Development of in vitro and in vivo models to characterize biofilm formation by a necrotizing strain of Group A Streptococcus

Anuradha Vajjala

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**DEVELOPMENT OF *IN VITRO* AND *IN VIVO* MODELS TO
CHARACTERIZE BIOFILM FORMATION BY A
NECROTIZING STRAIN OF GROUP A *STREPTOCOCCUS***

ANURADHA VAJJALA

**SCHOOL OF BIOLOGICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY
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A VAJJALA

ANURADHA VAJJALA

SCHOOL OF BIOLOGICAL SCIENCES

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in partial fulfillment of the requirement for the degree of
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2017

“To strive, to seek, to find, and not to yield”

*Alfred Lord Tennyson,
Ulysses.*

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Abstract

Group A *Streptococcus* (GAS) causes a wide variety of human diseases, ranging from mild, self-limiting infections to highly invasive life-threatening diseases such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS). In accordance, GAS is the fourth most common bacterial cause of human morbidity and mortality, accounting for more than half a million deaths annually. In recent years, a few molecular determinants and pathways that regulate GAS biofilm formation have been identified, based on various *in vitro* and a few *in vivo* models. However, we lack information as to whether the mechanisms identified have clinical relevance for biofilm-associated infection, especially acute infection. Biofilm is thought to play a role during GAS colonization of epithelial cells and structures of microcolonies were observed in the fascia of NF patients. Yet, the role of these aggregates in GAS infections remains uncertain. In this study, we establish and characterize an *in vitro* model of GAS biofilm formation on mammalian cells and further validate our findings in an *in vivo* mouse model of human soft tissue infection. We found that GAS forms dense aggregates on a variety of epithelial cells that display biofilm characteristics, including a 3D architecture, extracellular matrix, enhanced tolerance to antibiotics and late stage biofilm dispersal. In contrast to GAS biofilm on abiotic surfaces, host-associated biofilm required the expression of GAS secreted toxins streptolysins O and S (SLO, SLS). These toxins trigger endoplasmic reticulum (ER) stress that leads to host cell apoptosis and necrosis. Further, during the early stages of biofilm development, GAS SLO and SLS induced cell death cascade results in the release of a host-associated factor that augments subsequent biofilm development on necrotic mammalian cells. Supernatants from GAS-infected mammalian cells or from cells treated with ER stressors restored biofilm formation of a streptolysin null mutant that is otherwise attenuated in biofilm formation on mammalian cells. Similar behavior was detected in a mouse model of GAS soft tissue infection. While wild type GAS formed dense aggregates that concentrated mainly in the fascia and subsequently spread beyond the initial infection site, the streptolysin null mutant formed considerably fewer microcolonies and the infection was confined to the site of infection, without progressing into deeper tissues. Most striking, pre-treatment of the infection site with the ER stress-inducing drug thapsigargin restored the ability of the streptolysin null mutant to cause wild type-level infection. Taken together, I have demonstrated a novel role for streptolysins SLO and SLS mediated ER stress that plays a major role in *in vitro* and *in vivo* GAS biofilm formation and disease progression.

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Abbreviations

AMPs	antimicrobial peptides
ASNS	asparaginase synthase
AspA	AgI/II type polypeptide
ATF6	activating transcription factor 6
CDC	cholesterol-dependent cytolysin
CFU	colony forming units
CHOP	C/EBP-homologous protein
CMT	cytolysin-mediated translocation
CLSI	Clinical and Laboratory Standards Institute
CLSM	confocal laser scanning microscopy
CovRS	control of virulence two-component system
DAMPs	damage-associated molecular patterns
DTT	dithiothreitol
ECM	extracellular matrix
eDNA	extracellular DNA
EPS	extracellular polymeric substances
ERAD	ER-associated degradation
ER	endoplasmic reticulum
ERES	ER exit sites
FasL	Fas ligand
FbaA	fibronectin binding protein A
FBP54	fibronectin binding protein
FCT region	fibronectin-binding collagen-binding T antigen region
FnBPs	fibronectin binding proteins
Fn	fibronectin

GAS	group A <i>Streptococcus</i>
hpi	hours post-infection
IdeS	immunoglobulin G degrading enzyme of <i>Streptococcus</i>
IFN- γ	interferon γ
IL	interleukin
IRE1	inositol-requiring enzyme 1
IVIg	intravenous immunoglobulin
LDH	lactate dehydrogenase
LTA	lipotechoic acid
MBEC	minimum biofilm eradication concentration
MFI	mean fluorescence intensity
Mga	multiple gene regulator
MIC	minimum inhibitory concentration
MMP	matrix metalloproteinase
MOI	multiplicity of infection
Mrp	M-related proteins
MSCRAMMS	microbial surface components recognizing adhesive matrix molecules
NETS	neutrophil extracellular traps
NF	necrotizing fasciitis
NSTI	necrotizing soft tissue infections
PAMPs	pathogen associated molecular patterns
PCD	programmed cell death
<i>Pel</i>	pleiotropic effect locus
PERK	protein kinase R (PKR)-like ER kinase
PFA	paraformaldehyde
PFT	pore forming toxins

PMNs	polymorphonuclear monocytes
protein F/Sfb	streptococcal Fn- binding protein
PrtF1	protein F1
QS	quorum sensing
RBCs	red blood cells
ROS	reactive oxygen species
<i>Sag</i>	SLS-associated gene
Scl1	streptococcal collagen-like protein1
ScpA	streptococcal C5a peptidase
Sda1	streptococcal DNase1
SHP	short hydrophobic peptides
Sic	streptococcal inhibitor of complement
Sil	streptococcal invasive locus
Ska	streptokinase
SlaA	streptococcal phospholipase A2
SLO	streptolysin O
SLS	streptolysin S
Sof	serum opacity factor
SpeB	streptococcal pyrogenic exotoxin B
SpnA	streptococcal cell wall-anchored nuclease A
SPN	<i>S. pyogenes</i> NAD ⁺ -glycohydrolase
SpyCEP	<i>Streptococcus pyogenes</i> cell envelope protease
Srv	streptococcal regulator of virulence
SSA	streptococcal superantigen
STS	staurosporine
Sse	secreted streptococcal carboxylic esterase
STSS	streptococcal toxic shock syndrome

TCS	two component systems
TGF β 1	transforming growth factor β 1
TLRs	toll like receptors
TNF- α	tumor necrosis factor α
UPR	unfolded protein response

CHAPTER 1

Introduction

1.0 Introduction

Group A *Streptococcus* (GAS) is an exclusive human pathogen with a recorded history dating back to the 5th century BCE when Hippocrates first described the association between necrotising soft-tissue infection and injury (1). GAS has a global disease burden of more than 18 million cases per year, and deaths associated with invasive infections exceed half a million per year (2-5). GAS causes a wide spectrum of diseases due, in part, to the genetic diversity and antigenic variability of GAS strains (6, 7). Infections range in location and severity from superficial infections such as impetigo and pharyngitis, to systemic infections like streptococcal toxic shock syndrome (STSS) and bacteremia, to fulminant soft tissue infections such as necrotizing fasciitis (NF) (6, 8, 9). A hallmark of NF is rapid proliferation and spread of bacteria accompanied by extensive tissue destruction affecting skin, fascia and muscle (10). Spread of necrosis is fast, rendering early diagnosis and treatment crucial to favourable outcomes for the patient (11). With a global disease burden of nearly 3000 NF cases annually, and a mortality rate exceeding 50% in some cases, there is a pressing need for the discovery of novel therapies for this serious human disease (10).

Figure1: An overview of Group A *Streptococcus*/*Streptococcus pyogenes*

1.1 Epidemiological versatility of group A *Streptococcus* (GAS)

Streptococcus pyogenes (group A *Streptococcus*; GAS) is an important species of gram-positive bacterial pathogens. *Streptococcus pyogenes* are beta-hemolytic streptococci typed as group A based on the serological reactivity with their group specific carbohydrate. GAS is further classified into more than a hundred serotypes based on the variable N-terminus of surface-associated M proteins that serve as antigenic targets for serological typing, or based on analyses of the 5' *emm* gene sequences (12, 13). Other classifications based on expression of serum opacity factor (SOF) and T and R proteins are also used (14). FCT (fibronectin-binding collagen-binding T antigen) region based typing also allows to discriminate GAS serotypes based on the variability in gene content and sequence of the different genes encoded within this region (15).

Decades of epidemiological studies have paved the way to a distinct tissue-tropic pattern of serotypic distribution, with some serotypes exclusively infecting the throat, and some with a predilection to cause only skin diseases (8). Population analysis of more than 4000 GAS isolates across six continents shows a common pattern in which GAS serotypes corresponding to the *emm* pattern A–C group have a strong propensity to cause infections of the throat, and *emm* pattern D strains have a strong tendency to cause impetigo, while *emm* pattern E are designated as “generalists” as they cause both infections in equal frequency (16).

1.2 Overview of GAS pathogenesis

GAS infections can progress from superficial to invasive, and may culminate in systemic spread (5). An overview of GAS pathogenesis therefore encompasses initial adherence and colonization of GAS, followed by invasion and intracellular survival in the host tissue. This may in some cases progress into an acute phase characterized by invasive infection and systemic spread. A brief summary of GAS factors that mediate such an infection cascade has been described below in **Figure 2**.

Figure 2: Temporal and spatial regulation of GAS virulence factors during infection

A) During early stage infection, adherence and immune evasion factors, crucial for establishing infection are overexpressed. **B)** During intracellular survival and persistence, host factors play a major role in internalization of GAS, along with GAS factors like M protein, capsule, and FnBPs that aid in cellular invasion. **C)** Acute invasive infection entails expression of a plethora of surface-bound and secreted factors that mount massive tissue destruction including host proteases. **D)** During bacteremia, factors associated with systemic spread, like CovR/S and anti-phagocytic M protein are expressed, with Ska and Sda enhancing GAS survival in blood.

Abbreviations: Sic, streptococcal inhibitor of complement; SlaA, streptococcal phospholipase A2; Sof, serum opacity factor; ScpA, streptococcal C5a peptidase; FbaA, fibronectin-binding protein A; PrtF1, protein F1; Ska, streptokinase; Sda1, streptococcal DNase; MMP-2/9, matrix metalloproteinase 2/9; Sls, streptolysin S; Sse, secreted streptococcal carboxylic esterase; IdeS, immunoglobulin G degrading enzyme of *Streptococcus*; SpyCEP, *Streptococcus pyogenes* cell envelope protease; SpeB, secreted streptococcal cysteine protease B; Scl1 streptococcal collagen-like protein 1.

Figure adapted from Olsen *et al.*, 2010 (10)

1.2.1 Adherence and colonization

Attachment of GAS to oropharyngeal or dermal epithelial cells, the two primary niches of GAS in the human host, is the most important step to initialize colonization. GAS uses specific surface components (adhesins) to mediate attachment to their respective host surface-associated receptors. The first adhesin that was purified and found to block attachment of GAS to host cells was lipoteichoic acid (LTA) and the first receptor to be identified was fibronectin (Fn) (17, 18). Since then, many additional surface components have been found to

play a role in adhesion of GAS. Predominant among these are the M protein (19-21), hyaluronic acid capsule (22, 23), protein F/Sfb (streptococcal Fn-binding protein) (24), protein F2, FBP54 (Fn-binding protein 54) (25), R28 protein (26) and collagen binding protein (27) that mediate adhesion to host cells. Very recently, GAS pili-like cell surface structures were shown to be the central players in pharyngeal epithelial cell adherence and biofilm formation (28, 29). Courtney and Hasty and colleagues have proposed a hypothetical model for adhesion, which requires two steps: 1) relatively weak adhesion, mediated by LTA that serves to overcome electrostatic repulsion (30), and 2) adhesion involving M protein, FBP54, protein F, serum opacity factor (SOF), and any number of other adhesins which have been described (30, 31).

Adherence paves the way to two well established events, which may or may not be mutually exclusive. The first is colonization and superficial infection of tissues, that may persist asymptomatically and result in long term carriage, or cause secondary invasion of host tissue resulting in acute infection, dissemination into deeper tissues, and systemic spread (32). Colonization is a multifaceted event that entails more than just adherence, although for successful colonization, adhesion to host surface receptors is a crucial first step. Secondly, adhesion enhances cytolysin mediated toxicity that has a very important role in tissue damage and subsequent spread of infection (33, 34). Furthermore, GAS modulates the expression of genes encoding transporters, signalling molecules, two component systems (TCS), transcriptional regulators, and other virulence factors to enable nutritional and environmental adaptation, immune modulation of the host and to optimise fitness, survival and proliferation of bacteria under infection conditions (32).

The M protein and hyaluronic acid capsule are key surface adhesins that modulate GAS colonization of upper respiratory mucosa of mice (23, 27, 35-37). The cell wall anchored C5a peptidase prevents clearance of GAS by reducing the influx of neutrophils and thereby promoting colonization (38). Receptor-adhesin recognition triggers downstream signalling cascades that result in a number of host-modulatory effects. Upon binding to Toll like receptors (TLRs) on monocytes, GAS M protein induces inflammation resulting in the release of cytokines like IL-6, IL-1 β and TNF- α that in turn result in the severe pathophysiology of GAS invasive infections (39).

1.2.2 Invasion, intracellular survival and persistence

Classically, GAS was considered to be predominantly an extracellular pathogen, but several studies have shown over the past decade that GAS may invade and persist within epithelial cells (40). Invasion allows bacterial refuge from host defences by providing an intracellular protective niche enabling carriage and persistence of streptococci (41) with low virulence associated with downregulation of virulence factors (42). Additionally, internalization may lead to invasion of deeper tissues (43). This intracellular status also contributes to the failure of antibiotics to eradicate GAS (44). During the process of internalization, GAS first binds to cell surface integrins that leads to extensive actin cytoskeletal rearrangement, facilitating GAS internalization (45). Many of the proteins involved in GAS epithelial cell adherence also participate in cellular invasion, including FnBPs, M protein, and streptococcal collagen-like protein1 (Sc11) (46). GAS interacts with integrin-bound fibronectin (Fn), resulting in the upregulation of transforming growth factor β 1 (TGF β 1), which in turn upregulates cell surface expression of α 5 integrin and Fn, priming the cells for enhanced streptococcal binding (47). Laminin has been shown to bind GAS and induces ingestion of M1 GAS, independent of fibronectin (48). Hyaluronic acid capsule contributes to intercellular translocation by disrupting intercellular junctions, thereby, facilitating GAS dissemination into deeper tissues (31).

