INVESTIGATING THE SEROLOGICAL BACKGROUND OF NECROTIZING SOFT TISSUE INFECTIONS

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Table of Contents

List of Figures

Introduction

Materials and Methods

Results

List of Tables

Materials and Methods

Results

Abbreviations

Abbreviation	Full name
%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromole
3’	Three prime end
5’	Five prime end
A	Adenine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
APS	Ammonium persulfate
aq.	Aqueous
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cfu	Colony forming units
CO_2	Carbon dioxide
CRP	C-reactive Protein
dH_2O	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate

Abbreviation	Full name
DTT	Dithiothreitol
E. coli	*Escherichia coli*
e.g.	*Exempli gratia* (latin ‘for example’)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
G	Guanine
g	Gram
GAS	Group A *Streptococcus*
h	Hour
HA	Hyaluronic acid
Hyl	Hyaluronidase
IPTG	Isopropyl-β-d-thiogalactopyranoside
IVIG	Intravenous Immunoglobulins
kb	Kilobase
L	Litre
LB	Luria bertani
LTA	Lipoteichoic acid
M	Molarity
MCS	Multiple cloning site
mg	Milli gram
MHC	Major histocompatibility complex
min	Minute(s)
ml	Milli liter

Abbreviation	Full name
mm	Milli meter
mM	Milli mole
MOI	Multiplicity of Infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MQ	Milli-Q water
MW	Molecular weight
n.s.	Non-significant
NETs	Neutrophil extracellular traps
NF	Necrotizing Fasciitis
NSTI	Necrotizing Soft Tissue Infections
OD_{600}	Optical density at 600 nm
p value	Probability value
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween-20
PCR	Polymerase chain reaction
PMNs	Polymorphonuclear leukocytes
RNase	Ribonuclease
rpm	Rotations per minute
S. pyogenes	*Streptococcus pyogenes*
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDSE	*Streptococcus dysgalactiae* subspecies *equisimilis*

Abbreviation	Full name
sec	Second(s)
SfbI	Streptococcal fibronectin binding protein I
sic	Streptococcal inhibitor of complement-mediated lysis
SLO	Streptolysin O
SLS	Streptolysin S
Spe	Streptococcal pyrogenic exotoxin
T	Thymine
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with tween-20
TCR	T cell receptor
TE	Tris/EDTA
TEMED	N,N,N,N-tetramethylethylenediamine
THY	Todd Hewitt broth with yeast extract
™	Trademark
U	Units
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

1a. Abstract

Necrotizing Soft Tissue Infection (NSTI) is a fulminant disease of the deeper layers of skin and subcutaneous tissue that is mostly caused by the gram-positive pathogen *Streptococcus pyogenes.* Due to the aggressive and fast development, NSTIs require intensive care with rigorous medication, ablative surgery and adjunctive therapies like intravenous immunoglobulin administration (IVIG). Considering the discussed functional effects of an IVIG treatment, it was hypothesized that a lack of protective antibodies against the causative bacterial pathogen and its specific spectrum of secreted toxins represents a risk factor towards the development of a severe NSTI.

To address the role of bacterial and exotoxin-specific antibody titers during the NSTI pathogenesis, a serologic approach was pursued using plasma samples and bacterial isolates of clinical NSTI cases and non NSTI control cases of severe skin and tissue infections that did not develop a necrotic stage, collected during the early and late stage of infection. The antibody mediated protection potential of the collected plasma samples against the corresponding bacterial pathogens was measured by an established Opsono-Phagocytosis assay. It was obvious that the adaptive immune system of all analysed NSTI patients was not able to provide efficient protection against the corresponding bacterial isolate during the early stage of infection, although an applied IVIG treatment was able to compensate this susceptibility. This was in contrast to the results obtained with the non NSTI control cases wherein all plasma samples mediated effective opsonisation and bacterial killing. The analysis of the patient plasma samples towards antibody mediated protection against pathogen specific exotoxin sets identified by a multiplex PCR screening was addressed with an established ELISA system in combination with specialized functional assays. This approach not only confirmed the crucial role of the adaptive immune system during the confrontation with *S. pyogenes,* it also demonstrated the potential of an IVIG treatment to compensate this stage of immunologic susceptibility.

The presented data clearly illustrate that a lack of protective antibodies against the invading bacterial pathogen and its specific set of secreted toxins represents a significant risk factor for the development of a severe NSTI. Additionally, it could be shown that the applied IVIG treatment is able to compensate this serologic susceptibility. Consequently, the presented work not only provided a valuable insight into the complex interactions during the pathogenesis of an NSTI, it also represents a first step towards the development of a specialized, more targeted treatment strategy.

1b. Zusammenfassung

Nekrotisierende Weichgewebeinfektionen (NSTIs) sind schwere Entzündungen der tieferen Schichten der Haut und des subkutanen Gewebes meist hervorgerufen durch den Gram-positiven Erreger *Streptococcus pyogenes*. NSTIs erfordern eine aggressive medizinische Behandlung mit erheblicher Medikamentierung, ablativer Chirurgie und dem Einsatz spezialisierter Therapieformen wie der intravenösen Verabreichung von Immunglobulinen (IVIG). Unter Berücksichtigung der beschriebenen funktionellen Wirkung einer IVIG-Behandlung wurde die Hypothese aufgestellt, dass ein Mangel an protektiven Antikörpern gegen den ursächlichen bakteriellen Erreger und das ihm inhärente Spektrum sekretierter Toxine einen Risikofaktor darstellt, der zur Entwicklung einer schweren NSTI beiträgt.

Um die Rolle von bakterien- und exotoxinspezifischen Antikörpern während der Pathogenese einer NSTI zu untersuchen, wurde eine umfassende serologische Studie durchgeführt, bei der Plasmaproben und bakterielle Isolate sowohl klinischer NSTI Patienten, als auch einer Kontrollgruppe von Patienten mit schweren Haut- und Gewebeinfektionen, die sich nicht zu einer NSTI entwickelten, analysiert. Die Plasmaproben wurden mit Hilfe von Opsono-Phagozytose-Experimenten auf ihr Potential untersucht, die spezifischen bakteriellen Erreger mit Hilfe von Antikörpern für eine effektive Immunantwort zu markieren. Dabei konnte gezeigt werden, dass das adaptive Immunsystem aller analysierten NSTI Patienten nicht imstande war, einen wirksamen Schutz gegen das jeweilige Bakterienisolat während der frühen Phase der Infektion zu gewährleisten, wobei eine IVIG-Behandlung diesen Mangel kompensieren konnte. Demgegenüber waren die Antikörper in den Plasmaproben der Kontrollgruppe in der Lage, eine effektive Opsonisierung der Bakterien zu vermitteln. Die Analyse der Plasmaproben in Hinsicht auf einen antikörpervermittelten Schutz gegen erregerspezifische Exotoxinkombinationen, die durch eine Multiplex-PCR Analyse identifiziert wurden, konnte mit Hilfe eines etablierten ELISA-Systems in Verbindung mit spezialisierten funktionellen Experimenten adressiert werden. Dieser Ansatz bestätigte die entscheidende Rolle des adaptiven Immunsystems während der Konfrontation mit dem Erreger *S. pyogenes* und demonstrierte das Potenzial einer IVIG-Behandlung, den beobachteten Mangel an schützenden Antikörpern erfolgreich zu kompensieren.

Die vorgestellten Daten zeigen, dass ein Mangel an protektiven Antikörpern gegen den bakteriellen Erreger und sein Spektrum an Toxinen einen bedeutenden Risikofaktor für die Entwicklung einer schweren NSTI darstellt und dass die verabreichte IVIG-Behandlung in der Lage ist, diesen Risikofaktor zu kompensieren. Damit trägt die vorliegende Arbeit nicht nur zum Verständnis der komplexen Wechselwirkungen bei der Pathogenese einer NSTI bei, sondern stellt auch einen ersten Schritt bei der Entwicklung einer spezialisierten, gezielteren Behandlungsstrategie für nekrotisierende Weichgewebeinfektionen dar.

2. Introduction

2.1 Necrotizing Soft Tissue Infections (NSTI)

Necrotizing Soft Tissue Infections like Necrotizing Fasciitis (NF) are life-threatening infections of the deeper layers of skin and subcutaneous tissue, spreading across the fascia[1]. These infections were first reported by Jones in 1871 and were named as 'Hospital gangrene' at that time. Later in 1951, Wilson observed inflammation and necrosis of the subcutaneous tissue and fascia with and without cutaneous gangrene and introduced the term 'Necrotizing Fasciitis' for such infections[2]. Even though it can impinge any part of the body, it is most commonly seen in the lower extremities[3]. Generally, cases of NSTI without prompt diagnosis may lead to 100% mortality, therefore, early diagnosis, along with aggressive surgical treatment, are critical factors for a reduction of the mortality rate. Unfortunately, it is often difficult to diagnose NF due to its close similarities with cellulitis during early days of infection. Patients are erroneously treated for cellulitis until the site of infection turns black, becomes necrotic and starts to degrade rapidly[4]. Antibiotic therapy and early surgical debridement are mandatory for ensuring a decreased mortality rate; that generally varies from 25-35% but can even go as high as 73%[5]. NSTI is thought to be associated with some risk factors including diabetes, chronic diseases, immunosuppressive drugs, malnutrition, age more than 60 years, intravenous drug misuse, peripheral vascular disease, renal failure, underlying malignancy and Obesity, but also with small wound infections or trauma[4]. Various pathogens have been associated with Necrotizing Fasciitis and thus NF can be either a poly-microbial or mono-microbial infection (2.1.1).

2.1.1 Pathogens associated with Necrotizing fasciitis

NF is not associated to a specific pathogen, rather can be caused by a wide spectrum of aerobic, anaerobic or mixed flora. Due to this diverse etiology, NF can be classified into three distinct types: Type I, II and III. A Type I infection of NF refers to poly-microbial infections as it is caused by a combination of anaerobic bacteria and facultative anaerobic bacteria, such as *enterobactericeae* and *streptococci* other than group A. In almost all cases of Type I NF, one anaerobic organism was cultured in combination with a facultative anaerobe. Type II are mono microbial infections largely dominated by *Streptococcus pyogenes* (Group A *Streptococcus*), therefore this type is also known as 'Streptococcal gangrene'. In some cases *Staphylococcus aureus*

can also be responsible for a Type II NF. Lastly, Type III is known as 'gas gangrene', due to the production of gas at the site of infection as a result of glucose fermentation[6]. It is generally associated with an infection of *Clostridium* species; especially by *Clostridium myonecrosis*, *Clostridium perfringens*, *Clostridium septicum* and *Clostridium novyi.* Irrespective of the pathogen causing NF, there are some common clinical manifestations. (2.1.2)

2.1.2 Clinical Manifestations of Necrotizing fasciitis

Necrotizing Fasciitis is a rapidly progressing insidious disease and is therefore difficult to diagnose. The mortality rate is directly dependent on the time spent for the identification of NF. The longer is the time frame, the higher is the predicted mortality rate. Hence, early diagnosis is a crucial factor in reducing the mortality rate of NF. As the disease progresses, distinct signs and symptom of the disease appear. Initial phase (Approximately day 1-2) is generally demarcated by local pain, swelling and erythema. At this stage, it mimics closely to cellulitis but the necrotizing infection is located in deeper skin layers therefore difficult to recognize. Unlike cellulitis, the margins of infection are poorly defined, with tenderness extending beyond the apparent area of involvement. On day 2-4, the infected area develops tense edema, extending beyond the margin of erythema. The affected skin area becomes dis-colored, progressing to grey/black necrotized skin to tear off (skin abrasion and avulsion of the sub cutaneous region). Pain sensation may progress from intense tenderness to anesthesia as the nerves are destroyed. If there is an open wound, probing the edges with a blunt instrument produces easy dissection of the superficial fascial planes well beyond the wound margins. Till day 4-5, hypotension and toxic shock develops and the patient becomes confused and apathetic.

2.1.3 Diagnostics of necrotizing fasciitis

Establishment of fast diagnosis for NF is not easily possible, since it resembles closely to cellulitis during early days. Resultantly, a delayed identification lead to a dramatic increase in the prognosticated mortality rate. Therefore, a proper test is essential for its confirmation and thence treatment. Unfortunately, there is no definitive test available and therefore, surgery is requisite to confirm or exclude the possibility of NF. However, initial standard clinical tests are performed for diagnosing NSTI as mentioned below.

- Blood tests: Usually, patients with NF infections show abnormal blood chemistry due to systemic effects of infection. As there is excessive involvement of inflammatory responses, concentration of C-reactive protein (CRP) (>16 mg/ml) and White blood cell count (>15.4 x 10^9/L) are elevated in NSTI patients. In addition, blood samples may show acidosis, leukocytosis, altered coagulation profile, hypo-albuminaemia, and abnormal renal function[7]. Other changes in blood parameters, such as reduced serum sodium (<135 mmol/L), raised CK level (> 600 U/L) and urea levels (>18 mg/dL) can also be observed.

- Bedside finger test: Surgical interventions to explore NSTI affected soft tissue is considered to be a reliable option by the physicians and should be performed as soon as possible. For this test, local anesthesia is executed and an incision of 2 cm is made into the deep fascia. Gentle probing with the index finger is performed to screen for any signs of NF. Some of the signs include lack of bleeding, malodorous "dishwater pus", and lack of normal tissue resistance to blunt finger dissection. Alternative procedures are taking a bedside incisional biopsy of the affected fascia and perform cryo-sectioning and Gram staining.

- Microbiology: Includes blood culturing, wound swabs, Gram staining and culture of affected tissues (from surgery or biopsy). Fungal culturing is important in immunocompromised and trauma patients. Since classical microbiological examinations take few days for confirmation for NSTI and its causative pathogen, it generally delays the targeted therapeutic interventions, thereby increasing the prognosticated mortality rate.

- Radiology: Plain X-ray or Computed tomography (CT) scan is able show accumulation of soft tissue gas[8]. Magnetic resonance imaging (MRI) scans can show the extent of tissue involvement[9], but may not be accurate and should not delay a surgery. Similarly, Ultrasound has also been used to show subcutaneous gas[10]. However, there are many possibilities of false positives and therefore Radiological techniques are not the first/preferred choice of Physicians in initial diagnostics of NF.

- Other tests: Tissue oxygen saturation measured by near-infrared spectroscopy can be used as a bedside test. A cut off value of tissue oxygen saturation reading of less than 70% is confirmation of NSTI[11].

The development of an NSTI is interplay between the host immune system and the invading pathogen. Delays in the identification can significantly increase the prognosticated mortality rate. To identify risk factors for the disease development, it is important to understand the role of the host immune system and the mechanisms through which the pathogen counteracts it. It is a crucial step for the development of specific diagnostics and therapeutics.

2.2 The role of the immune system during necrotizing fasciitis

The human immune system comprises an enormous variety of cells and molecules that specifically recognize and eliminate foreign invaders. These cells and molecules act together in a dynamic network for successful elimination of pathogens. Functionally, the immune system can be divided into two major but related activities: *Recognition* and *Response*. Recognition by the immune system is performed by exquisite chemical differences that distinguish self from non-self and thus identifies an invading pathogen. Once it is recognized as a foreign, the immune system recruits 'always ready and available' cells and molecules that escalate an appropriate response, called an effector response, to eliminate or neutralize the foreign organism (*Innate Immune System*). If the pathogen eludes the capture by the innate immune system, or if there is an antigenic confrontation, the second part, called the *Adaptive Immune System*, is activated that constitutes a set of specialized cells recruited which identifies the pathogen, eliminate it and retains memory of the encounter for future (Memory Response). Later exposure to the same foreign organism induces a memory response that is described as more rapid and heightened immune reaction to eliminate the pathogen. In this way, the immune system is able to convert the initial recognition event into a variety of effector responses, each one being exclusively suited for eliminating a particular type of pathogen.

2.2.1 Functionality of the innate immune system during Necrotizing Fasciitis

The innate immune system represents the first line of defense against invading organisms as most components of it are present before the onset of infection and constitute a set of disease-resistance mechanisms that are not specific to a particular pathogen but include cellular and molecular components that recognize classes of molecules peculiar to frequently encountered pathogens. For a proper functioning of the immune system, it requires three sets of components: A *recognition* system to identify the pathogen, a *disposal* system to kill/eliminate the pathogen and a

communication system to coordinate activities of various recognition and disposal elements.

Generally, the *Physical and Anatomical Barriers* of the body (like the skin) act as a very effective obstruction against invasion by most pathogens. However, pathogens like *Streptococcus pyogenes* can enter the host generally through wounds/ burns/ scratches or abrasions that disrupt the continuity of the skin. Once the physical barriers are broken and *S. pyogenes* successfully enters the host, the *Physiological barriers* contribute in its recognition and elimination through various soluble and cell associated molecules. Many of these molecules of the innate immune system have the property of **Pattern Recognition,** the ability to recognize a given class of molecules. Because some surface molecules are unique to *S. pyogenes* that are not present on the surface of the host cells, the innate immune system is able to immediately recognize and combat the invader. Molecules of Pattern recognition could be soluble, like the complement system (described later) or cell surface receptors, for example, **Toll like Receptors** (TLRs). TLRs are single, membrane spanning, non-catalytic receptors, usually localized on the surface of cells of innate immune system (like macrophages or polymorpho-nuclear cells, PMNs) that detect the presence of microbial pathogens via the conserved microbial structures called pathogen-associated molecular patterns (PAMPs)[12]. TLRs can be differentiated into distinct types, based on their location and recognizing target, including TLR1-13, although TLR 11-13 are not described in humans. Different TLRs respond to different PAMPs, for example, TLR2 functions as a receptor for lipopolysaccharide (LPS), an integral component of the outer membranes of gram-negative bacteria[13] and for Lipoteichoic acid (LTA), a major constituent of the cell wall of gram positive bacteria like *S. pyogenes*. The exact mechanistic action of TLRs in the recognition of S. *pyogenes* is not known, however some studies in mice suggest that *S. pyogenes* is initially recognized by TLR2 and subsequently by TLR13. However, phylogenetic analyses revealed that TLR13 occurs only in few mammals, including mice and rats, which are naturally resistant against *S. pyogenes*[14]. Hence, the presence of TLR13 in mice and not in humans has functional consequences for the recognition of *S. pyogenes* in these organisms. The interaction of TLRs with their target motifs initiates a cascade of intracellular signaling events that results in the production of proinflammatory cytokines (TNF-α, IL-l, IL-8), chemokines, and costimulatory molecules that are important to coordinate the host defenses[12]. These molecules attract neutrophils and macrophages, the main participants of the innate immune response that help in eradication of the bacteria at the site of infection through

Phagocytosis: a process by which bacteria/solid particles are engulfed into the cell through membrane invaginations (pseudopodia) and formation of vesicles called 'phagosomes', that subsequently fuse with lysosomes to form a 'Phagolysosome' where the bacteria are killed by reactive oxygen and nitrogen species (ROS/RNS) and digested by various proteases and DNases in a bacteriostatic acidic environment (Figure 2.1).

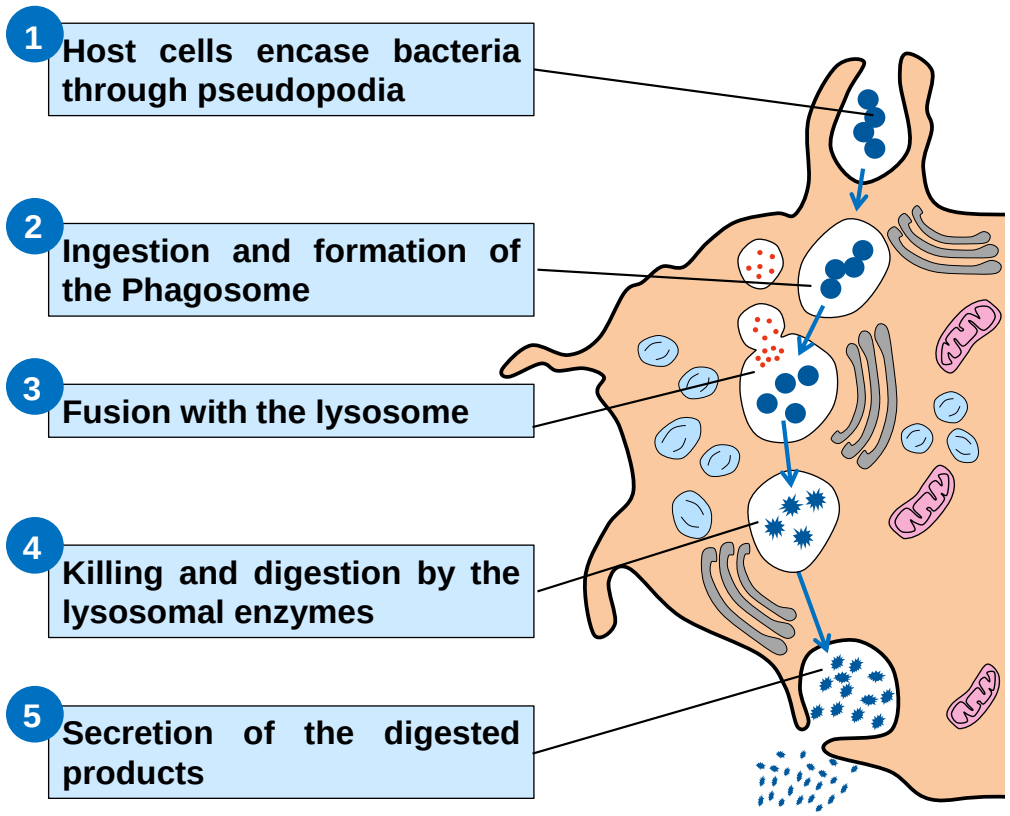

Figure 2.1: Schematic illustrating the different steps in phagocytosis of bacteria by professional phagocytic cells of the innate immune system. (1) The bacteria get attached to the membrane of the phagocytic cell, which induces invagination by pseudopodia. (2) The bacteria are ingested into the cell, enclosed in a Phagosome. (3) The phagosome fuses with a lysosome that contains more than 50 digestive enzymes and forms a phagolysosome. (4) Bacteria are killed by reactive oxygen and nitrogen species (ROS/RNS) and digested by various proteases and DNases in an acidic environment (5) Degraded bacterial components are secreted from the cell. [Modified from Kuby, 5th Edition]

Tissue damage due to a wound and the subsequent recognition response triggered by TLR binding to an invading pathogen usually leads to an "Inflammatory response". The cardinal signs of inflammation can be reflected by three signs:

- Vasodilation: An increase in the diameter of the blood vessels and nearby capillaries occurs along with the constriction of capillaries that carry blood away from the affected site, resulting in the engorgement of the capillary network, causing tissue redness (erythema) and increased tissue temperature.

- Increase in Capillary permeability: High influx of fluids (exudate) containing higher protein content than the fluid normally released into the tissue from Vasculation leads to tissue swelling (Edema).
- Influx of Phagocytes: Due to increased permeability of capillaries towards the tissue, phagocytic cells like neutrophil granulocytes are recruited from the blood circulation system and accumulate at the tissue site of infection to attack bacterial invaders. Finally, the accumulation of dead immune cells with their digested material and fluid forms Pus.

Once the inflammatory response subsides, most of the developed debris is cleared by phagocytic cells of the innate immune system. Subsequently, tissue repair and regeneration of new tissue structures begins. As aforementioned, apart from TLR-associated recognition of pathogens that is receptor based, soluble proteins of Pattern recognition, like the Complement system, also play an important role in identification and labelling of bacterial invaders for phagocytosis.

2.2.2 The role of the complement system during necrotizing fasciitis

The Complement system is a major component of the innate immune system that is involved in identification and eradication of pathogens by complement factors that aid in opsonization, chemotaxis and killing. The complement system is a general term assigned to more than 30 soluble plasma proteins and to a number of cell receptors and control proteins found in blood and tissues that support the innate and adaptive immune response in recognition and eradication of invading bacteria. The complement system can be activated by the classical (Antibody-Antigen interactions), alternative (Bacterial Surface), or lectin pathway (Microbial carbohydrates). Activation of the complement system by one of the three pathways leads to an accumulation of complement opsonins, C3b and C3bi, on the bacterial surface thus opsonizing it. Opsonization is a process of 'labeling' or 'targeting' the bacterial cells by the immune system for phagocytosis. The complement system not only labels *S. pyogenes* for disposal by neutrophils, but also leads to formation of the Membrane Attack Complex (MAC) that causes a pore formation in the bacterial cell membranes. However, the MAC complex is considered to be not very efficient in case of *S. pyogenes* as it is a gram positive bacterium carrying a thick peptidoglycan cell wall[15].

Along with the innate immune system and the complement system, second line of defense or the Adaptive immune system is activated that boosts the clearance of

pathogen by generation of specific antibodies and memory cells that retains memory of the encounter for future.

2.2.3 The role of the adaptive Immune system during necrotizing fasciitis

The adaptive (or acquired) immunity actively contributes to the recognition and selectively eradication of foreign antigens/pathogens. In contrast to the innate immune system, which is a broad spectrum defense system, the acquired immune system varies according to specific antigenic challenges encountered. An antigen (*Anti*body *gen*erator; Ag) can be any structural substance that serves as a target for the receptor regions of Immunoglobulins (Ig). Ig are Y-shaped glycoproteins produced by differentiated B-cells (activated B-cells/Plasma cells; B-cells that have encountered Ag). All immunoglobulins have a common structure consisting of two heavy (H) and two light (L) protein chains, with constant (C) and one variable (V) regions. Constant regions of heavy chains (C_H1, C_H2 and C_H3; C_H4 in case of IgE and IgM) or light chain (C_L) are connected to the variable regions of Heavy chain (V_H) or light chain (V_L) by protein bonds called Hinges. The heavy and the light chain are interconnected to each other by di-sulfide bonds. Additionally, the antibody can be horizontally divided into two regions: Fab and Fc. Fab (Fragment antigen binding) is the region where an antigen is bound and is composed of one constant and one variable domain of each heavy and light chain. The Fc (Fragment crystallizable) region, on the other hand, forms the tail region of the antibody which interacts with cell surface receptors (Fc receptors) or the complement factors (C1q) to induce the immune system (**Figure 2.2A**).

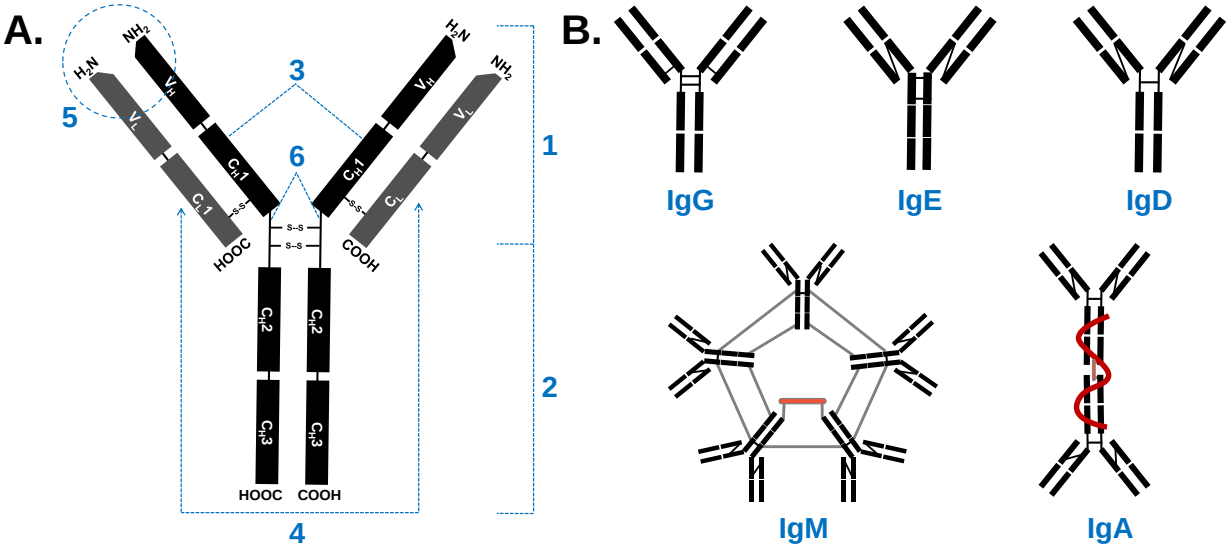

Figure 2.2: Schematic diagram showing the types of antibodies generated by B-cells of the adaptive immune system. (A) General structure of an antibody produced by activated B-cells. Different regions have

been demarcated with different numbers. 1: Fab Region, 2: Fc region, 3: Heavy chain (Black bars) with one variable (V_H) domain followed by a constant domain (C_H1), a hinge region and two/three more constant (C_H2, C_H3 and C_H4) domains, 4: Light chain (Grey bars) with one variable (V_L) and one constant (C_L) domain, 5: Antigen binding site (paratope), 6: Hinge region. (B) Different types of antibodies produced by human B-cells: IgG, IgE, IgD are produced and function as monomers. IgM is known to act in pentamers whereas Secretory IgA as dimers. The oligo/polymerization of IgM and IgA is done by Junction-chains (orange). The secretory component of sIgA has been shown in red [Modified from Kuby, 5th Edition[16]].

Antibodies could be differentiated into five different types (**Figure 2.2B**), based on their location of action and type of Ag confronted:

- IgG: IgG is the main antibody found in human blood, constituting up to 70-80% of total Ab[16]. IgG is able to cross the placenta and therefore be transferred (acquired) from mother to newborn. It is widely distributed to the blood and tissue and plays a crucial role in combatting *S. pyogenes* and its secreted toxins in local tissue and systemic infections through several mechanisms: (1) Binding to pathogen causing their debilitation by agglutination[17], (2) coating the surface of pathogen, thus activating the complement system via the classical pathway[17] (3) Binding to exotoxins to neutralize them[17].

- IgM: IgM is the type of antibody that is produced during the initial encounter of the pathogen with the immune system and actively functions as pentamers, thereby aiding in crosslinking and restricting the pathogen from further dissemination. Due to its polymeric nature, IgM is difficult to diffuse, but possess high avidity. IgM contributes greatly to opsonization by activating complement and causing C3b to bind to the antigen[16].

- IgA: IgA consists of two types (IgA1 and IgA2) that exist in dimeric form called the secretory IgA or sIgA. Secretory IgA plays an important role in mucosal immunity and functions as a dimer (made of 2 antibodies), joined together by the Junction-chain (J-chain) and are distributed in serum, nasal discharge, breast milk and bowel fluid[16]. The secretory component of sIgA protects it from proteolytic enzymes, thus sIgA can survive in harsh environments and provide protection against pathogens[18].

- IgD: IgD comprises of only 0.25% of the total immunoglobulins and form approximately 1% of proteins found in the plasma membrane of B-cells. It plays a role in the induction of antibody production[16].

- IgE: IgE is believed to be related to immune reactions to invading parasites, and has recently become known as a key factor of allergies such as pollinosis[16].

The Adaptive immune response exhibits four distinct characteristics on encountering a pathogen: Antigenic specificity, diversity, immunological memory and self/non-self-recognition. *Antigenic specificity* refers to the ability of the adaptive immune system to differentiate exquisite distinctness among antigens. Antibodies are able to differentiate between two proteins that vary only in one amino acid[19]. *Immunological diversity* of the adaptive immune system allows it to identify unique structures of millions of pathogens/antigens. Once the immune system has responded to a particular antigen, it displays an *immunological memory*. A second encounter with the same antigen would therefore produce a rapid and intensive immune reactivity. This attribute helps in the development of a long-term immunity to that particular antigen. Finally, the adaptive immune system is able to distinguish between *self* (host) and *non-self* (foreign)-antigens; thus responding only to the foreign molecules. This is an important feature of the adaptive immune system as any response to self-antigens could lead to auto-immune diseases.

Once the pathogen or its fragments/secreted products are recognized, they are taken up by professional phagocytic cells, wherein the antigens are broken down into peptides/fragments. These fragments are displayed on the surface of an altered self-cell (antigen presenting cell) bound to MHC (Major Histocompatibility Complex) molecules which are subsequently recognized by T-cells in combination with T-cell receptors (TCR). MHC class II molecules tend to bind to T-helper cells (T_H) that are activated once they bind to the MHC-II. Activated T_H cells secrete cytokines that contribute to activation of B-cells. Activation of B-cells through T_H cells is known as *cell-mediated response*[16]. In contrast, B-cells can also interact with antigens independently (*humoral response*) and differentiate into antibody producing plasma cells. These antibodies can bind to antigens and facilitate clearance[16]. However, all different types of immune responses (innate/complement/adaptive) are always dependent on each other and the antigen clearance is a coordinated interplay of all three.

The generated antibodies are able to bind to antigens on the surface of bacteria and can act as 'opsonins' together with factor C3b of the complement system. This opsonization increases phagocytosis and hence helps in bacterial clearance. Antibody-mediated activation of the complement system induces a localized production of immune effector molecules that help in developing heightened inflammatory response. C3a, C4a and C5a act as anaphylatoxins that induce mast-cell degranulation and hence causes vasodilation and extravasation of lymphocytes

and neutrophils from the blood into the tissue (**Figure 2.3**). Other complement factors serve as chemotactic factors that attract numerous neutrophils and macrophages at the site of infection.

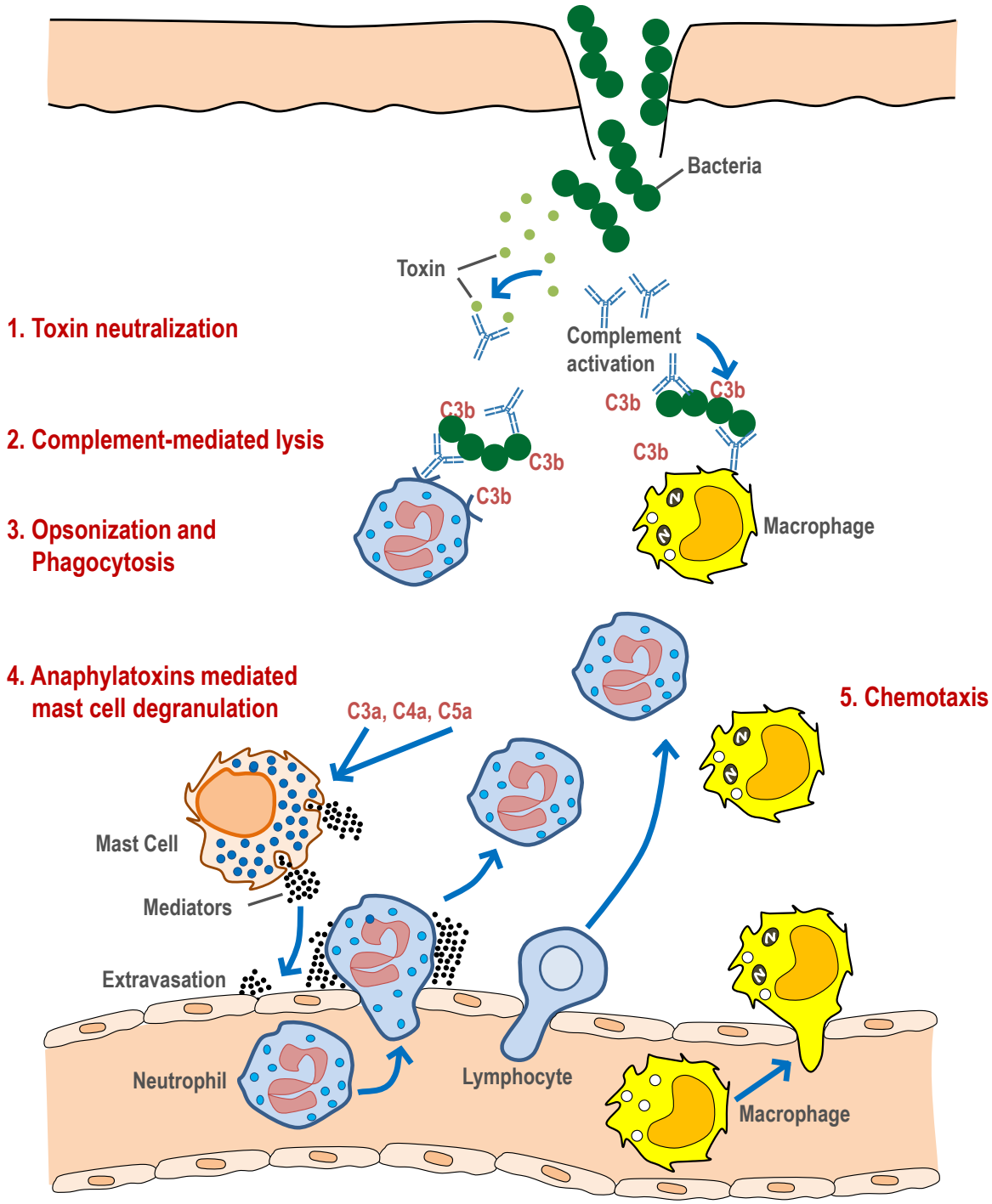

Figure 2.3: Antibody-mediated mechanisms for combating infections by invading bacteria. (1) Antibodies neutralize bacterial toxins. (2) Complement activation on the bacterial surface leads to complement-mediated lysis of bacteria by MAC. (3) Antibodies and the complement product C3b bind to the bacterial surface, serving as opsonins to increase phagocytosis. (4) C3a, C4a, and C5a, generated by antibody initiated complement activation, induce local mast cell degranulation, releasing substances that mediate vasodilation and extravasation of lymphocytes and neutrophils. (5) Other complement products are chemotactic for neutrophils and macrophages. [Taken from Immunology, Kuby 5th Edition[16]]

Antibodies are able to boost the host immune system by coating the surface of the pathogen, thus activating the complement system via the classical pathway and neutralizing bacterial exotoxins. The presence of antibody titers specific for the invading bacteria and its set of toxins during early phase of infection can lead to faster and efficient eradication of the pathogen.

