in 1ml of

75% ethanol, and centrifuged at 7,500g for 5 minutes. The ethanol was removed and the RNA pellet dissolved in 100µl DEPC treated water by heating at 60°C for 10 minutes.

RNA from all tissue samples was treated to remove any trace proteins or contaminants using the Qiagen RNeasy mini kit RNA clean up protocol. To each 100µl RNA sample 350µl of RLT buffer was added and mixed, followed by 250µl of 100% ethanol. Each sample (700µl) was applied carefully to a clean RNeasy mini spin column seated in a 2ml collection tube. Samples were centrifuged at 10,000g for 15 seconds and flow-through discarded. 500µl RPE buffer was added to each sample and centrifuged for 15 seconds at 10,000g. Flow-through was discarded, and a second application of RPE 500µl performed. Samples were centrifuged for 2 minutes (10,000g) and then transferred to a fresh collecting tube and centrifuged for a further minute (10,000g) to remove all residual RPE buffer. Spin columns were transferred to fresh 1.5ml tubes and 30µl RNase free water applied. RNA was eluted by centrifugation at 10,000g for 1 minute, and the final step repeated with a further 30µl water to maximise RNA yield. RNA concentration was measured by spectrophotometry (Picodrop, Saffron Walden, UK), and samples stored at -80°C until needed.

2.2.3.6 *First strand cDNA synthesis*

RNA samples were DNase treated by incubating at 37°C with Turbo DNase (Applied Biosystems, California, USA), up to 200µg RNA per reaction. After 30 minutes stop solution was added and samples centrifuged for 90 seconds at 10,000g. Supernatant was transferred to a fresh tube and RNA concentration confirmed. Concentrations were adjusted (as listed in individual chapters) and RNA samples heated for 10 minutes at 65°C with random hexamers. Reverse transcription was performed using Transcriptor reverse transcriptase (Roche, Burgess Hill, UK) with 10mM dNTP's and RNase inhibitor (RNaseIn, Sigma). Samples were incubated for 10 minutes at 25°C, 30 minutes at 55°C and 5 minutes at 85°C. Samples were frozen at -20°C until needed. Reverse transcriptase free (RT negative) controls were run for each sample and RNA free (water) controls for each batch.

2.2.3.7 Plasmid construction

A plasmid was constructed to contain one copy each of the following genes, to allow for accurate analysis in real-time RT-PCR: superantigens *smeZ* and *speA*, and the streptococcal housekeeping gene *proS* following previously published methods (Turner, Kurupati, Jones et al. 2009). Primer sequences were created for *smeZ* and *speA* by comparing multiple strain types using Clustal W gene alignment of published gene sequences. Primers were created to amplify a 94bp segment (*speA*) and 209bp segment (*smeZ*), with a melting point of approximately 64°C. Sequence for amplification of a 93bp segment of prolyl-tRNA synthetase (*proS*) was taken from previously published methods (Salim, Cvitkovitch, Chang et al. 2005; Virtaneva et al. 2003). A list of primers used in all plasmid RT-PCR experiments is listed in Table 6. Purified PCR products of all 3 genes were created using the following PCR protocol: 95°C (5 min), 30 cycles of 95°C (30s), 60°C (30s), 72°C (45s), then final extension 72°C for 5 min, using PFU polymerase (Promega). PCR products were analysed by electrophoresis on a 2% agarose gel, and purified (Qiagen). Products were blunt-end ligated together using T4 ligase (Promega) and incubated overnight at 14°C. Ligated segments were then amplified by PCR and the relevant band cleaned using a PCR Gel Purification Kit (Qiagen). The resultant product was cloned into the plasmid pCR2:1 using the TA cloning kit (Invitrogen), and transformed into Top 10 competent *Escherichia coli* cells according to manufacturer's instructions (Invitrogen). Colonies containing the kanamycin resistance gene (carried on the plasmid) were identified by growth on LB agar containing Kanamycin 50µg/ml. Colonies were grown in LB Kanamycin 50µg/ml broth, and plasmid extracted using the Qiagen plasmid mini-prep kit. Presence of target genes was confirmed by PCR for each gene of interest, and those colonies which were positive by PCR for all target genes were sent for sequencing by the MRC sequencing centre, Imperial College London. Sequences were analysed using Bioedit sequence alignment software.

The plasmid concentration was measured by spectrophotometry (Picodrop), and the plasmid was then linearised using the restriction enzyme BamH1 (New England Biolabs, Hitchin, UK) and diluted to a known concentration of 2×10^7 copies/µl, and 1:10 serial dilutions performed. The PCR product melting temperature was assessed and a plasmid standard curve created using SYBRGreen (Sigma) on

the Stratagene MXP3000, according to manufacturer's instructions. A representative plasmid map of this plasmid and the one containing *gyrA* and *cepA* (Turner et al. 2009) are shown in Figure 10.

Gene	Primer Sequence
<i>speA</i> Forward	GAGGGGTAACAAATCATGAAGG
<i>speA</i> Reverse	TCAAATGATAGGCTTTGGATACC
<i>smeZ</i> Forward	TCCCTTCTAAGGAATATCTATAGTACGATTG
<i>smeZ</i> Reverse	TTCCAATCAAATGGGACGG
<i>gyrA</i> Forward	AGCGAGACAGATGTCATTGCTCAG
<i>gyrA</i> Reverse	CCAGTCAAACGACGCAAACG
<i>proS</i> Forward	TGAGTTTATTATGAAAGACGGCTATAGTTTC
<i>proS</i> Reverse	AATAGCTTCGTAAGCTTGACGATAATC
<i>cepA</i> Forward	ACACGGTATGCATGTGACAG
<i>cepA</i> Reverse	GATAAAGAGTGATTGAGGTGATCC

Table 6: Primers used for qRT- PCR

Primers for *speA* and *smeZ* were designed to amplify conserved regions across the different alleles. Primers for *gyrA*, and *cepA* were as previously described (Turner et al. 2009). Primers for *proS* were also previously described (Virtaneva et al. 2005; Virtaneva et al. 2003).

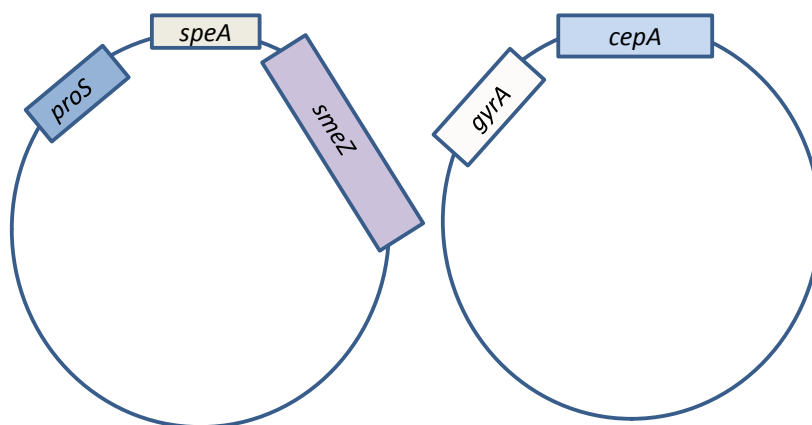


Figure 10: Plasmid maps

A representation is shown of the two plasmids created and used for qRT-PCR. A new plasmid containing the genes for *proS*, *speA* and *smeZ* was created as described above. The second plasmid containing the genes *gyrA* and *cepA* was previously created by Dr C. Turner (Turner et al. 2009).

2.2.3.8 Quantitative realtime RT-PCR using a standard curve

Reaction efficiencies for each primer pair were optimised with different concentrations of primers, from 50 – 400nM per reaction. For *proS* efficiency was best using 400nM of each primer, for all others a concentration of 200nM was optimum. To create a standard curve, linearised plasmid was adjusted to a high standard of 2×10^8 copies (final reaction concentration) and 1:10 dilutions made to a low standard of 10^2 copies. For each standard, a 50µl reaction was performed containing 25µl SYBRGreen Jumpstart Taq readymix (Sigma), 10µl plasmid, 13µl λDNA (to stabilise the plasmid and mimic test sample conditions) and 1µl of appropriate primers. A 50µl test reaction contained 25µl SYBRGreen, 2µl cDNA, 21µl H₂O and 1µl of appropriate primers. The PCR cycle was run with a 2 min denaturation at 94°C and 40 cycles of 94°C (15s) and 58°C (1 min), with a pause to read fluorescence at 76°C (81°C for *gyrA*) on an MXPro3000 (Stratagene, California, USA). A standard curve was generated and values calculated for test samples. Copies were calculated as copies of target gene per 10,000 copies *proS/gyrA*. Plasmid samples were run in duplicate and test samples in triplicate, RT negative samples once. A new standard curve was used for each plate and each gene of interest.

For the clinical study “Virulence factors in streptococcal tonsillitis”, 0.5µg of first strand cDNA generated from each throat swab, and quantitative real time RT-PCR was performed using 10ng of template per reaction against a plasmid-generated standard curve. Positive controls included RNA was extracted from bacterial pellets of the same isolate in overnight and late log growth in Todd-Hewitt broth and from a CBA plate, for which 2.5µg cDNA generated and 100ng run in a 50µl reaction. Each value was expressed as the ratio of the mean value of experimental triplicates for each sample.

For tissues from patients with invasive disease, quantitative real time RT-PCR was performed using 20ng of template per reaction against a plasmid-generated standard curve, with the same controls as for throat swabs. Each value is expressed as the ratio of the mean value of experimental triplicates for each sample. As there were not repeat samples available for the swabs or the tissues / fluids, and only sufficient RNA to run once in triplicate for each gene, a single value was generated for each sample.

For tonsil cell suspension co-cultures, quantitative bacterial RT-PCR for bacterial superantigen genes *speA* and *smeZ* was performed, using 100ng of cDNA per 50µl reaction, and results normalised to *proS*.

2.2.3.9 Quantitative realtime RT-PCR TaqMan protocol

Quantitative real time RT-PCR for human genes was performed using TaqMan gene expression assays (Applied Biosystems). 10ng of cDNA was used per 20µl reaction, with 10µl of TaqMan Gene Expression master mix (Applied Biosystems) and 1µl of probe/primer mix. The following PCR cycle was used: 50°C for 2 mins, 95°C for 10 mins and 50 cycles of 95°C (15s) and 60°C (60s).

Housekeeping genes used were 18s, GAPDH, β actin, and at least 2 were used to normalise each sample. Each sample was run in triplicate and RT negative samples once. Individual probes used were IGHG (immunoglobulin heavy chain gene), MS4A1 (CD20), AID (activation induced cytidine deaminase), PDRM1 (Blimp-1, B lymphocyte inducible maturation protein 1), XBP1 (X box binding protein 1) and BCL6 (B cell lymphoma 6).

From clinical throat swabs, expression of the human RNA transcripts for 18s ribosomal RNA (as a reference gene) and the Immunoglobulin heavy chain gene (IGHG) were assessed using Taqman probes (Applied Bioscience) with 10ng of template in a 25µl reaction.

For tonsil suspension cultures, quantitative RT-PCR for lymphocyte regulatory genes RNA was extracted from a minimum of 10×10^6 cells at the end of culture (days 0 and 5), and cells were separated into B and T cells before RNA extraction for some studies.

Results were analysed by relative expression using REST 2009 software, which normalises results by cycle threshold for the target genes against the housekeeping genes and compares relative expression from test samples to controls. Further analysis was performed using Graphpad Prism 5.

2.2.4 Human cell culture

2.2.4.1 Tonsil cell suspension cultures

Tonsil tissue was dissected into small pieces and passed through a 70µm cell sieve into a sterile Petri dish, using the end of a 1ml syringe plunger (all BD Falcon), to create a single cell suspension. Cells were collected and suspended in Tonsil Media to a volume of 50 mls. Cells were pelleted by centrifugation at 300g for 10 minutes and the supernatant discarded. The pellet was re-suspended and washed in twice more in fresh Tonsil Media. Cells were stained with 0.4% Trypan Blue (Sigma), counted on a modified Neuberg haemocytometer, and suspended at a final concentration of 2×10^6 viable cells/ml. Cells were then incubated at 37°C in a 5% CO₂ humidified atmosphere in culture flasks, 12, 24 or 96 well plates (Giger et al. 2004).

When appropriate, 50% of the culture media was removed at 48-72 hrs of incubation, and stored at -20°C for future analysis. The same volume of Tonsil Media was replaced +/- 20iu/ml recombinant human IL2 supplementation (Milltenyi).

At the end of experiments cells were harvested into sterile tubes, centrifuged at 300g for 10 minutes, and the supernatant collected and stored at -20°C. Cells were either used immediately, stored at -80°C in Freezimix (90% Foetal Calf Serum (Invitrogen), 10% DMSO (Sigma) for later flow cytometry analysis or mixed in Trizol reagent (Invitrogen) for RNA extraction.

For tonsil cell culture stimulation with recombinant superantigens, recombinant superantigens Streptococcal pyrogenic exotoxin (SPE) A, Staphylococcal enterotoxin (SE) B and C, toxic shock syndrome toxin (TSST)-1 (all Toxin Technologies) SPEJ or Streptococcal mitogenic exotoxin Z (SMEZ) were added to tonsil cell suspension cultures at a variety of different concentrations. Concanavalin A (Sigma) and anti CD3 and anti CD28 (Milltenyi) were used as T cell mitogen controls. Anti CD3/CD28 antibodies were used at 1µg/ml, as per manufacturers' recommendations.

2.2.4.2 Tonsil cell suspension live bacterial co-cultures

For live bacterial co-cultures, cells were prepared as above, and incubated in culture flasks overnight. Cells were harvested and washed 4 times in Antibiotic Free Media, before being re-counted and re-suspended in Antibiotic Free Media at a concentration of 2×10^6 viable cells/ml. Presence of residual antibiotics was tested for by applying 5µl of washed cells to a CBA plate (Oxoid) seeded with 1×10^6 CFU/ml M1 strain *S. pyogenes*. Contamination was excluded by plating 5µl cell suspension onto CBA and incubating for 48 hours at 37°C 5% CO₂. Cells were then co-cultured in 12 well plates with live bacteria grown in Antibiotic Free Media, to ensure no endotoxin contamination. Bacteria were prepared from an overnight growth of the relevant bacterial strain in antibiotic free media. This was then sub-cultured 1:5 into fresh media and incubated at 37°C for 4-6 hours, before adding to the cell suspension or to the same volume of fresh media alone for control wells. The bacterial inoculum was calculated as CFU after 18 hours incubation, and was divided by the initial inoculum to create a multiplication factor (MF).

2.2.4.3 Tonsil cell suspension proliferation assays

For proliferation assays, human tonsil cell suspensions were cultured for 56 hours in the presence of recombinant superantigens, filter sterilised bacterial culture supernatants or negative controls at a concentration of 2×10^5 cells per well in flat bottom 96 well plates. 20µl of stimulant was added to a final volume of 200µl cells, and incubated at 37°C, in a 5% CO₂ humidified incubator. For the final 18 hrs of incubation 1µCurie/well H³-Thymidine was added, and then cells harvested onto glass fibre mats and analysed on a Wallac Trilux (USA) Scintillation Beta counter. A 4 parameter curve fit equation of log transformed data was performed using Graphpad Prism 5.0 software, to give an R² goodness of fit value for concentration curves.

2.2.4.4 Transfer of cell culture supernatants

To determine whether SPEA exposed tonsil cells produced a secreted factor that could negatively impact on immunoglobulin production, cell-free supernatants from SPEA exposed tonsil cells were transferred to naive tonsil cell cultures. Cell free supernatants from two different tonsil donors were chosen, from days 1, 7 and 14 of culture from one donor, days 4, 9 and 14 from the second, either

with or without SPEA 100ng/ml stimulation, and frozen at -20°C immediately after harvesting, until use. Before these supernatants were used, the levels of total IgG were assessed in each supernatant, to confirm inhibition of IgG production in the SPEA supernatants compared to the negative controls. These supernatants were then applied to fresh tonsil cell cultures of two different tonsil donors at a concentration of 1% (10µl in a 1ml culture), in triplicate, with or without SPEA 100ng/ml. To confirm that there was no influence from transferred SPEA in relevant supernatants, control wells were set up containing rabbit anti-SPEA antibody (10µg/ml) or normal rabbit serum (both Toxin Technologies, USA).

2.2.4.5 *Inhibitory antibodies and ELISAs*

Cytokine inhibition was achieved using 10µg/ml of neutralising goat anti-human IL2, 4, 10, TNFα, and INFγ antibodies (all R&D systems) to tonsil cell suspension cultures with/without SPEA 100ng/ml, and repeated in 2 (IL2 and 4) or 3 separate donors (IL10, TNFα and INFγ). Antibody was added at the start of culture and at days 2 and 5 of culture, before cells were harvested on day 7 – a protocol and concentration found to be necessary after testing several different tonsil donors. Normal goat serum was used as a control (Sigma). Confirmation of cytokine inhibition was performed using ELISA kits (R&D Systems). Human total IgG production was measure by ELISA in culture supernatants (Bethyl Labs). Levels of soluble TNF receptor 1 (sTNFR1) was detected in cell culture supernatants by ELISA (R&D systems). Details of ELISA protocol as are in methods section 2.2.5.

2.2.4.6 *Tonsil cell separation*

Human tonsil cell suspension cultures were separated using AutoMACS bead technology (Milltenyi biotech), either before or after culture. T cells were isolated using CD2 positive selection. B cells were then isolated using the B cell negative selection kit, which positively selected for CD2, CD14, CD16, CD36, CD43 and CD235a, leaving only B cells remaining. The protocols were run according to specific instructions for each bead set, briefly: harvested cells were counted and centrifuged at 200g for 10 minutes and pellets resuspended in ice-cold MACS buffer (sterile PBS with 0.5% filtered BSA and 2mM EDTA). Samples were centrifuged at 300g for 10 minutes and supernatant completely removed. Cells were resuspended in the recommended concentration of MACS buffer (40µl per 10⁷

cells) and appropriate concentrations of antibodies added (usually 10 μ l per 10⁷ cells). Cell-antibody mixes were incubated at 4°C for 10 minutes with occasional mixing. Further buffer (30 μ l per 10⁷ cells) was then added followed by the magnetic beads (20 μ l per 10⁷ cells), and the mix incubated at 4°C for 15 minutes with periodic mixing. Cells were washed in MACS buffer, and centrifuged at 300g for 10 minutes. Cells were resuspended in 500 μ l MACS buffer and run through the AutoMACS on the recommended programme.

As large volumes of cells were required for separation after stimulation with superantigens (or unstimulated controls), cells for these experiments were incubated for 5 days in 50 ml flasks at a concentration of 2 x 10⁶ cells/ml, with or without SPEA 100ng/ml, and 50% of the media was carefully changed at 48 hours and supplemented with 20 iU/ml recombinant human IL2 (Milltenyi).

Confirmation of the quality of separation was performed using surface staining for CD3 (T cells) and CD20 (B cells), and gave a >99% cell purity on each occasion.

2.2.4.7 Tonsil histocultures

This method was adapted from that previously published by Glushkova et al. (Glushakova, Baibakov, Margolis et al. 1995; Grivel & Margolis 2009). Tonsils were collected as above, capsule, connective tissue and any damaged tissue removed and remaining tissue dissected into 2-3 mm blocks using a scalpel (disposable Swann Morton size 23 curved blade, VWR). Gelfoam constructs were assembled in a 12 well plate as follows: 1x1x2cm pieces of sterile Gelfoam (Upjohn, Pfizer, UK) were cut, and placed into each well of a 12 well plate. The foam was then soaked in 500 μ l Tonsil Media +/- stimulant for 30 minutes, or until fully moist. A 1cm diameter 5 μ m Isopore membrane (Millipore, MA, USA) was placed onto each block of foam, with the edges not touching the media below. 3-4 blocks of tonsil tissue were pre-exposed to the correct concentration of stimulant (or media alone), and then placed onto each Gelfoam-membrane construct, and incubated in a 37°C, 5% CO₂, humidified for up to 1 week. A demonstration of this is displayed in Figure 11. At 48-72 hrs of incubation 200 μ l media was removed from the bottom of each well (and stored at -20°C), and replaced with 200 μ l fresh Tonsil Media supplemented with 20 iu/ml recombinant IL2 (Milltenyi).

Culture supernatants were collected from the Gelfoam constructs at the end of the experiment and stored at -20°C. Histocultures were harvested into formalin for histopathology examination, disrupted directly into Trizol for RNA extraction, or disrupted into a single cell suspension in PBS through a 70µm cell sieve for flow cytometry.

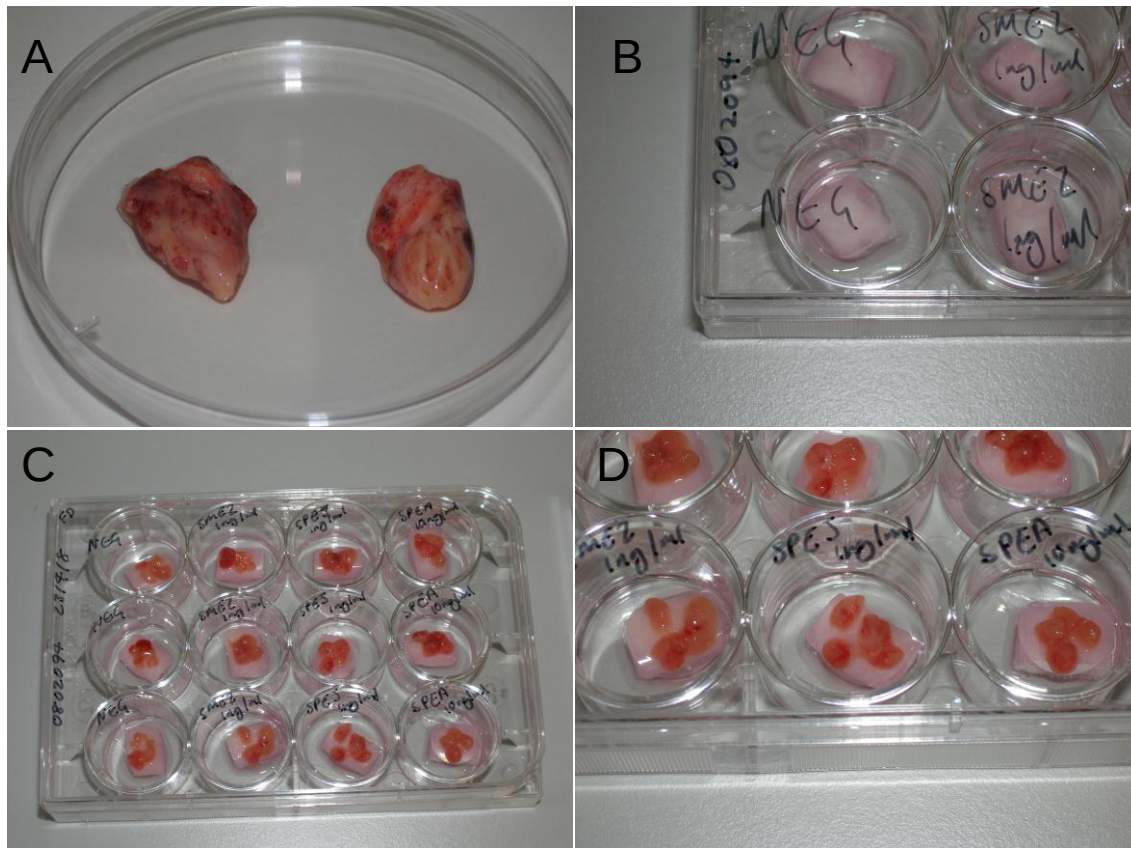


Figure 11: Tonsil histoculture construction

A/ tonsil before dissection. **B/** Gelfoam construct before tonsil added. **C+D/** Finished construct. Purified superantigens were added to the media for each well at the correct concentration for each situation before the gel was soaked, to ensure even distribution of superantigen to each well. Blocks were also pre-soaked in the appropriate superantigen media solution for 10 minutes before being applied to the histoculture construct, to ensure adequate antigen exposure.

2.2.4.8 Histoculture live bacterial co-cultures

In order to successfully culture live bacteria on the surface of tonsils, there could be no antibiotics remaining in the tonsil histoculture as this would falsely alter the results, and endogenous flora had to be minimised. Therefore two different methods were developed to allow this. The first method was to expose the tonsil block to 2 x 10 minute washes in antibiotic-containing Tonsil media, before washing

6 times in Antibiotic Free media. Then the tonsils were checked for the presence of residual antibiotics (Figure 12). Although there was no contamination using this system, and infection could be reproducibly created, on some occasions there was evidence of residual antibiotic in the blocks, which could falsify the results or reduce the degree of infection. To counteract this, a second method of tonsil histoculture was developed where there was no antibiotic exposure, but tonsil blocks were submerged in 60% Ethanol solution for 30 seconds before washing 4 times (10 minutes each) in antibiotic free media. Blocks were then applied to the scaffold in Antibiotic Free media and bacteria applied as above. These cultures had no antibiotic content but were at high risk of endogenous flora contamination, and so were only viable for a maximum of 72 hours before complete necrosis had occurred. Only tonsils which looked healthy at the time of surgery with few tonsoliths (small stones of bacteria and dead inflammatory cells which develop deep inside tonsils during chronic infection) or evidence of active infection were deemed suitable for live-bacterial co-culture infection.

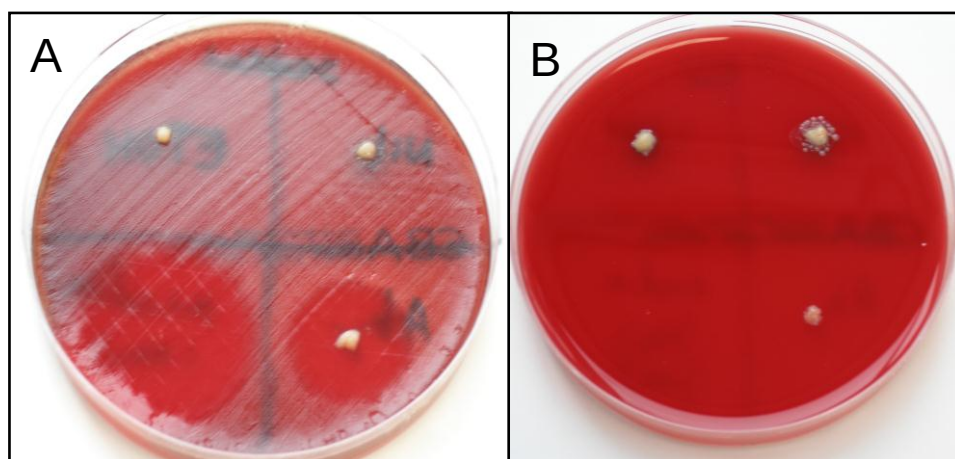


Figure 12: Detection of antibiotics and endogenous flora in tonsil histocultures

A/ antibiotic inhibition plate, seeded with a semi-confluent growth of *S. pyogenes* M1 strain H305. No inhibition of growth of GAS from the untreated block (top right), or from the ETOH soaked block (top left), 21mm zone of inhibition from 6x washed antibiotic-soaked block (bottom right) and 33mm zone of inhibition from 5 μ l of antibiotic-containing tonsil media (bottom left). **B/** purity plate. Overnight incubation shows growth of colonies around the tonsil block which was untreated (top right). A few colonies are growing from the ETOH treated block (top left), no growth from the washed antibiotic-treated block (bottom right) and no contamination of antibiotic free media (bottom left).

Bacterial suspensions were prepared as described in section 2.2.2.4, and applied at 5µl per block (3 blocks per well), with each well repeated in triplicate. Blocks pre-treated with Tonsil Media were infected immediately and again after 18 hours of incubation. Ethanol treated blocks were infected once only. Residual antibiotic effect and contaminating bacteria were checked as for cell suspensions on replicate blocks. Infected/control blocks of tissue were harvested at different time points, and either immersed in formalin for histopathology examination, disrupted into 500µl PBS and plated onto CBA plates for colony counting (dilutions ranging from neat to 1×10^{-9}), or processed for RNA extraction in Trizol. Controls included the same amount of bacteria grown in the same volume of media as the histoculture, for both bacterial quantification and qRT-PCR. A schematic representation of the histoculture infection is shown in Figure 13

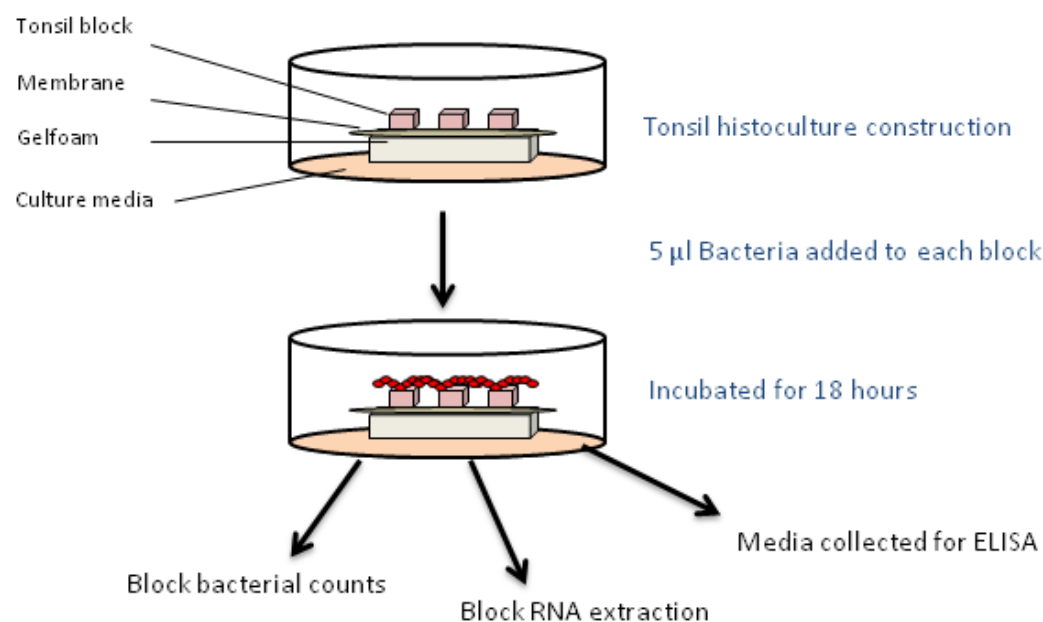


Figure 13: Schematic of histoculture live bacterial co-culture system

Three blocks sized $2\text{-}3\text{mm}^3$ of human tonsil were placed onto a $5\mu\text{m}$ Millipore membrane balanced on top of a square of Gelfoam in a sterile 12 well plate well. The construct was soaked in $500\mu\text{l}$ of the appropriate culture media. Bacteria were applied to the top of each tonsil block in a volume of $5\mu\text{l}$ and incubated for the indicated time (usually 18 hours, up to 6 days). Bacterial counts were established by homogenising blocks in sterile PBS and plating onto CBA. Blocks could also be used for RNA extraction. Residual culture media was harvested from the bottom of the well and from the Gelfoam after blocks were removed from the system.

2.2.4.9 Histopathology of histocultures

Tonsil histocultures were immersed in 3 mls of Formalin at the end of experiments. Paraffin embedded blocks were prepared and sectioned by Mahrokh Nohadani (C&C laboratory services) and sections were stained with Haematoxylin and Eosin (H&E) or Gram stained. Immunohistochemical staining was attempted using a polyclonal goat anti-Group A carbohydrate antibody (Abcam, ab9191), at a range of dilutions from 1:100 to 1:50,000, again by Mahrokh Nohadani. Slides were analysed with the help of a consultant histopathologist, Dr Joseph Boyle (Imperial College, London).

2.2.4.10 Human PBMC isolation

Human PBMCs were collected from healthy volunteers. The appropriate volume of venous blood was collected into heparinised tubes (BD Falcon). Blood was diluted 1:2 with sterile PBS (Invitrogen) and layered 15-25mls over a 15mls Ficoll-Paque solution (GE Healthcare) in sterile falcon tubes. Cells were centrifuged for 25 minutes at 800g (22°C) and the buffy coat containing PBMCs removed. Cells were washed to remove any traces of plasma, counted (section 2.2.4.1) and re-suspended in Standard Culture Media at a concentration of 1×10^6 viable cells/ml in 96 well plates.

2.2.4.11 PBMC bioassay (proliferation)

Cell suspensions were prepared as described above. Cells were cultured in 96 well flat or round bottom plates at a concentration of 2×10^5 cells/well, in triplicate wells. Cells were incubated and harvested as described for tonsil cell suspension proliferation assays, in section 2.2.4.3.

For bioassays using invasive disease tissues, tissues which had been homogenised in PBS were centrifuged at 10,000g for 5 minutes. The supernatant containing both human and bacterial proteins was then carefully removed and used as a stimulant at a 10%. Culture supernatant from the matching bacterial isolate from that patient was used as a control. Experiments were performed in triplicate wells for each experimental condition on 3 different PBMC donors. Negative control wells contained no supernatant.

2.2.5 Enzyme-linked immunosorbent assays (ELISA/ELISpot)

2.2.5.1 Immunoglobulin ELISA

ELISAs were performed on cell culture supernatants from Tonsil and PBMC cultures, which were either freshly harvested or which had been aliquoted and stored at -20°C. ELISAs were performed for total human IgG, IgA, IgE and IgM using kits according to manufacturer's instructions (Bethyl laboratories, Texas, USA). Briefly, plates were coated using capture antibodies provided, diluted in coating buffer, 100µl/well. Plates were incubated for 1 hr at room temp and washed x5 in wash buffer (PBS/0.05%Tween 20, Sigma) on an automated 96 well plate washer. Plates were blocked for 30 minutes using 1% BSA in PBS (300µl/well), and washed x5. Samples/standards were applied (100µl/well) and incubated for 2 hours, and then washed x5. HRP-Conjugate antibody was applied at a concentration of 1:150,000 IgG, 1:50,000 IgA and 1:60,000 IgM, 100µl/well, and incubated for 1 hr, room temperature in the dark. Plates were washed x5, and the substrate TMB (100µl/well) added to each well (incubated in the dark, room temperature). After approximately 10 minutes 100µl/well 1M H₂SO₄ was added as a stop solution, and plates read on an ELISA plate reader at wave length of 450nm with 570nm wavelength correction. Results were blanked to control media, and analysed on KC Junior software, using a 4 parameter curve fit equation. Sample dilutions in PBS were optimised for each assay, and each sample type.

2.2.5.2 Cytokine ELISA

All other ELISAs (Human cytokines IL1β, 2, 4, 6, 10, 12, 17, Interferon γ, TNFα, TNFβ, TGFβ and CXCL13) were performed using Duoset kits obtained from R&D Systems (R&D systems, UK). Plates were coated with capture antibody overnight at room temperature at the concentration specified for each test, 100µl/well, diluted in PBS. Plates were washed x3 as above, and plates blocked for 1hr with 1% BSA/PBS (300µl/well). Plates were washed x3, samples/standards applied (100µl/well) and incubated for 2 hours at room temperature. Plates were washed x3 and detection antibody applied for 2 hours, diluted in 1% BSA/PBS to the specified concentration (100µl/well). Plates were washed x3 and Streptavidin-HRP applied to each well (1:200 dilution, 100µl/well) for 20 minutes, room

temperature in the dark. Plates were washed x3 and developed with TMB as above. For all ELISAs, supernatants were titrated to find the optimum dilution for measurement.

For TGF β , samples were treated with a solution of 1N HCl of 10 minutes and then neutralised with 1.2N NaOH/0.5M HEPES before analysis, according to manufacturer's recommendations. As a control, tonsil media was also treated in the same way, and the treated suspension used as a diluent for the standard curve, again according to manufacturer's recommendations, as there are detectable background levels of TGF β in the media.

Differences between median cytokine/immunoglobulin productions were analysed for statistical significance using Graphpad Prism 5 software. Mann Whitney test was performed for comparisons for groups with small numbers of tonsil donors, but where there were sufficient donors to allow for effective pairing, Wilcoxon signed rank pairs test was performed (usually N=7 different donors or above). For analysis of multiple paired groups, a Friedman Test (one way ANOVA) was performed. Frequency of distribution testing was performed to ensure that the distribution was not Gaussian, where necessary.

2.2.5.3 *Specific anti-streptococcal IgG ELISA*

A 10 ml overnight Todd-Hewitt broth culture of the relevant bacterial strain was washed and heat killed as described (section 2.2.2.4) for each ELISA plate. The heat killed bacterial pellet was resuspended in 10 mls of coating buffer and incubated overnight at room temperature, 100 μ l/well. Spare plates could be stored for up to 1 week at 4°C without any loss of accuracy or sensitivity of results. The next day plates were washed x5 in PBS/0.05% Tween 20 and blocked for 3 hours with 1% BSA in PBS. The plates were then washed x5 and the samples applied in volumes of 100 μ l, diluted in PBS. To establish accurate antibody dilution titres, 10 doubling dilutions were performed starting at a dilution of 1:50, where there was sufficient serum to allow. If there was insufficient serum a single dilution of 1:100 was used. Samples were incubated for 2 hours at room temperature or overnight at 4°C, before being washed x5 in PBS/Tween 20.

100µl/well of secondary antibody, biotinylated goat anti-mouse IgG (Sigma, UK) was used at a dilution of 1:80,000 in 1% BSA/PBS, and incubated for 2 hours at room temperature. After washing 3 times plates were incubated with 100µl/well streptavidin at 1:200 dilution (R&D Systems) for 20 minutes in the dark, room temperature. Plates were washed x3 and then developed as above with TMB. A positive result was classed as a blanked (to wells with no serum) reading of optical density greater than 0.5 for titrated samples, optical density alone was read and compared for samples where there was insufficient serum to titrate. Control sera was used from mice which had been vaccinated against SpyCEP or saline vaccine controls (Turner, Kurupati, Wiles et al. 2009).

2.2.5.4 B cell ELISpot

PVDF 96 well plates (Diacclone) were washed in 70% Ethanol solution for 10 minutes at room temperature. 100µl/well heat killed *S. pyogenes* (prepared as in section 2.2.2.4) re-suspended in sterile PBS was applied to the plate and incubated at 4°C overnight. The following day the plate was washed in 100µl/well sterile PBS and incubated with a blocking solution of 100µl/well 2% skimmed milk solution in sterile PBS for a minimum of 2 hours at room temperature, while cells were prepared, and then washed in 100µl/well sterile PBS.

Lymph node or spleen cells were harvested from freshly culled mice and dispersed into a single cell suspension by gentle disruption through a 70µm cell sieve into standard culture media. Cells were washed once in media, counted on a haemocytometer (using 0.04% Trypan blue staining) and re-suspended to the correct concentration of live cells. 100µl/well of cells was carefully applied to triplicate wells for each mouse, at concentrations of 10^5 cells/well for lymph nodes and 10^6 cells/well for spleens. Plates were incubated for 5 days at 37°C in a humidified 5%CO₂ atmosphere, without moving them for the duration of the incubation.

After 5 days incubation, cells were washed once with 100µl/well PBS/0.1%Tween 20 and incubated for 10 minutes at 4°C with 100µl/well PBS/Tween. Cells were washed x3 in PBS/0.1%Tween, and then incubated with goat anti-mouse IgG biotinylated antibody (Sigma, as above) at a dilution of 1:80,000 in 1%BSA/PBS for 2 hours at 37°C. Plates were washed x3 in PBS/0.1%Tween and

100µl/well streptavidin-ALP conjugate (Diacclone) added at a dilution of 1:5000 in 1% BSA/PBS, and incubated at 37°C for 1 hour. Plates were washed 3 times with PBS/Tween, and then developed with 100µl/well BCIP/NBT buffer (Diacclone) for 20 minutes at room temperature, until spots had developed and the green colour was lost. The plates were washed extensively in distilled water and dried for 2 hours at room temperature and then overnight at 4°C. Plates were read on an automated ELISpot plate reader, with settings adjusted for cell size and background colouration. Counts were confirmed by checking each well by eye, and readings from any damaged/faulty wells excluded. Each spot detected represents one IgG producing B cell, so the mean results from the triplicates for each mouse were plotted as B cell counts. An example of the ELISpot appearance is shown in Figure 14.

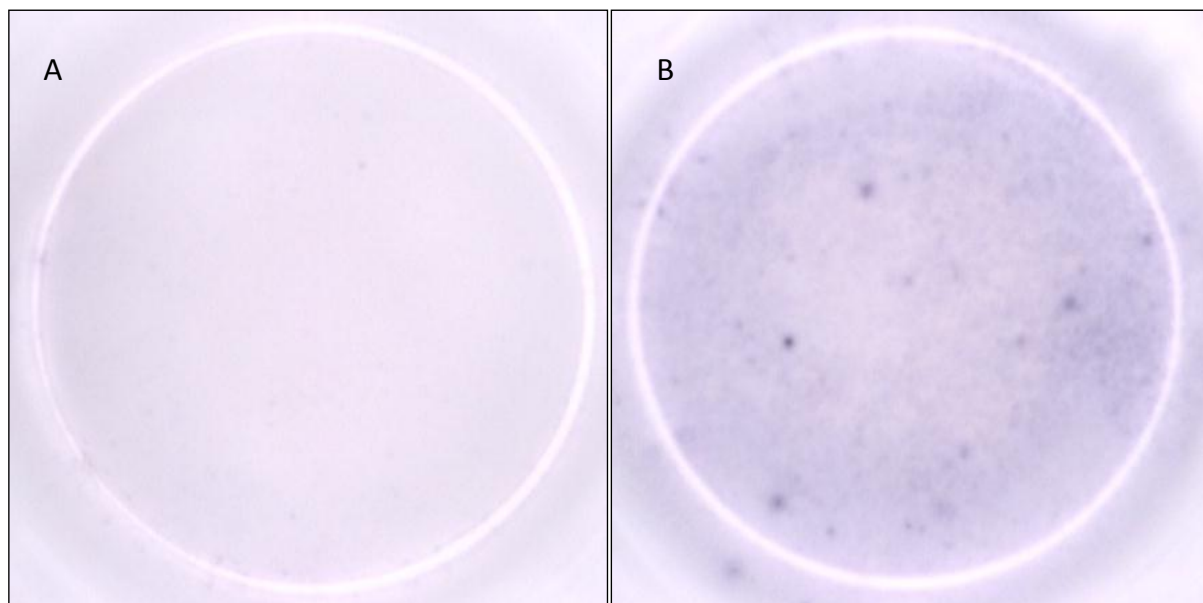


Figure 14: Example of lymph node anti-streptococcal IgG ELISpot

HLA DQ8 mouse lymph nodes were dissected and re-suspended to a concentration of 1×10^5 cells per well. Spleens were incubated at a concentration of 1×10^6 cells per well. Cells were incubated for 5 days in ELISpot plates pre-coated with heat killed *S. pyogenes*. Specific IgG producing B cell counts were detected by developing the plates with an anti-mouse IgG antibody. Each spot represents one anti-*S. pyogenes* B cell. Results were analysed on an automated ELISpot plate reader corrected for background, and checked manually. A / Representative lymph node from an *S. pyogenes* naïve mouse, 1 spot detected. B/ Representative lymph node from an *emm1 S. pyogenes* vaccinated mouse pre-exposed to *speA* negative strain and rechallenged with wild type *speA*⁺ strain, 61 spots detected.

2.2.6 Flow cytometry

2.2.6.1 Surface staining and general flow cytometry methods

Cells in single cell suspensions were either stained freshly or frozen at -80°C in Freezimix (10% DMSO in FCS) until use for surface staining. There were no discernible differences in surface staining of cells which had been frozen to those which were stained fresh from culture (either percentages or fluorescent intensity), unless specifically stated in the results. Stored cells were defrosted and washed once in PBS. For surface staining, cells were washed once in PBS with 10% FCS and re-suspended to a concentration of 1×10^7 cells/ml. Cells were aliquoted at 100µl per tube, and antibodies added (usually 5µl per million cells, titrated for optimum fluorescent intensity for each antibody). Cells and antibodies were incubated in the dark for 20 minutes at room temperature before washing twice in 10% FCS in PBS, and fixing in 100µl of 2% Paraformaldehyde solution (Sigma). Cells were run on a FACS Calibur flow cytometer (BD) within 24 hours of staining, settings adjusted to unstained cells and compensated with single stained cells. Isotype controls were used where appropriate. 10,000 gated lymphocytes or 100,000 total events were acquired when possible and analysed using FlowJo Software (Treestar inc, USA).

2.2.6.2 Intracellular staining

Intracellular staining was performed on freshly harvested cells using the intracellular staining kit from BD Bioscience. 10µg/ml Brefeldin A was added to cultures and incubated for 2-4 hours at 37°C in a 5% CO₂ incubator. Cells were washed twice in 10% FCS/PBS and then blocked by incubating in 100% FCS for 10 minutes (room temperature). Cells were then washed in 10% FCS/PBS before surface staining was performed as above, without fixing cells. Cells were washed twice in 10% FCS/PBS and then resuspended in Fix/Perm buffer (BD Bioscience) for 30 minutes at 4°C. Cells were washed twice in permeabilisation buffer and then stained with intracellular antibodies for 30 minutes, 4°C in the dark. Cells were washed twice in permeabilisation buffer before being fixed in 2% Paraformaldehyde solution. For intracellular FoxP3 staining, a similar protocol was followed except using the specific buffers and timings specified in the BD FoxP3 staining kit. Cells were analysed

within 2 hours of staining on a BD FACS Calibur, as above. Data analysis was performed using FlowJo software (Treestar inc.).

2.2.6.3 *Annexin V and Propidium iodide staining for apoptosis*

Cell apoptosis was assessed on freshly harvested cells using the Annexin V FITC / Propidium iodide staining kit (Beckton Dickenson, USA), according to manufacturer's instructions, with concentrations titrated for optimal staining. Briefly, cells were washed twice in ice cold PBS and resuspended in freshly prepared Annexin V binding buffer (diluted from 10 x concentrate in ddH₂O for use) and at 2×10^6 cells /ml. 2µl FITC Annexin V and 5µl Propidium iodide were added to each tube, containing 2×10^5 cells, mixed by gentle vortexing, and incubated for 15 minutes in the dark. 200µl binding buffer was added to each tube and cells analysed immediately on a BD FACS Calibur as above. Data analysis was performed using FlowJo software (Treestar inc.).

2.2.6.4 *Antibody selection*

A list of all antibodies used is in Table 7. All antibodies were purchased from BD biosciences conjugated to either FITC, PE, PECy5 or PerCPCy7, with the following exceptions: T cell Receptor variable β subunit (TCRV β) antibodies, isotype controls and TCRV β Repertoire kit (IOtest Beta Mark) were purchased from Beckman Coulter (Marseille, France), CXCR5 PE and isotype control were purchased from R&D systems, CXCR5 PE-Cy5 and isotype control was purchased from eBioscience (California, USA).

Analysis group	Purpose	Antibodies
T Cells	Cell characteristics	CD3; CD4; CD8; TCRV β 2, 8, 11, 14; IOTest TCRV β repertoire kit; CD25; FoxP3; CXCR5; TCR $\alpha\beta$
	Activation markers	CD69; CD27; CD278 (ICOS); CD134 (OX40); CD28
	Apoptosis markers	CD95
	Immune synapse	CD150 (SLAM); CD154 (CD40L); CD70
B Cells	Cell characteristics	CD19; CD20; CD21; CD23; CD27; CD38; HLADR; IgD; IgM
	Activation markers	CD69; CD80; CD86
	Apoptosis markers	CD95
	Immune synapse	CD150 (SLAM); OX40L; CD275 (ICOSL); CD40; CD70
Other	Cell type identification	CD14; CD68; CD11b; CD11c; CD56
	Apoptosis	Annexin V; Propidium iodide
	Intracellular cytokines	IL4; IL9; IL17; INF γ

Table 7: Flow cytometry antibodies

2.2.7 Mouse studies

2.2.7.1 Animals used

Female C57BL/10.DQ8 transgenic mice carrying genomic constructs for DQA1*0301 and DQB*0302 were bred and genotyped as previously described (Unnikrishnan et al. 2002). These mice have been shown to be uniquely sensitive to the effects of superantigens. All mice were bred and maintained according to Home Office standards. All mice were age and weight matched between different experimental groups.

2.2.7.2 Infection protocol – primary immune response to *S. pyogenes*

To establish the influence of superantigen exposure on immediate susceptibility to re-infection, mice were challenged with heat killed preparations of *speA*[±] *emm1* isogenic strains of *S. pyogenes* (see section 2.2.2.1). Groups of 5 mice were exposed to the different *S. pyogenes* strains twice by IM injection, 1 week apart, with heat killed bacteria, re-suspended in the culture supernatant to ensure exposure to superantigens. The exposure used was as follows: Group 1 – RPMI; Group 2 – Wild type *emm1*; Group 3 – WT *emm1* Δ *speA*; Group 4 – WT *emm1* Δ *speA* complemented. All concentrations of bacteria were between $0.2 - 8 \times 10^8$ CFU/mouse.

After nine days, serum samples were collected and then live bacterial infection instituted. Mice were injected into the right thigh with 50 μ l of live bacterial suspension from overnight culture of WT *emm1* Δ *speA* *S. pyogenes*, which was washed twice in sterile PBS and re-suspended to an optical density A₆₀₀ of 10, which yielded 7.45×10^7 CFU per mouse.

Mice were culled at 20 hours from the time of infection and blood harvested by cardiac puncture, of which 5 μ l was plated onto Columbia blood agar (CBA) and the rest processed for serum. Draining lymph nodes were homogenised in 100 μ l sterile PBS and plated onto CBA. Liver, spleen and infected thigh were harvested, homogenised in sterile PBS (volume adjusted for organ weight) and plated onto CBA. Plates were checked for the growth of β -haemolytic colonies at both 24 and 48 hours.

Serum was collected by tail bleeding before live infection or cardiac puncture when culled. Blood was centrifuged for 30 mins at 15,000g and serum removed from the pellet. Serum was frozen at -20°C until use in specific IgG ELISAs.

2.2.7.3 Impact of SPEA on recall antibody responses to *S. pyogenes*

Groups of 5 mice were vaccinated twice, two weeks apart, with preparations of heat killed WT *emm1* Δ *speA* *S. pyogenes*. On each occasion this was injected into the right thigh at concentrations of 12.3×10^7 CFU/mouse for the first injection and 5.2×10^7 CFU/mouse for the second injection, in volumes of 50 μ l per mouse. A control group of 5 mice were unchallenged. 4 weeks from the initial vaccination mice were rechallenged in the right thigh with heat killed bacteria in the following groups: Group 1 –

unvaccinated, unchallenged; Group 2 - WT *emm1*Δ*speA* vaccinated, RPMI challenged; Group 3 - WT *emm1* Δ *speA*, WT *emm1* challenged; Group 4 - WT *emm1* Δ *speA* vaccinated, WT *emm1* Δ *speA* challenged; Group 5 - WT *emm1* Δ *speA* vaccinated, WT *emm1* Δ *speA* complemented challenged. Challenge bacteria were used at a concentration of $4-12 \times 10^7$ CFU/mouse of heat killed bacteria. RPMI was used as a control for non-specific immune responses in group 2 as this was the media in which bacteria had been cultured and so formed the basis of the supernatant in which they had been re-suspended prior to injection. Serum was collected by tail bleeding prior to challenge.

48 hours after the challenge mice were culled and blood collected by cardiac puncture and serum separated by centrifugation. The draining lymph node (right) and spleen was harvested from each mouse and processed for ELISpot (see section 2.2.5.4).

2.2.8 Statistics

All statistically analyses, unless stated specifically elsewhere, were performed using Graphpad Prism 5 software. All data was non-parametric, as confirmed when numbers were high enough to test. For comparison of two groups, Mann-Whitney test was performed, or Wilcoxon signed ranked paired test for paired data for paired samples numbering 6 or more per group. For comparison of multiple groups, a 1 way ANOVA (Kruskal Wallis, or Friedman for paired data) test was performed. Statistical tests were not performed on groups with sample size less than 3. A probability value <5% was classed as significant ($p < 0.05$).

3 Expression of superantigens in different conditions

The most frequent infections caused by *Streptococcus pyogenes* are those of the pharynx, yet remarkably little is known about the events which occur in establishing an infection or the role of key virulence factors. Although experimental models of infection in Macaque monkeys (Sumbly, Tart, & Musser 2008; Virtaneva et al. 2005) have provided valuable information regarding the role of virulence factors in pharyngitis, it is not ethical or feasible to undertake experiments where an infection is induced, or followed from the time of acquisition, in humans. Quantitative real-time RT-PCR (qRT-PCR) and microarray data from the Macaque model have shown that expression of the superantigens SPEA and SMEZ were both enhanced in the colonisation phase of infection, suggesting a role in establishing disease (Virtaneva et al. 2005). Although one study has assessed the role of *S. pyogenes* regulatory genes by qRT-PCR in patients presenting with acute pharyngitis (Virtaneva et al. 2003), superantigens were not evaluated in this study. To date no studies have been performed to evaluate the role of streptococcal virulence factors in people with asymptomatic carriage, although nearly half of patients can be found to still harbour the infection up to 13 weeks after symptoms of infection have resolved (Johnson et al. 2010) and winter carriage rates can be as high as 16% in children and 2% in adults (Danchin et al. 2007).

Similarly, most of the information regarding the role of virulence factors in invasive *S. pyogenes* disease comes from animal models of infection (Graham et al. 2006). In one study, examination of tissues from patients with necrotising fasciitis showed high levels of Interferon γ , TNF- β and IL1, consistent with active superantigen production at the site of infection (Norrby-Teglund et al. 2001).

Therefore, this project set out to determine the contribution of bacterial superantigens both in pharyngitis and invasive streptococcal disease. For pharyngitis, children presenting with clinical tonsillo-pharyngitis and their healthy accompanying relatives (to find asymptomatic carriers) were recruited to a clinical study where qRT-PCR for bacterial superantigens was performed directly from their throat swabs. For invasive disease, qRT-PCR for bacterial superantigens and proliferation (H^3 -

thymidine incorporation) studies were performed directly on the clinical samples from patients with invasive *S. pyogenes* disease.

3.1 Results

3.1.1 Paediatric study demographics.

25 patients were recruited to the study, 12 children with pharyngitis/tonsillitis, and 13 adults, of whom 12 were mothers of the recruited children and one father. Unfortunately, of the children recruited, only 1 patient was positive on the rapid test (confirmed on laboratory culture media) and a further 2 children were positive on laboratory culture. No relatives were positive for *S. pyogenes* carriage. All cultures were confirmed with swabs sent to the diagnostic microbiology lab, and there was a 100% concordance between research and laboratory culture results. A summary of study participant characteristics is shown in Table 8.

Emm typing and superantigen profiling were performed on DNA extracted from the 3 positive isolates cultured *in vitro*. 16s ribosomal RNA PCR for *S. pyogenes* was positive on DNA extracted directly from all 25 throat swabs collected during the study. However the 16s rRNA PCR was found to be poorly specific, as a PCR product was also obtained using the same primers with DNA extracted from *Streptococcus gordonii* and *Streptococcus pneumoniae*; both bacteria which are commonly isolated amongst commensal flora in throat swabs. This PCR therefore confirmed the presence of bacterial DNA in the samples, but was not specific enough to act as an additional diagnostic test for *S. pyogenes*.

PCR for *proS* showed greater specificity with bacterial DNA (PCR product only obtained using *S. pyogenes* DNA) but this PCR was negative from all 25 throat swabs, including the 3 which had been positive on culture and/or rapid testing. As DNA extracted from these bacterial isolates *in vitro* yielded a PCR product for *proS*, this most likely reflects the poor yield of bacterial DNA from the throat swabs by the extraction method used. The three *emm* types from the bacteria cultured were *emm* 28, 12 and 89, which are representative of common circulating throat strains in the UK (Lamagni et al. 2008). None of the bacterial isolates were positive for the *speA* gene on PCR genotyping, but all were positive for *smeZ* and a variety of other superantigens (Table 8).