1.2.3 Acute invasive infection and systemic spread

As described earlier, GAS causes a broad spectrum of diseases, ranging from mild superficial skin and throat infections, to fulminant invasive infection that results in massive tissue destruction and systemic spread as observed in necrotizing fasciitis (NF), streptococcal toxic shock syndrome (STSS) and myositis. Among the 700 million cases of GAS mild infections reported per year, around 650,000 progress into invasive diseases with high rates of morbidity and mortality (5). Alarming, there has also been a resurgence in GAS invasive infections in the past 30 years, mostly traced to the rise of a globally disseminated GAS M1T1 subclone that is notorious for its virulence and unusual epidemiological features (49). In order to breach tissue barriers, subvert host immunity and clearance, and induce tissue damage and dissemination into deeper tissues, GAS actively mounts differential transcriptomic regulation, virulence factor expression, dysregulation of host immune clearance and other adaptive

mechanisms. Some of these are well understood and some still elusive (9) and hence a subject of current investigation.

The switch from superficial to invasive modes lies in differential gene regulation that is brought about at multiple levels including the activity of stand-alone transcription regulators as well as two component signal transduction systems (TCS) (50, 51) that govern the expression of large subsets of the GAS genome. The *ihk-irr* TCS actively protects GAS from immune clearance and neutrophil-mediated ROS toxicity, thereby enabling greater dissemination and bacteremia (52, 53). An important TCS that regulates almost 15% of the GAS genome is the CovR/S TCS, that positively regulates the broad spectrum protease SpeB, has an active role in invasive phenotype (54-56), and represses the expression of key GAS virulence factors namely hyaluronic acid capsule, streptokinase (Ska), streptolysin O (SLO), IgG degrading enzyme of *S. pyogenes* (IdeS), and the DNase Sda1(57). For this reason, CovRS mutants display hypervirulence in systemic infection in mice (58) and are more resistant to killing by neutrophils thereby enabling invasiveness (57). The standalone multiple gene regulator, *Mga*, undergoes phosphorylation under low carbohydrate availability, promoting GAS dissemination into deeper tissues. This in turn represses the expression of some key colonization factors like the M protein family (*emm*, *mrp*, *arp*, and *enn*), *scpA*, *sof*, *sic*, and *sclA* and enables GAS to switch from colonization to invasive mode (56).

The widespread tissue destruction observed during invasive GAS infection entails sequestration of the proteolytic activity of host cell surface plasmin that has been observed to be a prerequisite to systemic spread (59, 60). Plasminogen activation generates plasmin that degrades fibrin clots, connective tissue, ECM components, and adhesion proteins, contributing to widespread tissue damage (61). GAS factors that facilitate this process include the plasminogen activator streptokinase (*ska*) (62), plasminogen binding proteins (63), and fibrinogen binding proteins (64).

Other mechanisms that GAS has evolved to mount acute invasive infections include superantigen mediated hyperinflammation that is mediated by multiple GAS superantigens like streptococcal pyrogenic exotoxins (SpeA, SpeC, and SpeG to SpeM), the streptococcal superantigen (SSA), and streptococcal mitogenic exotoxin Z (SmeZ) and SSE (secreted streptococcal carboxylic esterase) (65). Severity of infection has a strong link to circulating levels of pro-inflammatory mediators of inflammation like IL-1 β , IL-6, IL-8, TNF- α and IFN-

γ (66). Another distinct clinical feature of GAS fulminant infections like NF is vascular thrombosis that results in hypoxia-induced tissue damage (67). Coagulopathy is a host defence against this damage by triggering either the intrinsic (at the cell surface by contact activation) (68) or the extrinsic (mediated by attachment of M protein to TLR 2 receptors) (69) coagulation pathways that induce vasodilation, vascular leakage and formation of clots and fibrin networks to limit the spread of bacteria. GAS factors, predominantly the M protein, mediate subversive events (56, 70) that results in the dysregulation of both early inflammation and coagulopathy hence aiding in invasive pathogenesis. Systemic spread of GAS to the vascular system results in bacteremia and sepsis syndrome (10). The M protein has been shown to bind to the soluble fragment of the cell surface complement regulator CD46, that is shed by apoptotic and necrotic host cells, thereby increasing GAS survival in blood and dissemination by subversion of host defence molecules (71). Factors like *ska* and the bacteriophage encoded DNase Sda1, shown previously to enhance GAS survival in blood are also upregulated during bacteremia (72). Sda1 in turn induces a selective pressure on CovR/CovS mutation that enhances the switch to dissemination mode (72).

1.3. Immuno-pathogenesis of streptococcal necrotizing fasciitis (NF)

1.3.1 Disease progression of NF

In the fifth century BCE, Hippocrates described the acute severity and tissue damage associated with necrotizing fasciitis (NF), colloquially called the “flesh eating” disease (10). Necrotizing fasciitis (NF) is characterized by rapid destruction of the skin, subcutaneous and deep soft tissue, and eventually muscle, with disease progression measured in hours to few days. Mortality rates exceed 50% in some cases and apart from antibiotic therapy, the only course of treatment is surgical debridement or limb amputation (10).

Figure 3: Histopathological features of human necrotizing fasciitis.

A) Arm of a patient with NF shows swelling, bruising and ulceration. B) Histopathological analysis of skin (2x magnification) shows epidermal erosion (*horizontal bracket*), dermal thickening due to local edema (*vertical bracket*), and infiltration of immune cells. C) Ulceration and dermal-epidermal junction is shown in higher magnification. D) Section from deep soft tissue shows extensive necrosis, acute inflammation, and bacterial aggregation along the major fascial planes (*arrows*). E) The necrosis extends beyond the fascia planes to breach along individual myofibres. F) Four severely damaged muscle fibres are seen at high magnification. G) Region showing vascular thrombosis and haemorrhage, typical of NF.

Taken from Olsen *et al.*, 2010 (10)

The infection may begin at a breach in the epithelial or mucosal surface which quickly results in spread of bacteria along the fascial planes that separate myofibres. Rapid GAS proliferation results in high titres of bacteria that in turn elicit a strong immune response brought about by neutrophil and PMN recruitment. The widespread tissue damage that is a hallmark of NF is mediated by multiple factors, both GAS and host-derived. Potent cytolytins and other degradative proteases that are secreted by GAS enable breaching of the epithelial barriers, resulting in GAS invasion and extensive colonization within the damaged tissue (10). A subset of virulence factors, including SSE (secreted streptococcal carboxylic esterase) (73), SLO (streptolysin O), SlaA (streptococcal phospholipase A2) (74) and streptokinase (Ska) (75, 76) directly damage host tissue. Other virulence factors such as SLS (streptolysin S), Mac1/IdeS (immunoglobulin G degrading cysteine protease), and SpyCEP (*Streptococcus pyogenes* cell envelope protease) indirectly damage host tissue by inducing coagulopathy, inactivating PMNs and cleaving immune molecules (10, 77-80). In addition, the streptococcal DNase Sda1 allows GAS escape from neutrophil extracellular traps (NETS) (81) as well as serves as a selective force for a CovRS mutation that is crucial for the switch to invasive mode (72). Finally, broad-spectrum virulence factors such as SpeB (streptococcal pyrogenic exotoxin B, a secreted cysteine protease) contribute to pathogenesis by several different mechanisms (82-85). Disease progression also results in ischemia due to vascular thrombosis that causes regional hypoxia that further aids in tissue damage, but may also limit the spread of GAS from necrotic lesions (86, 87). Dissemination of GAS into the muscle layers results in a fulminant condition called myositis (88). Vascular infiltration of GAS due to systemic dissemination by further tissue damage culminates in bacteraemia and toxic shock that further aggravate the pathology (89). An overview of the histopathological events and GAS virulence factors that actively drive NF disease progression are listed in the schematic of **Figures 3 and 4**.

Figure 4: GAS virulence factors involved in NF disease progression

Virulence factors like SpeB, Sse, SlaA, Sda1 and Ska/Plasmin induce extensive tissue damage, degrade extracellular matrix proteins, and promote vascular dissemination by their enzymatic functions. Virulence factors like SpeB, SpyCEP and Mac1/IdoS indirectly damage host tissue by degrading immune molecules, inactivating or lysing polymorphonuclear (PMN) leukocytes, and triggering the release of proapoptotic molecules. Other factors like SLS, SLO, Capsule and M protein aid in PMN lysis, coagulopathy and resistance to phagocytosis. SpeB also cleaves host ECM proteins like fibronectin and vitronectin, thereby causing further tissue damage. Host associated factors like matrix metalloproteinases (MMPs) and coagulopathy contribute to the increased severity and dissemination of bacteria during the course of NF.

Abbreviations: ECM, extracellular matrix; Mac1/IdoS, immunoglobulin G degrading enzyme of *Streptococcus*; Sse, secreted streptococcal carboxylic esterase; SlaA, streptococcal phospholipase A2; Ska, streptokinase; Sda1, streptococcal DNase I; IgG, immunoglobulin G; IL, interleukin; SpeB, secreted streptococcal cysteine protease B; SpyCEP, *Streptococcus pyogenes* cell envelope protease; TNF- α , tumor necrosis factor alpha; Sls, streptolysin S; Slo, streptolysin O; FasL, Fas Ligand.

Figure adapted from Olsen *et al.*, 2010 (10)

1.3.2 Treatment strategies for streptococcal NF

The only current treatment strategies employed for NF are radical surgical debridement and broad-spectrum antimicrobial therapy (10). Since the spread of infection is mostly subcutaneous with minimal superficial manifestation, early diagnosis and the extent of surgical debridement of the necrotic tissues helps in limiting the spread of bacteria to sterile sites. Broad-spectrum antibiotic therapy is required, and the empirical therapy consists of four antimicrobials: penicillin G, clindamycin, vancomycin, and gentamicin (11). Adjunctive care includes specialized care like hyperbaric oxygen (HBO) treatment to limit edema, stimulate fibroblast growth, and enhance immune functions and antibiotic efficacy (56). Intravenous immunoglobulin (IVIg) administration from human donors can block exotoxins secreted by GAS and limit systemic spread (90).

1.3.3 Vaccine development against streptococcal infections

No commercial vaccine exists for the treatment of GAS infections (91). This is attributed to a number of causes, predominantly the extreme strain specificity and antigenic variability within serotypes as well as geographically segregated isolates that GAS manifests (92). Cross-reactivity of antibodies with human tissue leads to autoimmune post-strep sequelae (91), and very little is known about anti-GAS protective immunity for the development of a successful vaccine (91). The M protein remains the most widely studied vaccine candidate, but given the serotype-specific protection of this protein, broad-spectrum multivalent vaccines are being developed using the fused amino-terminal peptides taken from commonly occurring virulent and epidemiologically significant M serotypes (93, 94). Some efforts have also been made to validate the candidature of other non-M protein based vaccine targets. These include virulence factors described previously i.e. C5a peptidase (95), streptococcal pyrogenic exotoxin A (SpeA) (96), streptococcal pyrogenic exotoxin B (SpeB) (97), streptococcal pyrogenic exotoxin C (SpeC) (98), fibronectin-binding proteins (99), R28 protein (26), and group A carbohydrate (100). Extracellular GAS lipoproteins have also been used to raise vaccines (101). Another promising class of vaccine candidates are anchorless surface proteins like arginine deiminase and trigger factor, which are conserved across GAS serotypes and that have been shown to elicit minimal cross reactivity with human tissue (92). New approaches

like combinatorial synthetic peptide strategy that uses part M protein and part SpyCEP for vaccine development against CovRS mutants renders complete immunity against bacteremia and skin infection (102). With multiple screenings for new and more effective candidates that increase vaccine efficacy and reduce associated secondary risks currently being performed on a large scale by numerous groups, the future for a GAS vaccine seems promising.

1.4 Role of biofilms in group A streptococcal infection

1.4.1 Biofilm formation by bacteria

Biofilm formation is one of many pathogenic strategies that GAS employs for disease progression and persistence in hosts (7). Bacterial biofilms are densely packed microbial communities, encased in a self-produced matrix, that are often recalcitrant to antibiotic or immune clearance and are physiologically different from their planktonic counterparts (103). Bacterial biofilms are structurally complex and dynamic, with different cell populations organized in a community that aggregates on both abiotic surfaces (plastic, glass, metal, and minerals) and biotic surfaces (plants and animals) in three developmental steps. Planktonic or free swimming bacteria first adhere to surfaces for primary attachment. During the second step, primary colonizers proliferate into a mature biofilm. At the same time, most biofilm bacteria produce extracellular polymeric substances (EPS), (mainly composed of biopolymers like carbohydrates, proteins and extracellular DNA (eDNA)), which stabilize the biofilm architecture in addition to conferring enhanced antibiotic tolerance. In the final step, previously sessile members of the mature biofilm detach by active or passive dispersal mechanisms and spread to other sterile sites for secondary colonization (104). The consortium of bacteria within a biofilm communicates to one another by a mechanism called quorum sensing (QS) (105). The gene expression profile, cellular physiology and other phenotypic traits of bacteria in biofilms are markedly distinct from their planktonic counterparts (105). The schematic in **Figure 5** describes the different stages of biofilm formation by bacteria.

There are numerous reports that link microbial pathogenesis of many pathogenic and nosocomial bacteria with the ability to form biofilms that result in acute and chronic infections. Examples of biofilm-associated diseases are dental caries, gingivitis, periodontitis,

and endocarditis (103). Biofilm formation has also been associated with antibiotic treatment failure (106, 107). Therefore, biofilm formation is recognized as an important virulence factor for pathogens.

Figure 5: Developmental program of a bacterial biofilm

Planktonic (free-swimming bacteria) adhere to a surface for primary or reversible attachment, followed by secondary or non-reversible attachment with the help of adhesins. The nascent biofilm then grows, and forms a mature biofilm that is encased in a self-produced matrix, that confers physical protection and antibiotic tolerance to the biofilm. A fully mature biofilm, upon various cues, triggers dispersal, during which previously sessile members of the biofilm detach and form secondary colonizers at other sites.

Figure adapted from Monds *et al.*, 2009 (104)

1.4.2 Characterization of biofilms based on *in vitro* and *in vivo* models

Ever since Bill Costerton first observed and coined the term “biofilms”, extensive studies in this field have established that bacterial biofilms are ubiquitous, complex and heterogeneous in their physiological and genetic makeup (103). Owing to the immense variety of naturally occurring biofilms, be it on medical devices, other natural abiotic surfaces, or formed in the course of animal or human infections, the exact definition of what a biofilm truly means is a point of contention. Another significant issue is the relevance of the model under study, because concerns have been raised over the interpretation of *in vitro* results that poorly replicate *in vivo* infections (66).

Broadly speaking, the biofilm phenotype is based on certain basic criteria that are universally acknowledged. In *in vitro* settings, microtiter based assays are widely recognised, with crystal violet (CV) assay being a standard assay that enables biomass quantification of adherent biofilms (68). Other *in vitro* models that enable *in situ* biofilm characterizations are flow cells and other microfluidic devices that can be used to grow and examine the 3D architecture of biofilms under various experimental conditions (65). Scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM) is used extensively for imaging of fixed or fluorescently labelled biofilms grown in these devices (66, 108). Qualitative data from the images is processed using image analysis software like COMSTAT, IMARIS, bioImage_L and others that reconstruct the 3D architecture of biofilms, thus enabling visualization of the ultrastructure, biomass, thickness and other physical attributes of biofilms (87-89) .