Although host-defense mechanisms act at each and every step during bacterial invasion, bacteria have evolved strategies to circumvent some of these host defenses. Mentioned below is the role of the pathogen, *S. pyogenes*, in the manifestation of necrotizing fasciitis and how its virulence factors intervene in host defense mechanism.

2.3 Role of the pathogen *S. pyogenes* in the manifestation of NF

The beta hemolytic Group A *Streptococcus* (GAS) or *Streptococcus pyogenes*, belongs to the genus *Streptococcus* of the phylum *Firmicutes* and is an important human pathogen causing wide a spectrum of diseases ranging from mild infections of skin and mucosa to severe invasive infections generally associated with high mortality, such as necrotizing soft tissue infections and toxic shock syndrome[20]. *Streptococcus pyogenes* is negative for catalase and oxidase and is facultative anaerobe. Different *Streptococcus pyogenes* strains can be characterized based on various typing methods; the most widely used is the *emm* typing (deciphering the M-protein subtype). The M-protein was first identified by Rebecca Lancefield who named it "M-protein" because of the 'Matt' appearance of the colonies due to this surface linked protein. Later, it was reported to be involved in pathogenesis and crucial for the virulence of *S. pyogenes*[21]. M-typing can be performed through amplification and sequencing of the *emm* gene encoding for the M-protein. Till date, around 220 different serotypes of M–proteins have been reported. Besides the M-protein, *S. pyogenes* possess a huge array of virulence factors that are detrimental to the host and counteract the immune system defenses in various ways (2.3.1).

2.3.1 Virulence factors of *S. pyogenes* involved in NF

The host-defense mechanisms try to combat the pathogen at various stages of bacterial invasion and infection. However, *S. pyogenes* secretes a lot of factors/exotoxins to fight back the immune system and thus promoting proliferation and dissemination of the bacteria. There are four major stages of bacterial infection: Initial entry and attachment to host structures, bacterial proliferation, invasion of

host tissue and Toxin-induced damage to host cells; each stage been made successful by specific proteins and/or other bacterial components. Initial weak interactions are triggered by lipoteichoic acid (LTA) or pili with the cell surface or mucosa of epithelial surfaces, leading to bacterial colonization[22,23]. This initial attachment is pursed by stronger binding (carbohydrate and/or protein) between the epithelial cell layer and the bacteria. Proteins like the M-protein, SfbI, SfbII and ECM proteins like fibronectin are known to be involved in this process[24]. During these interactions, the bacteria already start to proliferate and spread into the host system but during all stages (attachment, proliferation and dissemination) they are challenged by the innate and adaptive immune system of the host. Different mechanisms developed by *S. pyogenes* that intervene in the immune responses are described below.

2.3.1.1 Virulence Factors intervening the innate immune response

The first strategy for a pathogen to evade the host immune system is to not get recognized. For this, all *S. pyogenes* strains are able to surround themselves by a thick **hyaluronic acid capsule**[25] that serve dual purpose: (1.) Since hyaluronic acid is a natural component of the human connective and epithelial tissue, it provides active protection against phagocytosis[20] as the host immune system is unable to differentiate it from the 'self'. (2.) The capsule is able to cover most of the potentially antigenic structures on the surface of *S. pyogenes* and thus do not elicit the immune response by posing an antigenic challenge to the system. Hyaluronic acid is a linear polymer of glucuronic-β-1,3-*N*-acetylglucosamine, produced by the highly conserved *hasABC* (hyaluron synthase) operon[26] and is also an important adhesion factor as it binds to CD44 receptors on epithelial cells[27]. Once the bacteria have successfully dodged the initial recognition and reached tissue structures, it produces **Hyaluronidase** (Hyl), an enzyme that cleaves hyaluronic acid and exposes all bacterial surface proteins for their function. Additionally, Hyl is also able to digest structurally identical hyaluronic acid in human tissue and improves bacterial spreading[28].

Once the hyaluronic acid capsule is dissolved, the bacterial cell surface is exposed, revealing all surface bound proteins. The **M-protein** is the most abundant protein found on the bacterial surface and is the primary virulence factor of *S. pyogenes*[29]. It is also one of the most important factors in the initial attachment of the bacteria to different human cell types[30] and contributes with its antiphagocytic function mediated by fibrinogen binding[31] to the bacterial survival. The M-protein has a hypervariable N-terminus, which is the basis for serological differentiation of

different *S. pyogenes* isolates. Interestingly, immunization with M-protein provides a strong protectivity toward Streptococcal infections in mice[32]. Hence, this protein has been the most important candidate for vaccine development strategies. Recently, a 30 valent vaccine, that covers 26 different M-types, is being tested in Phase-1 clinical trials and is seen to generate cross–opsonic antibodies also against non-vaccine serotypes of *S. pyogenes*[33].

As already mentioned, phagocytosis is a crucial mechanism of the host innate immune system to eradicate the invading pathogen with the help of professional phagocytic cells like Macrophages and Neutrophils. However, *S. pyogenes* is seen to cleverly evade these phagocytic attacks by the following mechanisms:

- Pore formation in phagocytic cells: Production of **Streptolysin O** (SLO) and **Streptolysin S** (SLS): The 69kDa streptolysin O (SLO) is a thiol-activated cholesterol dependent cytolysin that forms large pores of about 30 nm in eukaryotic cell membranes leading to apoptosis[34,35]. It is reported to cause apoptosis in epithelial cells, neutrophils and macrophages[36]. SLO also aids infiltration of superantigens (described later in the chapter) into stratified mucosa[37]. Since SLO is co-transcribed with NAD glycohydrolase, it is thus seen to mediate the translocation of NAD glycohydrolase into cells through the pores being formed. NAD glycohydrolase modulates the host cell signaling pathways; hence assisting in *S. pyogenes* internalization[38]. Interestingly, SLO expression is noticed to be higher in severe invasive cases when compared to non-invasive controls pointing towards their active role in the pathogenesis of *S. pyogenes* during tissue infections[39]. SLS, on the other hand, is non-immunogenic oligopeptide of ~2.8 kDa that is responsible for the hemolytic zone around colonies of *S. pyogenes* when cultivated on blood-agar[40]. Interestingly, cytotoxic effects of SLO and SLS were seen to enhance the virulence of poorly encapsulated *S. pyogenes*[41].

- Cleavage of NETs: Production of DNases: In order to promote the entrapment and consequent clearance of bacteria, neutrophils are able to release 'NETs' (Neutrophil Extracellular Traps) that are made from DNA and proteins[42]. *S. pyogenes* has evolved to produce various DNases that can cleave these NETs and thus lead to the escape of the bacteria. One of such DNases is the **Extracellular streptodornase D** (sda1/sdaD). SdaD cleaves the DNA network of NETs, thus helping the bacteria from the entrapment and killing[43]. Recently, it

has been reported that sdaD helps the bacterial evasion through by TLR9-dependent recognition and is done through auto-degradation of CpG rich DNA by bacterial DNase[44].

- Degradation of Chemokines (IL-8): Production of **SpyCEP**: SpyCEP (also known as ScpC, CepA or Prts) is a subtilisin-like protease secreted by *S. pyogenes*[45] that can cleave various chemokines like Interleukin-8 (IL-8), Grap-2 (GRB2- related adapter protein 2) and GCP-2 (granulocyte chemotactic peptide-2)[46,47]. Mature SpyCEP is a dimer of 2 subunits of 30 and 150 kDa. A stable interaction between both subunits is prerequisite for enzymatic activity of the protease[48]. Interestingly, like SLO, **SpyCEP** is also seen to be upregulated in invasive diseases pointing towards an active role in tissue infection manifestation[46].

- Dissolution of Fibrin Clot: Production of **Streptokinase** (Ska): Another common host defense mechanism is to trap the bacteria within a fibrin clot in order to block the dissemination of the bacteria. In case of a wound that causes blood vessel breakdown, a fibrin clot is formed by the action of the protease thrombin on fibrinogen which causes the latter to polymerize to stops further loss of blood. When the blood vessel/injury is healing, plasminogen is converted to plasmin that dissolves the fibrin clot. *S. pyogenes* has very cleverly utilized this host mechanism for self-defense, by the production of **Streptokinase** (Ska). Ska is a 50 kDa glycoprotein that is an important factor in fibrinolysis as it activates plasminogen[49]. Plasminogen is converted to "activated plasminogen" that is structurally plasminogen but functionally plasmin, thereby dissolving the fibrin clot thus allowing the bacteria to disseminate[50]. Interestingly, all strains of *S. pyogenes* are known to secrete streptokinase and are capable for acquisition of cell surface plasmin[51]. However, based on the pathway of cell surface plasmin acquisition by *S. pyogenes*, ska is differentiated into three clusters: *Cluster-1*, which combines with plasminogen to form a complex with plasmin activity; *Cluster*-2a, wherein streptokinase must form complex with plasminogen and fibrinogen to form a tri-molecular-complex that exhibits plasmin activity by binding to the cell surface by fibrinogen binding receptors and *Cluster-2b*, which forms a tri-molecular-complex (streptokinase-plasminogen-fibrinogen) to exhibit plasmin activity by binding to cell surface receptors such as the plasminogen binding group A streptococcal M-like proteins (PAMs)[51]

- Degradation of antimicrobial peptides: Production of **SpeB: Streptococcal pyogenic toxin B (SpeB)** is a broad spectrum cysteine protease released by most of the isolated *S. pyogenes* strains[52] that is seen to degrade anti-microbial peptides produced against the pathogen. Although almost all (<99%) isolated strains carry this gene in their genome, there is a considerable difference observed in the expression levels[53]. SpeB is initially produced as a 40kDa zymogen which is self-cleaved to generate a 23 kDa mature form[54]. Loss of the speB gene is a crucial factor for conversion of a local infection causing an *S. pyogenes* strain to be invasive. As SpeB is a broad spectrum protease, it also cleaves bacterial virulence factors.

- Interruption of complement activation: Production of **SIC**: Another aspect of the innate immune system is its interaction with the complement system to activate the Membrane Attack Complex (MAC). However, the **streptococcal inhibitor of complement (SIC)**, a highly polymorphic 31 kDa protein, is secreted by *S. pyogenes* and interfere with the formation of the MAC by constraining the interaction of complement complex C5b67 with the bacterial cell membrane[55]. It is also reported to inhibit other molecules of immune system like human cathelicidin LL-37, α-defensins, secretory leukocyte protease inhibitor and lysozyme[56].

All these mechanisms of combating the attack of phagocytic cells of the innate immune system like neutrophils have been illustrated below (**Figure 2.4**)

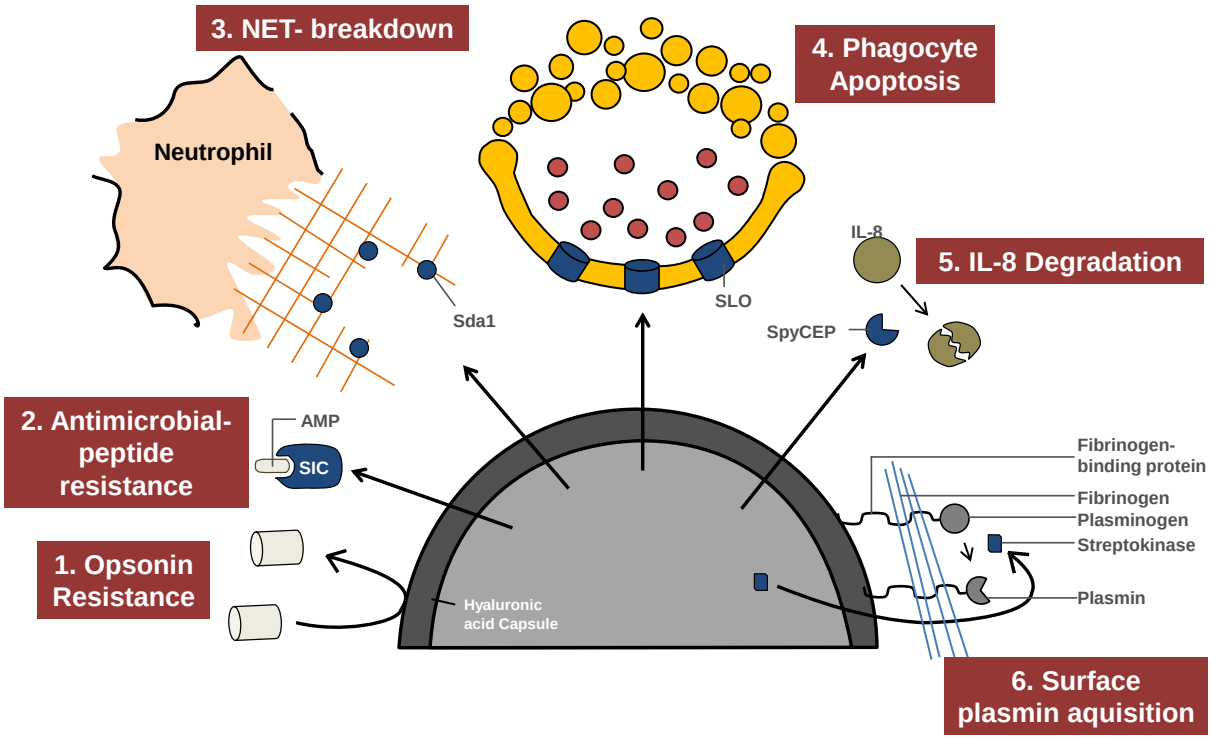

Figure 2.4: Virulence factors of *S. pyogenes* that interfere the cellular innate immune response by Neutrophils. *Streptococcus pyogenes* has evolved various defense mechanisms that intervene the innate and adaptive immune response by disabling professional phagocytic cells like neutrophils. (1) *S. pyogenes* strains are surrounded by Hyaluronic Acid Capsule that masks antigenic structures on the bacterial surface and resists Opsonization. (2) *S. pyogenes* secretes SIC (Streptococcal Inhibitor of Complement) that binds to Anti-microbial peptides (AMP) and constraints the interaction of complement complex C5b67. (3) SdaD/Sda1 is a DNase secreted by *S. pyogenes* that breakdown Neutrophil Extracellular Traps (NETs). (4) SLO or Streptolysin O is an active pore forming enzyme that punctures the cell membrane of neutrophils and other host cells to lead them towards Apoptosis. (5) SpyCEP is a subtilisin-like protease secreted by *S. pyogenes* that can cleave various chemokines like Interleukin-8. Lastly (6) Streptokinase (Ska) actively participates in fibrinolysis as it activates plasminogen to exhibit plasmin activity, that dissolves the fibrin clot thus free the bacteria for spreading. [Modified from Cole *et al.*, 2010[57]]

The host immune system is able to eliminate an infection though the interplay of innate immune system, the complement system and the adaptive immune system. The Adaptive immune system is more targeted towards the pathogen due to production of antibodies. However, *S. pyogenes* has developed mechanisms that can assist in its efficiently escaping the adaptive immune response.

2.3.1.2 Virulence Factors intervening the Adaptive immune system

B-cells are directly or indirectly (with help of T_H cells) activated to produce specific antibodies against antigens presented by the invading pathogens. These antibodies help in opsonization and therefore killing of bacteria by specialized phagocytes like neutrophils. Out of all types, IgG is the major form of antibodies produced against bacterial infections. *S. pyogenes* secretes **IgG endopeptidase** (IdeS; also known as Mac, MspA), a cysteine protease, that specifically cleaves human immunoglobulin G (IgG)[58]. It is a homologue of human leukocyte β2 integrins which are reported to inhibit the activation of neutrophils and production of reactive oxygen species by binding CD16B (a low-affinity Fc receptor)[56]. Two different variants of Mac have been described: Complex-I and complex-II, that differ in amino acid level (amino acids 112 to 205)[59]. Complex-I wields their function through proteolytic cleavage of IgG[58]; whereas Complex-II, which is a weaker endopeptidase, blocks interaction of the FcγIIIb receptor; thus inhibiting phagocytic killing[58,59].

Antigen presentation and antibody activation are complex mechanisms of the host immune system to elicit a response against any foreign structure or pathogen that is potentially harmful. Specific receptors for antigen recognition are situated on T-cell surfaces called T-cell Receptors (TCR) and on antigen presenting cells (APC). Generally, an antigen is cleaved in the lysosome into smaller fragments and is then displayed by the antigen presenting cells (APC). These antigen fragments finally interact with T-cell that carries a specific αβ TCR having constant and variable regions. The selectivity of a TCR to any conventional antigen mainly depends upon the variable regions: Vβ, Dβ, Jβ, Vα and Jα[60]. After binding to a specific TCR, the antigen induces a signal transduction and biochemical changes in the T-cell that activates it, which then proliferates to generate definite subpopulations of T-cells with peculiar variable regions specific to this particular antigen. Unlike the conventional antigens, superantigens can bind and stimulate enormous sub-population of T-cells (**Figure 2.5**) via the variable region of β chain (Vβ elements)[61].

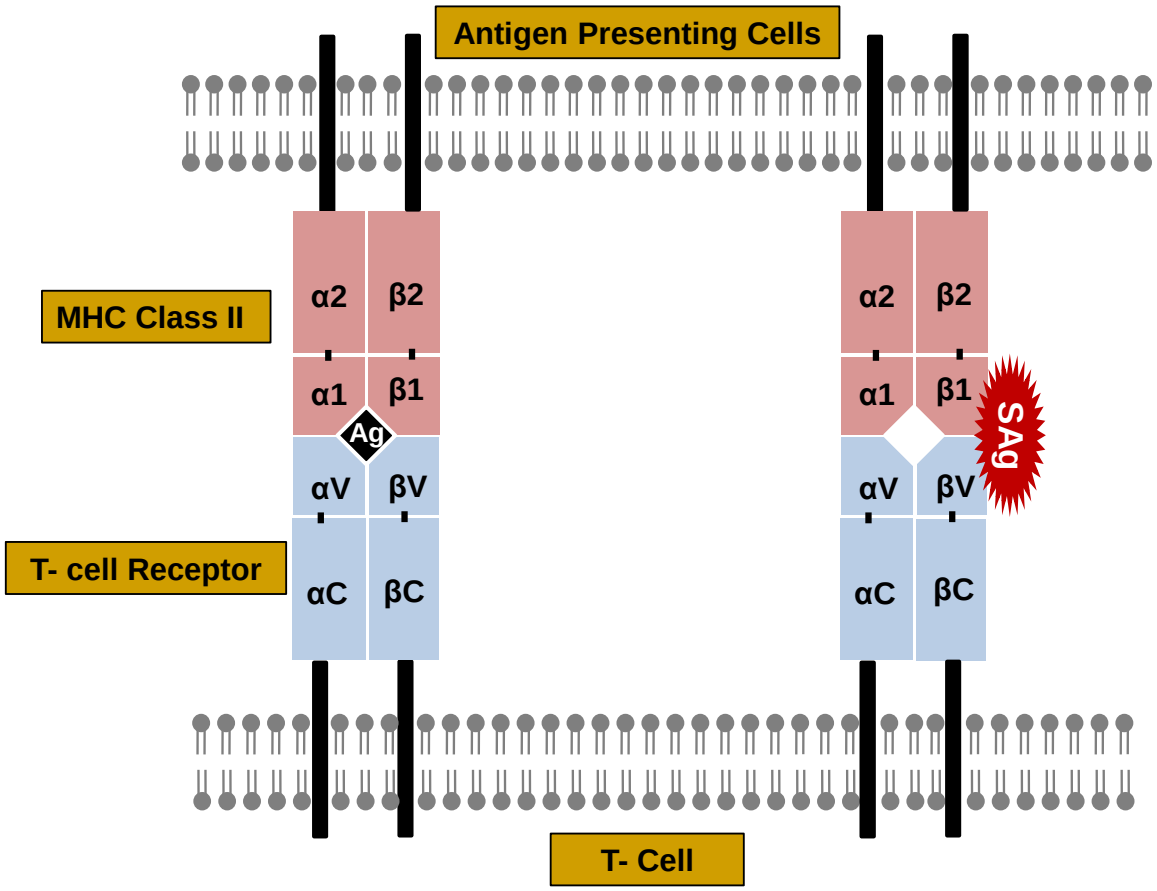

Figure 2.5: Differences in the receptor binding activity of a conventional antigen (Ag) and a superantigen (SAg). The Ag binds to a TCR via MHC-II receptors on the antigen presenting cells, whereas, a SAg bind to the T-cells via the variable β region, leading to non-specific stimulation of large number of T-cells. It should also be noted that different SAgs activate different Vβ subtypes [Modified from Streptococcal *Superantigens*[62]].

S. pyogenes secrete a number of different **Streptococcal pyogenic exotoxins** (SPEs), that share sequence similarity of about 17-48% , nevertheless are structurally related[20,63]. These exotoxins belong to the family of 'Superantigens' (SAgs)[64]. SAgs are small, but highly mitogenic protein molecules that bind to Major Histocompatibility Complex (MHC) class II receptors and TCR molecules leading to a co-stimulation of large number of T-cells[65]. They are known to bind MHC class II outside the peptide groove and to CDR2 and HV4 regions on TCR Vβ chains[66]. These superantigens are seen to bind laterally to the βV region and hence cause massive cytokine release[61]. Several SAg genes are located on mobile DNA elements, such as bacteriophages, integrated in the bacterial genome[67] and hence, are more likely to be genetically transferred among different Streptococcal species[68].

Binding of SAg to the T-cell receptor triggers biochemical changes in the cell that leads to non–specific activation of a copious amount of T-cells and release of inflammatory cytokines like IL-6 and IL-8. Interestingly, these molecules give an

edge to the pathogen by allowing it to circumvent the induction of specific adaptive immune response of the host. Superantigens are seen to deter the immune system by one of the three mechanisms: First, its capability to induce SDCC (Superantigen-Dependent Cellular Cytotoxicity) that eradicates cells hindering SAg-activated T-cell proliferation. Production of IL-8 and other inflammatory cytokines worsens the situation for the host, thus enhancing SDCC. Eradication of MHC-II expressing cells may also lead to anergy and further incapability of T-cell stimulation. Second, massive production of cytokines and proinflammatory chemokines may lead to specific apoptosis of T-cells carrying definite Vβ binding site for that Superantigen. Lastly, activation of nonspecific T-cells helps in "distracting" the host immune system, thus diverting the attention from a centered and specific immune response toward the pathogen.

Understanding the host immune responses and bacterial virulence mechanisms is an important aspect of applying therapeutic interventions in treating a disease. Below mentioned are few ways that are generally used by physicians to treat NF.

2.4 Therapeutic interventions to treat NF

Necrotizing Fasciitis is a rapidly progressing disease. Any delay in initiating suitable treatment causes significant increases in mortality rates. Once an NF is diagnosed, different specialized therapeutic treatments are provided depending on the severity and the site of infection.

2.4.1 Antibiotics and surgery

The first step in the treatment of NF is the administration of empirical antibiotics for which the causative pathogen is sensitive to. Till the time the pathogen is being identified through microbiological testing, initial antibiotics are generally broad spectrum, so as to cover both aerobic and anaerobic bacteria. The maximum doses given for a particular antibiotic is dependent on the patient's weight along with the liver and renal status. Generally, possible regimes include combination of Penicillin G and an aminoglycoside (if renal function permits), as well as clindamycin (to cover *streptococci, staphylococci*, gram-negative bacilli, and anaerobes). Clindamycin is generally preferred over Penicillin G, as it's efficacy is not affected by the inoculum size or stage of bacterial growth[69]. Broad-spectrum beta-lactam drugs such as Meropenem (Imipenem) cover Gram negative bacteria, especially *Enterobactericeae* like *Escherichia coli*. However, antibiotics are not as

efficient in case of NSTI as the infection is usually localized in deeper tissue layers and the blood supply to the site of infection is limited, thereby reducing the amount of antibiotics actively reaching the infection through translocation of antibiotics from blood.

Along with the antibiotic treatment, urgent surgical procedures are important to improve the survival rate of the patient. Even a few hours delay in controlling the infection cause significant increases in mortality rates. Surgical removal of infected tissue is advantageous for survival as it reduces inflammation and restricts the spread of the local infection. Surgery also provides an increase of the local oxygen pressure in the infected area, thus boosting the activity of host immune cells. The debridement must be extensive, with adequate margins so that no infected tissue remains. Following initial debridement, the wound must be observed closely on a daily basis until the infection is controlled. In addition to administrating antibiotics and surgery, the patients undergo specialized treatments like hyperbaric Oxygen therapy (HBO) and Intravenous Immunoglobulin administration (IVIG) that increase the chances for better recovery.

2.4.2 Hyperbaric Oxygen treatment (HBO)

Hyperbaric oxygen treatment is given as an adjunctive therapy in diseases like necrotizing fasciitis so as to decrease the morbidity and mortality. In this treatment, the patient breathes 100% oxygen inside a chamber with an oxygen concentration of 100% at a pressure of at least 1.4 atm. In regard to manage necrotizing fasciitis, numerous studies have been done that show a beneficial role of HBO treatment[70]. The region of infection is generally characterized by a low oxygen pressure and oxygen transportation is restricted due to reduced blood flow at the site. HBO treatment is seen to boost the host immune system for phagocytosis and eradication of the pathogen by increasing the oxygen pressure at the site of infection. The treatment includes initial therapy at 2.0 to 2.5 atm pressure given twice a day for 90 min till the day no further extension of necrosis is seen and the infection is controlled. In addition to HBO, patients also need extensive care and administration of intravenous immunoglobulins (IVIG); especially when the causative organism is *S. pyogenes*[71].

2.4.3 Intravenous Immunoglobulins (IVIG) as Adjunctive therapy

Intravenous Immunoglobulins (IVIG) are a sterile preparation of concentrated antibodies from pooled plasma samples of at least 1000 healthy donors. Even though IVIG is dominated by IgG, there might be few traces of IgA found as well. Since a lack of protective antibodies is hypothesized to be one of the risk factors in the development of diseases like Streptococcal toxic shock syndrome (StrepTSS) and necrotizing fasciitis, IVIG could be one of the potential collateral therapies[72]. Since, IVIG is a pool of all antibodies derived from thousands of healthy donors; it is non-specific and thus can cover various serotypes of *S. pyogenes* as well as the complete spectrum of superantigens and other streptococcal exotoxins. IVIG is also commonly used as a therapy for patients with immune deficiencies (hypogammaglobulinemia, X-linked agammaglobulinemia), low antibody titers, autoimmune diseases, and inflammatory diseases[73].

2.4.3.1 Mechanistic actions of IVIG in NF

Several different mechanistic actions of IVIG have been described, that contribute to its positive effect during Streptococcal infections. These comprise modulation of cytokine responses, variation in immune cell function, interaction with idiotype-anti-idiotypic network and antigen neutralization[74]. With respect to Superantigen (SAg) mediated diseases like StrepTSS, IVIG is seen to neutralize antigens (including SAgs), mediate bacterial opsonization, and modulate cytokine responses. IVIG contains antibodies that can opsonize the bacterial cells and hence lead to clearance by phagocytosis and subsequent killing[75]. Antibodies against streptococcal M-proteins have also been found in IVIG preparations[75] and analyses of plasma samples from patients pre- and post-IVIG treatment revealed a significant increase in anti-M-protein antibody titers[75]. Hence, bacterial opsonization and thence clearance is likely to be one of the mechanistic actions of IVIG, thus contributing to its effectiveness as an adjunctive therapy. Contrarily, when efficacy of IVIG was checked, in murine models of necrotizing fasciitis, no beneficial effect of IVIG was observed, thus failing to support the previous hypothesis[76]. However it could be anticipated that human antibodies were not efficient opsonins for murine phagocytes.

IVIG also contains potent neutralising antibodies against streptococcal superantigens. These antibodies are able to inhibit the massive superantigen induced T-cell proliferation and cytokine releases under *in-vitro* conditions[77]. Even though

IVIG was able to neutralise all streptococcal SAgs, the degree of inhibition varied. However, only SpeA and SpeC have been studied extensively in this regard. Hence, IVIG can be considered as a 'cocktail' of different antibodies against various virulence factors of *S. pyogenes*. As already mentioned, IVIG is primarily composed of IgG, but can also have varying amounts of IgA and IgM. However, using polyspecific IgA and/or IgM preparations showed an inhibitory effect on the opsonizing and toxin-neutralizing capability of IVIG[78]. IgA and IgM were inhibiting superantigens, especially SpeA, where the most efficient neutralisation could be observed by a mixture containing all three immunoglobulins (IgG, IgA and IgM)[78]. Overall, optimization of IVIG therapy still remains to be proved in a clinical setting.

2.4.3.2 Clinical studies of IVIG usage in NF

There are many clinical cases wherein IVIG was used as an adjunctive therapy to treat Streptococcal infections, especially in StrepTSS[79]. Many case reports[80,81,82], observational cohorts[83], case-control study[84] and multi-centered placebo-control study[85] were conducted to study the efficacy of IVIG in clinical settings. StrepTSS is generally seen along with Necrotizing Fasciitis thence increasing the mortality even further higher.

Recently, another clinical study called INSTINCT (Immunoglobulin for Necrotizing Soft Tissue Infection: a Randomized Control Trial) has been initiated by Rigshospitalet in Denmark that would estimate for the first time the effect of IVIG in a double blind study, taking 1:1 patients treated with IVIG and placebo on outcome of patients with NSTI (https://www.clinicaltrials.gov/ct2/show/study/NCT02111161). The study is currently in the process of patient recruitment (April 2014).

The data of these studies suggests IVIG is beneficial as a collateral therapy and is effective against wide varieties of strains and serotypes[80,81,82]. It has been seen to neutralize superantigens, activate the complement system and facilitate the opsonization of *S. pyogenes*. Clinical studies including cohort studies and case-control studies definitely substantiate the beneficial effects of administration of IVIG to patients of StrepTSS and necrotizing Fasciitis. Unfortunately, the efficacy of IVIG has only been reported *in-vitro* and there is nothing known about its efficiency *in vivo* with respect to the NSTI scenario. Additionally, IVIG is a cost-intensive therapy, usually accompanied by a wide variety of side effect like fever, increased blood pressure and in some cases, acute renal failure and hypersensitivity. There is, thus, a

need of development of more targeted and specific therapeutic treatments to rescue the NSTI patients.

2.5 Aim of the study

Necrotizing soft tissue infections (NSTI) including Necrotizing Fasciitis are rapidly progressing infections of the deeper layers of skin and subcutaneous tissue, spreading across the fascia. It requires intensive care with rigorous medication, ablative surgery and adjunctive therapies like hyperbaric oxygen therapy (HBO) and intravenous immunoglobulin administration (IVIG). IVIG is discussed to act by a variety of mechanisms like opsonization of the pathogen, activation of complement and neutralization of bacterial toxins. However, IVIG is a cost-intensive therapy, usually accompanied by a wide variety of side effect like fever, increased blood pressure and in some cases, acute renal failure and hypersensitivity. Considering the efficacy of IVIG treatment, we hypothesize that the lack of specific antibodies against the invading bacterial pathogen and/or against a certain combination of bacterial toxins could represent a risk factor for the development of a severe NSTI. This project also aids towards the development of more targeted alternatives to the common IVIG treatment.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals/ Solutions

Table 3.1: Chemicals used in the study

Chemical	Supplier
2-mercaptoethanol	Sigma
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	Roche
6× loading dye (agarose gel electrophoresis)	Thermo Scientific
Acetic Acid	Merck
Acrylamid-Bisacrylamid (Rotiphorese Gel 30)	Roth
Agarose Broad Range (NEEO Ultra Quality)	Roth
Ammonium persulfate (APS)	Roth
Ammonium Sulfate	J.T. Baker
Ampicillin	Applichem
Bacto™ Agar	Difco
Bacto™ Peptone	Difco
Bacto™Tryptone	Difco
Bacto™ Yeast Extract	Difco
Bovine serum albumin (BSA)	Applichem
Bradford reagent	Bio-Rad
Bromophenolblue	Merck

Chemical	**Supplier**
Calcium chloride	Merck
Chloroform	Sigma Aldrich
Citric acid	Roth
Columbia blood agar plates	Becton Dickinson
Coomassie Blue R-250	Serva
Dipotassium hydrogen phosphate	Merck
Disodium hydrogen phosphate	Merck
Dimetheylsulfoxide (DMSO)	Applichem
dNTP mix	Fermentas
Dithiothreitol (DTT)	Applichem
Ethanol	J. T. Baker
Ethidium Bromide (EtBr)	ICN-Biomedicals
Ethylene diaminetetraacetic acid (EDTA)	Applichem
Fetal calf serum (FCS)	PAP
Ficoll® PM400	Sigma
Gene Ruler (1kB ladder)	Fermentas
Glycerol	Fluka
Glycine	Roth
Glycogen	Roche
High fidelity polymerase	Roche
Hyaluronidase from *Streptococcus pyogenes*	Sigma

Chemical	**Supplier**
Hydrochloric acid	Fluka
Hydrogen peroxide	Applichem
Imidazole	Applichem
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Applichem
Kanamycin	Roth
Lysozyme	Sigma
Magnesium Chloride	Merck
Methanol	Baker
Mutanolysin	Fluka
Mac (IdeS) "FabriCATOR™"	Genovis
Ni-NTA sepharose	Qiagen
Page Ruler™ Plus Pre-stained Protein Ladder	Thermo Fischer
Ponceau S	Applichem
Potassium di-hydrogen phosphate	Roth
di- Potassium hydrogen phosphate	Roth
Proteinase K	Fluka
Protein G sepharose	Pharmacia
RNase (DNase-free)	Applichem
Restriction enzymes	NEB/Fermentas
Skim milk	Difco
SpeB "FabULOUS™"	Genovis

Chemical	**Supplier**
Sodium acetate	Merck
Sodium carbonate	Merck
Sodium chloride	Roth
Sodium dihydrogenphosphate	Roth
Sodium dodecylsulfate (SDS)	Roth
Sodium hydroxide	Merck
Streptolysin O (SLO) from *Streptococcus pyogenes*	Sigma
Sucrose	Fluka
T4 DNA ligase	New England Biolabs
Taq-polymerase	Qiagen
TEMED	Roth
Todd-Hewitt Broth	Beckton Dickinson
Tris (Trizma base)	Sigma
Trypan Blue	Sigma
Trypsin	Sigma
Tryptic Soy Broth	Beckton Dickinson
Trypsin-EDTA	PAN Biotech
Triton X-100	Sigma
Tween 20	Roth

3.1.2 Instruments

Table 3.2: Instruments used in the study

Instrument	Company
Agarose gel chambers	Horizon™ 58
Autoclave-steam sterilizer	Biomedis
Centrifuge 5417R	Eppendorf
Centrifuge 5804R	Eppendorf
CO_2 water jacketed incubator	Forma Scientific
Elisa plate reader	Varioskan (ScanLab)
French® pressure cell press (SLM Aminco, 40.000 PSI)	Thermo Fisher
Gel doc™ XR+ system	BioLab
Incubator	Kelvitron® Heraeus
Infors HT multitron shaking incubator	Tuttnauer
Leica MS 5 Microscope	Leica Microsystems
Microwave Pro 825	Sharp Electronics
Nano Drop®	Thermo Scientific
pH-meter 766	Calimatic Knick
Power pack 200	BioRad
Power pack 300	BioRad
SONICS®	VibraCell VC-50
Sorvall® RC 6 Plus, rotor SLA-3000	Thermo Electron Corporation
Sorvall® RC 6 Plus, rotor SS-34	Thermo Electron Corporation

Instrument	Company
Special accuracy weighing machine AE 260	Mettler
SZ TrinocularStero Microscope	Olympus
Thermocycler	Biometra
ThermolyneVari-Mix	Barnstead
Transferpette® S-8 (multichannel pipet)	Biohit
UV-transilluminatorHerolab	UVT 2020
Waterbath	GFL
Weighing machine PC 2000	Mettler
Zeiss Microscope Axiovert 25	Zeiss

3.1.3 Primers

Table 3.3: Primers/Oligonucleotides used in the study

Gene Name	Primer Sequence (5' to 3')	Reference
Typing of streptococcal Isolates		
emmA	TATTCGCTTAGAAAATTAA	Facklam *et al*(1999)[86]
emmB	GCAAGTTCTTCAGCTTGTTT	
*16s*_FP	AGAGTTTGATCCTGGCTC	Zhou *et al* (1995)[87]
*16s*_RP	GGTTACTTGTTACGACTT	
Multiplex PCR		
*speA*_FP	AAGAACC(A/T/G)AGAGATG(T/G)CAAC	Friães *et al* (2013)[88]
*speA*_RP	ATAG(G/A)CTTTGGATACCATC(G/A)	