Number	Date	Patient/ Volunteer	Rapid test	Culture	16s PCR	ProS PCR	Emm Type	Superantigen profile
1	09/03/10	P	-	-	+	-		
2	09/03/10	V	-	-	+	-		
3	11/03/10	P	-	-	+	-		
4	11/03/10	V	-	-	+	-		
5	19/03/10	P	-	+	+	-	28	SPEC/G/J/K/M, SMEZ
6	19/03/10	V	-	-	+	-		
7	25/03/10	P	-	-	+	-		
8	25/03/10	V	-	-	+	-		
9	27/04/10	P	-	+	+	-	12	SPEC/G/I/H, SMEZ, SSA
10	27/04/10	V	-	-	+	-		
11	12/05/10	P	+	+	+	-	89	SPEC/G/H/J, SMEZ
12	12/05/10	V	-	-	+	-		
13	03/06/10	P	-	-	+	-		
14	03/06/10	V	-	-	+	-		
15	01/07/10	P	-	-	+	-		
16	01/07/10	V	-	-	+	-		
17	15/12/10	P	-	-	+	-		
18	15/12/10	V	-	-	+	-		
19	15/12/10	P	-	-	+	-		
20	15/12/10	V	-	-	+	-		
21	15/12/10	V	-	-	+	-		
22	16/12/10	P	-	-	+	-		
23	16/12/10	V	-	-	+	-		
24	11/02/11	P	-	-	+	-		
25	11/02/11	V	-	-	+	-		

Table 8: Details of patients recruited to the clinical study

25 participants were recruited to the study between 9th March 2010 and 2nd February 2011. There were 12 Patients (P) and 13 healthy volunteer relatives (V). The rapid test for *S. pyogenes* was only positive in patient 11 (marked +), and laboratory culture was also positive in two additional patients (5 and 9, marked + and highlighted grey). 16s ribosomal RNA PCR was positive in all swabs (+) but the more specific *ProS* PCR was negative on swabs (-). *Emm* typing was performed on all positive cultures. Superantigens detected were SMEZ (Streptococcal mitogenic exotoxin Z), SSA (Streptococcal superantigen), and Streptococcal pyrogenic exotoxins (SPE) C, G, H, J, I, K and M.

3.1.2 Quantitative RT-PCR from throat swabs and matching bacterial isolates.

Quantitative real-time RT-PCR (qRT-PCR) for *smeZ* and the housekeeping gene *proS* was performed on the total RNA extracted directly from throat swabs. The swabs from children with clinical pharyngitis but negative for *S. pyogenes* on culture served as a control for culture positive swabs, and healthy volunteer swabs as negative controls. For comparison qRT-PCR was performed on the isolates grown in laboratory culture, on blood agar and in Todd-Hewitt broth at both late log and stationary (overnight) cultures.

A mean of 1226 ng of RNA was extracted from each swab (range 60 to 3906 ng per swab) after the RNA had been purified. No signal for the bacterial housekeeping gene *proS* was detected in RNA extracted directly from any of the throat swabs. Transcripts of *smeZ* were also not detected from the three swabs which were positive for *S. pyogenes* on culture and known to carry the *smeZ* gene. *smeZ* qRT-PCR was positive from late log growth in all bacterial cultures, with a mean of 671 copies of *smeZ* amplified per 10,000 copies *proS* (Figure 15), though detection in blood agar and stationary phase broth culture was variable.

In contrast, a signal for human 18s ribosomal RNA was detected from all throat swabs, with a mean CT (cycle threshold) value of 31.5 cycles per reaction (data not shown). As a comparison human tonsil cell cultures have a mean CT value for 18s rRNA of 18.5 cycles per reaction with 10ng of cDNA. This implies firstly that a large proportion of the RNA recovered was human RNA rather than bacterial RNA, and that the low yield from each swab might account for the lack of bacterial signal amplification.

However, despite the positive 18s rRNA result from throat swabs, the immunoglobulin heavy chain gene (IgHG) was only detected in swabs from 2 participants – patient 7 (*S. pyogenes* negative) and volunteer 23 (also *S. pyogenes* negative, data not shown). These swabs yielded a higher than average quantity of total RNA (2.8µg and 3.1 µg RNA respectively). It is interesting that there was not a

significant increase in the Immunoglobulin gene production in the patients over the volunteers, though this may represent the poor yield of RNA from the swabs rather than a true result.

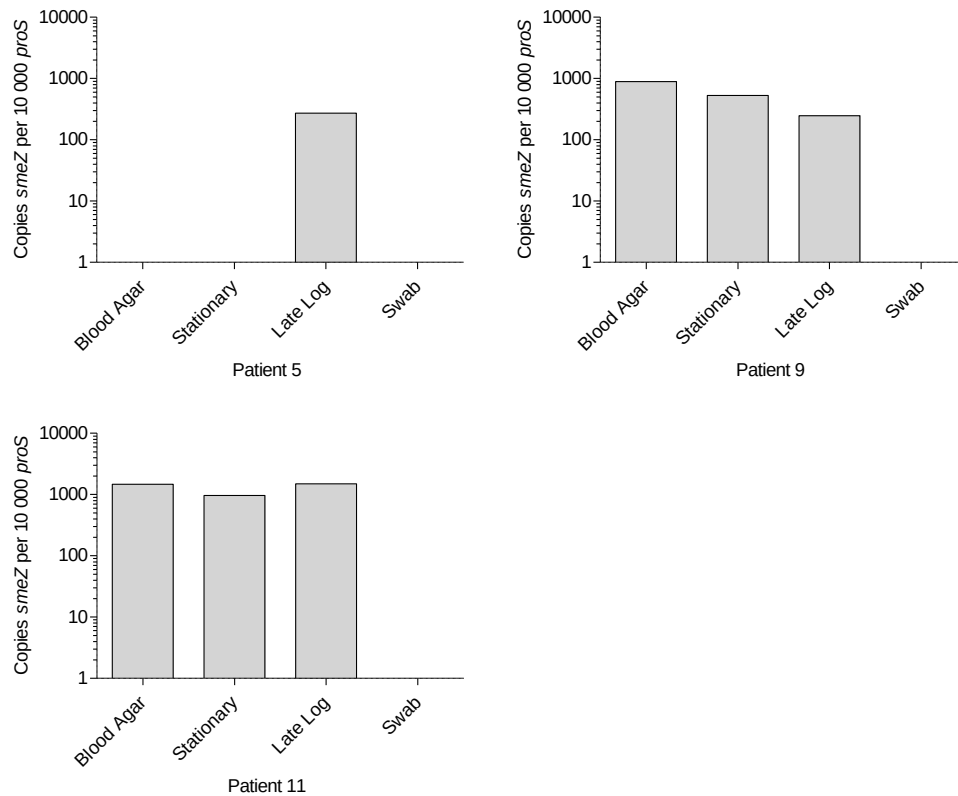


Figure 15: *SmeZ* qRT-PCR from throat swabs and matching bacterial isolates

Quantitative real-time RT-PCR from the three patients recruited to the clinical study with positive cultures for *S. pyogenes*. Bars represent the copies of *smeZ* per 10,000 copies of the housekeeping gene *proS* at three different *in vitro* growth phases for each strain (on blood agar, overnight stationary phase broth culture and late-log phase broth culture) compared to directly from the throat swab for each patient. For patient 5 *smeZ* transcripts were not detected in RNA extracted from Blood agar or overnight broth culture. No RNA transcripts for either *smeZ* or *ProS* were detected in RNA extracted directly from the throat swabs. Each experimental sample was run in triplicate for each gene, and the ratio determined using the mean copy numbers.

3.1.3 Expression of virulence factors in clinical samples from cases of necrotising fasciitis

Tissues were collected from 10 patients with necrotising fasciitis between 2007 and 2010. Of the 10 patients, 9 were operated on by the plastic surgery department at Chelsea and Westminster Hospital, and all 10 patients had *Streptococcus pyogenes* isolated from the site of infection and/or blood cultures by standard microbiology techniques in a Clinical Pathology Accreditation (CPA) scheme approved diagnostic microbiology lab. In total 23 tissue or body fluid samples were collected from the 10 patients. An example of the clinical tissue is shown Figure 16. This demonstrates that the tissue was markedly devitalised and greyish in colour, and there was a copious quantity of bacteria-filled exudate from the tissue – the characteristic “dish-water fluid” of necrotising fasciitis.



Figure 16: Example of debrided tissue

Tissue from patient H700. The left hand image shows the tissue as it arrived in the microbiology lab. The fluid around the tissue demonstrates the classic appearance of “dishwater fluid” which is seen in *S. pyogenes* necrotising fasciitis, and has exuded from the tissue after surgery. The right hand image shows the unfolded tissue, which is a piece of devitalised fascia measuring approximately 10 x 6cm.

The details of the clinical infections and tissues collected are listed in the tables in Figures 17 to 26, where the results for each patient are presented. All bacterial isolates represented the first sample from which the *S. pyogenes* was isolated, and with the exception of one case (H665) these were intra-

operative samples. Additional operative samples were available from repeated surgical debridement on days 2 and 4 for case H629. Antibiotic effect was noted on direct plating of all tissue samples which were positive for bacterial culture. For each clinical strain isolated, *emm* typing and superantigen genotyping was performed. Four of the isolates were found to be *emm* 1, two were *emm* 89 and one *emm* 3, reflecting the circulating *emm* types of invasive *S. pyogenes* in the UK (Luca-Harari, Darenberg, Neal et al. 2009).

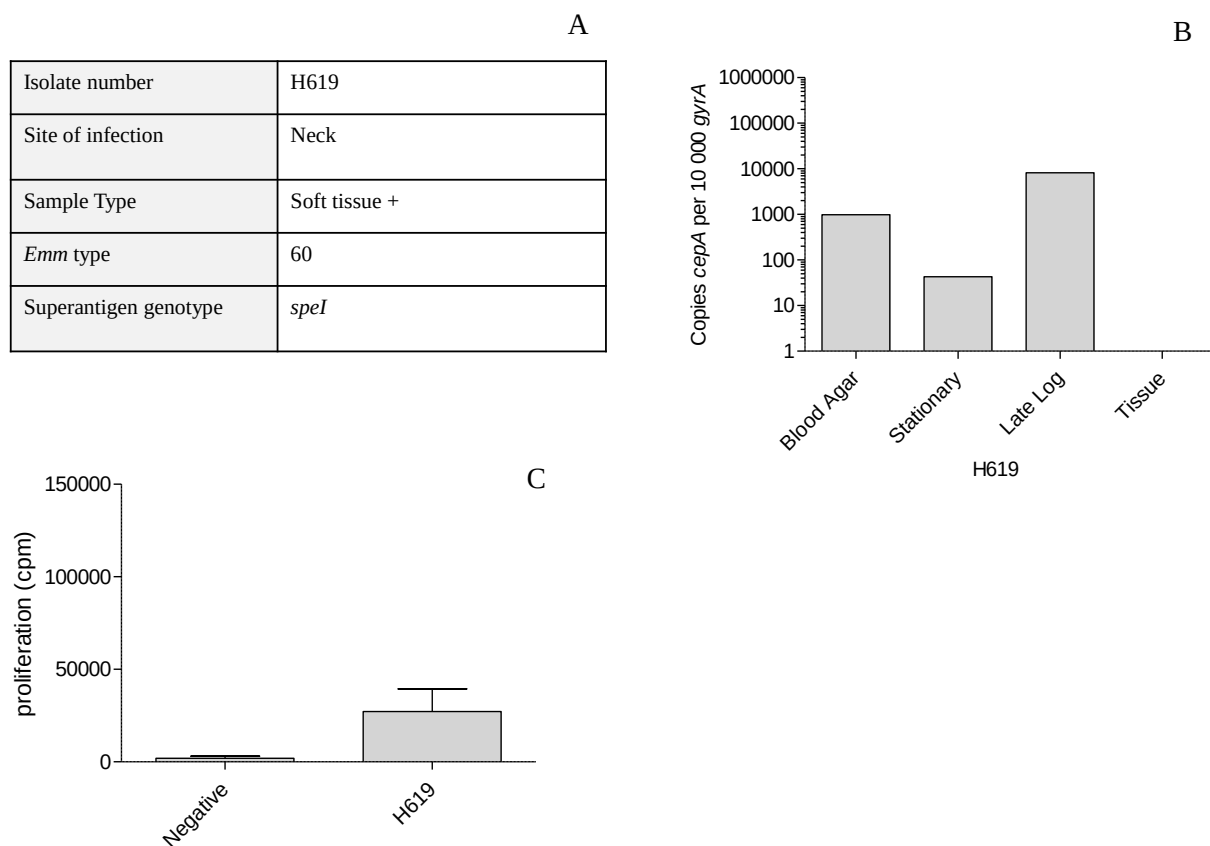


Figure 17: Clinical case H619

A/ details of clinical case H619. + represents positive bacterial culture from tissue sample on arrival in the laboratory. B/ expression of *cepA* *in vitro* and in the tissue sample, expressed as copies per 10,000 copies *gyrA* by qRT-PCR. C/ proliferation of human PBMC by isolate H619 bacterial culture supernatant. Mean and s.d. of 3 separate PBMC donors shown. No proliferation value was obtained from the tissue sample due to live bacterial contamination.

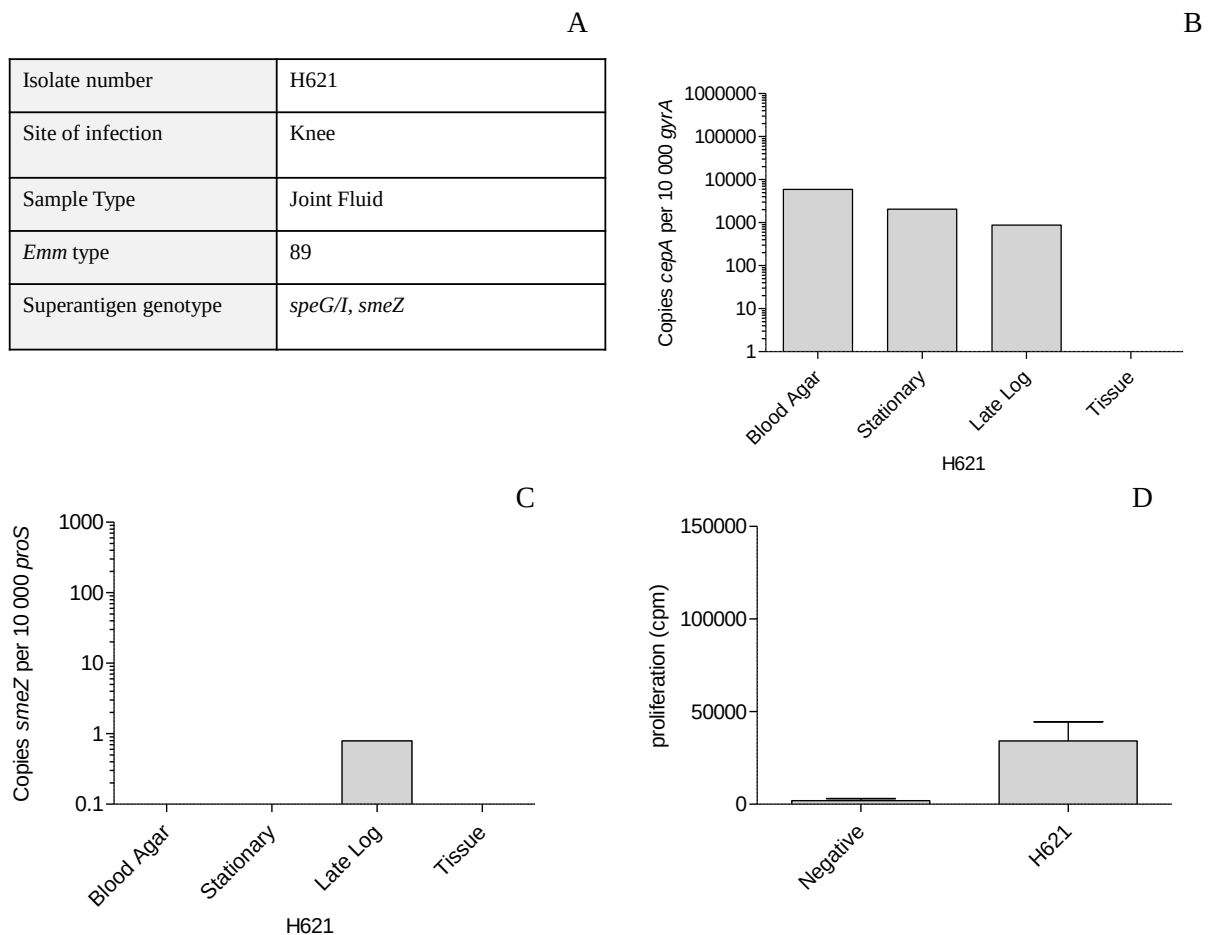


Figure 18: Clinical case H621

A/ details of clinical case H621. B/ qRT-PCR expression of *cepA* per 10,000 *gyrA* both *in vitro* and in the fluid sample. C/ transcripts of *smeZ* were only detected in late log growth *in vitro* by qRT-PCR, expressed as copies per 10,000 *proS*. D/ proliferation of human PBMC by isolate H621 bacterial culture supernatant. Mean and s.d. of 3 separate PBMC donors shown. No proliferation value was obtained from the fluid sample due to bacterial contamination.

For each set of clinical samples, the following investigations were attempted: qRT-PCR for *cepA* (the gene encoding SpyCEP, a *S. pyogenes* virulence factor which functions as an IL8 cleaving proteinase and is known to be associated with necrotising fasciitis (Kurupati, Turner, Tziona et al. 2010)) directly from tissues and from the same bacterial isolate culture *in vitro* on CBA and in stationary and late log broth cultures; qRT-PCR for superantigens *smeZ* and *speA* where the isolate was genotyped as positive for these superantigens from tissues and matching *in vitro* cultures; human PBMC proliferation assays using bacterial culture supernatants and supernatants from tissues homogenised in PBS. These results are presented in Figures 17 to 26.

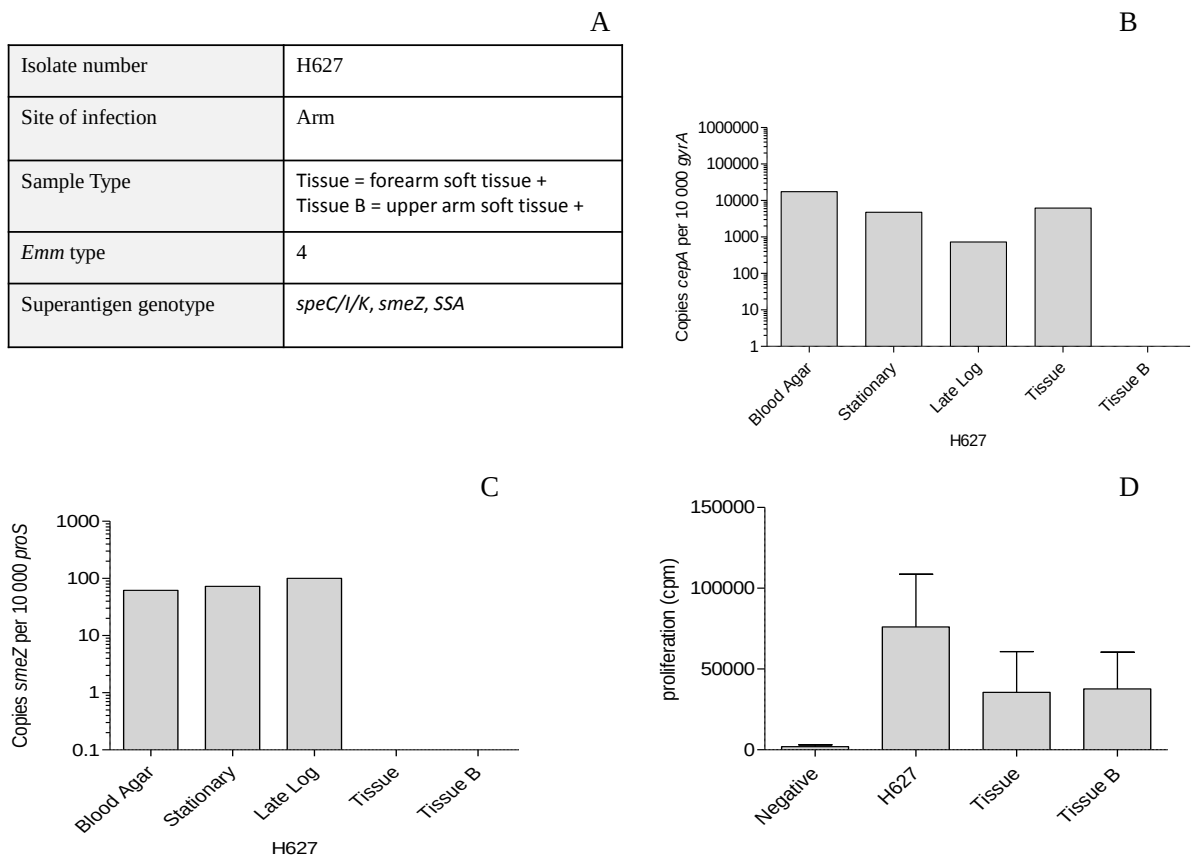


Figure 19: Clinical case H627

A/ details of case H627. + represents positive bacterial culture from the tissue sample when received in the laboratory. B/ expression of *cepA* *in vitro* and in tissue samples by qRT-PCR, expressed as copies per 10,000 *gyrA*. C/ expression of *smeZ* *in vitro* and in tissue samples by qRT-PCR, expressed as copies per 10,000 *proS*. D/ proliferation of human PBMC by overnight bacterial culture supernatant (labelled H627) and from tissue samples homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

The superantigens of interest for further study by qRT-PCR were the phage encoded streptococcal pyrogenic exotoxin A (SPEA) and the chromosomally encoded streptococcal mitogenic exotoxin Z (SMEZ). A gene product for *speA* was amplified by PCR in only 5 out of the 10 isolates; the *emm* 1 and *emm* 3 strains H629 (Figure 20), H665 (Figure 22), H669 (Figure 23), H749 (Figure 25) and H758 (Figure 26). This is in accordance with previously described frequencies of *speA* among clinical isolates (Ma, Yang, Huang et al. 2009). Primers for *smeZ* were designed to cross a conserved region of the gene amongst all known alleles. As *smeZ* has been described as present in virtually all strains (Proft et al. 2000), it was surprising that it was not amplified from the *emm* 60 strain H619. Further

analysis of this strain with full length *smeZ* gene sequencing would be necessary to confirm that the gene was genuinely absent, in case a mutation meant that the conserved sequence was not being amplified by the chosen primer pairs. However, the chosen primers for *smeZ* and *speA* relate to the sequences incorporated into the qRT-PCR plasmid, so for further analysis in these tissues, the results of genotyping those primers were the most relevant results for this work.

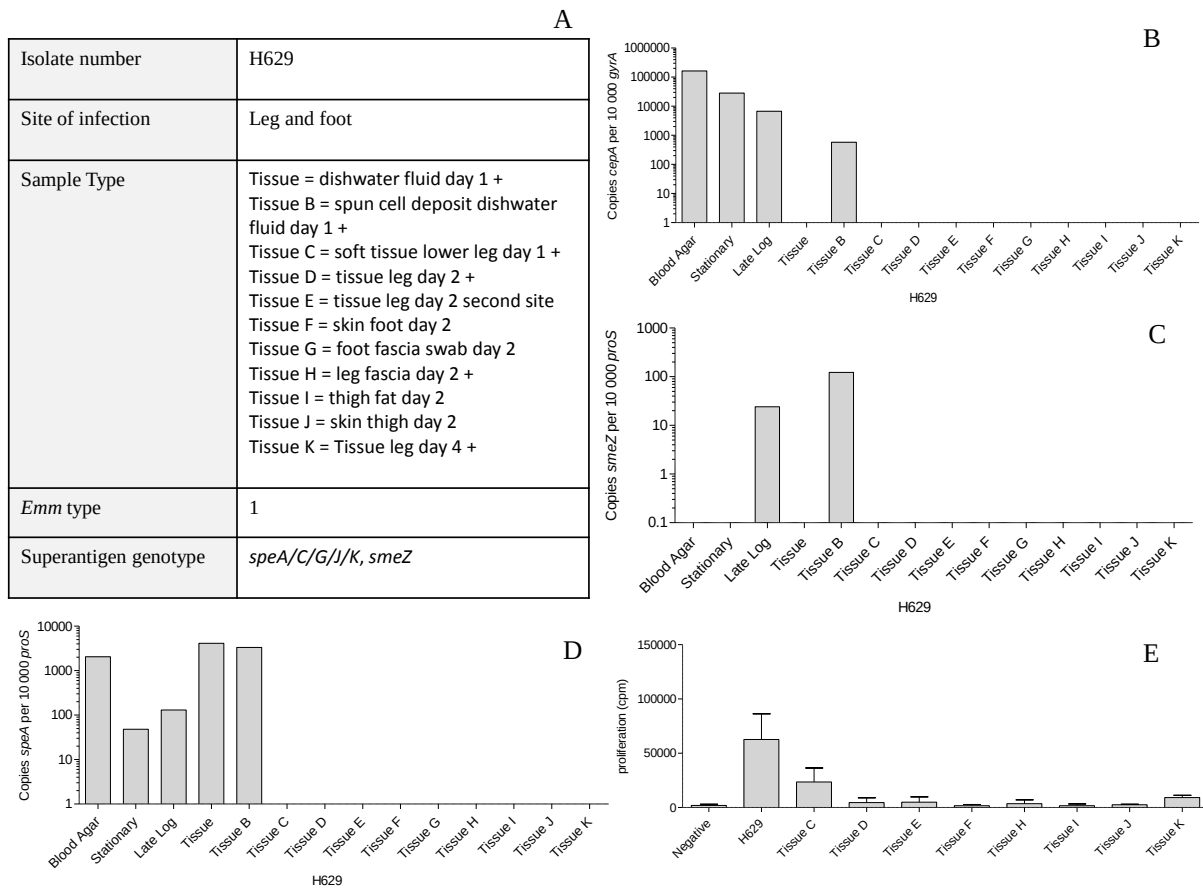


Figure 20: Clinical case H629

A/ details of case H629. + represents positive bacterial culture from the tissue sample when received in the laboratory. B/ expression of *cepA* as determined by qRT-PCR (expressed as copies per 10,000 *gyrA*) *in vitro* and in tissue samples. Expression of *smeZ* (C) and *speA* (D) as determined by qRT-PCR (expressed as copies per 10,000 *proS*). E/ proliferation of human PBMC with H629 bacterial culture supernatant and tissues homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

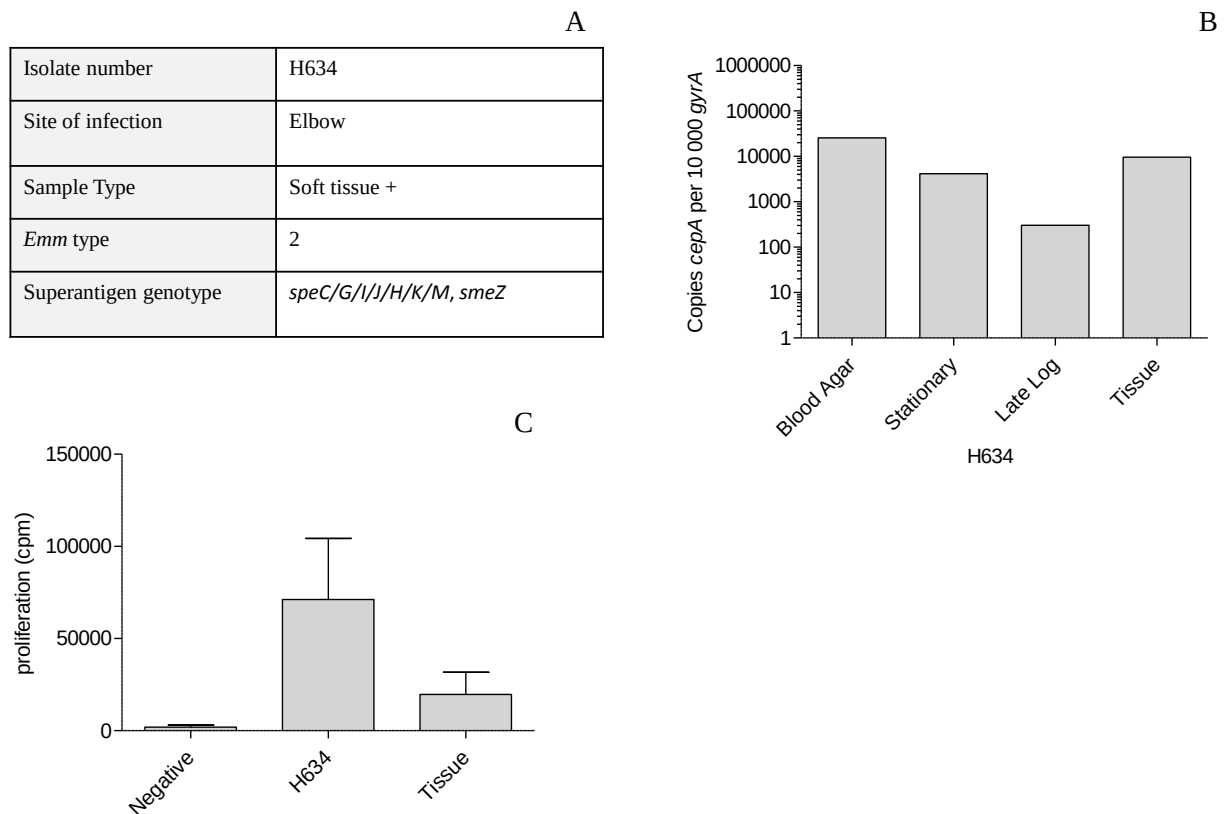


Figure 21: Clinical case H634

A/ details of clinical case H634. + denotes positive bacterial culture when the sample was received in the laboratory. B/ expression of *cepA* as determined by qRT-PCR (expressed as copies per 10,000 *gyrA*) *in vitro* and in the tissue sample. qRT-PCR data for expression of *smeZ* is not shown as no transcripts were detected *in vitro* or in the tissue sample. C/ proliferation of human PBMC by bacterial culture supernatant and the tissue sample homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

The transcription of *speA* has previously been found to be maximal at late log or stationary phases of growth (Unnikrishnan, Cohen, & Sriskandan 1999). To assess transcription of *speA* and *smeZ* in these clinical tissues, RNA was extracted from late log and stationary (overnight) growth in Todd-Hewitt broth and also directly from colonies grown on CBA plates. This was then compared to the result obtained by extraction of RNA directly from the patient tissue / fluid sample from which the isolate had been originally cultured. *CepA* (encoding the IL8 cleaving proteinase SpyCEP) expression was also measured in these samples as a control virulence factor that is known to be expressed by all strains of *S. pyogenes*, and is known to be important in the pathogenesis of necrotising fasciitis (Kurupati et al. 2010). Therefore RNA extracted from all clinical samples was tested for the presence

of transcripts the two housekeeping genes *gyrA* and *proS*, the IL8 cleaving proteinase *cepA*, and superantigens *smeZ* and *speA* (in isolates which were DNA genotype positive for *smeZ* and *speA* respectively).

Of the housekeeping genes, transcripts of both *gyrA* and *proS* were detected in only 7/23 tissue or fluid samples, and transcripts of *gyrA* alone were detected in a further 5 samples; H627 (Figure 19), H629 (Figure 20), H634 (Figure 21), H665 (Figure 22) and H749 (Figure 25). Where detected, *proS* transcript copy numbers were consistently higher than those of *gyrA* (median 35 times higher, range 9 to 159 times, data not shown). 10 clinical samples yielded no transcripts for either *gyrA* or *proS*. Further analysis of virulence gene transcription in these 10 samples was therefore not attempted.

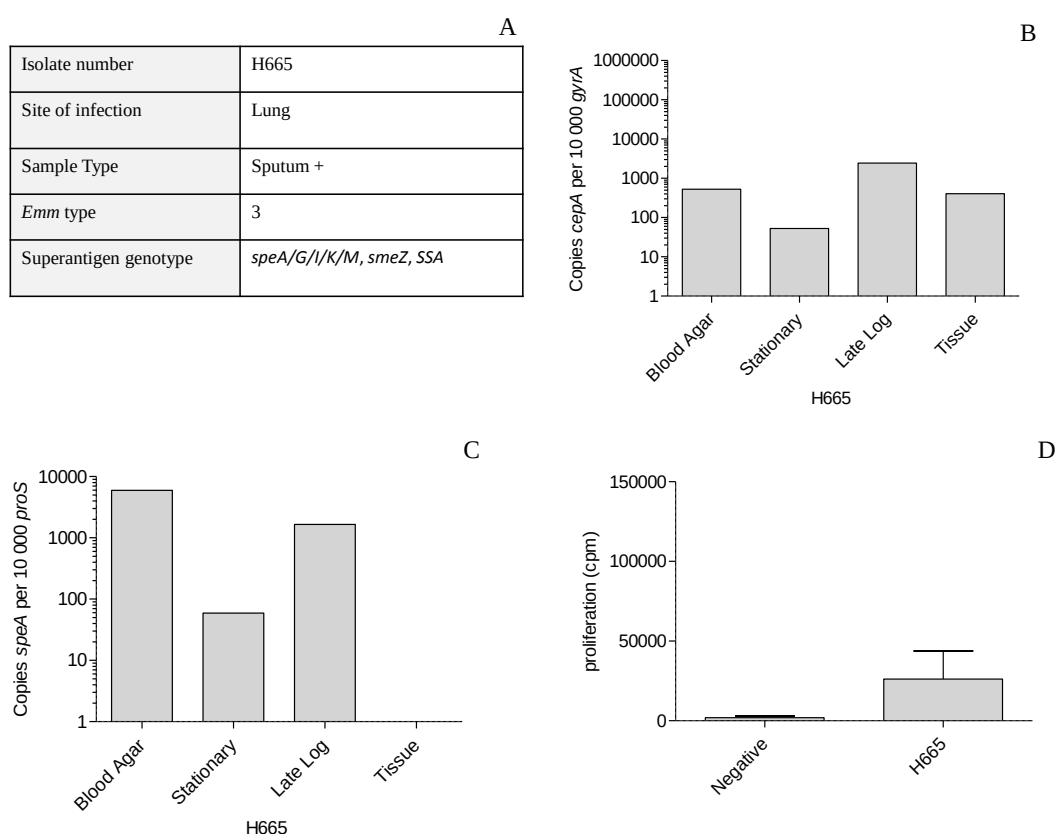


Figure 22: Clinical case H665

A/ details of clinical case H665. + denotes that the sputum sample was culture positive for *S. pyogenes* on reaching the laboratory. B/ expression of *cepA* *in vitro* and in the sputum sample (expressed as copies per 10,000 *gyrA*, by qRT-PCR). C/ expression of *speA* *in vitro* and in the sputum sample (expressed as copies per 10,000 *proS*, by qRT-PCR). Expression of *smeZ* is not shown as no transcripts were amplified either *in vitro* or in the sputum sample. D/ proliferation of human PBMC upon stimulation with H665 bacterial culture supernatant. No result is shown for the sputum sample due to contamination with fungal spores. Mean and s.d. of 3 separate PBMC donors.

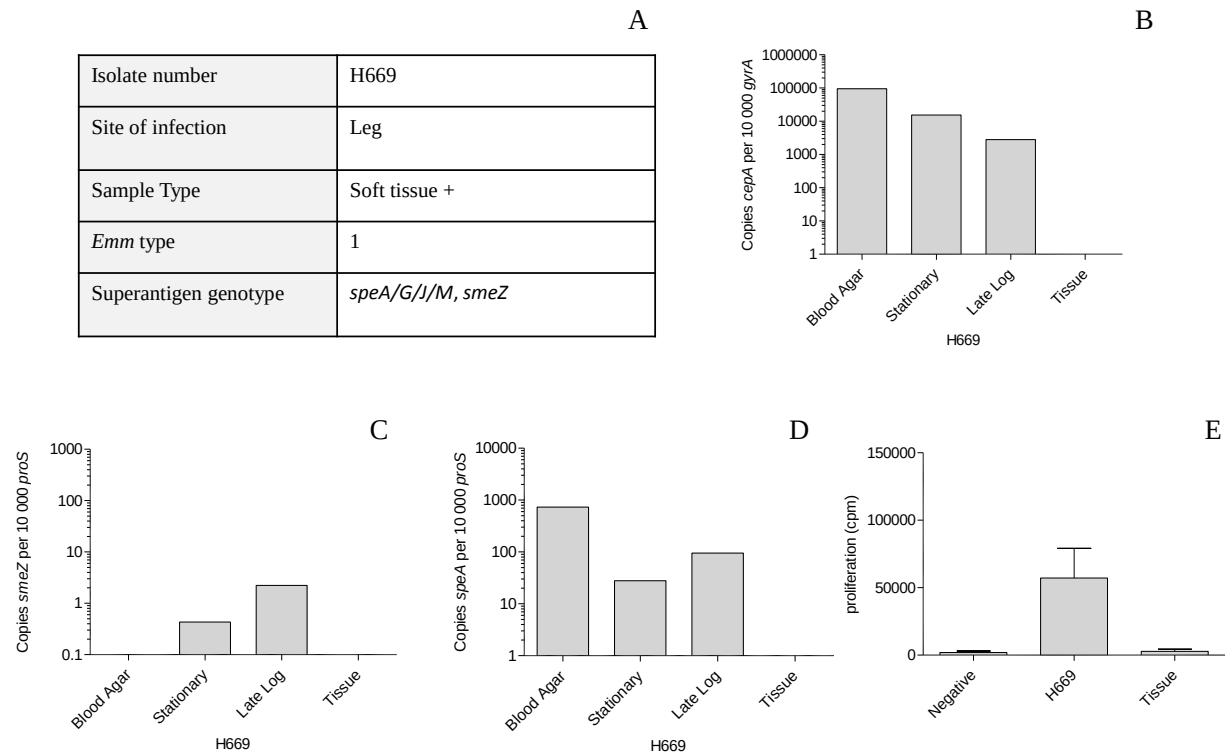


Figure 23: Clinical case H669

A/ details of clinical case H669. + denotes positive culture result for the tissue sample on receipt in the laboratory. Transcripts of *cepA* (B), *smeZ* (C) and *speA* (D) as determined by qRT-PCR, expressed as copies per 10,000 *gyrA* (B) or *proS* (C and D), both from the bacterial isolate *in vitro* and from the tissue sample. E/ human PBMC proliferation in response to the bacterial isolate H669 culture supernatant and the tissue sample homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

CepA transcripts were detected in 5 of the 7 clinical samples where both housekeeping genes were detected, and one where *gyrA* alone was detected (a total of 6/23 clinical tissue/fluids, Table 9). There was no correlation between the values of *cepA* (when normalised to either 10,000 *gyrA* or *proS*) detected in the clinical samples and the same isolate grown in any of the laboratory conditions tested. This lack of correlation most likely represents a difference in SpyCEP production *in vitro* compared to *in vivo* for these strains, presumably due to host factors or tissue storage factors before the tissue was received in the laboratory for RNA extraction; SpyCEP is predominantly regulated by *CovRS* and therefore is highly likely to exhibit differences between the two conditions, but expression may also have been affected by concomitant antibiotic administration (Olsen & Musser 2010).

Copies of <i>CepA</i> per 10,000 <i>GyrA</i>				
	Blood Agar	Stationary	Late log	Clinical sample
Minimum	528	42.67	171.7	402.9
Median	21500	10090	2620	6229
Maximum	349110	56246	17400	53474

Table 9: Range of *cepA* expression

Median and range of copies of *cepA* per 10,000 *gyrA* both *in vitro* (all 10 bacterial isolates) and in the 5 clinical samples where RNA transcripts were detected.

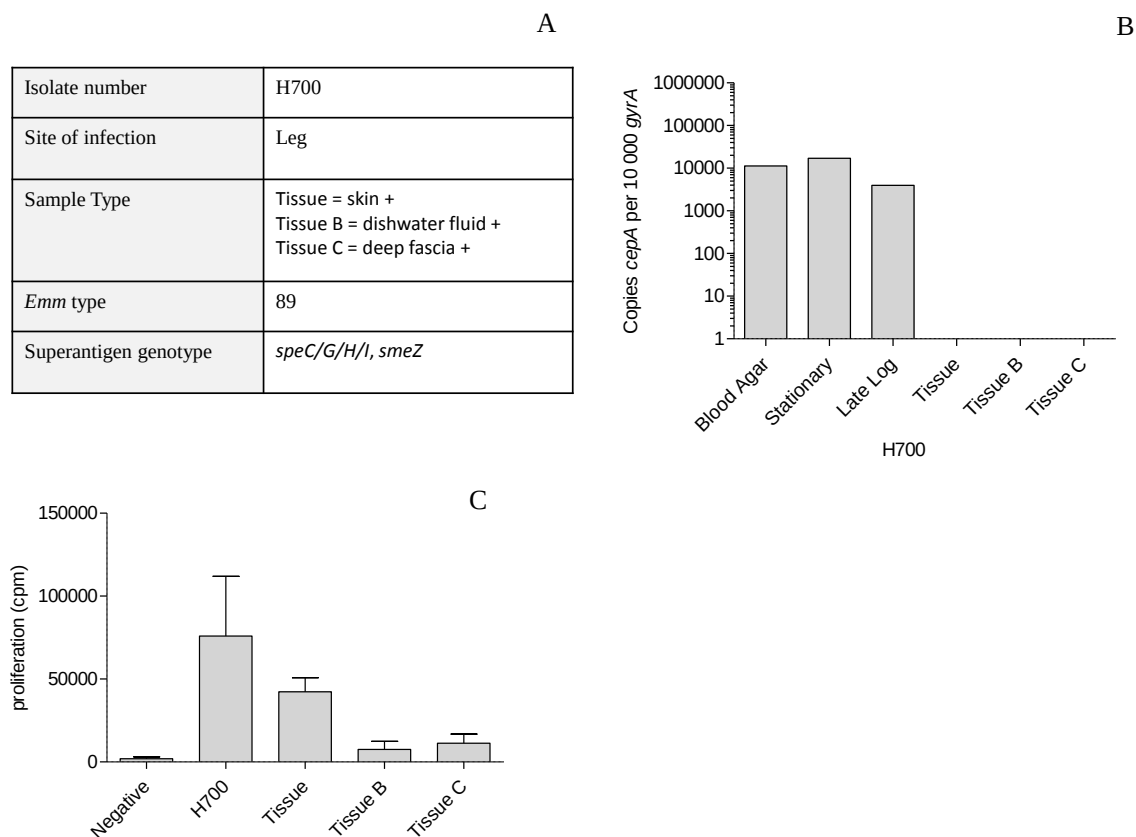


Figure 24: Clinical case H700

A/ details of clinical case H700; + represents positive bacterial culture from the tissue / fluid sample when received in the laboratory. B/ *cepA* expression as determined by qRT-PCR from the bacterial isolate cultured *in vitro* and directly from the tissue / fluid samples (expressed as copies per 10,000 *gyrA*). qRT-PCR results for *smeZ* expression are not shown as no transcripts were detected either *in vitro* or in the clinical samples. C/ human PBMC proliferation in response to stimulation with the bacterial isolate H700 culture supernatant or directly with tissues homogenised in PBS or fluid samples. Mean and s.d. of 3 separate PBMC donors.

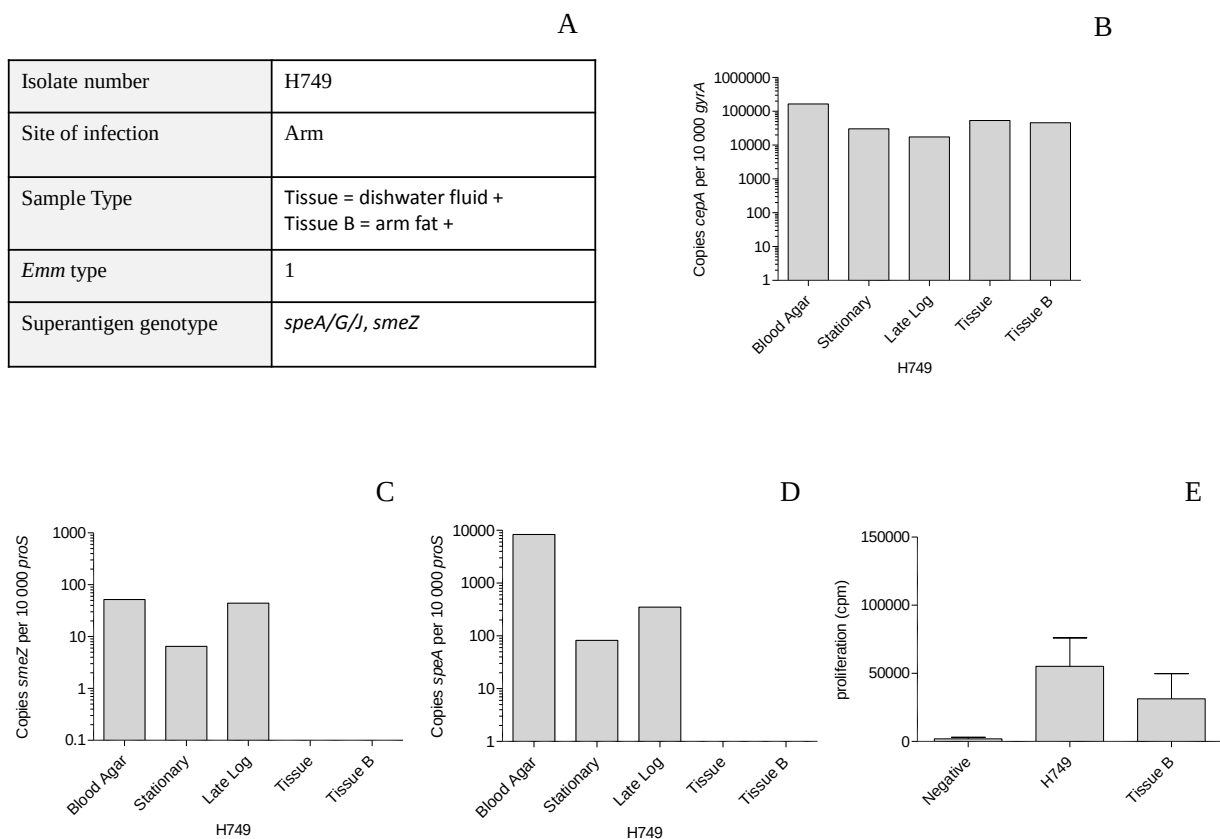


Figure 25: Clinical case H749

A/ details of clinical case H749; + denotes positive bacterial culture from the fluid / tissue samples on arrival in the laboratory. Expression of *cepA* (B), *smeZ* (C) and *speA* (D) from *in vitro* bacterial culture and in the fluid / tissue samples was determined by qRT-PCR (expressed per 10,000 copies *gyrA* (B) or *proS* (C and D)). E/ human PBMC proliferation with bacterial culture supernatant and tissue B homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

Expression of *smeZ* was variable *in vitro*, with expression being mainly detected in late log broth culture with a mean of 19 copies *smeZ* per 10,000 copies *proS*. For three samples, *smeZ* transcripts were not detected in any of the tested *in vitro* culture conditions (H634, H665 and H700). However, these *in vitro* results contrast strongly with the results of the throat isolates collected in the paediatric study (Figure 15), where the mean was 671 copies *smeZ* per 10,000 copies *proS* ($p=0.014$ Mann Whitney test). This is consistent with up-regulation of *smeZ* expression in the throat, although numbers are too small to draw firm conclusions.

SmeZ transcripts were detected only in one tissue sample which was the dishwater fluid from patient H629 (Figure 20C), with 122 copies *smeZ* per 10000 *proS*. *SmeZ* transcripts were also detected in two samples where no housekeeping gene transcripts were detected (H621 knee fluid, 114 copies and H627 arm tissue, 37 copies), but these could not be used for further analysis due to the lack of a comparator transcript.

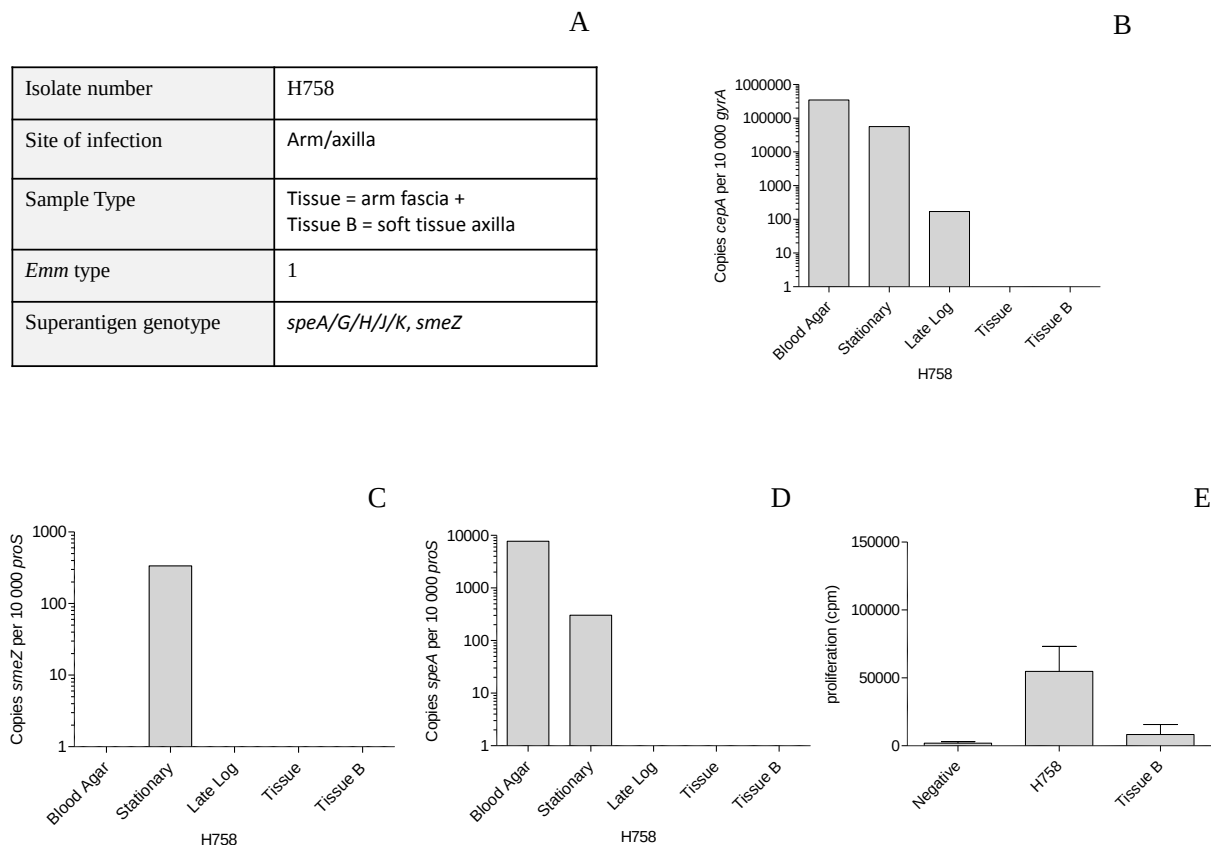


Figure 26: Clinical case H758

A/ details of clinical case H758; + denotes positive bacterial culture from tissues on receipt in the laboratory. Expression of *cepA* (B), *smeZ* (C) and *speA* (D) as determined by qRT-PCR are expressed as copies per 10,000 *gyrA* (B) or *proS* (c and D), for both bacteria culture *in vitro* and directly from tissue samples. E/ human PBMC proliferation on stimulation with bacterial culture supernatant (H758) and supernatant from tissue B homogenised in PBS. Mean and s.d. of 3 separate PBMC donors.

Expression of *speA* was significantly higher in RNA extracted from growth on blood agar (mean of 43,142 copies per 10,000 *proS*) than in stationary (mean of 12503 copies per 10,000 *proS*) or late log broth culture (mean of 5754 copies per 10,000 *proS*), $p=0.0055$ Friedman test of paired samples. However, as with *smeZ*, *speA* transcripts were only detected in the tissue samples from one patient, H629 (Figure 20D), where there were 3326 copies *speA* per 10,000 *proS*. The un-spun fluid sample from this patient also yielded 4123 copies *speA* per 10,000 *proS*. In this patient, *speA* expression exceeds that of *smeZ*, suggesting that the regulatory mechanisms that govern phage-encoded superantigen genes such as *speA* are distinct from those that regulate chromosomally-encoded superantigens.

3.1.3.1 Functional detection of bacterial superantigens in tissues

Although there was little superantigen gene transcription detected in the clinical samples on qRT-PCR, this does not necessarily represent the functional status of the bacterial superantigens in the tissues. Previous *in vivo* studies have shown that SPEA could be detected in the liver of mice which had been infected with SPEA producing bacteria (Sriskandan et al. 1996), suggesting that there is likely to be production of superantigens at some point during the course of natural clinical infection. Furthermore the mitogenic effect of superantigens can be detected in the blood of patients with streptococcal toxic shock syndrome (Proft et al. 2003).

As superantigens are known to cause marked T cell proliferation, the supernatants of tissues which had been macerated in sterile PBS were used to stimulate healthy donor PBMCs, and the resultant T-cell proliferation measured as H^3 -thymidine incorporation for the last 12 hours of culture (counts per minute). This was compared to the proliferation caused by the supernatant of the same bacterial strain after overnight growth *in vitro*.

All of the clinical tissue/fluid samples which had a viable laboratory culture of bacteria when received in the diagnostic microbiology lab, caused proliferation of the PBMCs above baseline levels. Three clinical samples were excluded from the analysis due to live bacterial (H619, Figure 17, and H621, Figure 18) or fungal contamination (sputum H665, Figure 22). Of the remaining tissues, the only ones

which caused no proliferation were those from the distal resection margins on the second day of surgery from patient H629 – tissues F, I and J, Figure 20E.

The proliferation data shows that although it was not possible to detect superantigen gene transcripts for *smeZ* and *speA* in the majority of the tissues in invasive disease, mitogenic effects of the tissues could be demonstrated, which could represent the presence of active superantigens within infected tissues.

3.2 Discussion

Both of the clinical studies presented here demonstrate the difficulties of working with patients to obtain meaningful *in vivo* data. Both studies showed difficulties in obtaining samples in a timely fashion – either with recruitment and sampling issues (paediatric study) or delays in sample processing (clinical tissues study). Without the active interest and participation of a large number of NHS employees in different departments, neither of these studies would have recruited any patients, and shows how reliant clinical studies are on the good will of the clinical colleagues.

3.2.1 Clinical paediatric study

There were several practical problems with the study, reflecting elements of the study protocol that were necessary for ethical approval or practical microbiological processing of the swabs. Firstly, recruitment of cases was poor, with only 3 children having positive throat swabs for *S. pyogenes*. The rapid test proved unhelpful, with only 1 in 3 of the positive cases being detected by the rapid test. The sensitivity of all bedside testing kits for *S. pyogenes* has been questioned in a number of trials (Clerc & Greub 2010), being dependent on achieving a good swab: as a result they are not routinely used in a clinical setting in the UK, with their poor cost effectiveness and low negative predictive value. The study was dependent on the Paediatric Ambulatory Unit staff members identifying appropriate cases, and a number of potential cases were seen and discharged by the ambulatory unit general practitioners rather than the paediatric staff, without recruitment to the study, due to the pressure of bed space and high patient turnover. In the UK, the majority of cases of tonsillitis and pharyngitis are reviewed and treated in a primary care setting, before attending hospital for review.