Surface attachment or bacterial adherence plays an important role in deciding the nature of biofilms, as is evident from studies where differentially coated plastic surfaces seeded with the same bacterial strain have given rise to different types of biofilms (108). Since bacteria express adhesins (discussed in Section 1.2.1) that specifically bind to the complementary receptors on the host surfaces, presence of these receptors (mostly ECM proteins) either coated on abiotic surfaces or expressed naturally on the cell membranes of eukaryotic cells is an important factor in biofilm formation (18, 21, 24, 25, 48, 109). An interesting model that is based on device-associated infections is when foreign-body insertions are examined for biofilms formed on their surfaces (90). These biofilms are formed when indwelling medical devices like central venous and urinary catheters and heart valves or artificial prosthetics get colonized by bacteria. These conditions are replicated *in vitro* and similar analysis using microscopy to study the biofilm architecture is done (110) .

Apart from surface adherence and 3D architecture, an important parameter for biofilm analysis is the characterization of the biofilm matrix. The matrix is highly heterogeneous, and comprises of extracellular polymeric substances (EPS) namely extracellular polysaccharides, proteins, nucleic acids and lipids with variable composition that is determined by the type of microorganisms, age of the biofilms and the different environmental conditions under which the biofilms exist (111). Determination of matrix composition is done by various methods, most notably enzymatic degradation of matrix components. Additionally, visualization of specific components of the matrix by immunostaining with fluorescently labelled conjugates and probes specific for those matrix components is also done extensively (73, 111-114).

There is generally no consensus regarding the composition of biofilm matrices formed by bacteria, and slight modifications in nutrient supply, nature of surface, age of the biofilm and shear forces etc. can alter the composition of the same.

Biofilms are also determined by their ability to display enhanced tolerance to antibiotics, a key feature that is attributed to the matrix and the physical protection that it offers against a host of external factors in addition to the inherent physiology of biofilm-embedded cells (115). The matrix also offers protection from host-defence elements elicited against *in vivo* biofilms, that is discussed later in Section 1.5 (101). A common methodology is to check for persistence of bacteria in biofilms when treated with antibiotic concentrations that are several fold higher than the concentration sufficient to kill planktonic bacteria, called the MIC (minimum inhibitory concentration) of the drug. Enhanced tolerance of biofilm-associated bacteria to such high levels of drug that are sufficient to kill planktonic bacteria is a universally accepted parameter defining the biofilm phenotype (116, 117).

In addition to the above biophysical and biochemical techniques of biofilm characterization, a genetics approach is also taken to determine the transcriptomic profile of biofilm-encased bacteria as compared to the planktonic counterpart (52). Significant differences between the two modes are reported, which underscores the difference in the physiological state of the cells that form the biofilm (52, 118). Additionally, there is extreme heterogeneity between the metabolic state of biofilm bacteria within the 3D architecture, with nutrient depletion forcing cells in the centre of the biofilm to enter a dormant state that makes them resistant to antibiotic killing and gives rise to “persistor” cells that act as secondary colonizers of a new biofilm after dispersal (117). A recent report has also established that the proteome of biofilm versus planktonic cells is very different, and may not truly reflect the changes evident from the transcriptome profile, thereby cautioning researchers not to base their conclusions on just one type of analysis owing to the immense heterogeneity that is an inherent hallmark of biofilms (52). Hence it is worth appreciating that biofilm bacteria are in a physiologically distinct state as compared to their planktonic counterparts.

All these techniques are also valid for testing the presence of biofilms or “microcolonies” that appear during *in vivo* infections (33, 42, 119, 120). In the host, bacterial biofilms encounter an additional level of resistance in the form of the host immune response that causes secretion of cytokines and other inflammatory molecules that may lead to bacterial clearance if the *in vivo* aggregates are not organised into biofilms (63, 121). Chronic infections wherein bacterial

microcolonies persist despite antibiotic treatment have been reported that substantiate the role of biofilm formation in *in vivo* conditions (107). Certain guidelines to detect biofilms in wound infections have been laid down, that are similar to the ones described above (63). A very recent report characterized GAS biofilms in biopsies from patients of necrotizing soft tissue infections (NSTI) for the first time (33). Interestingly they also reported the presence of GAS clusters that were categorised as biofilm versus “non-biofilm” based on the significantly higher bacterial load, aggregation, neutrophil infiltration, resistin, and IL-8 responses displayed by the biofilm positive strains (33). Extensive histological analysis and staining using fluorescent probes against GAS, extracellular polysaccharides, lipids and eDNA was also done, and matrix versus single-cocci staining was also used as a parameter for distinguishing the biofilm and non-biofilm formers. However, *in vivo* biofilms formed by the different clinical strains were very heterogeneous in their staining pattern, which raises a continued contention in the exact definition that holds good for *all* biofilms.

For instance, contradictory transcriptome profiles of GAS biofilms were observed from two different studies, one using GAS strain HSC5 and the other using GAS strain JRS4 (42, 118). The previous study used biofilms harvested from flow cells, and the latter from biofilms pre-grown on epithelial cells that had been PFA-fixed, leading to a disparity in the expression of virulence factors in these two conditions. It is worth noting that despite trying to address the pathogenesis of the two strains in the context of acute infection, neither study employed a relevant model, leading to results that proved to be difficult to interpret and extrapolate for physiological conditions. Also, GAS displays extreme strain specificity, thereby impeding the formation of a consensus biofilm phenotype (7). It is therefore imperative that studies related to biofilms be conducted under conditions closest to the physiological environment to circumvent such conjecture and uncertainty later.

1.4.3 Clinical significance of GAS biofilms:

GAS was considered a strict extracellular pathogen, until it was recently observed that GAS enables persistence and asymptomatic carriage by either adherence or invasion of host cells (41, 122). But this persistence was also found to be the result of enhanced antibiotic tolerance when GAS, instead of getting internalized, forms host-associated biofilms or microcolonies, as observed in a study where macrolide-susceptible bacteria that failed to internalize into cells

displayed persistence by forming biofilms (107). The clinical significance of GAS biofilms has been established by different groups (7). Clinical isolates of GAS from a range of serotypes, both invasive and non-invasive, have been tested for their ability to form biofilms using *in vitro* models including different substrates, media, and flow cells (107, 108, 123, 124) all of which indicate that there is considerable strain-specificity among GAS serotypes in their biofilm forming capacity. Furthermore, as discussed earlier, a substantial link has been established between antibiotic treatment failure and biofilm formation by various groups working with clinical strains in both *in vitro* and *in vivo* studies (41, 106, 122).

Figure 6: Evidences for the occurrence of host-associated GAS microcolonies/biofilms

A) GAS aggregates (green) in zebrafish model of myositis (125). B) ConA stained *in vivo* GAS microcolonies from impetigo are shown to possess a glycocalyx (119). (C) GAS aggregates resembling microcolonies within debrided section from patient with GAS NF (126). D) SEM of GAS microcolonies surrounded by glycocalyx in infected zebrafish muscle (118).

A number of observations suggest that biofilm formation in soft tissue may contribute to the pathogenesis of diseases caused by *Streptococcus pyogenes*. Hidalgo- Grass and colleagues (80) have observed that structured bacterial communities appear in necrotizing fasciitis

lesions in mice, and Neely *et al.* (125) found similar structures in a model of *S. pyogenes* myositis in zebrafish (**Figure 6A, C**). Akiyama *et al.* (119) reported that *S. pyogenes* communities from a murine model of impetigo were embedded in a glycocalyx, a feature also observed by Cho *et al.* in a zebrafish model of myositis (118) (**Figure 6B, D**). A significant event in biofilm development is dispersal, and this event has been associated with *in vivo* dissemination and spread of invasive infection (127, 128), further substantiating the relevance of biofilm formation in clinical conditions. In addition, GAS biofilms have been shown to confer natural competence to GAS, which makes it transformable by foreign DNA, and hence a clinical concern (42). These observations establish a role of GAS biofilm formation as a potent pathogenic strategy by means of which GAS can survive, persist, invade and disseminate in the host, and hence has been included as a parameter of epidemiological investigations of GAS pathogenesis (15).

1.4.4 GAS factors contributing to biofilm formation and its regulation

Figure 7: Regulatory networks in GAS biofilm formation

GAS factors (light blue) and transcriptional regulators (dark blue) co-ordinate to execute biofilm formation in conjunction with the external microenvironment (yellow). Arrows indicate direct or indirect induction; blocked lines indicate direct or indirect repression. The three common regulatory networks are represented as 1) SHP 2) RopB or CovR/S and 3) Mga mediated pathways.

Abbreviations: Srv, streptococcal regulator of virulence; CovRS, control of virulence two-component system; Mga, multiple gene regulator; SpeB, secreted streptococcal cysteine protease B; SHP, short hydrophobic peptides

Figure adapted from Fiedler *et al.*, 2015 (7)

More than fifty GAS virulence factors have been implicated in biofilm formation, most of which are differentially expressed and regulated based on either growth phase and environmental conditions (129), or the specific stage of biofilm development (130). Only two studies have been conducted to date to interrogate the expression profile of GAS virulence factors in biofilms as compared to planktonic bacteria (7). The first study described the upregulation of biofilm-associated virulence factors *speB*, *spd/mf* and capsule and the CovRS and Mga regulons, with limited changes in the M protein profile compared to planktonic bacteria, in an M14 HSC5 GAS strain (118). The second study differed substantially from these observations, probably due to the difference in the model of study and strain (M3 GAS strain MGAS315) wherein they first grew biofilms on fixative-killed keratinocytes and later transplanted those pre-grown mature biofilms on to live keratinocytes. Using this model, the authors observed a down-regulation in genes involved in streptolysin biosynthesis (*sagA*, *slo*), capsule biosynthesis (*hasA*), M-protein (*emm3*) and *speB*, while competence-associated *com* genes were upregulated (42). The authors reasoned that downregulation of virulence factors associated with invasion and dissemination could promote persistence and asymptomatic carriage in the infected tissue during oropharyngeal infections. These differences likely reflect both the adaptability of different GAS strains (15, 107), as well as differential effects of colonizing microenvironment and stage of infection which may influence biofilm formation in the host. One of the biggest caveats to deducing a general GAS biofilm-specific transcriptome is the extreme strain-specific behaviour of GAS described earlier (15, 107). Hence it is imperative to develop and study various models of GAS biofilm in association with the host to improve our understanding of the cellular and molecular basis of GAS colonization in the context of invasive infections.

Since aggregation of bacteria is brought about primarily by adherence to either abiotic or biotic surfaces, widely characterized GAS adhesins including M or M-like proteins and the FCT (fibronectin-binding, collagen-binding, T-antigen) region-encoded pili are actively involved in this process (15, 28, 29, 108, 131, 132). Additional surface adhesins driving biofilm formation include fibronectin binding proteins PrtF2 and Sc11 and the AgI/II type polypeptide AspA (124, 133). Secreted virulence factors are equally implicated in biofilm formation. The broad spectrum SpeB protease is involved in biofilm matrix degradation which is associated with dissemination of bacteria to deeper tissues and increased severity of infection (83, 127, 128). The hyaluronic acid capsule is another important virulence factor that has variable effects on biofilm formation. Initially, a thick capsule might mask surface

adhesins, thereby preventing bacterial aggregation and biofilm formation, but at later stages it is suggested to contribute to matrix production (118). Other reports indicate that the capsule is involved in pharyngeal colonization (23) and adherence to human keratinocytes (22).

Differential transcriptional regulation of about 25% of the GAS genome has been reported for GAS biofilms from one of the few biofilm-specific transcriptomic studies conducted to date (118). There are currently three regulatory processes defined for GAS biofilms as summarised in the schematic in **Figure 7**:

1) Peptide pheromone based quorum sensing mediated by the short hydrophobic peptides SHP2/SHP3 under the regulation of Rgg2 and 3 transcriptional regulators (134). In the GAS strain NZ131, it has been shown that whereas Rgg2 modulation by SHP2/3 enhances biofilm formation, alteration in Rgg3 expression leads to an attenuation in the same by unknown mechanisms (134).

2) Biofilm formation is also regulated by the repression of secreted and surface associated proteases and nucleases, including SpeB (135, 136), the regulation of which is directly and indirectly controlled by GAS regulators (137), most significantly by the CovR response regulator of the CovR/S TCS which directly represses the expression of SpeB (138) and consequently enhances biofilm formation (128). SpeB expression is positively regulated by another member of the Rgg regulator family called *ropB*, the deletion of which leads to downregulation of SpeB and promotes biofilm growth of GAS M49 strain NZ131 (139). CodY, yet another regulator that senses nutrient deprivation in many gram-positive bacteria (140), indirectly represses the expression of SpeB and other proteases and nucleases that influence biofilm formation by GAS (135). Srv (streptococcal regulator of virulence), another important virulence-associated regulator influences an indirect control over SpeB expression (141) and loss of *srv* in an M1T1 strain MGAS5005 results in enhanced SpeB production leading to loss of biofilm phenotype (120, 128, 142).

3) Transcriptional regulation of biofilm specific MSCRAMMS (microbial surface components recognizing adhesive matrix molecules), known as adhesins (described previously) also has a major impact on biofilm formation. The role of the stand-alone regulator Mga in regulation of *emm* or *emm*-like genes is well described (143), and Mga inactivation leads to loss of aggregation and consequently biofilm formation (118, 144). Additionally, strains that harbour the FCT-2, 3, 4 type pilus encoding regions are primarily driven towards biofilm formation by environmental cues like pH (131), which in turn are

speculated to be under the regulation of RofA-like regulators RofA and Nra (145), although there is no direct evidence for this.

In summary, the role of biofilm formation as a pathogenic strategy of GAS deserves due attention in current times for the immense scope and far reaching impact that it has and might have with more and more investigations adding to the current body of knowledge on GAS pathogenesis.

1.4.5 Current treatment strategies for GAS biofilm mediated diseases

Penicillin remains the primary drug for treatment of most skin and respiratory tract diseases associated with GAS (146) and although increasing cases of macrolide resistant strains have been reported and are of clinical concern (147, 148), GAS remains susceptible to β lactams like penicillin (149). Severe GAS invasive diseases like STSS and NF are treated with a combination of penicillin and clindamycin; the latter has been found to limit GAS toxin and enzyme production (55, 150). But several cases of treatment failures and increasing persistence either due to the intracellular survival of GAS or biofilm formation (discussed earlier) is posing a major clinical threat to antibiotic treatment strategies. The MBEC (minimum biofilm eradication concentration) for penicillin against GAS biofilms is several folds higher than the MIC (minimum inhibitory concentration) that is sufficient to eradicate planktonic bacteria. This amounts to near clinical levels of resistance (106-108), and hence it is necessary to approach this serious concern with an outlook that includes anti-biofilm therapeutics targeting signalling mechanisms, matrix degradation, dispersing agents and other biofilm-associated traits.