Gene Name	Primer Sequence (5' to 3')	Reference
*speB*_FP	TTCTAGGATACTCTACCAGC	Friães *et al* (2013)[88]
*speB*_RP	ATTTGAGCAGTTGCAGTAGC	
*speC*_FP	CAGTCATACTGATTTCTACTATTTCACC	
*speC*_RP	CAAGATAAATATCGAAATGACTAAAGTTC	
*speF*_FP	CGAAATTAGAAAAGAGGAC	
*speF*_RP	GGCTGAGCAAAAGTGTGTG	
*speG*_FP	GTATCTTTAGGCATTACTGATC	
*speG*_RP	GTTTACTATCTTTAGTAGCAAG	
*speH*_FP	TTCAAGCAAATTCTTATAATACAACC	
*speH*_RP	AAAGTCTCCATTGCCAAAATAATAC	
*speI*_FP	TATGAGATGAGTAGTGTGGGAGTTATTAA	
*speI*_RP	GTTCCTGAATCGTAACCTCTTTCTTATC	
*speJ*_FP	CGAGAGCTATATTACAACAAAG	
*speJ*_RP	CACTCCTTGTACTAGATGAGG	
*speK*_FP	TATCGCTTGCTCTATACACTACTGAGAGT	
*speK*_RP	CTTATCTTTAGCTGTTAATGTTCGTAATTC	
*speL*_FP	GGACGCAAGTTATTATGGATGCTCA	
*speL*_RP	TTAAATAAGTCAGCACCTTCCTCTTTCTC	
*speM*_FP	TATCGCTTGCTCTATACACTACTGAGAGT	
*speM*_RP	ATGAGTGAATAAATCGGTAAACTTTGTTG	

Gene Name	Primer Sequence (5' to 3')	Reference
*ssa*_FP	GTGTAGAATTGAGGTAATTG	Friães *et al* (2013)[88]
*ssa*_RP	TAATATAGCCTGTCTCGTAC	
*smeZ*_FP	TAGAAGTAGATAATAATTCC	
*smeZ*_RP	TTAGGAGT(C/T)AATTTCTATAT	
*spd3*_FP	ATCGTCGTACTTGGCAAGGTT	Borek *et al* (2011)[89]
*spd3*_RP	GCCGCTTCTTCAAACTCTTCG	
*sdc*_FP	AAGCTTAGAAACTCTCTCGCCA	
*sdc*_RP	AGTTCCAGTAATAGCGTTTTTCCGT	
*sdaB*_FP	TATAGCGCATGCCGCCTTTT	
*sdaB*_RP	TGATGGCGCAAGCAAGTACC	
*sdaD*_FP	TTTACGCTGAATCGGGCACT	
*sdaD*_RP	GGCTCTGGTTTGCTTTCCCA	
*spyCEP*_FP	GATCCGGCCCATCAAAGCAT	
*spyCEP*_RP	AGCTGCCACTGATGTTGGTG	
*scpA*_FP	GCTCGGTTACCTCACTTGTCC	
*scpA*_RP	CAATAGCAGCAAACAAGTCACC	
*mac*_FP	TCTTGCCCTGTTGAAAGTGT	
*mac*_RP	CGAGGTGGTATTTTTGACGCC	
*sic*_FP	TTACGTTGCTGATGGTGTATATGGT	
*sic*_RP	TTTGATAGAGGGTTTTCAGCTGGC	

Gene Name	Primer Sequence (5' to 3')	Reference
Cloning of Superantigens		
*speA*_FP	CGC**GGATCC**CAACAAGACCCCGATCCAAG	This study
*speA*_RP	CG**GTCGAC**TTACTTGGTTGTTAGGTAGACTTC	
*speC*_FP	CGC**GGATCC**GACTCTAAGAAAGACATTTCGAATG	
*speC*_RP	CG**GTCGAC**TTATTTTTCAAGATAAATATCGAAATGA	
*speG*_FP	CGC**GGATCC**GATGAAAATTTAAAAGATTTAAAAAG	
*speG*_RP	CG**GTCGAC**CTAGTACGTTTTTAAGTAGATATC	
*speH*_FP	CGC**GGATCC**AATTCTTATAATACAACCAATAGACA	
*speH*_RP	CG**GTCGAC**TTAGCTGATTGACACATCTACATG	
*speI*_FP	CGC**GGATCC**ATGAGTAGTGTGGGAGTTATTAATTTA	
*speI*_RP	CG**GTCGAC**TTATTTATTAAATTTAACTAAGTATATATC	
*speJ*_FP	CGC**GGATCC**AGTGAAAATATTAAAGACGTTAAGC	
*speJ*_RP	CG**GTCGAC**TTATTTAGTCCAAAGGTAAATATCAAA	
*speK*_FP	CGC**GGATCC**GATACGTACAATACAAATGATGTTAG	
*speK*_RP	CG**GTCGAC**CTAATCTTTAGAAAAATCTTCGTTTA	
*speL*_FP	CGC**GGATCC**GAAGAGACTATTAATATTAAGGATATA	
*speL*_RP	CG**GTCGAC**TTAATTTTCTTTGTTTGTGAATAAATAG	
*speM*_FP	CGC**GGATCC**GATGCTGTGTTGGTTAATAGCG	
*speM*_RP	CG**GTCGAC**CTAATTTTTAGAAAAATCTTCGTTTAAG	

Gene Name	Primer Sequence (5' to 3')	Reference
*ssa*_FP	CGC**GGATCC**AGTAGTCAGCCTGACCCTACTC	This study
*ssa*_RP	CG**GTCGAC**TTATTTTTTGGTAAGGTGAACCTC	
*smeZ*_FP	CGC**GGATCC**GAAGTAGATAATAATTCCCTTCTAAG	
*smeZ*_RP	CG**GTCGAC**TTAGGAGTCAATTTCTATATCTAAATG	
*ska*_FP	GC**GGATCC**ATTGCTGGATATGGGTGG	
*ska*_RP	GC**GTCGAC**TTATTTGTCTTTAGGGTTATC	
sic_FP	CGC**GGATCC**AAACGTATACATCACGCAATTTTG	
sic_RP	CG**GTCGAC**TTACGTTGCTGATGGTGTATATG	
***ska* Sequencing**		
*Ska*_FP	ACCTTGCCGACCCAACCTGT	McAurthur *et al* (2008)[51]
*Ska*_RP	GTGAACAGTTTCAAGTGACTGCGA	

3.1.4 Antibodies

Table 3.4: Antibodies used in the study

Antibody	Type	Dilution	Company
His Epitope-Tag (unconjugated) mAb IgG1	Primary	1:1000	Dianova
Mouse HRP Anti-Human IgG	Secondary	1:1000	BD Pharmingen™
Privigen® Intravenous Immunoglobulins (IVIG)	-	5mg/ml*	CSL Behring
Mouse Anti-SLO Antibody	Secondary	1:1000	Antibodiy Online
Mouse Anti-Hyaluronidase Antibody	Secondary	1:1000	Abcam

*Concentration of IVIG as in 100% Plasma; Diluted in 1x PBS

3.1.5 Kits

Table 3.5: Kits used in the study

Kits	Company	Catalogue Number
QIAprep® Spin Miniprep Kit	Qiagen	27104
DNeasy Blood and Tissue Kit	Qiagen	69581
Nucleospin® Gel and PCR clean up kit	Macherey-Nagel	740609.10
SLIDEX	Oxoid, Basingstoke, UK	DR0585A
PolymorphPrep™	Tebu-Bio	1114683

3.1.6 Antibiotics

Table 3.6: Antibiotics used in the study

Antibiotic	Purpose	Final Concentration
Ampicillin	Growing *E. coli* containing pQE30-TEV plasmid	100 µg/ml
Kanamycin	Growing *E. coli* strain M15	25 µg/ml

3.1.7 Buffers

Buffers used in the study:

1× PBS (pH 7.4)

NaCl	137 mM
KCl	2.7 mM
$Na_2HPO_4.2H_2O$	4.3 mM
KH_2PO_4	1.47 mM

1× TBS (pH 7.4)

Tris-HCl	50 mM
NaCl	150 mM

Tris/EDTA (TE) (10mM Tris/0.1mM EDTA)

Tris (pH 7.5)	10 mM
EDTA (pH 8.0)	0.1 mM

1× TAE (pH 7.9)

TrisHCl (pH 7.9)	40 mM
Sodium Acetate (CH_3COONa)	5 mM
EDTA	1 mM

Lysis Buffer (pH 8.0)

$NaH_2PO_4.H_2O$	50 mM
NaCl	300 mM
Imidazole	20 mM

Elution Buffer (pH 8.0)

$NaH_2PO_4.H_2O$	50 mM
NaCl	300 mM
Imidazole	250 mM

ACK Lysis Buffer

NH_4Cl	0.15M
$KHCO_3$	10mM
EDTA	0.1mM

3.2 Bacterial Strains, Media and culture conditions

3.2.1 *S. pyogenes* clinical isolates

Table 3.7: Selected *S. pyogenes* INFECT cases used in the study

Patient					Identified Pathogen	Isolated from	Treatment		Collection center
No.	Age	Gender	Co-morbidities	NSTI			IVIG	HBO	
2001	67	M	None	Yes	*S. pyogenes*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
2006	62	F	Hypertension tractatae	Yes	*S. pyogenes*	Blood	Yes	Yes	Rigshospitalet, Copenhagen
2015	70	F	C.Pulm 2012 Smoker	Yes	*S. pyogenes*	Blood	Yes	Yes	Rigshospitalet, Copenhagen
2017	38	F	None	Yes	*S. pyogenes*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
3005	65	F	Chronic obstructive lung Disease Hypertension	Yes	*S. pyogenes*	N.A	No	No	Karolinska University Hospital, Stockholm
3012	42	F	None	Yes	*S. pyogenes*	N.A	No	Yes	Karolinska University Hospital, Stockholm
5003	79	M	Malignancy colon Atrial fibrillation Chronic left leg edema	Yes	*S. pyogenes*	Blood	Yes	Yes	Sahlgrenska University Hospital Gothenburg

5004	41	F	None	Yes	*S. pyogenes*	Wound	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5006	37	M	None	Yes	*S. pyogenes*	Wound elbow Tissue right arm Wound right arm	Yes	No	Sahlgrenska University Hospital Gothenburg
6013	34	F	None	Yes	*S. pyogenes*	Blood Neck	Yes	Yes	University of Bergen Bergen
6016	41	M	None	Yes	*S. pyogenes*	Elbow aspiration	No	No	University of Bergen Bergen
6018	39	F	None	Yes	*S. pyogenes*	Axilla	No	No	University of Bergen Bergen
6025	37	M	Psoriasis	Yes	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6026	48	M	None	Yes	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6028	41	M	None	No	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6033	50	F	None	Yes	*S. pyogenes*	upper extremity	No	No	University of Bergen Bergen
6040	32	F	None	No	*S. pyogenes*	Upper Leg	No	No	University of Bergen Bergen

N.A: Not Available

3.2.2 Streptococcal Strains

Table 3.8: Streptococcal strains used in the study

Strain	HZI Number	*emm* type	Reference
***S. pyogenes* SF370**	A302	M1	Ferretti *et al* (2001)[90]
***S. pyogenes* MGAS315**	A570	M3	Beres *et al* (2002)[91]
***S. pyogenes* MGAS8232**	A571	M18	Smoot *et al* (2002)[92]
***S. pyogenes* AP-1**	A529	M1	Fiebig *et al* (2015)[93]
***S. gordonii* DL1**	GP202	-	Oggioni *et al* (1996)[94]

3.2.3 *E. coli* Strains

Table 3.9: *E. coli* strains used in the study

Strain	Plasmid	Gene Inserted	Selection marker
M15 [pREP4][§]	-	-	Kan
M15 [pREP4]	pQE30-TEV	-	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speA*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speC*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speG*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speH*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speI*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speJ*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speK*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speL*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*speM*	Amp (100µg/ml), Kan (25µg/ml)

Strain	Plasmid	Gene Inserted	Selection marker
M15 [pREP4]	pQE30-TEV	*ssa*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*smeZ*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*sic*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*ska-1*	Amp (100µg/ml), Kan (25µg/ml)
M15 [pREP4]	pQE30-TEV	*ska-2a*[#]	Amp (100µg/ml), Kan (25µg/ml)

[§]M15 [pREP4] cells were kindly provided by Guiseppe Gulotta but were initially bought from Quigen
ska-2a clone was kindly provided by Katharina Rox

The M15 strains contained a repressory system (pREP4) that inhibited any leaky expression of the exotoxins. This repressory plasmid contained a Kanamycin cassette and therefore M15 cells were propagated under Kanamycin.

3.2.4 Bacterial Cultivation

3.2.4.1 Media

All media used for bacterial cultivation was autoclaved prior to usage for 15 min at 121^{0}C and 15 psi above standard pressure. For growing bacteria on agar plates, 1.5% (w/v) agar was added to the required media before autoclaving.

Table 3.10: Media used for bacterial cultivation

Cultivation of *Streptococcus pyogenes*

THY Media

Todd Hewitt Broth	30 g/l
Bacto™ Yeast Extract	5 g/l

Cultivation of *Escherichia coli*

LB Media

Bacto™ Tryptone	10 g/l
NaCl	10 g/l
Bacto™ Yeast Extract	5 g/l

TSS Media

Polyethylene glycol (PEG 8000)	12.5 mM
Dimethyl Sulfoxide (DMSO)	700 mM
$MgCl_2$	20 mM

3.2.4.2 Cultivation of bacteria

Streptococcus pyogenes isolates were cultivated in THY-medium for 16 hours at 37°C without shaking. Bacteria were grown in sterile 50 ml falcon tubes with loose lids to allow air penetration. Working cultures were grown at 37°C until the desired optical cell density (ABS_{600nm}= 0.5; approximately 10^8 cfu/ml) for all assays unless stated otherwise. For solid media cultivation, isolates were grown on THY agar plates. Bacteria were stored at −80°C in THY medium containing 25% glycerol for long term storage.

E. coli strains were grown in LB Media containing appropriate antibiotics (Table 3.9). The strains were grown in conical flasks (with baffles) at 37^0C (or 30^0C after protein induction) with shaking at 150 rpm to increase aeration. Preparation of competent cells of M15 strain was performed using Transformation and Storage Solution (TSS) media. For long term storage, bacteria were stored at −80°C in LB medium containing 25% glycerol.

3.3 Molecular Biology Techniques

3.3.1 DNA Isolation

Bacterial genomic DNA was extracted from 15 ml THY overnight cultures. The cells were disrupted through Zirconia beads using a Fast Prep 24 device (MP Biomedical, USA) at 4 m/s for 30 sec. After removal of bacterial debris by centrifugation at 6000×g for 30 sec at room temperature, the DNeasy Blood and Tissue Kit (Qiagen, Germany) was used to extract the DNA from the lysate according to the manufacturer's instruction. The quality of the isolated DNA was examined by 1% agarose gel electrophoresis (section 3.3.3). The concentration of DNA was estimated by Nanodrop (peQLab Biotechnologie, GmBH) and adjusted to 1 μg in 1×TE for further use. Extracted DNA was stored at −20°C.

3.3.2 Plasmid Isolation

Plasmid DNA from *E. coli* strains was isolated with QIAprep® Spin Miniprep kit according to the manufacturer's protocol. Plasmid DNA was eluted in MilliQ and stored at −20°C until used. Isolated plasmid DNA was analyzed by agarose gel electrophoresis (section 3.3.3).

3.3.3 Agarose Gel Electrophoresis

The nucleic acid samples were analyzed by electrophoresis using 1% Agarose gels. Agarose gels were prepared by dissolving 1 g Agarose in 100 ml of 1×Tris-Acetate-EDTA (TAE) buffer by heating to boil. When temperature reaches around 50°C, the Agarose solution was poured into a casting tray with combs. The gel was allowed to solidify for 30 min at room temperature, combs were removed and the gel was placed into an electrophoresis chamber and covered with 1×TAE. A commercial 1 kb nucleic-acid size standard (Gene Ruler 1kb ladder, Fermentas) was added to estimate the molecular sizes of the nucleic acid samples. 5 μl of sample was mixed with 1 μl DNA loading dye and seperated for 1 hour at 90 Volt/cm. The gel was stained by immersing it in 0.5 μg/ml Ethidium Bromide (EtBr) solution for 10 min at room temperature with gentle agitation. Distilled water was used for destaining of the gels. The bands were visualized using an ultraviolet (UV) trans-illuminator.

3.3.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR), developed by Kary Mullis[95], is a technique used to amplify a specific DNA fragment using repeated cycles of DNA melting, hybridization of specific primer molecules and DNA replication *in vitro*. This process is generally carried out by heat stable DNA polymerase (like Taq polymerase; enzyme isolated from *Thermus aquaticus*) in presence of deoxy-ribonucleotides (dNTPs) and sequence specific primers. PCR was used throughout this study to amplify specific genes during the screening processes, cloning and sequencing analysis. Appropriate positive (containing the gene of interest) and negative controls (MilliQ) were used to verify the accuracy of the results.

3.3.4.1 Standard PCR

Amplification of specific genes from bacterial genomic DNA was performed using Primers as listed in **Table 3.3**. The PCR mixture consisted of 3 µl of template DNA (approximately 1 µg), 1×PCR Buffer containing 15 mM $MgCl_2$, dNTP-mix (10 mM per dNTP), 10 nmol of each primer, 1 unit of Taq DNA polymerase (Qiagen, Cat No. 201203) filled with PCR grade water to a final volume of 25 µl. The standard reaction protocol has been listed below (Table 3.11)

Table 3.11: Standard PCR Program

Step	Temperature	Time
Initial Denaturation	95^0C	5min
Denaturation	95^0C	1 min
Annealing	T^0C	1 min
Extension	72^0C	1 min
Final Extension	72^0C	10 min
No. of cycles	27	

***emm* typing**: Amplification of *emm* genes was performed using emmA and emmB primers (Table 3.3). The PCR was performed in accordance to the standard protocol of the Center for Disease Control and Prevention, CDC (http://www.cdc.gov/streplab/m-proteingene-typing.html). The PCR products were sequenced and *emm* type was allotted by strepBLAST (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp).

16S rRNA gene PCR: 16S rRNA gene from all bacterial isolates was amplified using primers 16s-FP and 16s-RP (Table 3.3). The annealing temperature used was 54^0C. A minimum of 1000 base pairs were covered. The PCR products were sequenced using Capillary Sequencing. NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for bacterial species classification.

***ska* sequencing PCR**: Variable region from *ska* were amplified at 50°C annealing temperature. The PCR products were sequenced using Capillary Sequencing. All the sequences were trimmed using BioEdit and a dendogram was prepared by ClustalW[96] analysis.

3.3.4.2 Multiplex PCR

Bacterial genomes were tested for the presence or absence of 21 genes coding for streptococcal exotoxins using a multiplex PCR system that consisted of five independent reactions. The PCR was established based on previous protocols[88,89] and was extended to the NSTI isolate collection. Primers are given in Table 3.3 whereas the controls used and the PCR conditions are given in Table 3.12 and Table 3.13 respectively. For Reaction-1, 2 and 3, Go Green Taq® DNA polymerase (Promega, Cat. No. M3178) was used whereas for reaction 4 and 5, Recombinant Taq DNA Polymerase (Fermentas, Cat. No. EP0402) was used. Genomic DNA of already genome sequenced *S. pyogenes* strains (SF370, MGAS315 and MGAS8232) was used as positive controls whereas, genomic DNA of *Streptococcus gordonii* strain Challis GP204 was used as a negative control in all of the reactions. Since one strain did not carry all the exotoxins amplified in one reaction, a combination of two strains was sometimes used as control.

Table 3.12: Multiplex PCR

Reaction	Gene	Size [bp]	*S. pyogenes* Controls	Reference
1.	*speF*	1,193	Sf370 MGAS315	Friães *et al* (2013)[88]
	speJ	386		
	speH	545		
	ssa	706		
	smeZ	628/629/616		
2.	*speA*	202	Sf370 MGAS8232	Friães *et al* (2013)[88]
	speB	300		
	speI	411/412		
	speL	460		
3.	*speC*	660	MGAS315 MGAS8232	Friães *et al* (2013)[88]
	speK	478/479		
	speM	587		
	speG	340		
4.	*sdc*	600	Sf370 MGAS315	Borek *et al* (2011)[89]
	sdaB	440		
	sdaD	295		
	spd3	784		

Reaction	Gene	Size [bp]	*S. pyogenes* Controls	Reference
5.	*sic*	150	Sf370	Borek *et al* (2011)[89]
	mac	389		
	spyCEP	786		
	scpA	622		

Table 3.13: PCR Program used in the Multiplex

Steps	Reactions									
	1		2		3		4		5	
	T	t	T	t	T	t	T	t	T	T
Initial Denaturation	95°C	05:00	95°C	05:00	95°C	05:00	95°C	03:00	95°C	03:00
Denaturation	95°C	01:00	95°C	01:00	95°C	01:00	95°C	00:15	95°C	00:15
Annealing	54°C	00:30	62°C	00:30	58°C	00:30	60°C	00:20	52°C	00:45
Extension	72°C	01:30	72°C	01:30	72°C	01:30	72°C	02:00	72°C	02:00
Final Extension	72°C	10:00	72°C	10:00	72°C	10:00	72°C	10:00	72°C	10:00
No. of cycles	40		40		40		40		40	

t = time (in min); T = Temperature

3.3.5 Preparation of Competent *E. coli* cells

Competence is the ability of bacterial cells to take up extracellular DNA. Competence can be natural (genetic ability of bacteria under natural conditions) or artificial. Artificial competence arises by exposing the cells to divalent cations (like Mg^{2+} or Ca^{2+}) in cold conditions that may change or weaken the cell surface structure, making it more permeable to DNA. The heat-shock (42°C for 90 sec) is thought to create a thermal imbalance on either side of the cell membrane, which forces the DNA to enter the cells through either cell pores or the damaged cell membrane. Competent cells were prepared for cloning of exotoxins in *E. coli. E. coli* M15 was grown in TSS media supplemented with 25 µg/ml kanamycin (Table 3.10) till mid-exponential phase (ABS_{600nm} = 0.4) at 37°C with shaking at 150 rpm for sufficient aeration. The flask was

incubated on ice for 10 min and then centrifuged at 4000×g for 10 min at 4°C. The pellet was resuspended in 20ml fresh TSS media. 100µl aliquots were prepared in 1.5ml micro-centrifuge tubes and shock-frozen in liquid nitrogen. The aliquots were stored at −80°C until further used.

3.3.6 Molecular Cloning

Molecular cloning is a technique to recombinantly express and purify a specific protein in a different background than the cell originally producing it. To identify antibodies against streptococcal exotoxins in NSTI patient plasma samples, a set of selected streptococcal exotoxins (n=14) along with the empty vector (background control) were cloned, over expressed and purified according to the outline mentioned below.

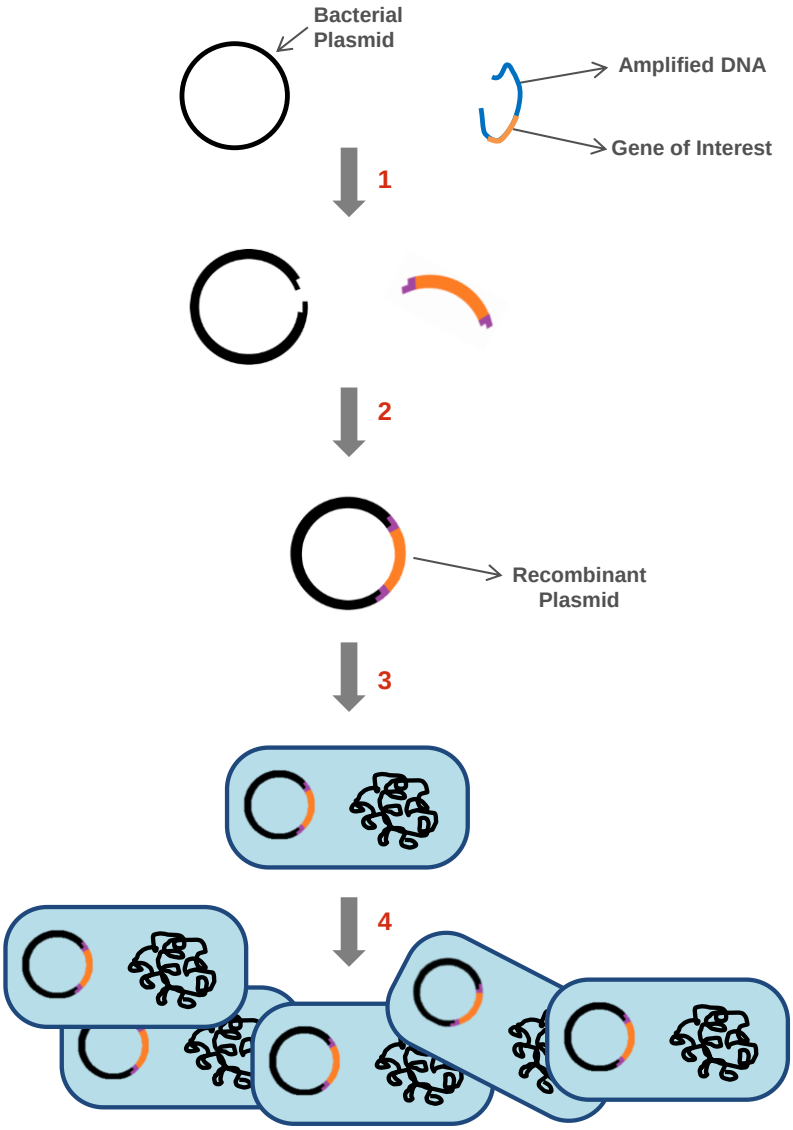

Figure 3.1: Outline of the cloning protocol. The vector (bacterial plasmid) and insert (gene of interest) are cut with restriction enzymes (1) to generate sticky ends. Ligation (2) of the sticky ends produces the recombinant plasmid. This plasmid is then transformed (3) in *E. coli* cells. Cell division (4) results in many identical copies of bacterial cells, all carrying the recombinant plasmid.

Step-1: PCR Amplification of the Insert

The gene of interest was amplified using standard PCR program (section 3.3.4.1). The size of the PCR product was confirmed by agarose-gel electrophoresis. The primers, product size and PCR program for cloning of superantigens are listed in Table 3.3. For removal of unused dNTPs and primers, PCR products were purified using Nucleospin® Gel and PCR clean up kit from Macherey-Nagel following manufacturer's instruction.

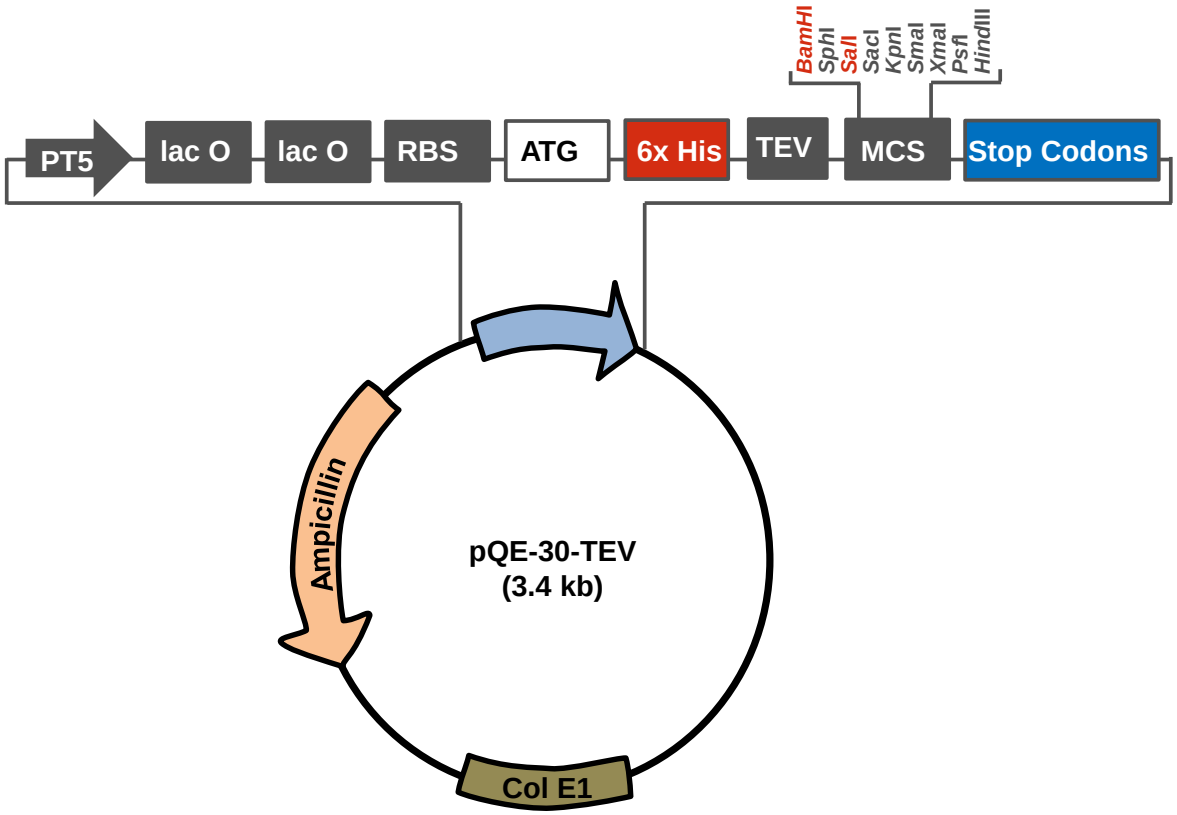

Figure 3.2: Map of the vector pQE-30 TEV. The vector pQE30 is modified to introduce a 6×His Tag cleavage site named as TEV[97]. The vector contains an Ampicillin resistance cassette. PT5 stands for Promotor region; lac O is lac operon site (that is inducible by IPTG); RBS is the Ribosome binding site and MCS is a multiple cloning site. ATG demarcates the start codon (Methionine). ColE1 is the site of origin of replication.

Step-2: Restriction Digestion

The restriction enzymes BamHI and SalI were selected to digest insert and vector pQE30-TEV in order to produce 'sticky ends' for ligation. The reaction mix consisted of 10 µl (around 1 µg) of template DNA, 1×BamHI-SalI buffer, 2 µl of each restriction enzyme filled with water to 35 µl. The restriction mix was incubated at 37°C for 1 h. The digested fragments were purified using Nucleospin® Gel and PCR clean up kit from Macherey-Nagel. A 1% agarose gel was used to check the digestion using uncut vector and vector cut with only BamHI and SalI as controls.

Step-3: Ligation and Transformation

The double digested vector and the insert were mixed in a ratio of 1:4 along with 1× T4 Ligase buffer and 1 µl of T4 Ligase to obtain a final volume of 11.5 µl. The mixture was incubated for 16 h at 16°C to allow the generation of the recombinant plasmid by ligation of generated 'sticky ends'. The ligated product (Recombinant plasmid) was added to 100 µl of prepared M15 competent cells (Section 3.3.5). The cells were incubated on ice for 30 min. Subsequently a heat shock at 42^0C for 90 seconds was applied and 700 µL of fresh LB broth was added. This culture was incubated at 37^0C for shaking for 1 h. Afterwards, it was centrifuged at 7500×g for 3 min at room temperature. 750 µL of the supernatant was discarded and the bacteria were spread on LA-Amp-Kan plate (Transformation Plate) and incubated for 16 h at 37^0C.

Step-4: Identifying positive Clones

The colonies from the transformation plate were transferred to a fresh LA-Amp-Kan plate (Master Plate) for further processing. The selection of positive clones was performed by **Colony PCR**. Therefore a single colony was picked from the antibiotic selection plate and dissolved in 30 µl autoclaved MilliQ. The sample was incubated at 95°C for 15 min to lyse the cells. 10 µl of this sample was used as a template for PCR. PCR was performed by the protocol mentioned below (Table 3.15) using the standard PCR program (Table 3.10)

Table 3.14: PCR Program used for Colony PCR

5× Taq Buffer containing $MgCl_2$	5 µl
10 mM dNTPs	0.6 µl
10 nmol Forward Primer	0.6 µl
10 nmol Reverse Primer	0.6 µl
5U/µl Taq Polymerase	0.2 µl
1 µg Template	10 µl
dH_2O	Up to 25 µl

The positive clones were picked from the master plate and were inoculated in 5 ml LB media containing Ampicillin (100 µg/ml) and Kanamycin (25 µg/ml). The plasmid was

isolated as mentioned in Section 3.3.2. The plasmids were analyzed by sequencing (Capillary Sequencing). Positive clones with the 100% similarity to the gene of interest were stored in a cryo-stock containing 25% glycerol at −80°C.

Step-5: Protein Over-expression and Purification

Since the applied cloning strategy allowed attaching a His-tag to the proteins of interest, their purification was done by Affinity Chromatography. Briefly, the constructed *E. coli* clones carrying the exotoxin genes within the pQE30-TEV vector were inoculated in 500 ml LB media containing Ampicillin (100 µg/ml) and Kanamycin (25 µg/ml) for 3 h at 37°C with shaking at 150 rpm for sufficient aeration. The proteins were induced for 16 h at 30°C with 1mM IPTG at mid-log phase (ABS_{600nm} = 0.4). For SpeG, the induction time was reduced to 4 h as a longer incubation time induced an insolubility of the over-expressed protein. Afterwards the bacterial cells were pelleted by centrifuging at 6000×g for 10 min at 4°C and resuspended in 10ml of Lysis Buffer (Table 3.7). The cells were lysed thrice by French® pressure cell press (18,000 psi) and centrifuged at 11000×g for 15 min at 4°C. The supernatant was loaded onto Ni-NTA columns to allow binding of the protein via the His-tag to the column matrix. The column was washed thrice with 5 ml of 20mM Imidazole (Washing Buffer same as Lysis Buffer, Table 3.7) and the protein was eluted by 3 ml of 200 mM Imidazole (Elution Buffer; Table 3.7). The eluted protein was dialyzed using a 3 kDa cut off membrane at 4°C for 16 h for buffer exchange with PBS.

3.4 Biochemical Techniques

3.4.1 Discontinuous Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (DISC SDS-PAGE)

SDS-Polyacrylamide Gel Electrophoresis (PAGE) is a method to separate proteins based on their molecular size. Sodium dodecyl sulfate (SDS) is an anionic detergent. Binding of SDS imparts uniform charge per unit mass to the proteins thus rendering the separation solely on the basis of molecular mass. Addition of β-mercaptoethanol or dithiotretol (DTT) further denatures all di-sulphide bonds present within a protein. Thus, due to the negative charge of SDS, proteins migrate towards the anode (the positive electrode) and are separated based on their molecular weight and independent of their charge. Large proteins are retained within the gel matrix and migrate slower than smaller proteins with lower molecular mass. DISC-PAGE is used in this study as pH values within the gel vary to change the average charge of the counter ions during the run to improve resolution. Proteins are first concentrated in a low percentage

stacking gel (5% acrylamide) with a pH of 6.8 and then they migrate into a higher percentage resolving gel (10% acrylamide) with a pH of 8.8 in which they are separated according to their size. Approximately, 5 ml of resolving gel (10% acrylamide) and 2 ml of stacking gel (5% acrylamide) were prepared for electrophoresis. For SDS-PAGE analysis, the protein samples were mixed with sample loading buffer in a ratio of 5:1 and kept at 94°C for 5-10 min. The samples were loaded on the SDS gel and separated using Mini-Protean3 cell (BioRad) by applying 30 mA (constant current). A PageRuler™ Prestained Protein Ladder (10 to 250 kDa, Thermo Fischer) was loaded as a standard for molecular mass. Following electrophoresis, the gel was either stained with Coomassie Brilliant Blue R-250 (Section 3.4.1.1) or used for western blot analysis as described in section 3.4.2.

SDS Loading Buffer

0.2 M Tris-Cl (pH 6.8)
0.02% β -Mercaptoethanol
3 % SDS
30 % Glycerol
0.2 % Bromphenolblue

Resolving Gel

10 % Acrylamide/Bisacrylamide (30:0.8)
0.37 M Tris/HCl (pH 8.8)
0.1 % SDS
0.5 µl/ml TEMED
0.5 mg/ml APS

Stacking Gel

5 % Acrylamide/Bisacrylamide (30:0.8)
0.125 M TrisHCl pH 8.8
0.1 % SDS
0.5 µl/ml TEMED
0.5 mg/ml Ammonium peroxodisulfate (APS)

5X Running buffer

25 mM Tris
192 mM Glycine
0.1% SDS

3.4.1.1 Coomassie Staining

To visualize proteins on an SDS-PAGE gel, the gel was stained for 3 h in Coomassie Brilliant Blue R-250 staining solution. The gel was washed with dH_2O and incubated in destaining solution for 2 h. Gels were scanned using a HP DeskScan II 6100C/T and analyzed using GIMP 2.6.1.

Coomassie stain solution

0.25 % (v/v) Coomassie Brilliant Blue R-250
50 % (v/v) Methanol
10 % (v/v) Acetic acid
Added to distilled water

Destaining Solution

20 % (v/v) Methanol
7 % (v/v) Acetic acid
Added to distilled water

3.4.2 Immuno-Western Blot Analysis

For specific detection of individual proteins, immune-western blot analysis was performed. Proteins were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (BioRad) in transfer buffer at 100 V for 1 h at 4°C using a MiniTrans-Blot cell (BioRad). The protein transfer was confirmed by staining the nitrocellulose membrane with Ponceau S as described in section 3.4.2.1. After washing thrice with 25 ml TBST (TBS in 0.05% Tween 20), the membrane was incubated for 2 h with 25 ml of blocking buffer (5% skim milk dissolved in TBST) at room temperature to block non-specific binding sites on the membrane. Subsequently the membrane was incubated for 16 h at 4°C with 5 ml primary antibody, diluted 1:1000 in TBST containing 5% skim milk. Afterwards the membrane was washed twice with 20 ml TBST and incubated with 5 ml HRP conjugated secondary antibody (diluted 1:1000 in TBST containing 5% skim milk) for 1 h at room temperature. The blot was washed three times with 20 ml TBST for 10 min each and immune-reactive proteins were detected by X-ray films using ECL Western Blotting Detection Kit according to the manufacturer's instruction (Amersham).