This study would be better performed in a primary care setting, with well motivated general practitioners who were able to obtain the consent and acquire the swabs at the initial consultation

rather than calling a study doctor to appear. Although the ethics allowed the attending physicians to obtain consent and the research swabs (and supplies of information sheets and swabs were left in the department) the staff were unwilling to recruit patients to the study themselves, preferring the swabs to be taken by the study principal investigator (myself) in each instance. Although this improved the consistency of the sampling and level of information provided to participants, several potential recruits were lost as they were either unwilling to wait or the child refused to show their throat to a new doctor, having already submitted to one examination. This was particularly the case for younger children where there is often only a single chance to swab the throat, usually at the initial throat examination, and may have accounted for the poor yield from some throat swabs.

3.2.2 Quantitative RT-PCR from throat swabs

The yield and quality of both RNA and DNA extracted from the swabs was poor, with no *S. pyogenes* RNA or DNA being amplified from even those swabs which were positive on culture. Although other methods of RNA and DNA extraction from swabs have been described, this was the only method available which was practical with the clinical aspects of the study design and still extracted both RNA and DNA. The average of 1.2µg of RNA per swab compares poorly to other studies, where an average of 4.8µg of total RNA per sample was extracted. This may have rendered the mRNA levels below the limit of detection estimated as necessary to achieve a positive signal in real-time RT-PCR (Virtaneva et al. 2005). Although the cDNA synthesis reaction was standardised to a set amount for each swab, with low concentrations of RNA it is possible that error of measurement in the initial concentration occurred for some samples.

Despite the use of the Bacterial Max enhancement reagent, there was a proportionately higher yield of human than bacterial RNA, as demonstrated by the positive 18srRNA result for each samples. However the CT values for 18s were high, approximately double those from human cells in culture, which again reflects the poor RNA yield, and why the IgHG gene was below the limit of detection for

most of the samples. Unfortunately this resulted in no meaningful comparison of immunoglobulin responses in patients with or without *S. pyogenes* as a cause of pharyngitis. In future, the detection of salivary IgA levels might be a more practical way to measure immunoglobulin responses to superantigens *in vivo*, as this is very simple to collect, and has previously been used to show that *S. pyogenes* increases the level of salivary IgA produced (Brandt, Hayman, Currie et al. 1999).

3.2.3 Quantitative RT-PCR from necrotising fasciitis tissues

There are a number of possible explanations as to why superantigen RNA transcripts were not detected in the patient tissue samples. Firstly, they may be only produced at very low levels in clinical disease, below the limits of detection in these tissue samples by the qRT-PCR methods used. Secondly, the transcripts might be unstable or have been degraded by tissue RNases or storage at 4°C for several days prior to processing. Equally the impact might be that bacteria were able to increase in numbers in some samples during storage, those where the virulence factors were identified. Thirdly, it is possible that the other superantigens detected in the isolated on genotyping were being produced in preference to SMEZ and SPEA, depending on the HLA type of the patient (Norrby-Teglund, Nepom, & Kotb 2002). Fourthly, it is possible that superantigens had been produced early in the development of disease, but were no longer being produced as there was sufficient accumulated toxin already available. Fifthly, all samples demonstrated the presence of an antibiotic effect in the tissues / fluids. It is possible that the use of antibiotics, particularly protein synthesis inhibitors, had caused the bacteria to enter a growth phase where superantigens were not needed to promote bacterial survival. Similarly, it is possible that the storage process had altered the bacterial metabolism, causing a dormancy or persister state to occur, which would account for why the bacteria could be subcultured but products not detected directly from the tissues.

3.2.4 Functional assessment of superantigens in patient tissues

Despite the poor results from qRT-PCR, the functional assay for bacterial superantigens using PBMC bioassay did demonstrate mitogenicity in the clinical tissues. The results were very consistent between PBMC donors and there was a degree of proliferation in every sample where viable bacteria had been detected.

To conclusively prove that the proliferation was due to superantigens, it would be best to neutralise the superantigens by the inclusion of specific neutralising antibodies to each superantigen present on genotyping. However, antibodies to the majority of superantigens are not commercially available, and creating recombinant superantigens and raising neutralising antibodies to them was not within the scope of this project. For similar reasons, and because of concerns about sensitivity, toxin quantification was not attempted by ELISA or western blotting. An alternative strategy to determine if the mitogens present are superantigens, would be to co-incubate the supernatants with PBMC and determine, by flow cytometric methods, whether specific T cell subsets are expanded. This information could then be used to deduce the range of superantigens present, since the V β specificity of each streptococcal superantigen is known.

3.2.5 Analysis of superantigens in patients with clinical disease

With the large number of technical issues, it is easy to overlook the importance of these findings. Firstly, there was evidence of mitogenicity at the site of infection, as demonstrated by the PBMC proliferation data, which may indicate the presence of superantigens in the tissues. This was surprising as the samples were operative specimens, and so collected after the administration of first dose antibiotics. Standard antibiotic therapy for necrotising fasciitis in the United Kingdom currently includes the use of protein synthesis inhibitors, such as clindamycin, which should have stopped production of superantigens. These findings may add weight to the *in vitro* findings of Stevens et al

that administration of beta-lactam antibiotics can actually increase the production of bacterial toxins before bacterial killing is achieved (Stevens, Ma, Salmi et al. 2007).

The other finding of note was that *in vitro* production of *smeZ* was significantly higher from the throat strains than from the invasive strains. The numbers of samples tested were small, as they related only to the patients recruited to the two studies, and so confirmation would need to be made from a number of different strains and with more *emm* types. It would also be interesting to compare production of *speA* between a number of throat and invasive isolates, to see if this was a specific effect to SMEZ, or a more general superantigen effect. However, this supports the theory that superantigens are of greatest importance in the pathogenesis of pharyngeal disease rather than invasive disease.

Finally, the expression of *speA* was significantly higher when bacteria were grown on CBA than in broth culture. It is difficult to ascertain whether this would be due to the presence of factors in the blood (such as complement) or the difference between growth on solid media rather than liquid culture. To answer this question, further work would need to address the expression of *speA* in blood-containing liquid media or Todd-Hewitt agar plates, though there was not time to perform these experiments within this project.

To try to address the production of superantigens in the pharynx, an *ex-vivo* live bacterial – tonsil co-culture method has been developed. As well as the production of superantigens, the effects of those superantigens on the immune responses of tonsils are addressed in chapters 4 and 5.

4 An *ex vivo* system of experimental *S. pyogenes* tonsillitis

The human pharynx is the major site for disease due to *S. pyogenes*, and a large number of people remain as asymptomatic carriers of *S. pyogenes* in the pharynx, providing a potential reservoir for future disease. Despite this, the local immune responses in the human pharynx have been poorly investigated or described to date. This is mainly due to the inaccessibility of human tonsils, the major immune component of the pharynx, making it difficult to study their immunology during acute infection. Tonsillectomy during acute infection is relatively contraindicated, due to the risk of fatal haemorrhage if an acutely inflamed vascular area is operated on and subsequent operative bed infection (Ahmad, Abdullah, Amin et al. 2010); the only exception being quinsy (tonsillar abscess) where a discrete collection can be drained. Although some insights can be gained by analysing saliva and throat swabs of pharyngeal cells during infection (Lilja et al. 1999), this is only analysing the surface and not a true representation of the tonsil core. Similarly analysing serum and peripheral blood cells during acute infection can give an indication of the systemic response to the infection, but not the local response.

This project set out to create an *ex-vivo* system for analysing tonsils, and to subject them to infection with *S. pyogenes*, to allow for analysis of both bacterial and host responses in this system.

4.1 Results

4.1.1 Tonsil cell culture models

The underlying diagnosis/reason for surgery was collected for all tonsil donors. In the majority of cases there was chronic enlargement of the tonsils due to recurrent episodes of tonsillitis. Samples from the first 22 tonsil donors received for the experiments were independently reviewed by a histopathologist, with H&E stains being examined. In 5 cases there was follicular hyperplasia, 16 had reactive lymphoid hyperplasia, and 1 donor mixed acute and chronic inflammation with abscesses. 13 donors had the presence of Actinomycosis reported (see appendix 1).

Human tonsils inherently carry a large amount of endogenous bacterial flora, particularly when there are signs of chronic infection such as the presence of tonsoliths. The most frequent contaminating bacteria in the culture system developed were *Staphylococcus aureus*, followed by endogenous *S. pyogenes*, other beta haemolytic streptococci and *Candida* species (data not shown). The presence of Actinomycosis noted in 13 of the tonsils where formal histopathology was performed confirmed chronic infection in these donors. Normal upper respiratory flora such as *Moraxella catarrhalis*, alpha-haemolytic streptococci and diptheroids were also isolated from all tonsils, though in lower numbers than *S. aureus* or the beta-haemolytic streptococci. The recipe for tonsil media was adapted from “Dissection media” by Freshney (Freshney 2005), with alterations of the antibiotics to suit the contaminating flora – namely high doses of penicillin and streptomycin, kanamycin and Amphotericin B. In most cases this antibiotic mixture was sufficient to fully suppress bacterial growth, and so was used for cell suspension culture.

An alternative method would have been to pass the tonsil cells over a Ficoll-Paque Plus gradient (GE Healthcare Lifesciences, UK), and collect the lymphocyte layer (Johnston, Sigurdardottir, & Ryon 2009). However this was not done as there are a number of large structural cells in the tonsils, such as

follicular dendritic cells, which play an important role in antigen presentation and would have been removed by a Ficoll-Paque gradient.

Both cell suspension cultures and histocultures suffered high levels of contamination with residual flora when they were cultured without antibiotics. In the case of cell suspension cultures, even after 18 hrs incubation in Tonsil media, *S. aureus* or *Candida* species could re-grow within 24 hours of changing to Antibiotic Free media if the tonsils were heavily colonised initially. Therefore, for all immunology studies, cells were grown in Tonsil media throughout the duration of culture, and antibiotic-free culture was reserved for live bacterial co-culture experiments. The immunology results are presented in chapter 5.

For histocultures, two techniques were adopted for the live co-cultures – brief exposure to antibiotics or washing in 60% ethanol. Although the exposure to antibiotics gave better results for removal of endogenous flora, it was difficult to ensure that antibiotics were then removed adequately, and not contributing to the effect of tonsils on bacterial growth. Therefore, although initial histoculture experiments were performed using the antibiotic wash method, it was decided that the ethanol method provided a more robust technique for reliably ensuring co-culture infection.

4.1.2 Tonsil cell suspension cultures – baseline characteristics

The cell types obtained from the tonsils were very consistent: B cells median 48.02% (range 33-57%) and CD3+ T cells median 43.55% (range 36-59%). Within the T cell population the proportions of CD4 and CD8 T cells remained constant: CD4+ median 80.37% (range 78 – 88%) and CD8+ median 12.7% (range 11.6-18%). This is outlined in Figure 27.

The remaining cells were predominantly antigen presenting cells, particularly dendritic cells and macrophages, with up to 6% staining positive for CD11b, the Mac-1 integrin expressed on macrophages, dendritic cells, natural killer cells and granulocytes. These cells types were not further

differentiated for this project, but have been characterised in previous studies (Giger et al. 2004;Plzak et al. 2003). It has been previously noted that macrophages can only be detected for the first 24 hours of either histoculture or cell suspension culture by CD68 expression, and this may account for the lack of specific CD68 staining of cell suspension cultures(data not shown), despite CD11b staining (Giger et al. 2004).

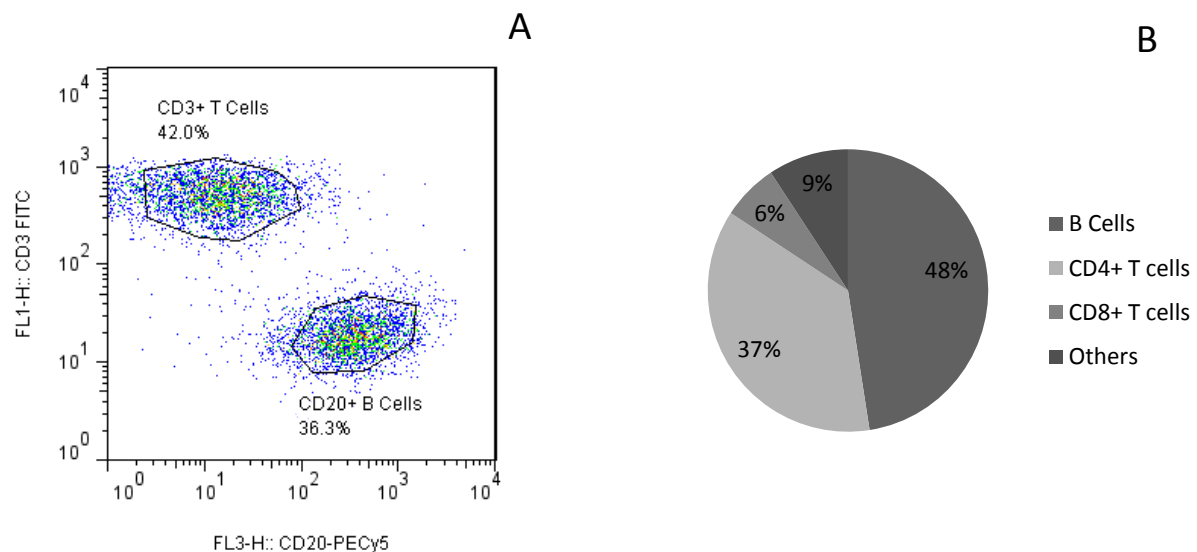


Figure 27: Typical lymphocyte populations in human tonsil

A/ typical flow cytometry plot, showing CD20+ staining B cells (vertical axis) and CD3+ staining T cells (horizontal axis). B/ tonsil lymphocytes have a mean of 48% B cells (s.d. +/- 12%) and 43% T cells (s.d. +/- 9%), of which 85% are CD4+ T cells (37% total lymphocytes) and 15% CD8+ T cells (6% total lymphocytes). N= 15 different tonsil donors

4.1.2.1 Characterisation of tonsil cell suspension populations over time

All tonsil populations examined showed two to three distinct lymphocyte populations, separated by forward and side scatter properties (Figure 28). The high side scatter and forward scatter populations contained mainly CD20 positive B cells (sets 1 and 3), with the T cells concentrated in the low forward and side scatter population (set 2, data not shown). All populations were included in subsequent analysis for B and T cells.

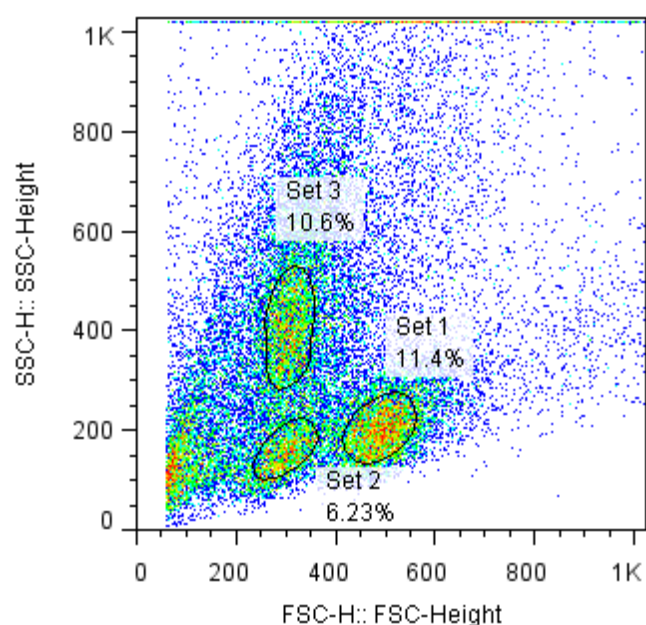


Figure 28: Typical flow cytometry plot of unstimulated human tonsil cells

A typical unstained flow cytometry plot of unstimulated tonsil cells after 1 week of culture. The cells can be seen to form three distinct sub-populations, separated by side and forward scatter properties. Set 1 and Set 3 contain mainly B cells (Staining CD20+) and Set 2 contains mainly T cells (CD3+), data not shown. In some tonsil donors there was no population in the Set 3 position.

Over time in culture, there was variation in the lymphocyte population in un-stimulated cells: as demonstrated in Figure 29, the cells become both larger and more granular over time in culture, along with a general increase in dead cells and debris. Although the total percentage of B and T cells in the whole cell population remained stable throughout culture, the T cells in particular increase in size, with a marked reduction in the low forward scatter cell population, Set 2 as described in Figure 28 above, by the end of 1 week. Lymphocyte activation and apoptosis in these different populations is investigated further in chapter 5.

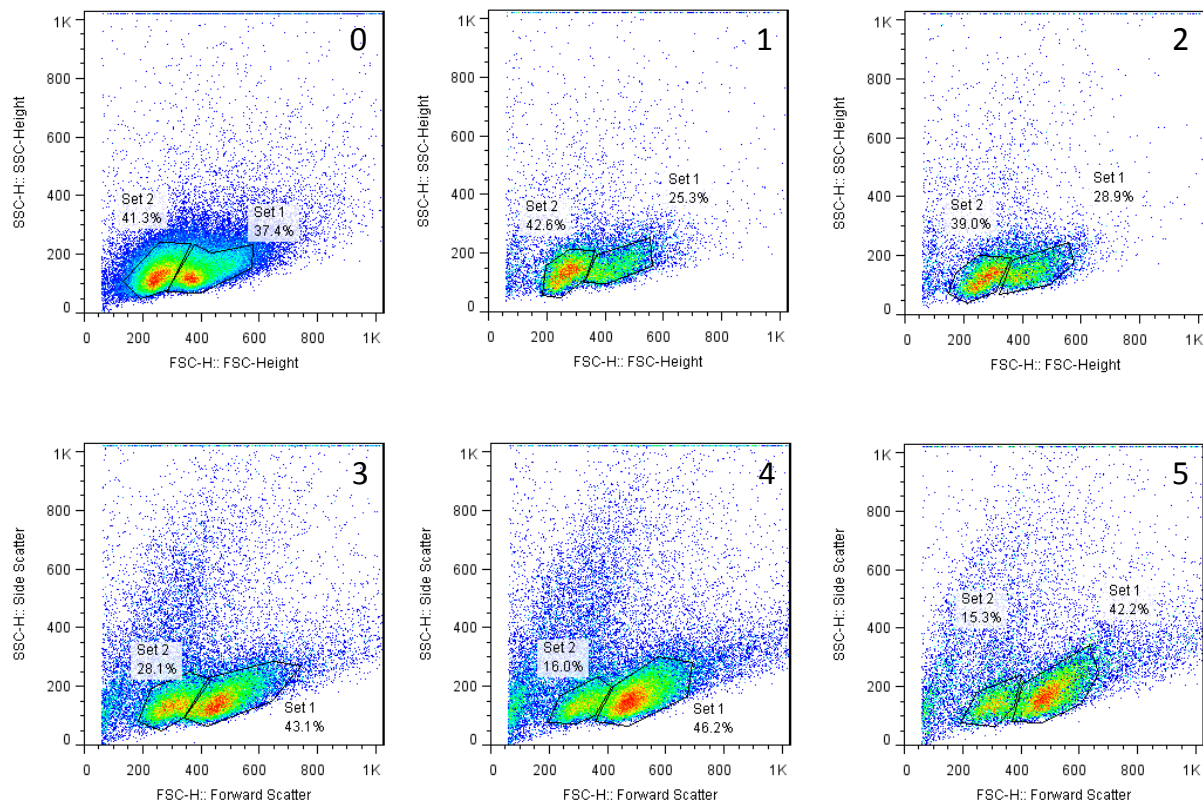


Figure 29: Appearance of lymphocyte populations over time

Unstimulated tonsil lymphocytes alter over time in culture – example of one typical tonsil donor. The days of culture are indicated in the top right corner of each flow cytometry plot, with 0 being the day of tonsillectomy. For the first 3 days of culture there is a depletion in the cells in the right hand group (Set 1), but from day 4 onwards there is an increase in both the forward scatter (representing cell size), and side scatter (representing cell granularity) of the cells. Plots 0 and 3-5 show 25,000 events, plots 1 and 2 15,000 events, all acquired with the same machine settings.

4.1.2.2 Cytokine ELISAs and immunoglobulin production

Cytokine production in unstimulated tonsil samples over the course of 1 week was evaluated by ELISA. There was minimal production detected of the following cytokines: IL1 β , IL2, IL4, IL6, IL10, IL17, TNF α , TNF β or INF γ (N=6 different tonsil donors), consistent with previous reports (Bonanomi et al. 2003).

However, the B cells in culture produced large amounts of Immunoglobulin, of classes IgA, IgG and IgM (Figure 30). Production of IgE was not detected in tonsil cell culture (N= 10 different tonsil donors, data not shown). Production of all classes of Immunoglobulin typically started after four days

of cell culture, with a maximum production between days seven and nine of culture. There was a wide variation in the total immunoglobulin production at one week, for all classes: IgG range 404.8-3914 ng/ml, median 1016 ng/ml; IgA range 561.2-1228 ng/ml, median 1029 ng/ml; IgM range 163-4808 ng/ml, median 1035 ng/ml. This is likely to reflect the relative number of B cell follicles and pre-formed plasma cells between different tonsil donors. The production of immunoglobulin in seven tonsil donors was delayed to after one week, and so these were excluded from subsequent analysis, which was performed at 1 week of culture. 18 donors were tested for IgG and nine for IgA and IgM.

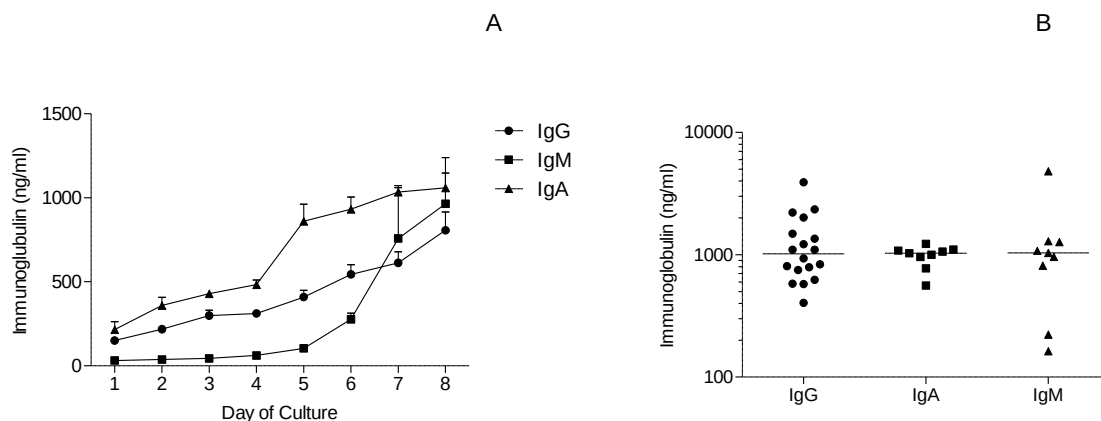


Figure 30: Immunoglobulin production in tonsil cell suspensions

Human tonsils grown in cell suspension culture are able to produce immunoglobulin. From day 4 of culture this increased, for IgA, IgG and IgM (A). Mean and standard deviation of experimental triplicate data for one representative tonsil donor are shown. For all classes of immunoglobulin the median immunoglobulin production at day 7 of culture was approximately 1000 ng/ml (B). N=18 different tonsil donors for IgG, N= 9 different tonsil donors for IgA and IgM.

4.1.3 Tonsil histocultures over time

Tonsil histocultures showed central follicle necrosis after 24 hours in culture, which became more pronounced over time (Figure 31). These findings are consistent with previous characterisation of the tonsil histoculture model (Giger et al. 2004). The main features of necrosis included an early loss of follicle structure and a loosening of the tonsil architecture changing from compact follicles to widely dispersed cells. By day six there were very few lymphocytes still visible on Haematoxylin and Eosin (H&E) staining, and no visible follicles.

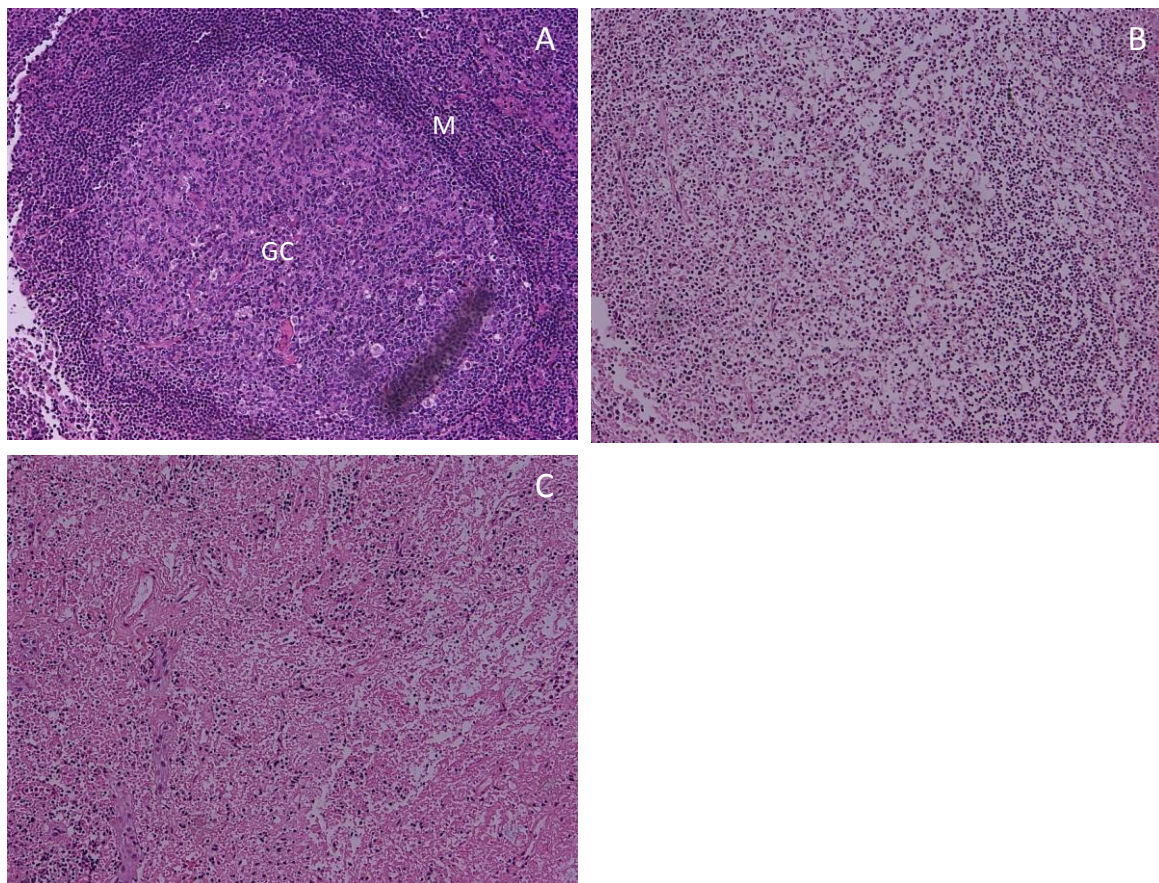


Figure 31: Tonsil histoculture necrosis over time

Haematoxylin and Eosin (H&E) stained formalin fixed tonsil blocks from one representative donor. Figure A shows the tonsil as it was received, and demonstrates a germinal centre (GC) with surrounding mantle zone (M, darker area). By 24 hours in culture there was marked destruction of the tonsil lymphocytes, and the germinal centre was poorly defined (B). After 6 days in culture there was complete necrosis, with no definable lymph node structure, and fibrotic changes (C). All images x100 magnification.

Despite the early necrosis, histocultures were able to produce large quantities of immunoglobulin (Figure 32). As the cell architecture remained intact, this was both faster and greater in magnitude than immunoglobulin production by cell suspension cultures, with the maximum production being reached by 48 of culture. Cytokines were not measured in histoculture media.

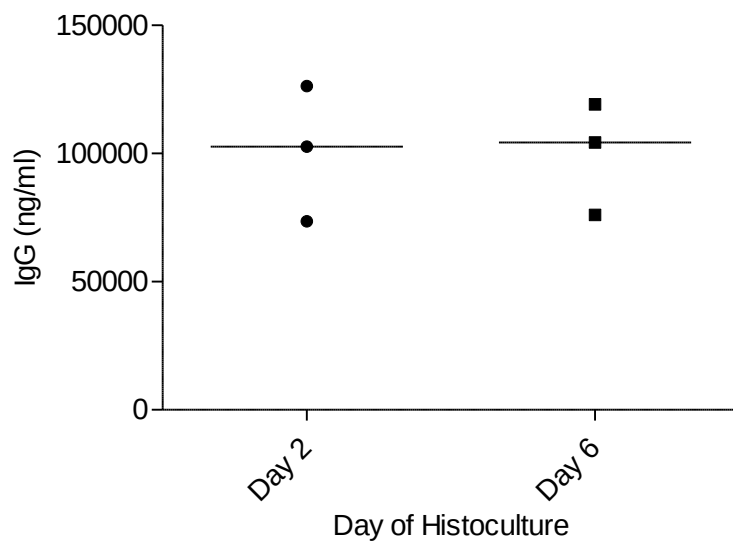


Figure 32: Immunoglobulin G production by tonsil histocultures

IgG production from tonsil histocultures was measured from the culture media on days 2 and 6 of culture. There was a high concentration of IgG in the media from all donors, which was sustained throughout the culture period. Bars represent median values.

4.1.4 Live bacterial co-cultures

4.1.4.1 Quantification of bacterial infection

There was a 2 log reduction in bacterial counts (CFU) cultured for 18 hours in the presence of tonsil cells compared to the same volume of media without cells, and a 5 log reduction at 18 hours in the presence of tonsil histocultures from the same tonsil donor (Figure 33a). Initial experiments with tonsil histocultures were performed on blocks which were briefly exposed to antibiotics. However, there were concerns that the low bacterial counts (Figure 33b) reflected the effect of residual antibiotics (as demonstrated in Figure 12, methods), rather than a direct action of the tonsils. Using the ethanol only washing method, it was difficult to accurately count the bacterial colony forming units at the end of the experiment, due to heavy contamination with endogenous flora. However, there was initially an increase in bacterial counts after 18 hours of co-culture, which fell over the subsequent days, as demonstrated in Figure 33c.

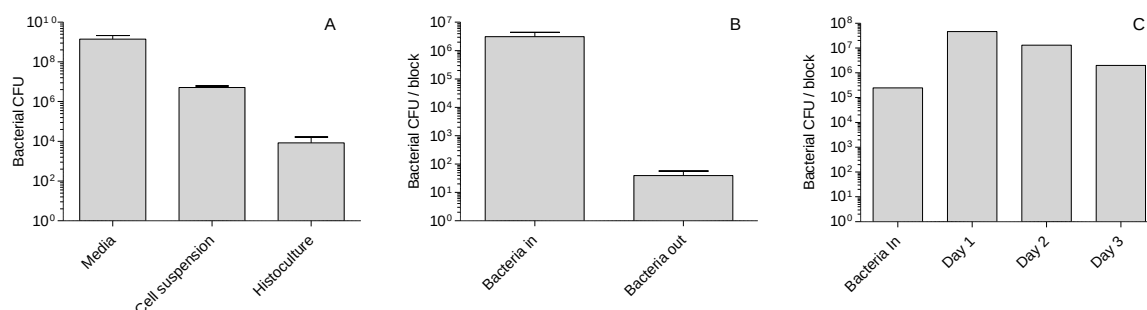


Figure 33: Bacterial co-culture with tonsil: impact on CFU

A/ reduction of bacterial growth in the presence of tonsil cells. Using a single donor tonsil, media alone, tonsil cell suspensions or histocultures were inoculated with 2×10^5 CFU *S. pyogenes* strain H305. Bacterial counts at 18 hours were calculated for each growth condition. Mean and standard deviation of 9 experimental replicates are shown for one tonsil donor. Bacteria grew, as expected, in media alone. There was a 2 log reduction in bacterial growth in the presence of tonsil cells compared to media alone, and 5 log reduction in histocultures (antibiotic method) $p=0.002$ Kruskal-Wallis test, data representative of 7 different tonsil donors. B/ reduction of bacterial counts in tonsil histocultures after 18 hours co-culture using the antibiotic exposure method (mean and standard deviation of 6 different donors, $p=0.0022$, Mann-Whitney test). C/ bacterial counts in tonsil histoculture after 18, 48 and 72 hours using the ethanol method. Results are counts from a single donor. Repeated in $N=5$ different tonsil donors, of which 2 were infected with endogenous β -haemolytic streptococci.

4.1.4.2 Tonsil co-culture histopathology

Tonsil histocultures were assessed for the pattern of bacterial infection by histopathological examination. There was no significant difference in the degree of necrosis between infected and uninfected tonsils at 18 hours. However, in successfully infected cultures, the presence of Gram positive cocci could be seen on Gram staining of formalin fixed, paraffin embedded blocks (Figure 34). The infecting bacteria could be seen to penetrate deep within the tonsil block, via the lymphovascular septae, with bacteria keeping away from the lymphoid follicles. On cut surfaces or sections of epithelium, bacteria would also cluster along the surface.

Although the presence of contaminating endogenous bacteria in histocultures could easily be identified by plating tonsil tissue onto agar, Gram staining of tonsil histopathological sections could not distinguish between Gram positive contaminants such as *S. aureus* and the experimental *S. pyogenes*. Immunohistochemical staining of blocks was attempted for identification of *S. pyogenes*, but the antibody selected was not specific enough: the commercial antibody was raised against whole killed *S. pyogenes*, thus there was cross-reactivity with the Gram positive cell wall of *S. aureus* (pictures not shown).

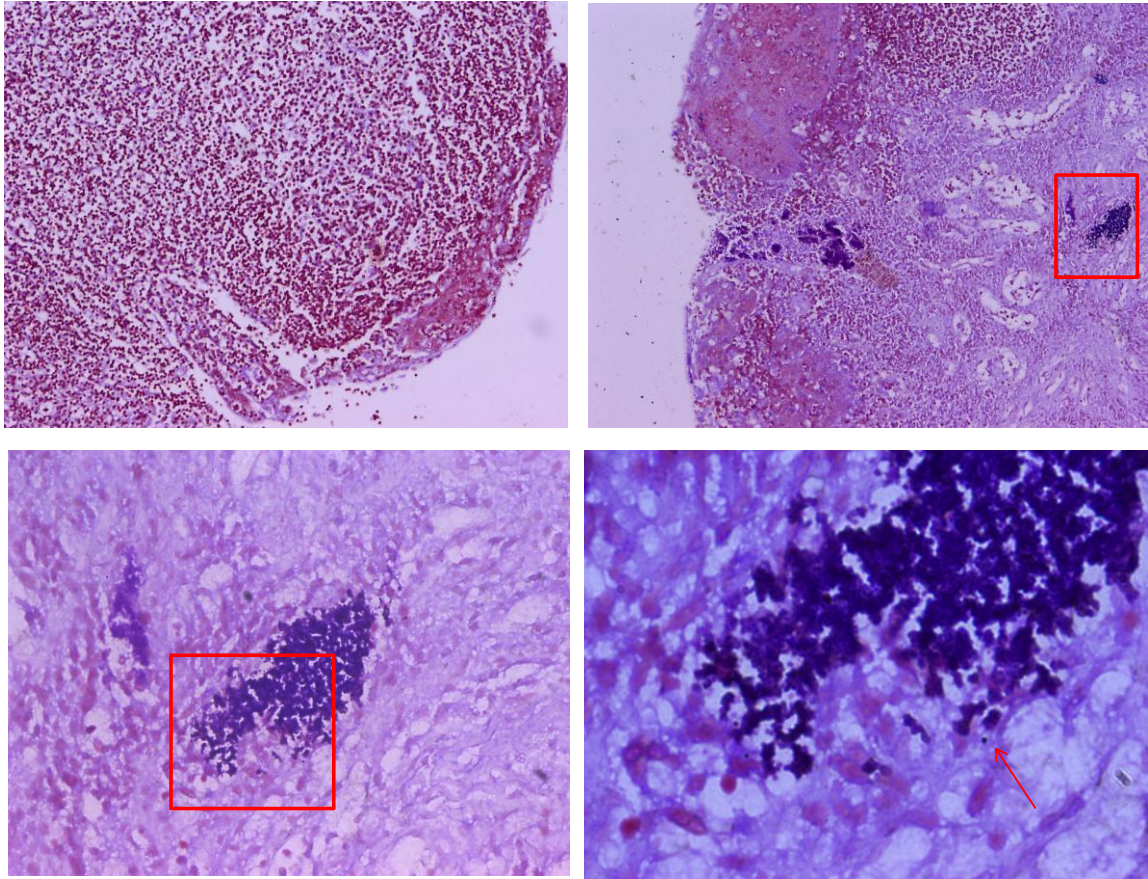


Figure 34: Histopathology of *S. pyogenes* infected tonsil co-cultures

Images show the successful infection of tonsil histocultures by live *S. pyogenes*. A/ uninfected tonsil after 18 hours of culture *in vitro*, Gram stain of formalin fixed block x100 magnification. There was no evidence of bacterial infection. B/ histoculture section from the same donor after 18 hours of co-incubation with *S. pyogenes*, Gram stain of formalin fixed block x100 magnification. It can be seen that there are numerous clumps of Gram positive cocci deep within the tonsil structure (stained deep purple colour), a representative block of which is highlighted by a red square. This section is shown at increased magnification in C (x400) and D (x1000 magnification). In D a red arrow shows that the purple clusters are compiled of Gram positive cocci.

4.1.5 Cell suspension live bacterial co-culture qRT-PCR

RNA was extracted after 18 hours of co-culture of live *S. pyogenes* with tonsil cell suspensions, histocultures or in the same volume of media alone. Superantigen gene expression was measured as described. For *speA*, transcripts were only detected in 3 of the 5 media-only cultures, while *smeZ* transcripts were only detected in 2 out of 5 media only cultures, although housekeeping gene *proS* transcripts were detected in all. This is similar to the results achieved previously with broth culture, as detailed in chapter 3. In contrast, transcripts of both *speA* and *smeZ* were detectable in each tonsil cell suspension co-culture (Figure 35). The wide range of superantigen transcript values in media alone makes the data difficult to interpret statistically, and so would need to be repeated on a greater number of controls to achieve an accurate result. There were 26 times more *speA* than *smeZ* transcripts produced in the tonsil cell suspension co-cultures, though this was not significant ($p=0.125$, Wilcoxon ranked pairs).

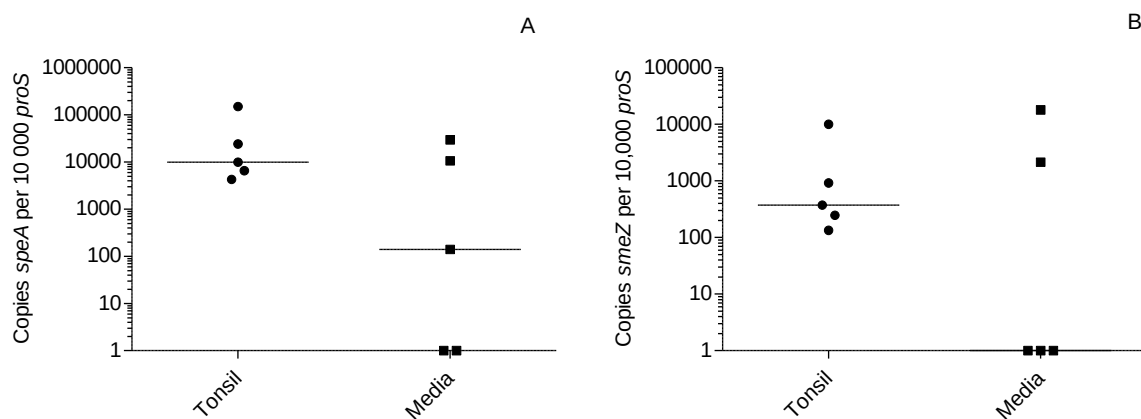


Figure 35: qRT-PCR of superantigen expression in live bacterial tonsil co-culture

Production of superantigen transcripts of *speA* (A) and *smeZ* (B) from *S. pyogenes* grown in the presence of tonsil cells or the same volume of media alone. N=5 separate tonsil donors and matching media alone cultures, each point being the mean of 3 experimental triplicates for each donor, run in triplicate for each gene of interest. Median production of *speA* in tonsil culture was 9893 copies *speA* per 10,000 copies *proS* (range 4277-149,808), compared to 140 copies *speA* per 10,000 *proS* (range 0-29,778) in media alone cultures ($p=0.29$, Mann Whitney U Test). In the same cultures there was 26 times less *smeZ* produced, with a median of 375 copies *smeZ* per 10,000 *proS* (range 133-10,000) in tonsil culture, compared to a median of 0 copies *smeZ* per 10,000 *proS* (range 0-18,094) produced in media alone cultures ($p=0.53$, Mann Whitney U Test). Bars represent median values.

Bacterial RNA was not detected when extracted from histoculture co-cultures, probably because the recovered bacterial load per block was <100 CFU, and therefore below the limit of detection by qRT-PCR (data not shown). This was performed using the antibiotic soaking method before washing and then inoculation of bacteria applied. This should ideally be repeated using the ethanol washing (fully antibiotic free) method, to guarantee a heavier infection of the histoculture blocks.

4.2 Discussion

4.2.1 Establishment of cell culture system

Two methods of tonsil culture had previously been described (Giger et al. 2004), and both have been tested in this project for their suitability as an *ex-vivo* model of streptococcal tonsillitis. This project has so far identified that there are advantages and disadvantages to each system, and both were successfully developed to allow for examination of responses to recombinant bacterial proteins and bacterial supernatants.

Cell suspension cultures are easier to prepare, and are less likely to become contaminated with endogenous flora, as the antibiotics and nutrients in the media have equal access to all the cells. Cells are also easy to access for analysis by flow cytometry when they have been cultured as a cell suspension as opposed to in solid blocks, and RNA extraction is similarly easier. However, the close cell-cell interactions, which appear to be so important in both speed and outcome of response to stimulation, are lost by this method. In contrast histocultures preserve the tonsil architecture and cellular interactions, but at a price of reduced nutrient availability to cells at the centre of the follicles, resulting in premature cell death and block necrosis. As demonstrated by histopathology, and in concordance with other studies, the viability of the histocultures was limited to about 72 hours. These differences were best demonstrated by the production of immunoglobulin, where it took 4-5 days of culture for immunoglobulin to start being produced in cell suspension culture, whereas in histocultures there was not only a ready production of immunoglobulin by 48 hours, but the quantity of immunoglobulin was far higher (Figure 32).

It might be possible to adapt the system so that tonsil histocultures were performed as thin slices in slide cultures rather than as blocks – this should still allow the major components of the lymph node structure to be preserved, but with better access to nutrients, and could also be used to observe real time movement of cells and bacteria within the system.

Another major flaw in the tonsil histoculture model is the lack of access to the systemic circulation and recruitment of neutrophils. For the cell suspension cultures, this could be easily remedied by the addition of neutrophils to the culture system at the appropriate time point, either directly or across a membrane to allow the observation of neutrophil migration. However, for histocultures, it would be necessary to add the neutrophils to the media and depend on chemotaxis and migration through the matrix and membrane scaffold, or directly apply neutrophils to the top of the block. Obtaining fresh neutrophils from the same donor as the tonsils would require revision of the approved ethics protocols, as tonsils obtained for this project were surplus to diagnostic requirement and obtained as a result of clinically-indicated surgery, without interaction with the donor. Use of neutrophils from patients would require explicit consent. It might, therefore, be necessary to use healthy volunteer neutrophils in the system if that were to be attempted.

4.2.2 Characterising cell populations over time

There was a high degree of consistency in B and T cell populations between tonsil donors. This was surprising as tonsillar hypertrophy (the commonest indication for tonsillectomy) is reported to be due to B cell follicle expansion (Alatas & Baba 2008), which perhaps might have suggested that larger sized tonsils would have a higher proportion of B cells. The variations in the cell populations over one week in culture (Figure 29) reflect the adjustment of the cell to culture *in vitro* – the appearances becoming similar to the original tonsil population around the same time as function such as Immunoglobulin production was restored (day 4-5).

The viability of tonsil histocultures has been previously assessed by both histopathology sections and flow cytometry (Giger et al. 2004), and confirms the degree of necrosis seen in this study at similar time points. By one week the appearance of tonsil blocks has changed, so that the cells have lost their pink colour, and become pale and yellowed, and the outer layers are easily crushed/ disintegrate. This suggests that considerable cell death is happening in the histocultures, to a degree not seen in the cell

suspensions. Interestingly the expansion of connective tissue observed (Figure 31b), is not dissimilar to that seen in previous streptococcal infection studies (Sriskandan et al. 1996). There is suggestion that fibroblasts play a vital role in preventing plasma cell apoptosis in tonsils (Merville, Dechanet, Desmouliere et al. 1996) and that this mechanism is reliant on close cell interactions. It is possible that some of the stromal changes seen are in response to the stresses on the tonsils when they are removed from the body and processed in the laboratory, representing a reaction to limit cell damage. The use of unstimulated controls in later experiments is therefore essential to ensure that any responses observed are due to superantigens rather than just due to the traumas of primary cell culture.

The lack of production of cytokines in unstimulated cell suspension cultures is similar to previous published results (Bonanomi et al. 2003), and is reassuring for future work that there is no residual influence on tonsil cells from the endogenous flora or recent infections.

4.2.3 Live bacterial-tonsil co-cultures

Technically it was difficult to achieve live bacterial co-culture, in comparison to the relative ease with which responses to bacterial proteins, heat-killed bacteria or supernatants could be examined. Initial experiments found that brief exposure to 60% ethanol was well tolerated by the tonsils, and markedly reduced the level of endogenous bacterial contamination at 24 hours. However, the only way to ensure that there was not excessive endogenous flora was to expose the tonsil blocks to antibiotics. Even after washing 6 times, there was still trace antibiotic residue detected in the blocks (Figure 12), and successful infection could rarely be achieved by the application of bacteria at this point. Infection was achieved by giving a second application of bacteria to the tonsil blocks after overnight incubation, but a potential residue of antibiotics remained, which could adversely alter bacterial characteristics. This was best evidenced by the marked reduction in bacterial growth in histocultures using this method (Figure 33). Live bacterial infection of cell suspension cultures was much more successful, though again was reliant on the pre-exposure of tonsil cells to antibiotics to remove contaminating

endogenous flora. Although it was easier to wash antibiotics from cell suspensions, there still remains the possibility that the reduction in bacterial counts in the presence of tonsil cells is a reflection of residual intracellular antibiotics rather than a true effect of the tonsil cells. The presence of aminoglycoside antibiotics in cell culture has been previously shown to reduce bacterial adhesion to tonsil epithelium even when the bacteria are not killed by the same concentration of antibiotic in broth culture (Abbot et al. 2007), suggesting that the poor histoculture colonisation results in this study may be antibiotic-related. The influence of high concentrations of antibiotics on human cell function could not be assessed in this work, though an influence on cell metabolism cannot be excluded. Multiple experiments attempting to measure this by culturing the cells for a prolonged period of time (i.e. longer than 24 hours) in antibiotic-free conditions prior to infection, were unsuccessful, due to contamination with either *S. aureus* or *Candida* species.

Washing the tonsil blocks in ethanol alone did allow for the successful infection of tonsil blocks with *S. pyogenes*, but this was always accompanied by a heavy growth of endogenous flora. When the endogenous flora included beta-haemolytic streptococci, this made distinction of the experimentally added *S. pyogenes* impossible, and as such the number of tonsil histoculture co-culture infections which had to be discarded was high. However, it could be argued that this is a more realistic system for measuring the early events of *S. pyogenes* infection of tonsils for two reasons: firstly, patients are rarely on antibiotics at the time of *S. pyogenes* exposure, and secondly the precise role of endogenous flora in protecting against infections with pathogens has not been established, but is likely to be significant (Shelburne, III, Sumby, Sitkiewicz et al. 2006).

Distinction of *S. pyogenes* from other bacteria in histopathology specimens should be easy to identify, but the currently commercially available antibodies lack adequate specificity for this to be performed. The use of a highly specific anti-*S. pyogenes* antibody created in-house (such as anti-SpyCEP antibody) could yield better results, but the degree of antibody stability required for staining formalin-fixed specimens is high. A combination of an improved specificity antibody and frozen section staining would improve histopathology results.

Although the model has significant flaws for live bacterial co-culture at present, there are several encouraging points. It has been possible to achieve live bacterial infection of tonsil histocultures, and the method of no antibiotic exposure is best for this, notwithstanding the risk of contamination with endogenous flora. The exploitation of artificially antibiotic resistant strains of *S. pyogenes* would be the simplest way to successfully achieve co-culture infection, in a reliable and repeatable method. Antibiotic resistance genes are incorporated into the systems used to alter specific gene expression in *S. pyogenes* (Unnikrishnan, Cohen, & Sriskandan 2001), and so the use of such bacterial strains would enable the study of both the bacterial infection on tonsils and the effect of the genetic manipulation. The ideal antibiotic resistance genes would confer resistance to macrolide or tetracycline groups, as incorporation of these antibiotics into the culture system would enable all significant endogenous flora to be eradicated, including *S. aureus*, *Moraxella* species, most alpha haemolytic streptococci and Actinomycosis. Testing of the available macrolide (erythromycin) resistant *S. pyogenes* strains developed in the lab has shown that there is inducible resistance to clindamycin (a related lincosamide group antibiotic), which would be the ideal choice of antibiotic for incorporation into the tonsil histoculture system. Amphotericin B could then be added to eliminate infection with fungi (either yeasts or moulds), and a successful uncontaminated histoculture system created.

The other bacterial manipulation which would significantly improve the output from live-bacterial tonsil co-cultures is the use of bioluminescent or fluorescent bacteria. Bioluminescent and fluorescent strains of *S. pyogenes* have been developed in the laboratory, and are being actively used in animal and cell culture models of disease. These bacteria are macrolide resistant, and so would be good candidates for use in the histoculture system. They would also allow imaging of infected histocultures using techniques not yet attempted, particularly fluorescence microscopy. The interaction of tonsils with fluorescent bacteria could potentially be examined in both histocultures and cell suspension cultures, leading to exciting new discoveries about host-pathogen interactions.

With the methods employed, it was not possible to achieve successful live bacterial infection without causing tonsil cell death, either in cell suspensions or histocultures. If immune cell responses to live *S.*

pyogenes infection were to be examined, it would be best if this were done at short time points, such as 3 to 6 hours of infection, in a totally antibiotic-free system.

4.2.4 Expression of bacterial superantigens in tonsil cell co-culture system

The successful development of the live bacterial co-culture system opens a number of possibilities for exploring the role of putative streptococcal virulence factors in tonsillitis. The focus of this project is the role of *S. pyogenes* superantigens, and whether they are of importance in establishing tonsil infection. In this system, in tonsil cell suspension co-culture RNA transcripts for the bacterial superantigens *speA* and *smeZ* were detected in 5 separate experiments. Unfortunately the matching media alone culture transcripts showed a wide variation, meaning that the results were not statistically interpretable. This may be because at 18 hours the bacteria had entered stationary phase, and were no longer producing superantigens (Unnikrishnan, Cohen, & Sriskandan 1999). The best way to overcome this would be to repeat the experiments at a number of different time points. However, the detection of *speA* transcripts in the tonsil co-culture system is consistent with previous studies in different systems (Virtaneva et al. 2005).

Unfortunately, there was a low level of bacterial infection of histocultures, as confirmed by colony counting. This meant that the level of bacterial RNA was below the limits of detection by RT-PCR. All these infections were attempted using the antibiotic exposure histoculture system, prior to development of the ethanol only live co-culture method. It would be interesting to repeat the experiments on a number of donors using the ethanol only system, as preliminary results show that the bacterial infection achieved is far higher.

Although the concept of organ culture is not new, and the tonsil histoculture model has been successfully used in the past only for the examination of responses to viral infections (Glushakova et al. 1995), the results presented here show that it is possible to infect human tonsils with the relevant live pathogenic bacteria for that site, and use them as a tool to examine bacterial responses to human

tissues. However, the limitations of the histoculture model mean that for examining immunological responses to bacteria, cell suspension cultures are still the best method. The immunological responses of human tonsil cells in suspension culture to *S. pyogenes* superantigens are presented in chapter 5.

5 Tonsil immune responses to streptococcal superantigens

Human tonsils provide a unique immune system – they have similar follicles to those found in other lymphoid organs, but they are covered in a specialised epithelium which allows direct contact with the pharynx. Unlike Peyer’s patches in the gut, the frond-like structure of tonsil tissue maximises the surface area available to mount a rapid immune response to infection. The T and B cell follicles lie a mere 200-300µm below the epithelium (Ohtani & Ohtani 2008), so rapid cytokine and immunoglobulin responses are possible when pathogens are detected.

S. pyogenes is widely recognised as the most important bacterial pathogen in the human pharynx, both in terms of direct disease burden and as a source for secondary infections and immunological sequelae (Carapetis et al. 2005). As mentioned in chapters 3 and 4, there is mounting evidence that among the virulence factors produced by *S. pyogenes*, superantigens are important in establishing pharyngeal disease. The mechanism by which superantigens activate immune cells has been extensively investigated since their discovery in the 1970s (Kim and Watson 1970). Instead of conventional antigen presentation, superantigens bind ectopically to both the T cell receptor and the MHC class II molecule, causing altered intracellular signalling. For T cells, the result of superantigen binding is clonal expansion and proliferation, depending on the T cell receptor variable beta chain subset to which the superantigen has bound, and marked pro-inflammatory cytokine release. In antigen presenting cells the effect of superantigen binding has yet to be fully established, though alterations in intracellular signalling have been demonstrated (Bueno et al. 2007). The advantage to the bacteria of producing such powerful immunomodulatory agents has yet to be explained.

Therefore, this project set out to establish the main immunological consequences of streptococcal superantigen exposure to human tonsils, using the human tonsil cell culture models described in chapter 4.

5.1 Results

5.1.1 Tonsil cell proliferation in response to superantigens

Whole tonsil cell preparations were cultured in the presence of varying concentrations of recombinant bacterial superantigens – the streptococcal superantigens SPEA, SPEJ and SMEZ, and as a control the staphylococcal superantigen Staphylococcal enterotoxin B (SEB). Tonsils cells were able to proliferate in response to bacterial superantigens in a dose dependent manner (Figure 36), as has previously been shown with peripheral blood mononuclear cells (PBMC's) (Llewelyn et al. 2006). The background level of proliferation with human tonsils is far higher than would be expected from PBMC's – approximately 20x higher (PBMC baseline approximately 2000 cpm, tonsil baseline 20-50000cpm). SPEA required higher concentrations to initiate a proliferative response than SMEZ, SPEJ or SEB (SPEA 1ng/ml, SMEZ and SPEJ 100fg/ml, SEB 1pg/ml,). These results are similar to the superantigen responses of PBMC's and mouse spleen cells that have previously been reported (Proft et al. 1999). It was determined that optimum discrimination of tonsil cell proliferation by H³-thymidine incorporation was achieved at 72 hours.

To support that the PBMC proliferation was due to the specific recombinant superantigens, tonsils were stimulated with a 1% bacterial supernatant from isogenic superantigen +/- *emm1* and *emm89* strains of *S. pyogenes*. There was considerable loss of proliferation using strains with targeted disruption of genes encoding *speA* (*emm1* strain, Figure 37A) and *smeZ* (*emm89* strain, Figure 37B), which was restored in the complemented strain. The addition of *speA* to the *emm89* strain further increased proliferation. These findings were consistent with previously published observations using the same strains in PBMC's (Russell & Sriskandan 2008;Unnikrishnan, Cohen, & Sriskandan 2001).