Several anti-biofilm strategies employed for other bacterial biofilms have already been adapted for GAS. Dispersal agents like the fatty acid messenger cis-2-decenoic acid produced by *Pseudomonas aeruginosa* can be used to induce dispersal of GAS biofilms in combination with antibiotic therapy (151). Endolysins are a class of peptidoglycan hydrolases that are phage-encoded and used by the phage to lyse the cell wall of the host bacterium to release the phage progeny (152). Endolysins have been used extensively to disrupt biofilms in a variety of gram-positive bacteria (153-155). In GAS, the streptococcal-specific bacteriophage C1-encoded bacteriophage lysin C (PlyC) has efficacy in disrupting GAS biofilms to degrees

exceeding that of penicillin (152). The use of resident microflora to modulate the detrimental effects of pathogenic bacteria is also a relatively novel concept (156, 157). *Streptococcus oralis* and *Streptococcus salivarius*, two resident bacteria of the upper respiratory tract, prevent GAS colonization by forming an impermeable biofilm that renders the host epithelium inaccessible for GAS adherence (157). Another study showed that *S.salivarius* secretes lantibiotics salivarin A2 and B that disrupt GAS biofilm (158).

In conclusion, although much is known about GAS virulence, biofilm formation as a robust pathogenic strategy has only begun to be appreciated. Most of the current data is from *in vitro* studies conducted in the absence of the host microenvironment, making it further difficult to interpret these results without the context of host-pathogen cross-talk that influence microbial pathogenesis. Detailed and relevant models need to be designed to fully comprehend the mechanisms and underlying regulatory processes that define GAS biofilm/microcolonies and their role in disease.

1.5 Host modulation and host responses during GAS infections

Within the host milieu, GAS encounters an array of host factors, including a robust immune response. In order to establish a niche within the host to cause disease, GAS must not only subvert these immune molecules, but also establish infection by induction of inflammatory and stress pathways that may lead to host cell death. GAS has a repertoire of virulence factors that elicit a broad range of host responses as described below for the establishment and propagation of infection.

1.5.1 GAS mediated immunomodulation of the host defence system

Neutrophils constitute the first line of defence against extracellular pathogens that cause opsonophagocytosis of pathogens. GAS in turn is armed with a plethora of factors that resist both opsonisation by the host complement pathway as well as macrophage-mediated phagocytosis. Primary GAS virulence factors that are involved in these processes are the M protein, the hyaluronic acid capsule, as well as *Streptococcal* inhibitor of complement-mediated lysis (SIC). The M protein enables resistance to opsonophagocytic clearance by

binding to and inactivating the host complement system (159-161). The capsule is an anti-phagocytic factor (162) that inhibits complement deposition (162) and enhances GAS survival in neutrophil extracellular traps (NETs) (163). Immunoglobulins bind to invading organisms to facilitate clearance by either phagocytosis or complement fixation and GAS expresses Ig-binding proteins that prevent complement activation. Members of such Ig-binding proteins include M proteins, M-related proteins (Mrp), and M-like proteins (Mlp or Enn) (164) that bind to the Fc region of IgA or IgG and prevent activation of the complement pathway (165, 166). GAS also secretes Ig-degrading enzymes, including the IgG-degrading enzyme of *S. pyogenes* (IdeS), resulting in subversion of immune responses (167). SpeB, the broad spectrum serine protease can degrade IgG, IgA, IgM, IgD, and IgE (168).

Antimicrobial peptides (AMPs) are small cationic molecules produced by host cells that have potent bactericidal properties. GAS factors that inactivate AMPs include SpeB, which cleaves and inactivates human cathelicidin LL-37 produced by skin keratinocytes, mucosal epithelium and neutrophils (169). GAS surface-bound plasmin activity and SIC mediate degradation and inactivation of LL-37, respectively (170, 171).

Another strategy employed by GAS is to impair neutrophil recruitment and prevent phagocytosis by cleaving chemokines IL-8 and C5a that act as chemoattractants to these immune cells. A key factor that mediates this impairment is a cell wall-associated peptidase called SpyCEP (ScpC) (78) that not only blocks neutrophil recruitment (172) but also promotes GAS dissemination in systemic disease (173). The streptococcal C5a peptidase (SCPA) is a cell wall-associated proteolytic enzyme that prevents phagocytosis (174). C5a peptidase is a subtilisin-like serine protease that specifically cleaves the polymorphonuclear leukocyte (PMN) binding site of C5a, an important member of the complement system that recruits and stimulates neutrophils (174). Neutrophil extracellular traps (NETs) are highly complex extracellular structures composed of chromatin, granule proteins, and AMPs released by neutrophils that bind and kill bacteria (175). GAS can degrade NETS through the production of bacteriophage-encoded DNase Sda1 in GAS M1T1 strain (81) and cell wall-anchored nuclease A (SpnA) (176) that also enhances survival of GAS in human blood. The versatile M protein and capsule also degrade NETs as well as increases resistance to LL-37 (163). **Figure 8** describes GAS factors that mediate all host immunomodulatory functions described above.

Figure 8: GAS proteins involved in subversion of host immune response.

Abbreviations: SpeB, cysteine protease; Sic, streptococcal inhibitor of complement; PrtF1, protein F1; PrtF2, protein F2; SdaD1, streptococcal DNase I; NET, neutrophil extracellular trap; Mac1/IdeS, immunoglobulin G degrading enzyme of *Streptococcus*; SpyCEP, *Streptococcus pyogenes* cell envelope protease; ScpA, streptococcal C5a peptidase

Figure adapted from Olsen *et al.*, 2009 (46)

1.5.2 Role of GAS secreted cytolytins in host immunomodulation and tissue damage

GAS secretes potent streptolysins that induce a variety of cellular responses in the host, including the activation of signalling pathways that result in host cell death leading to extensive tissue damage and the release of host molecules that can enhance GAS survival and proliferation (177). There are two well characterized streptolysins secreted by GAS, streptolysin O (SLO) and S (SLS).

Streptolysin O (SLO)

SLO, a 69-kDa oxygen-labile cholesterol-dependent cytolysin (CDC), is the best characterized member of the family of pore forming toxins (PFT), secreted by all tested GAS isolates (178, 179). SLO primarily binds to cholesterol on the host cell membrane and forms multi-subunit pores leading to the formation of membrane-penetrating toxin channels that allow the passage of very large molecules secreted by GAS to induce extensive host cell damage (178). Apart from pore-dependent functions (180), SLO has also been associated with pore-independent functions (181). SLO provides resistance against phagocytosis by inducing membrane damage and consecutive caspase-dependent apoptosis in neutrophils, macrophages and epithelial cells and enables GAS escape from within endosomes (182-184). SLO also suppresses the expression of host chemokines TNF α and IL- β leading to enhanced virulence in a mouse model of invasive infection (182), as well as proinflammatory cytokines IL-1 β , IL-6 and IL-8 in infected keratinocytes (185). Synergistic cytotoxicity is induced by co-expression of SLO and SPN (*S. pyogenes* NAD⁺-glycohydrolase), which together constitute the cytolysin-mediated translocation (CMT) machinery, analogous to the Type III secretion system in Gram negative bacteria (33). These toxins prevent internalization of GAS and induce keratinocyte apoptosis (183). Very recently, the specific activity of SPN, uncoupled from its co-toxin SLO was deduced and it was shown that SPN facilitates the intracellular survival of SLO deficient GAS (186). SLO is associated with extensive tissue damage at the sites of infection (182, 183), and it also augments superantigen mediated tissue damage in porcine vaginal mucosa in an *ex vivo* model of superantigen penetration (187).

Purified SLO is a potent cytotoxin and is lethal within a few minutes when injected into mice and rabbits (188, 189). At sublethal doses, SLO causes necrotic dermal lesions, vascular permeability, and other neurological abnormalities resulting in death (190, 191). In the realm of invasive systemic disease, SLO contributes to virulence in various subcutaneous, intravenous, and intraperitoneal mouse models (182, 189, 192).

Streptolysin S (SLS)

SLS is the other potent oxygen-stable hemolysin with potent cytopathic activity that is secreted by all GAS strains (193). SLS is 2.8kD and bears structural similarity to the bacteriocin class of microbial toxins (193) although structural elucidation of SLS has not been successful owing to its poor physiochemical properties (194). Moreover, the precise mechanism of its toxicity to mammalian cells is not known (195) except that it causes transmembrane pores via several intermediate complexes finally leading to the irreversible osmotic deregulation and subsequent cell lysis (195). SLS is encoded by a conserved operon called *sag* (SLS-associated gene) consisting of nine genes designated as *sagA-sagI* (193), sometimes called *pel* (pleiotropic effect locus) (196) since it regulates the expression of several secreted proteins including streptokinase and SpeB, hyaluronic acid capsule, as well as fibronectin and IgG-binding proteins. It is also responsible for the β hemolysis of RBCs that is characteristic of GAS and is observed as the classic zone of lysis around GAS colonies on blood agar (196).

The cytopathic and cytotoxic effects of SLS are manifold. SLS renders GAS more resistant to phagocytic killing by forming hydrophilic pores in cell membrane of neutrophils (197) and contributes to GAS virulence and pathogenesis by lysing several types of cells including lymphocytes, erythrocytes and platelets (198). At sublytic concentrations, SLS has other roles that contribute to virulence, notably influencing host-cell signalling for its own survival, dissemination, and pathogenesis. SLS contributes to GAS translocation across the epithelial barrier by disrupting the intracellular junctions via the recruitment of cysteine protease calpain that degrades host-proteins like occludin and E-cadherin (199). Like SLO, SLS also impairs immune cells like macrophages and neutrophils by inducing programmed cell death (197, 200). Very recently, SLS-dependent p38 MAPK activation leading to downstream NF- κ B signalling was reported, leading to programmed necrosis in keratinocytes (201). SLS acts in synergy with other GAS virulence factors like SpeB, neutrophil derived proteases, as well as SLO to cause cellular injury (202-204). In a subcutaneous mouse model of NF, deletion in the *sagA* gene impaired GAS proliferation, neutrophil recruitment, and induced enhanced vascular injury with the development of necrotic lesions that also reduced the weight of the mice (205). In another study in a murine dermo-necrosis model, SLS was attributed with inducing necrotic skin ulcers, systemic spread, and mouse lethality (204, 206). Since the

CovRS TCS regulates the expression of the capsule, SpeB and SLS, CovRS mutants in a murine skin infection model were attenuated in virulence, especially with respect to lesion formation where SLS and the capsule were attributed with subcutaneous spread and lethality, and SpeB was associated with dermal ulceration (36, 207).

Combined contribution of SLO and SLS to GAS virulence

A number of *in vitro* and *in vivo* models have been used to interrogate the effects of deletion of both SLO and SLS on GAS virulence. Compared to single mutants, the double mutant lacking both streptolysins displayed increased attenuation in inducing weight loss, lesion formation, and severity of lesion spread in a murine intraperitoneal GAS infection model (204), leading the authors to conclude that the streptolysins made “incremental” rather than “critical” contribution to disease severity. This “incremental” or additive contribution was also observed in another study wherein the double mutant failed to suppress immune responses in the peritoneal cavity of mice compared to the single mutants, and was significantly impaired in inducing cytotoxicity in bone marrow-derived murine macrophages, human blood monocytes, and neutrophils compared to the parent wild type strain that contributed to attenuation in inflammation, tissue damage, and bacterial dissemination (200). A more recent study by our collaborators extensively explored the role of these streptolysins in *in vivo* GAS survival, and more precisely the autoinduction of a unique locus called the *sil* (streptococcal invasive locus) locus (208-211). SLO and SLS were shown to induce endoplasmic reticulum (ER) stress in the host which triggered the release of asparagine that GAS M14 clinical isolate JS95 (210) utilized for its benefit and modulation of almost 17% its genome (177).

In summary, although these streptolysins are not co-regulated, nor work together functionally, there is a substantial redundancy in their effector functions. The effects of SLS are more complex, since its expression is linked to pleiotropic expression of other GAS virulence factors, that has also led to some inconclusive observations as to whether SLS contributes directly or indirectly to GAS virulence (204). Nevertheless, both SLO and SLS are expressed by all GAS strains, and are highly conserved across various M serotypes (193) and have been implicated in a number of distinct as well as overlapping functions.

1.5.3 Role of GAS factors and streptolysins in the induction of host programmed cell death (PCD) pathways

The host innate immune system has very effectively evolved a potent arsenal to combat pathogens. As described earlier, pathogens encounter antimicrobial peptides at mucosal surfaces, or are opsonised by the complement pathway in the blood. Upon infection, chemo-attractants recruit immune cells like neutrophils, macrophages and other phagocytic cells to the infection site and eliminate the pathogen by activation of inflammatory pathways. The host expresses specific receptors that recognize pathogen associated molecular patterns (PAMPs) that trigger these events. Furthermore, the host also has the ability to induce cell death by virtue of which it can jettison infected cells and clear the infectious niche of the pathogen. The outcome of cell death is wholly dependent on the type and infection load of the pathogen as well as the cell type of the site of infection, as epithelial cells, endothelial cells and phagocytic cells have distinct modes of inducing cell death (212).

Apoptosis remains the most common form of programmed cell death for epithelial cells as well as lymphocytes, and in some cases macrophages and dendritic cells, although they primarily undergo pyroptosis as a quick way of clearing pathogens. On the other hand, an overwhelmingly high bacterial burden might elicit other cell death pathways, such as oncosis and autophagy (212). GAS is capable of inducing all these PCD pathways (177, 213) based on differences in serotype, host cell type and bacterial load. The different PCD pathways induced by GAS on host cells are summarised in the schematic of **Figure 9**.

Figure 9. Pathways leading to host cell death.

Death inducing stimuli in the host, like cellular stress, infection and pathological conditions trigger a variety of molecular pathways leading to programmed cell death (PCD). **Apoptosis** is a very common PCD mediated by the activation of initiator caspases that cause effector caspases to cleave cellular substrates. Apoptotic cells are characterized by cytoplasmic and nuclear condensation, DNA damage, and formation of apoptotic bodies meanwhile maintaining an intact plasma membrane. Apoptotic cells may start expressing surface markers that brand them for phagocytic clearance by PMNs. In the absence of phagocytosis, apoptotic bodies may proceed to lysis and secondary or **apoptotic necrosis**. **Autophagy** entails the degradation of cellular components of the cell within autophagic vacuoles. Autophagy is represented by vacuolization, degradation of cytoplasmic contents, and chromatin condensation. Autophagic cells can also be cleared by phagocytosis. **Oncosis** is a pre-lethal pathway leading to cell death characterized by cellular and organelle swelling and membrane disintegration, with the eventual release of inflammatory cellular contents, culminating in **Necrosis**.