Transfer Buffer

50 mM Tris-Base
40 mM Glycine
20 % Methanol (v/v)

Blocking Buffer

5 % (w/v) Skim milk
Dissolved in TBST

3.4.2.1 Ponceau Staining

To verify the protein transfer efficiency and immobilize the proteins on the nitrocellulose, the membrane was stained with 20 ml Ponceau S stain for 5 min. Ponceau S is a sodium salt of a diazo dye of light red color. The staining of the membrane with Ponceau S allows the reversible staining of proteins on a nitrocellulose membrane by ionic interactions. The membrane was rinsed with 20 ml TBST until the signals disappeared and probed for western blot analysis as described in section 3.4.2.

Ponceau stain solution

0.2 % (v/v) Ponceau S
3 % (v/v) Trichloroacetic acid
3 % (v/v) Sulfosalicylic acid

3.4.3 Estimation of Protein Concentrations

The concentration of purified proteins was determined by Bradford Assay[98]. Briefly, 40 µl of Bradford solution (BioRad) was added to 10 µl of a protein solution in a microtiter plate; double distilled water was added to a total volume of 200 µl. The absorption at 595 nm was measured using an ELISA-Reader (Tecan Sunrise) without any prior incubation. The standard curve was plotted by generating a serial dilution standard curve using BSA. The absorbance of known concentrations of BSA was measured at 595 nm and the amount of protein in the solution was estimated by comparison with the standard.

3.4.4 IgG Depletion

To analyze the role of IgG mediated immunity during the development of NSTI, a pool of commercially available human plasma was depleted of IgG to serve as negative control for further experiments. For this, pooled human Li-Heparin Plasma (Innovative Research Inc.) was centrifuged at 14,000×g for 10 min at room temperature prior use to remove freezing induced precipitates of fibrinogen which may hinder the depletion process. The removal of the IgG content was performed using Sepharose Protein G beads (GE Healthcare). 1 ml of the resin was mixed with 2 ml of plasma and incubated at room temperature for 1 hour with continuous gentle agitation. The supernatant was collected, the beads were washed with 5 ml PBS and IgG was eluted using 2 ml of 0.2 M glycin. The efficiency of depletion was checked by western blotting as described in section 3.4.3

3.5 Immunological Techniques

3.5.1 Typing for Surface Antigen

Streptococcal isolates were streaked on a Blood Agar Plate (Columbia Blood Agar with 5% Sheep Blood, Thermo Scientific) and kept for 16 h at 37°C. A single colony was picked to determine the surface antigen type using a commercial SLIDEX kit (Oxoid, Basingstoke, UK) according to manufacturer's instruction.

3.5.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a common laboratory technique that can be used to measure the concentration of an analyte in solution by using enzyme immunoassays. ELISA is a multi-step process that involves at least one antibody with specificity for a particular antigen. The protein (either an antigen or an antibody) is immobilized by adsorption to a polystyrene 96 well plate. Coating is followed by blocking wherein all the unoccupied surfaces in the well are captured by either BSA or skim milk. The detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After a final washing step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. The established ELISA protocol is listed in Table 3.15.

Table 3.15: Established ELISA protocol

STEP	PROTOCOL
Coating	50 µl of 3 µg/ml protein in TBS was added per well and incubated for 16 h at 4°C
Washing	Each well was washed three times with 300 µl TBST
Blocking	250 µl 5% BSA in TBS was added to each well and incubated at 37°C for 1 hour
Washing	Each well was washed three times with 300 µl TBST
Primary Antibody	50 µl of each plasma sample (1:50 dilution plasma in TBS with 5%BSA) was added and incubated overnight at 4°C

Washing	Each well was washed three times with 300 µl TBST
Secondary Antibody	50 µl of secondary antibody in TBS (1:1000 dilution) with 5% BSA was added to each well and incubated at 37°C for 1 hour
Washing	Each well was washed three times with 300 µl TBST
Developing	200µl ABTS (800 µg/ml with 0.1% H_2O_2) was added to each well and incubated for 30 min at 37°C
Detection	The Absorption at 416nm was determined using a suitable plate reader (Varioscan, ScanLab)

For one serum sample, antibody titers against all selected exotoxins were tested in two seperate plates: one for superantigens and other for the second set of exotoxins. The plate layout is displayed in Figure 3.3. Empty vector purification was diluted in the same ratio as the corresponding superantigen and served as the 'background' control. The amount of coated toxin was verified by the use of an Anti-His antibody that served as the 'Toxin coating control'. The obtained absorbance values of antibodies in INFECT patient plasma against toxins and empty vector purification were normalized within different days using the absorbance values obtained from the 'Toxin coating control'. For statistical significance analysis, ANOVA test was applied between absorbance values obtained from toxins and empty vector purification ($p<0.05$, $n=3$).

Out of the four commercially available exotoxins (SpeB, Mac, SLO and Hyl), SpeB and Mac were His-tagged and hence an Anti-His antibody was used to generate 'Toxin coating control' as in described for the purified superantigens. An Anti-SLO and Hyl antibodies were used to control the coating efficiency.

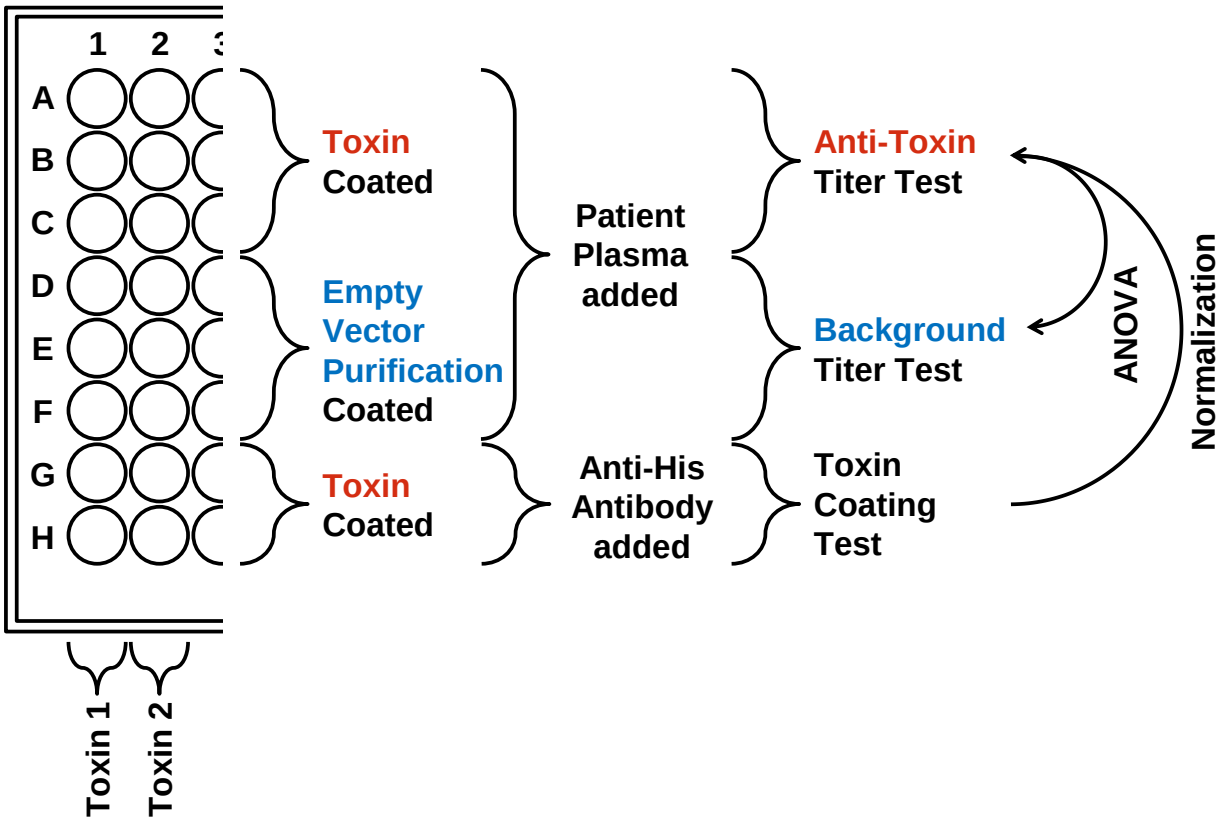

Figure 3.3: ELISA plate layout. Each column of the 96 well plates was coated with a different toxin. Same dilutions of the empty vector purified proteins were coated (row D-F) for each toxin to serve as the 'Background titer test'. For primary antibodies, row A-F was incubated with INFECT patient plasma whereas row G-H was incubated with Anti-His antibody for checking the efficiency of the coating. The obtained absorbance values were normalized against the absorbance values of 'Toxin coating test' and statistical significance test was done by applying ANOVA between normalized values of 'Anti-toxin titer test' and 'Background titer test'.

3.5.3 T-cell Proliferation Blocking Assay

A T-cell proliferation Assay was established to determine the protectivity of detected antibodies within the patient plasma samples against superantigens. Lymphocytes were isolated from Buffy coat, a fraction of anti-coagulated blood that contains leukocytes and platelets. The buffy coats were collected in accordance with rules of the Regional Ethics Committee of Lower Saxony, Germany and the declaration of Helsinki. Buffy coats from blood donations of healthy human volunteers who provided informed consent were obtained from the Institute for Clinical Transfusion Medicine, Klinikum Braunschweig, Germany. Buffy Coats were produced from whole blood donations on day 1 by using the Top & Bottom Extraction Bag System (Polymed Medical Devices™, Triple Blood Bag System, No. 7300; containing CPDA-1. 20 ml Ficoll Reagent (GE life Sciences) was poured in two separate falcon tubes and 30ml buffy coat were carefully layered on the top of the Ficoll fraction. The tubes were centrifuged for 20 min at 450×g at room temperature.

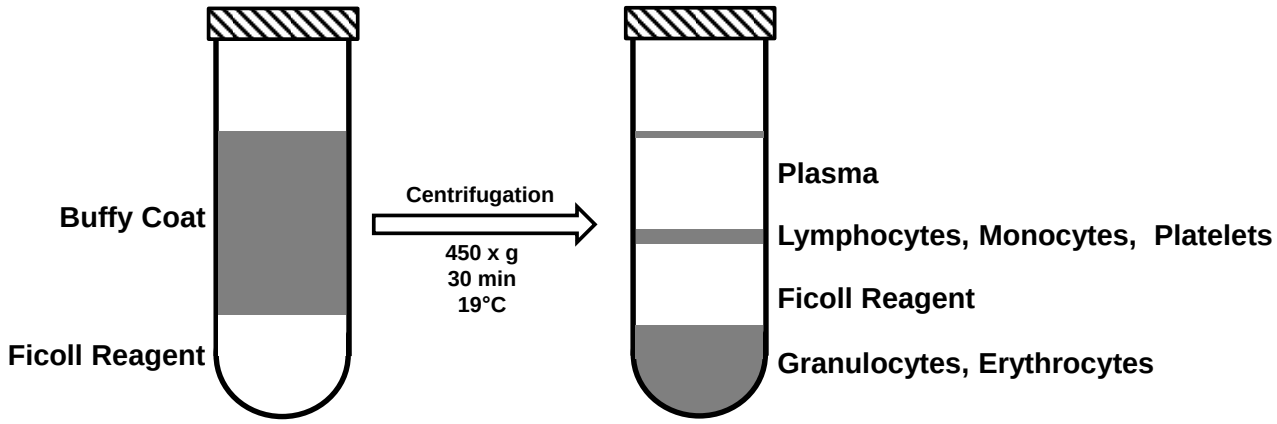

Figure 3.4: Ficoll gradient. Buffy coat was carefully layered on top of Ficoll regent and centrifuged at 450×g for 30 min at 19°C. Different fractions of blood are separated based on their densities and have been labelled beside the tube.

The gradient is formed as depicted in Figure 3.4. The cell layer containing lymphocytes, monocytes and platelets was transferred to a new Falcon tube containing 50 ml 1×PBS. The cells were washed at 400×g for 10 min. The supernatant was discarded carefully and the washing step was repeated two times. Erythrocyte contaminations in the lymphocyte fraction were removed by ACK buffer lyses (10ml, 37°C for 10 min) (section 3.1.7). The cells were washed two times with 50 ml PBS at 400×g for 6 min to remove erythrocyte cell debris. The purified lymphocytes were resuspended in RPMI containing 10% FCS and counted using Neubauer Cell Counter under Axiovert 25 microscope (Zeiss) after staining with Trypan Blue. The cell density of purified cells was adjusted to 2×10^6 cells/ml, 100 µl aliquoted in 96 well plate according to the plate layout described in Figure 3.5.

In a 96 well cell culture plate, a concentration gradient of superantigen was prepared in RPMI to determine the optimal concentration with maximum T-cell proliferation. To analyze protection potential of antibodies against the unspecific stimulation of T-cell proliferation of purified superantigens, 20% plasma (Day 0 and 3) diluted in PBS was incubated together with different superantigen concentrations and incubated at 37°C for 1 hour. 100µl of 2×10^6 cells/ml of isolated T-cells were subsequently added to the well and the plate was incubated at 37°C with 5% CO_2 for 6 days for T-cell activation and proliferation. IgG depleted plasma and IVIG served as the negative and positive control respectively.

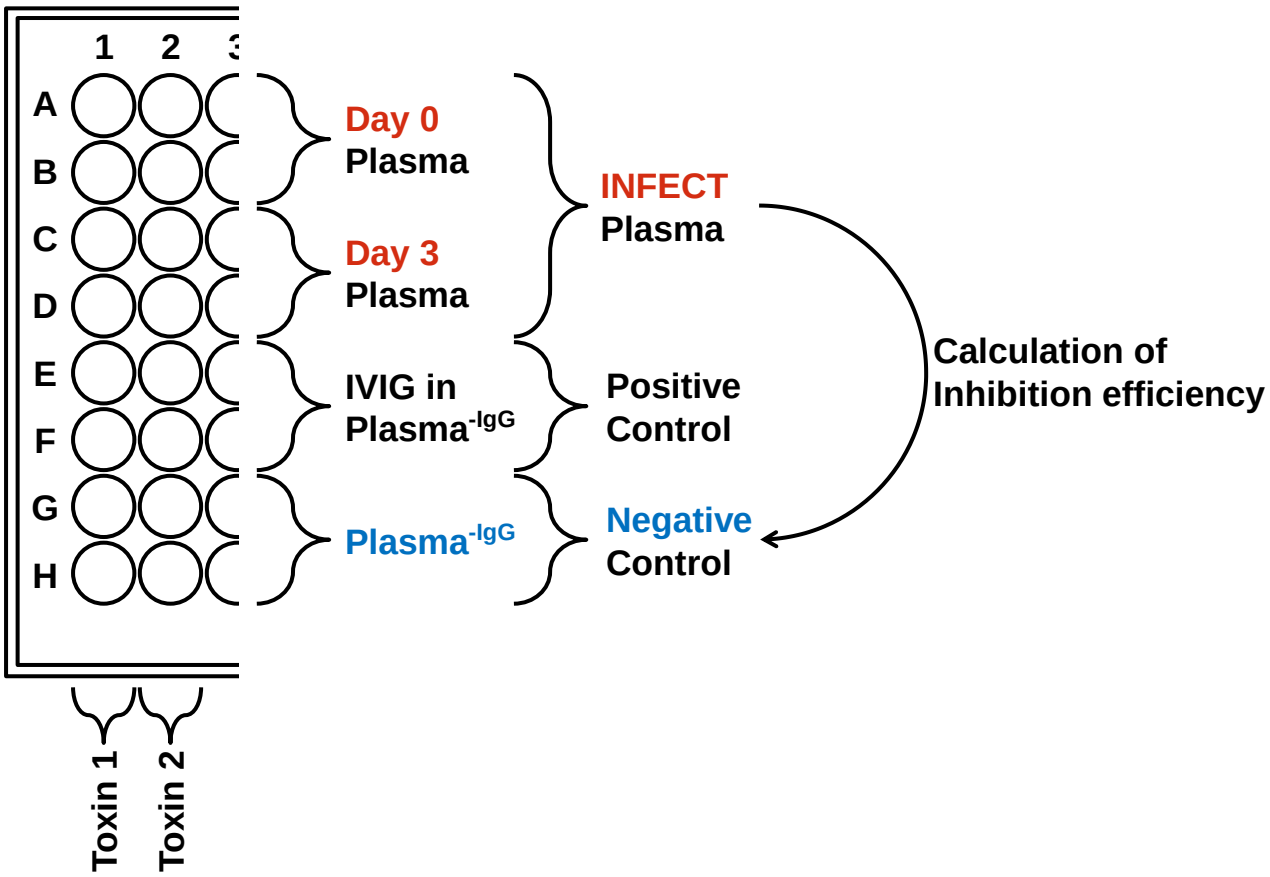

Figure 3.5: Plate Layout for T-Cell Proliferation Assay. Each column of the 96 well plates was tested with a different toxin. Defined concentrations of superantigens were incubated with 20% INFECT plasma (Day 0 plasma in row A-B and Day 3 plasma in row C-D) diluted in PBS and were kept at 37°C for1 h. Superantigens incubated with IVIG (row E-F) and IgG depleted plasma (row G-H) served as positive and negative controls. 100µl of 2×10^6 cells/ml of isolated T-cells were subsequently added to the well and the plate was incubated at 37°C with 5% CO_2 for 6 days for T-cell activation and proliferation. The inhibition efficiency was calculated by taking T-cell proliferation in untreated (negative control) as 100%, as mentioned in the figure.

For detecting the extent of T-cell proliferation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the wells to reach a final concentration of 1 mg/ml. MTT is a yellow tetrazole that is converted to purple formazan in living cells by NAD(P)H-dependent cellular oxidoreductase enzymes. The cells were further incubated for 3 hours at 37°C with 5% CO_2, and afterwards centrifuged at 3000×g for 10 min at 19°C. The supernatant was carefully removed and 100µl DMSO was added to dissolve the formazan crystals into a colored solution. The cells were incubated for 15 min at room temperature and the absorbance of the colored solution was measured at 560 nm with reference wavelength of 670 nm.

For statistical significance analysis, Student t-Test was applied between absorbance values obtained from determining proliferation by superantigens incubated in INFECT patients plasma or IgG depleted plasma ($p<0.05$, $n=3$). Percentage inhibition was

calculated by considering proliferation of T-cells by superantigens incubated in IgG depleted plasma as 100%.

3.5.4 Opsonophagocytosis Assay

To investigate if the antibodies against the bacteria itself would mediate efficient opsonization and subsequent killing, opsono-phagocytosis assay was established. Blood was always taken freshly from right median cubital vein of healthy mid-aged volunteers (Ethical Approval Number BO/07/2013) using Sodium Heparin Vacutainer® and Safety-Lok™system (BD Biosciences). *Streptococcus pyogenes* was grown as described in section 3.2.4.2 until mid-logarithmic growth phase (ABS_{600nm} = 0.4), washed twice with sterile PBS at 2000×g for 3 min and adjusted to ABS_{600nm} = 0.5 (~5 $x10^8$ cfu/ml) in PBS. 10^8 bacterial cells of a selected INFECT isolate were added to 1 ml of the corresponding INFECT plasma sample (20% in 1×PBS) and incubated at 37°C for 30 min with gentle agitation on test tube rocker (Thermo Scientific Vari Mix). Bacterial cells incubated in IgG depleted plasma with and without 20% IVIG diluted in PBS served as the positive and negative control respectively. Thereafter, the bacterial cells were washed twice with sterile PBS and diluted to $2×10^6$ cfu/ml in RPMI with 10% FCS. To analyze if the patient antibodies mediate efficient opsonization of bacterial cells, *in-vitro* phagocytosis assays were performed[99]. Human neutrophil granulocytes were isolated from fresh human blood using PolyMorphPrep™ system (Axis-Shield) following the manufacturer's instruction. The purified cells were resuspended in RPMI (with 10% FCS) and adjusted to $2×10^6$ cells/ml by trypan blue dye exclusion method using an Improved Neubauer chamber and Axiovert 25 microscope (Zeiss). 1 ml of RPMI (with or without isolated neutrophils) was infected with $2×10^6$ cfu/ml of bacteria (MOI=1) for 3 h and incubated slowly rolling in an Hybridization Oven (Model 1012, Shel Lab) at 37°C and 6 rpm. At hourly intervals, 50 µl aliquots were taken and added to 450 µl sterile PBS. 5 sec low power ultrasonic treatment (LabSonic U) was performed to disrupt bacterial aggregates as well as blood cells and samples were analyzed for remaining bacterial cell density on ten-fold serial dilution series in sterile PBS. Triplicates of 10 µl of each dilution were spotted on THY Agar plate and kept at 37°C for 16 h. The colonies were counted using the SZ TrinocularStero Microscope (Olympus) and cfu/ml was calculated.

4. Results

The human immune system constitutes the major line of defense against invading bacterial pathogens. During an infection event, the adaptive immune system is able to boost the innate immune response by specific antibodies that lead to complement-mediated opsonization of bacterial cells via the classical pathway to increase recognition and phagocytic clearance as well as neutralizing secreted bacterial toxins. Consequently the lack of specific antibodies against the invading bacterial pathogen and/or against a certain combination of bacterial toxins could represent a risk factor for the development of a severe NSTI.

4.1 Characterization of Bacterial Isolates

4.1.1 Selection and Typing of Isolates

To investigate the role of the serological background within the NSTI scenario, cases of Necrotizing Fasciitis were recruited and the corresponding bacterial isolates were collected in Rigshospitalet (Copenhagen), Karolinska University Hospital (Stockholm), Sahlgrenska University Hospital (Gothenburg) and University of Bergen (Bergen) under the European Union funded project named INFECT (http://www.fp7infect.eu/). Additionally control cases were enrolled wherein patients had suffered from severe skin and tissue infections that did not develop a necrotic stage. The bacterial isolates were obtained either by swabbing from the site of infection or through blood collected from infected patients. A total of 163 INFECT cases were collected over the period of two years (2013-2015), wherein, 59 cases were identified to be caused by members of the genus *Streptococcus*. To validate the bacterial species of the collected isolates, 16s rDNA sequence comparison was performed. INFECT Isolates identified as members of the genus *Streptococcus* were further analyzed by specialized typing methods like hemolysis screening and surface antigen (SLIDEX) determination. Furthermore, all streptococcal cases were characterized based on their M-protein. The M-protein is a surface bound virulence related protein that was first used in 1946 for characterizing and typing various *S. pyogenes* isolates[100]. Later, homologues of the M-protein were also found in *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) isolates[101]. PCR amplification and sequence database driven comparisons of sequences of various *emm* genes enabled assigning different M-types to the isolates (*emm* typing) (**Figure 4.1**).

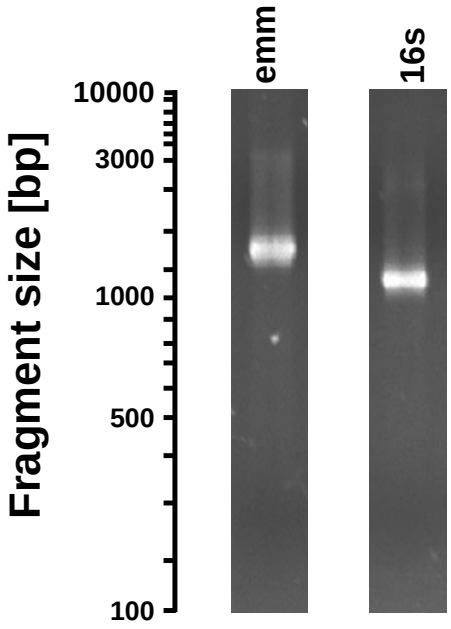

Figure 4.1: Agarose gel picture of PCR amplified *emm* and 16s rDNA genes from genomic DNA of NSTI isolate 2001 for sequencing. The *emm* gene and 16s rRNA gene were amplified from the genomic DNA of INFECT isolates for capillary sequencing. The PCR products were analyzed on 1% agarose gel, stained with Ethidium Bromide. The separation of DNA was based on the length/size of the PCR product. Size of the bands was estimated by comparison with standard 1 kb GeneRuler.

All generated typing results were compiled into a database, which enabled a comprehensive overview about the species and *emm*-type distribution of all streptococcal NSTI and non NSTI control cases enrolled within the collection time of two years and constitutes the starting point for the selection process towards further serological analyses (**Table 4.1.1**).

Table 4.1.1: Typing results of streptococcal INFECT Isolates

Number	Hemolysis	Slidex	16s rDNA sequencing	M-type
2001	beta	A	*S. pyogenes*	M1
2006	beta	A	*S. pyogenes*	M1
2015	beta	A	*S. pyogenes*	M87
2017	beta	A	*S. pyogenes*	M28
2028	beta	A	*S. pyogenes*	M3
3005	beta	A	*S. pyogenes*	M89
3012	beta	A	*S. pyogenes*	M77
5003	beta	A	*S. pyogenes*	M77

Number	Hemolysis	Slidex	16s rDNA sequencing	M-type
5004	beta	A	*S. pyogenes*	M28
5005	beta	G	*S. dysgalactiae* ssp. *equisimilis*	stG485.0
5006	beta	A	*S. pyogenes*	M1
5006	beta	A	*S. pyogenes*	M1
5006	beta	A	*S. pyogenes*	M1
6003	beta	A	*S. pyogenes*	M1
6003	alpha	C	*S. anginosus*	-
6004	beta	A	*S. pyogenes*	M3
6006	beta	C	*S. dysgalactiae* ssp. *equisimilis*	stG62647
6007	beta	G	*S. dysgalactiae* ssp. *equisimilis*	stG2078.0
6008	alpha	-	*S. constellatus*	-
6009	alpha	-	*S. anginosus*	-
6012	beta	F	*S. dysgalactiae* ssp. *equisimilis*	stG62647
6012	alpha	-	*S. oralis*	-
6013	beta	A	*S. pyogenes*	M1
6013	beta	A	*S. pyogenes*	M1
6016	beta	A	*S. pyogenes*	M1
6017	beta	G	*S. dysgalactiae* ssp. *equisimilis*	stG643.0
6017	beta	G	*S. dysgalactiae* ssp. *equisimilis*	stG643.0
6018	beta	A	*S. pyogenes*	M1
6020	beta	C	*S. dysgalactiae* ssp. *equisimilis*	stG62647
6025	beta	A	*S. pyogenes*	M1
6026	beta	A	*S. pyogenes*	M4
6028	beta	A	*S. pyogenes*	M4

Number	Hemolysis	Slidex	16s rDNA sequencing	M-type
6033	beta	A	*S. pyogenes*	M63
6040	beta	A	*S. pyogenes*	M28

From the applied typing of the INFECT isolates, it was found that all *S. pyogenes* isolates showed a β hemolytic phenotype, while growing on blood agar and expressing the 'A' antigen whereas SDSE isolates expressed either 'C' or 'G' surface antigen. Furthermore, all the *S. anginosus* isolates were α- hemolytic with no typical surface antigen.

On the basis of initial microbiological culturing and typing, the distribution of all species within the INFECT case collection (Appendix 6.2) was examined to determine occurrences of various causative pathogens of NSTI and frequencies of streptococcal infections (Figure 4.2).

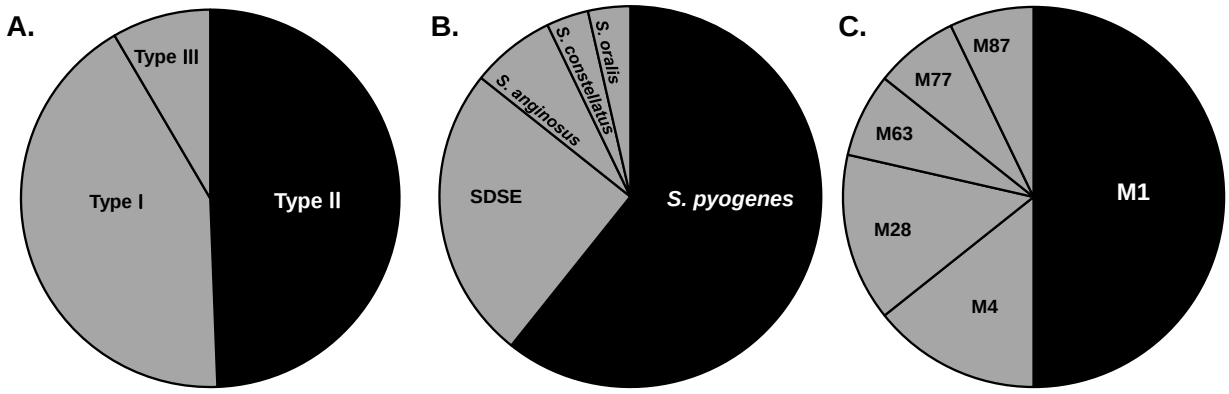

Figure 4.2: Pie-chart depicting the distribution of different NSTI types, causative bacterial species and streptococcal M-types of the NSTI case collection within two years (2013-2015). (A) Distribution of different types of NSTI cases collected under the project INFECT. Type I refers to poly-microbial infections, Type II are infections caused by *S. pyogenes* whereas clostridial species causing NSTI is assigned as Type III. Microbiological culturing was done from the infected site or from withdrawn blood of the patient. Each type is assigned to a particular case based on the causative organism. (B) Distribution of various species of the genus *Streptococcus* in the NSTI isolates collection. The 16s rRNA gene was amplified, sequenced and thereafter compared with existing databases to assign a particular species to the NSTI isolate collection. (C) Distribution of different M-types in the collected *S. pyogenes* isolates. The *emm* gene was amplified and sequenced to compare with CDC database to assign a particular M-type to the NSTI *S. pyogenes* isolates.

The enrolled NSTI cases can be divided into three types based on the causative pathogen: Infections caused due to diverse set of anaerobic and aerobic bacteria are categorized as "Type I" or poly-microbial infections; "Type II" are generally mono-infections dominated by *S. pyogenes* whereas "Type III" are infections by *Clostridium* species and are referred to as 'gas gangrene' due to gas formation at the site of infection. In the INFECT NSTI case collection, type II were seen to be dominating; followed by Type I (**Figure 4.2A**). Among all isolated streptococcal species, isolates identified as *S. pyogenes* (60%) were majoring the isolate collection followed by SDSE (15%); together summing up to about 75% (**Figure 4.2B**). The most frequent *emm* type of *S. pyogenes* was found to be M1, accounting for about 50% of the *S. pyogenes* collection (**Figure 4.2C**).

Within the analyzed NSTI case collection, isolates of the species *Streptococcus pyogenes*, especially of the serotype M1, were most frequently found to be the causative pathogen. Therefore the focus of the serologic analysis was set to cases of NSTI mono-infections with *S. pyogenes*. Emphasis on determining the antibody titer and their protective potential against bacterial toxins would provide insights to the role of adaptive immune system in NSTI pathogenesis and would aid in development of more targeted therapeutics. Since Intravenous Immunoglobulins (IVIG) is generally given as an adjunctive therapy in cases of NSTI with *S. pyogenes*, it was one of the criteria for the selection of cases to be analyzed in this study. Based on the IVIG treatment and disease development, cases were differentiated into 3 distinct groups (**Table 4.1.2**):

- NSTI cases that received IVIG treatment (**IVIG Treated Group**)
- NSTI cases that did not receive IVIG treatment (**non IVIG Treated Group**)
- Control group of cases with severe skin/tissue infections, that did not develop into an NSTI (non NSTI cases) and therefore also did not received any IVIG (**Control/Non NSTI Group**).

Analyzing antibody titers and their protective potential against bacterial toxins in these groups would provide insights in identifying factors that plays a crucial role in development of disease (NSTI cases versus non NSTI cases) and would aid in elucidating the role of the serological background in the development of an NSTI. Also, the comparison of IVIG-treated and non IVIG treated cases would decipher the efficacy of IVIG as an adjunctive therapy.

Table 4.1.2: List of Selected Isolates

IVIG Treated Group	Non IVIG Treated Group	Control Group
2001	3005	6028
2006	3012	6040
2015	6016	
2017	6018	
5003	6025	
5004	6026	
5006	6033	
6013		
n = 8	n = 7	n = 2

The number of cases in each group was dependent on the chronological order of arrival of these cases in the two years of collection, thereby differing in the number of collected isolates in each group. Hence, the complete serological study was conducted on 17 cases:

- 8 **IVIG treated NSTI cases**
- 7 **non IVIG treated NSTI cases**
- 2 **non NSTI/control cases**

The adaptive immune system constitutes specific antibodies that boost the bacterial killing by marking the bacterial cells for efficient phagocytosis which might play a crucial role in the development/retrogression of a severe NSTI. To investigate if the NSTI patient plasma samples carry antibodies against the causative bacterial pathogen, and the potential of these antibodies to mediate efficient opsonization and killing, an Opsono-Phagocytosis Assay was established.

4.2 Detection of antibodies in the INFECT plasma samples mediating opsonization and killing of bacterial cells in NSTI plasma samples

Opsonization is a process of pathogen recognition by the host immune system, which can be induced and increased by specific antibodies, which mark the bacterial cells and induce chemotactic signals for neutrophils and other phagocytic cells of the innate immune system to increase the efficiency of eradication by phagocytosis. To investigate if the NSTI patient plasma samples carry antibodies against the causative bacterial pathogen, and the potential of these antibodies to mediate efficient opsonization and killing, plasma samples from the patients recruited in the INFECT project were collected by clinical partners at two different time points: **Day 0**, when a tissue infection was rated as NSTI and the patient recruited in the study, and **Day 3**, which was the third day of treatment after the NSTI was identified (**Figure 4.3**).

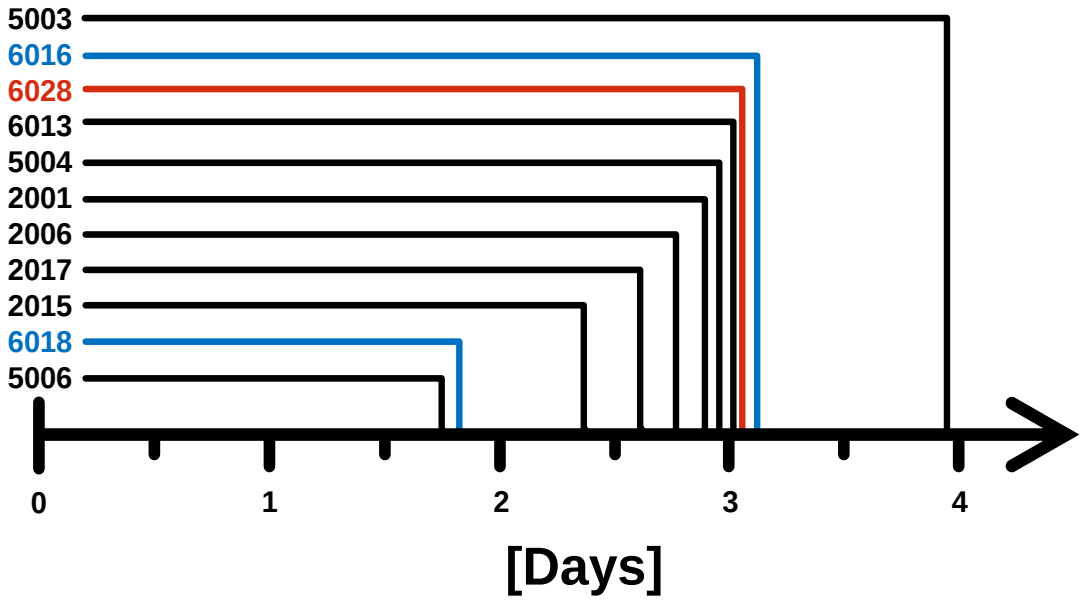

Figure 4.3: Time line depicting the time points of plasma collection under INFECT. Plasma samples were collected under the project INFECT on two different time points: Day 0 and 3. INFECT cases have been listed along with the time span between sample point Day 0 and Day 3. Different groups are shown with different colors: IVIG-treated (black), non IVIG treated (blue) and control non NSTI group (red).

From the time-line, it is apparent that all samples named as 'Day 3' were collected in a Gaussian distribution close to the third day of treatment irrespective of which study group they belong to. An Opsono-Phagocytosis assay was established to examine the Day 0 and 3 patient's plasma sample for their potential to opsonize and thereafter kill the bacterial cells.

4.2.1 Establishment of an Opsono-Phagocytosis Assay

To investigate the role of the adaptive immune system in the development/retrogression of a severe NSTI, the protective potential of the collected patient plasma samples against the corresponding bacterial isolate was determined. Therefore an Opsono-Phagocytosis Assay was established in which the bacterial cells were incubated in the corresponding plasma samples to allow available antibodies to bind and mediate opsonization. These opsonized bacterial cells were co-incubated with primary human neutrophil granulocytes (Poly-Morpho-Nuclear leukocytes; PMNs) and the bacterial killing was measured. As a first test experiment the NSTI isolate **2006** from the IVIG treated group was incubated with and without IVIG as it is thought that IVIG possesses antibodies that are able to recognize various serotypes of *S. pyogenes* and therefore mediate detectable opsonization. The bacterial survival rates were calculated after infection with human neutrophils (**Figure 4.4**).