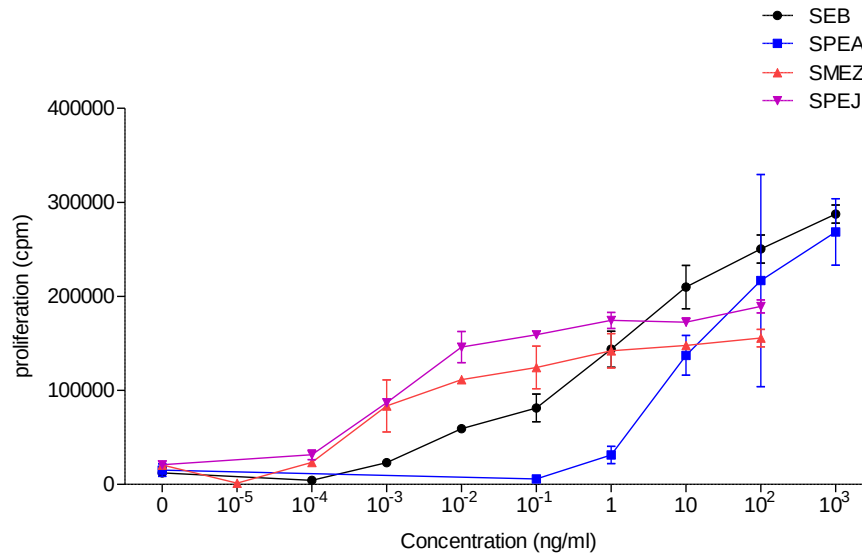


Figure 36: Tonsil cell proliferation in response to bacterial superantigens

Tonsil cell suspension culture for 72 hours in the presence of varying concentrations of recombinant superantigens. There was a dose dependent proliferation in response to the superantigens SPEA, SPEJ, SMEZ and SEB. A 4 parameter curve of log transformed data gives a regression value of $R^2=0.84$ for SPEA, $R^2 = 0.97$ for SPEJ, $R^2 = 0.94$ for SMEZ and $R^2= 0.99$ for SEB. Results are shown for a single tonsil donor (mean and standard deviation of 3 experimental replicates). Representative of experiments using N=9 different donors (SEB), N=8 SMEZ and SPEJ, N=6 SPEA.

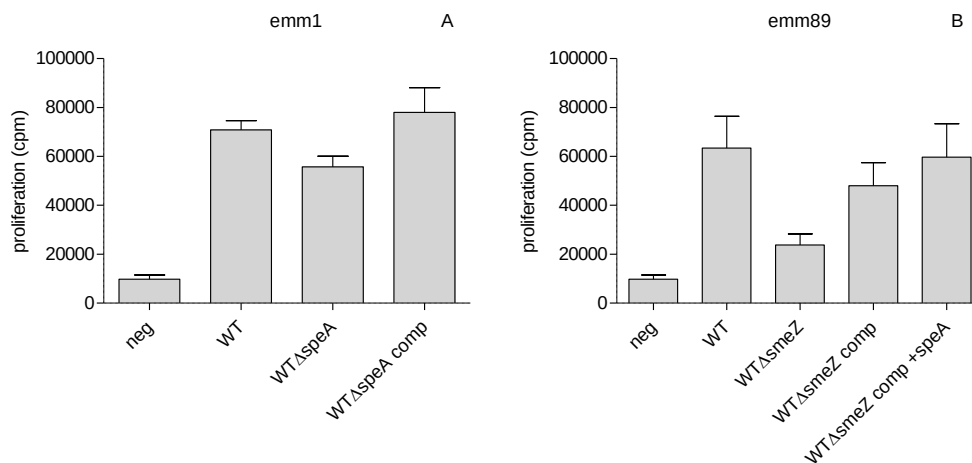


Figure 37: Tonsil proliferation with streptococcal culture supernatants

Human tonsil cells were incubated for 72 hours with 1% superantigen-containing streptococcal supernatants, H³-thymidine was added for the final 18 hours of culture. A: isogenic *emm1* *S. pyogenes* supernatants +/- *speA*. B: isogenic *emm89* *S. pyogenes* supernatants +/- *smeZ* and with the addition of *speA*. Bars show the mean and standard deviation of experimental triplicates in one tonsil donor, representative of N=6 different tonsil donors.

5.1.2 Global B and T cell populations in human tonsils stimulated with superantigens.

To determine which cells were proliferating in response to the superantigens, flow cytometry was performed on the cell cultures, and the percentages of B and T cells noted (Figure 38). Using SPEA as a model superantigen, there was consistently an increase in both the percentage and total number of T cells after 1 week of culture in the presence of superantigen, and a decrease in both the percentage and absolute number of B cells. A concentration of 100ng/ml was chosen for SPEA stimulation, because this represented the concentration which yielded approximately half maximum proliferative response in H³-thymidine incorporation assays (Figure 36).

To further investigate this shift in cell populations, both T and B cell populations were examined for their subset characteristics and their functional characteristics in terms of cytokine production (T cells) and immunoglobulin production (B cells), when exposed to bacterial superantigens.

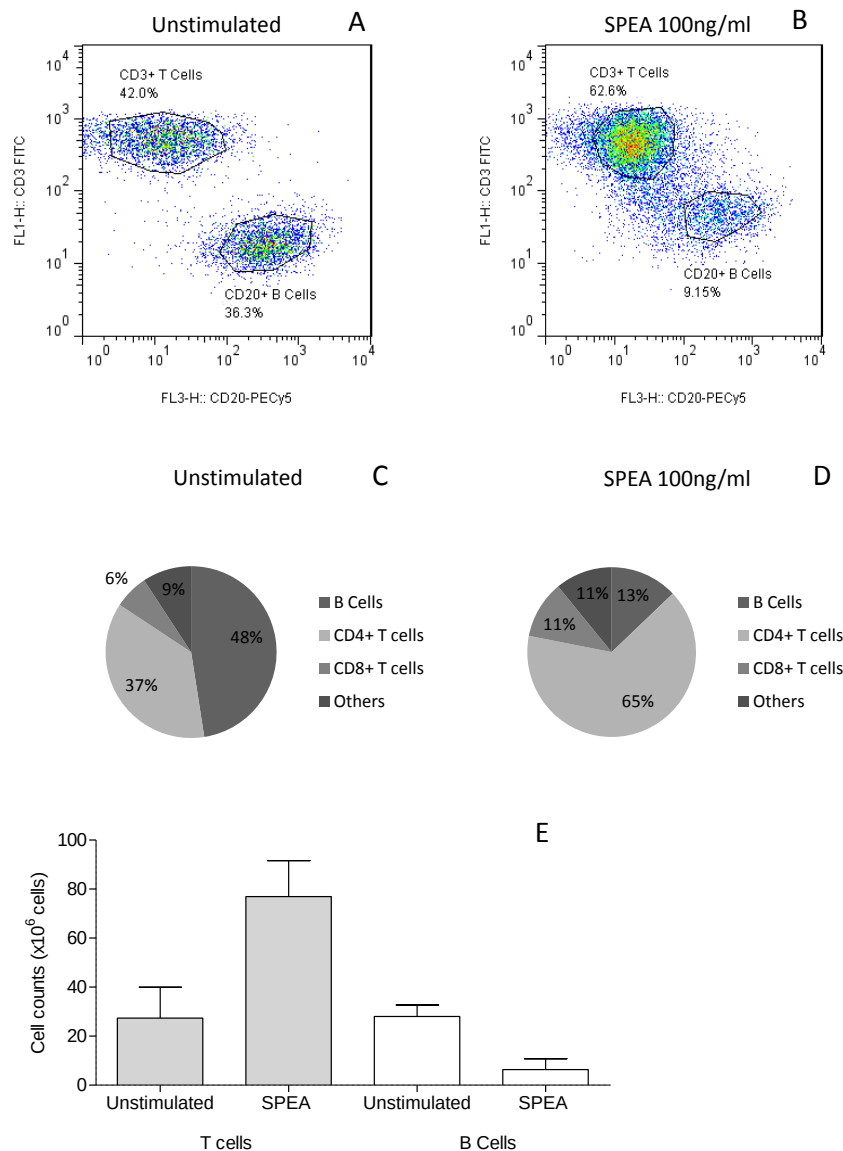


Figure 38: Tonsil cell populations following superantigen stimulation

When tonsil cells were stimulated with SPEA 100ng/ml for 1 week, there was a marked decrease in the percentage of B cells and an increase in the percentage of T cells, compared to unstimulated cells from the same donor (A and B, representative flow cytometry plots from one tonsil donor). This cell expansion was predominantly CD4+ T cells (C and D), which expanded from 37% (range 25 – 59%) to 65% (range 58 – 96%) of the total lymphocyte population after 1 week. B cells fell from 48% (range 30 – 62%) to 13% (range 2 – 26%). There was no significant alteration in the percentage of other cells. Mean of N= 16 donors unstimulated cells cultured for 1 week, N=8 donors stimulated with SPEA 100ng/ml for 1 week. E/ Cells were cultured for one week in volumes of 35 mls at 2×10^6 cells/ml, either unstimulated or in the presence of SPEA 100ng/ml. Cells were counted at the end of 1 week, and analysed for CD3 (T cells) and CD20 (B cells) by flow cytometry demonstrating a reduction in the absolute numbers of B cells and concomitant rise in the absolute numbers of T cells in cultures exposed to SPEA compared with unstimulated controls cultured for the same duration of time. Results were confirmed by cell separation (by AutoMACS, Chapter 6, data not shown). Mean + sd of N=3 different tonsil donors.

5.1.3 Tonsil T cell receptor variable β subset expansion with superantigens

One of the defining characteristics of bacterial superantigens is their ability to selectively expand T cell populations depending upon the T cell receptor variable β (TCRV β) subset to which they bind (Sundberg et al. 2002). As each superantigen is structurally different, they each have a preference for binding to different TCRV β subsets, resulting in different clonally expanded T cell populations (Llewelyn et al. 2006; Proft & Fraser 2003). To confirm that the expanded T cell populations in human tonsil cultures were due to selective TCRV β subsets, cells were cultured with recombinant SPEA (100ng/ml), SMEZ (1ng/ml) or SPEJ (1ng/ml) for 1 week, or in cell culture media alone. Cells were then surface stained for 24 different TCRV β subsets using the IOTest Beta Mark Kit (Beckman Coulter, France), which stains more than 95% of the TCRV β subset repertoire. Staining for individual TCRV β subsets 2, 8, 11 and 14 was performed when the kit was unavailable. TCRV β subset alterations were calculated by dividing the percentage of cells for each subset in superantigen stimulated cultures by the value in unstimulated cell cultures from the same donor (Figure 39).

SPEA and SPEJ exhibited a wide range of TCRV β subset expansions. SPEA demonstrated expansion of TCRV β 2, 7.1, 7.2, 12, 13.2, 14, 16, 18, 20, 22 and 23, though there was marked inter-donor variation in this (Figure 39B). For SPEJ, TCRV β 1, 2, 4, 13.2 and 16 were the predominant subsets expanded (Figure 39D), though characterisation of the whole panel with a greater number of donors would be needed to confirm this. The TCRV β repertoire for SMEZ stimulated cells was more restricted, with the majority of cells being expanded in subsets 4 (4.24 fold expansion) and 8 (8.29 fold expansion), and to a lesser degree, subsets 7.1, 13.2 and 23 (Figure 39C). These findings are consistent with previously published results in healthy PBMC donors (Proft & Fraser 2003), and control healthy human PBMC donors used to optimise cell culture methods and flow cytometry staining for this project (data not shown).

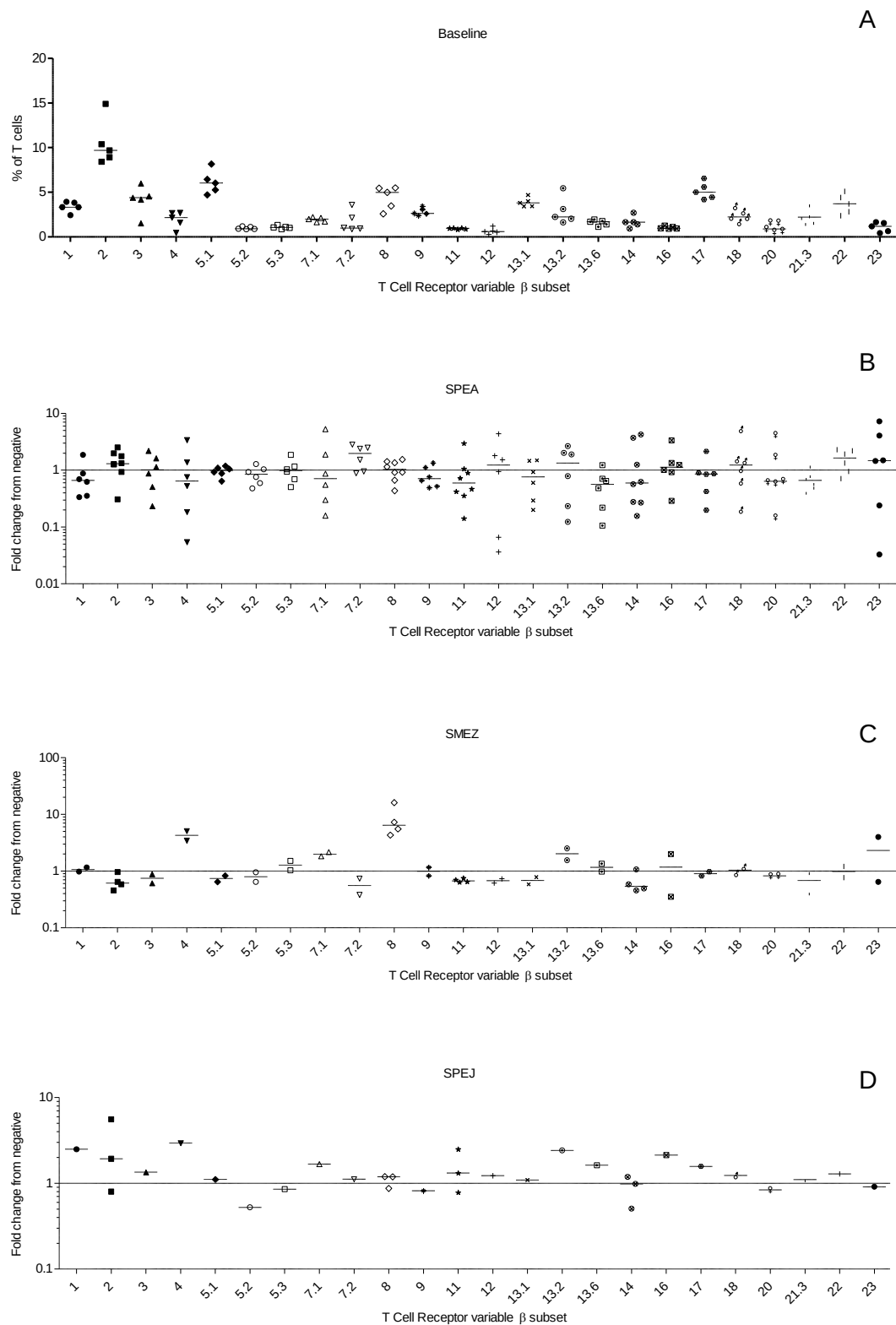


Figure 39 TCRV β subset changes with SPEA, SMEZ and SPEJ

A/ baseline TCRV β profile of 5 different tonsil donors. Fold change from baseline profile (from the same donor) is shown with superantigen stimulation for 1 week: SPEA (B), SMEZ (C) or SPEJ (D). All bars represent median values. N=8 different tonsil donors for SPEA, N=4 for SMEZ and N=3 SPEJ. For 2 tonsil donors (each superantigen) only 4 TCRV β subsets were checked; 2, 8, 11 and 14.

5.1.4 TCRV β clonal expansion in response to bacterial supernatants

Strains of *S. pyogenes* typically carry the gene for more than one superantigen, and the disruption of those genes can have marked effects on T cell proliferation (Figure 37). To assess the relative contribution of each superantigen to T cell proliferation, tonsil cultures were stimulated for 1 week in the presence of 1% bacterial supernatants from the *S. pyogenes emm 1* strain H305 and +/- *speA* isogenic mutant strains, or the *emm 89* strain H293 and +/- *smeZ* isogenic mutant strains.

The *emm1* strain H305 has been shown by multiplex PCR to carry genes for the superantigens *speA*, *speG*, *speJ* and *smeZ* (Russell & Sriskandan 2008). TCRV β expansion of subsets 2, 8, 11 and 14 only were examined, though these results are preliminary, as they have only been analysed for a single tonsil donor. There was expansion of tonsil T cell subsets bearing TCRV β 2, 8 and 14 on stimulation with the wild type strain compared to unstimulated tonsil cells, but no expansion of TCRV β 14 on stimulation with the *speA*- mutant (V β 2 and 8 expansion remained unchanged). TCRV β 14 expansion was restored using supernatant from the *speA*++ complemented strain, though at a lower level than when stimulated with wild type supernatant (Figure 40A). This implies that of the TCRV β subsets tested, SPEA was responsible for expansion of TCRV β 14, but not TCRV β 2 or 8. As recombinant SPEJ and SMEZ have been shown to expand TCRV β 2 and 8 respectively (Figure 39D and C) it is likely that these superantigens were responsible for the remaining expansion demonstrated. To further examine the contribution of different superantigens in the *emm 1* strain, these experiments would need to be repeated on more tonsil donors, using the whole TCRV β panel, and using the *emm 1* isogenic mutant *smeZ*+/- and *smeZ*+/-*speA*+/- strains now available in the laboratory, neither of which were available at the time these experiments were performed.

The *emm 89* strain has been previously shown to carry the genes for *speG*, *speH*, *speJ* and *smeZ*, though it is now thought that *speJ* is actually a pseudo gene in this strain (S. Sriskandan, personal communication). Cultures stimulated with wild type supernatant showed predominantly TCRV β 4, 7.1, 8 and 23 expansion comparative to unstimulated tonsil cultures (Figure 40B), in a pattern very

similar to the expansion with recombinant SMEZ (Figure 39C), though again these results are preliminary, having only been analysed for one tonsil donor. Stimulation with isogenic *smeZ*-supernatant showed that this was then changed to a profile of TCRV β 1, 2, 9, 13.2 and 18, though V β 23 remained unchanged. Complementation with *smeZ* restored this to the wild type TCRV β expansion profile (Figure 40B). This implies that SMEZ is the predominantly active superantigen produced by the *emm89* strain, and that the effects of the other superantigens are only seen when SMEZ was removed. To confirm this, the experiments would need to be repeated in a number of different tonsil donors, and ideally with recombinant SPEG and SPEH for comparison, as well as recombinant SPEJ and SMEZ.

Taken together, this preliminary data showed that tonsil cells respond to superantigens in a TCRV β -specific manner similar to peripheral blood mononuclear cells. Experiments with isogenic bacterial supernatants, containing native superantigens, suggest that TCRV β 14 was the main subset expanded by SPEA, while SMEZ expanded TCRV β 4, 7, and 8. This is broadly consistent with data obtained using recombinant superantigens (Figure 39).

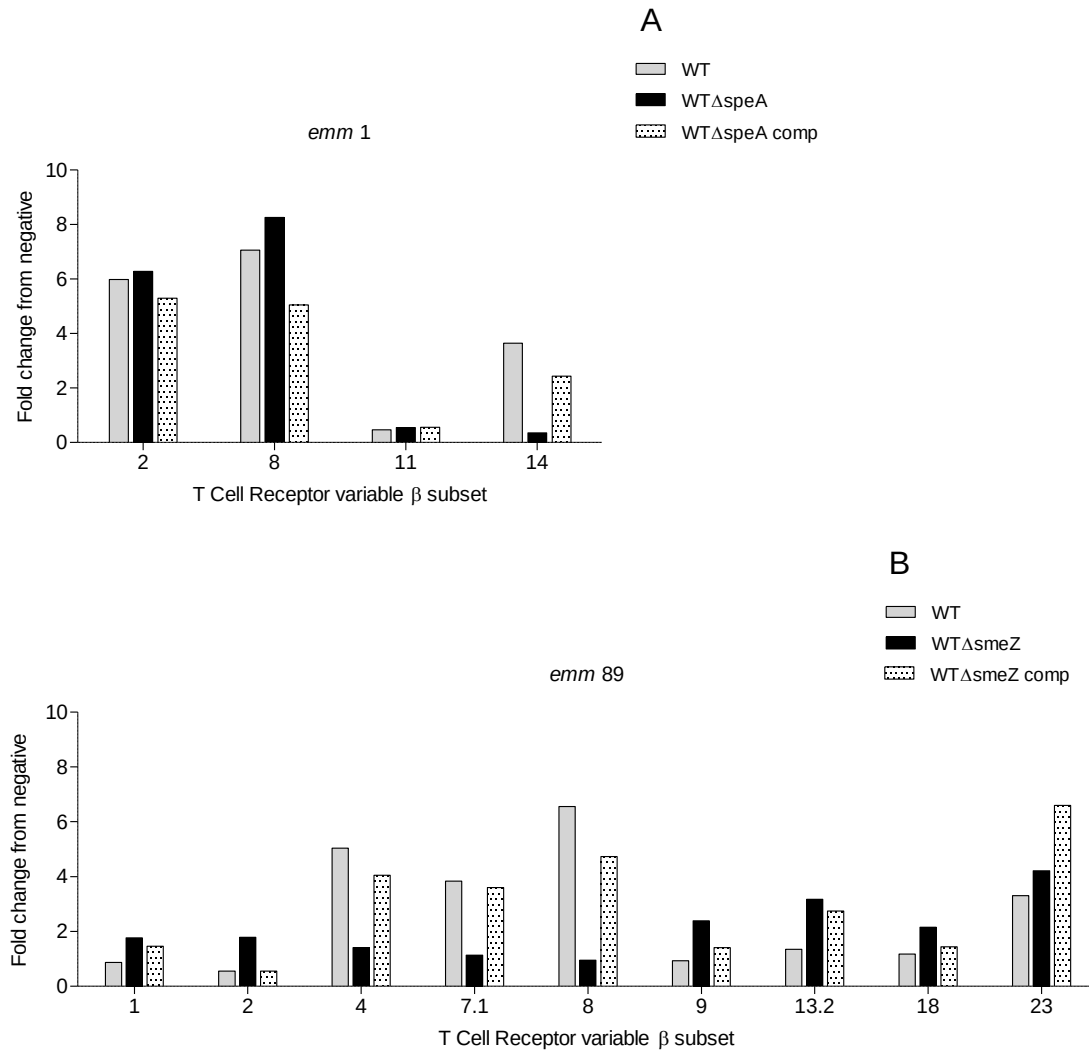


Figure 40: TCRVβ expansion of tonsil cells with bacterial supernatants

Tonsil cells were stimulated for 1 week with 1% bacterial supernatant, and the TCRVβ profile assessed. A: *emm1* bacterial supernatants, the wild type strain (WT) and isogenic *speA* negative (WTΔ*speA*) and complemented (WTΔ*speA* comp) strains were assessed for TCRVβ subsets 2, 8, 11 and 14 compared to unstimulated cultures from the same tonsil donor (results from 1 donor). B: *emm 89* bacterial supernatants, the wild type strain (WT) and isogenic *smeZ* negative (WTΔ*smeZ*) and complemented (WTΔ*smeZ* comp) strains were assessed for the whole TCRVβ repertoire compared to unstimulated cultures from the same tonsil donor (results from 1 donor). The results for the TCRVβ subsets 1, 2, 4, 7.1, 8, 9, 13.2, 18 and 23 only are shown, as there was no alteration from baseline with the other TCRVβ subsets.

5.1.5 CD4⁺ T cell subset changes with SPEA

Traditionally, CD4⁺ T helper cells can be divided into T helper (T_H) 1 and T_H2 cells, and the more recently described T_H17, Regulatory T cells (TReg), T_H9, $\gamma\delta$ T cells and Natural killer (NK) T cell subsets. These cell types are defined predominantly by the cytokines they produce in response to a stimulus (such as PMA and Ionomycin), cell surface characteristics and the active transcription of certain key regulatory genes. Although these subdivisions are helpful, particularly in mouse immunology, in human cells the distinction between subsets is often blurred, with multiple cell types producing similar cytokines (Crotty 2011). In tonsils and lymph nodes the situation becomes even less clear, as over half of the CD4⁺ T cells can be categorised as T follicular helper (T_{FH}) cells (with surface expression of CXCR5 and transcription of the regulatory gene BCL6), and yet they also express the characteristics of the other CD4⁺ T cell groups described (Crotty 2011).

In this project the cytokines produced in tonsil cell culture with or without the superantigen SPEA were assessed by ELISA, and flow cytometry (surface and intracellular staining) was performed to help define the major CD4⁺ T cell subsets present.

5.1.5.1 Cytokine production by tonsil suspensions in culture

There was a marked production of IL2, IL10, IL17, TNF α , TNF β and INF γ by tonsil cell cultures in response to stimulation with SPEA (Table 10, Figure 41, Figure 42), with minimal comparative production of these cytokines in the unstimulated cell cultures. No additional recombinant IL2 was added to cultures for time course experiments, to prevent alteration of the cytokine response to superantigens. Although there was a rise in all cytokines presented, the most significant difference was seen in the production of TNF α and TNF β .

It should be noted that there were very high baseline levels of TGF β 1 due to the presence of foetal calf serum in the cell culture media, which cross reacted with the ELISA. This meant that it was impossible to interpret low levels of TGF β 1, particularly from the first 5 days of culture. IL1 β , IL4, IL6, and IL12 were not detected in cell culture supernatants by ELISA.

These cytokines were chosen for examination as they are the cytokine where superantigens have been shown to most alter production in different *in vitro* models (Proft, Schrage, & Fraser 2007).

Cytokine	Peak day of production	Median unstimulated (pg/ml)	Median SPEA (pg/ml)	Number of tonsil donors	Significance
IL2	2	30	938	5	p=0.012
IL10	4	86	581	5	p=0.036
IL17	4-7	567	2241	7	p=0.0156
TNF α	4	145	1054	12	p=0.0005
TNF β	4	143	3616	8	p=0.0078
INF γ	5	10	535	7	p=0.0156
TGF β	7	129	480	7	p=0.0156

Table 10: Peak cytokine production in tonsil cell suspensions

The median values for production of each cytokine are shown on the peak day of production in both unstimulated and SPEA stimulated tonsil cell cultures. To test for statistical significance, IL2 and IL10 were assessed by the Mann Whitney Test (as numbers were too small for effective pairing), and all others were assessed using the Wilcoxon matched-pairs signed rank test.

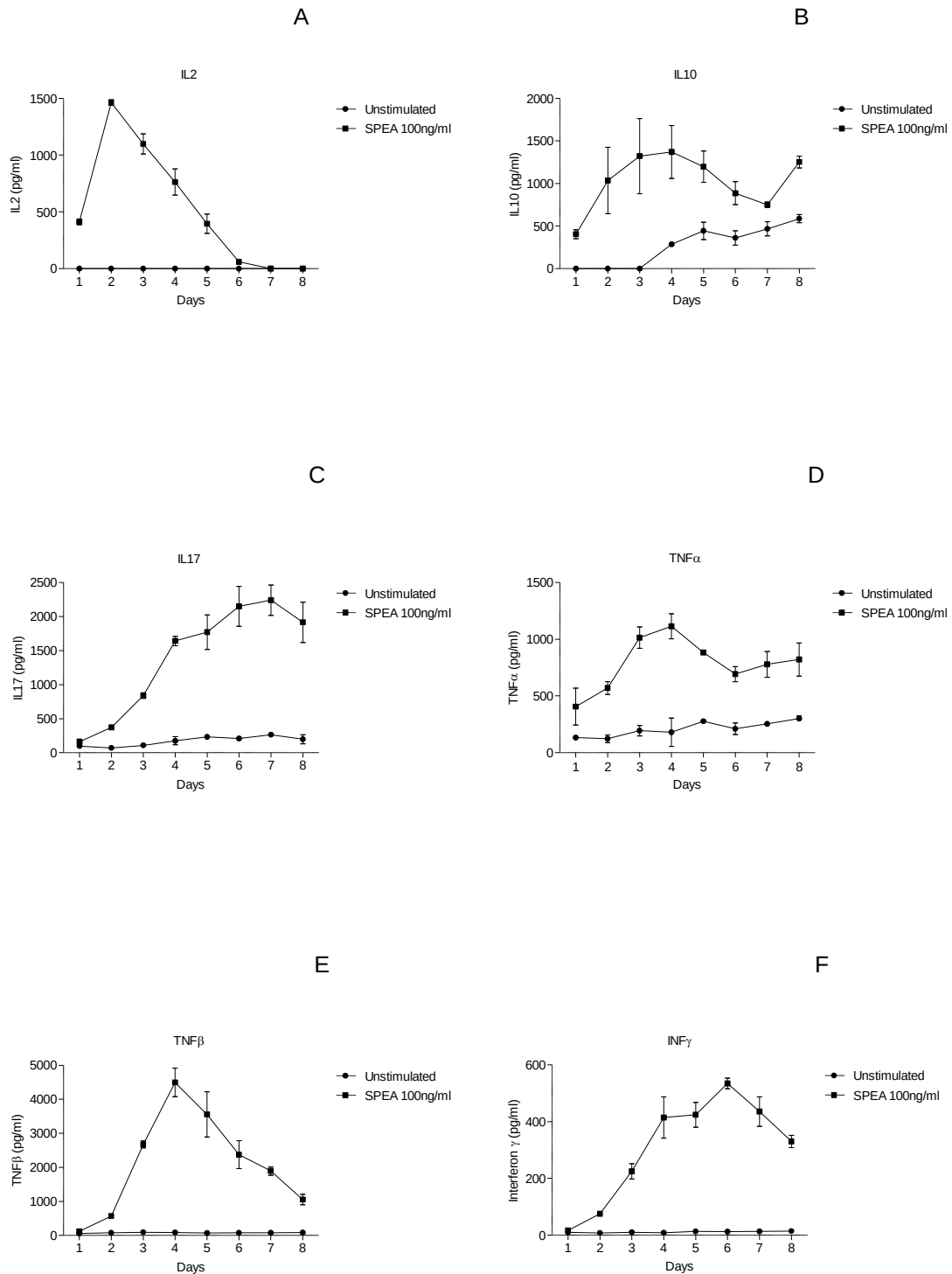


Figure 41: Tonsil cytokine production time course

Tonsil cells were stimulated for 1 week with recombinant SPEA (or unstimulated control) and supernatants harvested daily from days 1 to 8 of culture. Harvested supernatants were then analysed by ELISA for the presence of cytokines IL2 (A), IL10 (B), IL17 (C), TNFα (D), TNFβ (E) and INFγ (F). Mean and standard deviation of experimental triplicates for a single representative donor is shown for each cytokine.

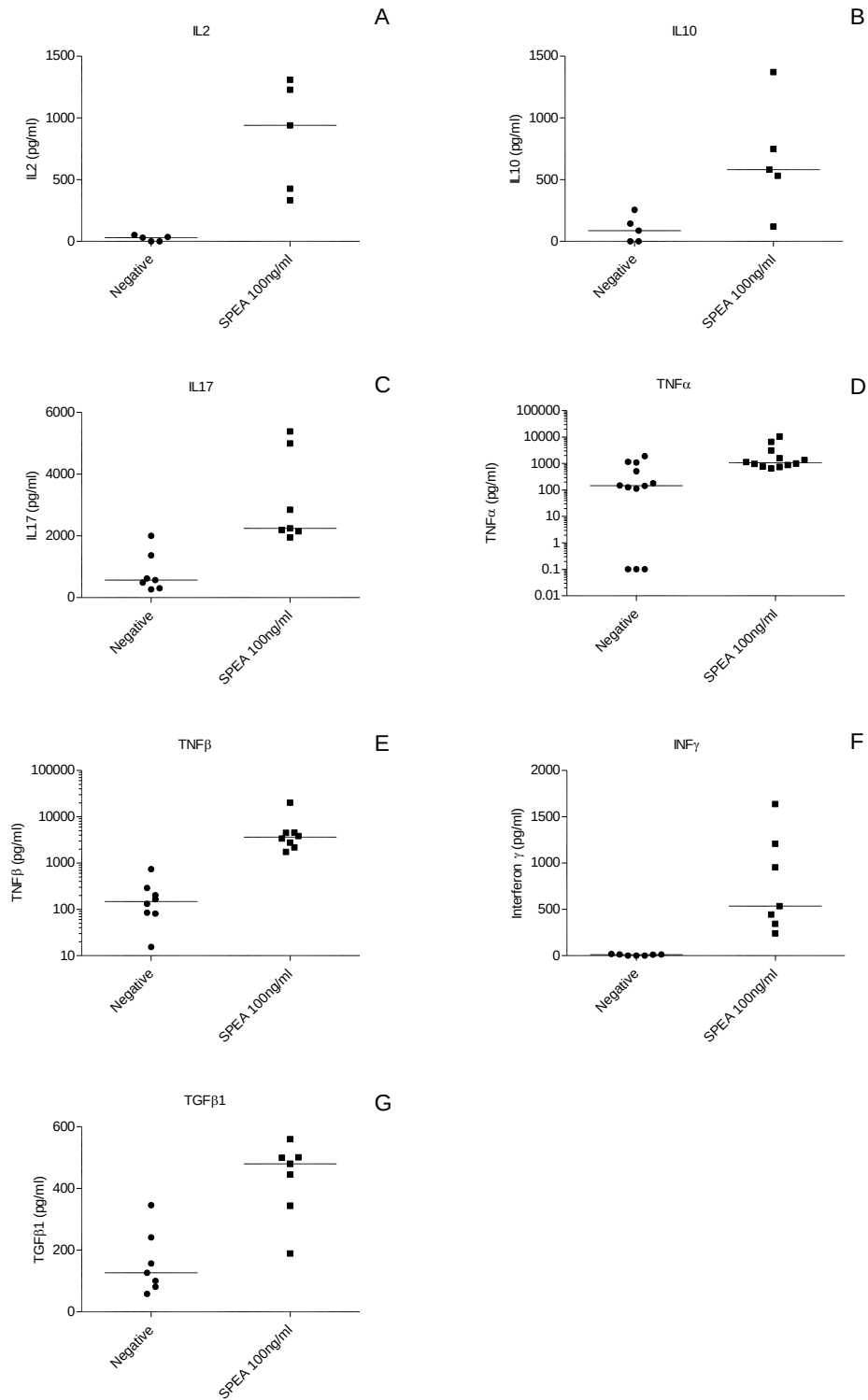


Figure 42: Median cytokine production

Median cytokine production for the major cytokines produced in the presence of SPEA or unstimulated cells cultures from the same day, as measured by ELISA. A/ IL2, N=5 different tonsil donors. B/ IL10, N=5 tonsil donors. C/ IL17, N=7 tonsil donors. D/ TNF α , N=12 tonsil donors. E/ TNF β , N= 8 tonsil donors. F/ INF γ , N= 7 tonsil donors, G/ TGF β 1, N=7 tonsil donors.

5.1.5.2 *T helper cell subset determination by flow cytometry*

The production of cytokines by T cells can be used as a marker of different T cell subsets, including T_H1 cells (INF γ), T_H2 cells, T_H17 cells (IL17) and T_H9 cells. Production of INF γ and IL17 had already been shown to be produced in cell culture supernatants, suggesting the presence of T_H1 and T_H17 subsets. Therefore tonsil cell cultures were stimulated for 5 days in the presence of SPEA 100ng/ml or unstimulated control cells from the same donor to confirm this. Due to a limited number of cells available from each tonsil donor, a single time point of day 5 was chosen for analysis, as this was the time at which there was maximal cytokine production as found by ELISA.

In contrast to ELISA results, there was no detection of INF γ or IL17 using flow cytometry in either unstimulated or SPEA stimulated cultures (N=2 different donors). The staining for intracellular IL4 showed an increase in the IL4 level in the SPEA stimulated cells in one donor, but there was no difference in the second tonsil donor tested (Figure 43A and B). To confirm which result is true, the experiment would need to be repeated on more tonsil donors. For IL9, there was no difference between the SPEA stimulated and unstimulated control cells in either donor tested (Figure 43C and D).

Regulatory T cells (TRegs) were identified by surface staining for CD4 and CD25, and intracellular staining for FoxP3 (Figure 44). Although there was a dramatic increase in the IL2 receptor CD25 expression on the CD4 T cells (mean 18.9% (+/- sd 7.5) of CD4 T cells expressing CD25 in unstimulated cells, 73.2% (+/- sd 17.7) in SPEA group, N= 5 different tonsil donors, p=0.0079 Mann Whitney test) in SPEA treated samples, there was no significant difference in the percentage of TRegs (CD4+ CD25+ FoxP3+ cells) between SPEA stimulated and unstimulated cell cultures (mean 6.69% of CD4 T cells unstimulated cells, 4.53% of CD4+ T cells SPEA treated cells, N=2 donors).

Less than 1% of T cells were lacking expression of the $\alpha\beta$ T cell receptor, and there was no change in this on superantigen stimulation (N=3 different tonsil donors). Therefore no further analysis for $\gamma\delta$ T cells (which lack expression of the $\alpha\beta$ component of the T cell receptor) was performed. Less than 1% of cells stained positive for the marker CD56, which is an indicator of NKT cells (data not shown).

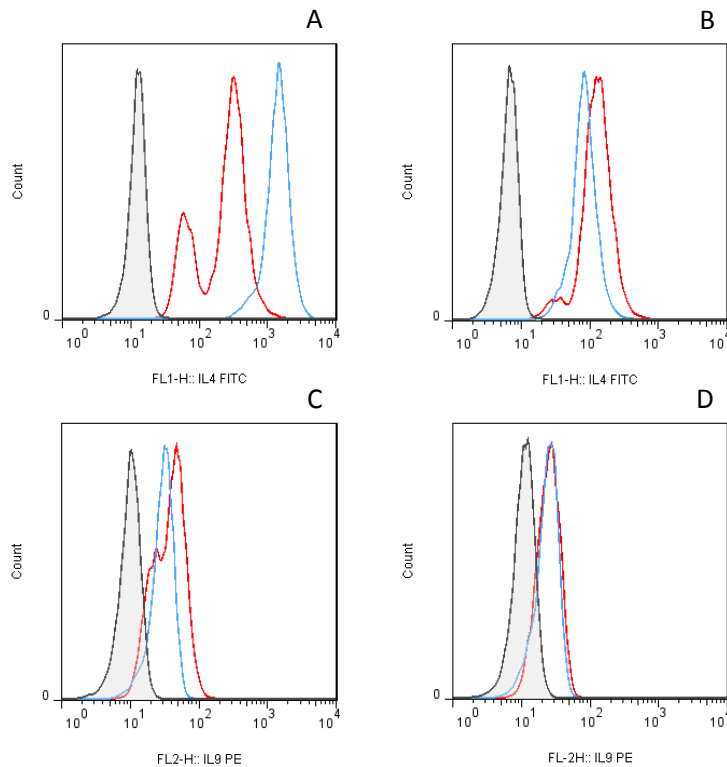


Figure 43: Intracellular staining for IL4 and IL9

Cells from two different tonsil donors were stained for intracellular IL4 (A and B) and IL9 (C and D). Grey = Isotype control, Red = Unstimulated cells, Blue = SPEA stimulated cells.

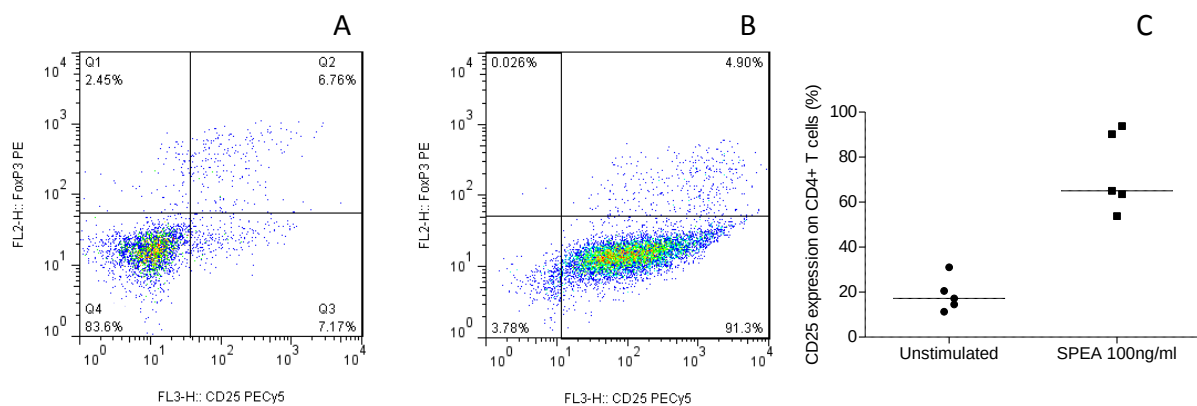


Figure 44: Regulatory T cells

There was no significant increase in the percentage of Regulatory T cells on stimulation of human tonsil cells with SPEA 100ng/ml. Flow cytometry plots shown are gated on CD4⁺ lymphocytes, FoxP3 determined with reference to an isotype control (not shown). There was a marked increase in the expression of CD25 in the SPEA treated group. A/ Unstimulated cells, B/ SPEA stimulated cells. Plots from one representative tonsil donor, N=2. C/ Median CD25 expression from 5 different donors.

Surface staining was performed for expression of CXCR5, the receptor for the chemokine CXCL13 expressed on T Follicular Helper cells (T_{FH}), as well as B cells and follicular dendritic cells. In human tonsils, expression of CXCR5 on T cells has been found to occur on cells in the mantle zones and throughout the germinal centres, and these cells have been identified as T_{FH} cells by confirmatory studies on expression of the regulatory genes BCL6 and BLIMP-1 (Crotty 2011).

In unstimulated tonsils, there was expression of CXCR5 on half of the CD4⁺ T cells (mean 47.2% $\pm$ s.d. 6.0), which dropped to 25% of cells ($\pm$ s.d. 5.5) in cells treated with SPEA 100ng/ml for 1 week (N= 3 different tonsil donors, Figure 45A). There was also a decrease in the fluorescence intensity of the cells with residual CXCR5 expression (Figure 45B, C). CXCR5 expression was not expressed on the clonally expanded TCRV β subsets of cells identified in Figure 39A (when the cells were counter stained for both TCRV β type and CXCR5, data not shown). Despite the loss of CXCR5 receptor expression, there was a significant increase in CXCL13 production in the cell culture supernatant (as measured by ELISA) in the SPEA treated group compared to unstimulated control cells (Figure 45D, $p=0.03$ Wilcoxon matched-pairs signed rank test, N=6 tonsil donors). This is despite there also being a loss of CXCR5 expressing non-T cells following SPEA stimulation (mean 55.8% $\pm$ s.d. 9.5 of the total lymphocyte population in unstimulated cells compared with 19.4% $\pm$ s.d. 9.1 in SPEA stimulated cells, N= 4 tonsil donors). Although specific staining for B cell and dendritic cell markers was not performed with CXCR5, this probably reflects the general reduction of B cells in the samples which decrease by a similar amount in the presence of SPEA (Figure 38).

Taken together these results lead to the conclusion that in the presence of SPEA, there is a clonal expansion of cells that are different in phenotype to the usual tonsil T cell population. These cells lack expression of CXCR5, a key receptor involved in tonsil and lymph node B-T cell interactions, they have increased expression of the IL2 receptor CD25, and are associated with a generalised pro-inflammatory cytokine release.

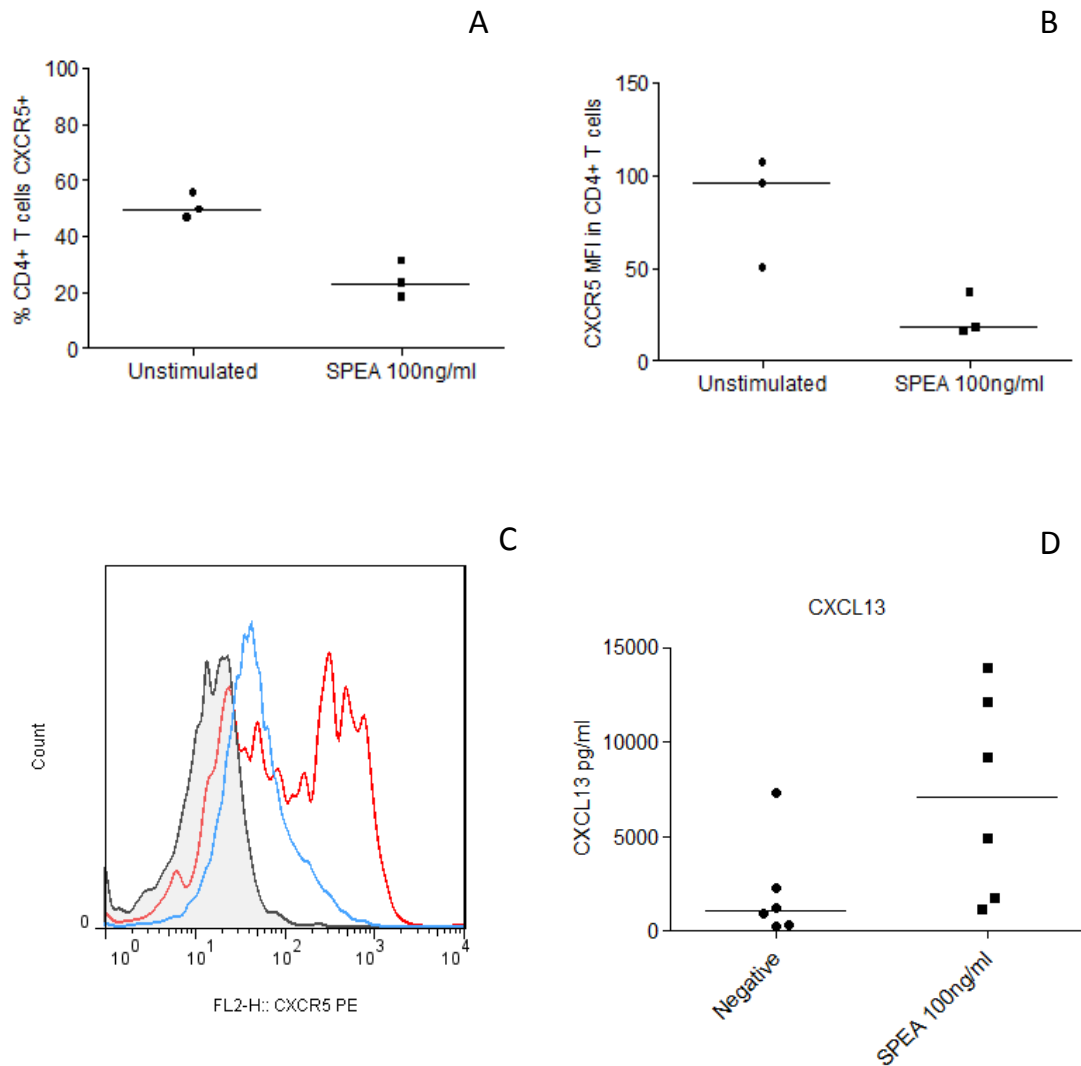


Figure 45: CXCR5 and CXCL13 expression

There was a reduction in CXCR5 expression in both percentage of CD4+ T cells (A) and Median Fluorescent Intensity (MFI, B), N=3 different tonsil donors. C/ an example of the flow cytometry plot is shown for one representative donor, Grey = Isotype control, Red = Unstimulated cells, Blue = SPEA 100ng/ml for 1 week. D/ CXCL13 expression in tonsil supernatants at 1 week, N=6 tonsil donors, $p=0.03$. All bars represent median values.

5.1.6 Effect of SPEA on tonsil B cell subsets

As shown in Figure 38, on stimulation with recombinant SPEA at a concentration of 100ng/ml, there was a marked reduction in the percentage and total number of B cells compared to unstimulated tonsil cell cultures. The low numbers of B cells present in SPEA stimulated cultures made accurate subset analysis by flow cytometry difficult. CD21, also known as complement receptor 2, is present on mature B cells and expression is lost on B cell receptor activation (Masilamani, Kassahn, Mikkat et al. 2003). CD21 was consistently expressed on >90% of CD20+ B cells (N=6 different tonsil donors), but in the presence of SPEA the percentage of the total lymphocyte population expressing CD20 and CD21 fell from a median of 55.4% of lymphocytes (range 43.1-67.1) to 25.5% of lymphocytes (range 11.2-35.4) in the SPEA stimulated group (N=5 tonsil donors, $p=0.0079$, Mann Whitney test, Figure 46), closely mimicking the general loss of B cells. There was no alteration in the expression of CD21 on the remaining cells on stimulation with SPEA compared to unstimulated cells.

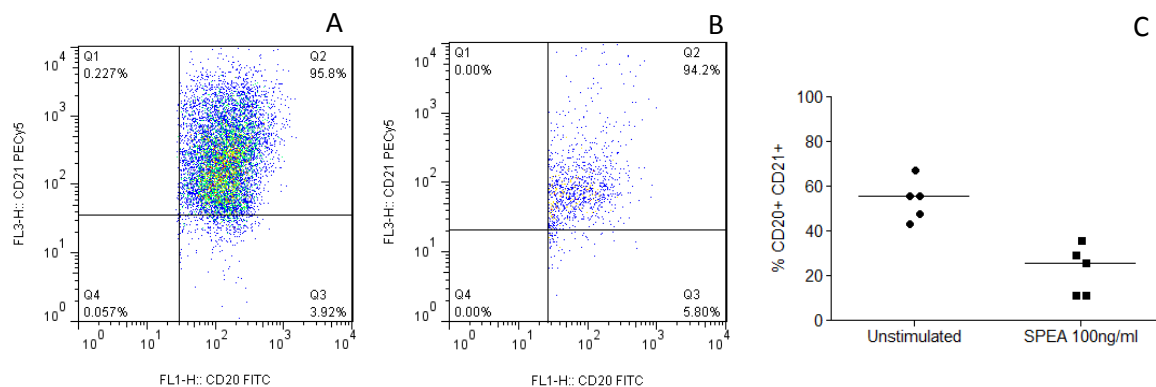


Figure 46: CD21 expression on tonsil B cells

CD21 expression on CD20 positive B cells remained constant at 94-95% of all B cells in the presence of SPEA (B) compared to unstimulated cells (A), though as a proportion of the total lymphocyte population CD21 expressing B cells fall from a median of 55.4% in unstimulated cultures at 1 week to 25.5% in SPEA stimulated cultures (C, $p=0.0079$). A and B are representative plots from one tonsil donor, N=5 different tonsil donors.

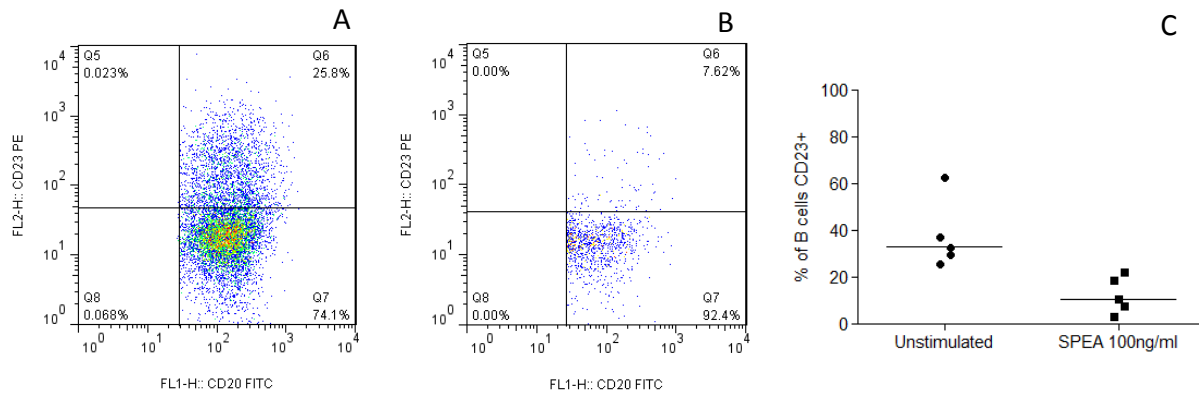


Figure 47: CD23 expression on tonsil B cells

The percentage of B cells expressing CD23, a marker of germinal centre B cells, falls from a median of 33% of B cells in unstimulated cultures (A) to a median of 10.6% of B cells in SPEA stimulated cultures (B), A and B are representative plots from one tonsil donor, N=5 different tonsil donors, as shown in C ($p=0.0079$).

There was a decrease in the proportion of B cells expressing CD23 (Fcε receptor II, indicative of germinal centre B cells); median percentage of B lymphocytes in unstimulated cells 33% (range 25.8-62.8%) compared to 10.6% (range 3.19-22.4%) in the SPEA stimulated group (N= 5 different tonsil donors, $p=0.079$, Mann Whitney test, Figure 47).

The expression of the B cell receptor on the surface of B cells is a marker of B cell viability, as loss of receptor expression on mature B cells is associated with inevitable B cell death (Goodnow, Vinuesa, Randall et al. 2010). The B cell receptor is comprised of surface expressed IgM and IgD. In 3 out of 4 tonsil donors tested there was a reduction in the surface expression of both IgM and IgD following exposure of cells to SPEA (Figure 48). Interestingly, in the fourth donor the expression of both surface IgM and IgD increased on the residual B cells in culture, even though the B cell remaining in culture represented only 11.7% of the total lymphocyte population (compared with a negative control of 62.2%). This may be consistent with increases in immunoglobulin gene function arising in those residual surviving B cells. The interaction between the co-receptor CD40 on B cells and the CD40 Ligand (CD154) on T cells is also essential for correct functioning of the B cell receptor. There was

no significant difference in the expression of the either CD40 on B cells or the CD40 ligand on T cells (data not shown).