Figure adapted from Fink *et al.*, 2005 (214)

Autophagy:

Autophagy is a well conserved PCD that involves the degradation of cytosolic components, damaged organelles or intracellular pathogens via formation of double-membraned cytosolic vacuoles called autophagosomes that eventually fuse with lysosomes (215, 216). One of the first studies to document the evidence of bacteria being targeted for autophagy-mediated clearance (217) showed that an M6 JRS4 GAS strain was cleared by autophagy in non-phagocytic HeLa cells which could be subverted in an SLO-dependent manner (218). In contrast, in another study, the highly virulent GAS M1T1 but not JRS4 strain, resisted

ubiquitylation and recognition by the host autophagy marker LC3 and ubiquitin-LC3 adaptor proteins in Hep-2 epithelial cells by SpeB mediated proteolytic degradation of these proteins (216). In yet another study, it was reported that since GAS can cause systemic infection, an understanding of its interaction with endothelial cells is also imperative to fully comprehend the role of autophagy in GAS clearance (219). The study shed light on the importance of acidification for autophagy-mediated clearance of GAS, but only in epithelial cells. In endothelial cells, GAS survives intracellularly by secreting SLO and its co-toxin SPN thereby preventing acidification of the phagolysosome, already reported in a previous study (220).

Necrosis:

Necrosis has only recently been categorised as a special case of PCD, since it was attributed to physio-chemical stress and hence is thought to be accidental and uncontrolled (221). The defining physiology of necrosis is swelling and increased permeability, as well as cell membrane damage that causes spillage of cellular contents that in turn leads to the release of damage-associated molecular patterns (DAMPs) and prompts rapid immune responses (213, 222). There are some differences as to how necrosis is understood, as some studies link it to apoptosis and report the occurrence of both in the same cell population, due to overlap of certain signaling pathways such as activation of death receptors, kinase cascades, and mitochondrial dysregulation (222). Others describe it as a caspase-independent cell death pathway with extensive crosstalk between several molecular events (221). The hallmark of GAS-mediated NF is extensive tissue necrosis of fascia and muscle (10). GAS streptolysins SLO and SLS have been implicated in the development of necrotic lesions in several murine dermonecrosis/subcutaneous skin infection models (204-207).

Oncosis:

Oncosis is defined as a rapid, pre-lethal pathway leading to necrosis, characterized by cytoplasmic vacuolization, organelle swelling, clumping of nuclear chromatin and loss of membrane integrity (213). GAS was shown to induce rapid SLO and SLS mediated oncosis in

resident peritoneal and bone marrow derived macrophages, which altered the mitochondrial membrane potential, intracellular ATP depletion, leading to the activation of calpains and production of reactive oxygen species (ROS) (200), in line with previous reports (223).

Apoptosis:

Pathogen-induced apoptosis has been extensively studied, and is a common end to different pathogenic interactions in both phagocytic as well as non-phagocytic or epithelial cells (224). Genes that comprise a common apoptosis differentiation program in human PMNs after phagocytosis of various pathogenic bacteria have been described (225). GAS induces apoptotic cell death in epithelial cells, although the effectors vary for different GAS serotypes and cell type of the host. SpeB induced apoptosis in epithelial cells via activation of the caspase pathway, which was impaired when GAS internalization was blocked using cytochalasin D (226). Distinct from exotoxin-dependent apoptosis, the role of M protein and the fibronectin binding protein F1 (that aids in internalization) mediated mitochondrial dysfunction and the subsequent activation of caspase 9 (227) has been reported. In contrast, extracellular GAS was able to induce apoptosis in keratinocytes by dysregulating the calcium signalling mediated by SLO that forms transmembrane pores that permit extracellular calcium influx into the cytosol (228). SLS expression causes caspase7 mediated apoptosis in thioglycolate-induced murine PMNs (197). Dendritic cell maturation is impaired by GAS via SLO-mediated apoptosis (229). Timmer *et al* showed that in contrast to work by Goldman (200) (where streptolysin mediated mitochondrial membrane depolarization led to oncosis in macrophages), SLO mediated membrane damage elicited the intrinsic apoptotic pathway mediated by caspase 3 and 7 (182). From these reports, it is evident that no uniform apoptosis induction pattern exists for GAS serotypes, and hence makes it beguiling as a host modulatory mechanism.

From these observations, the direct role of GAS streptolysins in PCD is evident and it underscores the cytopathic and cytotoxic effects of these streptolysins in inducing host injury and death, facilitating GAS in disease progress.

ER stress:

Figure 10: Cellular responses to endoplasmic reticulum stress.

Upon accumulation of unfolded proteins in the ER lumen, the unfolded protein response (UPR) cascade is activated mediated by three sensors resident in the ER membrane- protein kinase R (PKR)-like ER kinase (**PERK**), inositol-requiring enzyme 1 (**IRE1**) and activating transcription factor 6 (**ATF6**). During homeostasis, all three sensors are repressed by the binding immunoglobulin protein (**BiP**), which gets relieved when BiP gets recruited away by the unfolded proteins, thereby activating these sensors and triggering UPR. This leads to stalling of mRNA translation, increasing protein folding capacity, and ER-associated degradation (**ERAD**). Activation of the PERK-eIF2-ATF4-CHOP pathway leads to intracellular calcium dysregulation, ROS production, perturbation of mitochondrial membrane potential leading to cytochrome c release that culminates in apoptosis. The PERK pathway gets activated upon GAS secretion of SLO and SLS, which in turn causes the upregulation of asparaginase synthase (ASNS) as part of the amino acid metabolism changes mediated by ATF4. The host secretes asparagine which is utilized by GAS for its own proliferation and growth.

Figure adapted from Celli *et al.*, 2014 and Baruch *et al.*, 2014 (177, 230)

Although not strictly part of the PCD pathways described above, ER stress is emerging as an important cellular response to pathogenic bacteria, culminating in apoptosis in some cases (230). The endoplasmic reticulum (ER) is involved in the synthesis, processing, and folding of secretory and membrane proteins. Upon dysregulation in protein folding, unfolded proteins accumulate and induce ER stress that triggers the unfolded protein response (UPR), a well

conserved cyto-protective pathway that serves to relieve this stress by stalling mRNA translation, increasing protein folding capacity, and ER-associated degradation (ERAD) (230). These effects are brought about by three sensors that are located in the ER membrane: inositol-requiring enzyme 1 (IRE1), double-stranded RNA-dependent protein kinase R (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). When this stress goes unmitigated, it results in programmed cell death. Activation of the PERK-eIF2-ATF4-CHOP pathway leads to intracellular calcium dysregulation, ROS production, perturbation of mitochondrial membrane potential leading to cytochrome c release that culminates in apoptosis (230).

Intracellular bacteria induce ER stress in the host when they evade autophagosomal degradation and fuse with the ER which is a safe niche for their survival as the ER does not harbour any bactericidal mechanisms (230). Quite a few intracellular bacteria have been reported to reside in ER-derived vacuoles and organelles that closely interact with the ER, namely, *Brucella* spp, *Legionella* spp, *Chlamydia trachomatis* and the *Chlamydia*-related bacterium *Simkania negevensis* (230). Both *Brucella* spp and *Legionella* spp form BCVs (*Brucella* containing vacuoles) and LCVs (*Legionella*-containing vacuoles) that intercept early secretory vesicles containing sub-compartments of ER called ER exit sites (ERESs) trafficking between the ER and the Golgi apparatus. Prolonged and sustained interactions of these bacterial vacuoles and ERES results in formation of ER-derived vacuoles that provide a safe niche for these intracellular bacteria to proliferate and spread disease (230).

Extracellular bacteria, on the other hand, induce ER stress in the host by secreting a host of toxins, especially a family of pore-forming toxins (PFTs) that activates the UPR as a host defence strategy (231). In GAS, SLO (which belongs to the PFT family) and the capsule have been implicated in extracellular induction of ER stress, obviating the need for GAS to establish an intracellular niche as described above, culminating in caspase 9 and 3 mediated apoptosis in keratinocytes (228). GAS streptolysins SLO and SLS also play a role in extracellular ER stress induction in host epithelial cells as shown by our collaborator's work (177). The activation of the PERK-eIF2-ATF4 pathway of UPR leads to increased expression of asparagine synthase (ASNS) enzyme, causing the host to secrete asparagine that GAS then uses for its own proliferation and expression of a substantial subset of its genome that eventually enhances GAS survival and virulence (60, 61, 177, 232).

These observations indicate the many mechanisms that GAS uses not only to subvert host defence and surveillance, but to also modulate the host cellular machinery for its own persistence, propagation and disease progression.

1.6 Significance of my study

In conclusion, while much is known about the virulence mechanisms of GAS infections, there remain gaps in the complete understanding of how host responses, including bacterial intra- and extra-cellular recognition, immune surveillance, and other pathogen and host signalling pathways leading to host cell death can augment and define GAS pathology. An understanding of these host responses is crucial to the development of novel diagnostic and therapeutic interventions. Furthermore, the clinical significance of biofilm formation as an integral part of GAS pathogenesis is just beginning to be explored. Caveats to this end include divergent host responses that are elicited by disparate GAS strains in different host cell types impeding a consensus; a dearth of physiologically relevant models of study that ineffectively recreate the infection biogeography that is essential for a correct analysis of the underlying cellular and molecular events of pathogenesis; and lastly, lack of a comprehensive exploration of *in vivo* models where both persistent and acute infections can be studied. Unlike host-pathogen interaction-based studies that can easily be replicated *in vitro*, biofilms, which are defined primarily by their spatio-temporal makeup, need to be studied *in situ* with relevant analytical tools and with a minimum disruption of their innate 3D architecture. Therefore, the approach towards a better analysis of biofilm-driven pathogenesis needs to be drafted judiciously, and the resulting data needs to undergo careful and relevant interpretation in the context of infection.

With these issues in mind, the primary aim of my thesis has been to establish and characterize an *in vitro* acute infection-based biofilm model to interrogate the molecular determinants, both from GAS and the host, required for biofilm-mediated pathogenesis. I extended my investigation by studying the *in situ* architecture, spatial distribution, and spread of *in vivo* tissue-associated GAS microcolonies. My findings attribute GAS toxins SLO and SLS with a previously undefined role of modulating the host cellular machinery, by induction of ER stress in the host, thereby eliciting the secretion of a biofilm-promoting factor that augments biofilm formation. Most importantly, the histopathology of these microcolonies not only mirrors previous observations of acute infection studies, but further extends our understanding of NF disease progression by establishing a link between pathogenesis and biofilm formation.

CHAPTER 2

Materials and Methods

Materials

General chemicals:

Todd Hewitt (TH) medium	Sigma
Yeast extract	Becton Dickinson
Bacto™ Agar	Becton Dickinson
Kanamycin	Sigma
Erythromycin	Sigma

Tissue Culture reagents

Dulbecco's modified eagle medium (DMEM)	Gibco, Life Technologies
Fetal bovine serum (FBS)	PAA laboratories
Penicillin/streptomycin	Gibco
Thapsigargin (TG)	Sigma
Etoposide (ET)	Sigma
Staurosporine (STS)	Sigma
Dithiothreitol (DTT)	Sigma
Saponin	Sigma
Triton X 100	Sigma
Hydrogen peroxide	Sigma
Tissue culture grade DMSO	Sigma

Fluorescent conjugates, antibodies and kits for immunofluorescence

goat polyclonal anti- <i>S.pyogenes</i> Group A Carbohydrate	Abcam, UK
rabbit anti-goat AF 488	Life Technologies

rabbit polyclonal anti-GADD153 (CHOP)	Santa Cruz
donkey anti-rabbit AF 594	Life Technologies
ConcanavalinA AF 488 conjugate	Life Technologies
ConcanavalinA AF 594 conjugate	Life Technologies
DAPI	Life Technologies
LDH Cytotoxicity Determination kit	Clonetech
LIVE/DEAD® BacLight™ Viability Kit	Life Technologies
NucView 488 and RedDot 2 Apoptosis and Necrosis Kit	Biotium, Inc.
Phalloidin AF 350 conjugate	Life Technologies
Phalloidin AF 594 conjugate	Life Technologies
TUNEL Assay Kit	Roche
VECTASHIELD Mounting Medium	Vector Laboratories
Wheat germ agglutinin (WGA) AF 555	Life Technologies

Materials for animal work:

Insulin syringes (12.7 mm needle, 1ml)	Becton Dickinson
Disposable Biopsy Punches - 8.00 mm	Robbins Instruments. Inc
Optimum cutting temperature (OCT) compound	Sakura® Finetek, INC

Methods

Bacterial strains and growth conditions

All bacterial strains used in the study are described in **Table I**. For culturing of GAS, we used Todd-Hewitt medium (Sigma Aldrich) supplemented with 0.2% yeast extract (Becton, Dickinson) (THY media) with incubation at 37°C in sealed tubes without agitation for 18 h in 5% CO₂. Bacto™ Agar (Becton Dickinson) was added to a final concentration of 1.4% for solid media. Antibiotics were added at the following concentrations where appropriate: 250 µg/ml kanamycin (Km) and 1 µg/ml erythromycin (Em).

Table 1

SN	GAS strains	Description	Antibiotic resistance	Ref
1	JS95 WT	GAS Clinical isolate from PPBS in Israel	-	(210)
2	$\Delta emm14$	<i>emm</i> deletion mutant of JS95	Km250	(210)
3	<i>speB</i> ⁻	<i>speB</i> insertion-inactivated derivative of JS95	Em1	-
4	<i>hasA</i> ⁻	<i>hasA</i> insertion-inactivated derivative of JS9	Em1	-
5	Δslo	<i>slo</i> deletion mutant of JS95	Km250	(177)
6	<i>sagI</i> ⁻	<i>SagI</i> insertion-inactivated derivative of JS95	Em1	(177)
7	$\Delta slo sagI$ ⁻	<i>slo</i> deletion and <i>sagI</i> insertion-inactivated mutant of JS95ATG	Em1Km250	(177)
8	MGAS5005	Representative of GAS MITI serotype, highly invasive clinical strain	-	(142)
9	JRS4	GAS M6 serotype, streptomycin-resistant derivative of strain D471	-	(233)

Cell culture and media

Cell lines used in this study; mouse embryonic fibroblasts (MEFs), human foreskin fibroblasts (HFFs), and HaCat human keratinocytes were cultivated in Dulbecco's modified eagle medium (DMEM+ high glucose (1X) with L glutamine, without sodium pyruvate; Gibco) with 10% fetal bovine serum (FBS; PAA) at 37°C in 5% CO₂ in 24 well polystyrene cell culture plates (Corning).