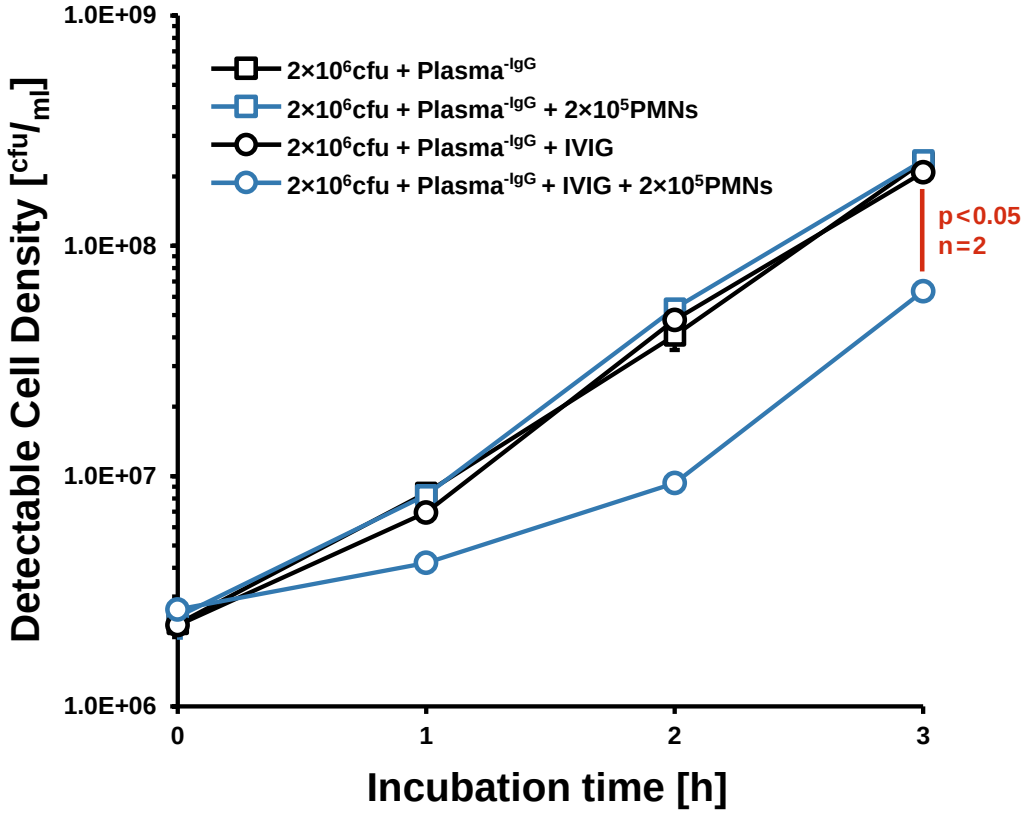

Figure 4.4: Opsono-Phagocytosis Assay with primary human PMNs using the *S. pyogenes* NSTI isolate 2006 in combination with IVIG treatment. Three hour infection of 2×10^6 cfu/ml of isolate 2006 opsonized in IgG depleted human plasma with and without IVIG for 30 min at 37°C in combination with or without 2×10^5 PMNs. Statistical significance was calculated by applying Student's T-test between detectable cell densities of INFECT 2006 with and without IVIG ($p < 0.05$, n=2).

No bacterial killing was mediated in non IVIG opsonized infection as well as in the growth controls wherein no PMNs were added. However, a significant decrease in the

bacterial cell density was observed in the infection with applied IVIG opsonization (Figure 4.4). This validates the functionality of the established Opsono-Phagocytosis assay and proves the presence of antibodies reacting specifically against *S. pyogenes* isolates in the applied IVIG mixture.

4.2.2 Screening of INFECT plasma samples using the established Opsono-Phagocytosis Assay

The established opsono-phagocytosis assay was initially applied on three INFECT isolates: Isolate **2001** (IVIG-treated), **6018** (non IVIG treated) and **6028** (non NSTI control) (**Figure 4.5**).

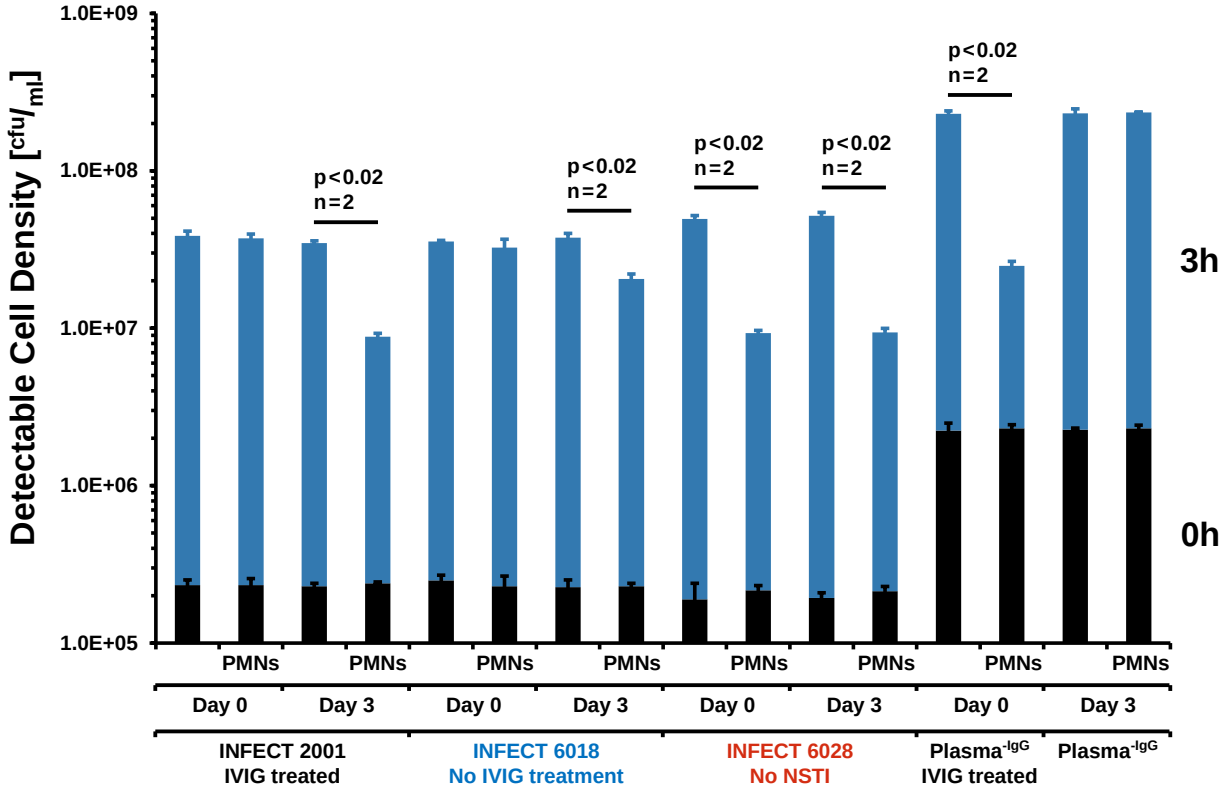

Figure 4.5: Results of the Opsono-Phagocytosis Assay using three bacterial INFECT isolates (2001, 6018 and 6028) in combination with the corresponding patient plasma samples. Three hours infection of 2×10^6 cfu/ml of selected INFECT isolates after 30min opsonization at 37°C in corresponding patient plasma samples (20%, diluted in PBS) collected at day 0 and day 3 with 2×10^5 PMNs. Detectable cfu/ml at time point zero (black bars) and after three hours of infection (blue bars) has been depicted in the figure. Infection in presence and absence of IVIG in IgG depleted plasma served as positive and negative controls respectively. ($p < 0.02$, n=2; T-Test).

As shown in Figure 4.5, no significant reduction in the bacterial cell number could be observed for the NSTI isolates **2001** and **6018** when opsonized with the corresponding Day 0 plasma. However, the Day 3 patient plasma was able to mediate efficient killing for both tested isolates. Contrarily, the Day 0 patient plasma sample of the non NSTI control case **6028** was able to opsonize and mediate killing of the corresponding

bacterial isolate already on Day 0. IgG depleted Plasma with and without IVIG served as positive and negative controls respectively and verified the previous observations. Since the selected patients received antibiotics as therapeutic treatment, a decrease in the initial bacterial counts could be observed after incubation with the plasma samples, which is a possible explanation for the observed differences between the bacterial counting results obtained with the patient plasma and the IgG depleted plasma controls.

The assay was further extended to the selected cohort to address the question if the observed effect can be rated as general (**Figure 4.6**).

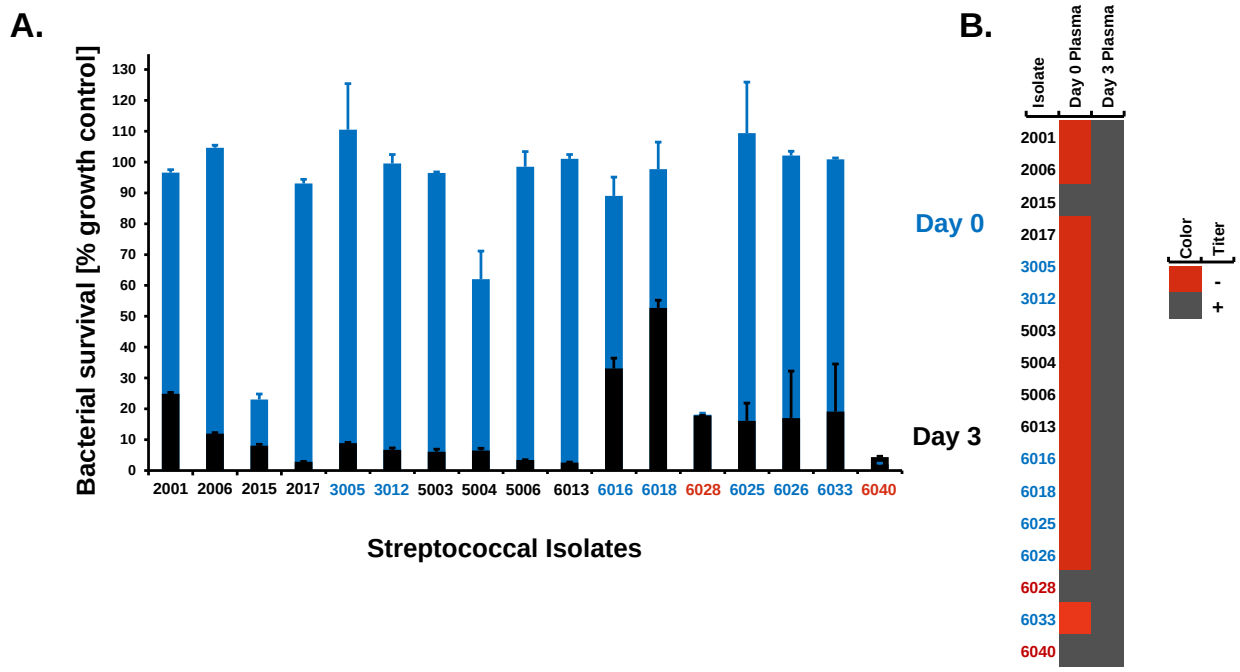

Figure 4.6: Results of the Opsono-Phagocytosis Assay of all selected INFECT isolates in combination with the corresponding patient plasma samples. (A) Three hours infection of 2×10^6 cfu of representative INFECT isolates after 30 min opsonization at 37°C in corresponding patient plasma samples collected at day 0 (black bars) or day 3 (blue bars) with 2×10^5 PMNs. Percent bacterial survival was calculated by growth controls wherein no PMNs were added. (B) A significant reduction in the bacterial cell number when compared to growth control has been marked as grey while non-significant titers are marked in red ($p < 0.02$, n=2; T-Test).

Out of the 15 analyzed NSTI cases, 14 showed no significant opsonization potential against the corresponding bacterial isolate in the Day 0 plasma samples, indicating a susceptibility of these patients towards the causative pathogen. Contrastingly, the Day 0 plasma sample of the non NSTI case **6028** mediated bacterial killing of the corresponding isolate up to 80% and the Day 0 plasma of control case **6040** enabled up to 95% killing. Out of all NSTI cases, the plasma sample of the IVIG treated NSTI case **2015** was the only one that mediated efficient bacterial killing already on Day 0. However, all analyzed NSTI cases showed a significant increase in the antibody mediated bacterial killing when using the Day 3 plasma samples for opsonization. This

could be accounted to the applied IVIG treatment or a rapid adaptive immune response of the host. Finally, it can be concluded that the adaptive immune system of all analysed INFECT NSTI patients is not able to mediate efficient protection against the corresponding bacterial isolate during the early stage of infection, where IVIG is able to compensate this susceptibility.

In conclusion, the data generated with the Opsono-Phagocytosis Assay approach indicate a crucial role of the adaptive immune system in the development of a severe NSTI. All analysed NSTI cases showed a significant susceptibility against the corresponding bacterial pathogen during the initial stage of infection whereas the data obtained from the analyses of the non NSTI control cases showed protectivity on the Day 0 itself. However, bacterial killing does not reduce their detrimental effects as *S. pyogenes* secretes a wide array of exotoxins that remain active and can further deteriorate the patient's condition. To also investigate the protective potential of the adaptive immune system against streptococcal exotoxins during the development of an NSTI, an ELISA based serology screening was performed. The first step in this serology approach was to identify which exotoxins are present in the genomes of the selected *S. pyogenes* INFECT isolates.

4.3 Detection of Exotoxin genes in the genomes of *S. pyogenes* INFECT isolates

It is known that *S. pyogenes* secretes exotoxins that impair the host immune response in different ways like activation of non-specific T-cell sub-populations (superantigens), cleavage of IgG (Mac), degradation of chemokines (spyCEP) or inactivation of complement system (SIC). Screening for the genes encoding exotoxins in the genomes of *S. pyogenes* INFECT isolates would provide insights on which exotoxin genes are prevalent and is a further step towards understanding the influence of the serological background in the development of NSTIs.

4.3.1 Establishment of a Multiplex PCR

To determine the presence or absence of genes coding for secreted toxins in the genomes of the selected *S. pyogenes* isolates, a multiplex PCR was established. The multiplex PCR was set up by modifying and combining previous screening methods of Friães *et al*[88] that screened for superantigen genes and Borek *et al*[102] that could amplify DNase genes from the genome of *S. pyogenes*. The established multiplex PCR system is able to efficiently detect 21 exotoxin genes within the genomes of *S. pyogenes* isolates in

5 different reactions. The genomic DNA of genome sequenced *S. pyogenes* strains (SF370, MGAS315 and MGAS8232) was used as positive control whereas the genomic DNA of the genome sequenced *S. gordonii* strain Challis (GP204) was used as a negative control in all five reactions. Since one strain did not carry all the exotoxins amplified in one reaction, a combination of two strains was sometimes used as control for a particular reaction (**Figure 4.7**)

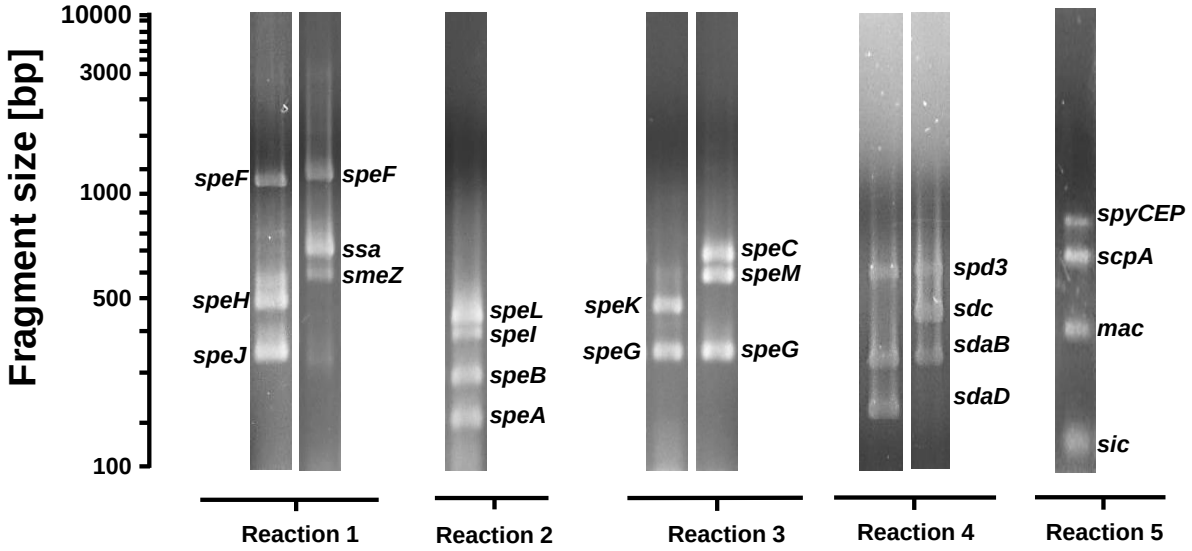

Figure 4.7: Agarose gel picture of all 21 exotoxin genes amplified out of the genomes of selected positive controls used in the multiplex PCR. Conserved regions from 21 exotoxin genes were amplified from control strains in five different reactions: reaction 1-3 amplifies superantigens while reaction 4-5 amplifies a second set of streptococcal exotoxins. The amplified PCR products were separated by size on a 1% agarose gel and stained with Ethidium Bromide. Mobility of the bands is expressed in terms of base-pairs (left side) determined by standard GeneRuler 1 kB ladder (Fermentas). Each signal in the figure is demarcated by name of the corresponding gene being amplified.

4.3.1 Application of the Multiplex PCR on selected INFECT isolates

After the successful optimization of the multiplex PCR, it was used to determine the presence or absence of exotoxin genes in the genomes of the selected *S. pyogenes* INFECT isolates (**Table 4.3.1).**

Table 4.3.1: Repertoire of exotoxin genes within the genomes of selected *S. pyogenes* INFECT isolates

Isolate	*speA*	*speB*	*speC*	*speF*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speL*	*speM*	*ssa*	*smeZ*	*sdc*	*sdaB*	*sdaD*	*spd3*	*spyCEP*	*mac*	*sic*	*scpA*	emm
2001	+	+		+	+			+			+	+			+	+	+	+	+	+	+	M1
2006	+	+		+	+			+			+				+	+	+	+	+	+	+	M1
2015	+	+		+	+			+			+			+	+		+	+	+		+	M87
2017		+		+	+			+	+	+	+				+	+		+	+		+	M28
3005		+	+	+	+					+	+		+		+		+	+	+		+	M89
3012		+		+	+		+			+	+		+		+		+	+	+		+	M77
5003	+	+		+	+								+		+	+	+	+			+	M77
5004	+	+		+	+			+	+	+	+				+	+		+	+		+	M28
5006	+	+		+	+			+	+				+		+	+	+	+	+	+	+	M1
6013	+	+		+	+			+	+				+		+	+	+	+	+	+	+	M1
6016	+	+		+	+			+	+				+		+	+	+	+	+	+	+	M1
6018	+	+		+	+			+	+				+		+	+	+	+	+	+	+	M1
6025	+	+		+	+		+			+		+	+		+	+	+	+		+	+	M1
6026	+	+		+	+			+				+	+		+		+	+	+	+	+	M4
6028	+	+		+	+							+	+		+		+	+	+	+	+	M4
6033	+	+		+	+	+					+	+	+		+		+	+		+	+	M63
6040		+	+	+	+	+		+	+						+	+		+	+		+	M28
	Superantigen	Protease	Superantigen	DNase	Superantigen	Superantigen	Superantigen	Superantigen	Superantigen	Superantigen	Superantigen	Superantigen	Superantigen	DNase	DNase	DNase	Dornase	Chemokine degradation	IgG cleavage	Complement inhibition	Complement inhibition	

The screening for exotoxin genes in the genomes of *S. pyogenes* INFECT isolates showed that some of the exotoxins are distributed in a serotype specific pattern. Out of the 21 exotoxins, 11 Streptococcal pyrogenic exotoxins have **super antigenic properties**: they can cause nonspecific activation of copious amount of T-cells. Interestingly, only the gene coding for the superantigen SpeG was present in all the tested *S. pyogenes* isolates, while other superantigen genes like *speJ*, *speK*, *speL* and *speM* were not restricted to any specific serotype. The gene coding for SpeA and SmeZ were

the most abundant superantigens, covering about 93% and 60% of *S. pyogenes* isolates respectively, while the *speC* and *speH* genes were the least common. SpeB was initially thought to be a superantigen but was later found to be a Cysteine-Protease[103]. The gene coding for SpeB could also be detected in all the tested *S. pyogenes* isolates, irrespective of the serotype of the isolate. DNases are known to degrade extracellular traps released by neutrophils (NETs) and hence are important for the escape of entrapped bacteria[56]. While the gene coding for SpeF (mitogenic factor) and SdaB were recurring in all isolates, others were sporadic. Interestingly, the *sdc* and *sdaD* genes were seen excluding each other; the former being present only in one of the INFECT isolates (INFECT **2015**). The genes of the immune-modulating exotoxins, *scpA* and *spyCEP* were always present while the genes coding for Mac and SIC did not show any serotype specificity.

To investigate the degree of similarity among different *S. pyogenes* isolates based on the distribution of detected exotoxin genes and the serotype determined by M-typing, a resemblance matrix was generated within the three selected groups (**IVIG-treated**, **non IVIG treated** and **non NSTI control group**) (**Figure 4.8**).

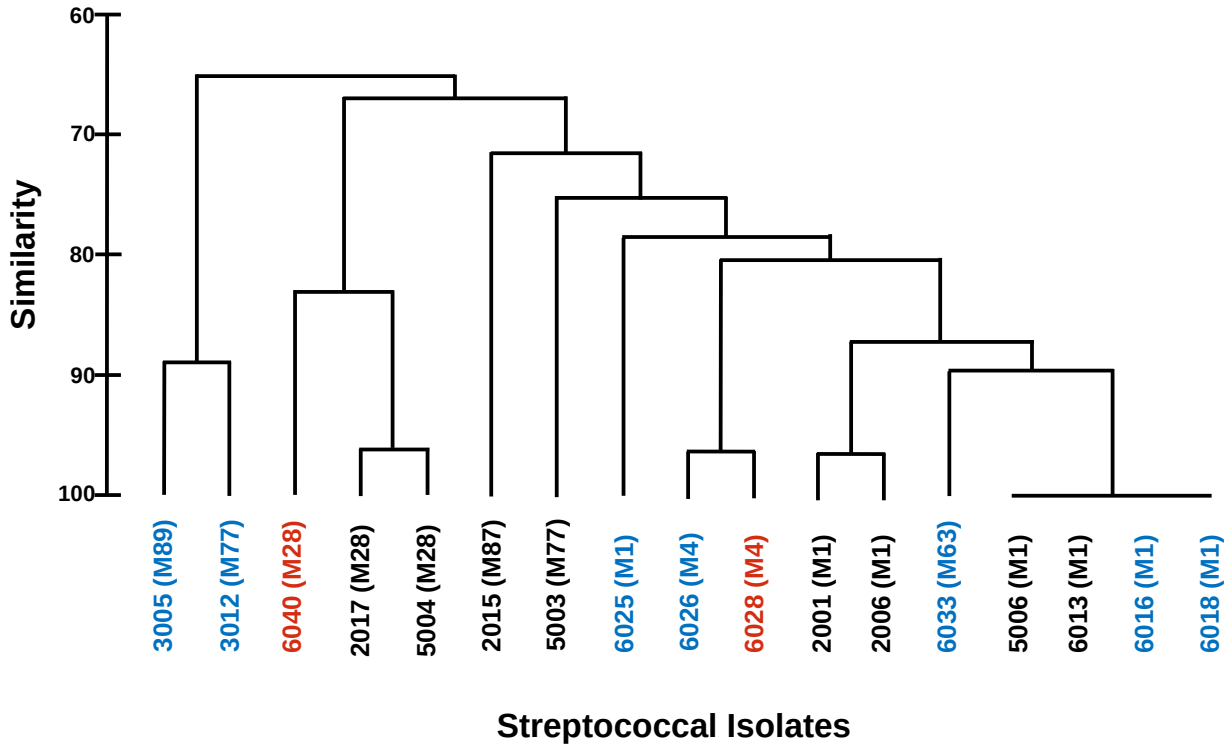

Figure 4.8: Dendrographic plot of a resemblance matrix of *S. pyogenes* INFECT isolates based on their genetic exotoxin profile. The resemblance matrix was generated by S17 Bray Curtis method calculating the group average values. IVIG treated cases are highlighted in black, non IVIG and non NSTI cases are highlighted in blue and red respectively. The corresponding M-type is given in parenthesis.

Most of the *S. pyogenes* INFECT isolates of a particular serotype clustered together, thus showing a high similarity quotient within the serotype. Interestingly, there was no distinct clustering within different groups (**IVIG treated NSTI isolates, non IVIG treated NSTI isolates** and **non NSTI control cases**) being studied.

Most of the screened *S. pyogenes* exotoxin genes are present in various Single Nucleotide Polymorphism alleles (SNP). Despite these polymorphisms, they possess conserved structure with no change in function or epitope. For example, the gene coding for streptokinase, Ska, is ubiquitously present in genomes of all *S. pyogenes* isolates and is found to occur in three different forms: *ska-1*, *ska-2a* and *ska-2b*. These *ska* subtypes have different mechanism of action for plasmin activity formation[51]. For the determination of *ska* types present in the genomes of the analyzed INFECT isolates, the *ska* gene was sequenced and a dendogram based on sequence comparisons was constructed (**Figure 4.9**).

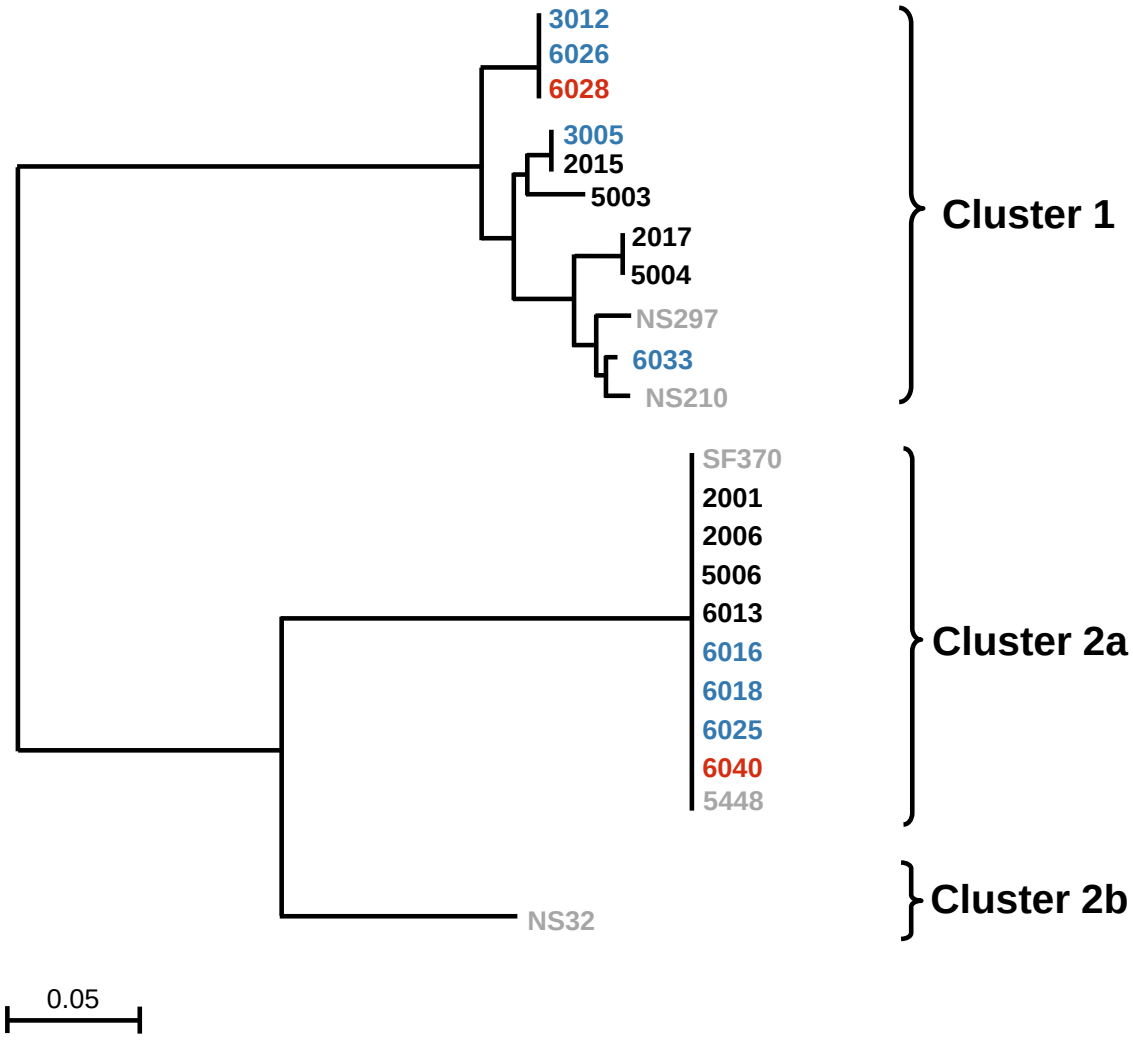

Figure 4.9: Dendogram visualizing different clusters of the *ska gene* as obtained by sequence comparison. The variable region of the *ska* gene was sequenced from all selected INFECT *S. pyogenes* isolates and a

dendogram was generated by sequence comparison using ClustalW (Number of Bootstraps=1000). IVIG treated patients are marked in black, non IVIG in blue and non NSTI controls in red. The reference sequences for different clusters are marked in grey and were obtained from McArthur *et al* [51].

The *ska* gene of all analyzed isolates belonged to Cluster 1 or 2a. The obtained sequences of INFECT isolates belonging to cluster 2a were 100% similar to the reference sequences of *S. pyogenes* SF370 and *S. pyogenes* MGAS5448 whereas there were differences in percent similarities in cluster 1, ranging from 100% to as low as 53% with the reference sequences. Interestingly, the presence of *ska* gene of cluster type 2b could not be observed in the genomes of the analyzed *S. pyogenes* isolates.

The screening for presence or absence of exotoxin genes in the genomes of *S. pyogenes* isolates provides an insight of which exotoxin genes may play a critical role in the NSTI scenario. Therefore all detected exotoxins (n=18) were either cloned purified or purchased if commercially available to determine corresponding antibody titers in the plasma samples of the selected INFECT cases.

4.4 Detection of antibodies against exotoxins in INFECT plasma samples

The host adaptive immune response is crucial for the efficient identification and elimination of pathogens. Specific antibodies are able to boost the process of eradication of the pathogen by initiating efficient opsonization of the bacteria and by neutralizing bacterial toxins. To further investigate the role of the adaptive immune system during the development of an NSTI, an extensive serology approach was performed wherein antibody levels against exotoxins were measured in plasma samples of NSTI and non NSTI patients. Therefore all streptococcal exotoxins identified by the multiplex PCR screening could to be potentially involved in the pathogenesis of the analyzed INFECT cases and therefore, were, if not commercially available cloned and purified to be used in an ELISA system.

4.4.1 Cloning and Purification of Exotoxins

The results of the established multiplex PCR clearly showed which exotoxin genes are present in the genomes of the selected *S. pyogenes* INFECT isolates. To measure antibody titers against these exotoxins in the collected plasma samples all genes, if not commercially available toxins, were cloned in the pQE30-TEV vector in an *E. coli* M15 background, overexpressed and the corresponding exotoxin purified (**Figure 4.10**).

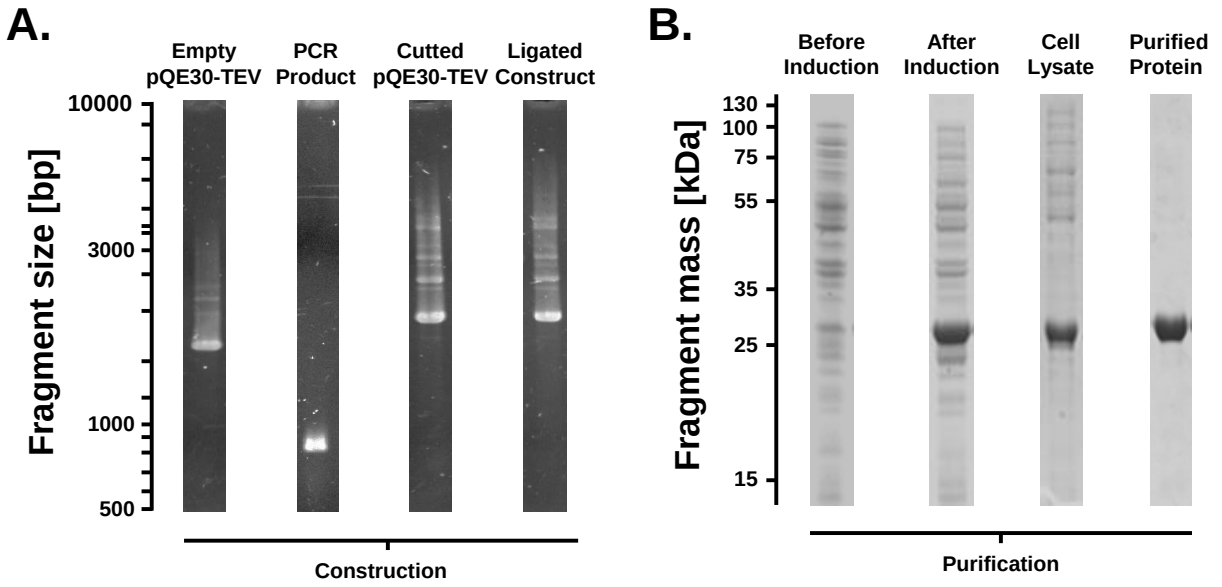

Figure 4.10: Electrophoretic gel pictures of exotoxin gene cloning and protein purification. (A) Selected exotoxins were cloned in pQE30-TEV vector (Lane 1). For this, the exotoxin genes (Insert) were amplified by PCR (Lane 2) using primers that contained restriction sites for BamHI and SalI. The insert and Vector were double digested with BamHI/SalI (Lane 3) and ligated using T4 DNA Ligase (Lane 4). The ligated products were transformed in *E. coli* M15 cells. Different lanes have been demarcated with the product as analyzed on 1% Agarose gel electrophoresis, stained with Ethidium Bromide. The sizes of the fragments were estimated by comparison with Standard DNA Ladder (1 kB GeneRuler). (B) The expression of the cloned exotoxin genes were induced with 1mM IPTG (Lane 1 and 2). The cells were lysed using French Press and the cell debris was removed by centrifugation. The cell lysate (Lane 3) was incubated with Ni^{2+}-NTA Sepharose beads for protein binding. The proteins were purified by washing thrice with wash buffer containing 20 mM Imidazole and elution with 250 mM Imidazole (Lane 4). The proteins were analyzed with 10% SDS-PAGE, stained with Coomassie. The sizes of the fragments were estimated by comparison with standard protein ladder. Here, the cloning, overexpression and purification of SpeA (Superantigen) has been shown as an example.

Some of the exotoxins (SpeB, Mac, SLO and Hyl) were purchased from commercial sources. Since the bacterial exotoxins are overexpressed and purified in an *E. coli* background, there are possible contaminations with the *E. coli* proteins that could result in false positive antibody titres. To control this possible *E. coli* background contamination, the empty pQE30-TEV vector was cloned and the protein background pattern, that unspecifically binds to the used Ni^{2+}-NTA Sepharose was purified and diluted in the same way as the target exotoxin and served as the 'background' control.

4.4.2 Establishment of an ELISA detection system for exotoxin specific antibodies

After cloning and purification of streptococcal exotoxins, an efficient detection system to identify IgG titers against the purified exotoxins in the NSTI patient's plasma was essential. Therefore, an Enzyme-linked Immunosorbent Assay (ELISA) was established. ELISA is a common laboratory technique that can be used to measure the concentration of an analyte by using enzyme immunoassays. An ELISA is a multi-step

process that involves at least one antibody with specificity for a particular antigen. The analyte (either an antigen or an antibody) is immobilized/coated by adsorption to a polystyrene 96 well plate. Coating is followed by a blocking reaction wherein all the unoccupied surfaces of the well material are captured by a non-interacting protein. The detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is washed using a mild detergent solution to remove proteins or antibodies that are non-specifically bound. After a final washing step, the plate is 'developed' by adding an enzymatic substrate to produce a detectable signal, which indirectly indicates the quantity of the analyte in the sample. Hence, establishing an ELISA needs optimization of various factors: amount of analyte being coated, dilutions of primary and secondary antibody, concentration of enzymatic substrate (ABTS), all in accordance with the linear range of detection system (Varioscan) (**Figure 4.11**).