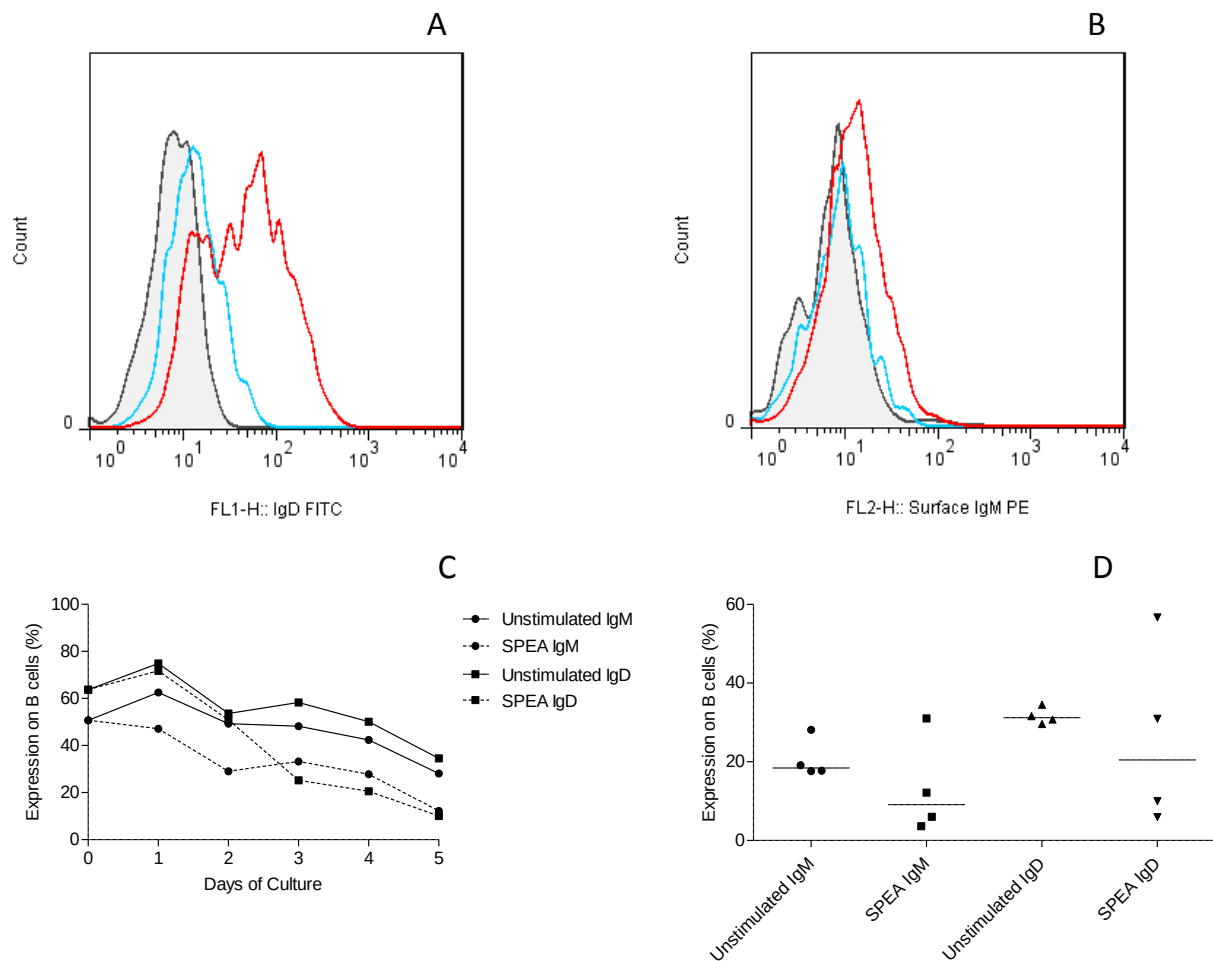


Figure 48: Expression of surface IgD and IgM

The B cells remaining in culture at 1 week demonstrated a marked loss of expression of the B cell receptor components, surface IgD (A) and IgM (B), Grey = isotype control, Red = unstimulated, Blue = SPEA 100ng/ml. Over time, this fell steadily for both classes of surface immunoglobulin (C, representative plot from one tonsil donor). These results were consistent for 3 out of 4 donors tested (D), with median values for IgM of 18.4% of B cells (Unstimulated) falling to 9.11% (SPEA) and IgD falling from 31.2% (Unstimulated) to 20.5% (SPEA) after 1 week of culture.

Two markers of B cell progression to immunoglobulin secreting (plasma) cells, are increasing expression of CD38 (the glycoprotein cyclic ADP ribose hydrolase) and CD27 (a member of the TNF receptor superfamily, expressed on mature B cells), with a loss of CD19 and CD20 expression on

reaching full plasma cell maturity. In unstimulated cultures, a mean of 10.22% (+/- s.d. 3.4) of total lymphocytes stained double positive for CD20 and CD27, representing mature B cells (data not shown). There was no significant difference in the percentage of B cells which were expressing CD27 between unstimulated tonsil cell cultures (median 31.6% of cells, range 9.2-35.2%) and those stimulated with SPEA (median 20.6% of cells, range 15.9-24.3%, $p=0.4206$ Mann Whitney test $N=5$ different tonsil donors, Figure 49). There was also no significant difference in the percentage of B cells expressing CD38 between unstimulated tonsil cell cultures (median 39.4% of B cells, range 31-62.8%) and those stimulated with SPEA (median 59% of B cells, range 46.2-76.7, $p=0.0952$ Mann Whitney test, $N=5$ different tonsil donors).

Overall this means that in the presence of SPEA, the predominant B cell populations (of those tested) which decreased were those B cells expressing CD23, the mature germinal centre B cells. The few B cells remaining still expressed CD21, CD27 and CD38, but represented a lower percentage of the total lymphocyte population than in unstimulated cultures.

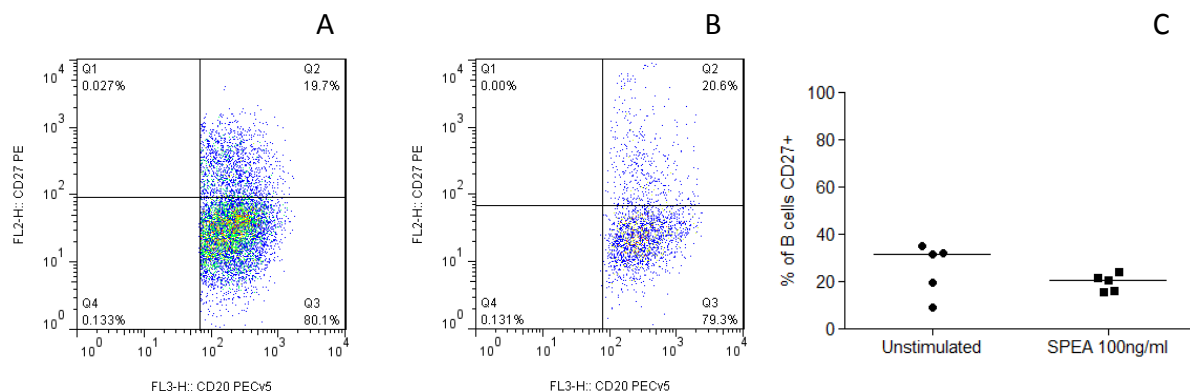


Figure 49: CD27 expression in tonsil B cells

There was no significant change in the percentage of B cells expressing CD27 (a marker of memory B cells) between tonsil cells cultured without stimulation (A) or in the presence of SPEA (B). Representative plots are shown for 1 donor in figures A and B, median for 5 different tonsil donors shown in C, ($p=0.42$).

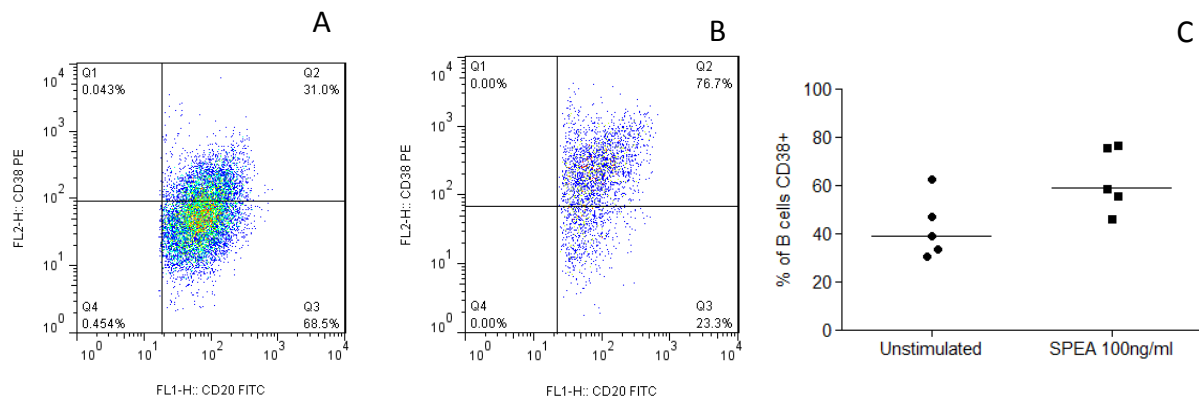


Figure 50: CD38 expression in tonsil lymphocytes

There was no significant difference in the expression of CD38 between tonsil B cells which were unstimulated (A) compared with those which were cultured in the presence of SPEA (B). CD38 expression was calculated with reference to an isotype control (not shown). Representative plots for 1 donor are shown in figures A and B. The median values for 5 different tonsil donors in shown in C ($p=0.0952$).

5.1.7 Immunoglobulin production in the presence of SPEA

One of the predominant functional outputs of B cells is immunoglobulin. As previously outlined in chapter 4 (Figure 30) approximately 1000 ng/ml of IgG, IgA and IgM was produced after one week of normal tonsil cell suspension culture *in vitro*. As shown by flow cytometry earlier in this chapter, in response to superantigens, there was a loss of B cells from tonsil cultures in the presence of SPEA, and the phenotype of the proliferating T cells changes from a T_{FH} phenotype with high expression of CXCR5 (which promotes B cell function) to one which expresses no CXCR5 but produces copious cytokines. To see what the functional consequences of these cellular alterations were, immunoglobulin expression was assessed in culture supernatants from tonsil cells treated with SPEA 100ng/ml, as compared to unstimulated cells.

In the presence of SPEA, there was no increase in the production of all classes of immunoglobulin tested (IgG, IgA or IgM, Figure 51) above the baseline value produced in early culture. For IgG the median production fell from 1099ng/ml (range 576.4 to 3914 ng/ml) in unstimulated cultures to 491.1ng/ml (range 95.3 – 977.5ng/ml) with SPEA stimulation ($p=0.0005$ Wilcoxon matched-pairs signed rank test, N=16 different tonsil donors). For IgA this fell from 1032ng/ml (range 561.2 to 1228ng/ml) in unstimulated cultures to 513.4ng/ml (range 140.8-751.8ng/ml) in the presence of SPEA ($p=0.0078$ Wilcoxon matched-pairs signed rank test, N=8 different tonsil donors). For IgM the median in unstimulated cultures was 1035ng/ml (range 222.8 – 4809ng/ml) compared to a SPEA stimulated value of 291.4ng/ml (range 75.2 – 507.6ng/ml, $p=0.0156$ Wilcoxon matched-pairs signed rank test, N=7 different tonsil donors). The same 7 tonsil donors were tested for IgM, IgA and IgG. Additional tonsil donors for IgA (1) and IgG (9) were the result of different experiments where IgM/A was not tested

Baseline values of immunoglobulin varied widely, and probably reflected the number of mature plasma cells already present in the different tonsils when they were collected.

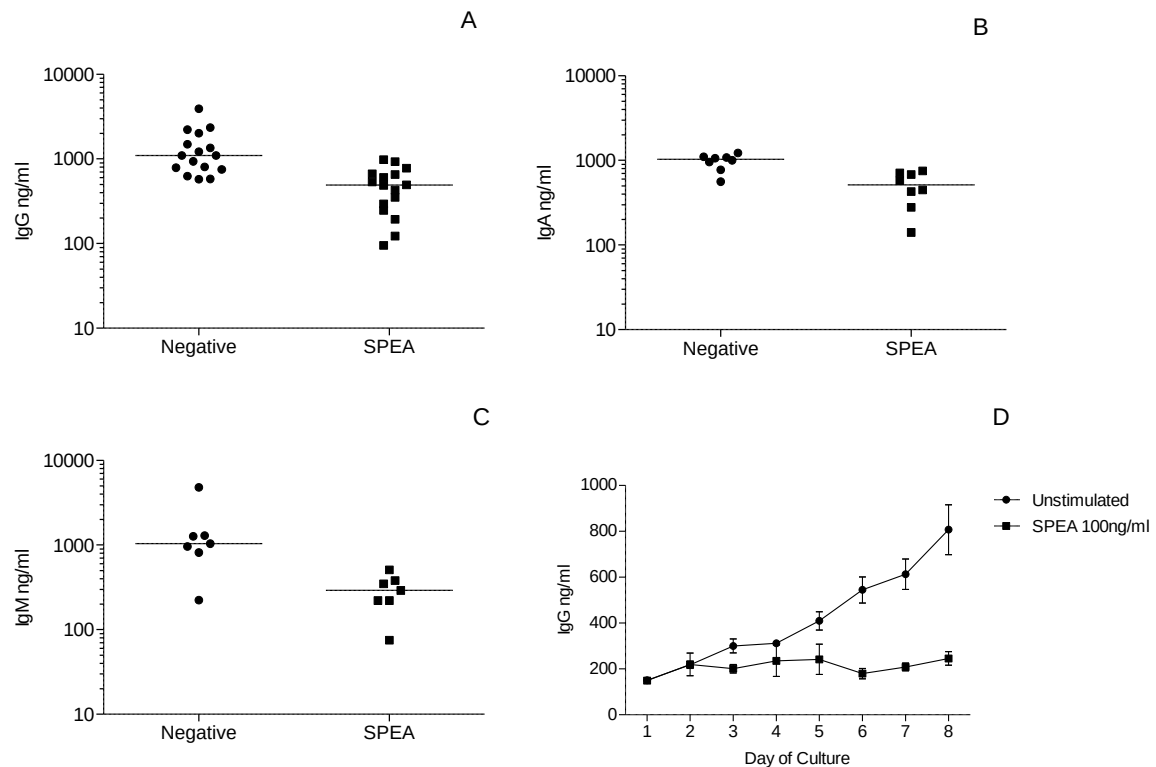


Figure 51: Production of immunoglobulin in the presence of SPEA

SPEA significantly reduced the production of IgG (A, N=16 donors) IgA (B, N=8 donors) and IgM (C, N=7 donors) from tonsil cell culture suspensions in comparison to unstimulated cultures after 7 days of culture. A typical plot from one donor of IgG production over time is shown in figure D (representative of N=5 different donors).

5.1.8 IgG production with other T cell mitogens

To see if this effect of reducing immunoglobulin production was specific to SPEA, a number of different T cell mitogens were tested. These included the streptococcal superantigens SMEZ and SPEJ, and the staphylococcal superantigens SEB, SEC and Toxic shock syndrome toxin (TSST) 1. In addition, a non-superantigen T cell mitogen Concanavalin A was tested. As T cell proliferation is known to occur following stimulation with combined anti-CD3 and anti-CD28 antibodies, these antibodies were also used to assess the impact of T cell proliferation on B cell immunoglobulin production in tonsil cell cultures (Figure 52).

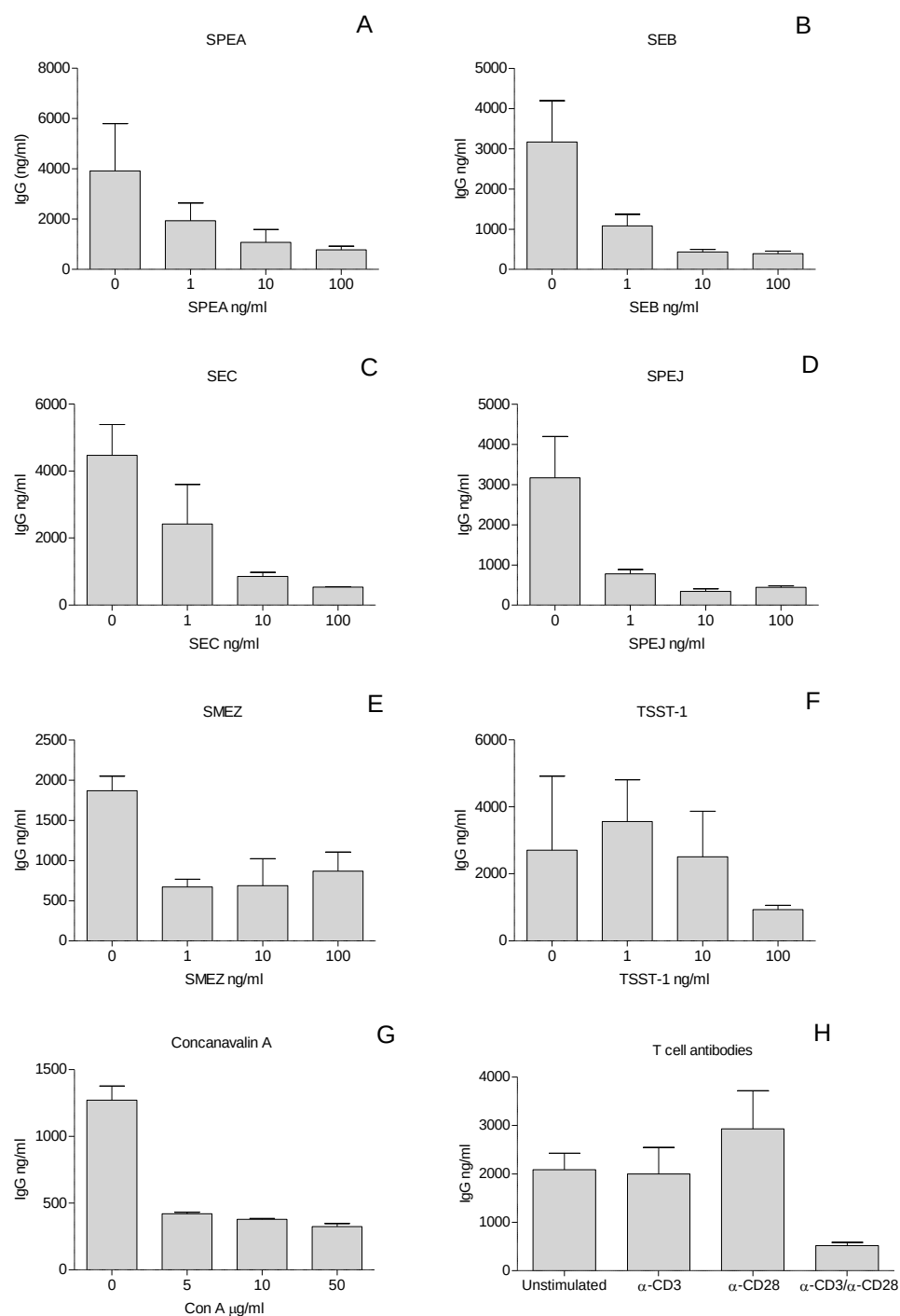


Figure 52: Immunoglobulin production with a range of T cell mitogens

IgG production in the presence of different concentrations of a variety of bacterial superantigens was tested: SPEA (A), SEB (B), SEC (C), SPEJ (D), SMEZ (E) and TSST-1 (F). For comparison, the T cell mitogen Concanavalin A was tested (G) and T cell stimulation using anti(α-) CD3 and CD28 antibodies (H). All mitogens inhibited immunoglobulin production in cultures at 1 week. Each graph shows the mean and standard deviation of experimental triplicates from one representative tonsil donor (Data representative of experiments using N=3 donors for the different superantigens, N=2 for Concanavalin A and α-CD3/28).

It was found that a similar inhibition of IgG production occurred in response to all of these T cell mitogens, though the concentration required to produce the effect varied between mitogen. The superantigens SEB, SEC, SPEJ and SMEZ all reproduced the effect in similar concentrations to those of SPEA (partial inhibition at 1ng/ml, full inhibition with 10ng/ml or greater, Figure 52A-E). However TSST-1 required a minimum of 100ng/ml to inhibit IgG production compared to unstimulated cultures (Figure 52F). In contrast to one previous study which noticed a similar effect with TSST-1 (Hofer, Newell, Duke et al. 1996) there was no significant increase in immunoglobulin production noted with any of the superantigens when used at lower concentrations (N=3 different tonsil donors).

As superantigens are known to cause T cell expansion and cytokine production it was investigated whether other known T cell mitogens could produce the same effect on immunoglobulin production. Anit-CD3 and anti-CD28 monoclonal antibodies (both singularly and combined, according to manufacturers' recommended concentrations) and Concanavalin (Con) A were tested. It was found that either high concentrations of Con A (Figure 52G) or combined anti-CD3/28 (Figure 52H) stimulation could recreate this effect. Anti-CD28, when used alone, enhanced immunoglobulin production, highlighting the importance of this co-stimulatory molecule in normal immunoglobulin responses.

The data imply that the inhibition of immunoglobulin production by superantigens is a class effect of T cell mitogen stimulation rather than a specific toxic effect of superantigens. As shown earlier with flow cytometry, the phenotype of T cells is altered away from T_{FH} cells in the presence of mitogens, with a dramatic loss of CXCR5 expression. Similar flow cytometry studies would be useful to confirm that the mechanism of inhibition and T cell phenotype change is the same with all mitogens used, but this was outside the time restraints of this project.

5.1.9 Immunoglobulin with bacterial supernatants

In order to confirm that the changes in IgG production in the presence of T cell mitogens were a true result, and also physiologically relevant, IgG production was assessed in tonsil cell cultures treated with bacterial culture supernatants from the isogenic superantigen +/- *emm* 1 and *emm* 89 strains of *S. pyogenes* mentioned previously (section 5.1.1, 5.1.4). Bacterial strains were grown in the same media as tonsil cultures (without antibiotics) and filter sterilised for use, with media alone used as a control.

There was a significant difference in IgG production with the tonsil cultures treated with *speA* +/- *emm* 1 *S. pyogenes* supernatants ($p=0.0087$, Friedman Test, $N=5$ different tonsil donors). There was an increase in the production of IgG in the *speA*- strain compared to the wild type strain, which reduced when *speA* function was restored by complementation (Figure 53A). There was no significant difference, however, in cultures treated with the *smeZ* +/- *emm* 89 strain supernatants, or when *speA* expression was added ($p=0.0543$ Friedman Test, $N=6$ different tonsil donors, Figure 53B).

It is interesting to note that there was no increase in the IgG production on stimulation with the wild type *emm* 1 supernatant. This is surprising, because the normal response of lymphoid cells to bacterial antigens would be to produce more immunoglobulin, and suggests that the quantity of superantigen produced must be significant to reduce this effect. It should also be noted that a mutation a regulatory gene (*CovRS*) has arisen in the *speA*- *emm* 1 strain; the regulatory gene mutation can abrogate expression of the cysteine protease SPEB through mechanisms that are not understood (Shelburne, Olsen, Suber et al. 2010). As one of the described functions of SPEB is to cleave immunoglobulin it is possible that the observed differences between the *emm*1 parent strain of *S. pyogenes* and the *speA*- *emm* 1 mutant could be accounted for by changes in SPEB protease activity rather than the superantigen SPEA. However, the mutation is also present in the *speA* complemented strain which diminishes immunoglobulin production, thus the observed differences must be related to SPEA rather than SPEB having the predominant effect on IgG levels.

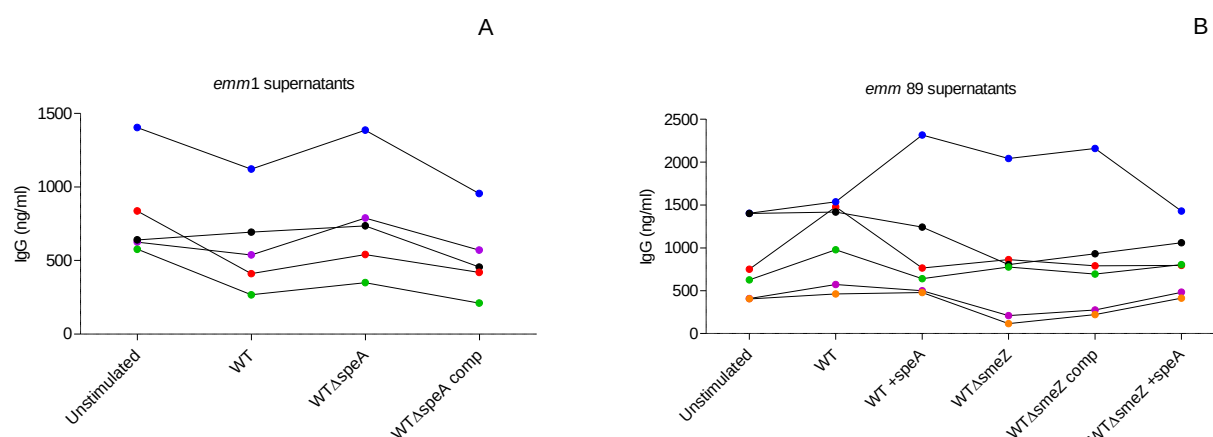


Figure 53: IgG production with bacterial supernatants

IgG production was measured in the supernatants from tonsil cell suspensions treated with bacterial culture supernatants from *emm 1* (A) and *emm 89* (B) strains of *S. pyogenes*. For the *emm1* strain, results are shown for the wild type parent strain (WT), isogenic *speA*- mutant *emm 1* strain (WTΔ*speA*) and *speA* complemented strain (WTΔ*speA* comp), for 5 different tonsil donors (results for each individual donor linked by a line). For the *emm89* strain results are shown for the wild type parent strain (WT), complemented with *speA* (WT+*speA*), isogenic *smeZ*- mutant (WTΔ*smeZ*), and complemented strains with *smeZ* (WTΔ*smeZ* comp) or *speA* (WTΔ*smeZ* +*speA*), for 6 different tonsil donors (results for each donor linked by a line).

5.1.10 IgG production in histocultures treated with superantigens

As previously described, tonsil histocultures are able to produce immunoglobulin much faster and at a greater magnitude than cell suspension cultures. Therefore, histocultures were exposed to superantigens in the culture media and also briefly before being mounted onto the blocks. The IgG responses at 48 hours of culture were examined in the media. There was a significant reduction in immunoglobulin production with SPEA and SPEJ, ($p=0.0174$, Friedman test). Although not significant, a reduction in IgG production with SMEZ was observed in two of the three donors tested. These findings with histocultures substantiate the results obtained using cell suspension cultures.

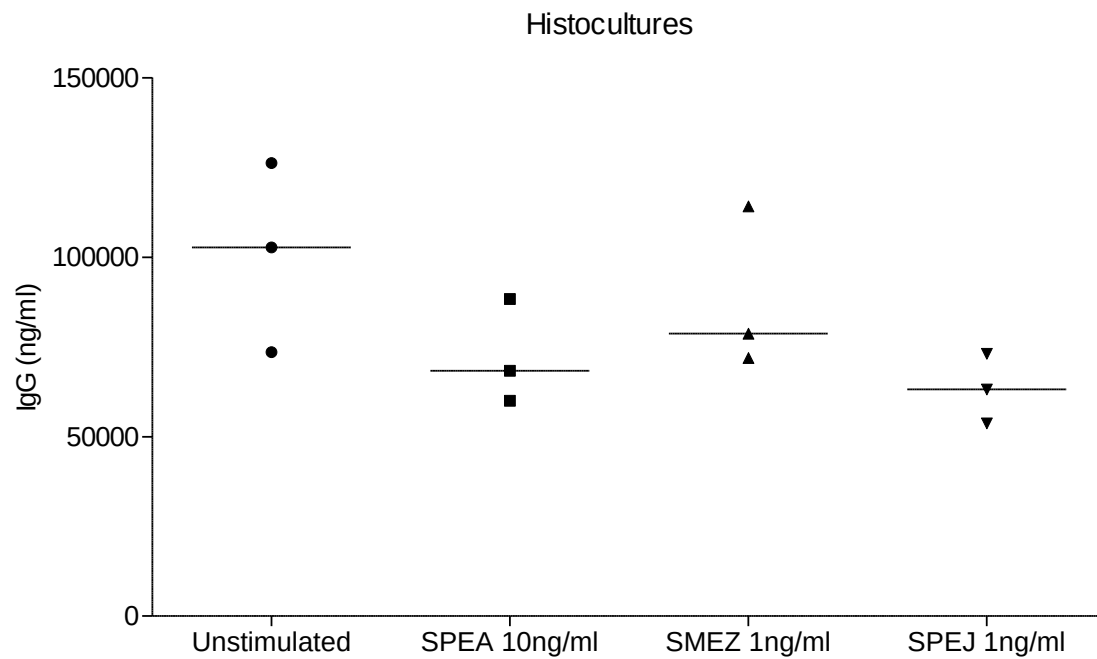


Figure 54: IgG production in histocultures with superantigen

Tonsil histocultures were treated with superantigen for 48 hours, before culture media were collected and analysed for IgG production. The bars represent the median of 3 different tonsil donors, each tested as experimental triplicates. $p=0.011$, ANOVA.

5.2 Discussion

The known mechanism of action of superantigens, by binding to the T cell receptor, makes it unsurprising that the predominant proliferating cell type in SPEA stimulated cultures was T cells. However, the loss of B cells is not a widely recognised effect of conventional superantigen stimulation. Although B cell loss in the spleen of mice injected with SPEA and a subsequent reduced antibody response was one of the earliest observations of the effects of superantigens (Cunningham and Watson 1978a), no satisfactory explanation for the mechanism of this has been made. By using human tonsils rather than PBMC's to examine human immune responses to superantigens, this effect has again been noted as a predominant feature of superantigen stimulation, and re-examined with the benefit of recent immunological discoveries.

5.2.1 Tonsil T cell proliferation

The baseline proliferation values achieved from tonsil cell suspension cultures are far higher than those previously reported from the same concentration of peripheral blood mononuclear cells (PBMC's). As the percentage of T cells is lower in tonsil cell suspensions than in PBMC's (tonsil T cells represent about 40% of the normal lymphocyte population, compared to approximately 75% of PBMC's), this is unlikely to be the cause of increased proliferation. However, the proportion of MHC class II expressing cells is far higher in tonsils than PBMC's (in a ratio of nearly 1:1 with T cells), and this perhaps means that the relative proportion of the T cells present which are able to proliferate is subsequently higher. Despite the high background readings, tonsil cell suspensions still demonstrated a fine degree of discrimination to different concentrations of superantigens, producing near perfect biological concentration curves (Figure 36).

It is unlikely that there are cells other than T cells proliferating in response to superantigens, as the flow cytometric analysis shows that the cells which expand in number in response to superantigens

are T cells. Other possible reasons for the increased T cell proliferation rates in tonsils include the possibility that tonsil cells are already in a “primed” state, with higher levels of background activation than PBMC’s. Certainly, all patients having a tonsillectomy are doing so because of an underlying disease state, generally caused by recurrent throat infections. Both *S. pyogenes* and *S. aureus* produce superantigens, and these are among the commonest bacteria to be isolated from tonsil core biopsies, and have been shown to cause inflammation rather than just colonisation (Zautner et al. 2010). Another explanation is the different T cell phenotype in tonsils compared to PBMC’s. As noted in section 5.1.5, a high proportion of T cells express the T follicular helper phenotype, a cell subset present in low proportions in peripheral blood T cells.

Establishing that this tonsil cell culture system was able to respond in a similar manner to previous *in vitro* superantigen work was important for proof of concept, and setting this model against the previous benchmarks of PBMC and mouse work. Having shown that proliferation was both possible and discriminatory in this culture system, it was possible to reliably explore the more detailed T lymphocyte responses associated with superantigens.

5.2.2 Tonsil TCRV β subset expansion

Initial TCRV β expansion experiments were focussed around the 3 key subsets V β 2, 8 and 14 previously shown to respond to the superantigens of interest SPEJ, SMEZ and SPEA respectively. TCRV β 11 was included as a negative control, to show that the proliferation seen was not a generalised non-specific T cell subset expansion, as TCRV β 11 is not known to be expanded by any superantigens to date (and is usually used as a marker for invariant NKT cells). Subsequent analysis using the Beckman-Coulter IO test beta mark kit provided a far greater degree of detail, but time restraints and a restricted number of tonsil donors meant that this has not been performed on as many donors as would be ideal. Certainly for SPEJ and the bacterial culture supernatants, it would be

important for these experiments to be repeated on a number of different tonsil donors to confirm the key TCRV β subset changes.

Previously published work looking at the isogenic +/- *speA* and *smeZ* strains of *S. pyogenes* used here show that SPEA and SMEZ do not have suppression effects on the production of other superantigens or secreted proteins (Russell & Sriskandan 2008). However, the TCRV β profile attained with the *emm* 89 wild type strain was very similar to the profile with purified SMEZ. This suggests that although the production of other superantigens is not suppressed, they have little functional effect on the T cells compared to SMEZ. Since the start of this project, a range of new *S. pyogenes* strains has been developed, in particular, an isogenic mutant strain of the parent *emm*1 strain H305 which does not express either *smeZ* or *speA*. Further characterisation of TCRV β responses both in tonsils and in PBMC's using these strains would provide valuable information about the balance of superantigen production, and consolidate the findings presented here.

Recent studies have considered the use of TCRV β profiles of the blood of patients who are clinically septic to try to determine if they have toxic shock syndrome (Ferry, Thomas, Bouchut et al. 2008; MacIsaac, Curtis, Cade et al. 2003; Thomas, Perpoint, Dauwalder et al. 2008). However, although an individual superantigen may show preference for a limited number of TCRV β subsets, this has not been fully worked out for all superantigens, and there is a large degree of cross-over between the profiles of both staphylococcal and streptococcal superantigens. This is not even taking into account the human factors that may cause variation in TCRV β profiles, such as chronic inflammatory conditions or haematological malignancy. The preliminary work presented here using the bacterial isogenic superantigen +/- mutant strains has shown that even when a known superantigen profile exists, the individual contribution of each superantigen can vary in the effect it produces. With the variation of superantigen genes carried even in a single *emm* type of *S. pyogenes* (Turner et al. 2011), there is little replacement for clinical and conventional laboratory diagnostic techniques using TCRV β profiling of septic patients until this has been explored in greater detail.

5.2.3 Cytokine production in response to SPEA

When tonsils were stimulated with 100ng/ml of SPEA, there was a profound release of T_H1, T_H2 and T_H17 cytokines, as measured by ELISA. The predominant cytokines measured were TNF α and TNF β at day 4 of culture, followed by a later peak of IL17 at day 6 of culture. IL2, IL10, INF γ and TGF β were all produced at lower levels, though still clearly detectable. This supports the findings by previous investigators, that show a variety of cytokines produced, with considerable inter-donor and inter-superantigen variation (Proft, Schrage, & Fraser 2007). IL1 β , IL4 and IL6 were not detectable in culture supernatants, which is slightly surprising as they are noted among the cytokines produced by superantigens. This may be because the cell distributions in tonsils are different from PBMC's, with some of the cytokine production coming from follicular dendritic cells rather than monocytes/macrophages.

IL17 production has been previously shown in response to whole killed *S. pyogenes* preparations (Wang, Dileepan, Briscoe et al. 2010), and the streptococcal superantigens SPEC and SMEZ (Li, Nooh, Kotb et al. 2008). IL17 has also been shown to be produced *in vitro* in response to the staphylococcal superantigens SEB and SEC after 3 to 6 days of culture (Islander, Andersson, Lindberg et al. 2010; Purvis, Stoop, Mann et al. 2010). In this project, although IL17 and INF γ were detected by ELISA, they were not detected by intracellular staining. The cells were specifically not re-stimulated with PMA/ Ionomycin or anti-CD3 antibodies before staining, as cells stimulated with superantigens should not be in a resting state on day 5 of culture, a time when they are still actively proliferating. Although this may be the cause for reduced detection of the intracellular cytokines, and would be appropriate to try with future donors and at different time points, this may not be the answer as to why intracellular staining failed to detect IL17 or INF γ . Studies in PBMC's with SEB have shown that intracellular IL17 and INF γ were only detectable when low concentrations of superantigen were used (<10pg/ml SEB), despite high concentrations in the culture supernatant being detected by ELISA at the same time point (Purvis et al. 2010). Also, PBMC studies have shown that the level of intracellular INF γ detected in response to SEB drops dramatically after 24 hours culture (Mehta and

Maino 1997), so it is likely that although the cells were analysed at the time of peak INF γ detection in the culture media, this was too late for peak detection within cells.

The measurement of TGF β -1 was difficult in this culture system, as the kit used cross-reacts with bovine TGF β , which is present in the foetal calf serum used in the media. The best way to overcome this, to allow the measurement of a time-course of TGF β , would be to culture the cells in serum free media. Preliminary experiments using AIM V media showed good tonsil cell viability, but there was insufficient time to repeat the experiments in this media for the purposes of measuring TGF β . However, there was a clear difference in the peak amount of TGF β produced in the presence of superantigens (as measured by correcting for the baseline level in the media), and this is shown in Figure 42G. TGF β 1 has multiple regulatory functions and, in the context of *S. pyogenes* infection, it promotes bacterial invasion of epithelial cells (Wang, Li, Southern et al. 2006). There is the suggestion from a mouse NALT model of infection that TGF β and IL17 production are important in the formation of cellular immunity to *S. pyogenes* (Wang et al. 2010). TGF β has been found to have marked effect on T cell proliferation, differentiation and survival, depending on the presence of other cytokines and regulatory signals (Li and Flavell 2008). This being the case, then the TGF β 1 and IL17 responses demonstrated here would support a theory that the responses shown to superantigens are part of a healthy immune response rather than an abnormal immune response.

IL21 has been identified as a key cytokine produced by T_{FH} cells, and is involved in tonsil B cell responses (Bryant, Ma, Avery et al. 2007; Eto, Lao, DiToro et al. 2011). Although levels of this cytokine were not examined for this project, it would be interesting to see how levels changes with the introduction of SPEA, especially as *in vivo* mouse studies have shown that levels increased in the presence of SEB (Rajagopalan, Tilahun, Asmann et al. 2009).

5.2.4 Loss of T cell follicular helper phenotype

On examination of the T cell subsets, the most striking change was the loss of CXCR5 expression on the T cells in response to SPEA exposure, both in percentage of cells and fluorescent intensity, particularly in the T cells which were actively proliferating. CXCR5 was initially described as the G-protein coupled Burkitt's-Lymphoma Receptor-1 (BLR1), and was found to be highly expressed in both B cells and CD4⁺ T cells of lymphoid tissue origin. It was noted that expression could be rapidly down regulated on both B and T cells in response to stimulation with CD40 and CD3 monoclonal antibodies respectively (Forster, Emrich, Kremmer et al. 1994). Expression on B cells was predominantly on mature B cells, but was lost as they developed into immunoglobulin-secreting cells. On T cells, expression of CXCR5 was not found on activated CD25 (IL2 receptor) expressing cells. In human tonsil sections, CXCR5 expression on B cells was most pronounced in the mantle zones, but on T cells was also expressed on cells in the inter-follicular regions and throughout the germinal centres (Forster et al. 1994). These T cells expressing CXCR5 in tonsils have now been identified as T_{FH} cells and are characterised by transcription of the gene BCL-6 and suppression of BLIMP-1 transcription (Crotty 2011).

CXCR5 acts as the receptor for the B cell chemokine CXCL13, and in lymph nodes is important in communication between B and T cells. Despite the loss of CXCR5 expression on the tonsil T cells, there was a marked increase in CXCL13 detected in the culture supernatants of SPEA stimulated cells compared to unstimulated controls (Figure 45D). Although CXCL13 is produced by T_{FH} cells, another major source of this chemokine is follicular dendritic cells. Experiments using SEB have previously shown that CXCL13 production in response to superantigen stimulation was all produced by T_{FH} cells, and none was produced by B cells or dendritic cells, and that the production was dependent on B-T cell contact. Similar results were obtained with the use of both CD28 and CD3 antibodies for T cell stimulation. Interestingly, when T_H1 or T_H2 cells were substituted for the T_{FH} cells (i.e. T cells not expressing CXCR5) at the start of the experiment, there was no production of CXCL13 in response to SEB (Kim, Lim, Kim et al. 2004). However, the expression of CXCR5 was not assessed after

stimulation in those experiments, unlike the experiments performed here. Recent work has shown that CXCR5/CXCL13 interaction acts in conjunction with B cell receptor ligation to cause activation of B cells, without interfering with and independent from the B-T cell immune synapse (Saez de, Barrio, Mellado et al. 2011).

From the results presented here, it can be concluded that SPEA, as a model superantigen, is disrupting the normal T and B cell responses to antigen stimulation. The result is abnormal stimulation of T cells, with increased proliferation but decreased CXCR5 expression. Despite this CXCL13 production continues and is increased in response to SPEA, but without the end effect of increasing B cell immunoglobulin production. These experiments have not explained the mechanisms behind this, and to further investigate the mechanism of CXCR5 expression loss, the expression of key regulatory genes for T cells and B cells are examined further in Chapter 6, as well as activation and apoptosis markers and detailed examination of the T-B cell immune synapse.

5.2.5 Loss of B cells and immunoglobulin with SPEA

The other profound effect noted on stimulation of tonsil cultures with SPEA was the loss of B cells and abrogation of IgG, IgA and IgM production. There was a loss specifically of B cells expressing CD23, with down regulation of IgD and IgM expression, representing those cells located in germinal centres and which classically react in a T-cell dependent fashion. This B cell loss is consistent with the degree of T cell proliferation observed with superantigens, and deviation away from a T_{FH} phenotype. Those B cells remaining expressed CD21, a marker of mature B cells which have not been activated through the B cell receptor (CD21 expression being lost on BCR activation), also known as marginal zone B cells, and which exhibit more T-independent immune responses, but which have also been linked to antigen presentation to T cells (Pillai, Cariappa, & Moran 2004). There were also remaining CD20+ cells which were expressing CD38, a plasma cell marker and CD27, a memory cell marker (Sanz, Wei, Lee et al. 2008). CD20 expression is generally lost on development of B cells into

mature plasma cells, but in tonsils B cells in the germinal centre expressing low/intermediate levels of CD38 have been identified, as a immunoglobulin secreting plasma cell precursor subset of cells (Arce et al. 2004). The use of either CD19 or CD20 as a pan-B cell marker may exclude from analysis those B cells which have terminally differentiated and matured, but this alone does not account for the loss of B cells and does not explain the lack of immunoglobulin production, and the similarly low numbers of recovered B cells when separated by negative selection which incorporated plasma cells (Chapter 6). Follicular dendritic cells share a number of surface molecules with B cells, including CD20, and they may account for some of the residual CD20⁺ cells after SPEA stimulation. Confirmatory staining with CD138 for Plasma cells and CD11c for dendritic cells as well as CD3 co-staining of CD38 cells would help to further define the nature of the remaining cells. Definition of B cell subsets could have been improved in this project with the use of multi-parameter flow cytometry, but this was not available until the end of the project.

The effect of immunoglobulin inhibition was not restricted to SPEA, but was found to be a concentration-dependent class effect of superantigens, and was also seen in response to other known T cell mitogens, Concanavalin A and combined CD3/CD28 antibody stimulation, suggesting that this effect was a result of T cell proliferation. The loss of Immunoglobulin production was also seen in histocultures stimulated with SPEA and SPEJ, though not with SMEZ, though this may due to the concentration of SMEZ used. Similarly there was a reduction of IgG production in response to the *emm 1 S. pyogenes* supernatants which contained SPEA compared to SPEA negative supernatants, though this effect was not noted with the *emm 89 S. pyogenes* supernatants.

Among the earliest described effects of streptococcal superantigens were both *in vitro* and *in vivo* effects on the production of immunoglobulin and cognate immune responses to antigenic stimulus. It was noted that mouse spleen cell plaque forming ability and subsequent liver and peritoneal macrophage clearance of ⁵¹Cr-labelled sheep red blood cells was delayed after the administration of superantigens. It was theorised that this was due to a suppression of the antibody response, and hence reduced phagocytosis, rather than direct effects on the mechanism of phagocytosis (Cunningham & Watson 1978a). Subsequent work showed that these abnormal responses were due to altered T cell

function and subsequent alteration in the T cell dependent antibody response. This response was similar to previously described with other generic T cell mitogens such as Concanavalin A (Cunningham and Watson 1978b). Further work with staphylococcal superantigens repeated these observations, and showed that the effect could be replicated in human PBMC's cultured with TSST-1. There was a degree of toxicity to both T and B lymphocytes detected in the cultures (B cell viability reduced from 66 to 43%, T cells 48 to 40%), and the effect could be propagated when TSST-1 stimulated cells or large volumes of culture supernatants were transferred to fresh cultures. As the transferred supernatants did not contain sufficient contaminating quantities of TSST-1 to reproduce the effect, this was felt to be due to some produced suppressing factor by TSST-1 activated PBMC's (Poindexter and Schlievert 1986). B cell viability was later thought to be reduced due to apoptosis and increased expression of the Fas antigen CD95. The apoptosis could be partially inhibited by anti-Interferon γ antibodies in the culture, though this was not the whole answer, as the addition of exogenous Interferon γ to cultures with low concentrations of TSST-1 (insufficient to inhibit immunoglobulin) did not reproduce these findings (Hofer et al. 1996). To confirm the significance and importance of cytokines in reproducing the immunoglobulin-suppressing effect of superantigens on tonsil cultures, various supernatant transfer and cytokine inhibition experiments were performed, the results from which are presented in chapter 6.

Superantigens have been shown to suppress production of IgG, IgA and IgM, and a similar effect was produced by the use of an anti-HLA DR antibody in culture (Moseley and Huston 1991). However, whereas these antibodies inhibited general B cell proliferation and enhanced IL-2 receptor expression, superantigens neither inhibited or enhanced B cell proliferation, and did not change IL2 receptor expression. Also, the superantigen responses were dependent on T cell presence in culture, where HLA DR antibodies can inhibit immunoglobulin production in pure B cell cultures, and the effect was not due to competitive binding of superantigens to HLA DR molecules (Moseley & Huston 1991). Low concentrations of staphylococcal enterotoxins were shown to have an antigenic effect on B cells, resulting in proliferation and immunoglobulin production, as demonstrated when T cells in culture are irradiated or pre-treated with Mitomycin C (Fuleihan, Mourad, Geha et al. 1991). Overall,

superantigens were shown to induce B cell proliferation and immunoglobulin production in an antigenic manner in the presence of inert T cells, but when the T cells were driven to proliferate by superantigens, immunoglobulin production was completely stopped. Increased levels of cyclic AMP were detected in pure B cells stimulated with TSST-1, though no other intracellular markers for B cell activation were detected (no Ca^{++} influx, *c-Myc* mRNA increase or proliferation as seen after B cell receptor ligation with PMA or anti- μ antibodies). No change to this was made by the addition of exogenous cytokines (Fuleihan et al. 1991). The work presented here and in chapter 6 confirms these previous findings, and starts to explore the mechanisms of B cell loss and T cell activation further.

As the superantigen effects on B cells were previously found to be T cell dependent, some work has been done looking to the B-T cell interactions, to look for clues as to the mechanism. Mainly this work was directed at seeing if superantigens could help to enhance specific antibody responses by increasing T cell numbers, despite work showing that superantigen exposure reduced survival of subsequent infection (Sriskandan et al. 1996). From these studies it can be seen that superantigens (SEA) increase CD80 (B7-1) and CD86 (B7-2) expression on B cells, key accessory molecules involved in T-B cell interactions (Ingvarsson, Lagerkvist, Martensson et al. 1995). This has led to many authors trying to use superantigens to enhance specific antibody responses, though with limited success if the T cells are still functional. The concentrations of superantigen chosen to stimulate cells in the experiments presented here are not near to saturation concentrations, as shown by proliferation assays, yet there is still a significant drop in the IgG level produced by SPEA. This would suggest that there is possibly an important role in the mechanism of binding of superantigens to both antigen presenting cells and T cells (Tiedemann & Fraser 1996).

It was interesting to note that in contrast to the superantigens and combined CD3/CD28 antibody stimulation, treatment of cultures with CD28 antibody alone caused an increase in IgG production. CD28 is a co-stimulatory molecule on T cells, and ligation is essential for normal T cell function. However, superantigens possibly utilise CD28 to enhance the proliferative effect through the T cell receptor signalling (Bueno et al. 2007). The expression of CD28 is therefore also examined in chapter 6, along with other key co-stimulatory molecules.

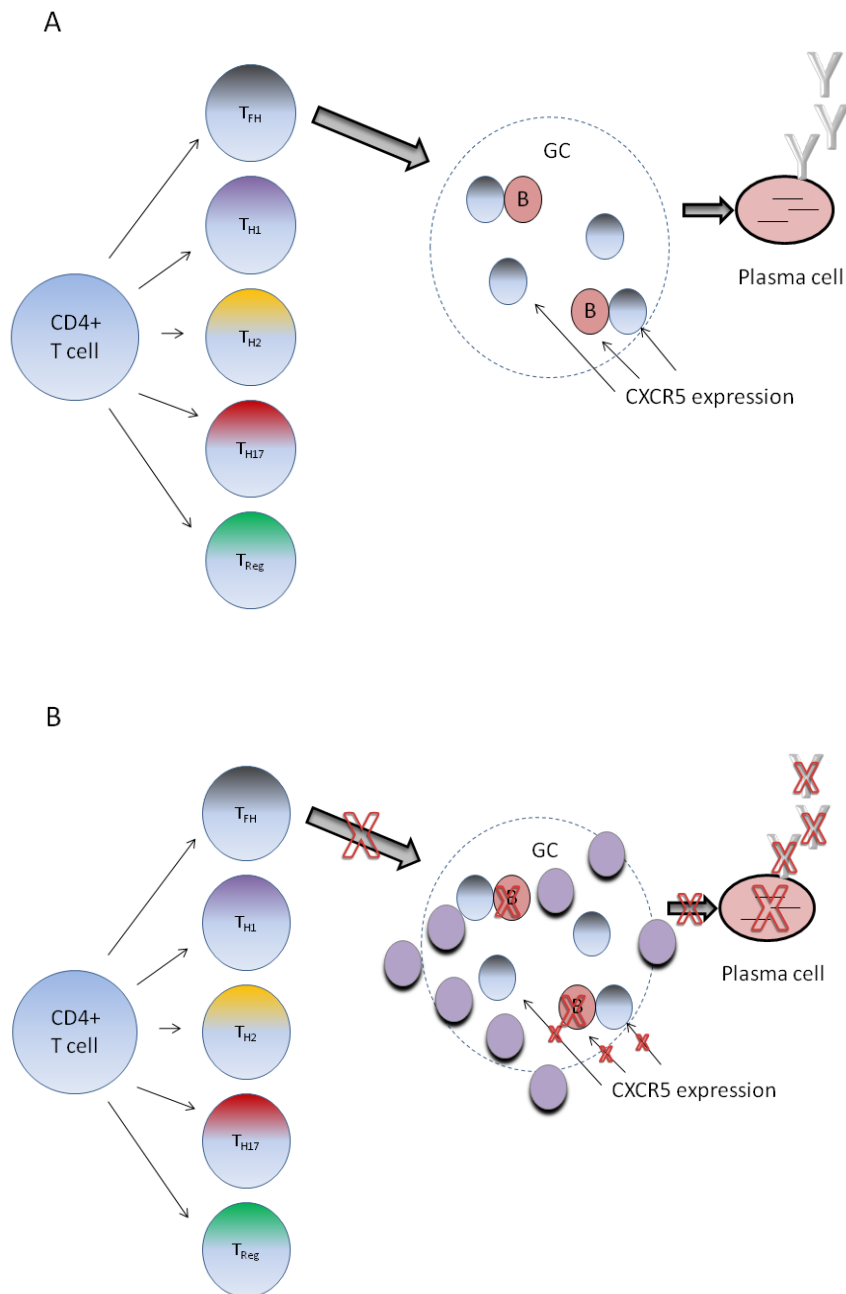


Figure 55: Proposed mechanism for superantigen immunoglobulin suppression effects in tonsils

A/Tonsils contain the full range of T helper cell subsets, with approximately 50% of T cells in the T follicular helper (T_{FH}) phenotype, expressing CXCR5. On antigen stimulation, these T cells migrate to the germinal centre (GC, dashed line) where B cells are also expressing CXCR5. Close contact between B and T cells in the presence of antigen causes B cells to mature into immunoglobulin secreting plasma cells. B/ In the presence of superantigens, T_{FH} cells lose CXCR5 expression and begin to proliferate into a poorly defined T helper phenotype (purple cells). These cells do not migrate normally to the germinal centre to initiate cognate immune responses, resulting in B cell death and loss of immunoglobulin production.

In conclusion, two main effects of superantigen stimulation were seen on tonsil cell cultures: a profound proliferation of T cells with altered phenotype, and a loss of B cells with reduced immunoglobulin production. Taken together, the findings suggest that the proliferating T cells are no longer able to provide adequate B cell help, resulting in B cell apoptosis. As superantigens form abnormal links between the T cell receptor and MHC class II molecules (which in the case of tonsils are predominantly expressed on B cells or follicular dendritic cells), immune synapse interactions between B and T cells as well as cell activation and apoptosis and other putative mechanisms for the effects form the focus of the work presented in chapter 6. A proposed schematic diagram of the changes taking place in tonsils in response to superantigen exposure is shown in Figure 55.

6 Mechanism of superantigen B cell inhibition

Immunoglobulin production in response to infection is one of the fundamental components of the adaptive immune system, yet the work presented here has shown that the influence of a specific bacterial product, a superantigen, can inhibit this process. The regulation of immunoglobulin production is complex, with a wide number of signals determining the fate of a B cell – what type and quantity of immunoglobulin to produce, development of memory cells and ensuring that antibody is not directed against self. Inside the B cell, the final editing step is the class switch recombination system, where the immunoglobulin gene is twisted and edited to result in the correct immunoglobulin form being produced. This is tightly controlled by a number of transcription factors ensuring that once a B cell starts on the path to becoming an immunoglobulin secreting plasma cell, the process cannot be reversed.

The signals which initiate B cell development into an immunoglobulin secreting (plasma) cell are multiple. The presence of different cytokines can alter the type of immunoglobulin produced and the fate of the B cell, but the fundamental trigger is ligation of the B cell receptor. However the B cells cannot act on these signals alone – and without the close contact of dendritic cells and T follicular helper cells, the B cells remain passive. There are multiple co-stimulatory molecules which form the immune synapse between T and B cells or B cells and dendritic cells, expression of which can alter B cell destiny.

Therefore, to try to establish the main mechanism by which superantigens cause a loss of B cells and immunoglobulin production from tonsil cultures *in vitro*, a number of these factors were explored: the expression of the key regulatory transcription factors determining B and T cell fate; the activation state of cells and degree of apoptosis; the expression of key inflammatory and immune synapse molecules; the influence of transferred cytokines and blocking the action of specific cytokines.

6.1 Results

6.1.1 Tonsil cell regulatory gene expression

To establish whether the production of immunoglobulin was being switched off by superantigens at a genetic level the transcription of the immunoglobulin heavy chain gene (IgHG) and the CD20 molecule (as a marker of general B cell viability) were quantified by real time RT-PCR, along with transcripts of BCL6, BLIMP-1, AICDA and XBP1, with reference to the house keeping genes GAPDH and 18S or β -Actin.

In RNA prepared from un-separated tonsil cells from 5 different donors, there was a significant decrease in the expression of AICDA (mean decrease by factor 0.218, $p=0.001$), IgHG (mean decrease by factor 0.052, $p<0.001$) and CD20 (mean decrease by factor 0.046, $p<0.001$) transcripts in whole tonsil populations. In contrast, expression of BCL6 was significantly increased (mean increase by factor 1.881, $p<0.001$), but there was no change in the expression of BLIMP-1 or XBP-1 (Figure 56A). In one additional tonsil donor there were no transcripts detected for AICDA, XBP1 or BLIMP-1 in the unstimulated samples, although they were detected in the SPEA stimulated cells; this donor was excluded from the analysis. As BLIMP1 and BCL6 can both be produced by both B and T cells, cell cultures from 4 of these donors were separated at the end of 1 week of stimulation, and RNA from B and T cells isolated and analysed separately.

Conflicting results were obtained between different donors when separated B cells were analysed. No RNA transcripts were detected for AICDA, BLIMP-1 or BCL6 in the SPEA treated cells from two tonsil donors, XBP-1 in one of these donors, and AICDA was not amplified in the unstimulated sample either from one of these donors. In the other two donors tested there was an increase in AICDA expression by a factor of 3.48 in SPEA stimulated cells ($p=0.001$, REST analysis), though no change was detected in the expression of BCL6 (factor 0.991) or BLIMP-1 (factor 0.942). In these donors there was no significant change in XBP1 expression (factor 0.562, $p=0.25$ REST analysis).

Interestingly there was no decrease in CD20 expression (factor 0.609, $p=0.143$ REST analysis) or IgHG expression (factor 1.115, $p=0.777$ REST analysis) in these remaining B cells in the SPEA treated group in all 4 donors (Figure 56B). The increase in AICDA expression in 2 donors might indicate that the remaining B cells in SPEA stimulated cultures were maturing into plasma cells, in preparation to produce immunoglobulin, and so were functioning normally, albeit a small minority. The fall in IgHG and CD20 expression in the whole cell population is a reflection of the proportionate loss of B cells.