Cultivation of biofilms

Overnight cultures (18 h) of GAS strains grown in THY medium were diluted 20-fold in a 4:1 mixture of THY (Todd Hewitt Yeast extract) broth and DMEM supplemented with 10% FBS and grown until early log phase ($OD_{600}=0.2$). The bacterial cells were centrifuged at 5000 g for 10 minutes, and the resulting pellet was washed in phosphate-buffered saline (PBS) twice and then resuspended in PBS to an OD_{600} of 0.8, equivalent to 1×10^8 CFU/ml. Finally, 5×10^5 CFU of bacteria were seeded into 24 well polystyrene plates containing DMEM with 10% FBS for growth on polystyrene or into 24 well polystyrene plates already seeded with 5×10^4 MEFs/well after overnight attachment in DMEM with 10% FBS for an MOI (multiplicity of infection) of 10. The biofilms were grown statically at 37°C with 5% CO₂ for the indicated times, with a media change after every 24 h to prevent accumulation of metabolic byproducts released during cellular growth.

Biofilm quantitation

Biofilm biomass was quantified using crystal violet (CV) staining of adherent biofilms on plastic, as previously described (123) with the following modifications for biofilms growing on mouse embryonic fibroblasts (MEFs) (234). After biofilm growth, media from the wells was removed, the plates were washed 2 times with PBS, and adherent bacteria were stained with 0.1% crystal violet for 15 min and washed 2 times with PBS to remove the excess stain.

To extract CV from the underlying MEFs, 1 ml ethanol:acetone (1:1) decolorizer was then added to the wells and removed immediately. Wells containing MEFs without bacteria or media alone were used as controls. The CV from the bacterial biofilms was eluted with 500 ml of 96% ethanol and the density of crystal violet stain was measured by absorbance at 590 nm using a Tecan F200 Microplate reader. For quantification of viable bacteria in GAS biofilms, media was aspirated from biofilms formed either on plastic or on MEFs, and the biofilms were gently washed with PBS. The biofilms were then gently scraped from the respective surfaces, resuspended in PBS, and quantified for CFU by dilution plating on THY agar plates.

Scanning electron microscopy (SEM) analysis

Biofilm samples were fixed using 2.5% glutaraldehyde (prepared in 0.1 M PBS; pH 7.4) for 48 h at 4°C and then washed thrice with 0.1 M PBS. Samples were then post fixed in 0.2% osmium tetroxide (prepared in 0.1 M PBS; pH 7.4) for 2 h at room temperature and washed thrice with 0.1 M PBS. Fixed samples were then dehydrated with a graded ethanol series in distilled water (once with 30%, 50%, 70%, 80%, 90% and thrice with 100% ethanol for 15 minutes at each step) together with gentle agitation. Samples were then treated with a 1:2 EtOH : hexamethyldisilane for 20 minutes, then with a 2:1 EtOH : hexamethyldisilane for a further 20 minutes with gentle agitation. Samples were then treated with 100% hexamethyldisilane twice for 20 minutes before air-drying in a fume hood overnight. Following chemical drying, samples were then deposited onto the SEM specimen stubs using one side of a double sided adhesive NEM Tape (Nisshin Em. Co. Ltd, Tokyo, Japan). Samples were then sputter coated with gold using a Bal-Tec SCD 005 sputter coater (Bal-Tec AG, Balzers, Liechtenstein). Samples were viewed using a JSM-6360LV (JEOL, Tokyo, Japan) scanning electron microscope.

Immunofluorescence and microscopy of *in vitro* biofilms

For confocal laser scanning microscopy (CLSM) studies, GAS biofilms were grown on 35 mm μ dishes (ibiTreat surface, ibidi), either directly on the polystyrene surface or on MEFs seeded at 5×10^4 cells/ μ dish. Biofilms grown for 24, 48, 72, 96 and 120 h were washed with PBS twice, fixed with 4% paraformaldehyde (PFA) for 10 minutes, and permeabilized for 10 minutes with 0.1% saponin in PBS. Next, non-specific sites were blocked using 5% BSA in PBS. Incubation with goat polyclonal anti-*S.pyogenes* Group A Carbohydrate (Abcam, UK; 1:100) primary antibody at 37°C for 3 h was followed by incubation with rabbit anti-goat Alexa Fluor (AF) 488 (Life Technologies; 1:500) secondary antibody for 45 minutes to stain GAS biomass. MEFs were stained either with phalloidin AF 350 conjugate or ConcanavalinA AF 594 conjugate for 15 minutes (Molecular Probes, Life Technologies) to stain F actin or surface glycoproteins on MEFs surface. Wheat germ agglutinin (WGA) AF 555 (Molecular Probes, Life Technologies) was used at a concentration of 1 μ g/ml for 20 minutes to stain biofilm exopolysaccharides. Excess dye was removed by washing with PBS, and the dishes were air dried and mounted with VECTASHIELD mounting medium (Vector Laboratories).

Antibiotic susceptibility testing and activity against bacterial biofilms

Minimum inhibitory concentration (MIC) for ampicillin against GAS was determined by the microdilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations (235). Antibiotic activity was also evaluated against 24, 48 and 72 h old biofilms. At the indicated timepoints, biofilm culture medium was removed and immediately replaced by fresh media (control) or media containing ampicillin at increasing concentrations (0.5 to 10-fold the MIC) and incubated for 24 h at 37°C. Biofilms were then disrupted and viable CFU enumerated by plating on THY agar plates.

LDH assay

MEFs infected with GAS in DMEM lacking phenol red with 5% FBS in 24 well plates were centrifuged at 400 g for 10 minutes and 100 µl of the cell free supernatant (filtered through a 0.22 µm Millex[®] syringe filter) was collected from each well at various time intervals. The lactate dehydrogenase (LDH) assay was performed using the Clontech LDH cytotoxicity determination kit according the manufacturer's directions. For negative controls, we used supernatants from uninfected MEFs and for positive controls, we used supernatants from MEFs treated with 0.2% Triton X for complete cell lysis. Additional controls included the assay medium alone to assess basal LDH levels in the DMEM with 5% FBS, and GAS bacterial suspension containing 5×10^5 CFU in DMEM with 5% FBS in the absence of MEFs. The assay plate was read at 490 nm using a Tecan F200 Microplate reader.

Detection of viability of MEFs infected with GAS

Viability of MEFs infected with GAS at an MOI of 10 was tested using the LIVE/DEAD[®] BacLight[™] Viability Kit, for microscopy. At the indicated time points, media was removed from the wells, and GAS infected MEFs were stained according to the manufacturer's instructions for 10 minutes at room temperature. Epifluorescence images were acquired using the Axio ObserverZ1 widefield microscope (Carl Zeiss, Thornwood, NY) at x20 magnification.

Detection of Apoptosis and Necrosis by microscopy

Cell death by apoptosis was assessed using a NucView 488 and RedDot 2 Apoptosis and Necrosis Kit (Biotium, Inc). Culture media was removed from infected MEFs at the indicated time points, cells were washed with PBS, and stained according to the manufacturer's protocol. Sample images were acquired using the Axio Observer Z1 widefield microscope (Carl Zeiss, Thornwood, NY) at x20 magnification.

ER stress, apoptosis, and supernatant complementation experiments

MEFs were incubated for indicated times with 1.0 μ M thapsigargin (TG) (Sigma), 10 μ M etoposide (ET) (Sigma), 0.5 mM dithiothreitol (DTT) (Sigma), 0.1 μ M staurosporine (STS) (Sigma) or dimethyl sulfoxide (DMSO) (Sigma) as the solvent control. Filtered cell free supernatants, referred to as conditioned media (CM), were collected at indicated time points and used as resuspension media for Δ *slo sagI*⁻ biofilm assays on MEFs. Effects of drugs on GAS viability were assessed by incubating 5 x10⁵ CFU planktonic GAS with the indicated concentration of drugs for 24 h, with subsequent enumeration of viable bacteria by CFU plating on THY agar plates. Bacteria free CM were also filtered and collected from wild type or Δ *slo sagI*⁻ mutant GAS strains grown on polystyrene or MEFs for the indicated time points, and used as resuspension media for Δ *slo sagI*⁻ mutant biofilm assays. For testing the effects of asparagine on biofilm growth, the culture media was supplemented at a final concentration of 15, 60 or 100 mg/l of asparagine for Δ *slo sagI*⁻ biofilm assays on MEFs.

Animal experiments

The ability of GAS to form *in vivo* (tissue-associated) biofilms was assessed by injecting GAS WT and the Δ *slo sagI*⁻ mutant strains in a murine model of soft-tissue infection as described previously (79, 80, 210). An inoculum of GAS at mid log phase containing 1x10⁸ CFU/ml was injected subcutaneously in the rear flank of female BALB/c mice aged 3-4 weeks weighing 10-12 g. All animals were purchased from InVivos Pte. Ltd., Singapore.

For pre-induction of ER stress or non-ER stress mediated apoptosis, respectively 2 mg/kg body weight of TG and 320 mg/kg body weight of ET was injected subcutaneously in shaved mouse flanks 12 h prior to GAS injection at the same site. Biopsies of lesions at site of injection were collected at 12 hpi. The biopsies were fixed in 4% PFA for 4 h at room temperature followed by 30% sucrose at 4°C for 16 h before freezing in optimum cutting temperature (OCT) compound, Sakura® Finetek, USA INC. The frozen blocks were sectioned using a cryostat into 10 μ m thick sections and stained for histological analysis by hematoxylin and eosin (Merck) dyes following the manufacturer's protocol, or immunological analysis using an antibody specific to GAS (goat polyclonal anti-*S.pyogenes*

Group A Carbohydrate (Abcam, UK; 1:100), and counterstained with DAPI and phalloidin AF 594 (Molecular probes, Life Technologies) to visualize the surrounding tissue. Wheat germ agglutinin (WGA) AF 555 (Molecular Probes, Life Technologies) was used at a concentration of 1 µg/ml for 20 minutes to stain biofilm exopolysaccharides. To ascertain that the drugs as well as GAS induced ER stress or apoptosis, tissue sections were immunostained with rabbit polyclonal anti-GADD153 (CHOP) antibody (Santa Cruz) followed by AF 594 donkey anti-rabbit IgG secondary antibody. For apoptosis, the same tissue sections were co-stained using the TUNEL Assay Kit (Roche). For CFU quantification of GAS in the lesions, the tissue biopsies were homogenized in sterile PBS and plated on THY plates with or without Kan 250.

Image analysis

Imaris (Bitplane AG, Belfast UK) was used to calculate biovolume of the biofilm. ImageJ (NIH, USA) was used to quantify mean fluorescence intensity of biopsy sections after immunostaining.

Statistical analysis

All experiments were repeated at least three times, with three technical replicates each (unless stated otherwise). Statistical significance for data from all experiments was determined using the Prism 5 software (Graph Pad). Data are expressed as mean ±SEM (unless stated otherwise) and p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****), “ns” denotes no significance).

CHAPTER 3

Establishment and characterization of biofilms formed by a necrotizing GAS strain on mammalian cells

(in vitro model)

3.0 Establishment and characterization of GAS (JS95 strain) biofilms

3.1 Aim and overview of study

The clinical significance of GAS biofilms has been discussed at length in Chapter 1 of this thesis. GAS biofilms have been associated with persistence and asymptomatic carriage, enhanced tolerance to antibiotics, increased severity of infection and dissemination of bacteria into deeper tissue.

The role of biofilm formation as a pathogenic strategy in the propagation of GAS NF has not been previously explored in mechanistic detail, in part due to a lack of relevant models that allow for a full analysis of the host-pathogen interaction that shape the biofilm developmental program. Hence, we aimed to interrogate whether GAS clinical NF strain JS95 is capable of forming biofilms by establishing and characterizing an *in vitro* biofilm model. JS95, the clinical strain that we used for all our studies, was isolated during a prospective population based study (PPBS) of GAS invasive infections in Israel (210). Molecular analysis of this M14 strain led the investigators (our collaborators) to identify and characterize a unique virulence-conferring locus in JS95 called the streptococcal invasion locus, “*sil*”, which is involved in GAS dissemination into deeper tissues and is highly homologous to a regulon of *Streptococcus pneumoniae* involved in bacterial signalling (209-211). This necrotizing strain was inoculated on mouse embryonic fibroblasts (MEFs), a cell line known to mimic fascia and express many GAS-related receptors.

My aim was to establish a robust biofilm model that enabled us to recapitulate acute GAS infection conditions *in vitro*. This would be supported by an extensive and comprehensive characterization of GAS biofilms with respect to their biofilm traits to distinguish them from mere aggregation on these mammalian cell lines. Additionally, I also aimed to interrogate the molecular determinants that mediate host-pathogen interaction driven biofilm formation. I also aimed to study the dynamics of biofilm formation on live host cells compared to abiotic surfaces to better understand the contribution of host cellular processes that GAS has evolved to modulate for its own advantage, survival, growth and disease progression.

3.2 GAS strain JS95 forms biofilms on mammalian cells

To examine whether GAS biofilms form during the course of host-pathogen interactions, we inoculated GAS strain JS95 on mouse embryonic fibroblasts (MEFs) at a multiplicity of infection (MOI; a ratio of number of bacteria to the number of host cells) of 10, 1, and 0.1 and monitored biomass accumulation over time. All three MOIs displayed similar trends of biomass accumulation as described below for MOI 10; however, the kinetics of biofilm formation was slower at lower MOIs (data not shown). Thus, we chose MOI 10 for subsequent studies, changing the growth media every 24 hpi (hours post-infection) to limit the exposure of MEFs to acidified media arising as a result of GAS replication. We assessed CFU and biomass accumulation of GAS on MEFs as well as on abiotic tissue culture polystyrene plates (referred to as plastic) over time using a modified crystal violet (CV) staining protocol to distinguish bacterial from mammalian cell biomass (234). We observed increasing biofilm biomass until 72 hpi (**Fig 11A**), which corresponded with equivalent increase in viable bacteria within the biofilm (**Fig 11B**) on both the surfaces. By 96 hpi and 120 hpi, biofilm biomass on MEFs decreased significantly, by approximately 50% and 90% respectively (**Fig 11A, B**), which was not significant on plastic. GAS biofilm formation on MEFs was not dependent on cell-type, because both human foreskin fibroblasts (HFFs) as well as human keratinocytes (HaCat cells) supported the growth of GAS biofilms, although biomass accumulated more rapidly on HaCaT cells, with peak biomass aggregating by 24 hpi (**Fig 11C, Appendix, supplementary fig 1**).

Figure 11: GAS strain JS95 forms biofilms on mammalian cells

Time course of biofilm development by GAS JS95 on tissue culture plastic and mouse embryonic fibroblasts (MEFs) as quantified by (A) crystal violet (CV) staining and (B) CFU enumeration of viable bacteria. (C) Biomass quantification by CV assay of GAS JS95 biofilms grown on MEFs, human foreskin fibroblasts (HFFs), and human keratinocytes (HaCaT cells). Control bars represent only media or only mammalian cells as per condition. Data show the mean $\pm$ SEM values of 3 biological replicates, with 4 technical replicates each. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****), "ns" denotes no significance).