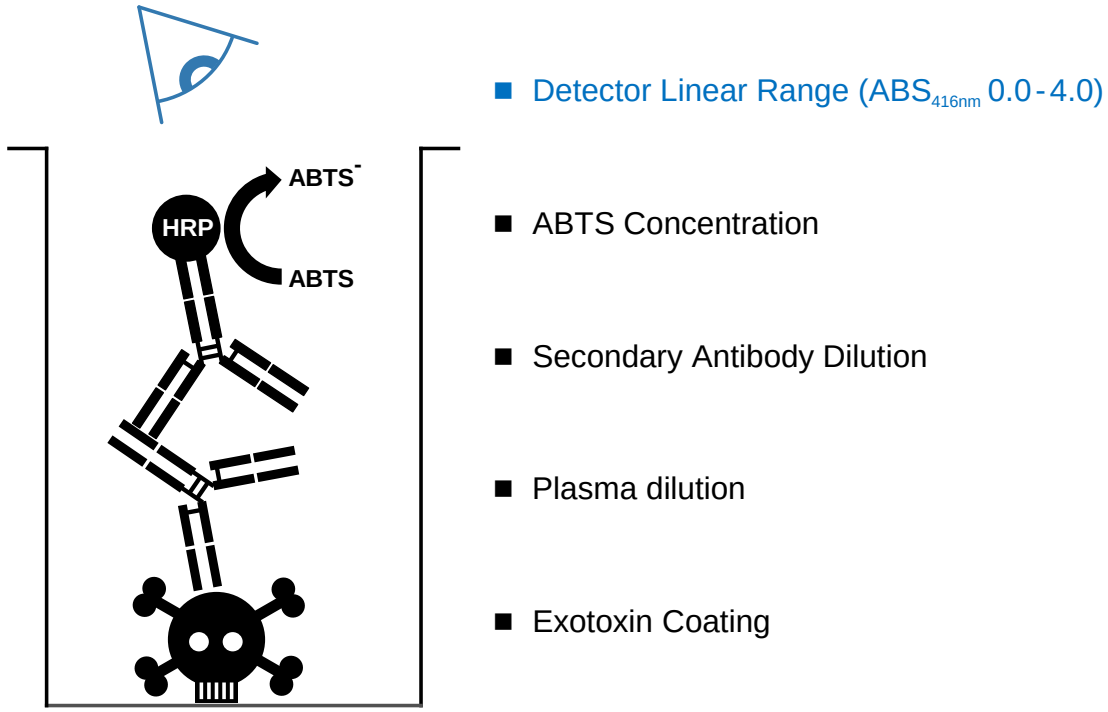

Figure 4.11: Schematic depiction of various parameters to be addressed during the establishment of an ELISA system. The standardization of an ELISA requires optimization of four factors all influencing each other: the exotoxin coating, the dilution of primary antibody (plasma), the concentration of the secondary antibody, and the ABTS concentration. The already fixed parameter (Detector's Linear Range) has been highlighted in blue.

- **Linear Range of the Detector:** The secondary antibody was conjugated with Horse Radish Peroxidase (HRP), hence ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] was used as a substrate. ABTS changes color from light green to dark green with an absorption maximum around 416 nm after chemical conversion

by HRP. The linear range of the selected detector (Varioscan) to measure absorption at 416 nm as stated by the manufacturer reached from 0.0 to 4.0.

- **ABTS concentration and Secondary Antibody dilution:** To cover the complete linear range of the detection system, the ABTS concentration as well as the concentration of the secondary antibody should not be limiting factors. To estimate the optimal dilution of the secondary antibody and ABTS concentration, a double-gradient experiment was performed (**Figure 4.12**).

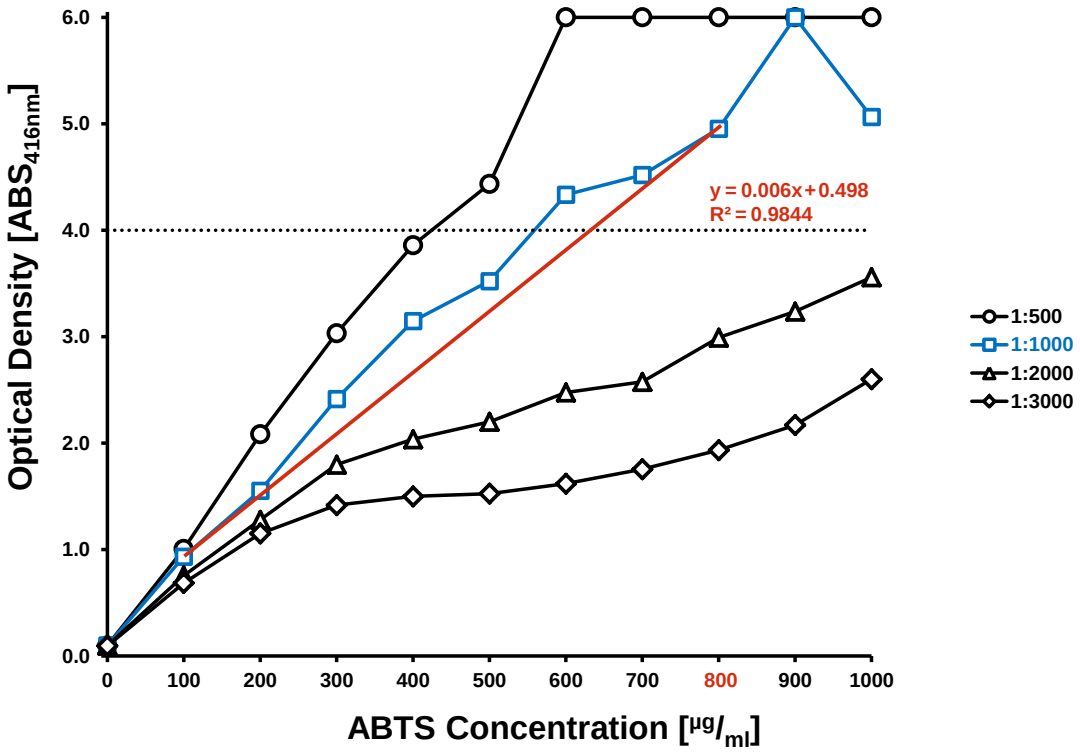

Figure 4.12: Dependency of secondary antibody dilution and ABTS concentration towards the signal intensity obtained by the ELISA system. A double gradient of secondary antibody dilution and ABTS concentration was performed. Different dilutions of HRP labeled secondary antibody were added to varying concentrations of ABTS in order to estimate the optimum secondary antibody-ABTS combination. The graph was plotted as gradient of ABTS concentration (in µg/ml) versus optical density (Absorbance measured at 416 nm). Different graphs depict different secondary antibody dilutions. The linear correlation of the selected antibody dilution was calculated and is shown next to the corresponding graph. A dotted horizontal line indicates the upper limit of linear range of the detection system.

Higher secondary antibody dilutions (1:2000 and 1:3000) were not sufficient to receive a signal intensity, that covers the complete dynamic range of the detection system, while the lowest antibody dilution (1:500) was able to produce a sufficient signal intensity with a minimum of 400 µg/ml of ABTS, but with the drawback of increased experimental costs. Hence, 1:1000 (0.2 µg/ml) was selected as the optimum dilution for the secondary antibody in combination with an ABTS concentration of 800 µg/ml.

- **Concentration of Exotoxin protein coating:** Once the optimal ABTS concentration and secondary antibody dilutions were fixed, the next variable to be optimized was the amount of analyte being coated. To estimate the optimal antigen concentration required for coating, a gradient of different protein concentrations was used. For this, the His-tag of the exotoxins was targeted to simulate an exotoxin specific antibody response (**Figure 4.13**).

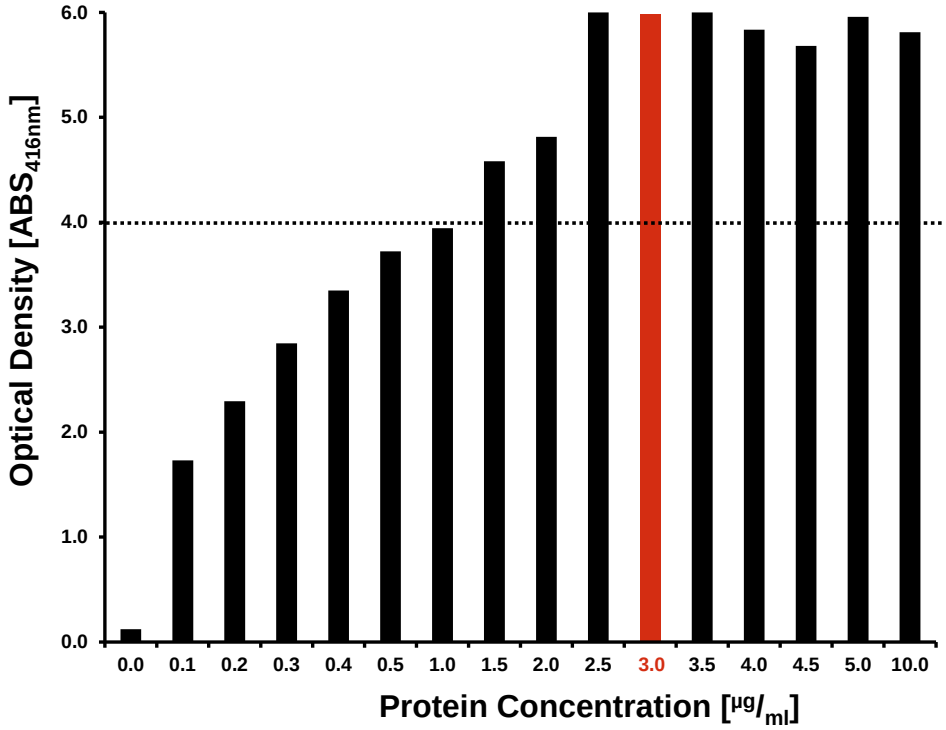

Figure 4.13: Dependency of the coating protein concentration towards the received ELISA signal intensity. A concentration gradient of His-tagged protein coated to the ELISA plate well was analyzed in the ELISA system. The coating was done by incubating the ELISA plate wells with different concentrations of protein in PBS (50 µl) overnight at 4°C. The standardized secondary antibody dilution (1:1000) and ABTS concentration (800 µg/ml) in combination with a mouse Anti-His antibody (dilution of 1:1000) as primary antibody was used in this experiment. The graphs were plotted as gradient of coated protein concentration (in µg/ml) versus the obtained optical density (Absorbance measured at 416 nm). The protein concentration selected for coating is highlighted in red. A dotted horizontal line depicts the upper limit of linear range of the detection system.

The applied concentration gradient of coated protein showed a linear effect towards the optical density measured at 416 nm. The system reached the maximum possible range at a minimum concentration of 2.5 µg/ml of protein. To avoid the limiting effect of the coated analyte concentration, 3 µg/ml was selected for the ELISA.

- **Plasma (Primary Antibody) Dilution:** The ELISA was established by determining optimal concentrations of all variables *viz.* coating of the antigen, secondary antibody concentration, ABTS concentration, all in accordance with the dynamic range of the detector. The primary antibody (plasma) concentration was the only variable left in this system that needs to be adjusted to a reasonable value. Since the

plasma of the selected NSTI patients is a limiting factor, a primary antibody (plasma) dilution of 1:50 was used. This concentration allowed an optical density to cover the complete dynamic range of the system with the established parameters (**Figure 4.14**).

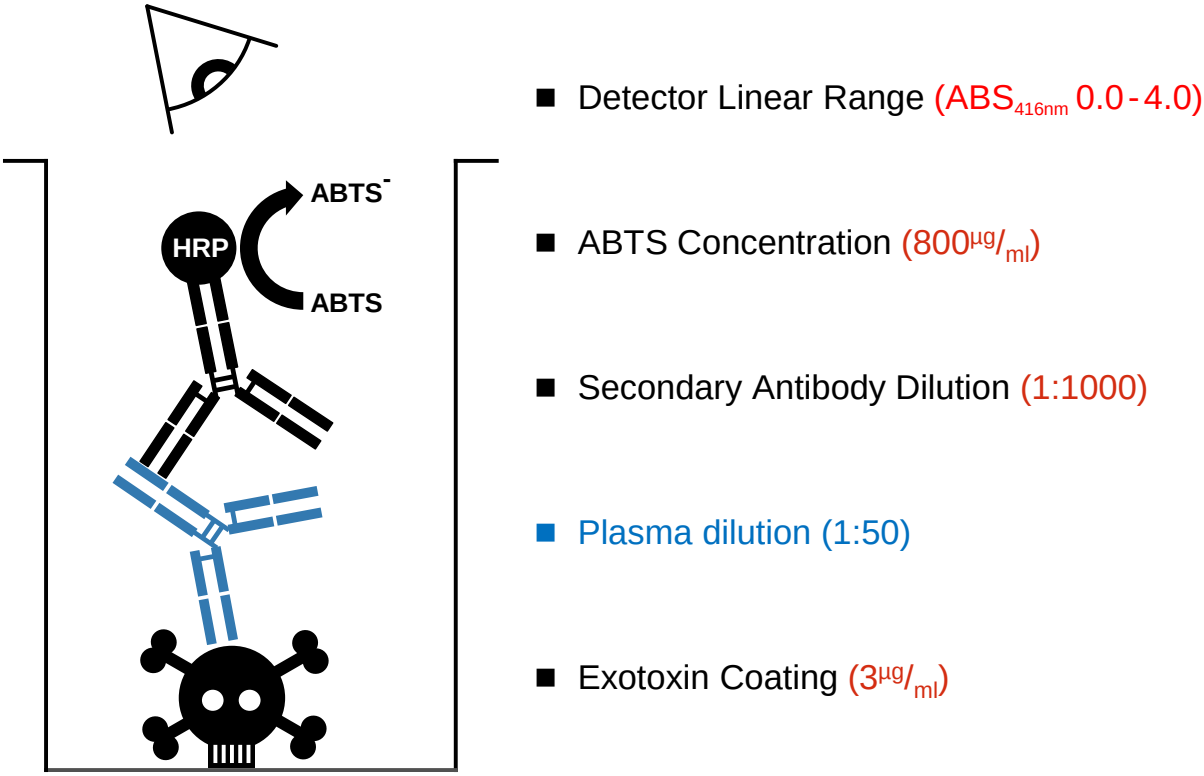

Figure 4.14: Schematic depiction of all parameters standardized in the established ELISA system. All parameters influencing the antibody detection capabilities of the established ELISA system are listed along with their optimized values in parenthesis.

4.4.3 Verification of established ELISA system

To test the potential of the established ELISA system (Section 4.4.2) in the detection of antibodies specific to different streptococcal exotoxins, an antibody mixture usually given as Intravenous Immunoglobulin treatment (IVIG) was used. IVIG are thought to contain antibodies specific to various serotypes of *S. pyogenes* as well as the complete spectrum of streptococcal exotoxins. 5 mg/ml of IVIG was used in accordance to the standardized protocol of the clinical trial called INSTINCT (Immunoglobulin for Necrotizing Soft Tissue Infections: a Randomized Controlled Trial) at Rigshospitalet Denmark (Trial Number NCT02111161). This clinical trial is a randomised study to evaluate the efficacy and efficiency of IVIG being administered to NSTI patients compared to a placebo treatment. Our study has been set in accordance to the INSTINCT programme, using the same manufacturer and concentration of IVIG as done within this programme. Since the bacterial exotoxins are overexpressed and

purified in an *E. coli* background, there are possible contaminations with the *E. coli* proteins that could provide false positive antibody titres (**Figure 4.15**).

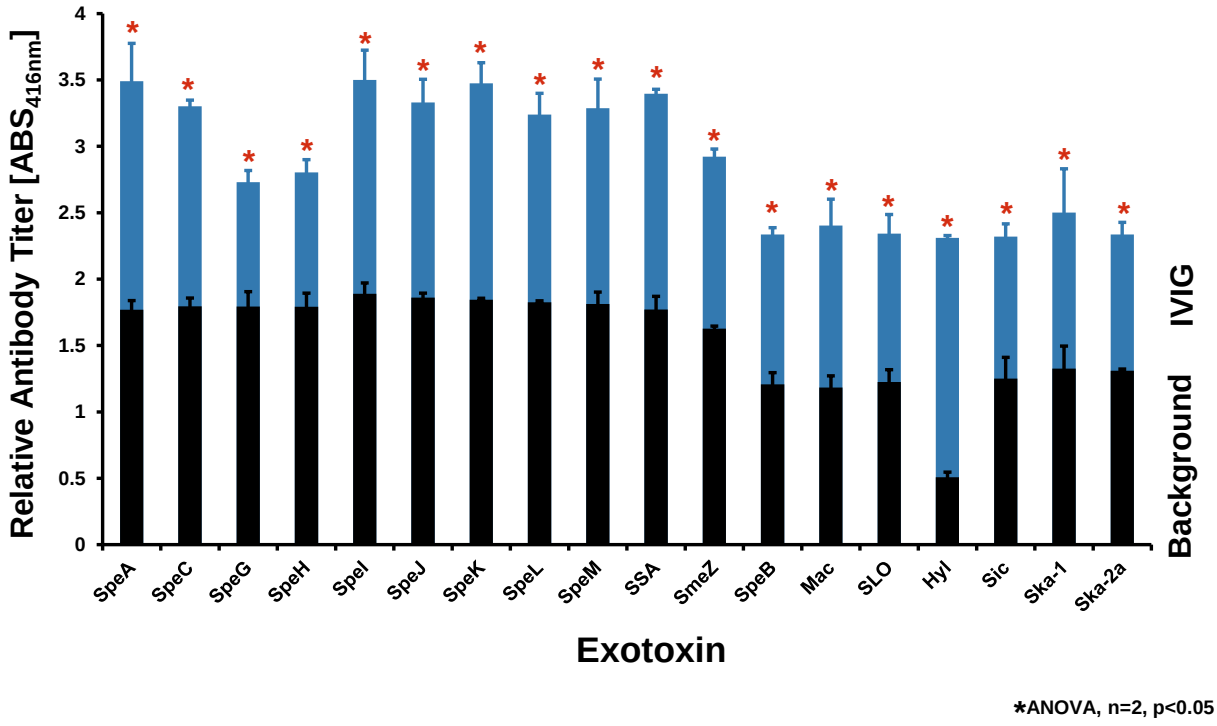

Figure 4.15: Bar graph diagram depicting the antibody titers against exotoxins in IVIG as detected by the established ELISA. The established ELISA system was tested for its potential to detect antibodies specific for streptococcal exotoxins using 5 mg/ml IVIG as the primary antibody. 3 µg/ml of each exotoxin were coated on 96 well high binding ELISA plate. Already standardized secondary antibody dilutions (1:1000) and ABTS concentrations (800 µg/ml) were used. To control the background (*E.coli*) titer, an empty vector protein purification was used in the same dilution as the target exotoxin. The relative antibody titer (Absorbance at 416 nm) was plotted against each exotoxin and its corresponding *E.coli* background. The statistical significance testing between relative antibody titer of exotoxin when compared to background *E. coli* protein, was done by applying an ANOVA test with a *p* value cutoff set to 0.05 (n=2) and are marked with red asterisks.

To control the possible *E. coli* background contamination, the empty pQE30-TEV vector was cloned and the protein background pattern, that unspecifically binds to the used Ni^{2+}-NTA Sepharose was purified and diluted in the same way as the target exotoxin. The established ELISA system was able to detect significant antibody (IgG) titers in the tested IVIG dilution against all 18 exotoxins ($p < 0.05$, ANOVA, n=2). This confirmed not only the presence of specific antibodies against streptococcal exotoxins in the used IVIG product, but also the potential of the established ELISA system to detect IgG titers against streptococcal exotoxins in human plasma samples.

4.4.4 Determination of antibody titers in INFECT plasma samples

After the establishment and validation of the ELISA system was successfully concluded, it was ready to be used with INFECT patient plasma samples to identify antibodies titers against the selected streptococcal exotoxins. The plasma samples to be analysed were collected by the INFECT clinical partners at two different time points : **Day 0**, when a tissue infection was rated as NSTI and the patient recruited in the study, and **Day 3**, which was the third day of treatment after the NSTI was diagnosed (Section 4.2, Figure 4.3).

Initially, the established ELISA system was tested on one set of plasma samples from each group: INFECT NSTI case **5006**, which was IVIG treated, INFECT NSTI case **6018** that was a non IVIG treated patient and INFECT case **6028** which was a member of the control group of non NSTI patients (**Figure 4.16**).

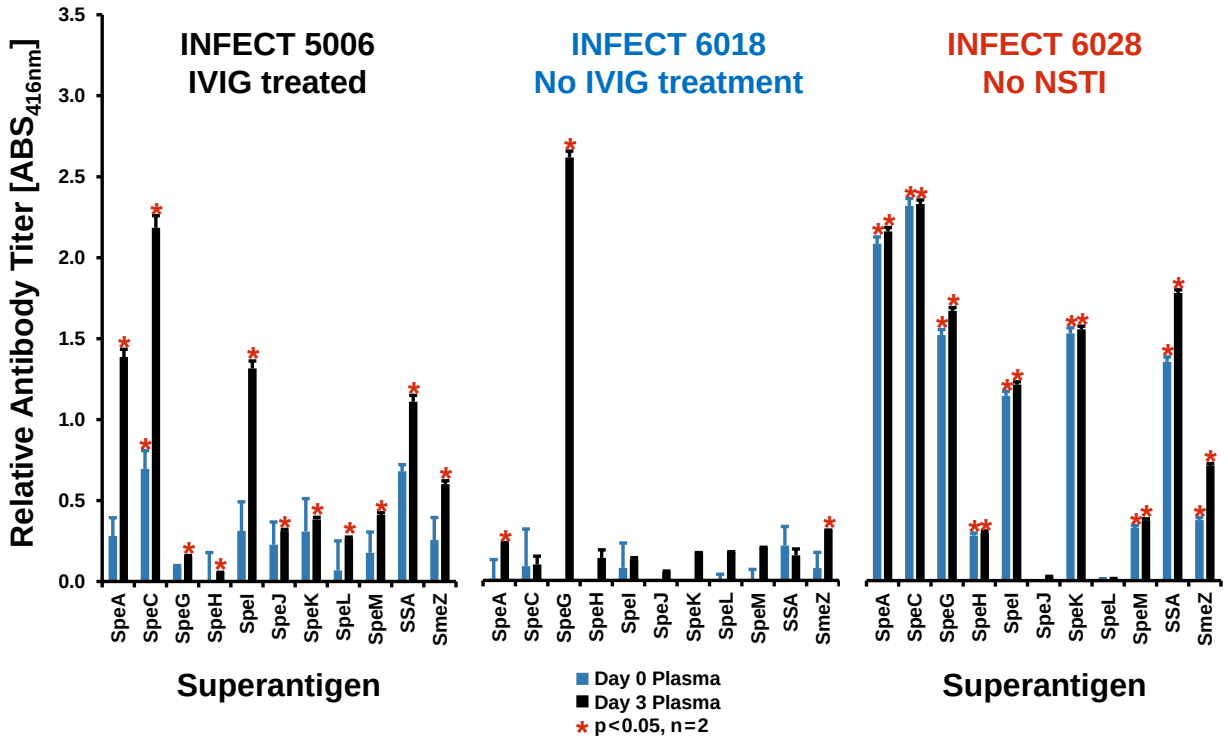

Figure 4.16: Bar graph diagram depicting relative antibody titers against 11 superantigens in the plasma sample sets of three selected INFECT cases. The established ELISA was used to analyze the Day 0 and Day 3 plasma sample sets of three selected INFECT cases, one from each group (IVIG-treated, non IVIG treated and non NSTI case) to detect antibody titers against all 11 streptococcal superantigens. Relative antibody titers were detected against superantigens on Day 0 (blue bars) and Day 3 (black bars). A statistical significance test was performed against the background *E. coli* empty vector purification ($p < 0.05$, n=2; ANOVA). Significant titers, when compared with the background control, are marked with red asterisks.

As shown in Figure 4.16, the initial antibody titers in the Day 0 plasma samples against 11 streptococcal superantigens were rare in NSTI patients as compared to the non NSTI

control. The Day 0 plasma sample of the NSTI case **5006** showed a significant antibody titer only against SpeC, while the plasma of NSTI case **6018** had no significant titers at all on Day 0. Contrarily, the non NSTI control case **6028** showed significant antibody titers against 9 of the 11 tested superantigens (SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeM, SSA and SmeZ) in the Day 0 plasma sample. On Day 3, there was a significant increase in the presence of antibody titers against all superantigens in the plasma sample of the NSTI case **5006,** probably due to the applied IVIG treatment. The non IVIG treated NSTI case **6018** developed significant antibody titers only against 3 of the 11 tested superantigens (SpeA, SpeG and SmeZ); while no change in the antibody titer pattern of the control **6028** could be observed. The comparative analysis of these three cases provided an insight to the adaptive immunological susceptibility of NSTI patients at Day 0 compared to non NSTI cases. All Day 0 plasma samples of NSTI cases lacked antibody titers against most of the tested superantigens, which is the opposite of what could be observed in the Day 0 plasma sample of the non NSTI control case. Additionally, the applied IVIG treatment was able to compensate the detected lack of antibody titers, which could be seen in the analysis of the Day 3 plasma sample of the IVIG treated case **5006**.

These results confirmed the successful establishment of the ELISA system to detect IgG titers against streptococcal exotoxins in NSTI patient's plasma samples. Hence, the ELISA based analysis was extended to the remaining samples of the cohort including 8 **IVIG treated NSTI cases,** 7 **non IVIG treated cases** and 2 **non NSTI control cases**. These results were compiled with the PCR data obtained through multiplex PCR to be able to examine significant pattern of particular exotoxin genes in combination with the presence of antibody titers against the corresponding toxin in all three study groups (**Figure 4.17**).

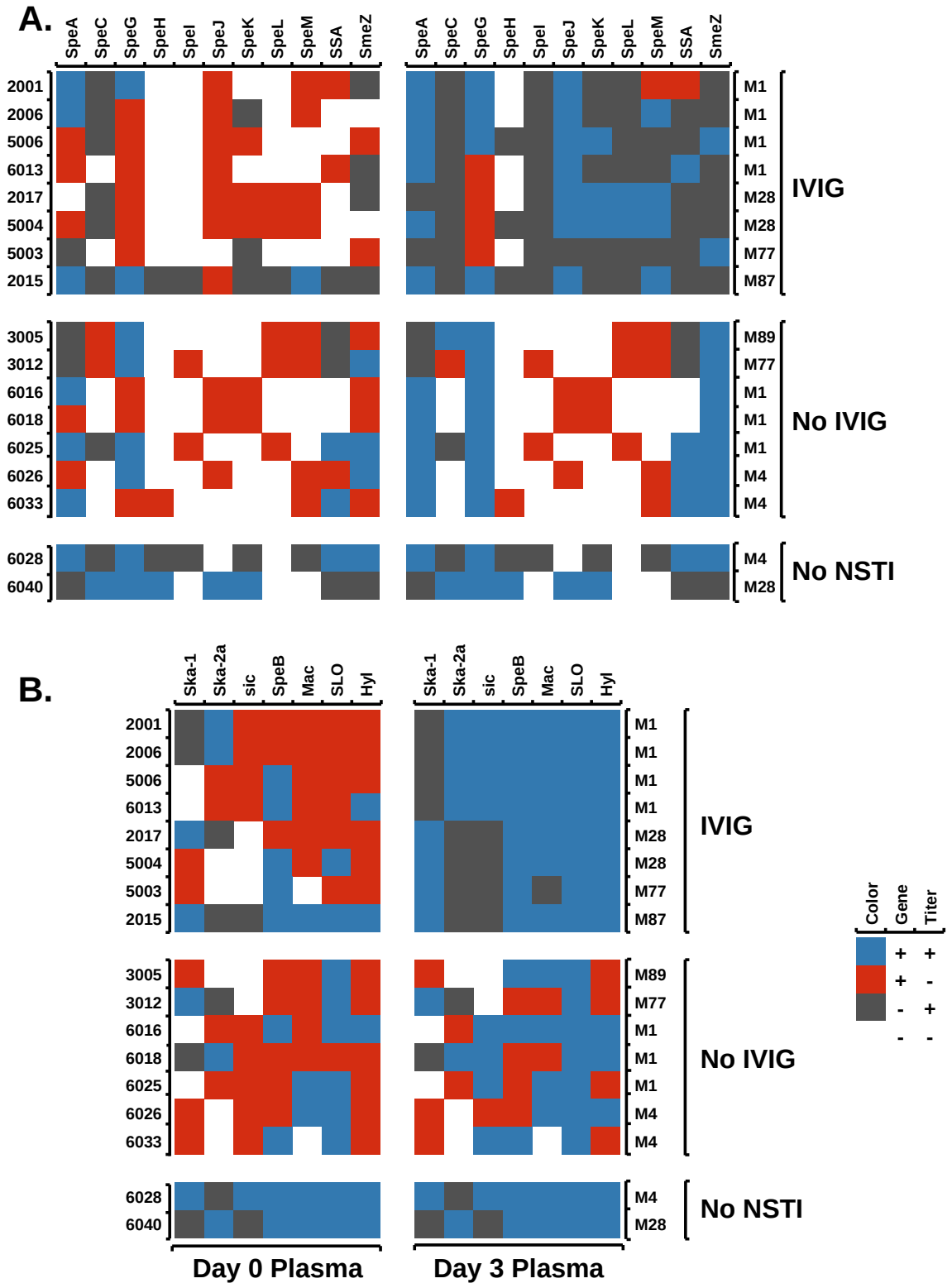

Figure 4.17: Serologic analysis of selected INFECT NSTI and non NSTI cases against streptococcal exotoxins of the corresponding isolate. Relative antibody titers of 11 superantigens (A) and a second set of 7 exotoxins (B) in Day 0 (left grid) and Day 3 (right grid) patient plasma samples combined with the Multiplex PCR results, for 8 IVIG treated cases, 7 non IVIG treated cases and 2 non NSTI control cases. Plasma IgG titers against a streptococcal exotoxin calculated as statistical significant against the corresponding *E. coli* purified empty vector background control ($p<0,05$, $n=2$) that matches to a positive PCR result within the genome of the corresponding bacterial isolate are marked with blue squares. Not significant titers against an exotoxin, whose gene was identified

in the genome of the corresponding pathogen during the screening PCR, are marked with red squares. Significant titers against a superantigen not present in the corresponding bacterial genome are marked with grey squares. The INFECT case number is given on the left vertical axis while the M-type of the corresponding streptococcal isolate is shown on the right vertical axis. The different analyzed exotoxins are given on top of each grid. The selected INFECT cases are sorted by their groups: IVIG-treated NSTI cases, non IVIG-treated NSTI cases, and non NSTI control cases.

The compiled results showed that the adaptive immune response plays a crucial role in development/retrogression of NSTI. All analysed NSTI cases lacked a specific antibody titer against at least one of the exotoxin present in the genome of the corresponding isolate and therefore very likely involved in the NSTI pathogenesis. Contrarily, the non NSTI control cases showed significant antibody titers against all superantigens present in the genome of the causative pathogen. Since IVIG is a solution of concentrated antibodies from healthy donors, it was able to compensate for the deficiencies of exotoxin specific antibodies in IVIG treated NSTI patients, as seen on the Day 3 samples. For non IVIG treated patients, raised antibody titer against only a subset of exotoxins (SpeA, SpeG, SSA, SmeZ, as well as SIC, SpeB, Mac, SLO, and Hyl) could be observed in the plasma samples taken on the third day of infection. Interestingly, no change in the antibody response was seen against SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, and Ska in any of the non IVIG cases, while the antibodies against Ska-1 and Ska-2a could not be differentiated by the developed ELISA system. To conclude, all patients that developed an NSTI, lacked titers against at least one of the streptococcal exotoxins present in the genome of the corresponding bacterial isolate in the Day 0 plasma samples. Contrarily, the plasma samples of the non NSTI control patients showed antibody titers against all necessary exotoxins, already on Day 0. The next step in the serological approach was to determine if the antibody titers, detected in the patient's plasma, were mediating protectivity against their respective exotoxin. To start with the analysis, the superantigens were selected as a first subset of exotoxins. Therefore, a T-cell Proliferation Blocking Assay was established that would provide insights in the activity of the purified superantigens and the potential of the identified antibody titers to block the toxin function.

4.5 Determination of protectivity of the detected antibody titers against superantigen function

Antibodies specific for a particular exotoxin are potentially able to block its toxic effects by binding to its active site, thereby neutralizing the toxin and providing 'protectivity' to the host. As already mentioned, superantigens specifically target T-cells, leading to a non-specific activation of large amounts of T-cells through binding and cross-linking MHC-II receptors on antigen presenting cells with T-cell receptors (TCR), thus forming

a MHCII-SAg-TCR complex. To evaluate if the detected antibody titers are mediating protectivity against all analyzed superantigens, a T-cell Proliferation Assay was established.

4.5.1 Development of a T-cell proliferation Assay

T-cells are an active part of the adaptive immune system and generally activated by specific antigens. Superantigens, on the other hand, lead to an unspecific activation of a huge subset of T-cells to distract the host immune system from establishing an adaptive response. A proliferation assay with human T-cell was established to analyze the protectivity of the detected antibody titers in the plasma samples. As a first step, a two-fold dilution series of all 11 superantigens was tested towards their potential to mediate unspecific T-cell proliferation (**Figure 4.18**).

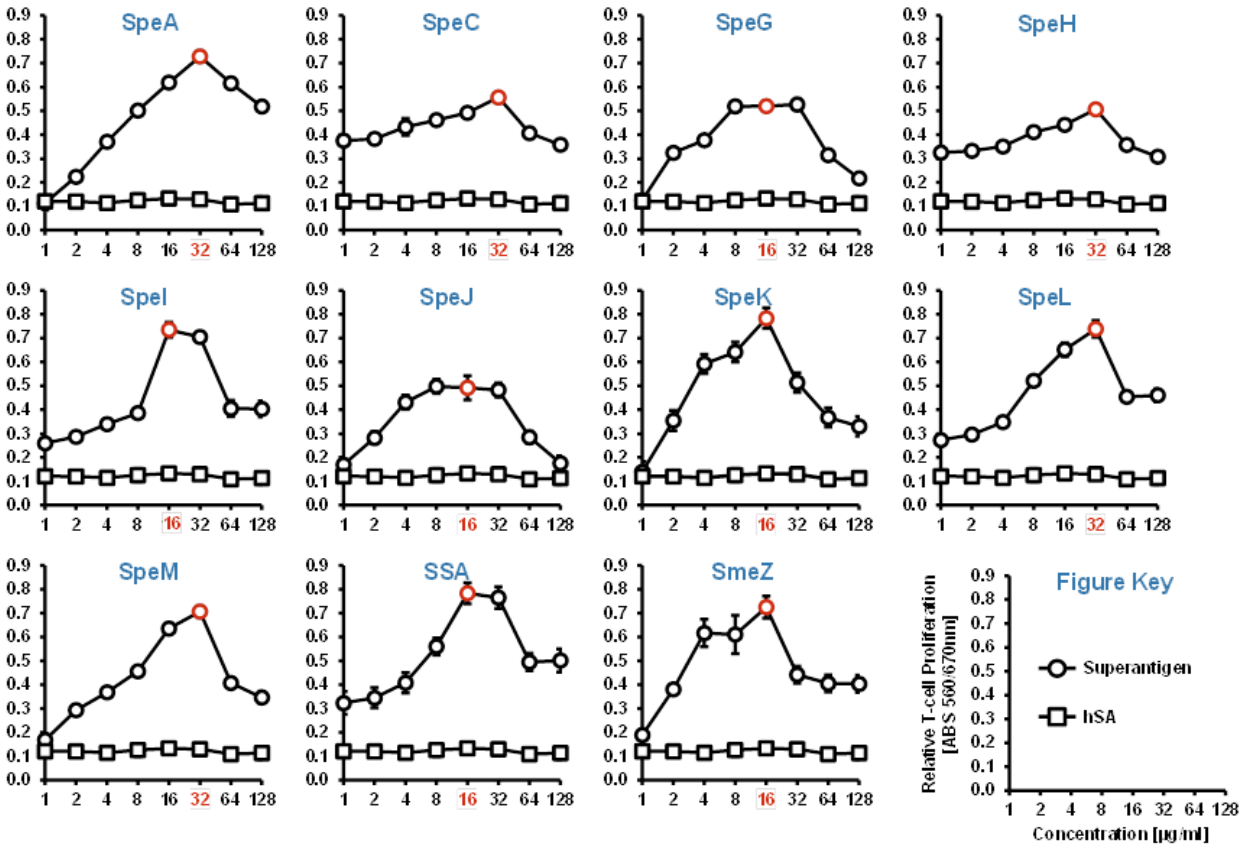

Figure 4.18: T-cell proliferation Assay to test the *in vitro* stimulation of human T-cells by 11 streptococcal superantigens. The proliferation of human primary T-cells derrived from two different healthy donors by 11 streptococcal superantigens was measured. A two fold dilution series of different superantigens in PBS was prepared and added to 2×10^6 purified human T-cells and incubated at 37°C with 5% CO_2 for 6 days. The T-cell proliferation was estimated using an MTT based assay. Human Serum Albumin was used as a non-stimulating negative control. The relative T-cell proliferation was plotted against the concentration of the corresponding superantigen (in µg/ml). The concentration of superantigens for which the highest response was the obtained (marked with a red circle) was used as defined concentration for furher experiments.

In most of the cases, the T-cell proliferation shows a parabolic effect with the increasing concentration of the tested superantigen, which clearly indicates the functionality of all purified superantigens. For each tested superantigen the concentration, where the stimulating effect towards unspecific proliferation of T-cells reached its maximum was determined and used for further experiments. For SpeA, SpeC, SpeH, SpeL and SpeM, a concentration of 32 µg/ml was selected whereas for SpeG, SpeI, SpeJ, SpeK, SSA and SmeZ, 16 µg/ml was selected for further experiments.