In the separated T cells there was a 2.28 fold increase in BCL6 transcription in SPEA stimulated cells compared to unstimulated cells at 1 week ($p=0.0001$ REST analysis). Surprisingly there was also an increase in expression of BLIMP1 by 1.57 fold ($p=0.038$ REST analysis), despite these two genes being antagonistic (Figure 56C). High expression of BCL6 in SPEA stimulated cells was unexpected given the loss of CXCR5 expression on flow cytometry (Figure 45), as both are characteristics of T_{FH} cells, and it would be expected that loss of CXCR5 expression would be reflected in all other phenotype defining characteristics, including master regulatory gene expression.

Overall, the transcription results supported the theory that there was a loss of immunoglobulin production from un-separated tonsil cell preparations treated with SPEA which was predominantly due to a loss of B cell numbers, though the picture of regulatory genes in separated B and T cells remains unclear.

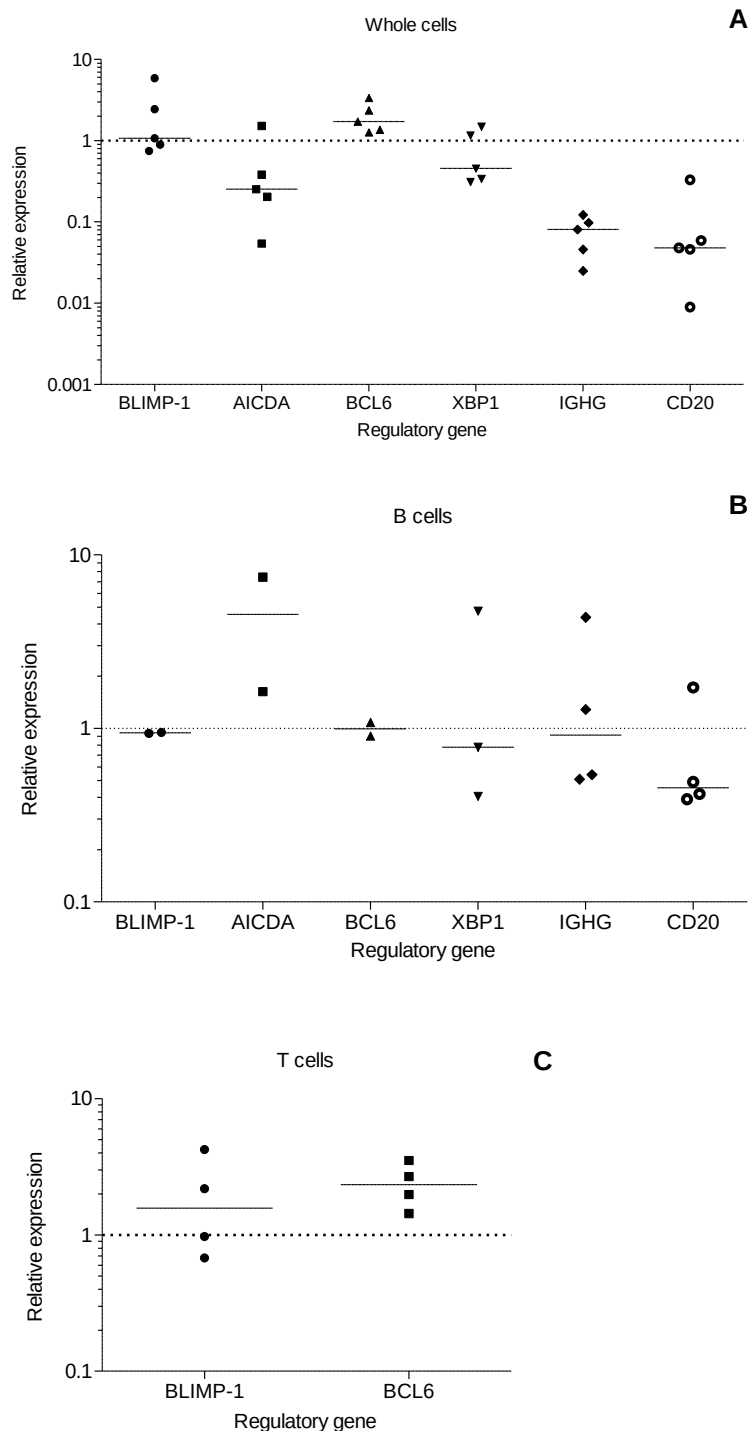


Figure 56: Expression of regulatory genes in un-separated tonsil cells, B cells and T cells following exposure to SPEA in mixed cell culture

Expression of B and T cells regulatory genes was analysed in un-separated cells (A, N=5 different tonsil donors), separated B cells (B, N=2 to 4 donors per gene) and separated T cells (C, N=4 donors). Results for each individual donor were analysed using the REST analysis, and figures show the relative expression of each gene in SPEA stimulated cells compared to unstimulated control cells from the same donor, normalised to two house-keeping genes (GAPDH and 18S or β -Actin). Relative expression of 1 represents no change. Bars represent the median expression value for each gene.

6.1.2 Tonsil cell activation and apoptosis

6.1.2.1 Activation markers

The molecule CD69 is one of the earliest molecules to be expressed on activated lymphocytes, and is expressed on both T and B cells as they become activated. Expression of this molecule is well recognised to be increased on peripheral blood T cells stimulated with both streptococcal and staphylococcal superantigens, with expression increasing from about 4 hours, and rising to a peak at 24 - 48 hours (Hutchinson, Divola, & Holdsworth 1999; Lindsey, Lowdell, Marti et al. 2007; Muller-Alouf et al. 2001; Reddy, Eirikis, Davis et al. 2004). In comparison, the IL2 receptor CD25 has been shown to be expressed later, with a peak at 72 hours (Reddy et al. 2004).

In tonsil cultures, CD69 expression on T cells peaked at 24 hours of culture, and then fell so that there was no difference in expression from unstimulated cells at the end of one week (Figure 57). At 24 hours of culture, in SPEA stimulated cells there was a median of 64.2% (range 60-72%) of CD3+ T cells expressing CD69, compared to 51.5% (range 33-57%) of unstimulated cells (N= 3 different tonsil donors). At 1 week of culture, there was no difference between CD69 expression (either percentage of cells or MFI) in SPEA stimulated compared to unstimulated T cells (SPEA median 22.7% of T cells, range 10.1-36.9%, unstimulated 32.8% of T cells, range 16.2-47.5%, $p=0.104$ Wilcoxon matched pairs signed rank test, N=8 tonsil donors; MFI median 18.3 (range 7 – 137) in unstimulated cells, median 17.5 (range 8.6 – 169) in SPEA stimulated cultures, N=6, $p=0.6875$ Wilcoxon matched pairs signed rank test). Baseline expression of CD69 in tonsil T cells was far higher than previously reported for PBMC's, with a mean of 66.9%, in cells which were freshly stained (N=3 tonsil donors), but expression was lower at 42.1% in cells which were frozen immediately after processing (N=3 tonsil donors). This may be an artefact of the freezing process reducing CD69 expression, or conversely, it may be an artificially high level on the freshly stained cells, due to the trauma of cell processing followed by immediate staining, as CD69 expression levels had fallen back to the same level as the frozen cells after 24 hours of culture in the freshly stained cells.

CD69 expression on B cells also increased with SPEA stimulation, however expression was sustained at a high level from 24 hours until the end of culture at 1 week, despite falling numbers of B cells in the tonsil cell population (Figure 57). By 1 week of culture this difference in CD69 expression on B cells was present in all donors tested, as assessed by both percentage of cells (median 22.5% of unstimulated B cells (range 3.25 – 47.5%) compared to a median of 51.8% in SPEA stimulated B cells (range 18.8 to 92.7%), $p=0.0078$ Wilcoxon matched pairs signed rank test, $N=8$ tonsil donors) and MFI (median MFI 17.1 (range 12-103) for unstimulated B cells, compared to 35.1 (range 14.1-254) for SPEA stimulated cells, $N=6$ tonsil donors, $p=0.0313$ Wilcoxon matched pairs signed rank test). As with T cells, the B cells which were frozen immediately on processing had a lower expression of CD69 at baseline than freshly stained cells (mean of 27.5% of frozen cells ($N=5$ donors) compared to 74.5% in fresh cells, $N=2$ donors).

As already demonstrated in Figure 44 (p156), expression of tonsil CD4⁺ T cell CD25 was significantly up-regulated in response to SPEA stimulation at 1 week. Time course evaluation showed that this expression increased steadily over the course of a week until a peak expression was reached by day 5 (data not shown).

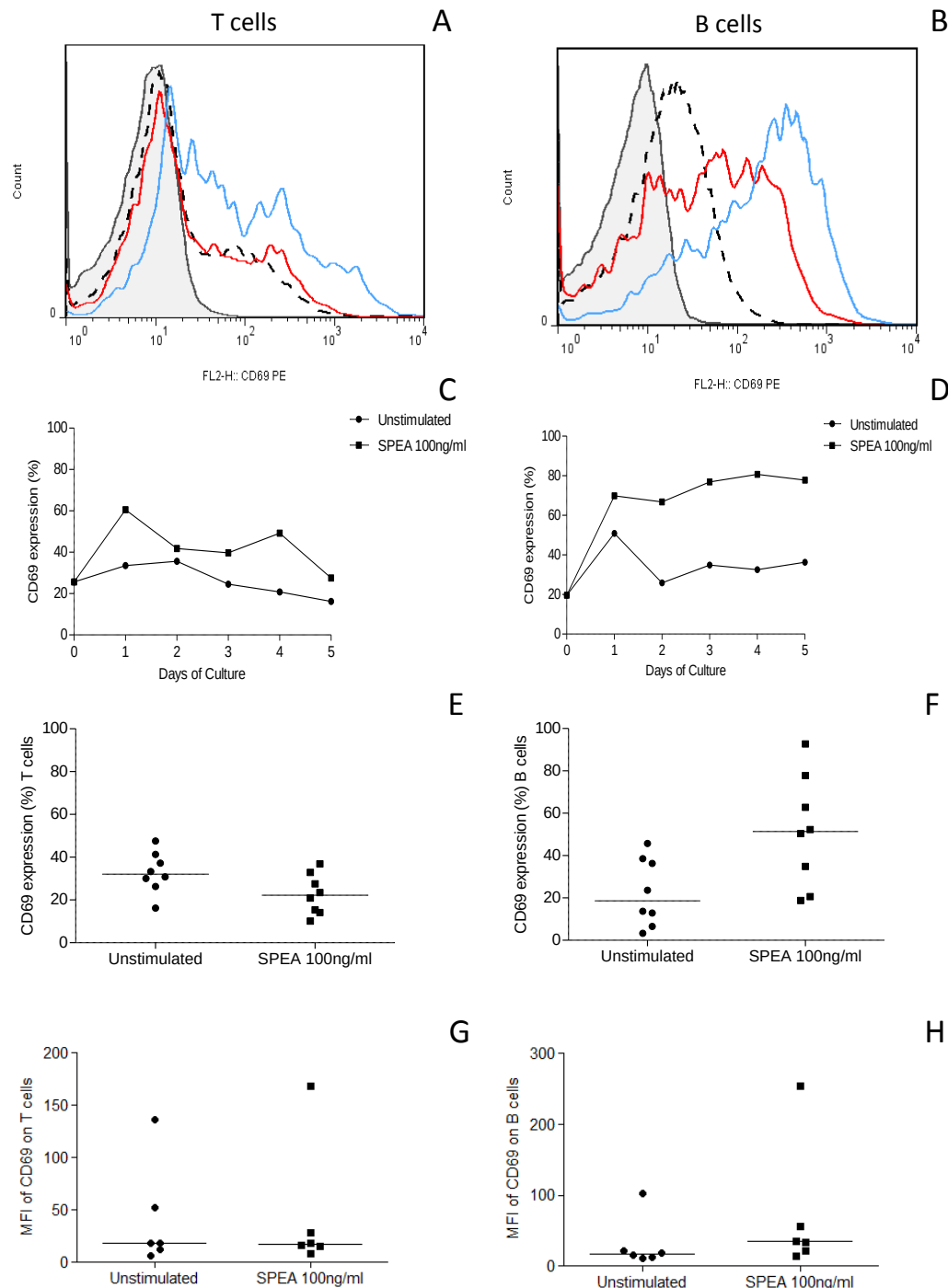


Figure 57: Expression of CD69 on T and B cells

There was an increase in CD69 expression in both T cells (A) and B cells (B) after 24 hours of stimulation with SPEA. Grey shaded = Isotype control, Black dashed line = baseline sample, Red line = unstimulated cells, Blue line = SPEA 100ng/ml stimulation. Over time, in T cells CD69 expression fell back towards unstimulated levels (C), but in B cells the high level of CD69 expression was maintained (D). Same donor shown in A - D, data representative of N=3 different donors (days 1 to 3) and N= 8 different donors (day 5-7). A summary of CD69 expression on donors at 1 week is shown for T cells (E/ % of T cells (N=8, p=0.1), G/ MFI on T cells (N=6, p=0.69)) and B cells (F/ % of B cells (N=8, p=0.0078), H/ MFI on B cells (N=6, p=0.0313)).

6.1.2.2 Apoptosis markers

CD95, the Fas antigen, is one marker of a cell starting to progress towards apoptosis, predominantly via the caspase 8 pathway, although as a member of the TNF receptor family it can also be a marker of cell activation by alternative signalling through NF κ B and MAP kinase signalling pathways (Strasser, Jost, & Nagata 2009). In baseline tonsil cultures a median 81.2 % of T cells expressed CD95 (range 33.6 – 82.4%); this figure was lower for B cells, with 44.8 % of B cells expressing CD95 (range 13.5 – 70.4%, N=3 different donors). As with CD69 there was a wide range in the baseline expression of CD95 on the tonsil donors tested, and this may represent differences in the response to surgery and the health of the patients at the time of surgery, as well as the difference between freezing cells and using them fresh.

Over time, there was an increase in the expression of CD95 on both B and T cells, whether they were cultured in the presence of superantigens or not. In the SPEA stimulated groups, this increase occurred more rapidly than in the unstimulated cells, with >80% of B and T cells expressing CD95 by 24 hours of culture compared to 3 days of culture for unstimulated cells (N=3 tonsil donors, Figure 58). In donors with a high starting expression of CD95 there was no alteration in the expression in culture either with or without SPEA. By one week, there was no significant difference in CD95 expression in the presence of SPEA from unstimulated cultures, for either B or T cells, either in percentage of cells expressing CD95 or the MFI (Figure 58): unstimulated T cells median 89.5% (range 64.5 – 97.7%); SPEA treated T cells median 97.9% (range 82-100%), $p=0.4688$ Wilcoxon matched pairs signed rank test (N=7); unstimulated B cells median 80.8% (range 52.4 – 95.2); SPEA treated B cells median 79.2% (range 51.3-92.5), $p=0.6929$ Wilcoxon matched pairs signed rank test (N=7); median MFI unstimulated T cells 172 (range 80-410), median MFI in SPEA stimulated T cells 517 (range 42-656), $p=0.0625$ Wilcoxon matched pairs signed rank test (N=6); median MFI unstimulated B cells 67.6 (range 52.4-216), median MFI in SPEA stimulated B cells 73.35 (range 34.3-642), $p=0.5625$ Wilcoxon matched pairs signed rank test (N=6). Overall, expression levels of CD95 in tonsil B and T cells were high and did not change significantly with superantigen exposure, though there was an earlier increase in expression on exposure to SPEA from baseline levels.

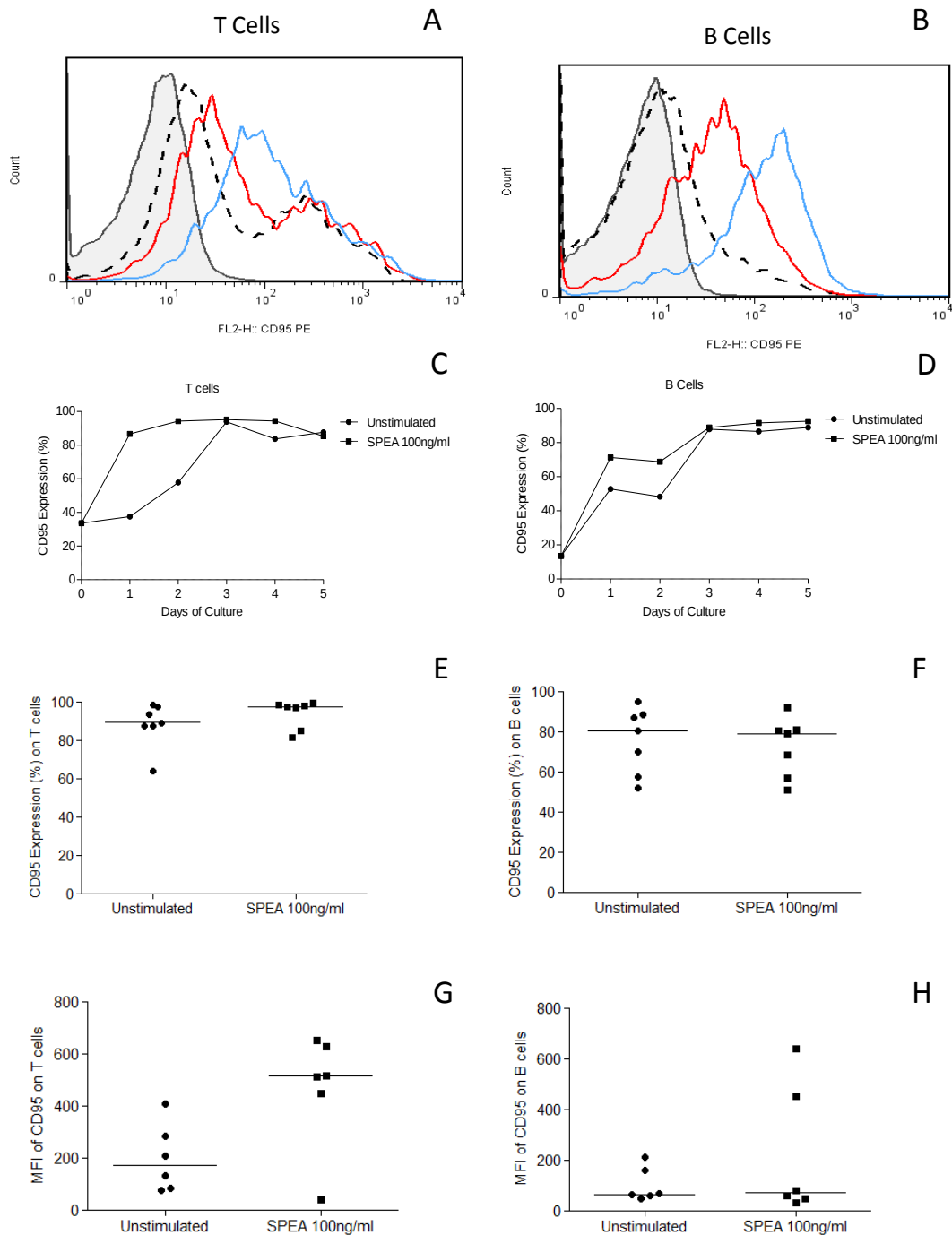


Figure 58: CD95 expression on B and T cells

Expression of Fas (CD95) increased more quickly on SPEA stimulated T cells (A and C) and B cells (B and D) than unstimulated controls, though all cells reached a plateau of high expression by day 3 of culture. A and B: Grey = isotype control; Black dashed line = baseline sample; Red line = unstimulated cells at 24 hours; Blue line = SPEA stimulation for 24 hours. Representative plots for N=3 different tonsil donors. There was no significant difference noted at 1 week of culture in the expression of CD95, measured as percentage of cells or MFI, on either T cells (E and G) or B cells (F and H) in SPEA stimulated cells compared with unstimulated controls (N=7 different donors for percentage, $p=0.4688$ for T cells and $p=0.2969$ for B cells; N=6 different donors for MFI, $p=0.0625$ for T cells and $p=0.5626$ for B cells).

Therefore, to assess whether the expression of CD95 represented cells entering apoptosis or activation, staining of cells with Annexin V (AV) and Propidium iodide (PI) was undertaken. Staining with both AV (which is expressed during blebbing of the inner cell membrane on cells about to undergo apoptosis) and PI (a nuclear stain, which does not stain healthy cells but stains cells which are dying due to apoptosis or necrosis), could accurately identify a population of cells in the tonsils which were about to enter apoptosis (Figure 59, labelled Dead cells). In cells both at baseline and at 24 hours of culture, these cells were all located in the low forward and side scatter cell population as described previously (chapter 4), with very few cells in the high forward and side scatter groups exhibiting staining with either AV or PI (Figure 59, marked Lymphocytes). There was no significant difference in the total percentage of cells staining positive with AV/PI in superantigen stimulated cells compared to unstimulated cells or baseline populations at 24 hours (unstimulated mean 9.06%, SPEA mean 11.6%, N= 2 different tonsil donors, Figure 59).

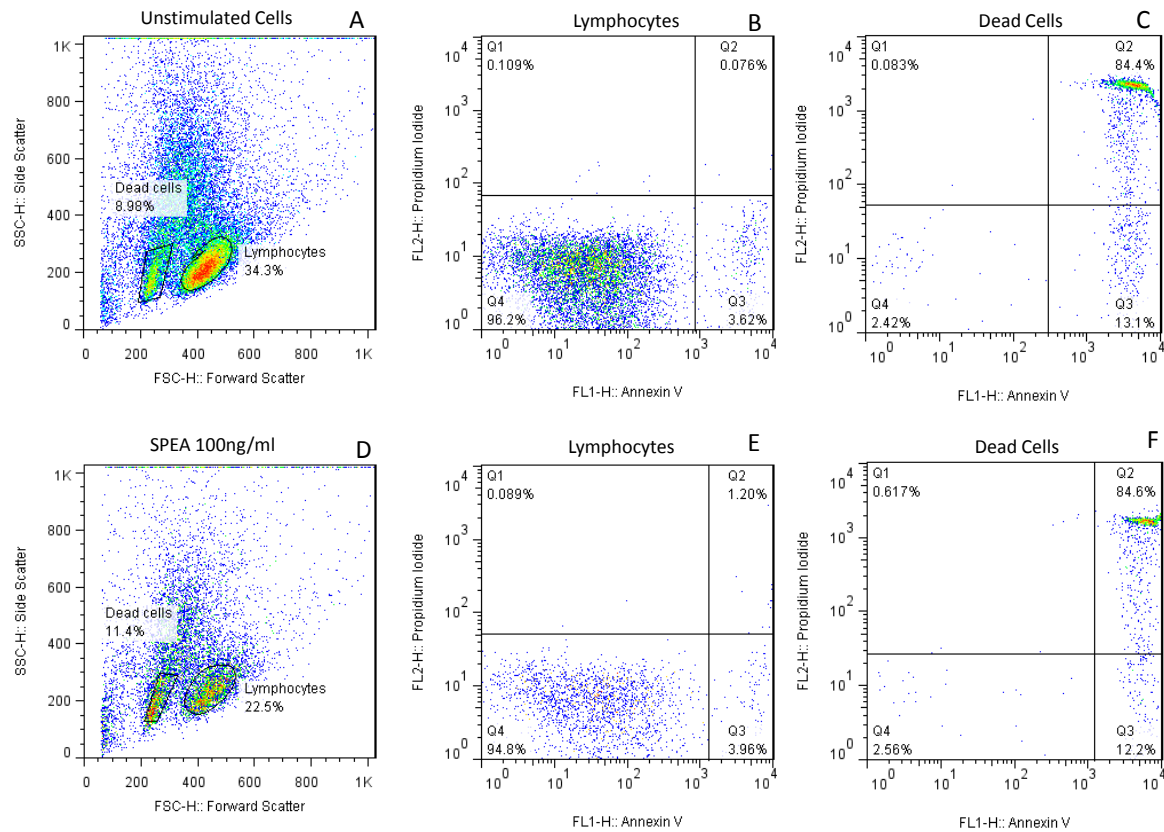


Figure 59: Tonsil cell apoptosis after 24 hours culture

Representative plots of Annexin V (horizontal axis) and Propidium iodide (vertical axis) staining of tonsil cells, after 24 hours in unstimulated culture (A-C), or with SPEA 100ng/ml (D-F). A and D show plots of unstained cells, with two easily distinguishable populations, labelled lymphocytes or dead cells. The lymphocyte gate shows no AV/PI double positive cells (B and E), but 85% of the cells in the dead cells gate are positive for AV and PI in both groups (C and F). Representative of N=2 different tonsil donors.

As there was no difference in apoptosis at the start of culture, at the same time as CD95 expression began to increase, cells were re-examined for apoptosis after 1 week of culture. Again there were no significant differences between unstimulated and SPEA stimulated cells in terms of the percentage of total cells that were AV/PI positive (mean unstimulated 47.6%, mean SPEA 43.4%, N= 2 different tonsil donors, Figure 60 A and D).

As it is not possible to counter-stain cells for other surface markers when staining with AV and PI, to determine whether the majority of cells undergoing apoptosis were B cells or T cells, cells were then

separated by AutoMACS after 1 week of culture and then stained for AV/PI. In both donors, the number of B cells isolated was very small in the SPEA stimulated group compared to the unstimulated groups (as demonstrated in chapter 5) and the absolute number of T cells, in comparison had increased. For T cells there was a small reduction in the percentage of cells which were AV and PI positive (mean unstimulated 43.9%, mean SPEA stimulated 39.3%, N= 2 donors, Figure 60 B and E). For B cells, there was an increase in the percentage of cells positive for both AV and PI (mean unstimulated 19.5%, mean SPEA stimulated 44%, N=2 donors, Figure 60 C and F).

This confirms that B cell numbers reduced in the presence of SPEA due to apoptosis while in T cells the degree of apoptosis decreased. These results do not fully explain the CD95 expression though – as T cells are not apoptosing in the face of continued high CD95 expression, and by the time there is demonstrable apoptosis in B cells in the presence of SPEA, there is no difference in CD95 expression from unstimulated cells.

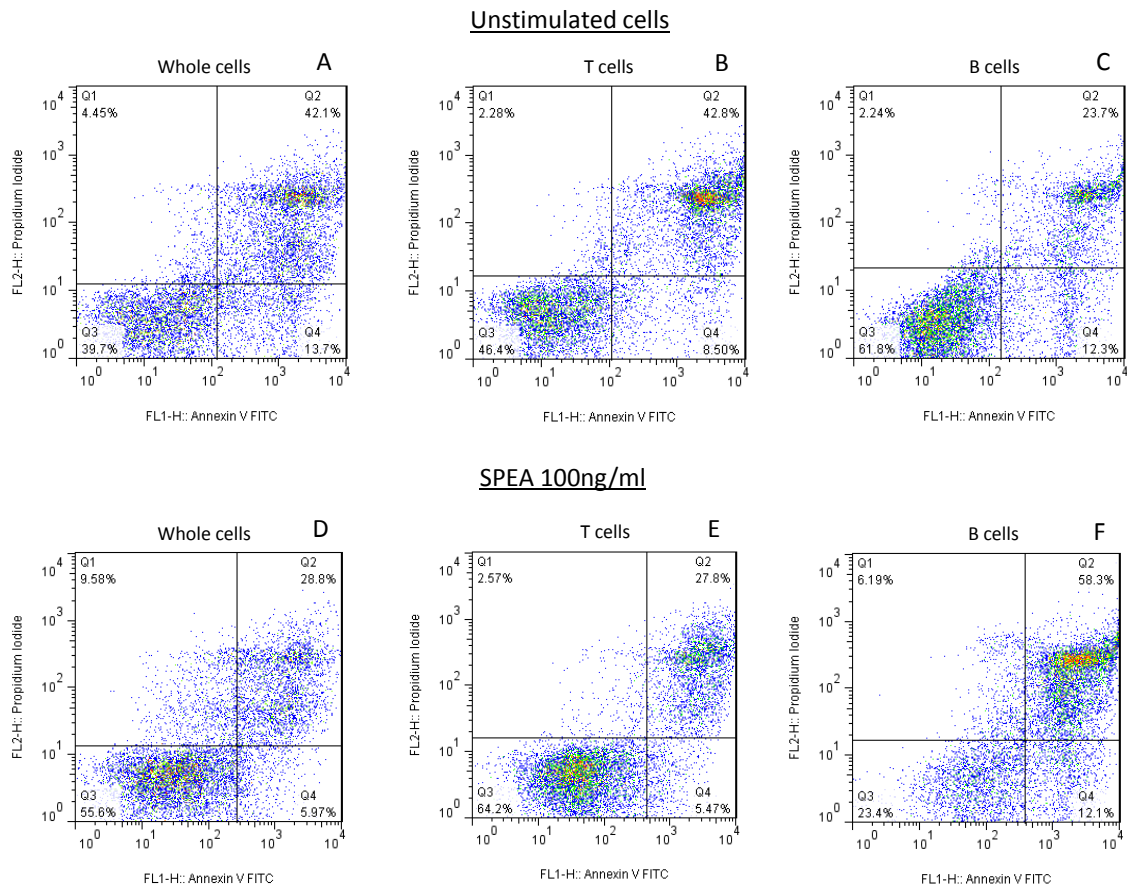


Figure 60: Tonsil cell apoptosis at 1 week

Tonsil cells were cultured for 1 week either unstimulated (A-C) or in the presence of SPEA 100ng/ml (D-F). T cells (B and E) and B cells (C and F) were separated from the total cell population, and all cultures were stained with Annexin V (horizontal axis) and Propidium iodide (vertical axis). Plots show staining for the whole cell population. Representative plots from 1 tonsil donor, N=2 different tonsil donors.

6.1.3 TNF receptor superfamily expression

The family of TNF receptors have a wide variety of functions on all lymphocytes, and play an important role in the control of activation and lymphocyte fate. Among these receptors, expression of CD95 (Fas) has already been shown to be increased in both tonsil B and T cells in response to superantigens (Figure 58), but does not necessarily relate to increased apoptosis. As there were marked increases in both TNF α and TNF β (lymphotoxin α) in the tonsil cell culture supernatants following SPEA exposure (Figure 41, Chapter 5), other key members of the TNF receptor superfamily which play an important role in the fate of both B and T cells were examined: CD27, OX40 (CD134) and ICOS (Inducible T cell Co-stimulator, CD278), their respective ligands (CD70, OX40L and CD275) and soluble TNF receptors in culture supernatants.

On T cells, there was an increase in expression of all members of the TNF receptor superfamily examined – CD27, OX40 and ICOS. For CD27, two donors had very high levels of expression in negative cultures (80 and 87% of T lymphocytes respectively) – in these cultures, there was no difference in CD27 percentage or MFI between negative and SPEA treated cells at 1 week. However, in the cultures where CD27 expression was <70% in the negative culture, there was a rise in CD27 expression in the presence of SPEA, both in percentage of cells expressing CD27 (median 44.9%, range 8.83-68.9 to 88.4%, range 38.9-91.5) and MFI (median 78.4, range 11.4-263 increasing to 368.5, range 15.6-894, N=4 donors, Figure 61A), though this was not statistically significant (p=0.1 Mann Whitney test). As mentioned in chapter 5, CD27 expression on B cells is a marker of mature B cells, with increasing expression corresponding with plasma cell development, and the population of CD27 expressing B cells was seen to decrease in the presence of SPEA (Figure 49). There was no significant change in the expression of the CD27 ligand, CD70, noted in the presence of SPEA for either T cells (median unstimulated cells 3.66%, range 0.34-5.44%; median SPEA cells 5.85%, range 3.12-8.96% of T cells) or B cells (median unstimulated cells 4.13% range 1.03-5.72%; median SPEA cells 4.29% range 2.57-16.8%; N= 4 different tonsil donors, data not shown).

OX40 expression in T cells started to increase from day 1 of culture. By 1 week there was a significant increase in the expression of OX40 in the presence of SPEA (median unstimulated cells 9.2%, range 4.13-16.4% of T cells, median SPEA 61.7%, range 47.2-68.3% of T cells, $p=0.028$ Mann Whitney test, $N=4$ different tonsil donors, Figure 61B). There was no change in OX40 ligand expression on B cells (data not shown).

ICOS, a T cell co-stimulatory molecule, is normally expressed on T_{FH} cells, and is one of the key markers for this subset of T cells along with CXCR5 expression. It can also be induced on other T cell subsets upon activation. Expression of ICOS (CD278) started to increase from day 1 of culture, and at 1 week of culture there was expression by 39.9% of SPEA treated cells (range 17.3-45.3%) compared to 12.2% (range 4.37-12.6%) of unstimulated cultures ($N=4$ different tonsil donors, $p=0.029$ Mann Whitney test, Figure 61C). There was no change in the ICOS ligand (CD275) expression noted on B cells (data not shown).

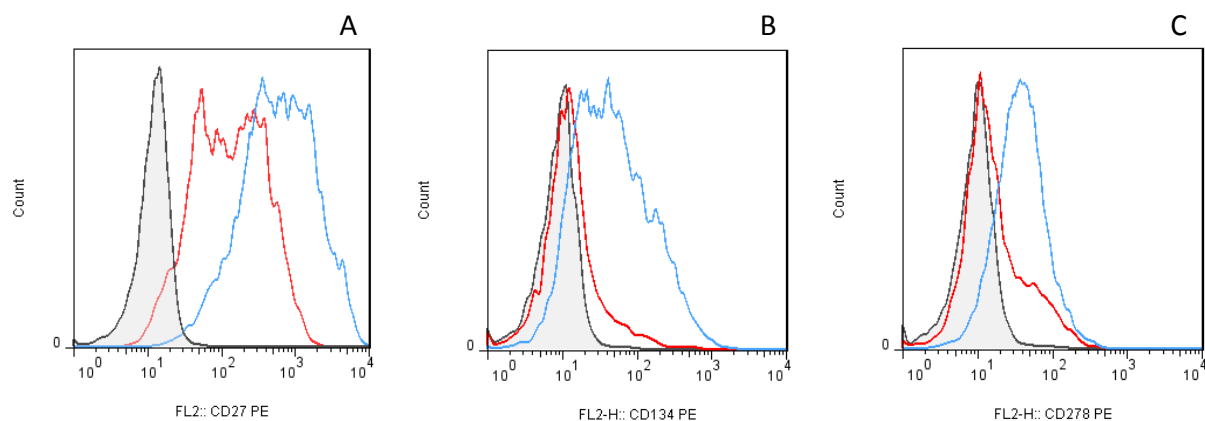


Figure 61: TNF receptor superfamily expression on T cells stimulated with SPEA

In T cells stimulated with SPEA there was a marked increase in the percentage and MFI of cells expressing CD27 (A), OX 40 (CD134, B) and ICOS (CD278, C). Representative plots of gated T lymphocytes are shown from 1 donor each. Grey shaded = isotype control, Red line = unstimulated cells at 1 week, Blue line = SPEA 100ng/ml for 1 week. $N=6$ different tonsil donors for CD27, $N=4$ different tonsil donors for OX40 and ICOS.

CD40 is a member of the TNF receptor superfamily expressed on B cells and antigen presenting cells. On B cells it plays a critical role in stimulating cells to undergo class switch recombination and develop into plasma cells. CD40 was expressed at high levels on all B cells, and there was no alteration in expression in the presence of SPEA over 1 week (median expression on 81.3% of B cells in both groups, N=4 different donors, data not shown). It is possible that any alterations in CD40 expression would have been noticeable only in the first 24 hours of culture in the presence of SPEA, as with CD95 expression. Early expression was only tested on 1 donor, but starting expression was 95% of all B cells, and this did not alter over time. There was no change in the CD40 ligand (CD154) expression on T cells or other cells in the presence of SPEA (data not shown).

Soluble portions of the TNF receptor are shed during TNF activation. Therefore, levels of soluble TNF receptor 1 (sTNFR1) were assessed in culture supernatants. Over time, expression started to be detected at day 4 of culture, with a peak at day 8 in SPEA stimulated cultures. There was no sTNFR1 detected in any of the unstimulated cultures compared to a median of 136.2 pg/ml (range 114.3-235.9 pg/ml) in SPEA cultures at 1 week (N=7, p=0.01 Wilcoxon matched-pairs signed rank test).

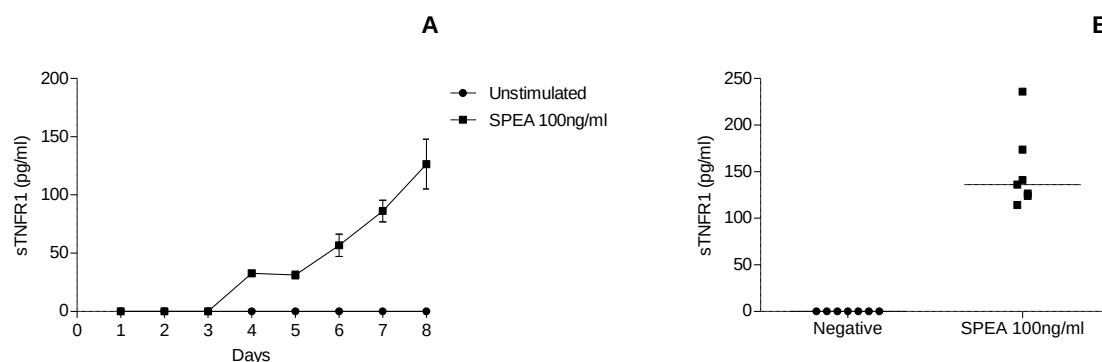


Figure 62: Soluble TNF receptor expression

sTNFR1 levels were measured in tonsil culture supernatant by ELISA. A/ Representative time course from 1 tonsil donor, mean $\pm$ s.d. of experimental triplicates in one representative donor shown (N=5). B/ median production at 1 week in unstimulated or SPEA treated cultures.

6.1.4 Immune synapse markers in response to superantigens.

Some of the key molecules involved in T-B cell communication are closely associated with the T cell receptor (TCR) and the HLA class II molecules on B cells, and the interactions between CD27/CD70 and CD40/CD154 as mentioned above.

There was no alteration in the expression of the $\alpha\beta$ components of the TCR in SPEA stimulated T cells, and no alteration in the percentage of cells expressing the $\alpha\beta$ components of the receptor (data not shown). There was a decrease in the percentage of cells expressing HLADR following SPEA stimulation at 1 week (unstimulated cells median 60.5%, range 46.5 to 66.1, SPEA stimulated cells median 31.5%, range 24.8 to 42.1%, data not shown). As 45-90% of the HLADR expressing cells were B cells in unstimulated cultures, this probably reflects the loss of B cells.

All T cells in tonsil cell suspension cultures were positive for CD28 expression on the cell surface. There was no alteration in the intensity of the expression of CD28 (as determined by measuring the MFI) on exposure to SPEA (N=4 different tonsil donors, data not shown). Similarly there was no significant alteration in the expression of CD80 or CD86 on B cells (the ligands for CD28) after one week of culture with or without SPEA (data not shown). Expression of all of these receptors was assessed after one week of culture, and it is possible that if cells were checked at an earlier time point (such as 24 to 48 hours of culture) then differences in expression would have been noted. This would be worth confirming in future cultures.

Signalling lymphocytic activation molecule (SLAM) is an important molecule involved in the activation and proliferation of both B and T lymphocytes, classically causing the production of T_H2 -type cytokines by T cells upon stimulation. There was no alteration in the expression of SLAM on B cells in SPEA treated cultures compared to unstimulated ones, although there was a wide variation in the level of expression between donors (45 – 90% of cells, N=3 different tonsil donors). However, in all donors tested there was an increase in both percentage and MFI of SLAM expression on the

surface of T cells in the presence of SPEA: median unstimulated T cells 18.3% (range 18.1-24.7%), SPEA stimulated T cells median 32.4% (range 29.5-55.9%, N=3 different tonsil donors, Figure 63).

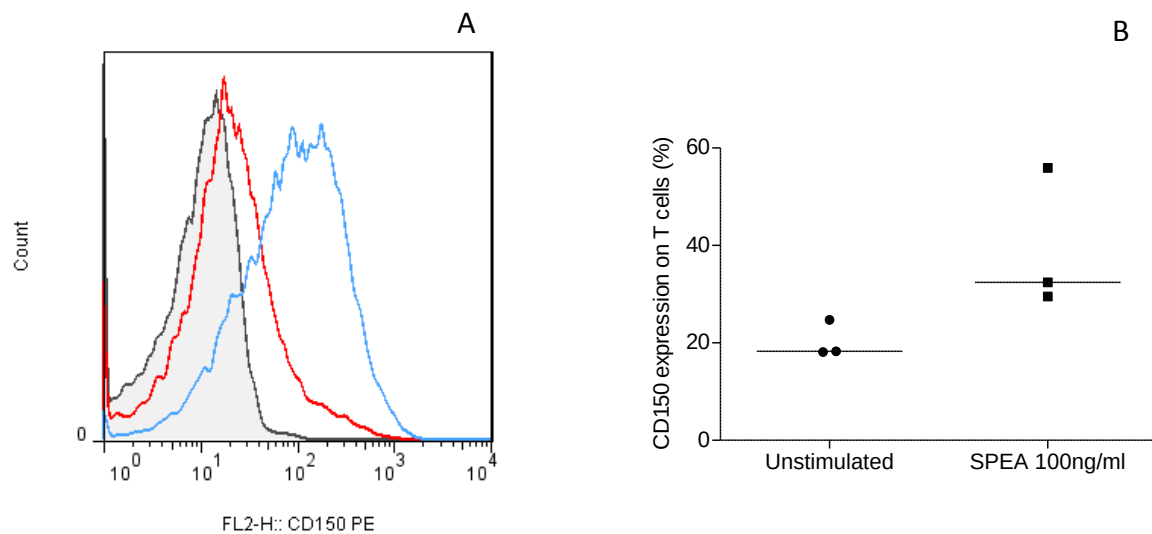


Figure 63: Expression of CD150 (SLAM) on T cells

A/ representative histogram from 1 tonsil donor of SLAM (CD150) expression on CD3+ gated T cells after 1 week of culture. Grey shaded = isotype control, Red line = unstimulated cells, Blue line = SPEA 100ng/ml. N=3 different tonsil donors. B/ median CD150 expression after one week of culture either unstimulated or in the presence of SPEA 100ng/ml (N=3 donors).

6.1.5 Mechanism of B cell loss - cytokine blockade and transfer of supernatants

In order to try to establish the mechanism of immunoglobulin synthesis inhibition, it was important to establish whether the effect was due to a soluble substance or cytokines in the culture media or whether the effect was cellular. On the basis that previous experiments had found some alteration in immunoglobulin synthesis with transferred supernatant from TSST-1 stimulated cells (Poindexter & Schlievert 1986) or interferon gamma blockade (Hofer et al. 1996), experiments were designed using the tonsil cell suspension culture system to establish 1) whether the effect of supernatant transferred from a culture where immunoglobulin production was completely inhibited could reproduce the effect

with fresh cells 2) whether the inhibition of either a pro-inflammatory (TNF α , INF γ or IL2) could reproduce the effect and 3) whether blocking B cell helper cytokines (IL4 and IL10) could reproduce the SPEA effect.

6.1.5.1 *Transfer of supernatants*

Supernatant from tonsil cell cultures known to inhibit production of immunoglobulin production due to prior SPEA exposure, and paired negative control supernatants, were transferred into fresh tonsil cell preparations, with or without the presence of fresh SPEA. Neutralising rabbit-anti-SPEA antibodies were also included into some cultures to ensure neutralisation of any residual SPEA. There was no abrogation of immunoglobulin production noted with the transferred supernatants, irrespective of the day of culture of the transferred supernatants (Figure 64, N= 2 different tonsil donors, each tested with transferred unstimulated and SPEA exposed supernatants from 3 different time points from two separate previous experiments, see methods for details). There was no demonstrable effect of transferred SPEA in previously treated supernatants on IgG production, and no alteration in IgG levels in the presence of anti-SPEA antibody or normal rabbit serum control. The antibody did partially stop the IgG inhibitory effect of SPEA 100ng/ml in fresh tonsil cultures (from 44% reduction in IgG levels to 31% reduction with anti-SPEA antibody, mean of N=2 tonsil donors), though there was insufficient antibody available to try this at higher concentrations (data not shown). This indicated that mechanism of immunoglobulin production inhibition was not likely to be due to a factor secreted into the cell culture medium.

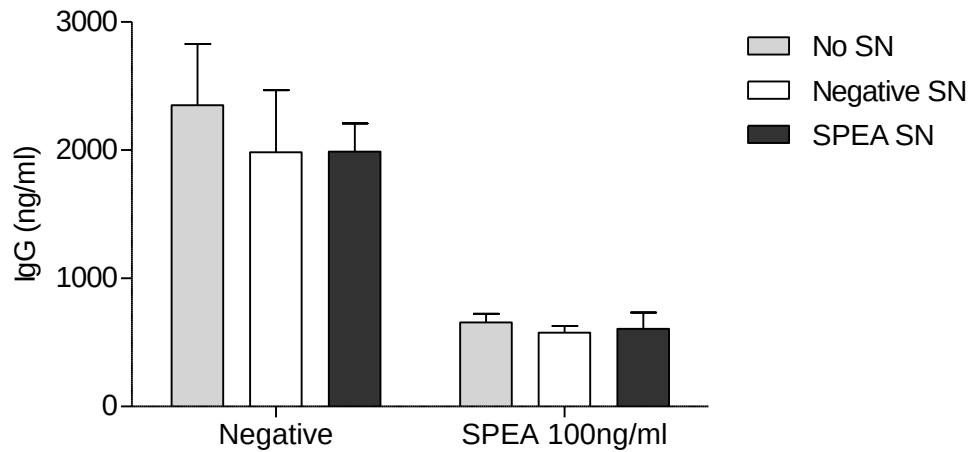


Figure 64: Effect of transferred supernatants on IgG production

Supernatants were transferred to fresh tonsil cultures from previous tonsil cultures that had been exposed to either SPEA 100ng/ml (black bars, SPEA SN) or not exposed (white bars, Negative SN). Fresh tonsil cultures without transferred supernatants are depicted by grey bars. Fresh cultures were then either unstimulated (Negative group on horizontal axis) or exposed to SPEA 100ng/ml (SPEA 100ng/ml group on horizontal axis). Bars represent mean $\pm$ s.d. of 1 new tonsil donor with transferred supernatants from day 7 of 1 previous culture. Repeated on N=2 fresh tonsil donors, with supernatants from 2 different previous cultures each at 3 different time points.

6.1.5.2 Inhibition of T_H1 cytokines

The levels of cytokines produced in response to superantigen stimulation of cells meant that full inhibition of a selected cytokine was difficult to consistently achieve with a single administration of neutralising antibody at the start of culture. However, when neutralising antibodies were administered repeatedly throughout culture (at the start, day 2 and day5), there were no detectable levels of the relevant cytokine detected (data not shown).

There was no reversal of SPEA inhibition of IgG production observed by blocking either $TNF\alpha$, or $INF\gamma$. Neutralising IL2 reduced IgG production in unstimulated cultures to the same levels as in SPEA treated cultures (Figure 65).

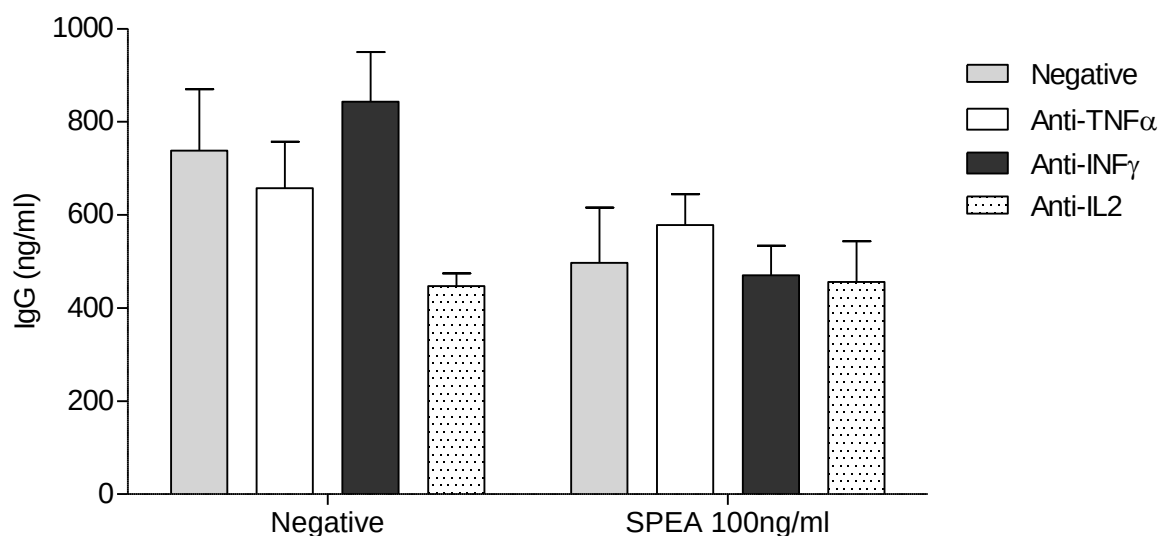


Figure 65: Effect of inhibiting T_H1 cytokines on IgG production

Tonsil cultures were either unstimulated (Negative group, horizontal axis) or stimulated with SPEA 100ng/ml (SPEA 100ng/ml group, horizontal axis) at the start of culture. The following inhibitory antibodies were added at days 0, 2 and 5 of culture: Negative/normal goat serum = grey bars, goat anti- $TNF\alpha$ = white bars, goat anti- $INF\gamma$ = black bars, goat anti-IL2 = spotted bars. Data show mean and SD of 3 measurements from single donor. Data representative of N=3 different tonsil donors.

6.1.5.3 Inhibition of T_H2 cytokines

Using the same method as for the inhibition of T_H1 cytokines, there was no replication of the SPEA IgG reduction effect produced by inhibiting IL4 in cells without SPEA. There was a partial reduction in IgG production with IL10 inhibition in one donor tested, though not to the same extent as with SPEA, and less than was observed with anti-IL2 antibody. It was not possible to check that inhibition of IL4 production had been complete as no IL4 could be detected by ELISA in tonsil culture supernatants.

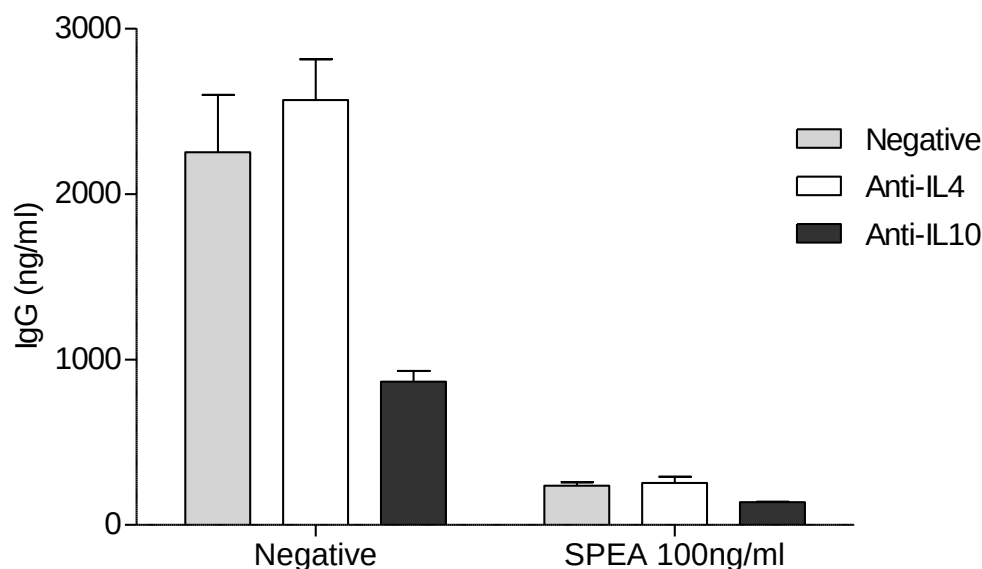


Figure 66: Effect of inhibiting T_H2 cytokines on IgG production

Tonsil cultures were either unstimulated (Negative group, horizontal axis) or stimulated with SPEA 100ng/ml (SPEA 100ng/ml group, horizontal axis) at the start of culture. The following inhibitory antibodies were added at days 0, 2 and 5 of culture: Negative/normal goat serum = grey bars, goat-anti IL4 = white bars, goat anti-IL10 = black bars. Data show mean and SD of 3 experimental replicates. Data representative of N=2 donors for IL4, N=3 donors for IL10.

6.2 Discussion

Determining the immunoglobulin response to a specific antigen is not easy to accomplish *in vitro*, as the majority of immunoglobulin produced during *in vitro* viability of cells is dependent on memory responses, and relates to antigens having been met previously. Using human tonsils to investigate immunoglobulin responses to *S. pyogenes* overcomes some of these problems, in that almost all patients undergoing tonsillectomy will have encountered *S. pyogenes*, and immune cells against them will be concentrated in the site of maximal future exposure – the tonsils. Although the specificity of tonsil immunoglobulin to *S. pyogenes* was not assessed in this project, the observations about the effect of superantigens on the production of immunoglobulin (Chapter 5) were global effects on the loss of B cells and abrogation of immunoglobulin production. To try to determine the main cause for this effect, activation and apoptosis states of both B and T cells were examined, their underlying regulatory gene functions examined, the influence of secreted cytokines investigated, and key B-T cell communication molecules explored.

6.2.1 Regulatory gene expression

The decision to use Taqman gene expression arrays to investigate the genetic control of B and T cell fate meant that it was only possible to analyse the results in two ways: a qualitative assessment of gene transcription, or, when gene transcription was detected, quantitative comparison between two conditions with reference to the transcription of house-keeping genes, using the REST analysis. The lack of a standard curve for each gene (due to insufficient RNA from untreated samples to use as a control) meant that it was not possible to plot exact transcript copy numbers, as had been performed for the superantigen genes. The time necessary to design and create a plasmid containing the genes of interest was outside the scope of this project. Even so, using the Taqman probe system, it was possible

to form an idea of the nature of the transcription events occurring in both B and T cells in response to SPEA.

The genes examined in this work for mRNA expression in B cells were BLIMP-1 (or PDRM-1, encoding a zinc-fingered transcriptional repressor, responsible for promoting B cell development into plasma cells), AICDA (activation induced cytidine deaminase, the key enzyme involved in the genetic editing of class switch recombination), BCL-6 (a transcriptional repressor, with actions antagonistic to BLIMP-1 and important in promoting germinal centre B cell survival), and XBP-1 (expression enhanced by BLIMP-1, promotes MHC II expression and IgJ chain formation). The immunoglobulin heavy chain gene (IGHG) constant region was assessed as a global marker of B cell immunoglobulin production. CD20 (encoded for by the MS4A1 gene) was used as a marker of general B cell health. In retrospect, for completeness PAX5 should have also been examined, which is also antagonistic in function to BLIMP-1, and is important for maintaining B cell identity and production of key functional proteins such as CD19 (Fairfax et al. 2008). An overview of the interactions between these genes and their role in determining B and T cell fate is shown in Figure 67.

It was interesting to note, that in the presence of SPEA, there was actually no change in the level of IGHG or CD20 transcript expression in B cells compared with unstimulated cells. This is despite the loss of immunoglobulin produced in cultures, and suggests that the mechanism is due to absolute B cell loss rather than functionally abnormal remaining B cells. The transcription of the other key genes in SPEA treated B cells was conflicting – in 2 donors tested there was no alteration in the production of BLIMP-1 or BCL-6 in the SPEA treated B cells, but in the other 2 donors, there was no transcription of either gene detected, suggesting it was down-regulated. In the same 2 donors there was no detection of AICDA transcripts in the SPEA treated groups (or the unstimulated group for 1 donor), yet the other 2 donors showed a significant increase in AICDA transcription in SPEA stimulated cells (as shown in Figure 56 B), despite housekeeping genes being detected in all donors. In one donor XBP-1 was also not detected in SPEA treated cells, though there was no change in the other 3 donors. The enhanced AICDA expression in the two donors where transcripts were detected implies that the B cells which had not undergone apoptosis were still viable and functioning. Several

more donors would need to be tested to confirm the reliability of the observations for BLIMP-1, BCL6, XBP1 and AICDA expression.