3.3 GAS exhibits strain-specificity during biofilm formation

GAS displays strain-dependent phenotypic heterogeneity and virulence potential (7, 57). Therefore, we tested whether GAS strains of different serotypes also formed biofilms on MEFs. We observed that GAS strains JS95 (M14) (210), MGAS5005 (M1T1) (142) and JRS4 (M6) (233) could all form biofilms on MEFs, although the biomass of strain MGAS5005

(M1T1 serotype) peaked at 24 hpi and had lost significant biomass by 72 hpi (**Fig 12**). While this is the first study implicating strain JS95 in biofilm formation, the highly invasive strain MGAS5005 has been previously characterized for the role of GAS cysteine protease SpeB in biofilm dispersal in both *in vitro* and *in vivo* models (120, 128, 142) and strain JRS4 forms biofilms on pre-fixed epithelia (42) as well as abiotic and biotic surfaces (236, 237). It is noteworthy that at all time points, GAS strain JS95 has significantly higher biomass compared to the other two strains (Fig 12), indicating that JS95 can form robust biofilms on MEFs compared to other virulent GAS strains. Taken together, these data show that GAS can accumulate biomass and proliferate after inoculation onto live mammalian cells, thereby establishing an efficient model to study *in vitro* GAS biofilms on host cells.

Figure 12: GAS exhibits strain specificity during biofilm formation on MEFs

Biofilm biomass of GAS strains JS95, MGAS5005 and JRS4 on MEFs quantified by CV assay over time. Data show the mean $\pm$ SEM values of 3 biological replicates with four technical replicates each. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****)).

3.4 GAS aggregates on MEFs exhibit biofilm characteristics

3.4.1 GAS biofilm 3D ultrastructure is more complex on MEFs than on polystyrene

Classical parameters to certify a biofilm phenotype are a complex three-dimensional (3D) ultrastructure distinct from planktonic bacteria including- in this case- adherence to abiotic or biotic surfaces, presence of a matrix-like encasing and enhanced tolerance to antibiotics compared to planktonic GAS.

To examine whether GAS growing on MEFs had a complex 3D architecture indicative of biofilms, we performed confocal laser scanning microscopy (CLSM) of GAS biofilms over the course of biomass accumulation. Using an antibody specific to the group A carbohydrate of GAS to visualize the bacteria (green) together with phalloidin to stain F actin in the underlying MEFs (blue), we recorded and quantified the temporal development of GAS biofilm on MEFs by CLSM and again observed peak biomass at 72 h (**Fig 13A, B, C**), consistent with CV and CFU data (**Fig 11A, B**). At 72 h, GAS was preferentially growing on top of MEFs and had a 3D architecture (**Fig 13A,C**) indicative of mature biofilms (104). The reduction of MEF-associated GAS biomass at 96 and 120 h corresponded to large areas of clearing, presumably where MEFs and MEF-associated biofilms had detached, exposing the underlying polystyrene surface and suggesting a preferential adherence of GAS to mammalian cells compared to polystyrene. On the other hand, GAS biofilm biomass and 3D architecture observed on tissue culture plastic (**Fig 13A, C**) is thinner and less complex, with a uniform density and spread, and persists until 120 h without any indication of drastic dispersal or disruption, probably due to the absence of the host cells that led to detachment in MEF-associated biofilms (**Fig13A**). Bio-volume quantification of fluorescence intensity from the CLSM z stack images of GAS biofilms on MEFs and plastic substantiated the previous data that GAS accumulates significantly higher bio-volume (obtained by using Imaris based image quantification) on MEFs compared to on plastic (**Fig 13B**). Uninfected MEFs, on the other hand, did not show any detachment throughout the time course of the experiment. However, a rapid increase in the cell confluency was observed (**Appendix, supplementary fig 2**).

C)

Figure 13: GAS biofilm ultrastructure is more complex on MEFs than on polystyrene

(A) Time course of GAS JS95 biofilm development on tissue culture plastic or MEFs by CLSM. Bacteria were immunostained with anti-GAS antibody (stains group A carbohydrate of GAS; green) and MEFs with phalloidin AF 350 (stains F actin of MEFs; blue). CLSM orthogonal images of Z stacks of biofilms were acquired at x63 magnification and scale bars represent 20 μm. (B) Bio-volume quantification of the same CLSM orthogonal images of Z stacks of biofilms comparing GAS biofilms grown on plastic vs MEFs by Imaris software. (C) 3D representation of GAS biofilms grown on plastic vs MEFs at peak time point of 72 hpi from CLSM orthogonal images of Z stacks of biofilms acquired at x63 magnification. Biofilms on MEFs were observed to be 3x times thicker than biofilms on plastic (based on scale). Data show the mean $\pm$ SEM values of 5 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) ; $p < 0.0001$ (****)).

3.4.2 Proteinase K and DNase I disrupt GAS biofilms

Having established that JS95 is capable of forming a complex 3D architecture, we next wanted to determine whether the biofilm was encased in a protective matrix. A direct approach to characterize the biofilm matrix is to assess enzymatic disruption of the biofilm as matrices are often rich in biopolymers of sugars, proteins and/or extracellular DNA (eDNA) (112, 114). Mature 72 h biofilms formed on MEFs and polystyrene were treated with Proteinase K, DNase I and sodium metaperiodate (commonly employed as a carbohydrate oxidizing agent) (114) for 2 h at 37°C and subsequently processed for either CLSM microscopy (**Fig 14A, B**) or CV assay (**Fig 14C**). These analyses revealed that the biofilm matrix involved a protein component as treatment resulted in almost complete disruption of the biofilm growing on plastic upon treatment with Proteinase K (**Fig 14A**). However, control experiments where we treated MEFs alone with Proteinase K also showed complete sloughing off of cells (**Appendix, supplementary fig 3**), and so we cannot interpret whether proteins are a component of the biofilm matrix on MEFs (**Fig 14B**). Treatment of planktonic GAS with Proteinase K was not included in the set of controls as the purpose was to record the disruption of biofilm matrix aggregated on a surface and planktonic GAS do not have a matrix to begin with. Partial disruption of plastic-grown biofilms occurred after treatment with DNase I, suggesting that eDNA is a component of the matrix, and may be a more critical component for the biofilms grown on plastic (**Fig 14A**). Metaperiodate treatment did not cause any apparent disruption (**Fig 14 A, B**). However, since we lack a positive control, this data was deemed inconclusive and therefore we adopted an alternative approach to determine whether the JS95 biofilm matrix contains any polysaccharides.

Figure 14: Proteinase K and DNaseI disrupt GAS biofilms

GAS JS95 biofilm (72 h old) growing on plastic (**A**) and MEFs (**B**) either untreated (buffer control), or treated for 2 h with Proteinase K, DNase I or sodium metaperiodate at 37°C. Bacteria are stained with anti-GAS antibody (green) and MEFs with phalloidin AF 350 (blue). CLSM images are acquired at x63 magnification and scale bars represent 20 μ m. (**C**) Quantification of 72 h GAS biofilm biomass by crystal violet (CV) assay, after enzymatic treatment under same conditions, on plastic (black bars) or on MEFs (grey bars). Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) $p < 0.0001$ (****)).

3.4.3 GAS biofilm display signs of a “matrix”

Numerous reports have relied on techniques like scanning electron microscopy (SEM) and staining for extrapolymeric substances (EPS) followed by confocal laser scanning microscopy (CLSM) to study matrix components (42, 63, 108, 112, 113). GAS biofilms on MEFs were fixed and processed for SEM analysis. The images show that MEF-associated biofilms have a thick and multi-layered aggregation of *Streptococcal* chains on MEFs. Moreover, certain regions display bulbous aggregates that appear to be encased in some kind of a “cementing material” that might be a matrix (**Fig 15A**). Additional high magnification SEM images of biofilms on MEFs demonstrate the possible presence of a matrix-like material (**Fig 15B**). Further analysis of matrix components needs to be done to make substantial conclusions.

Another common technique to visualize matrix components, especially exopolysaccharides is by staining the biofilms with lectins that are proteins or glycoproteins capable of binding reversibly and specifically to carbohydrates without altering their structures (70). Common fluorescently labelled lectins usually employed for streptococcal biofilm matrix analysis include concanavalin A (Con A) and wheat germ agglutinin (WGA) that stain the exopolysaccharides of the matrix in addition to the GlcNAc and sialic acid residues found in the GAS capsule (33, 73), as was observed when planktonic GAS was stained with WGA (**Appendix, supplementary fig 4**). WGA co-staining with Syto9 (DNA dye that stains all bacterial cells) was performed on unfixed GAS biofilms grown on MEFs (stained with F-actin- binding stain phalloidin) to visualize the matrix. A very heterogeneous staining pattern was observed for different samples, but the extensive WGA positive staining at certain regions indicated the presence of exopolysaccharides in the biofilms (**Fig 15C**). Biofilms on plastic also displayed a similarly heterogeneous but less robust pattern of WGA staining (**Fig 15D**).

As there is a complete lacuna regarding any information on GAS JS95 biofilms from previous studies, it is imperative that additional genetic and biochemical analysis be performed on these biofilms to understand their genetic and biochemical makeup. Towards this investigation, our lab is already well into screening a transposon mutant library in GAS JS95 background, to look for genetic determinants of biofilm formation on host cells compared to plastic-grown biofilms. Once some genetic markers are earmarked, PNA FISH probes can be synthesized against different regions of the biofilm for further analysis.

A) SEM of GAS JS95 biofilms on MEFs

B) SEM of GAS JS95 biofilms on MEFs (x5000)

C)

D)

Figure 15: GAS matrix analysis using SEM and CLSM

A) SEM images of GAS biofilms grown on MEFs at magnifications of x2500, x5000 and x1000. White arrows indicate thick “cementing material” like substance encasing GAS chains (red arrows) indicative of matrix. **(B)** Additional SEM images of GAS biofilms grown on MEFs at magnification of x5000 that highlight the “matrix” like material. **(C, D)** 3D split view of GAS biofilms on either MEFs or plastic stained with WGA, Syto 9 and phalloidin AF 350 to visualize the exopolysaccharides, live bacteria and F actin of MEFs respectively. Images were acquired at x63 magnification using CLSM and processed using ZEN software.

3.4.4 GAS biofilm is more tolerant to antibiotic treatment compared to planktonic cells

We next tested whether GAS biofilms growing on MEFs acquired any enhanced tolerance to antibiotics, which is a hallmark of biofilms (107, 116). Ampicillin and other penicillin derivatives have been the first choice of treatment for GAS infections, as most strains remain susceptible to these antibiotics (238). The minimum inhibitory concentration (MIC) of ampicillin for planktonic GAS JS95 is 0.5 µg/ml (data not shown). To test whether GAS biofilms on MEFs were more tolerant to ampicillin than planktonic cells in their early log phase, we exposed pre-formed GAS biofilms growing on MEFs to increasing concentrations of ampicillin, and counted the surviving CFU. Compared to planktonic cells, GAS biofilms were two to ten-fold more tolerant to ampicillin (**Fig 16**). This is a key biofilm-phenotype that ascertains the formation of biofilms, and distinguishes them from mere surface aggregation.

Figure 16: GAS biofilm is more tolerant to antibiotic treatment compared to planktonic cells

Enumeration of viable GAS JS95 in planktonic growth vs pre-formed 24 h, 48 h and 72 h old biofilms on MEFs upon treatment with increasing concentrations of ampicillin for 24 h. Viable bacteria were quantified by CFU enumeration after antibiotic treatment. Dotted line represents the initial inoculum for all samples (5×10^5 CFU). Data show the mean $\pm$ SEM values of 3 biological replicates with 8 technical replicates each.

3.4.5. GAS biofilms on mammalian cells display dose dependent dispersal/disruption

The MOI (multiplicity of infection) is the ratio of the number of bacteria that are infecting a given number of host cells. The MOI that we employed in all our experiments was 10, meaning, we infected 5×10^4 MEFs/well with 5×10^5 bacterial cells. This bacterial load is sufficient to form a robust biofilm on MEFs, but we questioned whether it would affect the viability of the MEFs, leading to their detachment from the surface of the tissue culture well, and in the process, causing the sloughing off of the biofilm itself. In order to test this, we next carried out a comparison of the biofilm biomass formed by employing MOIs of 10, 1 and 0.1 on MEFs by CV assay (**Fig 17B**) followed by microscopy (**Fig 17A**) at late time points from 72-120 hpi. CV assay results indicate that whereas MOI 10 and 1 cause a significant drop in the biofilm biomass from 72-96 hpi, reducing the MOI to 0.1 attenuates this drop, and the biofilm biomass is maintained through 120 hpi and possibly beyond that (not tested). Microscopic images taken at 60x magnification indicate a similar trend, with biofilms from MOI 0.1 persisting until 120 hpi. Images were also taken (**Fig 18A**) and analysed by manual counting (**Fig 18B**) for the number of adherent MEFs left during the course of the biofilm development at later time points between 72-120 hpi. Whereas MOI 10 causes MEFs to detach as early as 96 hpi, MOI 0.1 delays this detachment, and consequently, sloughing off of the biofilm on top of them. In the absence of GAS, uninfected MEFs remain attached and appear healthy throughout the time course of the experiment (**Appendix, supplementary Fig 2**).

These observations suggest that detachment is a consequence of prolonged exposure to bacterial cells and is dependent on the MOI and that there is a critical biomass that must be reached for detachment to occur. Alternatively, MEF detachment could be an effective host response to jettison adhered bacteria to clear infected cells. Additionally, this could also be a bacterial-driven response aiding bacterial dispersal and dissemination during infection. Unfortunately, the specific mechanism is rather difficult to deduce currently given the technical challenges of this experiment. The notable fact remains that this *in vitro* model is sustainable for a period of 72 h, as against some previous reports where infection with planktonic GAS resulted in cell death and detachment of mammalian cells as early as 4 hpi, requiring the biofilms to be grown on pre-fixed mammalian cells (2). In order to test whether this detachment/disruption is a direct consequence of cell death, we carried out cell viability assays that are discussed in the following chapter in detail.

Figure 17: GAS biofilms on mammalian cells display dose dependent dispersal/disruption

(A) Microscopic analysis of GAS JS95 biofilms growth kinetics on MEFs at MOIs 10, 1 and 0.1. Bacteria are stained with anti-GAS antibody (green) and MEFs with phalloidin AF 350 (blue). CLSM orthogonal images of Z stacks of biofilms are acquired at x63 magnification and scale bars represent 20 μ m. **(B)** Biomass quantification of late biofilms grown on MEFs at MOIs 10, 1, 0.1 by CV assay for 72, 96 and 120 hpi. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***) ; p < 0.0001 (****)).

Figure 18: Bacterial dose affects the adherence of MEFs that form the biofilm substrate

(A) CLSM images of uninfected or GAS infected MEFs at different MOIs after immunostaining with ConA AF 488 (green) and (B) subsequent enumeration of adherent MEFs at late time points using ImageJ software by picking 10 random fields of vision at x20 magnification. Scale bars represent 50µm. Data represents two experiments with two technical replicates; error bars represent SD values.