4.5.2 Development of a T-cell Proliferation Blocking Assay

To investigate the protective role of the antibody titers detected in the INFECT plasma samples, a Blocking Assay was established, which based on the determined maximum cellular proliferation concentration of each of the 11 streptococcal superantigens. The results of the analysis of IVIG treated NSTI case plasma samples with the established ELISA system suggests that IVIG carries significant antibody titers against all tested superantigens (Section 4.4.3). Therefore, a set of different dilutions of IVIG, co-incubated with the determined maximum proliferation concentration of each superantigen was used to measure the percentage of inhibition of each superantigen activity (**Figure 4.19**).

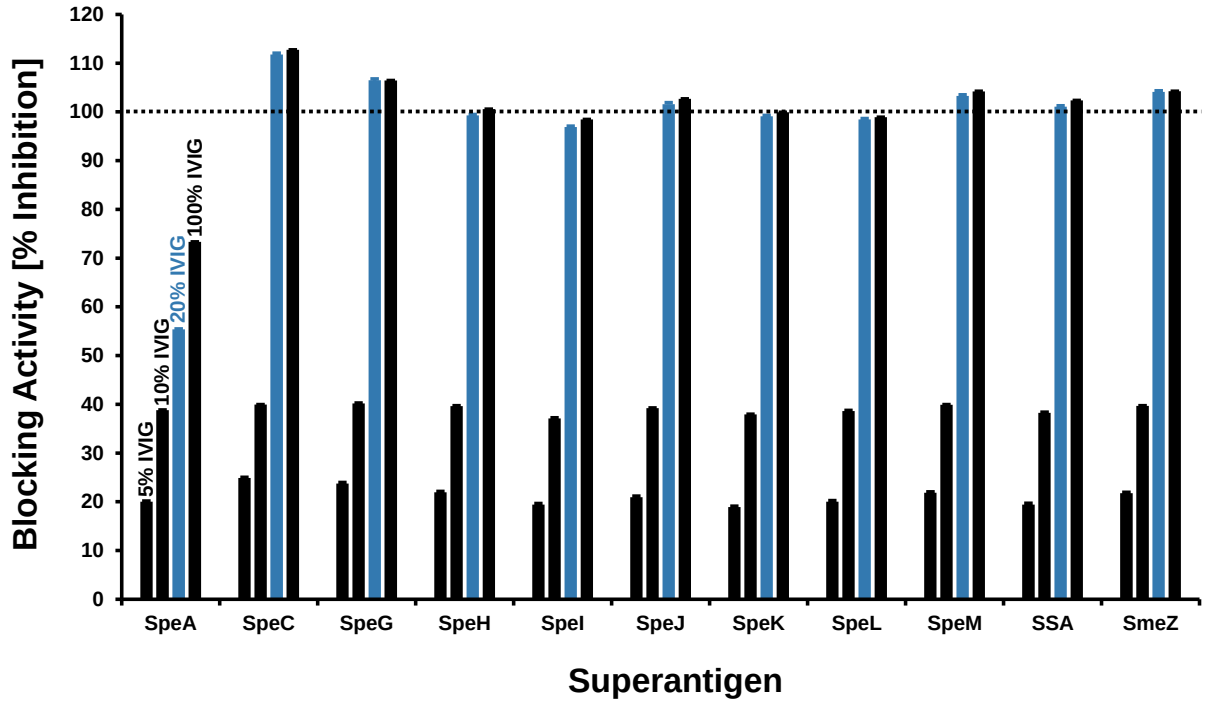

Figure 4.19: Bar graph depicting the blocking activity of antibodies in IVIG against 11 streptococcal superantigens. Superantigens were diluted in selected concentrations in IgG depleted plasma and co-incubated with different dilutions of IVIG (100% = 5 mg/ml) for 1 hour at room temperature. Subsequently 2×10^6 purified human T-cells were added to the Superantigen-IVIG mixture and incubated at 37°C with 5% CO_2 for 6 days. The T-cell proliferation was estimated using an MTT based Assay. IgG depleted plasma containing the same amount of human serum Albumin was set as negative control and the percentage of inhibition was calculated by substracting the

obtained IVIG blocking values with the proliferation values of T-cells with human albumin. Different concentrations of IVIG are depicted in black bars. Blue bars represent the concentration of IVIG selected for further experiments.

A concentration dependent inhibition of the superantigen activity by antibody mediated blocking of IVIG was observed. 100% IVIG (5 mg/ml) was able to completely block all superantigen activity except SpeA. As previously described, complete inhibition of SpeA cannot be achieved in this set up[104]. 5% IVIG and 10% IVIG were not able to completely block the activity of the tested superantigens. However, 20% IVIG showed the same effect *in vitro* as 100% IVIG with respect to its blocking efficiency. Thereafter, 20% IVIG in IgG depleted human plasma was used as positive control in further experiments.

4.5.3 Determination of the protectivity against superantigens of antibody titer detected in NSTI case plasma samples

The established T-cell Proliferation Blocking Assay was used to determine the protection potential of detected antibody titers in NSTI patient plasma sample sets. Due to constraint of limited plasma sample volume, only a few selected cases from the cohort were used in this approach. The blocking assay was initially tested on two INFECT cases: **5006** (**IVIG treated group**) and **6018** (**non IVIG treated group**) (**Figure 4.20**).

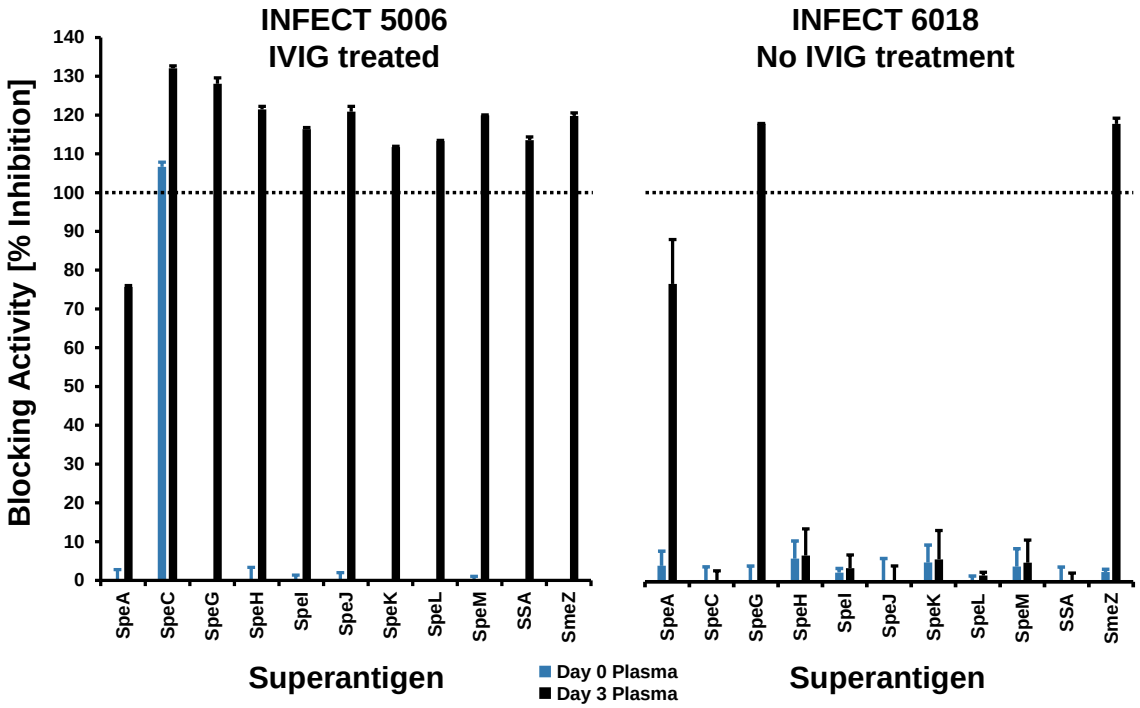

Figure 4.20: Blocking of superantigen activity by antibodies from NSTI patient plasma samples of two selected INFECT cases. Superantigens were diluted in selected concentrations in INFECT patient's plasma (20%, diluted in PBS) for 1 hour at room temperature. 2×10^6 purified human T-cells were added to the Superantigen-INFECT patient plasma mixture and incubated at 37°C with 5% CO_2 for 6 days. T-cell proliferation was estimated

using a MTT based Assay. IgG depleted plasma containing the same amount of human serum Albumin was set as negative control and the percentage of inhibition was calculated by substracting the obtained antibody blocking values with the proliferation values of T-cells with human albumin. Percentage inhibition of 11 streptococcal superantigens by 20% Day 0 plasma is indicated in blue bars and of 20% Day 3 plasma in black bars.

In the Day 0 plasma samples of the two selected NSTI cases no significant blocking activity could be observed, except a 100% functional inhibition of SpeC by the plasma of the case **5006**. However, the Day 3 plasma sample of this case showed 100% antibody mediated blocking of all superantigens, except SpeA, whose function was inhibited by around 70%. This increase of antibody mediated blocking of the T-cell proliferation inducing function of the tested superantigens could be explained by the applied IVIG treatment. IVIG was obviously able to compensate the observed deficiencies in antibody titers and antibody mediated protectivity. In the Day 3 plasma samples of the non IVIG treated NSTI case **6018** protective antibody titers could only be detected against SpeA, SpeG and SmeZ.

The proliferation assay was extended to a subset including the plasma samples of three IVIG treated cases (**5004, 5006** and **6013**) and two non IVIG treated cases (**6016** and **6018**) (**Figure 4.21**).

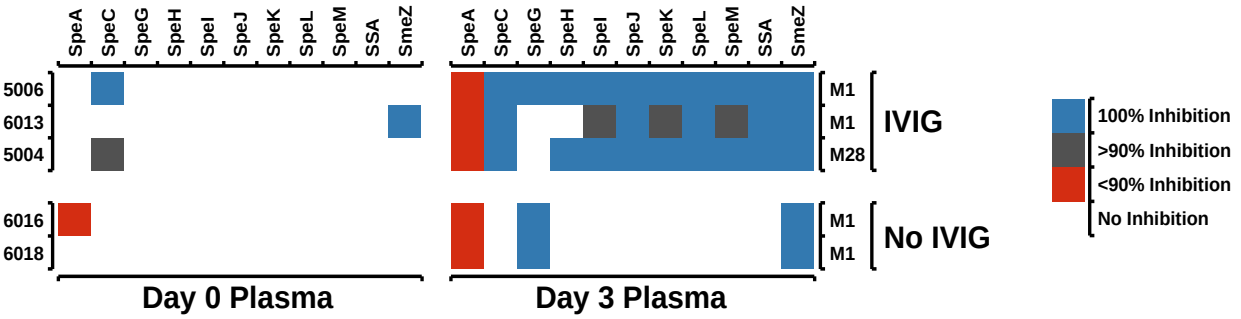

Figure 4.21: Antibody mediated blocking activity of NSTI plasma samples against 11 streptococcal superantigens. Percentage inhibition of 11 streptococcal superantigens by 20% Day 0 (left grid) and Day 3 (right grid) plasma was calculated against 20% IgG depleted plasma. Blue squares are marked in case of 100% blocking, grey squares show more than 90% inhibiton and red squares depict inhibition less than 90%.

All detected antibody titers were able to block the superantigen activity by more than 90%, except for SpeA wherein inhibition above 80% was not possible.

To generate a comprehensive overview about the presence of exotoxin genes in the genomes of the analysed INFECT isolates (Section 4.3), in combination with the antibody titers detected in the corresponding patient plasma samples (Section 4.4) and the determined antibody mediated protection against these toxins(Section 4.5), the results obtained with the developed ELISA system were linked to the results of the T-

cell proliferation blocking assay, and combined with the Multiplex PCR screening results (Figure 4.22).

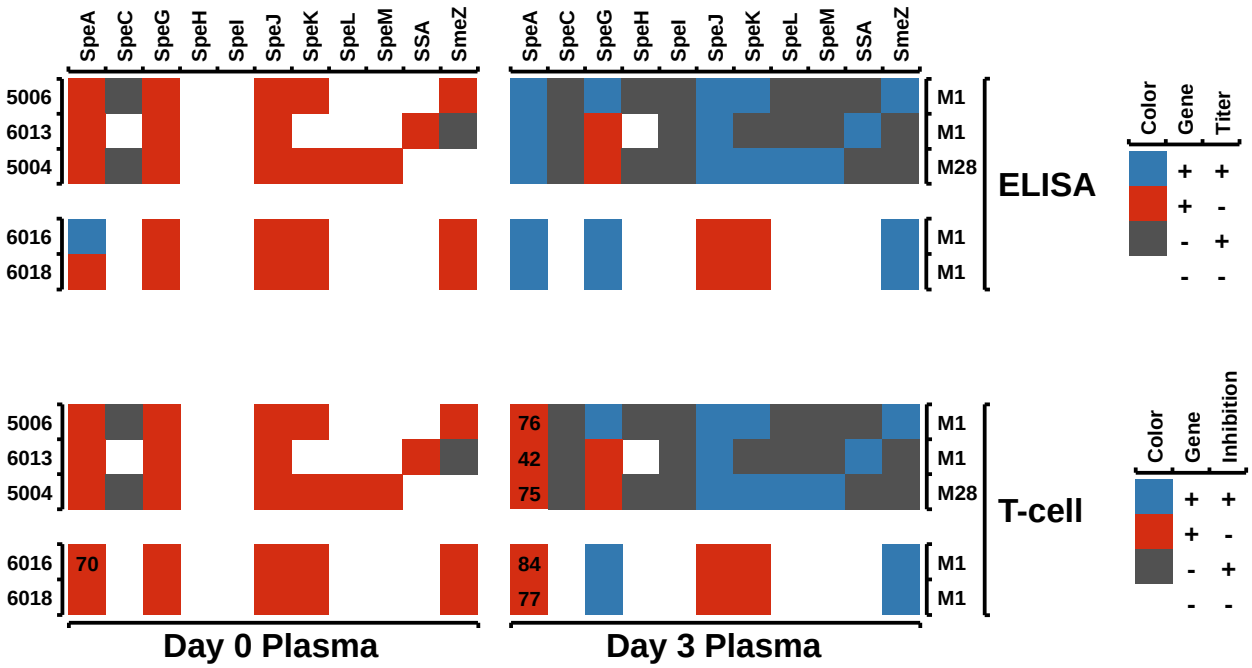

Figure 4.22: Comparative plot combining the results of antibody titer screening by the ELISA system, antibody mediated superantigen blocking determined by T-cell proliferation, and the exotoxin gene screening by Multiplex PCR. Antibody titers and their protection potential of three representative IVIG and two non IVIG treated cases were studied. The upper grid shows the detected antibody titers determined by ELISA in combination with the results of the bacterial Multiplex PCR genome screening. Plasma IgG titers that are considered to be significant ($p < 0.05$, n=2; ANOVA) are shown as blue square (including a positive PCR result). In case of no significant titer but a positive PCR result, the squares are colored red. While, a positive antibody titer but no corresponding gene in the genome of the causative bacterial isolate is marked in grey. The lower grid displays the protection potential of the detected antibodies against 11 streptococcal superantigens measured by the established T-cell proliferation blocking assay. Blue squares are marked in case of 100% inhibition of superantigen function when the gene coding for the corresponding superantigenis present in the genome of the causative bacterial isolate. Red squares depict that there was no inhibition when the corresponding gene was present, while grey squares show that there was an antibody mediated inhibtion but no corresponding gene present. Numbers given in red squares represent the percentage of inhibition values compared to the positive control treated with IgG depleted human plasma.

On comparative analysis of antibody titers detected with the ELISA system and their protection potential against the superantigen function determined by the T-cell proliferation blocking assay, it was quite apparent that all detected IgG titers considered as statistical significant when compared to the background control were mediating 100% protection against the corresponding superantigen, with the exception of SpeA, which cannot be blocked completely. If these results are extrapolated to other NSTI and non NSTI patients, the detected antibody titers in the Day 0 plasma samples of the analyzed non NSTI control patients could most probably inhibit the function of

most superantigens probably produced by the corresponding *S. pyogenes* isolate during the initial stage of infection, while the NSTI cases suffered from a fatal serologic susceptibility towards the superantigen repertoire of the causative pathogen. Hence it can be concluded, that the adaptive immune response against streptococcal exotoxins, especially against superantigens also plays a crucial role in development of a severe NSTI.

5. Discussion

Necrotizing Soft Tissue Infections (NSTIs) like necrotizing fasciitis (NF) are life threatening infections of the deeper layers of skin and subcutaneous tissue, spreading across the fascia[1]. NSTIs are generally associated with medical risk factors like diabetes mellitus, cardiovascular disease, or recent surgery, but also with small wound infections or trauma and have a mortality rate of 12-35%, when properly treated[5]. It is often difficult to diagnose an NSTI due to its close symptomatic similarities with cellulitis, during early days of infection, thus posing a threat to life of the patient due to delays in specialized treatment and simultaneous rapid spread of the pathogen[105]. NSTI affected patients need intensive care unit treatment with rigorous medication, surgery to remove infected tissue and specialized treatment strategies like HyperBaric Oxygen therapy (HBO) and IntraVenous Immuno-Globulin administration (IVIG). IVIG is a sterile preparation of concentrated antibodies recovered from the pooled serum of at least 1000 healthy donors that is discussed to work by a variety of mechanisms like activation of complement, facilitation of opsonisation, and neutralization of bacterial toxins, and therefore frequently applied by NSTIs although a positive effect on the recovery of affected patients has not been shown yet.

The development of an NSTI is a complex interplay between the host immune system and the invading pathogen that produces various virulence factors and exotoxins with which it is able to manipulate or evade the immune response. Generally, the first line of defense is provided by the innate immune system as an initial response against the invading pathogenic bacteria. However, along with the innate immune system, the second line of defense, the adaptive immune system, is activated and provides specific antibodies, which help boosting the innate immune response through mediating opsonization of bacteria and toxin neutralization. Antibody dependent opsonization of bacteria by the complement system acts as chemoattractant towards phagocytic cells of the innate immune system like neutrophils and thus increases the recognition and phagocytic clearance. Consequently, the lack of specific antibodies against invading bacterial pathogens and against bacterial toxins could represent a risk factor for the development of a severe NSTI. In this perspective, the here presented study was designed to investigate the contribution of the host immune system, especially the adaptive immune system, particularly focusing on the NSTI scenario. Therefore, cases of Necrotizing Fasciitis were recruited and the corresponding bacterial isolates were collected under the European Union funded project named INFECT (http://www.fp7infect.eu/). In parallel, control cases were enrolled wherein patients

suffered from severe skin and tissue infections that did not develop a necrotic stage. As a starting point, all collected INFECT cases within the time frame of two years (2013-2015), were typed on the basis of the causative pathogen. This allowed the distribution of the collected NSTI cases into three subtypes: type I, caused by a diverse mixture of different bacterial species; type II mainly caused by *Streptococcus pyogenes* and type III, caused by *Clostridium* species. This typing confirmed the complicated nature of NSTIs caused by bacteria, which are quite diverse. However, type II infections, dominated by *S. pyogenes*, were found most frequent compared to others subtypes of NSTIs. These results have been in agreement to previous studies[106]. Hence, the main focus of this study was set to the investigation of type II NSTIs caused by *S. pyogenes*. Therefore, various specialized typing methods were applied, which confirmed the species affiliation of all analyzed *S. pyogenes* INFECT isolates by their β-hemolytic behavior and their expression of the 'A' antigen on the bacterial surface. Furthermore, all *S. pyogenes* isolates were characterized based on their M-protein (*emm* gene), a surface bound virulence related protein and the primary antigen of *S. pyogenes*[29]. Sequence driven comparisons of the *emm* gene (M-typing) further classified the *S. pyogenes* INFECT isolates by different serotypes (M-types). A dominance of isolates of the serotype M1 in the analyzed collection was observed, which is in accordance to other publications that suggest the geographical dominance of *S. pyogenes* M1 in Scandinavian countries[107,108,109,110]. The serotype M1 has been seen to procure the ability to undergo rapid changes in severity and the disease frequencies[111,112]. However, the exact reason for its dominance still remains undetermined.

The human innate and adaptive immune system comprises of vast variety of cells and proteins that work in a well-coordinated manner during the course of an infection. In this perspective, antibodies perform a crucial function in bacterial clearance and toxin neutralization. Due to such indispensable role of antibodies, IVIG that are concentrated antibodies, are generally given as an adjunctive therapy in case of an NSTI caused by *S. pyogenes*. However, administration of IVIG is the sole choice of the physician treating the patient, thereby some patients receiving it and others not. Even though there are many reports that prove IVIG contains protective antibody titers against *S. pyogenes*, its efficacy in the NSTI scenario still remains ambiguous. IVIG treatment, in addition, is expensive and might not only cause mild side effects like fever but also severe repercussions such as anaphylaxis and renal failure. Taking the assets and drawbacks of IVIG as a specialized treatment during the acute phase of an NSTI in consideration, a comprehensive analysis of existing or missing antibody titers and their protective potential in serum samples of NSTI patients would provide valuable insights into the

role of the adaptive immune system during the development of this dramatic infection. Therefore 3 distinct groups of serum samples of recruited INFECT cases were analyzed: NSTI cases that received an IVIG treatment (IVIG Treated Group), NSTI cases that did not received an IVIG treatment (Non IVIG Treated Group), and a control group of cases with severe skin and tissue infections, that did not develop into an NSTI (non NSTI cases/Control Group) and therefore also did not received any IVIG. The determination of antibody titers and their protective potential against the pathogenic bacterial cells as well as the bacterial toxins in these three groups would not only identify bacterial factors that play a crucial role in the development of the infection (NSTI cases versus non NSTI cases), but would also aid in elucidating the role of the serological state of the patient in the development of an NSTI. In addition, the comparison of IVIG-treated and non IVIG treated cases would decipher the effectiveness of IVIG as an adjunctive therapy. The study was therefore concentrated on 17 INFECT cases: 8 IVIG treated NSTI cases; 7 non IVIG treated NSTI cases and 2 non NSTI control cases.

During the course of infection, bacteria can pose certain antigenic challenges to the host, that, if targeted by specific antibodies indirectly contribute to an increased clearance of the bacteria by phagocytic cells of the innate immune system. Therefore, studying the presence of protective antibody titer in patient sera against the bacteria itself during the early stage of infection would identify risk factors towards an increased susceptibly of NSTI patients and would represent a first step into the development of an optimized IVIG treatment strategy. Therefore, Opsono-phagocytosis assays were performed to determine if specific antibodies are present against expressed immunogenic proteins on the surface of the corresponding bacterial pathogen. These antibodies can boost the process of pathogen recognition with help of the complement system through clearance by Opsonization. To investigate if the antibodies against the bacteria itself would mediate efficient opsonization and subsequent killing, an opsono-phagocytosis assay was established using patient's plasma that was collected on two different time points: Day 0, when a tissue infection was rated as NSTI and the patient recruited in the study, and Day 3, which was the third day of treatment after the NSTI was diagnosed.

The establishment of a reproducible Opsono-phagocytosis assay implied certain mechanistic limitations. First of all, plasma components other than immunoglobulins and complement factors may also influence the process of bacterial opsonization[113]. To develop a suitable control system to address potential side effects of other plasma

factors a pool of commercially available human plasma was depleted from IgG by specific binding to Protein G Sepharose. This IgG depleted plasma was used on one hand as negative control but also in combination with a physiological amount of IVIG as positive control for bacterial opsonization. Secondly, due to the use of Li-heparin as anticoagulant in the experimental setup any factors of the complement system that are known to boost the opsonization and phagocytosis process *in vivo* are potentially excluded[114]. Therefore, all bacterial killing that is observed in the assay is potentially mediated by Fc receptor binding on the surface of the used phagocytic cells. Consequently, the real *in vivo* effect of the observed recognition and killing would be potentially higher than *in vitro* because of an active complement system The established Opsono-phagocytosis assay was validated with the INFECT isolate 2006, opsonized with IVIG in IgG depleted human plasma, which is known to potentially possess specific antibodies against *S. pyogenes*[115]. Interestingly, IVIG was able to mediate significant bacterial killing with respect to the growth controls. These results not only validated the functionality of the established Opsono-Phagocytosis assay but also proved the presence of antibodies reacting specifically against *S. pyogenes* isolates in the applied IVIG mixture. The established assay was extended to the complete set of selected INFECT isolates and their corresponding plasma samples. All NSTI cases did not show significant bacterial killing by human neutrophils when opsonized with Day 0 plasma samples. Contrarily, a significant bacterial killing could be mediated with Day 0 plasma samples of the non NSTI control cases. These results point towards a potential susceptibility of the analysed NSTI patients due to a lack of IgG titers in their plasma that are able to mediate efficient killing of the corresponding *S. pyogenes* isolate. However, all NSTI cases, irrespective of the IVIG treatment, showed significant bacterial killing mediated by the Day 3 plasma samples. The treatment with IVIG was obviously able to enhance the bacteria killing as compared to Day 3 plasma samples of non IVIG treated cases, thus accounting for its enhanced efficiency to target bacteria probably due to presence of specific antibodies against the pathogen.

The applied Opsono-Phagocytosis assay clearly showed an efficient reduction of bacterial counts mediated just by opsonization with plasma samples of NSTI and non NSTI patients, which are obviously carrying different antibody titers against the respective causative pathogen. Nonetheless, the antigenic presentation of the bacteria might vary significantly *in vivo* compared to the proteins presented *in vitro* (in THY media)[116]. Moreover, the bacterial capsule plays an important role *in vivo*, as it usually masks most of the exposed surface proteins by a thick Hyaluronic acid layer[117]. Hyaluronic acid is also a natural part of the host tissue and thus bacteria can easily

evade immunogenic responses. However, while growing *in vitro* (in THY), *S. pyogenes* downregulates the capsule[118], exposing the bacterial-surface bound-antigens like M-protein and SfbI that could lead to their enhanced killing while encountering human neutrophils.

Out of all 15 analyzed INFECT NSTI cases, 14 showed a very similar pattern towards an efficient opsonization and subsequent killing of the causative bacterial pathogen only mediated by Day3 plasma samples. Possible explanations for this observation are either the applied IVIG administration or in case of the non IVIG treated patients a rapid adaptive immune response by an activation of already present Memory T-cells. Memory T-cells are generally activated on second encounter of a pathogen which leads to a rapid production of specific antibodies. Surprisingly, the plasma sample of one of the NSTI cases (INFECT 2015) was able to mediate significant bacterial killing already with the Day 0 plasma sample, which is in agreement with the results obtained with the developed serology ELISA system against the corresponding exotoxins. 2015 closely resembled non NSTI cases with respect to titers and their protectivity. Unfortunately, due to the lack of detailed patient data, it is unsure if the patient 2015 received the IVIG treatment before the Day 0 plasma collection or if 2015 is a kind of 'on the edge' case, where the complex interplay between immune system and invading bacterial pathogen is located very close to the fine "borderline" that differentiates NSTI cases from non NSTI cases. This case also indicates that possessing a protective IgG titer against the invading bacterial pathogen is not the sole reason of not developing an NSTI. It is quite possible that phagocytic cells are unable to efficiently utilize the opsonization potential of the detected antibodies against the pathogen due to restricted oxygen pressure at the site of infection. Since the inflammation is immense, the blood supply to the necrotic site is hindered, leading to reduction in oxygen pressure and efficiency of phagocytic cells to eliminate the pathogen. To boost the oxygen pressure and on that way the activity status of immune cells, HBO is generally given as the adjunctive therapy and might not have been sufficiently provided. Other factors, like host HLA type[119], general immune status, and underlying medical risk factors, should also be considered to be involved in the development of the infection.

The applied Opsonophagocytosis assays revealed a significant susceptibility of the analyzed NSTI patients against the corresponding bacterial pathogen during the initial stage of infection whereas the data obtained from the analyses of the non NSTI control cases showed an IgG dependent protection potential in the Day 0 plasma samples. However, killing of the bacterial pathogen does not reduce their detrimental effects as

S. pyogenes is known to secrete a wide array of exotoxins[20] that remain active and can further deteriorate the patient's condition. Hence, the second aim of the study was to investigate the protective potential of an adaptive immune response against secreted bacterial toxins. Therefore, a comprehensive serology approach was applied, that should examine the question if specific antibody titers against all exotoxins encoded in the genomes of the causative bacterial isolate are present in the plasma samples of the selected INFECT patients and if these titers mediate efficient protection against the specific toxin function. This would elucidate if the absence of antibodies against a specific exotoxin or a group of exotoxins represents a risk factor in development of an NSTI.

It is known that *S. pyogenes* secretes exotoxins that impair the host immune response in different ways like activation of non-specific T-cell sub-population (superantigens)[120], cleavage of IgG (Mac)[121], degradation of chemokines (spyCEP)[122] or inactivation of complement factors (sic)[123]. Screening the genomes of all selected *S. pyogenes* INFECT isolates for the presence of various exotoxin genes would be the first step towards understanding the serological background of the selected NSTI cases towards the potential exotoxin repertoire of the causative pathogen. Therefore, a multiplex PCR was established that is able to detect 21 exotoxin genes in five separate reactions. The PCR genome screening of the selected *S. pyogenes* INFECT isolates showed that only some of the exotoxin genes were distributed in a serotype specific pattern, while other exotoxin genes like *speB*, *speF*, *speG*, *scpA* were always present; irrespective of the serotype of the bacteria. This could be indicative of their essential function in pathogenicity of the bacteria during the development of an NSTI. There are many previous researches that show similar exotoxin profiles of various M types[124],[125]. Interestingly, *sdc* and *sdaD* were seen excluding each other; the former being present only in one of the isolates (INFECT **2015**), which could represent a possible protection mechanism of *S. pyogenes* to eliminate redundancy in case of extracellular DNase activity. The resemblance matrix generated though the exotoxin gene repertoire clearly showed that the non NSTI isolates clustered together with the NSTI isolates, thus ruling out the possibility that NSTI cases carried a unique set of exotoxins that made it more virulent towards the development of a necrotic tissue infection like an NSTI. Since the occurrence and distribution of all known streptococcal exotoxins within the analyzed *S. pyogenes* INFECT collection was determined, the next logical step was the detection of antibody titers against the indicated exotoxins within the INFECT plasma samples.

As already mentioned, the host immune system eradicates the invading pathogen by a variety of mechanisms. The adaptive immune system helps boosting the process of pathogen recognition and clearance by the generation of specific antibodies. To investigate the contribution of the adaptive immune response by specific antibodies against streptococcal exotoxins in the NSTI scenario, all exotoxins identified by the applied PCR screening were either cloned, overexpressed and purified in an *E. coli* background or purchased if commercially available. An empty vector cloning batch was purified in parallel to address potential false positive readings induced by antibody titers against *E. coli* background contaminations. All variables possibly influencing the results of an ELISA system like antigen coating, primary and secondary antibody concentrations, ABTS concentrations, and the dynamic range of the detection system were sequentially standardized and a test experiment was performed using IVIG diluted in IgG depleted human plasma following the standardized procedure used in the Randomised Controlled Trial (INSTINCT) at Rigshospitalet Denmark (NCT02111161). This experiment clearly showed that the tested IVIG mixture contains specific antibody titers against all selected exotoxins, which, on one hand demonstrated the potential of the established ELISA system to detect antibody titers against streptococcal exotoxins, but on the other hand also proved the notion that IVIG contains specific antibodies against *S. pyogenes* exotoxins[77]. Extending the established ELISA system towards the analysis of all selected INFECT cases illustrated the existence of only rare antibody titers against streptococcal exotoxins in the Day 0 plasma samples, which were only increasing in the Day 3 samples independent of the IVIG treatment status of the patient. Contrarily, the plasma samples of the non NSTI cases showed significant antibody titers against the tested streptococcal exotoxins already at Day 0. Compiling the results of multiplex PCR screening with the data obtained with the ELISA system, it was obvious that all NSTI patients showed a potential susceptibility towards the exotoxin set present in the genome of the corresponding bacterial isolate during the early stage of infection. The administration of IVIG was able to increase the antibody titers against these exotoxin sets, proving the hypothesis that IVIG treatment potentially compensates a lack of specific antibodies against exotoxins of *S. pyogenes*. The non IVIG treated cases, showed an increase of specific antibody titers in the Day 3 plasma samples for only a few exotoxins. Especially the superantigens, SpeA, SpeG, SSA and SmeZ obviously seem to play a role in pathogenesis of *S. pyogenes* during development of NSTI as their titers increased significantly during the course of infection. This rapid antibody generation is most probably induced by a memory T-cell response of adaptive immune system. As mentioned earlier, the memory T-cells are generally activated on second encounter of

the pathogen and boost the production of specific antibodies rapidly so as to quicken up the annihilation of bacterial infection and neutralisation of secreted toxins. Other superantigens like SpeH, SpeI, SpeK, SpeL and SpeM did not seem to induce a detectable response of the host adaptive immune system, which can either be explained by a downregulation of these bacterial factors during the infection or by a lack of corresponding memory T-cells because the immune system of the patient was never before confronted with a streptococcal invader expressing this special setup of exotoxins. The second set of selected exotoxins includes various proteases, DNases and immune modulatory molecules. The antibody titers against SLO were seen to increase from the Day 0 towards Day 3 of infection in all non IVIG treated patients. SLO or streptolysin O, is an immunogenic cytolysin that ruptures the cell wall of host cells, thereby potentially causing failure of an immediate cellular immune response[41]. Streptokinase (Ska) is an important spreading factor of *S. pyogenes* that causes activation of Plasminogen by structural remodulation, which is subsequently able to dissolve fibrin clots thereby releasing bacteria entrapped in a thrombus probably induced by the initial injury[51]. Ska can be distinguished into three different classes on the basis of mechanism of plasminogen acquisition and activation: *ska-1*, *ska-2a* and *ska-2b*[51]. Sequencing the variable regions of *ska* revealed presence of only type 1 and 2a in the INFECT isolate collection. However, the established ELISA system was obviously not able to serologically differentiate between Ska-1 and 2b, as the variable region measures just 400 bp and might have lacked potentially accessible epitopes. In contrast to the analysed NSTI cases, the non NSTI control cases possess specific antibody titers against all exotoxins, for which the gene is present in the genome of the corresponding bacterial isolate already in the Day 0 plasma samples, which points towards a potential resistance of these patients against the development of an NSTI. Overall, the results obtained with the established ELISA system strengthen the hypothesis that a lack of specific antibodies against the causative bacterial pathogen and against corresponding bacterial toxins represents a risk factor for the development of an NSTI. The final step in the serological approach was to determine if the antibody titers, detected in the patient's plasma, are able to mediate sufficient protectivity against the respective exotoxin. As a starting point all streptococcal superantigens were selected as a first subset of streptococcal exotoxins and hence, a T-cell proliferation Assay was established that would provide insights in the activity of the purified superantigens and the potential of the identified antibody titers to block the toxin function.

Antibodies against a particular exotoxin block its toxic effects by binding to its active sites, thereby neutralizing the toxin and providing 'protectivity' to the host. The selected set of eleven *Streptococcal pyrogenic exotoxins* possesses super antigenic properties, which means they target human T-cells in combination with antigen presenting cells, which leads to a non-specific activation of large amounts of T-cell populations. In the first test experiment all purified superantigens were able to induce a non-specific T-cell proliferation response that follows distinct concentration dependent patterns. Hence, the concentration wherein maximum T-cell proliferation was observed was selected for further experiments. As confirmed by the applied ELISA measurements, the used IVIG mixture carries significant antibody titers against all eleven superantigens, therefore, a first blocking experiment was performed with different concentrations of IVIG in IgG depleted human plasma. As expected, IVIG was able to block 100% of the superantigen activity with a concentration of as low as 20% (1 mg/ml), wherein all the superantigens were successfully blocked except SpeA. Interestingly, it was not possible to completely block the activity of SpeA, irrespective of the concentration of IVIG used, which is in accordance to previous reports[104]. One possible explanation that is discussed points towards additional domains of SpeA targeting the T-cells in a way that cannot be efficiently blocked by antibodies, because unlike other superantigens, SpeA binds to the MHC-II α chain instead of β[126]. The mechanism by which SpeA escapes the blockage by IVIG is still not known. The expansion of the T-cell Proliferation Blocking Assay analyses on a selection of INFECT cases clearly demonstrated the protective effect of the detected antibody titers against the superantigens. Comparing the results from the multiplex PCR screening and the ELISA based serology approach with the data obtained with the T-cell proliferation blocking assay, displaced a similar pattern wherein all detected antibody titers considered to be significant were protective and were able to inhibit the superantigenic properties of the exotoxins. This does not only indicate the authenticity of the ELISA system, but also the functionality of the established T-cell Proliferation Blocking Assay to detect and determine the protectivity of antibody titers. All detected antibody titers were able to block the superantigenic activity by more than 90%, except SpeA wherein inhibition above 80% was not possible. From the combined results of the Multiplex PCR screening, the serologic ELISA approach and the T-cell proliferation blocking assays, it is quite evident that the adaptive immune response plays a crucial role in development of an NSTI. These results can henceforth be extrapolated to other NSTI and non NSTI plasma samples. For the second set of exotoxins that consists mainly of proteases and DNases with different immune-modulating functions, it is still unsure if the antibody titers detected in patient plasma samples mediate efficient protection.

Specialized blocking assays, which will address this open question, will be established in extension of this study.