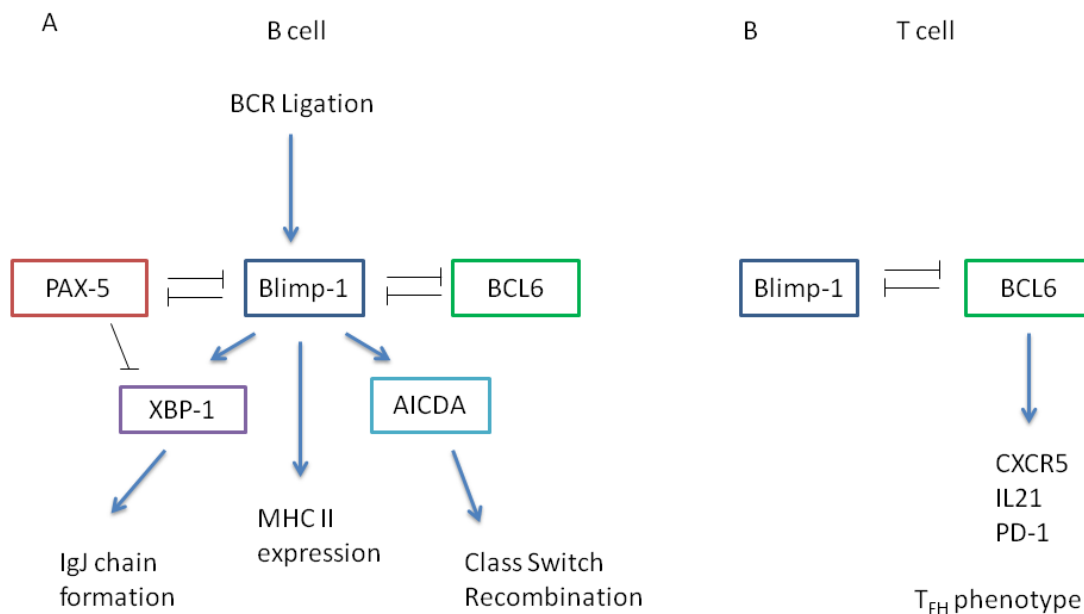


Figure 67: B and T cell regulatory genes

A/ B cell maturation to plasma cells is inhibited by expression of BCL6 and PAX5. On appropriate stimulation (e.g. B cell receptor ligation) BLIMP-1 is expressed, with negative regulation of BCL6 and PAX5, allowing for this start of plasma cell development and class switch recombination, leading to immunoglobulin production. Simplified and adapted from Fairfax et al. (Fairfax et al. 2008). B/ In T cells BLIMP-1 and BCL6 are counter-inhibitory. In T Follicular helper cells (T_{FH}) BCL6 is expressed, leading to cell surface CXCR5 and PD-1 expression and IL21 production.

B cells in the tonsils are all mature B cells, the stages of B cell maturation up to that point having been determined in the bone marrow. As described in chapter 5, the B cell subsets found in tonsils include a spectrum of cells from marginal zone B cells expressing CD21 and IgD, germinal centre B cells expressing CD23, and plasma cells expressing CD38, and various sub-stages of cell development in between. As there are no memory cells remaining after exposure to SPEA, as shown by flow cytometry in chapter 5 (CD27 cells), the activation of memory cells does not account for the gene transcription results.

Putting these results into context with the increase in B cell apoptosis and the flow cytometry results presented in chapter 5, it suggests that some of the residual B cells still have the potential to produce immunoglobulin, perhaps those cells which had already started to develop into plasma cells or T-independent marginal zone B cells before exposure to superantigens. Nonetheless, the overall B cell population in the presence of SPEA is not healthy, does not mount an effective immune response, and ultimately the B cells die prematurely.

BCL6 and BLIMP-1 are also expressed in T cells, and are similarly antagonistic. BCL6 is a master-regulator gene for T_{FH} cells, where as BLIMP-1 is expressed in the other T Helper subsets, T_{H1}, T_{H2} and T_{H17}. BCL-6 directly blocks the actions of the regulatory genes in these other T Helper subsets, including T-bet in T_{H1} cells, GATA3 in T_{H2} cells and ROR γ T in T_{H17} cells. This is despite evidence that T_{FH} cells themselves are capable of producing the characteristic cytokine profiles seen with the other T Helper subsets (Crotty 2011). The other characteristics of T_{FH} cells are CXCR5 expression, and expression of ICOS, SAP (SLAM associated protein), IL21 and PD1 (programmed death-1 receptor, a member of the CTLA-4 family of co-receptors). Therefore, with the loss of CXCR5 expression already noted on T cells in the presence of SPEA, it was surprising to find that BCL-6 transcription was significantly increased in the presence of SPEA. A recent study, published during the writing of this thesis, examined the responses of different human T_{FH} subsets to stimulation with CD3/28 and showed that the expression of BCL-6 in cells exhibiting the phenotype seen after SPEA stimulation (low CXCR5 expression and high ICOS expression) produced comparatively less BCL-6 and more BLIMP-1 transcripts and protein than classically described T_{FH} cells or pre-T_{FH} cells (located outside tonsil germinal centres) (Bentebibel et al. 2011). In a separate study, BCL6 expression in a mouse LCMV model of infection closely correlated with the development of T_{FH} cells, with expression peaking at 3 days after stimulation. The expression of BCL6 was dependent on ICOS signalling, which led to BCL6 transcription and subsequent CXCR5 development. Although early BCL6 signals were dependent on dendritic cell interactions, sustained signals were dependent on B cell interactions, including via SLAM (Choi, Kageyama, Eto et al. 2011). The same authors also found that BLIMP-1 signals took 3 days to reach a maximum strength in effector-T cells.

In the current work, increased T cell expression of ICOS and SLAM started to occur after one day of SPEA stimulation in the experiments presented here, and the expression continued to increase to a peak at the end of one week of culture. It is therefore possible that the enhanced BCL6 expression in T cells relates to signals from earlier during culture (when primed by dendritic cells), and that the level is decreasing as B cells (which help to maintain the signalling) are dying. Alternatively, as BCL6 is an important early signal for T cell proliferation rather than effector T cell differentiation, and expression occurs before BLIMP-1 regulation of T cell fate takes over (Crotty, Johnston, & Schoenberger 2010) the levels could reflect T cells being driven to proliferate before starting to differentiate. Certainly the increase in ICOS signalling should lead to increased BCL6 production, though why this has then led to a decrease in expression of CXCR5 on T cells, rather than an increase, is unclear. BLIMP-1 is usually expressed at relatively low levels in tonsils, in only 4-15% of cells (Angelin-Duclos, Cattoretti, Lin et al. 2000), so the increase seen in SPEA stimulated tonsil cells is certainly significant.

The results presented here are clearly preliminary, and enter into a rapidly developing area of immunology where the influence of each transcription factor and the contribution of alternative signals is yet to be fully worked out. Full investigation of the expression of BCL6 and BLIMP-1, both at the genetic and protein level, over time in both B and T cells in the context of SPEA and other bacterial superantigens would contribute greatly to the current understanding of the nature of lymph node cell interactions. Further important work in this area, and particularly in establishing the contribution of superantigens to the general tonsil response to *S. pyogenes*, would include similar experiments using the library of isogenic *S. pyogenes* strains with differing superantigen expression.

6.2.2 Cell activation markers

CD69 is widely recognised as one of the earliest markers of T cell activation in the context of superantigen exposure of cells, with expression preceding T cell proliferation and TNF α release (Marzio, Mael, & Betz-Corradin 1999). Therefore, the results presented here showing that the peak of T cell CD69 expression occurs at 24 hours after superantigen stimulation, are consistent with previous observations (Muller-Alouf et al. 2001). As CD69 expression is usually transient (Lauzurica, Sancho, Torres et al. 2000), the sustained expression by B cells in culture with SPEA was surprising, especially as B cell numbers were decreasing during that time. Sustained activation of eosinophils via a CD69 monoclonal antibody has previously been shown to lead to eosinophil apoptosis (Walsh, Williamson, Symon et al. 1996), though the mechanism for this was not explored. Although CD69 expression is usually linked to activation rather than apoptosis, it is possible that one of the effects that SPEA is having on B cells is sustained activation, which is contributing to B cell death. This is an area which could be explored further, to help establish the full mechanism of B cell death in the presence of superantigens.

There was a marked difference in baseline expression by T and B cells of both CD69 and CD95 when cells were stained directly after cells had been harvested rather than when they were stored at -80°C and then stained at a later date. Although it would seem logical to suggest that this is an artefact of the freezing process, this was not seen with any other cell surface markers which were stained on the same cells both freshly and from frozen. Furthermore, the expression of CD69 and CD95 had settled down to the same level as the frozen cells once they had been in culture for 24 hours. As CD69 and CD95 are both activated very quickly by cells (alterations cell surface expression can be detected within 3 hours of stimulation) and are non-specific responses to a variety of different stimuli, it is likely that this is actually an artefact of staining the cells after the trauma of processing rather than an artefact of cell freezing.

6.2.3 CD95 expression and apoptosis

Both B and T cells in tonsils expressed high levels of CD95 after 3 days of culture, but expression in SPEA stimulated cells increased more rapidly. Although changes in CD95 expression would account for increased apoptosis in B cells treated with SPEA, high levels of CD95 expression do not correlate with the continued T cell proliferation and population expansion in the face of SPEA stimulation or the fact that unstimulated B cells in culture expressed high levels of CD95 but were able to function normally. To confirm this, further staining of both B and T cells was performed using the Annexin V and Propidium iodide system, where double positive staining of cells confirms apoptosis. In B cells, this confirmed that SPEA exposure was leading to increased cell apoptosis, though in the unstimulated cells (where there was still a high level of CD95 expression) there was little apoptosis. For SPEA stimulated T cells, there was a marked decrease in the number of apoptotic cells with AVPI staining, despite increased CD95 expression.

The classic downstream signalling pathway from CD95 leads irreversibly to cell death by either the caspase-8 death receptor pathway or the BCL-2 regulated apoptosis pathway (Strasser, Jost, & Nagata 2009). Less commonly, increased CD95 expression has been reported in association with actively proliferating cells. Although the mechanism for this has not been fully identified, it may be due to increased NF κ B signalling via different cell surface receptors counteracting the effects of CD95 signalling, or the caspase-8 blocking action of cFLIP (Peter, Budd, Desbarats et al. 2007). Further examination of all these pathways would be appropriate in light of the findings presented here, to confirm the meaning of Fas expression on tonsil B and T cells both in the presence and absence of SPEA.

The expression of the B cell receptor is important for B cell survival, and loss of receptor expression can trigger B cell death to occur by increasing CD95 expression and increasing cytotoxic T cell mediated death (Pillai, Cariappa, & Moran 2004). This is the mechanism by which self-reactive B cells are deleted from the bone marrow during early development, and appears to be dependent on the proportion of B cell receptors ligated on a cell. In other words, occupancy of >80% of the BCRs on a

single B cell by an antigen makes it highly likely that this antigen is self derived, and so BCR expression is down regulated, and the signals through the BCR drive FOXO 1 transcription, directing the cell towards apoptosis (Goodnow et al. 2010). As superantigens have been shown to cause B cell receptor signalling in the presence of inert T cells (Moseley & Huston 1991), it is possible that the apoptosis of B cells with SPEA is also being driven by receptor saturation, as well as Fas mediated signalling. However, the experiments by Moseley and Huston used comparable doses of superantigen to the experiments presented here (SEA 1µg/ml), and saw that the cells were driven to produce more immunoglobulin rather than less on receptor ligation. Certainly there was a marked down-regulation of both IgM and IgD expression on the surface of SPEA stimulated B cells (Figure 48, Chapter 5) in the experiments presented here, though it may reflect B cell apoptosis rather than be the cause (Lam, Kuhn, & Rajewsky 1997). For completeness, investigating other causes of B cell death as well as Fas mediated B cell apoptosis in the context of superantigens, it would be worthwhile.

Intravenous Immunoglobulin (IVIG) preparations have been frequently discussed in the context of treatment of both streptococcal and staphylococcal superantigen-mediated shock. Although there is insufficient clinical trial data to conclusively support the use of IVIG as a standard treatment in cases of toxic shock, most authorities support the use of IVIG in the management of toxic shock syndrome if conventional therapies are failing (Anon 2010). One of the possible mechanisms of action of IVIG is prevention of Fas mediated death signals through anti-CD95 antagonistic antibodies. Though clinicians should treat this with some caution, as not only have levels of these antibodies been found to vary widely between different preparations of IVIG, they can contain CD95 agonistic antibodies as well as antagonistic ones, with the potential to enhance cellular apoptosis with IVIG therapy, especially in the liver (Reipert, Stellamor, Poell et al. 2008; von and Simon 2010).

6.2.4 TNF receptor superfamily and SLAM expression

For all the experiments that examined cell surface receptor expression, a majority of tonsil donor cells were examined at one week, although for all molecules at least one donor was examined on every day of culture. It is therefore possible that some of the earlier changes in expression of these molecules might have been missed, and if time were allowing then stored cells from earlier time points would be examined on a larger number of donors, to confirm that subtle changes had not been missed.

An increase in TNF α production is widely recognised as one of the key responses to bacterial superantigens, both *in vitro* (Muller-Alouf et al. 2001) and *in vivo* (Faulkner et al. 2005). *In vivo* TNF α production is rapid, and is associated with hepatocyte apoptosis in response to superantigen exposure in d-galactosamine sensitised mice (Faulkner, Altmann, Ellmerich et al. 2007). As demonstrated with CD95, TNF receptor expression is not always associated with cells proceeding to apoptosis, but can lead to cell activation. Soluble TNF receptors have been shown to increase in response to SEB *in vivo* (Faulkner et al. 2007), and that response was replicated here in the tonsil culture model, albeit after 4 days of culture rather than 90 minutes after superantigen exposure as seen in the mouse model of toxic shock.

Of the other members of the TNF receptor superfamily examined, expression of ICOS and OX40, were significantly increased on tonsil T cells in the presence of SPEA. Unlike CD95, these TNF receptors do not lead to cell death, but promote cell differentiation and survival (Watts 2005). ICOS expression on T cells has been identified as one of the defining markers of T_{FH} cells (Bentebibel et al. 2011), so it was surprising that expression of this was present on only 12.2% of unstimulated T cells, where CXCR5 was expressed on 47.2% of unstimulated tonsil T cells in this culture model. These figures were reversed in the presence of SPEA 100ng/ml for 1 week, with ICOS expression rising to 39.9% of T cells and CXCR5 falling to 25% of cells expressing low levels, with <10% remaining T cells expressing high levels of CXCR5 (data not shown). The initial experiments which led to the identification of ICOS showed that the level of ICOS expression in tonsil T cells when freshly harvested from donors was nearly 50% of all tonsil T cells and restricted to those in the germinal

centres (Hutloff, Dittrich, Beier et al. 1999) – far higher than the levels demonstrated here. The tonsils used in those experiments were all collected from children (aged 3 to 18 years), whereas the tonsils used in the experiments presented here were all from adults, which may account for the differences in the baseline levels of ICOS expression. The induction of ICOS expression is involved in the production of IL4, IL5, IL10, TNF α and INF γ by T cells (Hutloff et al. 1999), but B cell help via ICOS is dependent on interaction with the ICOS ligand (CD275) on B cells (Hu, Wu, Jin et al. 2011). In the experiments presented here, there was no expression of CD275 on B cells with or without SPEA exposure (on N=4 different tonsil donors), though there was a healthy production of immunoglobulin in the unstimulated cells. Although this may represent a technical issue, the lack of ligand expression on B cells for the other TNF receptors (OX40 ligand and CD70) and the lack of immunoglobulin production with SPEA suggests that this is not the case. Although superantigens have been used frequently by researchers wishing to non-specifically stimulate T_{FH} cells, few have looked specifically at what the effects of superantigens themselves are on ICOS expression. In one study mice exposed to both SEB and an inhibitory anti-ICOS antibody *in vivo* were shown to exhibit reduced early serum TNF α levels compared to SEB alone, and splenic T cells failed to proliferate when re-stimulated *in vitro* (Gonzalo, Delaney, Corcoran et al. 2001). This implies that ICOS is responsible for some of the characteristic T cell responses demonstrated on superantigen exposure.

Similarly, expression of OX40 was markedly increased in tonsil T cells exposed to SPEA, though there was no significant increase in OX40 Ligand expression on B cells. OX40/OX40L interactions are thought to promote B cell differentiation to plasma cells, in a similar way to CD40/CD40L interactions (Maxwell, Weinberg, Prell et al. 2000), and on T cells promote T cell survival after activation (Jensen, Maston, Gough et al. 2010). As with ICOS, OX40 expression on T cells is associated with increased production of cytokines: IL2, IL4, IL5 and INF γ , and also an increase in CD25 expression (Jensen et al. 2010). OX40 expression has been shown to increase on mouse T cells after exposure to SEA *in vivo*, but failed to prevent clonal T cell deletion unless the SEA was co-administered with LPS (Maxwell et al. 2000).

CD27 and its' ligand CD70 are both expressed on B and T cells. On B cells, the population of cells expressing CD27 completely vanished in the context of superantigen exposure, and the remaining cells expressed little CD70. Both CD27 and CD70 expression on B cells is thought to be induced on antigenic detection, and help to drive B cells towards plasma cells development (Arens, Nolte, Tesselaar et al. 2004; Jacquot 2000). On T cells, there was either a high starting level of CD27 expression or an increase on exposure to SPEA, consistent with previous findings using superantigens (Kai, Rikiishi, Sugawara et al. 1999), but again there was no alteration in the expression of CD70.

CD40 is a TNF receptor constitutively expressed on B cells, and the intensity of expression did not alter on exposure to superantigens. CD40 ligand expression on T cells did not increase in the presence of superantigens in the experiments presented here, which would be the normal immune response to antigens, to help drive plasma cell formation and immunoglobulin secretion by inhibition of B cell BCL6 expression (Vinuesa, Linterman, Goodnow et al. 2010). One previous study using TSST-1 found an increase in CD40 Ligand expression on T cells of up to 30% in PBMC's stimulated with TSST-1 by day 3-4 of culture, though again this was associated with decreased immunoglobulin production and B cell CD40 expression was not examined (Jabara and Geha 1996).

As well as the TNF receptor superfamily, the other molecules of note involved in B-T cell signalling include CD28, a T cell co-stimulatory molecule, and SLAM – a family of receptors which have an immunomodulatory role on B cells, T cells and NK cells by interaction with cytoplasmic SAP (SLAM associated protein). Deficiencies in SAP have been associated clinically with X-linked proliferative disorders and systemic lupus erythematosus, and reduced immunoglobulin production. SAP has been found to have an important role in the formation of normal germinal centres in lymphoid organs, by a number of different down-stream signalling events, probably important in cell adhesion (Schwartzberg, Mueller, Qi et al. 2009). SAP is known to be expressed on T cells as part of normal T_{FH} development and functioning, and is involved in the normal expression of T_H2 type cytokines (Crotty 2011). SLAMF1, also known as CD150, was examined in this study, and the expression on SPEA stimulated T cells increased both in percentage of T cells expressing it and fluorescent intensity on those T cells. A literature search could find no instances where SLAM or SAP had been

investigated previously in the context of superantigen exposure either *in vitro* or *in vivo*. Further investigation of SLAM and SAP signalling as well as downstream signalling would be useful to investigate the importance of these findings further.

In this study there were no differences noted in the expression of CD28 on T cells or reciprocal changes in CD80/CD86 expression on B cells on exposure to SPEA. Previous studies have suggested that CD28 or CTLA-4 co-stimulation by superantigens is not essential to the response but can enhance the potency of the superantigen effects on T cells (Bueno et al. 2007).

With all of the TNF receptors there is a common theme occurring – in the face of superantigen exposure there is marked up-regulation of TNF receptor expression, and appropriate subsequent cytokine production and increased T cell survival. However, there is no reciprocal increase in TNF receptor ligand expression, and B cells are being driven towards death instead of mounting the normal immune response and maturing towards plasma cells. Along with the increase in ICOS and OX40 expression, it would appear that the increase in SLAM expression is an attempt to produce the normal immunoglobulin response to antigenic stimulation, though abnormal superantigen signalling has prevented this. A summary of the alterations in T cell surface markers on exposure to SPEA are represented in Figure 68.

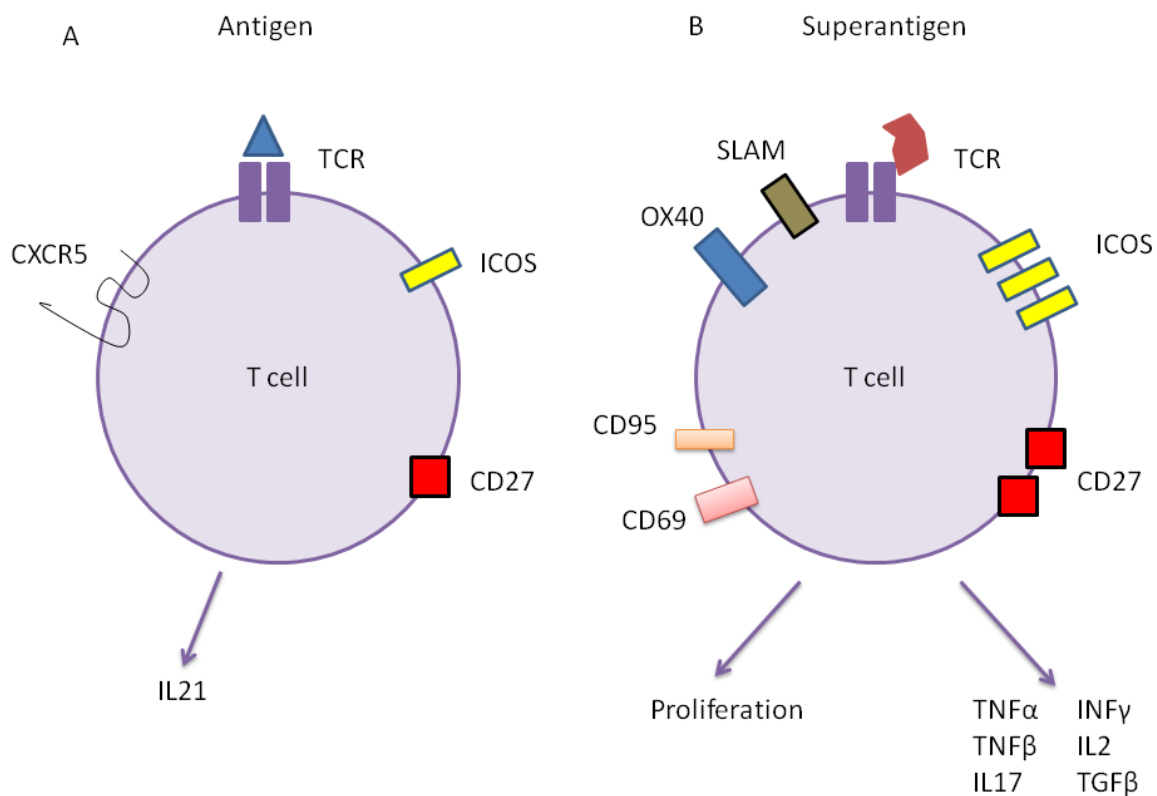


Figure 68: Alteration in T cell phenotype with superantigen stimulation

A/ with normal antigen presentation, tonsil T_{FH} cells maintain a normal T_{FH} phenotype, with CXCR5 expression. B/ with superantigen stimulation, tonsil T_{FH} cells proliferate and develop an altered phenotype with reduced CXCR5 expression, increased TNF receptor superfamily molecule expression, increased SLAM expression and the production of pro-inflammatory cytokines.

6.2.5 Supernatant transfer and cytokine inhibition

There was no replication of the inhibitory effect of SPEA on superantigens by transferring culture supernatants from previous SPEA treated cultures. These results are contradictory to the one paper which had previously attempted similar experiments, and found that transferred supernatant propagated the effect, even when it did not contain detectable quantities of superantigen (TSST-1) (Poindexter & Schlievert 1986). Aside from the choice of superantigen there were only two differences in methodology between the experiments performed by Poindexter and Schlievert and those presented here: their experiments were performed on PBMC's not tonsil cultures and secondly

that their outcome measure was formation of plaque forming cells rather than absolute immunoglobulin quantification. It is likely, therefore, that as the cell types tested were different, with different proportions of B cells and composed of different T cell mixes, that the supernatants contained different mixes of cytokines or other secreted proteins.

When inhibition of cytokines was attempted in cultures, immunoglobulin production was partially inhibited in the presence of anti-IL10, and completely inhibited in the presence of anti-IL2 antibodies, showing that both of these cytokines perform an essential role in normal immunoglobulin production by B cells. There was no alteration in IgG production in the presence of anti-IFN γ antibodies, again contrary to one previous study, which again used PBMC's and TSST-1 (Hofer et al. 1996), and one study which attempted to replicate those results and found a partial improvement in immunoglobulin production (Jabara & Geha 1996). However, Hoefer et al used lower concentrations of superantigens (TSST-1 1ng/ml rather than 1 μ g/ml) and the outcome measure tested was percentage of cells expressing CD95 or undergoing apoptosis, rather than immunoglobulin production. It is likely, therefore, that any superantigen induced cytokine effect is multi-factorial rather than due to a single cytokine, and that excessive pro-inflammatory cytokine production is not helpful to B cells, though some production of IL2 is essential for normal B cell function.

A recent publication looking at the action of T_{FH} cell subclasses in human tonsils on the immunoglobulin-secreting capabilities of either naive, germinal centre or memory B cells investigated the effect of inhibition of cytokines, and also Fas and ICOS inhibition (Bentebibel et al. 2011). In these experiments the authors separated the cells into different T_{FH} and B cell subsets before re-mixing them, and adding SEB to cultures of different cells mixes. The SEB was added to all cells to create an activated state but failed to include non-SEB stimulated cells as a control in their experiments (the only exception being when cells were stimulated with anti-CD3/28, again without a negative control, to examine T cell gene expression). Similarly to the experiments presented here the authors found that inhibition of IL10 partially reduced immunoglobulin production from the subset of cells that they found were able to produce immunoglobulin in these culture conditions (T_{FH} cells expressing either low levels of CXCR5 and ICOS or high levels of both). Interestingly these authors

also inhibited IL21 and ICOS, and found that there was a marked reduction in production of immunoglobulin of all classes, as seen with anti-IL2 in the experiments presented here. As noted in chapter 5, the presence of superantigen causes the T cells to adopt a phenotype of low-CXCR5 expression and high ICOS expression. In the experiments presented by Bentebibel et al, blocking the action of Fas in these cells restored immunoglobulin production, though as noted before there was no SEB negative control used (Bentebibel et al. 2011).

This supports the theory that the predominant mechanism of B cell death, and hence reduction in immunoglobulin production in superantigen exposed B cells, is by Fas mediated apoptosis, and that there may be an influence of some cytokines on induction of this process.

6.2.6 Summary of immune effects of SPEA on tonsil cells *in vitro*

In conclusion, the causes of B cell death in the presence of superantigens appear to be multi-factorial. There is clearly a role for CD95 driven apoptosis of B cells, but this cannot explain why equally high CD95 expression does not lead to apoptosis in T cells or unstimulated cells. Close B-T cell interactions are crucial to normal germinal centre function, and there is evidence of abnormal TNF receptor and ligand expression in the presence of SPEA, as well as alteration in the expression of a key B-T cell adhesion molecule SLAM. Certainly the cells appear to be skewing away from the normal T_{FH} phenotype classically found in human tonsils, towards a proliferating and high cytokine producing phenotype. There is likely to be some influence from the different cytokines produced, particularly IL10, IL21, IL2 and INF γ , but the action of no single cytokine can account for the B cell death seen with superantigens. Furthermore, a soluble factor was shown not to be the cause of reduced immunoglobulin production. Most of the B cells which survive in the presence of superantigens are probably those that do not rely on T cell support, and study of regulatory genes does not suggest that those cells are functioning abnormally. The results presented here offer some exciting new insights into the role that superantigens play in streptococcal tonsillitis, and warrant further investigation both

to explore the precise mechanism and their overall importance in the development of streptococcal disease. As these findings are all *in vitro*, it will be important to establish whether the same is true *in vivo*. Preliminary experiments to investigate this are presented in chapter 7.

7 The *in vivo* consequences of immunoglobulin inhibition by superantigens

The earliest experiments using streptococcal superantigens showed that that injections of purified superantigens into mice caused splenic B cell apoptosis (Cunningham & Watson 1978a), while *in vivo* superantigen exposure causes T cell expansion followed by T cell subset depletion after repeated superantigen administration (Muraille, De, Andris et al. 1997). The *in vivo* consequences of these specific events, in the context of streptococcal infection, are however poorly characterised. Reduced survival after infection was observed if mice were pre-exposed to SPEA (Sriskandan et al. 1996), consistent with a harmful effect of SPEA on resistance to infection. However, experiments using isogenic SPEA[±] strains in superantigen-sensitive HLA class II-transgenic mice did not show a major effect of SPEA on bacterial clearance in the acute setting, suggesting that any deleterious effects of SPEA must be on the adaptive rather than innate immune response (Llewelyn et al. 2004).

Having established that in the tonsil model of infection SPEA alters the magnitude of cognate immune responses *in vitro*, it was important to see if this was of physiological relevance in terms of immunity to infection *in vivo*. Using HLADQ8 transgenic mice, which are known to have enhanced susceptibility to the effects of SPEA (Sriskandan et al. 2001), a mock-infection model was developed to investigate the influence of SPEA on the development of local and systemic specific anti-streptococcal IgG responses to *S. pyogenes*. As discussed later in this chapter, the studies were constrained by a number of logistical problems so that the experiments were limited to two preliminary studies, and that we were unable to establish immunity to a known antigen in HLA-DQ8 mice, and then examine the impact of subsequent exposure to a superantigen such as SPEA on antibody responses.

7.1 Results

To determine whether the observations made in isolated tonsil cells could be reproduced *in vivo* it was necessary to measure immunoglobulin production in response to superantigen either in humans or in an animal model. Measurement in humans required the collection of saliva samples from patients with SPEA-positive *S. pyogenes* tonsillitis, or throat swabs that could be processed for immunoglobulin gene expression. Although these studies were initiated, over a 12 month period only 3 patients with confirmed *S. pyogenes* tonsillitis were recruited, and none of their isolated bacteria carried the SPEA gene. As mentioned in chapter 3, RNA quality and yield from the throat swabs was poor, and the immunoglobulin gene was only detected in one tonsillitis case and one healthy volunteer.

Animal models of tonsillitis are possible only in large mammals and non-human primates. Mice do not have pharyngeal tonsils but lymphoid aggregates known as nasal associated lymphoid tissue (NALT), which is poorly representative of tonsils and technically difficult to extract for processing. Therefore we opted to investigate the impact of SPEA on antibody production by studying the inguinal lymph node as a model of lymph node B cell function following intramuscular thigh infection, and serum for analysis of systemic antibody responses.

Measurement of anti-*S. pyogenes* responses in mice has not previously been undertaken, with the exception of antibody to specific vaccine targets (Turner et al. 2009). In humans, anti-streptolysin O (ASO) and anti-DNAse B titres are measured as an indication of recent exposure to *S. pyogenes* (Johnson et al. 2010), although not in the context of intramuscular infection. We hypothesised that exposure to heat-killed *S. pyogenes* would lead to a systemic antibody response to streptococcal cell wall, measurable by ELISA, and would also lead to a B cell response in the draining lymph node measurable by ELISpot. Exposure to SPEA-producing *S. pyogenes* was hypothesised to reduce serum antibody responses and local B cell immunoglobulin production.

7.1.1 Impact of *in vivo* SPEA exposure on primary antibody responses to *S. pyogenes*

To determine if exposure to SPEA *in vivo* might affect antibody production *in vivo*, it was necessary to find a way to expose mice to SPEA in a physiologically relevant manner, and also to try to identify an antibody response that could be measured. To emulate SPEA exposure, we chose to infect mice with a SPEA-producing strain of *S. pyogenes* (or isogenic SPEA non-producing and control bacteria) for a period long enough to stimulate antibody production, and measure the serum IgG response to whole *S. pyogenes* by ELISA.

Preliminary experiments showed that it was not possible to mimic a natural *S. pyogenes* infection and study humoral immune responses using live bacteria inoculated intramuscularly or subcutaneously, as even with antibiotic therapy the mice did not survive past 5 days - insufficient time to mount a specific anti-streptococcal antibody response. Therefore, a mock infection model was created, by injecting preparations of heat killed bacteria re-combined with the superantigen containing culture supernatant. As the aim was to mimic natural infection, Freund's adjuvant or other booster agents were not added to the cultures prior to injection. The aim of these experiments was then to assess the influence of exposure to SPEA-producing or SPEA-non-producing strains of *S. pyogenes* on the systemic antibody responses following an early re-challenge with live *S. pyogenes*, at 2-3 weeks from the initial exposure, when an anti-streptococcal antibody response should have been formed.

To assess the systemic antibody response, an anti-*S. pyogenes* IgG ELISA was performed on serum collected both pre-infection (after initial exposure to SPEA producing/non-producing bacteria) and after infection. Sera from mice which had been fully vaccinated with SpyCEP (an *S. pyogenes* vaccine candidate molecule) were used as controls for optimum antibody production, with a saline vaccinated control for no antibody response (Turner et al. 2009).

Unfortunately the antibody level to streptococcal cell wall was uniformly low among the experimental groups, and only the group with a value significantly higher than mice that had been exposed to saline or RPMI was the pre-infection serum from the group which had been exposed to the SPEA-non-

producing strain (WT Δ speA pre group, Figure 69A, $p=0.0031$ Kruskal-Wallis test). There were no significant differences between the median pre-and post-infection serum levels for any of the groups, including WT Δ speA pre and post infection samples (though the distribution of values altered between the two). These results contrasted with the control SpyCEP-vaccinated mice which had a significantly higher titre than the saline-vaccinated/RPMI exposed mice, with a median titre of 1:25600, compared to 1:400 to 1:1600 for all other groups ($p=0.0001$ Kruskal-Wallis test, Figure 69B), consistent with the reported potential of this cell wall protein as a vaccine candidate. This experiment showed that mice required a more prolonged immunisation period to mount a measurable immune response, though there was an indication that the SPEA non-producing strain of *S. pyogenes* might produce a greater primary antibody response.

Consistent with this, there were no significant differences in the bacterial counts between groups for blood, spleen, liver or thigh (Figure 70). All of the mice which had been pre-exposed to *S. pyogenes* had lower draining lymph node bacterial counts than the controls.

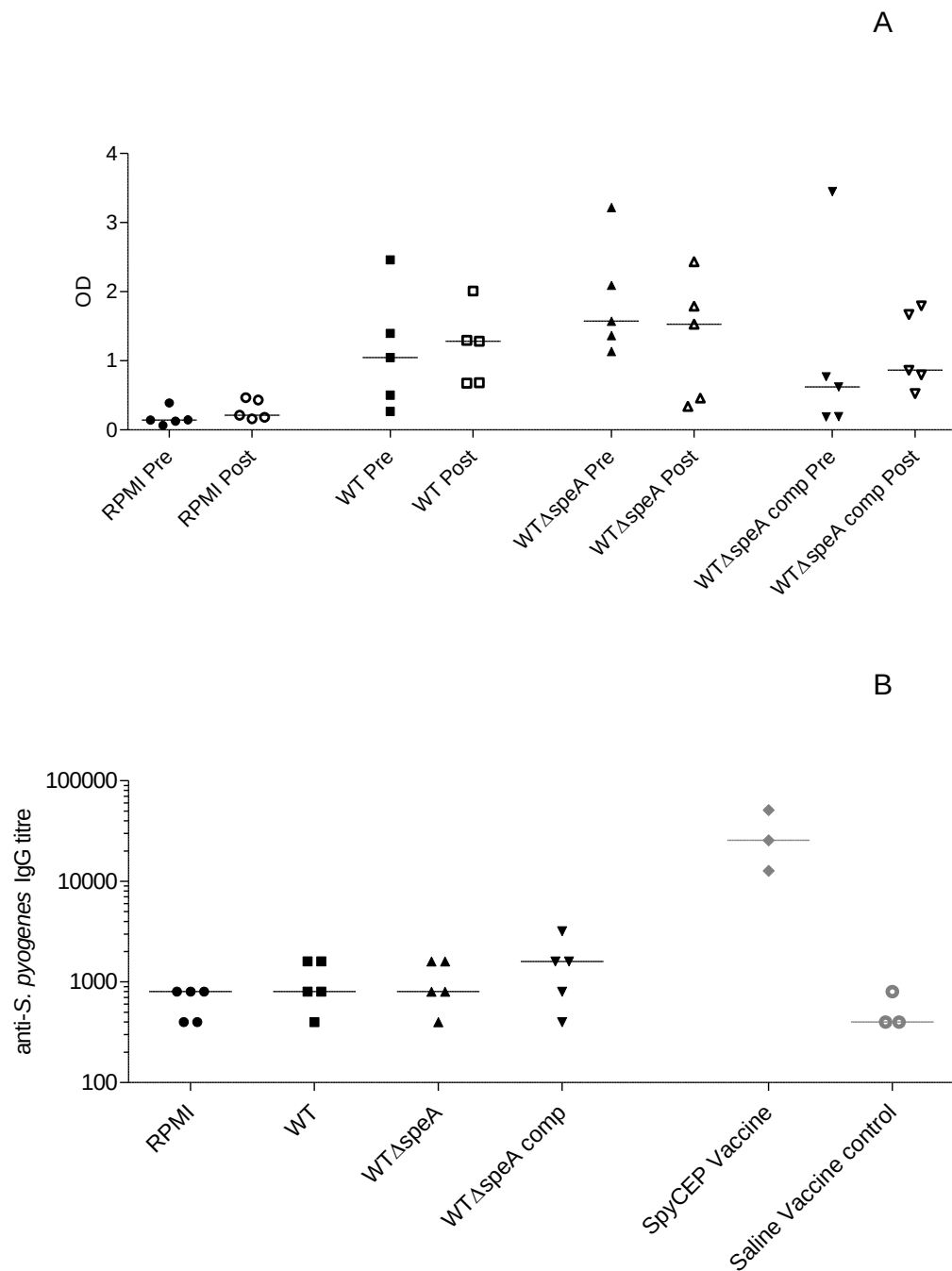


Figure 69: Anti-*S. pyogenes* IgG production: pre-exposure to SPEA+/- *S. pyogenes* (heat-killed) followed by challenge with live *S. pyogenes*

Anti-*S. pyogenes* IgG was calculated in the serum from mice exposed to wild type (WT) isogenic *speA*- (WTΔ*speA*) and complemented (WTΔ*speA* comp) strains of *emm1 S. pyogenes*. A/ serum from before (pre, closed symbols) and after (post, open symbols) infection with live bacteria was compared for each group (bars represent median values). Due to insufficient serum to titrate out levels in the pre-infection groups, all samples were diluted 1:100 and the optical density measured at 450nm. B/ The serum after subsequent live bacterial infection with the strain WT Δ *speA* was titrated from a starting dilution of 1:100. Control serum was from mice vaccinated with SpyCEP or saline and infected with the WT strain. RPMI was used as a negative control for vaccinations. Y axis = dilution titre that gave a positive colour change of optical density 0.5 or greater. Bars represent median values. Antibody levels were all tested against the WT strain.

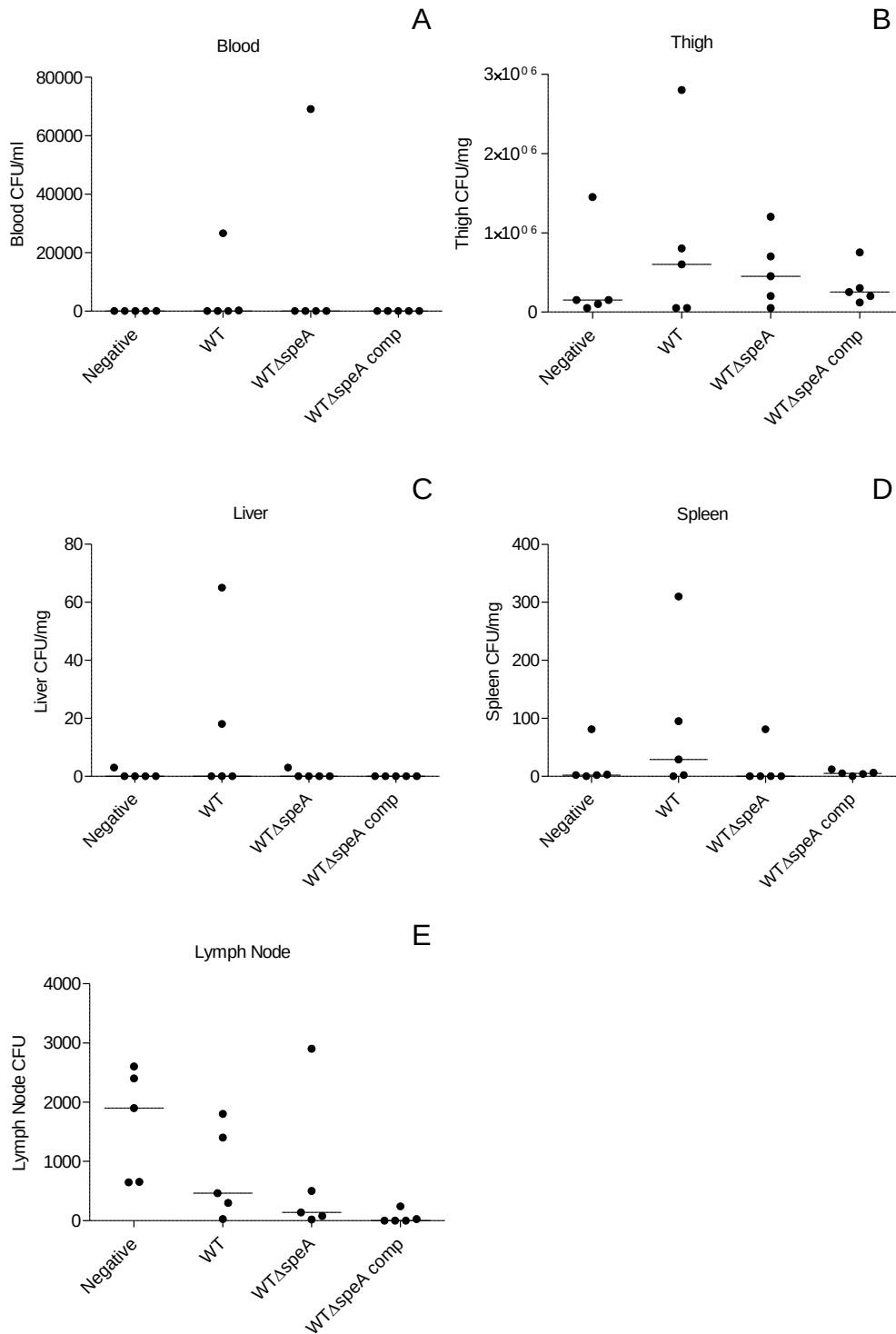


Figure 70: Bacterial counts – pre-exposure to SPEA+/- *S. pyogenes* (heat-killed) followed by challenge with live *S. pyogenes*

Groups of 5 mice were pre-exposed 1 week apart with RPMI (negative control) or heat killed bacteria with culture supernatant from the isogenic *emm1 S. pyogenes* strains varying in SPEA expression: WT = wild type strain, WTΔspeA = wild type strain not expressing *speA*, WTΔspeA comp = *speA* complemented WTΔspeA strain. Blood and organs were harvested 20 hours after infection with live WTΔspeA strain *S. pyogenes* bacteria. CFU = colony forming units.

7.1.2 Impact of SPEA exposure on *in vivo* antibody responses in immunised mice

To assess the local as well as systemic effects of SPEA on the production of antibody responses to *S. pyogenes*, mice were vaccinated twice (two weeks apart) in the right thigh with SPEA non-producing heat-killed *emm1 S. pyogenes* re-suspended in culture supernatant, in order to produce anti-streptococcal immunity. Exposure to SPEA was then achieved by injecting mice with heat killed SPEA-producing or non-producing *emm1 S. pyogenes* (re-suspended in culture supernatant) into the right thigh 2 weeks later (4 weeks from the initial bacterial vaccination) or RPMI as a control. A further group of 5 non-immunised/exposed mice were used as a control. Mice were then evaluated for local and systemic antibody production 48 hours later.

As anticipated, mice that had been immunised with *S. pyogenes* demonstrated measurable serum anti-streptococcal IgG titres at this later time point (Figure 71). Importantly IgG titres were lower in the mice exposed to the wild-type SPEA-producing strain (median titre 1:1600) compared with the RPMI or WT Δ speA re-challenged immunised mice (median titre 1:3200), and were not significantly higher than the un-immunised mice (median titre 1:200), which the other vaccinated groups were ($p=0.0067$ Kruskal-Wallis test). This result was in keeping with the hypothesis that SPEA exposure reduces antibody responses. However mice exposed to the SPEA-complemented strain did not recapitulate the results seen in mice exposed to the wild type (SPEA producing) strain – although the median titre was the same at 1:1600, the range was wider in this group, up to titres of 1:6400. This may suggest that the result is spurious, and unrelated to the effects of SPEA. The wild type strain is known to express more cysteine protease (SPEB) than the isogenic strains for reasons that are unknown (Unnikrishnan, Cohen, & Sriskandan 1999). As SPEB can degrade immunoglobulin (Collin & Olsen 2001) it is possible that this may reduce the serum immunoglobulin levels, though such an effect has not been observed for SPEB produced by live bacteria. All of the strains have a mutation in the regulatory locus *CovRS* (C. Turner, personal communication), and this is no different between the strains. Another explanation is that the complementation plasmid was poorly retained by bacteria as they were cultured *in vitro* without additional antibiotics, and the amount of SPEA in the challenge was not

measured. As previously, sera from SpyCEP and saline vaccinated mice were used as additional controls, and the SpyCEP vaccinated mice produced comparatively higher titres of anti-*S. pyogenes* IgG (median titre 1:12800).

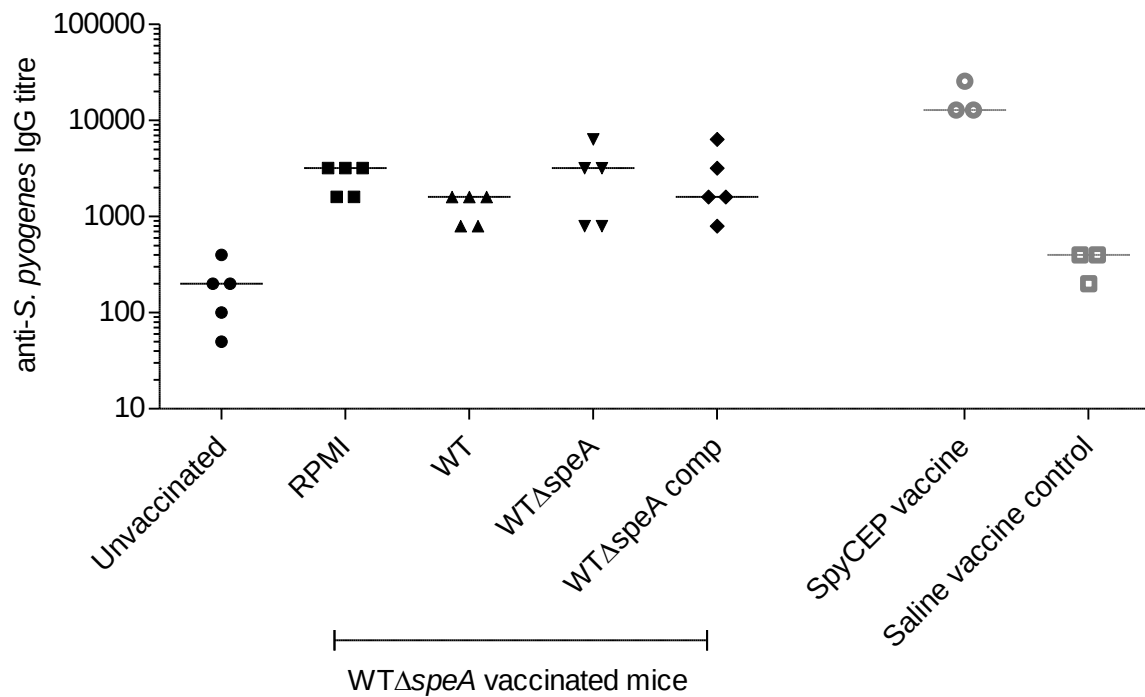


Figure 71: Anti-*S. pyogenes* IgG titres on re-exposure to *speA*[±] *S. pyogenes* strains

Serum anti-*S. pyogenes* IgG titres were assessed on mice pre-exposed to WTΔ*speA* *emm* 1 *S. pyogenes*, and then re-exposed to isogenic *speA*[±] strains (horizontal axis) or culture media (RPMI). Controls included mice never exposed to *S. pyogenes* (Unvaccinated) and mice previously vaccinated with SpyCEP or saline. Titres were tested against the WTΔ*speA* strain. Y axis = dilution titre that gave a positive colour change of optical density 0.5 or greater. Bars represent median titres.

The B cell ELISpot demonstrated that it is possible to detect specific anti-streptococcal B cell responses at the single cell level in a local draining lymph node. Anti-streptococcal B cell responses were observed in all of the lymph nodes from mice immunised with *S. pyogenes* compared to unvaccinated mice ($p=0.0014$ Kruskal-Wallis test, Figure 72A). In contrast to the serum IgG titres from the same mice there was, if anything, a paradoxical trend for the mice exposed to the wild type

S. pyogenes to have more anti-*S. pyogenes* IgG producing B cells, although this difference was not statistically significant. However the numbers of mice in the WTΔspeA and complemented groups were reduced to 4 each due to technical problems with the ELISpot, which may have influenced this result, meaning the data should be interpreted with caution as the numbers are small.

B cell ELISpot was also undertaken using spleen cells (Figure 72B), but no differences were seen between any of the groups, including between vaccinated and non-vaccinated mice. Interestingly, the number of cells producing specific anti-*S. pyogenes* IgG was generally lower in the spleen cells than the draining lymph node, despite there being 1 log more cells being used in each well. This suggests that the majority of the immune response at this stage is locally mediated.

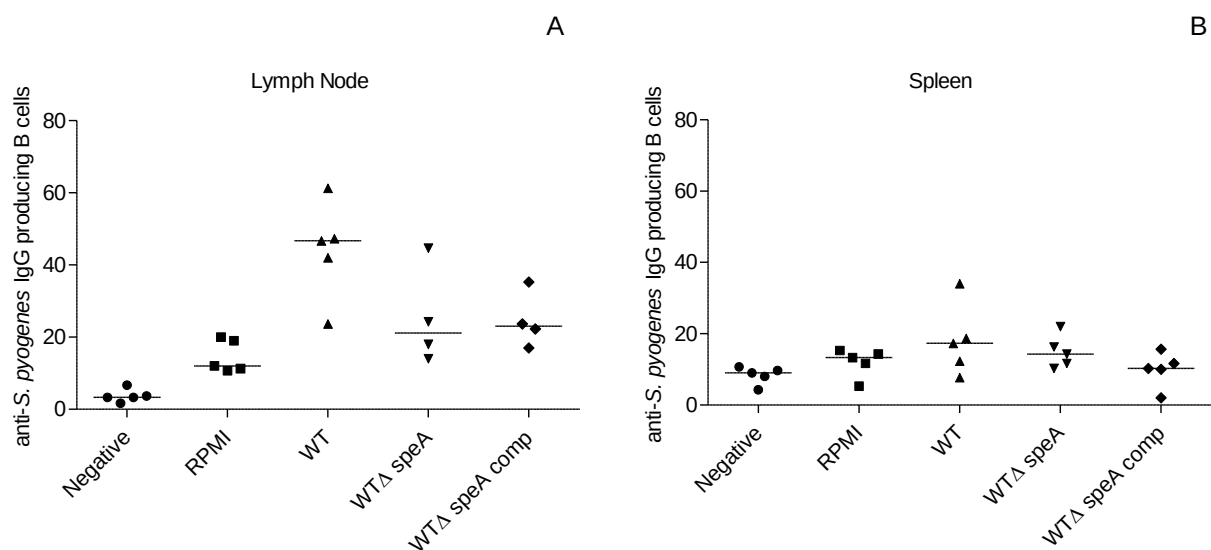


Figure 72: ELISpot responses to *S. pyogenes* in mice exposed to *speA*+/- supernatants

Mouse IgG responses to *S. pyogenes* (WTΔspeA) in the draining lymph node and spleen were assessed by ELISpot. Triplicates of 10^5 cells/well were used for each lymph node (A), 10^6 cells/well for each spleen (B). Y axis = each spot counted represents one IgG producing B cell. Bars represent the median spot count for each group of 5 mice. Results for 1 mouse each from the lymph nodes of WTΔspeA and WTΔspeA comp groups were excluded for technical reasons.

7.2 Discussion

7.2.1 Technical challenges

Transforming the *in vitro* findings of this project to *in vivo* significance presented a number of technical challenges, namely how to create a realistic model of natural infection and how best to measure specific antibody responses to streptococcal infection in that model. Poor recruitment to the paediatric clinical study and disappointingly low yields of RNA from throat swabs meant that a human trial failed to provide the *in vivo* answers. Re-creating pharyngeal infections in non-human primates was not an option, and so an *in vivo* mouse model of infection was developed.

7.2.1.1 Availability of mice

Although mice are not natural hosts for *S. pyogenes* infection, and do not respond well to bacterial superantigens which bind specifically to human MHC Class II molecules, the use of HLA-DQ8 transgenic mice overcame that problem, as they have been shown to have increased susceptibility to the effect of superantigens (Sriskandan et al. 2001), though only the female mice were suitable for *in vivo* infection studies (Faulkner et al. 2007).

These mice were bred in-house at Imperial College, and two major problems arose which meant that only limited numbers of mice were available. Initially there were fertility and health problems in the colony, and litter sizes were reduced to 1-2 surviving healthy offspring, most of which were needed to maintain breeding pairs and could not be used for experiments. Age-matching of mice to provide consistent data was particularly difficult. Then the breeding facility moved to a new location, and to ensure the health of animals in this new facility, the colony had to be re-established from previously frozen embryos into surrogate mice. Although this resolved the fertility and health issues, a period of 6 months was needed to fully re-derive the colony from the limited number (3 pairs) of healthy breeding animals which resulted from frozen embryo transfer procedures. Together these events meant that the number of animals available for experiments was severely limited for over a year after

the initial *in vitro* immunoglobulin findings were made, and only enough mice were bred from the new colony to perform two experiments before the end of this project.

7.2.1.2 Developing the model

The decision was therefore made to prioritise the available mice to re-create natural infections, using the isogenic *speA* +/- strains of bacteria, in order to emulate biologically relevant phenomena. However this further complicated the situation:

Firstly, using mouse nasal lymphoid tissue (NALT) to re-create a pharyngeal system of infection would not be a decent comparison to the human pharynx, as the lymphoid tissues in that area are lymphoid aggregates of cells, more similar to human gut lymphoid tissues and Peyer's patches than the lymph-node structure of the human pharynx. Therefore an intramuscular infection model was used, to test responses in the easily accessible and structurally relevant inguinal lymph node.