The combined results of this study clearly show that the lack of protective antibodies against streptococcal exotoxins and against the bacterial cells itself represents a significant risk factor for the development of a severe NSTI. Whenever the pathogen (here *S. pyogenes*) enters the healthy individual, the interplay between the host immune system and the pathogen decides their fate of encountering an NSTI. There are many factors that contribute to whether the person would develop the disease or not. This study identified one of the crucial factors to be presence or absence of protective antibody titers in the patient and can help to bridge the time lapse between identification of the disease and its effective treatment. Rapid serological screening methods can be developed that will help in a quick and efficient screening of plasma samples of patients to estimate their susceptibility status towards the causative pathogen during the very early stage of a developing NSTI. In case the patient lacks antibody titers against the invading pathogen, IVIG can be provided at early stage of the infection development to restrict the progression. As shown by this study, the applied IVIG treatment is able to compensate the detected lack of protective antibody titers against streptococcal exotoxins and the bacteria itself and on that way eliminate this risk factor. However, time is a critical factor in this treatment. As the disease develops, inflammation at the site of infection escalates, which will affect the penetration rate of IVIG into the tissue. Hence, administration of IVIG needs to started as early as possible. Other adjunctive therapies like HBO can also help in boosting the protective potential of these antibodies by enhancing the oxygen supply at the site of infection. As seen in INFECT case 2015, the presence of protective antibodies alone does not suffice to prevent the development of an NSTI. Other factors, especially the general immune status of a patient should always be considered to be involved in the infection development.

Although IVIG was seen to compensate the observed antibody titer deficiencies, its efficacy in clinical settings still remains questionable. Additionally, IVIG is a cost-intensive therapy, usually accompanied by a variety of side effects. As the results of this study show the crucial role of the adaptive immune system, especially the serological background of a patient within the NSTI scenario, it can contribute towards development of more specific and targeted therapies and constitutes the first step towards the development of an improved NSTI treatment.

6. Appendix

6.1 References

1. Rapini, R., Bolognia, J. & Jorizzo, J. *Dermatology, 2nd Edition*. (Mosby, Inc, 2007).
2. Wilson, B. Necrotising Fasciitis. *Am Surg* **18,** 416–31 (1952).
3. Wang, J. M. & Lim, H. K. Necrotizing fasciitis: Eight-year experience and literature review. *Brazilian J. Infect. Dis.* **18,** 137–143 (2014).
4. Puvanendran, R., Huey, J. C. M. & Pasupathy, S. Necrotizing fasciitis. *Can. Fam. Physician* **55,** 981–7 (2009).
5. Speers, D. & Shurr, D. Necrotizing Fasciitis: An Overview. *J. Prosthetics Orthot.* **13,** 83–86 (2001).
6. Chi, C. H., Chen, K. W., Huang, J. J., Chuang, Y. C. & Wu, M. H. Gas composition in Clostridium septicum gas gangrene. *J. Formos. Med. Assoc.* **94,** 757–9 (1995).
7. Hasham, S., Matteucci, P. & Stanley, P. Necrotising fasciitis. *BMJ.* **9,** 830–3 (2005).
8. Zacharias, N. *et al.* Diagnosis of necrotizing soft tissue infections by computed tomography. *Arch. Surg.* **145,** 452–5 (2010).
9. Ali, S. Z., Srinivasan, S. & Peh, W. C. G. MRI in necrotizing fasciitis of the extremities. *Br. J. Radiol.* **87,** 20130560 (2014).
10. Castleberg, E., Jenson, N. & Dinh, V. A. Diagnosis of Necrotizing Faciitis with Bedside Ultrasound: the STAFF Exam. *West. J. Emerg. Med.* **15,** 111–113 (2014).
11. Wang, T.-L. & Hung, C.-R. Role of tissue oxygen saturation monitoring in diagnosing necrotizing fasciitis of the lower limbs. *Ann. Emerg. Med.* **44,** 222–8 (2004).
12. Akira, S. & Takeda, K. Toll-like receptor signaling. *Nat. Rev. Immunol.* **4,** 499–511 (2004).
13. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11,** 443–451 (1999).
14. Fieber, C. *et al.* Innate Immune Response to Streptococcus pyogenes Depends on the Combined Activation of TLR13 and TLR2. *PLoS One* **10,** e0119727 (2015).
15. Coleman, W. & Tsongalis, G. *Essential Concepts in Molecular Pathology*. (Elsevier Ltd, 2010).
16. Kuby, J., Goldsby, R., Kindt, T. & Osborne, B. *Immunology, Fifth Edition*. (W. H. Freeman, 2002).
17. Ackerman & Nimmerjahn. *Antibody Fc: Linking Adaptive and Innate Immunity*. (Elsevier Ltd, 2013). at <http://store.elsevier.com/Antibody-Fc/isbn-9780123948182/>
18. Mantis, N. J., Rol, N. & Corthésy, B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* **4,** 603–611 (2011).
19. Alberts, B. *et al. Molecular Biology of the Cell.* (Garland Science, 2002).
20. Cunningham, M. W. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev* **13,** 470–511 (2000).
21. Bisno AL, Brito MO, C. C. Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* **3,** 191–200 (2003).
22. Courtney, H. S., Hasty, D. L. & Dale, J. B. Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. *Ann. Med* **34,** 77–87 (2002).

23. Nobbs, A. H., Lamont, R. J. & Jenkinson, H. F. Streptococcus adherence and colonization. *Microbiol. Mol. Biol. Rev* **73,** 407–450 (2009).

24. Nobbs, A. H., Lamont, R. J. & Jenkinson, H. F. Streptococcus Adherence and Colonization. *Microbiol. Mol. Biol. Rev.* **73,** 407–450 (2009).

25. Kendall F E, Heidelberger M, D. M. N. A serologically inactive polysaccharide elaborated by mucoid strains of group A hemolytic streptococcus. *J Biol Chem.* **118,** 61–69 (1941).

26. Crater, D. L. & van de Rijn, I. Hyaluronic acid synthesis operon (has) expression in group A streptococci. *J. Biol. Chem* **270,** 18452–18458 (1995).

27. Schrager H M, Alberti S, Cywes C, Dougherty G J, W. M. R. Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A streptococcus to CD44 on human keratinocytes. *J Clin Investig* **101,** 1708–1716 (1998).

28. Rivera Starr, C. & Engleberg, N. C. Role of Hyaluronidase in Subcutaneous Spread and Growth of Group A Streptococcus. *Infect. Immun.* **74,** 40–48 (2006).

29. Zampolli, D. M. and A. The M protein of group A Streptococcus is a key virulence factor and a clinically relevant strain identification marker. *Virulence* **2,** 402–412 (2011).

30. Okada, N., Liszewski, M. K., Atkinson, J. P. & Caparon, M. Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A Streptococcus. *Proc. Natl Acad. Sci* **92,** 2489–93 (1995).

31. Horstmann, R. D., Sievertsen, H. J., Leippe, M. & F. Role of fibrinogen in complement inhibition by streptococcal M protein. *Infect. Immun* **60,** 5036–41 (1992).

32. Dale, J. B. & Chiang, E. C. Intranasal immunization with recombinant group A streptococcal M protein fragment fused to the B subunit of Escherichia coli labile toxin protects mice against systemic challenge infections. *J. Infect. Dis* **171,** 1038–1041 (1995).

33. Dale JB, Penfound TA, Chiang EY, W. W. New 30-valent M protein-based vaccine evokes cross-opsonic antibodies against non-vaccine serotypes of group A streptococci. *Vaccine.* **29,** 8175–8 (2011).

34. Nilsson, M. *et al.* Activation of human polymorphonuclear neutrophils by streptolysin O from Streptococcus pyogenes leads to the release of proinflammatory mediators. *Thromb Haemost* **95,** 982–990 (2006).

35. Bryant, A. E. *et al.* Vascular dysfunction and ischemic destruction of tissue in Streptococcus pyogenes infection: the role of streptolysin O-induced platelet/neutrophil complexes. *J. Infect. Dis.* **192,** 1014–1022 (2005).

36. Timmer, A. M. et al. Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. *J. Biol. Chem.* **284,** 862–871 (2009).

37. Brosnahan, A. J., Mantz, M. J., Squier, C. A., Peterson, M. L. & Schlievert, P. M. Cytolysins augment superantigen penetration of stratified mucosa. *J. Immunol.* **182,** 2364–2373 (2009).

38. Madden, J. C., Ruiz, N. & Caparon, M. Cytolysinmediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152 (2001).

39. Ato, M., Ikebe, T., Kawabata, H., Takemori, T. & Watanabe, H. Incompetence of neutrophils to invasive group A Streptococcus is attributed to induction of plural virulence factors by dysfunction of a regulator. *PLoS One* **3,** e3455 (2008).

40. Wessels, M. R. Streptolysin S. *J. Infect. Dis.* **192,** 13–15 (2005).

41. Sierig, G., Cywes, C., Wessels, M. R. & Ashbaugh, C. D. Cytotoxic Effects of Streptolysin O and Streptolysin S Enhance the Virulence of Poorly Encapsulated Group

A Streptococci. *Infect. Immun.* **71,** 446–455 (2003).

42. Brinkmann, V. et al. Neutrophil extracellular traps kill bacteria. *Science (80-.).* **303,** 1532–1535 (2004).

43. Buchanan, J. T. et al. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr. Biol.* **16,** 396–400 (2006).

44. Uchiyama S, Andreoni F, Schuepbach RA, Nizet V, Z. A. DNase Sda1 allows invasive M1T1 Group A Streptococcus to prevent TLR9-dependent recognition. *PLoS Pathog.* **8,** e1002736 (2012).

45. Edwards, R. J., Taylor, G. W., Ferguson, M., Murray, S., Rendell, N., Wrigley, A., et al. Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of Streptococcus pyogenes. *J. Infect. Dis* **192,** 783–790 (2005).

46. Sumby, P., Whitney, A. R., Graviss, E. A., Deleo, F. R., and Musser, J. M. Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathog.* **2,** e5 (2006).

47. Chiappini, N., Seubert, A., Telford, J. L., Grandi, G., Serruto, D., Margarit, I., et al. Streptococcus pyogenes SpyCEP influences host-pathogen interactions during infection in a murine air pouch model. *PLoS One* **7,** e40411 (2012).

48. Zingaretti, C., Falugi, F., Nardi-Dei, V., Pietrocola, G., Mariani, M., Liberatori, S., et al. Streptococcus pyogenes SpyCEP: a chemokine-inactivating protease with unique structural and biochemical features. *FASEB J.* **24,** 2839–2848 (2010).

49. Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. Plasminogen activators, tissue degradation, and cancer. *Adv. Canc. Res* **44,** 139–266 (1985).

50. Ponting, CP., Marshall, J. M., and Cederholm-Williams, S. Plasminogen: a structural review. *Blood Coagul. Fibrinolysis* **3,** 605–614 (1992).

51. Jason D. McArthur, Fiona C. McKay, Vidiya Ramachandran, P. S., Amanda J. Cork, Martina L. Sanderson-Smith, Jason N. Cole, U. R. & Ulf Sjobring, Marie Ranson, and M. J. W. Allelic variants of streptokinase from Streptococcus pyogenes display functional differences in plasminogen activation. *FASEB J.* **22,** 3146–53 (2008).

52. Haataja, S. & Gerlach, D. The SpeB virulence factor of Streptococcus pyogenes , a multifunctional secreted and cell surface molecule with strepadhesin , laminin-binding and cysteine protease activity. *Mol. Microbiol.* **39,** 512–519 (2001).

53. Chaussee, M. S., Liu, J., Stevens, D. L. & Ferretti, J. J. Genetic and phenotypic diversity among isolates of Streptococcus pyogenes from invasive infections. *J. Infect. Dis* **173,** 901–908 (1996).

54. Carroll, R. K. & Musser, J. M. From transcription to activation: how group A streptococcus, the flesh-eating pathogen, regulates SpeB cysteine protease production. *Mol. Microbiol.* **81,** 588–601 (2011).

55. Fernie-King, B. A. et al. Streptococcal inhibitor of complement (SIC) inhibits the membrane attack complex by preventing uptake of C567 onto cell membranes. *Immunology* **103,** 390–398 (2001).

56. Cole, J. N., Barnett, T. C., Nizet, V. & Walker, M. J. Molecular insight into invasive group A streptococcal disease. *Nat. Rev. Microbiol.* **9,** 724–36 (2011).

57. Jason N. Cole, Timothy C. Barnett, V. N. and M. J. W. Molecular insight into invasive group A streptococcal disease. *Nat. Rev. Microbiol.* **9,** 724–736 (2011).

58. Agniswamy J, Lei B, Musser JM, S. P. Insight of host immune evasion mediated by two variants of group a Streptococcus Mac protein. *J Biol Chem.* **279,** 52789–96 (2004).

59. Lei B, DeLeo FR, Reid SD, Voyich JM, Magoun L, Liu M, Braughton KR, Ricklefs S, Hoe NP, Cole RL, Leong JM, M. J. Opsonophagocytosis-inhibiting mac protein of group a streptococcus: identification and characteristics of two genetic complexes. *Infect Immun.* **70,** 6880–90 (2002).

60. Prlic, M. & Jameson, S. C. Homeostatic expansion versus antigen-driven proliferation: Common ends by different means? *Microbes and Infection* **4,** 531–537 (2002).

61. Proft, T. & Fraser, J. D. in *Supernantigens anf Superallergens* **93,** 1–23 (2007).

62. Babbar, A. *Streptococcal Superantigens*. (Springer International Publishing, 2015). doi:10.1007/978-3-319-22455-8

63. Kotb, M. Bacterial Pyrogenic Exotoxins as Superantigens. *Clin Microbiol Rev.* **8,** 411–426 (1995).

64. Alouf H. Mueller-Alouf, and W. Koehler, J. E. Superantigenic Streptococcus pyogenes erythrogenic/pyrogenic exotoxins (ed.). *J. E. Alouf J. H. Free. Sourceb. Bact. protein toxins. Acad. Press. San Diego, Calif.* 567–588 (1999).

65. Proft, B. T. *et al.* Wide Allelic Variation , Mosaic Structure , and Significant Antigenic Variation. *J Exp Med* **191,** 1765–1776 (2000).

66. Sundberg, E.J., H. Li, A. S. Llera, J. K. McCormick, J. Tormo, P. M. Schlievert, K. Karjalainen, and R. A. M. Structures of two streptococcal superantigens bound to TCR-beta chains reveal diversity in the architecture of T cell signaling complexes. *Structure* **10,** 687–699 (2002).

67. Ferretti, J. J. et al. Complete genome sequence of an M1 strain of Streptococcus pyogenes. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4658–4663 (2001).

68. Kalia, A. & Bessen, D. E. Presence of streptococcal pyrogenic exotoxin A and C genes in human isolates of group G streptococci. *FEMS Microbiol. Lett.* **219,** 291–295 (2003).

69. Levison, M. E. & Levison, J. H. Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. *Infect. Dis. Clin. North Am.* **23,** 791–815 (2009).

70. Jallali, N., Withey, S. & Butler, P. E. Hyperbaric oxygen as adjuvant therapy in the management of necrotizing fasciitis. *Am. J. Surg.* **189,** 462–466 (2005).

71. Shah, J. Hyperbaric Oxygen Therapy. *J. Am. Col. Certif. Wound Spec.* **2,** 9–13 (2010).

72. Basma, H. *et al.* Risk Factors in the Pathogenesis of Invasive Group A Streptococcal Infections : Role of Protective Humoral Immunity. *Infect Immun.* **67,** 1871–1877 (1999).

73. Compagno, N., Cinetto, F., Semenzato, G. & Agostini, C. Subcutaneous immunoglobulin in lymphoproliferative disorders and rituximab-related secondary hypogammaglobulinemia: a single-center experience in 61 patients. *Haematologica* **99,** 1101–1106 (2014).

74. Mouthon, L. *et al.* Mechanisms of action of intravenous immune globulin in immune-mediated diseases. *Clin Exp Immunol* **104 Suppl,** 3–9 (1996).

75. Basma, H. *et al.* Opsonic Antibodies to the Surface M Protein of Group A Streptococci in Pooled Normal Immunoglobulins (IVIG): Potential Impact on the Clinical Efficacy of IVIG Therapy for Severe Invasive Group A Streptococcal Infections. **66,** 2279–2283 (1998).

76. Patel, R. *et al.* Lack of benefit of intravenous immune globulin in a murine model of group A streptococcal necrotizing fasciitis. *J. Infect. Dis.* **181,** 230–234 (2000).

77. Norrby-teglund, A. *et al.* Evidence for the Presence of Streptococcal-Superantigen-Neutralizing Antibodies in Normal Polyspecific Immunoglobulin G. *Infect. Immun.* **64,** 5395–5398 (1996).

78. Norrby-teglund, A. *et al.* Relative Neutralizing Activity in Polyspecific IgM , IgA , and IgG Preparations against Group A Streptococcal Superantigens. *Clin. Infect. Dis.* **31,** 1175–1182 (2000).

79. Jolles, S., Sewell, W. A. C. & Misbah, S. A. Clinical uses of intravenous immunoglobulin. *Clin. Exp. Immunol.* **142,** 1–11 (2005).

80. Schrage, B., Duan, G., Yang, L. P., Fraser, J. D. & Proft, T. Different preparations of intravenous immunoglobulin vary in their efficacy to neutralize streptococcal superantigens: implications for treatment of streptococcal toxic shock syndrome. *Clin. Infect. Dis.* **43,** 743–746 (2006).

81. Barry, W., Hudgins, L., Donta, S. T. & Pesanti, E. L. Intravenous Immunoglobulin Therapy for Toxic Shock Syndrome. *JAMA* **267,** 3315–3316 (1992).

82. Cawley, M. J. *et al.* Intravenous Immunoglobulin as Adjunctive Treatment for Streptococcal Toxic Shock Syndrome Associated with Necrotizing Fasciitis: Case Report and Review. *Clin. Transl. Sci.* **19,** 1094–98 (1999).

83. Norrby-Teglund, A. *et al.* Successful management of severe group A streptococcal soft tissue infections using an aggressive medical regimen including intravenous polyspecific immunoglobulin together with a conservative surgical approach. *Scand. J. Infect. Dis.* **37,** 166–172 (2005).

84. Kaul, R. *et al.* Intravenous Immunoglobulin Therapy for Streptococcal Toxic Shock Syndrome — A Comparative Observational Study. 800–807 (1995).

85. Darenberg, J. *et al.* Intravenous Immunoglobulin G Therapy in Streptococcal Toxic Shock Syndrome : *Clin. Infect. Dis.* **37,** 333–340 (2003).

86. Facklam, R. R. *et al.* emm Typing and validation of provisional M types for group A streptococci. *Emerg. Infect. Dis.* **5,** 247–253 (1999).

87. Zhou, J., Fries, M. R., Chee-Sanford, J. C. & Tiedje, J. M. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth of toluene and description of Azoarcus tolulyticus sp. nov. *Int. J. Syst. Bacteriol.* **45,** 500–506 (1995).

88. Friães, A., Pinto, F. R., Silva-Costa, C., Ramirez, M. & Melo-Cristino, J. Superantigen gene complement of Streptococcus pyogenes--relationship with other typing methods and short-term stability. *Eur. J. Clin. Microbiol. Infect. Dis.* **32,** 115–25 (2013).

89. Borek, A. L., Wilemska, J., Izdebski, R. & Hryniewicz, W. A New Rapid and Cost-E ective Method for Detection of Phages , ICEs and Virulence Factors Encoded by Streptococcus pyogenes. *Polish J. Microbiol.* **60,** 187–201 (2011).

90. Ferretti, J. J. *et al.* Complete genome sequence of an M1 strain of Streptococcus pyogenes. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4658–63 (2001).

91. Beres, S. B. *et al.* Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 10078–83 (2002).

92. Smoot, J. C. *et al.* Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4668–73 (2002).

93. Fiebig, A. *et al.* Comparative Genomics of Streptococcus pyogenes M1 isolates differing in virulence and propensity to cause systemic infection in mice. *Int J Med Microbiol* **accepted,** (2015).

94. Oggioni, M. R. & Pozzi, G. A host-vector system for heterologous gene expression in Streptococcus gordonii. *Gene* **169,** 85–90 (1996).

95. Mullis, K. Target amplification for DNA analysis by the polymerase chain reaction. *Ann*

Biol Clin **48,** 579–82 (1990).

96. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–4680 (1994).

97. Gulotta, G. Elucidating the molecular mechanisms of streptococcal M protein-induced autoimmune sequelae. (Technische Universität, Braunschweig, 2015).

98. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254 (1976).

99. Medina, E., Goldmann, O., Toppel, A. . & Chhatwal, G. S. Survival of Streptococcus pyogenes within Host Pahgocytic Cells: A pathogenic Mechanism for Persistence and Systemic Invasion. *J Infect Dis.* **187,** 597–603 (2003).

100. Lancefield RC, D. V. The properties of T antigen extracted from group A hemolytic streptococci. *J Exp Med* **84,** 449–71 (1946).

101. Takahashi, T. *et al.* Clinical aspects of invasive infections with Streptococcus dysgalactiae ssp. equisimilis in Japan: differences with respect to Streptococcus pyogenes and Streptococcus agalactiae infections. *Clin. Microbiol. Infect.* **16,** 1097–1103 (2010).

102. Borek, A. L., Wilemska, J., Izdebski, R., Hryniewicz, W. & Sitkiewicz, I. A new rapid and cost-effective method for detection of phages, ICEs and virulence factors encoded by streptococcus pyogenes. *Polish J. Microbiol.* **60,** 187–201 (2011).

103. Gerlach, D., Reichardt, W., Fleischer, B. & Schmidt, K. H. Separation of mitogenic and pyrogenic activities from so-called erythrogenic toxin type B (Streptococcal proteinase). *Zentralbl. Bakteriol.* **280,** 507–14 (1994).

104. Norrby-Teglund, A. *et al.* Evidence for the presence of streptococcal-superantigen-neutralizing antibodies in normal polyspecific immunoglobulin G. *Infect. Immun.* **64,** 5395–5398 (1996).

105. Sharkawy, A. *et al.* Severe group a streptococcal soft-tissue infections in Ontario: 1992-1996. *Clin. Infect. Dis.* **34,** 454–60 (2002).

106. Glass, G. E., Sheil, F., Ruston, J. C. & Butler, P. E. M. Necrotising soft tissue infection in a UK metropolitan population. *Ann. R. Coll. Surg. Engl.* **97,** 46–51 (2015).

107. Lynskey, N. N., Lawrenson, R. A. & Sriskandan, S. New understandings in Streptococcus pyogenes. *Curr. Opin. Infect. Dis.* **24,** 196–202 (2011).

108. Meisal, R. *et al.* Streptococcus pyogenes Isolates Causing Severe Infections in Norway in 2006 to 2007: emm Types, Multilocus Sequence Types, and Superantigen Profiles. *J. Clin. Microbiol.* **48,** 842–851 (2010).

109. Siljander, T. *et al.* Epidemiology, outcome and emm types of invasive group A streptococcal infections in Finland. *Eur. J. Clin. Microbiol. Infect. Dis.* **29,** 1229–35 (2010).

110. Luca-Harari, B. *et al.* Clinical and microbiological characteristics of severe Streptococcus pyogenes disease in Europe. *J. Clin. Microbiol.* **47,** 1155–1165 (2009).

111. Martin, D. R. & Single, L. A. Molecular epidemiology of group A streptococcus M type 1 infections. *J.Infect.Dis.* **167,** 1112–1117 (1993).

112. Muotiala, A., Seppala, H., Huovinen, P. & VuopioVarkila, J. Molecular comparison of group A streptococci of T1M1 serotype from invasive and noninvasive infections in Finland. *J. Infect. Dis.* **175,** 392–399 (1997).

113. Sandin, C., Carlsson, F. & Lindahl, G. Binding of human plasma proteins to Streptococcus pyogenes M protein determines the location of opsonic and non-opsonic epitopes. *Mol. Microbiol.* **59,** 20–30 (2006).

114. Makrides, S. C. Therapeutic Inhibition of the Complement System. *Pharmacol. Rev.* **50,** 59–88 (1998).

115. Reglinski, M., Gierula, M., Lynskey, N. N., Edwards, R. J. & Sriskandan, S. Identification of the Streptococcus pyogenes surface antigens recognised by pooled human immunoglobulin. *Sci. Rep.* **5,** 15825 (2015).

116. Kreikemeyer, B., McIver, K. S. & Podbielski, A. Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions. *Trends Microbiol.* **11,** 224–232 (2003).

117. Bisno, A. L., Brito, M. O. & Collins, C. M. Molecular basis of group A streptococcal virulence. *Lancet. Infect. Dis.* **3,** 191–200 (2003).

118. Wood, D. N., Chaussee, M. A., Chaussee, M. S. & Buttaro, B. A. Persistence of Streptococcus pyogenes in stationary-phase cultures. *J. Bacteriol.* **187,** 3319–28 (2005).

119. Kotb, M. *et al.* An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. **8,** (2002).

120. Proft, T. & Zealand, A. N. Bacterial superantigens. *Clin Exp Immunol* (2003).

121. Akesson, P., Moritz, L., Truedsson, M., Christensson, B. & von Pawel-Rammingen, U. IdeS, a Highly Specific Immunoglobulin G (IgG)-Cleaving Enzyme from Streptococcus pyogenes, Is Inhibited by Specific IgG Antibodies Generated during Infection. *Infect. Immun.* **74,** 497–503 (2006).

122. Kurupati, P. *et al.* Chemokine-cleaving Streptococcus pyogenes protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. *Mol. Microbiol.* **76,** 1387–1397 (2010).

123. Åkessont, P., Sjöholm, A. G. & Björck, L. Protein SIC, a novel extracellular protein of Streptococcus pyogenes interfering with complement function. *J. Biol. Chem.* **271,** 1081–1088 (1996).

124. Borek A.L Radosław Izdebski, Waleria Hryniewicz, Izabela Sitkiewicz, J. W. A New Rapid and Cost-Effective Method for Detection of Phages, ICEs and Virulence Factors Encoded by Streptococcus pyogenes. *Polish J. Microbiol.* **60,** 187–201 (2011).

125. Borek, A. L., Obszańska, K., Hryniewicz, W. & Sitkiewicz, I. Detection of Streptococcus pyogenes virulence factors by multiplex PCR. *Virulence* **3,** 529–33 (2012).

126. Sharma, P., Wang, N. & Kranz, D. M. Soluble T cell receptor Vβ domains engineered for high-affinity binding to staphylococcal or streptococcal superantigens. *Toxins (Basel).* **6,** 556–74 (2014).

6.2 INFECT Case collection

Patient					Identified Pathogen	Isolated from	Treatment		Collection center
No.	Age	Gender	Co-morbidities	NSTI			IVIG	HBO	
2001	67	M	None	Yes	*S. pyogenes*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
2002	64	M	Hypertension tractatae	Yes	*S. pyogenes* Yeast	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
2003	47	F	Fibromyalgia Short bowel syndrome	Yes	*E. faecium* *E. sakazakii* *C. perfringes* Yeast *C. albicans*	Outer abdomen fluid	Yes	Yes	Rigshospitalet, Copenhagen
2004	73	F	Hypertension tractatae	Yes	*Actinomyces* Yeast *C. albicans*	Neck Blood	Yes	Yes	Rigshospitalet, Copenhagen
2005	59	M	Diabetes Myocardial Infarction	Yes	*E. faecium*	N.A	Yes	Yes	Rigshospitalet, Copenhagen
2006	62	F	Hypertension tractatae	Yes	*S. pyogenes*	Blood	Yes	Yes	Rigshospitalet, Copenhagen
2008	53	M	HIV Positive	Yes	*S. pyogenes* Yeast *C. albicans*	Pus Tissue	Yes	No	Rigshospitalet, Copenhagen

2009	63	F	None	Yes	Yeast *C. albicans*	Blood	Yes	Yes	Rigshospitalet, Copenhagen
2010	66	M	None	Yes	*E. coli* *S. constellatus* *E. faecalis*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
2013	49	F	Deep vein thrombosis	Yes	*S. pyogenes* Yeast *S. aureus*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
2014	64	F	Alcohol Abuse	Yes	*E. coli*	N.A	Yes	No	Rigshospitalet, Copenhagen
2015	70	F	C.Pulm 2012 Smoker	Yes	*S. pyogenes*	Blood	Yes	Yes	Rigshospitalet, Copenhagen
2016	77	F	Hypertension tractatae	Yes	*S. anginosus* *S. warneri*	N.A	Yes	Yes	Rigshospitalet, Copenhagen
2017	38	F	None	Yes	*S. pyogenes*	Tissue	Yes	Yes	Rigshospitalet, Copenhagen
3001	47	M	Rheumatoid Disease	Yes	SDSE *S. aureus*	N.A	Yes	Yes	Karolinska University Hospital, Stockholm
3003	38	F	Hypertension	Yes	*S. pyogenes*	N.A	No	Yes	Karolinska University Hospital, Stockholm

3004	62	F	None	Yes	*S. pyogenes* *Staphylococcus*	N.A	No	Yes	Karolinska University Hospital, Stockholm
3005	65	F	Chronic obstructive lung Disease Hypertension	Yes	*S. pyogenes*	N.A	No	No	Karolinska University Hospital, Stockholm
3007	67	F	Asthma Hypertension	Yes	*S. anginosus*	N.A	No	Yes	Karolinska University Hospital, Stockholm
3008	69	F	Diabetes Hypertension	Yes	*S. epidermidis* *C. albicans*	N.A	Yes	Yes	Karolinska University Hospital, Stockholm
3009	85	M	Hypertension	Yes	*S. pyogenes*	N.A	Yes	Yes	Karolinska University Hospital, Stockholm
3010	73	F	None	Yes	*S. pyogenes*	N.A	Yes	No	Karolinska University Hospital, Stockholm
3012	42	F	None	Yes	*S. pyogenes*	N.A	No	Yes	Karolinska University Hospital, Stockholm
3013	58	M	Chronic Obstructive Lung Disease	Yes	*E. faecalis* *C. ramosum*	N.A	No	Yes	Karolinska University Hospital, Stockholm
3014	69	M	Chronic Obstructive Lung Disease	Yes	*E. faecalis* *B. fragilis*	N.A	No	Yes	Karolinska University Hospital, Stockholm

3015	58	F	Hypertension	Yes	*E. faecalis* *E. coli*	N.A	No	Yes	Karolinska University Hospital, Stockholm
3016	66	M	Hypertension	Yes	*S. pyogenes*	N.A	Yes	Yes	Karolinska University Hospital, Stockholm
4001	N.A	N.A	N.A	Yes	*E. faecalis* *S. saprophyticus*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
4002	N.A	N.A	N.A	Yes	*E. coli* *S: milleri* *B. ovatus*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
4003	N.A	N.A	N.A	Yes	*E. coli* *C. albicans*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
4004	N.A	N.A	N.A	Yes	*E. faecalis* *C. albicans*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
4005	N.A	N.A	N.A	Yes	*S. milleri* *C. perfringens* *E. coli*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
4006	N.A	N.A	N.A	Yes	*S. milleri* *Lactobacilli sp.*	N.A	N.A	N.A	Blekingesjukhuset Karlskrona
5001	51	F	Active Malignancy colon	Yes	*C. septicum*	Blood Wound	No	Yes	Sahlgrenska University Hospital Gothenburg

5002	76	M	Renal insufficiency	Yes	*C. septicum*	Tissue	No	Yes	Sahlgrenska University Hospital Gothenburg
5003	79	M	Malignancy colon Atrial fibrillation Chronic left leg edema	Yes	*S. pyogenes*	Blood	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5004	41	F	None	Yes	*S. pyogenes*	Wound	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5005	71	M	None	Yes	SDSE	Wound	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5006	37	M	None	Yes	*S. pyogenes*	Wound elbow Tissue right arm Wound right arm	Yes	No	Sahlgrenska University Hospital Gothenburg
5007	73	F	N.A	Yes	*S. constellatus*	Blood	N.A	Yes	Sahlgrenska University Hospital Gothenburg
5010	68	M	Pacemaker Artificial joint knee	Yes	*S. aureus* *S. pyogenes*	Wound	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5011	65	M	High Blood Pressure	Yes	*S. pyogenes*	Blood Tissue	Yes	Yes	Sahlgrenska University Hospital Gothenburg
5012	53	F	Malign Melanoma	Yes	*S. pyogenes*	Wound	Yes	Yes	Sahlgrenska University Hospital Gothenburg

5013	65	M	High Blood Pressure Alcoholism	Yes	*S. pyogenes*	Wound	Yes	No	Sahlgrenska University Hospital Gothenburg
5014	62	M	High Blood Pressure	Yes	*S. pyogenes*	Blood	Yes	No	Sahlgrenska University Hospital Gothenburg
5016	28	M	Hepatitis C	Yes	*S. massiliensis* *S. anginosus*	Tissue	No	Yes	Sahlgrenska University Hospital Gothenburg
5017	53	M	Diabetes mellitus type2	Yes	*S. anginosus*	Tissue	No	No	Sahlgrenska University Hospital Gothenburg
5019	52	M	Asthma	Yes	*E. coli* *Actinomyces*	Blood Wound	No	Yes	Sahlgrenska University Hospital Gothenburg
5020	48	M	None	Yes	*S. pyogenes*	Tissue	Yes	Yes	Sahlgrenska University Hospital Gothenburg
6002	40	M	Hepatitis C	Yes	*Enterobacter* sp. *Actinomyces*	Upper Leg	No	No	University of Bergen Bergen
6003	48	M	None	Yes	*S. anginosus*	Blood	No	Yes	University of Bergen Bergen
6004	55	M	None	Yes	*S. pyogenes*	Upper Leg	No	No	University of Bergen Bergen
6005	54	M	Diabetes mellitus	Yes	*S. aureus*	Blood	No	No	University of Bergen Bergen
6006	28	M	Active Malignancy	Yes	SDSE *S. aureus*	Thorax	No	Yes	University of Bergen Bergen

6007	57	F	Blunt trauma	Yes	SDSE	Lower Leg	No	No	University of Bergen Bergen
6008	48	M	Hepatitis C Blunt trauma	Yes	*E. coli*	Blood	No	No	University of Bergen Bergen
6009	71	M	Cardiovascular Disease	Yes	*E. coli* ESBL	Upper Leg	No	No	University of Bergen Bergen
6010	62	F	Asthma	Yes	*E. coli*	Infected Site	No	No	University of Bergen Bergen
6012	46	M	None	Yes	*E. coli* *S. milleri*	Blood	No	No	University of Bergen Bergen
6013	34	F	None	Yes	*S. pyogenes*	Blood Neck	Yes	Yes	University of Bergen Bergen
6015	37	M	Hepatitis C Drug User	Yes	Gram Negative species	Upper Leg	No	No	University of Bergen Bergen
6016	41	M	None	Yes	*S. pyogenes*	Elbow aspiration	No	No	University of Bergen Bergen
6017	72	M	Kidney Failure Liver Failure	Yes	SDSE	Blood Leg	No	No	University of Bergen Bergen
6018	39	F	None	Yes	*S. pyogenes*	Axilla	No	No	University of Bergen Bergen
6020	61	M	Cardiovascular Disease	Yes	SDSE	Axilla	No	No	University of Bergen Bergen

6023	54	F	Operated Breast cancer	Yes	*S. aureus*	Infected Site	No	No	University of Bergen Bergen
6025	37	M	Psoriasis	Yes	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6026	48	M	None	Yes	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6028	41	M	None	No	*S. pyogenes*	Infected site	No	No	University of Bergen Bergen
6029	62	M	Alcohol Abuse Chronic ulcer	Yes	*S. pyogenes* *S. aureus*	Infected Site	No	No	University of Bergen Bergen
6030	69	M	Hypertension	Yes	*S. aureus* *S. epidermidis*	Infected Site	No	Yes	University of Bergen Bergen
6031	74	M	Hypertension	Yes	*S. aureus*	Blood culture	No	No	University of Bergen Bergen
6032	54	M	Anal Cancer	Yes	SDSE *S. haemolyticus*	Infected Site	No	No	University of Bergen Bergen
6033	50	F	None	Yes	*S. pyogenes*	upper extremity	No	No	University of Bergen Bergen
6034	43	M	None	Yes	*S. pyogenes*	Blood	No	No	University of Bergen Bergen

6035	23	M	Alcoholism	Yes	*E. coli* *Citrobacter sp.*	Infected Site	No	No	University of Bergen Bergen
6036	37	M	Malignant melonoma	Yes	SDSE	Blood	No	No	University of Bergen Bergen
6037	80	F	Cardiovascular Disease	Yes	SDSE	Blood	No	No	University of Bergen Bergen
6038	60	M	Cardiovascular Disease	Yes	*S. pyogenes* *S. aureus*	Upper Leg	No	No	University of Bergen Bergen
6039	59	F	Psoriasis	Yes	*S. pyogenes*	Blood	Yes	No	University of Bergen Bergen
6040	32	F	None	No	*S. pyogenes*	Upper Leg	No	No	University of Bergen Bergen
6041	27	F	Recent dentist surgery	Yes	*S. milleri*	Neck	No	Yes	University of Bergen Bergen
6042	31	M	Recent dentist surgery	Yes	*S. anginosus* *S. intermedius*	Neck	No	Yes	University of Bergen Bergen

N.A: Not Available

6.3 Ethical Permissions

Task	Ethical Permit Number
Cloning of exotoxins	Funktionelle Charakterisierung von Virulenzfaktoren aus Gruppe A streptokokken (*Streptocccus pyogenes*) Projekt Nr: 70744 Anlagen Nr: 915101 S-Stufe: 2
Withdrawl of Blood	B0/07/2013
Hospitals:	
Stockholm, Sweden	2006/321-31/4 and 2012/2110-31/2
Copenhagen, Denmark	1151739
Gothenburg, Sweden	930-12
Bergen, Norway	2012/2227/REK vest

Acknowledgment

It is not the happy people that are thankful;

It is thankful people who are happy

-Anonymous

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