Secondly, pilot experiments showed that it was not possible to infect mice intramuscularly or subcutaneously and them to survive the six weeks that were shown to be necessary to generate detectable serum anti-streptococcal IgG. This resulted in a heat-killed bacterial model, with the addition of bacterial supernatants to ensure adequate exposure to superantigens. As mice were not being vaccinated to survive infection, immune boosting agents such as Freund's adjuvant were not used. Although the serum antibody response was not detectable at the early time point (3 weeks), there was a good antibody response detected at 6 weeks, even though the differences between groups were not significant on re-challenge. Future experiments using Freund's adjuvant or tetanus toxoid to bolster the general immune response might provide a clearer picture of responses for future experiments.

Thirdly, the decision to use whole bacterial preparations rather than purified superantigens meant that the contribution of one individual superantigen, SPEA, was difficult to assess. The strains used are known to also produce SMEZ, and it is unclear what influence this had on antibody production. There is now available an isogenic *smeZ* negative strain which matches the strains used in these experiments, and a double *smeZ* and *speA* negative strain, and complemented strains of these. The use

of these strains would help to remove the effects of conflicting superantigens from the equation, and allow the specific superantigen of interest to be studied more easily. The most straightforward experiment would be to establish immunity to a known antigen, for example SpyCEP, in all animals, and then examine the impact of subsequent exposure to a superantigen such as SPEA on anti-SpyCEP responses. It would be interesting to then see if any alterations in antibody responses were in specific streptococcal antibodies or general antibody production.

7.2.1.3 *Developing methods to measure specific anti-streptococcal IgG responses*

In humans, kits for testing *S. pyogenes* immune responses are readily available in the form of anti-streptolysin O (ASO) titres and anti-DNAse B. However these kits are specific for human antibodies, and do not have cross-reactivity with mouse antibodies. No previously published streptococcal work had directly tested anti-streptococcal antibody production, just antibodies against specific vaccine targets (Turner et al. 2009). Therefore new methods had to be devised to measure the anti-streptococcal immune responses in mice.

The anti-streptococcal IgG ELISA worked well, though the lack of a known standard meant that determining a specific value could not be done, only establishing antibody titres. For human serum, IVIG could provide a control to some extent (as it is known to contain various anti-streptococcal antibodies (Basma, Norrby-Teglund, McGeer et al. 1998)), but this would not be recognised by the mouse-specific detection antibody. There is a risk of error by just measuring optical density, which would be operator dependant, and would only allow for comparison within a plate, not between plates. Therefore doubling dilution titres were performed on samples, much as they would be for human ASO titres. After blanking values to the background, a measurement of optical density of 0.5 or greater was found to consistently represent a true positive titre. Purchase of an appropriate secondary antibody would allow this technique to be easily adapted to measuring different classes of immunoglobulin (i.e. IgM or IgA) and could be easily adapted to different species, including humans. It would be interesting to use this technique to measure streptococcal specific antibody responses in tonsils exposed to the different bacterial supernatants, which generally increase immunoglobulin production over baseline levels. The concentration of antibody in the culture supernatants was too low

to cause latex agglutination in the ASO titres test, but the ELISA developed here is very sensitive, and so should, in theory, work. It would be particularly interesting to see if the rise seen in IgG levels with SPEA negative bacterial strains was predominantly streptococcal specific – and this could be determined by comparing the results to total IgG levels in the same supernatants. Control human IgG or IVIG could also be used to create a standard against which supernatants could be measured in this model.

Assessing the local draining lymph node for specific anti-streptococcal antibody production was more difficult. Flow cytometry would provide good data about the number and health of B cells in each lymph node, and whether they were producing general IgM, IgG or IgA, but it would not demonstrate whether the antibody was general or specific to streptococcus. ELISpot seemed to provide the answer (Lycke and Coico 2001), but again there were few instances where this had been performed to test anti-bacterial titres. One publication described an ELISpot to test hybridoma production of antibody against *Neisseria* sp. (Cooper and Kirkpatrick 1997) and one other publication was found where ELISpot was used to test specific responses to the Human Papilloma Virus (HPV) vaccine (Giannini, Hanon, Moris et al. 2006). Adapting the methods used for the streptococcal ELISA, general ELISpot protocols were modified to allow for the measurement of anti-streptococcal IgG production from B cells, using the methods listed above. Ideally more cells would have been used for each lymph node, but the lymph nodes were not acutely inflamed as they would have been during live bacterial infection, so in order to perform the test in replicate wells, the cell number was limited to 10^5 cells per well. Even so, this concentration of cells generated clear spots which were easy to read, allowing the development of specific local immune responses in mice exposed to *S. pyogenes* to be evaluated. As with the serum responses, boosting the natural immune response or exposing the mice to higher concentrations of SPEA may make any subsequent differences between the groups on re-challenge clearer.

Future flow cytometry studies on draining lymph nodes and spleen cells would provide important information to back up the *in vitro* tonsil model TNF receptor superfamily expression and SLAM

expression data, and also to look at the T follicular helper cell phenotype in mice exposed to superantigens both singly and in the wider context of *S. pyogenes* infection.

7.2.2 Importance of the *in vivo* findings

The results presented here are clearly very preliminary, and do not answer the question “what influence does SPEA have on the production of specific anti-streptococcal antibody?” However, they have demonstrated that with some modification of the methods, the answer should be attainable. The serum IgG results hint that the *in vitro* findings will be confirmed and that SPEA will reduce the IgG level produced. The ELISpot seems to suggest that a paradoxical effect is happening at a local level, and that SPEA is having more of an antibody stimulating effect than an antibody inhibitory effect.

The influence of other streptococcal virulence factors, such as SPEB, cannot be excluded as playing a part in this. In the wild type M1 strain used, SPEB can be detected in culture supernatant, when it cannot in the isogenic strains. It is known that SPEB can cleave immunoglobulin (Collin & Olsen 2001), but also that it can impair superantigen functions by degrading them (Kansal, Nizet, Jeng et al. 2003). This may also explain some of the differences seen between the wild type and complemented SPEA strains.

In a wider context, the regulation of immunoglobulin production by superantigens is important in the management of toxic shock syndrome. The mechanism by which IVIG is useful in streptococcal toxic shock is multi-factorial, and a large proportion of it is a directly neutralising effect of superantigens and other bacterial virulence factors. However, even in the context of superantigen negative infections, IVIG can have a role in helping to clear the infection locally (Davies, Charnock, Turner et al. 2012). Therefore, if superantigens impair the production of immunoglobulin, this adds further weight to the argument in favour of using IVIG for the management of patients with toxic shock syndrome.

8 General Discussion

This project started with three aims:

- 1) To assess superantigen production *in vitro* and in patients with clinical disease
- 2) To investigate the main immune effects of superantigens in human tonsils
- 3) To establish the effects of superantigens on immunological memory *in vivo*

These aims were generated from the central hypothesis that superantigen production was important in the pathogenesis of streptococcal tonsillitis, and that the action was in some way mediated by the effects of superantigens on the normal functions of the immune system in the pharynx.

The project required a number of novel techniques to be developed, and examined the broader picture of tonsil immune responses both to superantigens and whole *S. pyogenes* rather than emulating previous results from different infection models. Surprisingly the most pronounced immunological finding, T cell-dependent B cell death in the presence of superantigens, led back to the earliest observations made about superantigens back in the 1970's, the mechanism for which had never been fully explained. This led to a detailed exploration of how B and T cells were communicating in tonsils, and ended up entering one of the most rapidly developing areas of immunology – the role of lymph node T follicular helper cells in the formation of cognate immune responses in tonsils.

8.1 Expression of superantigens by *S. pyogenes* *in vivo* and *in vitro*

The investigation of virulence factor expression in samples from patients with disease is never easy: the influence of patient factors such as co-morbidities, medication (including antibiotics) and time to presentation all mean that the samples received cannot be regulated in the way that *in vitro* or animal experiments are. Furthermore, in this project there was a significant delay in receiving the tissue samples from the laboratory, which may well have caused altered physiology, or even dormancy, in bacteria compared with examination straight from operation. In spite of this, bacterial RNA transcripts for the superantigens SMEZ and SPEA were detected in at least one tissue sample each, and transcripts for SpyCEP were detected in six tissues from five different patients.

The difficulties in examining patient tissues means that there are very few published studies to compare the results to; in the case of *S. pyogenes* there is only one study which has attempted to establish the role of virulence factors in patient tissues (Norrby-Teglund et al. 2001). In that study tissue biopsies from patients with necrotising fasciitis demonstrated increased levels of IL1, TNF β and INF γ in the tissues consistent with the presence of superantigens, but tissue mitogenicity and qRT-PCR for bacterial factors were not attempted.

In this project, functional assessment of superantigens in tissues provided more information about superantigen presence in the tissues than qRT-PCR. There was an association between the proliferation and culture results, suggesting that only the tissues where live bacteria were still present contained superantigens. In some cases the mitogenicity of the tissues lasted for several days after the initial surgical debridement, despite antibiotic therapy including protein synthesis inhibitors (which should have stopped superantigen production) being evident in the samples. The tissues where there was no live bacterial culture acted as control samples for proliferation, though to fully exclude the influence of tissue cytokines on the effects seen, cytokine measurements would need to be made on both culture positive and negative tissues.

The degree of proliferation attributable to superantigens in the tissues was considerably lower than the results achieved using overnight broth cultures from the same strain, possibly reflecting degradation of superantigens in the tissues, or reduced production *in vivo* compared to *in vitro* either due to fewer bacteria, bacterial self regulation or the presence of protein synthesis inhibiting drugs in the samples. The relative mitogenicity of each strain cultured *in vitro* corresponds with known variations in mitogenicity between different *emm* types (Turner et al. 2011). The reduced mitogenicity of the tissue samples from streptococcal necrotising fasciitis contrasts with the enhanced concentration of superantigens that can be found *in vivo* in cases of staphylococcal toxic shock (Davies, Smith, Ellington et al. 2012). This demonstrates that although the superantigens themselves are structurally similar, the diseases caused by the different bacteria which produce them are not the same, and this is reflected in the different clinical presentations of staphylococcal versus streptococcal toxic shock syndrome, and the differences in morbidity and mortality attributable to each.

To confirm conclusively that the mitogenicity was due to superantigens, cytokine levels in the tissues should be measured and, if possible, selective superantigen neutralisation performed by the incorporation of neutralising antibodies into the proliferation assay. Assessment of TCRV β expansion in normal donor peripheral blood T cells co-cultured with the tissue samples could indicate the presence of specific superantigens, if compared to the TCRV β profile obtained using culture supernatant from the same strain grown *in vitro*. This would also provide information about the level of expression of each superantigen in the samples relative to the other superantigens present on genotyping, for example predominant TCRV β 8 expansion would suggest that SMEZ was the predominant functional superantigen present in the infected patient tissues.

The patient studies presented here were further hampered when it came to the paediatric study that aimed to detect superantigen effects during tonsillitis. There was poor quality RNA and DNA obtained from the throat swabs, so the samples did not give a definitive answer to the hypothesis that superantigen expression by *S. pyogenes* would be enhanced in children with active tonsillitis compared to healthy carriers. The number of patients recruited was also small, and of the three cases of confirmed *S. pyogenes* infection, none of those strains carried the key superantigen gene of interest

in the project, *speA*. The project had aimed to recruit 15 patients with confirmed *S. pyogenes* tonsillitis, and assumed that the molecular epidemiology of tonsillitis would match that of invasive *S. pyogenes* disease; hence it was assumed that nearly all cases would be caused by *emm1* or *emm3* *S. pyogenes*, both types that carry the *speA* gene. However subsequently, this laboratory has conducted the only UK study of molecular epidemiology of tonsillitis using strains from the hospital diagnostic laboratory and demonstrated that *emm1* and *emm3* strains are under-represented among non-invasive diseases isolates; the commonest *emm* types were *emm12* and *emm6* strains (personal communication from S. Sriskandan, unpublished data). The technical procedures both of sample collection and processing could doubtless be improved, and patient recruitment in a primary care setting would be likely to yield both more disease recruits and healthy volunteers for this disease.

Although superantigen expression was not detected in RNA extracted directly from the throat swabs of children with *S. pyogenes* tonsillitis, the production of SMEZ by non-invasive clinical isolates *in vitro* was far higher than the values obtained from invasive clinical isolates associated with necrotising fasciitis, at 617 copies *smeZ* per 10,000 *proS* (throat isolates) compared to 19 copies *smeZ* per 10,000 *proS* (necrotising fasciitis isolates). As the number of isolates tested in this work were small, it would be important to repeat these experiments using a larger number of clinical isolates from both invasive disease and pharyngitis to ensure that there was no bias introduced by the methods of collecting the strains from these two clinical studies. Corresponding mitogenicity data would also be important, to confirm that the throat isolates were producing more functional superantigen as well as higher levels of transcription. However these preliminary results do tie in with the suggestion from monkey models of infection that superantigens are important in establishing throat infections (Virtaneva et al. 2005). There is a precedent for phenotypic changes occurring in clinical strains as a result of genetic changes in regulatory loci, particularly among *emm1* strains of *S. pyogenes* (Turner et al. 2009), which could be one explanation for these findings.

Human tonsils have been used in immunology investigations for many years, mainly as they provide a ready source of B and T cells. More recently tonsils have been used as a good model of a working lymph node, and so have formed the basis of many studies looking at the function of germinal centres. Despite this, tonsils have rarely been used to investigate bacterial pathogens (Wang et al. 2010), particularly *S. pyogenes* which is the most important cause of bacterial tonsillar disease, though some studies have looked at short term surface interactions between the tonsil epithelium and live bacteria (Abbot et al. 2007).

The difficulties involved in re-creating an *in vitro* model of tonsillitis are readily apparent from this project, particularly achieving a balance between contamination with endogenous flora and residual antibiotics which impaired laboratory introduced *S. pyogenes* infection. This was particularly problematic for the histocultures, where tissue necrosis after 24 hours provided an additional challenge. However, even cell suspension cultures were susceptible to infection from residual endogenous flora when antibiotics were washed away from the cells. The techniques were successful when using tonsils which had a lower level of contaminating flora at the outset, and it could be seen that the experimentally introduced bacteria clustered along the lympho-vascular septae and surfaces of the tonsil blocks, away from the lymphoid follicles. Whether this is truly representative of the situation during acute *S. pyogenes* tonsillitis in a patient is uncertain, due to the difficulties of obtaining deep tonsil samples from patients with acute disease. However the histopathology findings are broadly consistent with the core biopsy samples from tonsils which have been surgically removed (Lindroos 2000). Future experiments are planned to investigate this further, including with the addition of exogenously sourced neutrophils, to investigate the role they play in acute disease.

When measuring superantigen expression in the live bacterial-tonsil co-culture system, the problem was not measuring the superantigen expression in tonsil suspensions but a lack of superantigen expression in control wells, where *S. pyogenes* was incubated with media alone instead of tonsil cells. This may have been related to the timing of culture in the media, and future studies should use additional time points to ensure that the broth grown bacteria are sampled during log-phase of growth, when expression of superantigens is likely to be highest.

Despite the problems with live bacterial culture, in tonsil histocultures and cell suspensions, experiments using bacterial supernatants or recombinant superantigens worked consistently and it was from these experiments that the majority of results were obtained. The degree of block necrosis meant that the histocultures were more difficult to use for flow cytometry, but secreted factors in the culture media could be examined. Cell suspension cultures were easily generated and survived well for up to a week in culture, though a lag period of 1-2 days did seem to occur in cell responses to superantigens, probably due to the mechanical disruption of the tonsils during processing.

8.2 Immune effects of superantigens on tonsils

Of the three predominant cell types present in human tonsils, T cells, B cells and follicular dendritic cells, only T and B cells were examined in this project, due to the profound alterations which were seen in these cell populations. It was quickly established that the tonsil model provided a globally comparable superantigen response to that obtained from PBMC studies: T cells were proliferating, and the expansion of these cells was TCRV β subset specific, as previously described depending on the presence of specific superantigens (Proft & Fraser 2003); the cytokine production by tonsil cells was broadly similar to the mix of T_H1, T_H2 and T_H17 cytokines previously described (Li et al. 2008; Proft, Schrage, & Fraser 2007). However this was where the similarities to more recent PBMC work ended, and the different T cell and B cell proportions and subsets in the tonsils started to make an impact on the results.

The predominant T helper cell type in tonsils (as with other lymph nodes) is the T follicular helper (T_{FH}) subset. These T cells have a number of unique characteristics which distinguish them from other CD4 positive T cell subsets: they express CXCR5 on their surface, the receptor for CXCL13 with an important role in B-T cell communication; the main transcription regulatory gene is BCL6 compared to BLIMP-1 in all other T helper cell subsets; they have an innate expression of ICOS, SLAM-associated protein (SAP), and programmed death 1 (PD1) and there is a high expression of IL21 by these cells (Crotty 2011). These cells are thought to be highly adaptable in their responses to stimuli, and are easily able to alter their function to mimic the other helper T cell groups (King, Tangye, & Mackay 2008), though this concept is still controversial. In the presence of SPEA, the phenotype of T cells in this work, as assessed by cell surface marker and regulatory gene transcription, was found to be altered. The SPEA exposed T cells expressed significantly higher levels of ICOS, OX40 and SLAM, and significantly lower levels of CXCR5 than controls. There was increased expression of BLIMP-1, but interestingly also increased expression of BCL-6, perhaps reflecting cell proliferation as well as phenotype changes. The main outcome of this was T cell dependent B cell death by apoptosis and a subsequent loss of immunoglobulin production. Furthermore, this was found to be a

class effect not only of superantigens, but also of other T cell mitogens (Concanavalin A and CD3/28 antibodies), again suggesting that this was probably a T cell dependent effect resulting from proliferation. Apoptosis affected all B cell subsets, with the cells remaining continuing to express CD21 (representing marginal zone B cells), suggesting that they were cells able to operate in a T-independent fashion, possibly with a role as antigen presenting cells (Pillai, Cariappa, & Moran 2004), and some CD38 and CD27 expressing cells which probably represent terminally differentiated plasma cells in the tonsils when they were harvested, and accounted for the low baseline level of immunoglobulin production (Sanz et al. 2008). There was a significant reduction in the percentage of B cells expressing CD23, a marker of germinal centre B cells which function in a T-dependent manner (Pillai, Cariappa, & Moran 2004), in the presence of SPEA.

The mechanism of B cell death has not been fully established in the work presented here, though expression of Fas (CD95) is likely to play a part. Although CD95 expression occurred earlier in SPEA treated cultures, there were high levels of CD95 expression on B and T cells in unstimulated cultures too, which did not result in apoptosis. The downstream signalling of the caspase pathway would need to be examined to confirm that the predominant cause of B cell death was Fas-mediated apoptosis.

All of the flow cytometry experiments performed in this project were executed on a FACS Calibur machine, which restricted the analysis of samples to 3 colours per sample, and may appear out-moded compared to more recently reported approaches. Multi-parameter flow cytometry became available for use half way through this project, and so analysis could have been extended to give more information about cell types, which would have been useful for the characterisation of B and T cell subsets in particular, where the classification is complex and multi-factorial. However, by this time a number of the baseline characterisation experiments, including TCRV β profiling, had already been performed successfully on a number of different samples. The antibodies to analyse a number of the cell surface molecules were not commercially available until the end of the project, and then usually only conjugated to PE. This was particularly true for the TNF receptor superfamily molecules OX40 and ICOS and their reciprocal ligands, and SLAM.

These observations replicate some of the earliest *in vivo* and *in vitro* work with superantigens, observations which have been largely ignored in the last 20 years of superantigen research. The choice of human tonsils, as a physiologically relevant model for investigating superantigens, has again highlighted the importance of these effects and suggests that superantigens might play an important role in the ability of *S. pyogenes* to establish infection in the pharynx. Furthermore, these findings have important implications for the routine use of superantigens as T cell mitogens in immunology research unless superantigen-free controls are also used. No better illustration of this occurs than when superantigens are used to investigate T_{FH} cells and lymph node biology (Bentebibel et al. 2011).

8.2.1 Tonsil innate immune system responses to superantigens

The role of the innate immune system was not successfully examined in this project, and the preliminary results have not been presented. However, the role of the innate immune system in human pharyngeal defences against streptococcal infections cannot be ignored. There are three main components of the innate immune system in the pharynx which would ideally be examined in more detail in future work. Firstly the keratinised epithelium which covers the surface of tonsils: this was not removed from the surface of tonsils in the experiments presented here, but is known to cover the whole tonsillar surface. There are a number of important cell surface markers present on epithelial cells that are used by *S. pyogenes* as targets for attachment. Superantigens have been shown to be able to penetrate epithelia even when there is no locally invasive disease (Brosnahan & Schlievert 2011). Although this is clearly of importance in menstruation-related staphylococcal toxic shock syndrome, the relevance of these observations in the formation pathogenesis of pharyngitis is yet to be explored. In tonsils the epithelium is separated from the follicles by 200-300µm (Ohtani & Ohtani 2008), and so it is conceivable that local production of superantigens in the pharynx could have a profound effect on tonsil lymphoid follicles if they can penetrate the epithelium.

The second important part of the innate immune system which has not been investigated in this work is the production of antimicrobial peptides in the pharynx. The human beta defensins (HBD) 1-3, cathelicidin (LL37) and LEAP1 and LEAP2 (Liver expressed antimicrobial peptides) have been shown to be present in human tonsil epithelium, and are possibly reduced in chronic infections (Ball et al. 2007;Schwaab, Gurr, Hansen et al. 2009). LL37 has been shown to be present in large amounts in tissues from patients with invasive *S. pyogenes* disease and associated with SPEB production (Johansson et al. 2008) and degradation (Schmidtchen et al. 2002) but has also been shown induce *S. pyogenes* capsule expression (Gryllos et al. 2008), presumably as a bacterial defence mechanism. In this project I observed that the inhibition of bacterial growth was higher from tonsil suspension supernatants that had been exposed to superantigens than unstimulated controls. Unfortunately the concentrations of defensins in the culture supernatants were below the limit of detection by ELISA and no alteration was found in the RNA expression for HBD1-3 or LL37 of tonsils exposed to live *S. pyogenes* compared to negative controls (data not shown). Repeated attempts to culture cells in large volumes in antibiotic free media were hampered by infections from endogenous flora, so supernatants could not be fractionated and concentrated to examine this effect further. Previous studies have been able to detect defensins by ELISA from total tonsil protein extracts or by immunofluorescence (Schwaab et al. 2009) and so these are two other methods that could be employed, particularly with tonsil histoculture live infections.

Thirdly, the influence of neutrophils was not investigated in this model. Human tonsils are only removed when they are relatively un-infected, to reduce the risks of bleeding and surgical site infection. Future work with tonsils could examine the role of neutrophils in tonsillitis by using histocultures to measure exogenous neutrophil chemotaxis to the site of infection either in response to live bacteria or heat killed bacterial preparations.

8.3 Effects of superantigens on immunological memory

Two different experiments systems were used to try to establish the importance of superantigens in the generation of immunological memory: immunoglobulin production in the throat swabs of children recruited to the paediatric study and the generation of specific anti-streptococcal IgG responses in the animal model of infection. As the recovery of RNA and DNA was poor from throat swabs, the collection of salivary samples would give a better indication of immunoglobulin production in future human studies, and specificity to *S. pyogenes* could be examined using the ELISAs developed in chapter 7. There were considerable problems with the availability of the HLADQ8 transgenic mice necessary for examining the role of superantigens in *S. pyogenes* infection models. Although the decision to recreate a mock live infection seemed appropriate, the results were disappointing with only small and insignificant differences appearing between the groups. Future experiments to measure the effects of superantigens on immunoglobulin production *in vivo* would necessarily involve boosting the immune response to make the role of superantigens clearer.

It would be interesting to see if the generation of memory T cells was impaired as T cells were driven to proliferate by superantigens. The easiest way to do this would be to stain cells for CD45RO, which is a marker associated with the development of memory T cells.

Notwithstanding the results, two methods were successfully developed which have wide applicability to the examination of specific immune responses to bacterial infections both *in vivo* and *in vitro*. The anti-streptococcal ELISA showed that specific serum responses to a pathogen could be established as easily as those using commercially available human diagnostic tests, and could be tailored to suit different experimental conditions. The ELISpot successfully showed that bacteria-specific immune responses could be analysed at the single cell level. Neither of these techniques have previously been published in the context of *in vitro* or *in vivo* anti-streptococcal responses, and have already been adapted for use in a number of different experimental settings by other members of the Gram positive pathogenesis group.

8.4 Importance of experimental findings in *S. pyogenes* infections

The underlying hypothesis for this project was that superantigens were important in the pathogenesis of streptococcal tonsillitis by altering adaptive immune responses. Certainly the *in vitro* findings from exposing human tonsils to superantigens would support this hypothesis. Alteration in the characteristics of both T cells and B cells was observed in the presence of SPEA, with an increase in cytokine production, change in T cell phenotype and B cell failure to produce immunoglobulin and ultimately apoptosis. This implies that superantigens may have been developed by bacteria in order to target adaptive immune responses, which would help to either establish or propagate an infection. If this is the case, then the disease manifestations attributed to superantigens (such as scarlet fever and toxic shock syndrome) may be the side effects of superantigens produced either to excess or in a host who is more susceptible to their effects. This may have important implications on the transmission of infection and survival of bacteria between epidemics, as suggested by previous studies (Beres et al. 2006), and would be worth examining in animal transmission studies.

The implications of these results to patients are not difficult to conjecture. Although not proven, exposure to a high superantigen producing strain of *S. pyogenes* would not only increase the likelihood of developing infection but it would make it more difficult to clear the infection as the immune responses were dampened. Increased inflammation could increase transmission to others, and superantigens specifically could leave an individual vulnerable to early re-infection with the same or another strain of *S. pyogenes* if adaptive immunity had not formed correctly. Taking the results presented here in context with previous findings showing T cell depletion and anergy on repeated superantigen exposure (Cornwell and Rogers 1999; Eroukhmanoff, Oderup, & Ivars 2009), would indicate that the effects of superantigens are not limited to T cells but also the cells they interact with to form cognate immune responses.

For the future, superantigen neutralising TCRV β components have been described, which appear to have an impressive effect *in vitro* (Yang, Buonpane, Moza et al. 2008), though clinical use of these

products is clearly a long way off. Of greater relevance to current practice is the presence of superantigen neutralising antibodies in IVIG (Norrby-Teglund, Basma, Andersson et al. 1998). If these could be isolated and reproduced as a specific anti-streptococcal or anti-superantigen product then they could potentially play a role in treating pharyngeal disease as well as toxic shock syndrome, though the risks of IVIG preparations far outweigh the potential benefits from this at present. The *emm* types of *S. pyogenes* which are most associated with SPEA production are also among the most pre-eminent at causing outbreaks and epidemics in the UK, *emm1* and *emm3*. This supports the theory that SPEA has a role in causing epidemics and promoting bacterial transmission.

If the findings in this project are confirmed *in vivo*, then this would suggest that antibody responses are constantly downgraded by *S. pyogenes*, and even a vaccinated individual could remain susceptible to infection.

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Ref Type: Unpublished Work

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Appendix 1

Virulence factors in streptococcal tonsillitis – supporting documents.

Study flow diagram

Study protocol

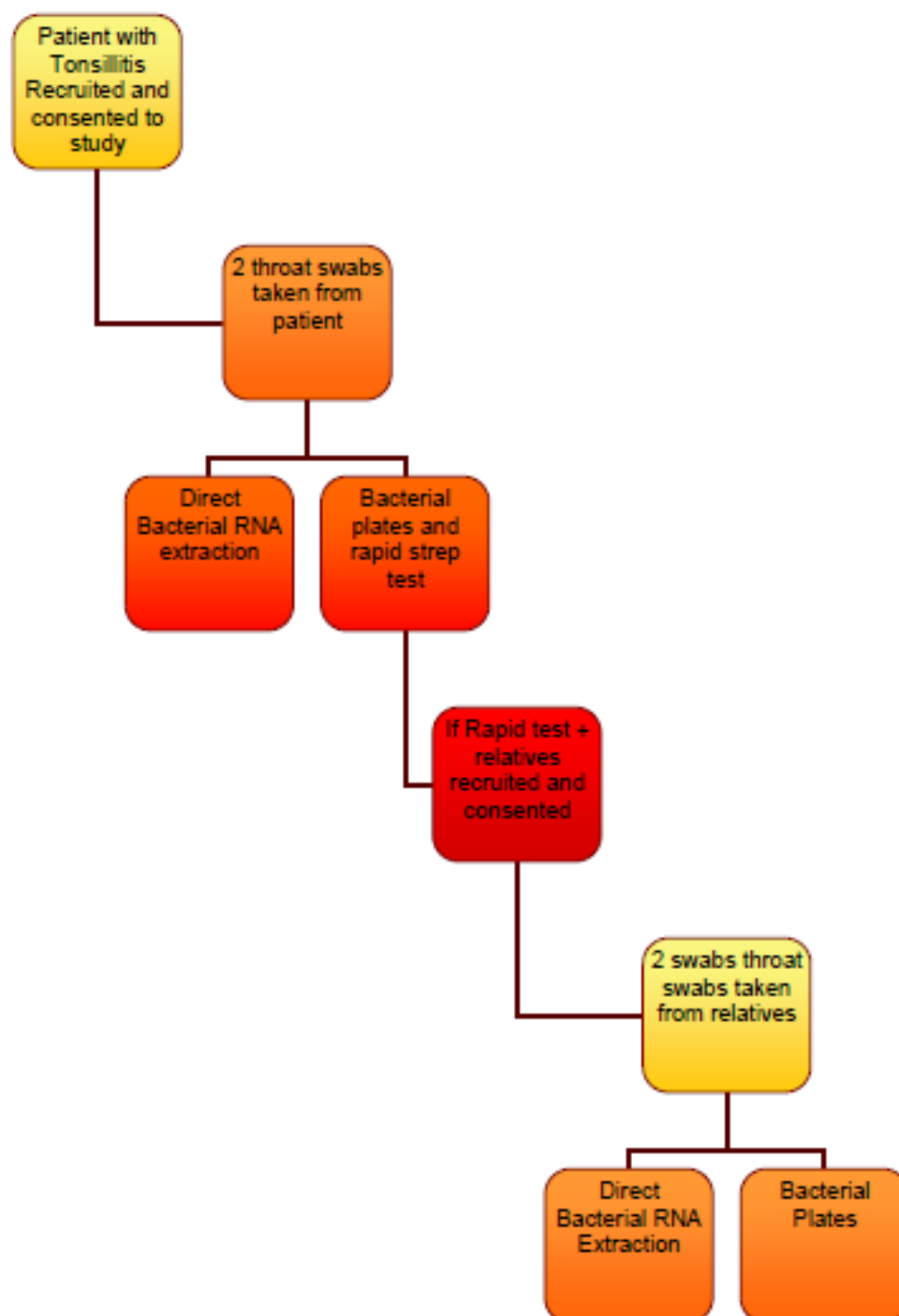
Study recruitment poster

Example of information sheets – child patient recruit

Example of consent form – child patient recruit

Healthy volunteer results sheet

Study flow diagram of protocol:



Virulence Factors in Streptococcal Tonsillitis Flow Diagram, Version 1
20080827, 09/H0708/46

Imperial College
London



Virulence Factors in Streptococcal Tonsillitis

Version 2, 28/10/2009

MAIN SPONSOR: Imperial College London

FUNDERS: Medical Research Council, Hammersmith Hospitals Trust Research Committee

STUDY COORDINATION CENTRE: Hammersmith Hospital, London

NRES reference: 09/H0708/46

Protocol authorised by:

Name & Role	Date	Signature
Frances Davies Chief Investigator	28/10/2009	

Study Management Group

Chief Investigator: Dr Frances Davies

Co-Investigators: Professor Shiranee Sriskandan, Dr Dayo Ajayi-Obe

Clinical Queries

Clinical queries should be directed to Dr Ajayi-Obe for children and Professor Sriskandan for adults, who will direct the query to the appropriate person.

Sponsor

Imperial College is the main research sponsor for this study. For further information regarding the sponsorship conditions, please contact the Research Governance Manager at:

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<http://www3.imperial.ac.uk/clinicalresearchgovernanceoffice>

Funder

Medical Research Council
Hammersmith Hospitals Trust Research Committee

This protocol describes the "Virulence Factors In Streptococcal Tonsillitis" study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

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GLOSSARY OF ABBREVIATIONS

[illegible]

KEYWORDS

Streptococcus pyogenes

Virulence factors

Tonsillitis

Children

STUDY SUMMARY

TITLE	Virulence Factors in Streptococcal Tonsillitis
DESIGN	1) Patient with tonsillitis is identified in outpatients or when admitted to a ward and briefly informed about the project 2) Research doctor called 3) Research doctor explains project to and obtains consent from patient and parent/legal guardian as appropriate. 4) 2 swabs taken from throat of infected patient by research doctor or clinical doctor. 5) One swab used to inoculate 3 bacterial culture plates: a) 2 plates given to hospital microbiology lab for routine processing, identified by patient name and hospital number. b) 1 plate for research lab, labelled by research number only. c) Same swab then used for rapid Group A streptococcal identification test. d) Second swab used for bacterial RNA extraction, labelled only with research number. Swab disposed of if both rapid test and plate culture are negative for Group A streptococcus. 6) Clinical doctor informed of rapid strep test result, to help make a treatment decision. 7) If the rapid test is positive, consent will be obtained from participating relatives, and 2 throat swabs taken from them: for bacterial culture (research lab only), rapid Group A streptococcal testing and bacterial RNA extraction. 8) Swabs from relatives will only be identified by research number, and will only be used for plates for research and bacterial RNA extraction. Relatives will be informed of their rapid test result, and given a form with this result to give to their GP should they become unwell. 9) Swabs for RNA processed in the research laboratory for production of virulence factors by real-time PCR. 10) At end of processing, all patient samples will be destroyed, and only bacteria and bacterial RNA retained for research use. No human material will be retained.
AIMS	To detect and measure the production of toxins by <i>Streptococcus pyogenes</i> bacteria in inflamed tonsils, compared to healthy carriers and the same bacteria grown in laboratory conditions.
OUTCOME MEASURES	1) Evidence of <i>S. pyogenes</i> by rapid screening methods from throat swabs, or by culture. 2) Bacterial virulence gene RNA transcripts as measured by real time RT-PCR
POPULATION	Children attending the paediatric emergency clinic with symptoms of tonsillitis, or adult patients admitted to the Hammersmith Hospital with tonsillitis.
ELIGIBILITY	Patient with confirmed streptococcal throat infection, and at least one healthy accompanying relative.

DURATION 2 years 1 month

1. INTRODUCTION

1.1 BACKGROUND

Streptococcus pyogenes causes 600 million cases of tonsillitis worldwide each year. It is known to produce a number of powerful toxins, including those known as superantigens, and the cell surface protein SpyCEP. The reasons for toxin production by *Streptococcus pyogenes* are completely unknown, but it seems likely they are made because of some advantage to the bacteria living in the throat. Previous research has shown that the toxins are made in invasive skin and blood infections, but the majority of infections with *S. pyogenes* occur in the throat. This research sets out to determine if the toxins are in fact made in the throat during tonsillitis, and will compare production with similar bacteria grown in different settings. It is important because firstly the cause of the symptoms of severe tonsillitis are poorly understood, and if these toxins play a role in causing tonsillar swelling, we may be able to develop tools to treat the swelling and infection in a more efficient manner. Secondly, Streptococcal tonsillitis is strongly associated with a serious immunological illness which can follow the infection, known as Rheumatic Fever, which can lead to severe heart disease. If superantigens are involved in causing tonsillitis, and are made during tonsillitis, then there is a strong chance that they are involved in triggering Rheumatic Fever. At present there is poor understanding of how Rheumatic Fever is triggered, and suppression of the acute illness and any relapses relies on the use of simple anti-inflammatory drugs, which are often not successful. If superantigen toxins are involved, then suppressive treatments should include those targeting the toxins themselves, which is not currently done.

1.2 RATIONALE FOR CURRENT STUDY

The purpose of this study is to detect and measure the production of toxins by *Streptococcus pyogenes* bacteria in inflamed tonsils, compared to bacterial growth in different laboratory conditions. We hypothesise that the expression of virulence factors will be increased in patients with acute tonsillitis compared to healthy carriers or bacterial growth under laboratory conditions, and are essential for causing streptococcal disease.

2. STUDY OBJECTIVES

This project aims to see if streptococcal toxins are responsible for the symptoms of acute tonsillitis – high fever and swollen tonsils. We plan to find out if these toxins are being made by the bacteria growing on the tonsils during tonsillitis, and if so how production differs from when the bacteria grow in other situations, such as when they are simply carried in the throat (as is often the case in family members) or grown in the laboratory.

This study will also show the prevalence of *Streptococcus pyogenes* as a cause of tonsillitis in the local population, and the carriage rates in family members of infected individuals. This will help assess the risk of spread of tonsillitis around the community and the risks to adults, who often carry the bacteria in their throat prior to developing invasive disease.

3. STUDY DESIGN

Study Design:

- This is an observational study, which aims to recruit between 10 and 15 patients with streptococcal tonsillitis, and at least 1 healthy relative for each patient, who are carrying the same bacteria in their throat.

- Cases will be selected from children attending the paediatric emergency clinic, with symptoms and signs suggestive of bacterial tonsillitis, and their accompanying relatives/guardians (collaborator Dr Ajayi-Obe, Consultant Paediatrician).
- Adult cases will be identified and recruited from patients admitted to hospital wards with tonsillitis
- Consent will be obtained from participating patients, and accompanying adults/guardians/relatives as appropriate.
- Two swabs will be taken from all participants in the study
- One swab will be used to grow bacteria on agar plates, the other swab will be used to directly extract bacterial RNA (reference 1).
- The bacterial plates from unwell patients will be cultured both in the diagnostic laboratory (allowing results to be reported onto the Trust pathology system) as well as anonymously in the research laboratory.
- All researchers taking swabs from patients will be trained doctors or nurses.
- All researchers inoculating bacteriology plates will be trained to the standard of a CPA approved microbiology laboratory.
- The Rapid Strep A detection test takes approximately 5 minutes to perform, can be done in the clinic where patients are being seen, and will help clinicians to make an early decision regarding antibiotic therapy, as well as allowing the research staff to proceed with collecting samples from relatives.
- A Rapid Strep A test will also be performed on the samples from relatives, and they will be informed of this result, in-case they become unwell at a later date. However, asymptomatic carriage does not warrant treatment (reference 2), so healthy volunteers will not be prescribed treatment if the rapid test is positive for Group A streptococcus.

Study Methods:

1. Two throat swabs will be taken from participating patients.
2. One swab will be used to create three bacterial plates: two of which will be delivered to the diagnostic laboratory for standard processing, the other will be anonymised for processing in the research laboratory.
 - This swab will then be used to perform a Clearview Exact Strep A rapid detection test, and the results will be written in the patients hospital notes.
3. The second swab will be anonymised and bacterial RNA immediately extracted from the swab (reference 1).
4. If a patient has a positive rapid test result, we will take two throat swabs from participating relatives.
5. These swabs will be anonymised and processed for bacterial culture (research laboratory only), rapid Group A Streptococcal testing and direct bacterial RNA extraction as above.
6. The amount of toxin production by the bacteria will then be compared in the research laboratory, between bacteria grown on a plate and the RNA extracted from the swab, and between patients with tonsillitis and healthy relatives.
7. No human tissue will be retained, and all research samples will be anonymised.

3.1 STUDY OUTCOME MEASURES

- 1) Evidence of *S. pyogenes* by rapid screening methods from throat swabs, or by culture
- 2) Bacterial virulence gene RNA transcripts as measured by real time RT-PCR

4. PARTICIPANT ENTRY

4.1 PRE-REGISTRATION EVALUATIONS

Patients will have symptoms and signs suggestive of bacterial tonsillitis. The initial throat swab will be performed in clinic, and tested by a Rapid Strep A test to confirm eligibility for the study.

4.2 INCLUSION CRITERIA

- Clinical diagnosis of tonsillitis as the reason for attending the clinic in index patients (condition to be studied)
- Positive group A streptococcus rapid test throat swab (to avoid unnecessary samples being collected from participating relatives if screening test is negative)
- Participating relatives who do not have symptoms or signs suggestive of active bacterial pharyngitis / tonsillitis.

4.3 EXCLUSION CRITERIA

- Patients who do not have tonsillitis as their primary reason for attending the clinic
- Patients who have received any antibiotics since the onset of the symptoms of this clinical episode (as this is likely to render the results invalid)
- Patients who do not have at least 1 accompanying relative
- Relatives who have symptoms of a sore throat or tonsillitis, as being asymptomatic is part of their inclusion criteria
- No exclusions will be made on the grounds of disability, race, sex, religion or sexual orientation

4.4 WITHDRAWAL CRITERIA

Study participants may withdraw from the study at any time, by contacting the principal investigator. Details for contact are provided on the participant information sheets.

5. ADVERSE EVENTS

5.1 DEFINITIONS

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- Results in death
- Is life-threatening – refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- Requires hospitalisation, or prolongation of existing inpatients' hospitalisation
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

5.3 REPORTING PROCEDURES

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

5.3.1 Non serious AEs

All such events, whether expected or not, should be recorded.

5.3.2 Serious AEs

An SAE form should be completed and faxed to the Chief Investigator within 24 hours. However, relapse and death due to Streptococcal disease, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the Brompton, Harefield and NHLI Research and Ethics Committee where in the opinion of the Chief Investigator, the event was:

- 'related', ie resulted from the administration of any of the research procedures; and
- 'unexpected', ie an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies.

Local Investigators should report any SAEs as required by their Local Research Ethics Committee and/or Research & Development Office. All SAEs will be reported by the Chief Investigator to the Clinical Research Governance Office as sponsor.

Contact details for reporting SAEs
Fax: 0208 3833394, attention Dr Frances Davies
Please send SAE forms to: Department of Infectious Diseases, 8th floor Commonwealth Building, Hammersmith Hospital, Du Cane Road, London, W12 0NN.
Tel: 0208 3832065 (Mon to Fri 09.00 – 17.00)

6. ASSESSMENT AND FOLLOW-UP

Clinical management decisions and follow-up of all patients will be provided by the attending clinician. There will be no follow-up arranged by the research team, and all follow-up will be arranged as necessary by their clinician. Advice for healthy relatives is provided on the information sheet about where to seek medical help, should they become unwell subsequent to participating in the study.

7. STATISTICS AND DATA ANALYSIS

This pilot study aims to recruit 10-15 patients with tonsillitis, and one or more healthy relative from each. This was estimated from the number of patients likely to be seen within the study period.

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study, including the follow-up period.

8. REGULATORY ISSUES

8.1 ETHICS APPROVAL

The Chief Investigator has been given a favourable opinion from the Brompton, Harefield and NHLI Research Ethics Committee. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the SSA approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

8.2 CONSENT

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best

interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

8.3 CONFIDENTIALITY

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

8.4 INDEMNITY

Imperial College London holds negligent harm and non-negligent harm Insurance policies which apply to this study.

8.5 SPONSOR

Imperial College London will act as the main sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

8.6 FUNDING

The Medical Research Council and the Hammersmith Hospitals Trust Research Committee are funding this study. There are no participant or investigator payments for this study.

8.7 AUDITS

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

9. STUDY MANAGEMENT

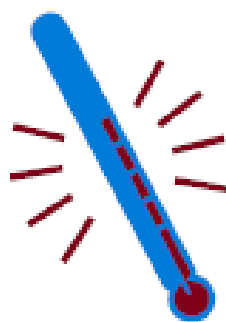
The day-to-day management of the study will be co-ordinated by Dr Frances Davies.

10. PUBLICATION POLICY

Any publications of the results from this study will be published in peer reviewed journals.

11. REFERENCES

- 1) Viriáneva K, Graham MR, Porcella SF, Hoe NP, Su H, Graviss EA, Gardner TJ, Allison JE, Lemon WJ, Bailey JR, Parnell MJ, Musser JM. Group A Streptococcus gene expression in humans and cynomolgus macaques with acute pharyngitis. *Infect Immun*. 2003 71(4):2199–2207
- 2) Bisno AL, Gerber MA, Gwaltney JM, Kaplan EL, Schwartz RH. Practice Guidelines for the diagnosis and management of Group A Streptococcal Pharyngitis. *Clin Infect Dis* 2002; 35: 113–125
- 3) Danchin MH, Rogers S, Kelpie L, Selvaraj G, Curtis N, Carlin JB, Nolan TM, Carapetis JR. Burden of acute sore throat and Group A Streptococcal pharyngitis in school-age children and their families in Australia. *Paediatrics* 2007; 120: 950–957
- 4) Unnikrishnan M, Altmann D, Proft T, Wahid F, Cohen J, Fraser J, Sriskandan S. The streptococcal superantigen SMEZ is the major immunoreactive agent of Streptococcus pyogenes. *J. Immunology*. 2002; 169: 2561–2569



Fever?



Entry
Criteria

Sore Throat? Big Tonsils?

We are doing a study to try to find out why some people get tonsillitis but others do not.

It involves having a throat swab taken while you are here.

If you have a sore throat and if you and a relative here with you are willing to get involved in the study please let your doctor or nurse know.

Recruitment poster, Version 3 20091019, 09/H0708/46
This study has been given a favourable opinion by the Brompton,
Harefield and NHLI Research Ethics Committee

Child patient information sheet:

What's in it for me?

If you take part in the study, a quick test will be done on your swab to see if you have the Group A strep bug. This takes about 5 minutes and can help the doctors decide how to treat you. This test is not usually available in the clinic.

What are the risks?

You may need to cough when the swab is taken, but it does not hurt. If you take part in the study you will have 2 swabs done. Normally you would only have one, or none.

Will other people know I have taken part in the research?

- The doctor who has seen you today will be told that you have decided to take part. They will be given the result of the quick test, so it can help them to plan your treatment.
- A copy of the consent form which you have signed will be kept in your medical notes.
- The only person from the research team who will know you have agreed to take part is the research doctor you have seen today.
- Any information which goes to the research lab will not contain your name, address or hospital number, and it will not be possible to identify you from it.
- A representative from Imperial College or the hospital may wish to look at your hospital notes to check the research is being conducted correctly.

What do I do if I change my mind and don't want to do the research any more?

Tell the adult who is with you today, and they will tell the research doctor that you no longer want to do the research. Any research samples which have been taken from you will be destroyed. You will still be treated in the same way by your doctor.

What happens to the results of the research?

When the study has finished, the results will be written about and published in a medical paper. Important results may be presented at a conference. Your details will not be published.



What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Dr Frances Davies, Contact no. below). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Governance Office.

Thankyou for reading this.

This study has been reviewed by the Brompton, Harefield and NHLI Research and Ethics committee, reference 09/H0708/46. Funding provided by the Medical Research Council and Hammersmith Hospitals Trust Research Committee.

Dr Frances Davies
Imperial College London
Department of Infectious Diseases
8th Floor Commonwealth Building
Hammersmith Hospital
Du Cane Road
London W12 0NN
Phone: 0208 3832065

ChildPatient Version 4/20090303

Dr Frances Davies
Imperial College
London
Virulence Factors in
Streptococcal Tonsillitis

Sore Throat Study



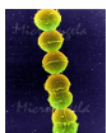
Patient information sheet

Imperial College
London
Tel: 0208 3832065



You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take your time to read the information in this leaflet carefully and discuss it with others if you wish.

What is the Sore Throat Study?



A picture of Group A Strep.

We are investigating the bugs that cause tonsillitis. These bugs are called Group A strep. We want to see what toxins they are making at the time they cause infection.

Dr Frances Davies
Imperial College London
Department of Infectious Diseases
8th Floor Commonwealth Building
Hammersmith Hospital
Du Cane Road
London W12 0NN
Phone: 0208 3832065

What is tonsillitis?

Tonsillitis is an infection of the throat. People with tonsillitis have a high temperature, sore throat, swollen red lumps at the back of the throat (tonsils) and sometimes a red rash on the skin. Some people with tonsillitis feel very unwell, and it can be difficult to swallow food. Tonsillitis sometimes needs to be treated with antibiotics, and some people have tonsillitis lots of times.

What are Toxins?

Toxins are small natural poisons made by living cells. Toxins are able to cause disease, which can range from mild to severe.

Why is this research important?

We do not know why some people can have Group A strep in their throat without any illness, while other people develop tonsillitis. We know that the bug can make toxins, and we are trying to work out how important these toxins are in making people feel ill. Hopefully this will one day mean that we can make the treatment of tonsillitis better.

Why have I been asked to take part?

We are asking you to take part in this study because the doctor or nurse who has seen you today thinks you may have tonsillitis caused by the Group A strep bug.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you can still change your mind at any time and without giving a reason. If you change your mind and don't want to take part any more, this will not change the standard of care you get given.

If I want to take part, what do I do next?

If you want to take part, tell the adult who is with you today, and they will tell the research doctor.

- We will then take 2 swabs from the back of your throat. This is a simple test which does not hurt, though it may make you want to cough.
- We will do a quick test on one of the swabs to see if you have the Group A strep bug in your throat.
- We will give this result to your doctor, who may want to give you some medicine.
- We might also ask your family and friends to have a throat swab taken to see if they "carry" the bug in their throats but it is not making them ill.
- We will then take all of the swabs to the lab and test them to see how the bugs grow, and what toxins they are making.



Virulence Factors in Streptococcal tonsillitis. Consent Form.

Trial Identification Number:

Name of Researcher:

Please Initial

1. I confirm that I have read and understand the information sheet for this study. I have had time to think about the information, ask questions and have had these answered satisfactorily.

☐

2. I understand that my participation is voluntary and that I can withdraw at any time without giving any reason, without my medical care or legal rights being affected.

☐

3. I give permission for responsible people from Imperial College or regulatory authorities to access my records that are relevant to this research.

☐

4. I agree to take part in the above study.

☐

Name of Patient	Date	Signature
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Name of Guardian	Date	Signature
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Name of Person taking consent	Date	Signature
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When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Healthy volunteer rapid test result sheet:



Virulence Factors in Streptococcal Tonsillitis Study

Volunteer Result Sheet

This form is to be handed to your doctor in the event of illness following participation in the study.

Dear Doctor

This is to confirm thathas been recruited into this study on(date) as a healthy volunteer, and has been screened for throat carriage of Group A Streptococcus.

People recruited to this study have been identified as close contacts of a confirmed case of Group A Streptococcal throat infection, and so are more likely to develop an infection with Group A Strep, which may require antibiotic therapy. Volunteers with a positive screening result have not been treated, as they are asymptomatic at the time of recruitment to the study. Volunteers with a negative result are still at risk of developing Group A Strep disease.

A Clearview Rapid A Strep test was performed today and the result was:

POSITIVE ☐

NEGATIVE ☐

Thank you for taking this result into consideration in your review of this person. Should you require more information please do not hesitate to contact the study doctor.

Yours Sincerely

Dr Frances Davies

Dr Frances Davies
Clinical Research Fellow

Department of Infectious Diseases
Office: 0208 3832063

Volunteer results sheet
Version1 20091008
09/H0708/46

Appendix 2

Table 11: Characteristics of tonsil donors

ID Number	Age	Sex	Clinical diagnosis	Histopathology diagnosis
702241	26	M	Infection occurs every 2 months, requires antibiotics	Reactive follicular hyperplasia, Actinomyces present
702242	31	F	Tonsillitis 4x/year, requiring antibiotics	Reactive follicular hyperplasia, Actinomyces present
702250	25	F	Frequent tonsillitis with pus	Reactive lymphoid hyperplasia.
702251	27	M	Sore throats and fevers	Reactive lymphoid hyperplasia Actinomyces present
702252	24	F	Nasal obstruction, recurrent and frequent sore throats	Reactive lymphoid hyperplasia Actinomyces present
702270	20	F	Recurrent tonsillitis	Reactive lymphoid hyperplasia.
702272	36	M	To reduce snoring	Reactive lymphoid hyperplasia Actinomyces present
702283	22	F	Recurrent tonsillitis	Acute-on-chronic inflammation, Actinomyces and abscesses present
702317	23	F	Recurrent tonsillitis, 5 episodes in 6 months	Follicular hyperplasia
702326	40	F	Recurrent tonsillitis, 3-4 episodes/year for many years	Reactive lymphoid hyperplasia Actinomyces present
702327	28	F	Recurrent tonsillitis, almost monthly sore throat	Reactive lymphoid hyperplasia.
702329	31	F	Sore throat for 4 weeks, acute onset	Reactive lymphoid hyperplasia Actinomyces present
702333	29	F	L sided quinsy, recurrent sore throats/tonsillitis	No evidence of malignancy
702346	42	F	Food/debris lodged on tonsil, sore throats, halitosis	Reactive lymphoid hyperplasia Actinomyces present
702368	26	F	Chronic fatigue, recurrent sore throats every 2 weeks	Reactive lymphoid hyperplasia Actinomyces present
702369	32	F	Recurrent tonsillitis more that 5x/year, sever episodes	Reactive lymphoid hyperplasia.
702393	26	F	Sore throat, 1/month	Reactive follicular hyperplasia
802019	36	M	Post nasal drip, chronic fatigue, sore throat, grade 1 tonsils, concerned about reactive arthritis	Reactive lymphoid hyperplasia Actinomyces present
802018	24	F	Recurrent tonsillitis, 8x in last year, grade 3 tonsils	Reactive lymphoid hyperplasia.

802043	35	F		Reactive lymphoid hyperplasia Actinomyces present
802078	34	F		Follicular lymphoid hyperplasia, no malignancy
802079	32	F	Sore throats, mostly right side	Follicular lymphoid hyperplasia, no malignancy
802094	34	F		Reactive lymphoid hyperplasia Actinomyces present
802116	25	F	L. Sided quinsy, recurrent sore throats	
802117	26	F	Recurrent tonsillitis >10 time in last year	
802118	19	F	Sore throat, every month	
802126	39	M	L sided quinsy	
802127	17	F	Sore throats every 2 months, cervical lymphadenopathy, grade 3 tonsils	
802128	19	F		
802129	25	F	Recurrent tonsillitis	
802170	34	M	Sleep disorder – sleep apnoea	
802184	18	F	Chronic disease of tonsils and adenoids – chronic tonsillitis.	
802201	37	M	Chronic tonsillitis, thyrotoxicosis and goitre, asthma	
802202	29	F	Chronic tonsillitis.	
802215	22	F	Chronic tonsillitis	
802220	25	F	Chronic tonsillitis.	
802283	24	M	Hypertrophy of tonsils.	
902030	36	F	Acute tonsillitis – streptococcal tonsillitis.	
902031	32	F	Chronic tonsillitis, Diabetes	
902119	41	F	Chronic tonsillitis, rhinitis, and pharyngitis	
902120	30	F	Chronic tonsillitis.	
902121	27	F	Chronic tonsillitis.	
902135	37	M	Chronic tonsillitis.	
902136	28	F	Chronic tonsillitis, penicillin allergic	
902141	29	F	Chronic Tonsillitis	
902142	46	F	Chronic Tonsillitis	
902175	59	M	Acute tons, COPD, sleep apnoea. Hypertrophy of tonsils.	
902176			Acute tonsillitis,	

			Dysphagia	
902191	25	F	Acute tonsillitis	
902201	19	F	Hypertrophy of Tonsils	
902202	34	F	Chronic tonsillitis	
102012	27	F	Chronic disease of tonsils, chronic rhinitis	
102013	26	F	Chronic tonsillitis, Hyperhidrosis	
102053	30	F	Acute tonsillitis	
102054	20	F	Acute tonsillitis	
102065	17	F	Acute tonsillitis	
102066	37	F	Acute tonsillitis	
102067	32	F	Chronic tonsillitis	
102090	24	M	Chronic tonsillitis	
102102	48	F	Acute tonsillitis	
102120				
102121				
102128	42	M		
102129	25	F		