3.5 Conclusion:

Based on this extensive characterization of GAS JS95 biofilms, we conclude that GAS JS95 is able to form robust biofilms on different mammalian cell lines that exhibit hallmark biofilm traits like 3D aggregation, matrix formation, and enhanced tolerance to antibiotics as well as late stage dispersal/disruption. What is noteworthy is that despite differences in all these parameters between biofilms growing on MEFs versus plastic, they both serve as models to study biofilm formation, underscoring the heterogeneity of biofilms grown on various surfaces and different models as discussed in previous studies (65). However, it is highly imperative that relevant models are used to address the question at hand. For instance, a human pathogen like GAS would have to be studied in the context of the host and therefore, all further analysis hereafter were performed on MEF-associated biofilms, with plastic-grown biofilms serving as mere controls to highlight the differences between the two surfaces.

Based on these guidelines, the comprehensive characterization carried out in this study is deemed satisfactory for substantiating the biofilm nature of GAS aggregates growing on MEFs and plastic (42, 108, 112). Additional matrix analysis is required to further substantiate the biofilm phenotype; although the current data strongly implies that the GAS aggregates growing on both plastic as well as MEFs possess a biofilm phenotype. Moreover, these characterizations also serve to establish a robust *in vitro* biofilm model that enable subsequent interrogation of GAS factors and host responsive events that shape the cellular and molecular dynamics of GAS biofilm formation on host cells during the course of acute infection.

CHAPTER 4

Determination of cellular and molecular factors responsible for *in vitro* and *in vivo* GAS biofilm formation

4.0 Determination of cellular and molecular factors responsible for *in vitro* and *in vivo* GAS biofilm formation

4.1 Overview and aim of study

Much is known about GAS virulence factors and pathogenic mechanisms that enhance its virulence potential and contribute to the severity of disease, evasion of the host defence system and establishment of a persistent niche in the host. Recently, researchers have begun to question and examine the clinical relevance of biofilm formation in GAS infections, and a few GAS virulence factors have been implicated in biofilm mediated pathogenesis. However, the results from different reports suggest that GAS strain-specificity may make identifying general biofilm-specific properties difficult. Hence, it is imperative to use relevant models using GAS strains pertinent to the type of infection at hand, as well as tissue specific cell lines or animal models to closely replicate infection conditions, for a better understanding of the cellular and molecular mechanisms of GAS biofilm formation.

My aim was to identify GAS virulence factors and host responses that participate in biofilm-specific events in a relevant host-pathogen *in vitro* model designed to mimic acute infection that I established and described in Chapter 3. Furthermore, I extended my *in vitro* findings to an *in vivo* mouse model of subcutaneous GAS NF to better understand the disease pathogenesis *in situ*. This is a first report wherein GAS JS95 strain was tested for *in vivo* microcolony formation, followed by a comprehensive characterization of these *in vivo* microcolonies to establish a link between pathogenesis and biofilm formation during acute GAS infections.

4.2 GAS virulence factors contribute to biofilm formation

The differential biomass and ultrastructure of GAS JS95 biofilms grown on MEFs compared to polystyrene (**Fig 11A, B** and **13A, B, C**) suggested that cues arising from the host-pathogen interaction contribute to robust GAS biofilm development on mammalian cells. To further probe GAS factors important for biofilm formation on MEFs, we examined a panel of GAS mutants (**Table 1, Chapter 2**) lacking the M protein, cysteine protease SpeB, hyaluronic acid capsule, and streptolysins SLO and SLS (82, 118, 132, 182, 206, 239) for biofilm accumulation on MEFs. This panel of mutants was chosen with the exclusive aim to test the role of previously established virulence genes in biofilm formation thereby linking pathogenesis and virulence with formation of biofilms in the host.

The GAS M protein is a cell wall anchored protein involved in anti-phagocytic functions (240) and its N-terminal hypervariable epitopes serve as the basis for GAS serotyping (13). Previous studies of GAS biofilms on abiotic surfaces demonstrated that a deletion in the *emm* gene in M1, M5, M6 and M24 serotypes caused a significant reduction in biofilm biomass (132). In contrast, an *emm14* deletion in the M14 serotype strain JS95 exhibited enhanced biomass on MEFs compared to the wild type (WT) strain (**Fig 19**). A *speB* mutant also displayed enhanced biomass compared to the WT (**Fig 19**), consistent with previous reports implicating SpeB in biofilm dispersal (127, 128). Similarly, a *hasA* mutant strain that lacks the hyaluronic capsule had significantly reduced biofilm biomass (**Fig 19**), consistent with earlier studies (22, 118).

Finally, we tested mutants lacking streptolysin O (SLO) and/or S (SLS) cytotoxins, which are important GAS virulence factors (182, 197, 200, 206, 241), for their role in biofilm formation on MEFs. The Δslo mutant was constructed by our collaborator and failed to produce detectable SLO activity as determined by Western blotting using anti-Streptolysin O antibody (177). The *sagI* gene is part of a nine-gene operon that codes for SLS (193). Deletion of *sagI* deletion was verified when *sagI* mutants failed to produce detectable SLS activity as determined by loss of β -hemolysis on blood agar plates (177).

The streptolysin null $\Delta slo sagI^-$ double mutant was severely attenuated for biofilm formation on MEFs (**Fig 19**) indicating that, in addition to their well-established cytotoxic effects, they are also involved in biofilm formation on host cells. Interestingly, single mutants Δslo or *sagI*⁻ lacking either SLO or SLS, respectively, did not differ in biofilm formation compared to WT

(Fig 19), indicating a functional redundancy between the two, in concordance with previous reports (177). Because of this marked defect of the streptolysin null mutant, we focused the remainder of our study on the role of SLO and SLS in biofilm formation.

Figure 19: GAS virulence factors contribute to biofilm formation

Biofilm biomass of GAS WT and isogenic mutants lacking GAS virulence factors on MEFs by CV assay. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance of mutants against the WT biomass was calculated by one-way Anova using the Dunnet's multiple comparison test. p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****); "ns" denotes no significance).

4.3 GAS streptolysin expression is necessary for biofilm formation on host cells.

To further investigate the roles of SLO and SLS in biofilm development, we examined the kinetics of the $\Delta slo sagI^-$ mutant biofilm growth on both polystyrene as well as MEFs over time. Whereas WT GAS biomass on MEFs accumulated over time, the $\Delta slo sagI^-$ mutant on MEFs was attenuated at every time point examined (**Fig 20A**). Strikingly, we observed that the attenuation of biomass accumulation by the SLO/SLS mutant occurred on mammalian cells only and not on the abiotic polystyrene. In contrast, on polystyrene, $\Delta slo sagI^-$ produced significantly more biofilm biomass at 48 and 72 hpi compared to WT, as well as compared to its biomass on MEFs at the same time points (**Fig 20A**). To rule out the possibility of a growth defect in the double mutant strain on MEFs, we monitored the number of viable bacteria in both the planktonic and biofilm fractions of the WT and the $\Delta slo sagI^-$ mutant infected MEFs. Compared to the biofilm fraction, we observed no difference in growth between WT and the $\Delta slo sagI^-$ mutant in the planktonic fraction (**Fig 20B**).

Next we examined the architecture and biomass of the biofilm formed on MEFs infected by WT, $\Delta slo sagI^-$ or $\Delta slo sagI^-$ harboring plasmids complementing either *slo* or *sagI*. CLSM revealed a densely packed GAS biofilm on top of MEFs for the WT and significantly less dense biofilm for $\Delta slo sagI^-$ (**Fig 20C, D, E**). The corresponding complemented mutants expressing either SLO or SLS restored the biofilm architecture and biomass similar to that of the WT, demonstrating that either SLO or SLS are sufficient to complement $\Delta slo sagI^-$ (**Fig 20C, D, E**).

Taken together, a streptolysin null strain was significantly and specifically attenuated for biofilm formation on MEFs, but not on an abiotic surface, indicating that streptolysins act through specific molecular pathways to modulate GAS biofilm growth on mammalian cells.

C)

centre view of z stack

WT

$\Delta slo sagI^{-}$ $\Delta slo sagI^{-}$ pLZ *slo* $\Delta slo sagI^{-}$ pLZ *sagl*

D)

bottom view of z stack

WT

$\Delta slo sagI^{-}$ $\Delta slo sagI^{-}$ pLZ *slo* $\Delta slo sagI^{-}$ pLZ *sagl*

Figure 20: GAS streptolysin expression is necessary for biofilm formation on host cells.

(A) Time course of biofilm biomass development of wild type (WT) and the $\Delta slo sagI^-$ double mutant strains on plastic or MEFs by CV assay. (B) Comparison of viable bacteria in WT and $\Delta slo sagI^-$ strains grown on MEFs for 7 hpi and 24 hpi in both the supernatant/planktonic fraction and adherent/biofilm fraction by CFU plating. (C, D) CLSM orthogonal images of z stacks of 72 h biofilms formed by GAS WT, $\Delta slo sagI^-$ double mutant, and $\Delta slo sagI^-$ harboring plasmids complementing either SLO or SLS at x63 magnification. Panel C shows the centre slice of the z stack, and panel D shows the bottom most z stack containing MEFs to ascertain that number of MEFs seeded originally is same. Scale bars represent 20 μm . Bacteria are stained with anti-GAS antibody (green) and MEFs with ConA AF 594 (red). (E) Comparison of biofilm biomass formed by GAS WT, $\Delta slo sagI^-$ double mutant, and complemented strains on MEFs by CV staining. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test (A, B, E). p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****); "ns" denotes no significance).

4.4 The streptolysin-null mutant biofilm on MEFs is significantly attenuated in biofilm traits compared to WT biofilm

To further analyse the 3D architecture of the WT and streptolysin null mutant, we compared the 3D view of the two biofilms (**Fig 21A**). WT was able to form a four-fold thicker biofilm on MEFs whereas the streptolysin null mutant was significantly attenuated in both thickness and bio-volume quantified from CLSM images (**Fig 21A, B**). Further, the ultrastructure of the two biofilms was compared by SEM analysis. The results indicate that the WT biofilms on MEFs at 24 hpi show regions of bulbous, multi-layered aggregations with the presence of a “matrix-like” cementing material encasing the streptococcal chains and aggregates (**Fig 21C**). On the other hand, the null mutant shows colonization of GAS on MEFs, but aggregation is limited and no evidence of any “cementing material” was observed (**Fig 21D**). Matrix analysis of the two biofilms by WGA staining also indicated that the double mutant displays lesser exopolysaccharides in its matrix as compared to the WT (**Fig 21E, F**).

A)

B)

C)

D)

E)

F)

Figure 21: Streptolysin-null mutant biofilm is significantly attenuated in biomass, complexity and exopolysaccharide content compared to WT biofilms on MEFs

(A) 3D view of 72 h WT and $\Delta slo sagI^-$ biofilms on MEFs stained with anti-GAS antibody (GAS, green) and ConA AF 594 (MEFs, red). Images were acquired at x60 magnification using CLSM and processed using ZEN software. WT biofilms were observed to be 4x times thicker than double mutant biofilms on MEFs based on the 3D scale. (B) Biovolume quantification of the same CLSM orthogonal images of Z stacks of WT and mutant biofilms by Imaris software. (C, D) Representative SEM images of WT and $\Delta slo sagI^-$ biofilms on MEFs acquired at x2500, x5000 and x10,000 magnifications. Black boxes enclose areas that display excessive aggregation with or without presence of a matrix-like material in the WT and $\Delta slo sagI^-$ biofilms respectively. (E, F) 3D split view of CLSM images showing WGA AF 555 (red) staining of WT and double mutant biofilms for exopolysaccharides and co-staining with anti-GAS antibody (GAS, green) and phalloidin AF 350 (MEFs, blue). Data show the mean $\pm$ SEM values of 5 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test (A, B, E). p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****); "ns" denotes no significance).

4.5 GAS induces host cell death during early stages of biofilm development

To determine the fate of MEFs during the initial stages of biofilm formation, we monitored LDH release into the culture supernatants as a measure of GAS-mediated cytotoxicity during the first 12 hpi. We observed progressive MEF-associated cytotoxicity starting at 4 hpi and more than 90% of the cells were killed by WT GAS by 10 hpi. In contrast, the $\Delta slo sagI^-$ mutant caused only 30% cytotoxicity at the same time point (**Fig 22A**). The limited host cell death caused by the $\Delta slo sagI^-$ double mutant at early time points correlated with the attenuation of biofilm formation in this strain over the same period, whereas the WT was already accumulating in biomass during these times (**Fig 22B**). These observations suggest that host cell death in the initial stages of infection might be important for subsequent biofilm formation.

To further understand the SLO/SLS-dependent mammalian cell death pathway during early biofilm formation, we probed GAS-infected MEFs for the apoptotic marker Caspase 3 and for necrosis-associated membrane damage using a membrane impermeable nuclear dye. In WT-infected MEFs, we observed apoptosis as early as 6 hpi, consistent with LDH release at 4 hpi (**Fig 22A**), and necrosis by 12 hpi (**Fig 22C**). In contrast, apoptosis in MEFs infected with the $\Delta slo sagI^-$ double mutant was delayed and necrosis did not occur during the time course examined. We confirmed the delayed apoptosis of MEFs after infection with the $\Delta slo sagI^-$ mutant by cell viability staining (**Appendix, supplementary fig 5**). We observed that the eventual apoptosis of MEFs caused by this strain at 24 hpi is due to the drop in pH as a result metabolic byproduct accumulation in the media by GAS (**Appendix, supplementary fig 6**). Briefly, acidified conditioned media (CM) generated from MEFs infected with *both* WT or the double mutant was observed to kill fresh MEFs after treatment for 8h or more in the *absence* of GAS.

Taken together, these results indicate that rapid GAS streptolysin-induced mammalian cell death is an early event during the course of biofilm formation with probable impact on early GAS adherence and biofilm proliferation thereafter.

A)

B)

C)

D)

Figure 22: GAS induces host cell death during early stages of biofilm development

(A) GAS-mediated cytotoxicity of MEFs quantified by release of LDH into the media, compared to LDH released by planktonic GAS or uninfected MEFs. Cytotoxicity is represented as a percentage of total LDH release from cells lysed with Triton X-100. Data show the mean $\pm$ SEM values of 3 biological replicates. (B) Biofilm biomass quantification of WT and $\Delta slo sagI^-$ double mutant at early (4, 8, 12 hpi) and late stages (24 h) of infection on MEFs by CV assay. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. p value < 0.05 was considered significant ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****)). (C) Epifluorescence images (x20) of MEFs either uninfected or infected with GAS WT and $\Delta slo sagI^-$ double mutant and subsequently stained with the Apoptosis & Necrosis Kit for Microscopy. MEFs with nuclei stained green represent apoptotic population by cleavage of Caspase3 substrate and MEFs with nuclei stained red represent necrotic population due to membrane permeability to the Red Dot2 dye. Scale bars represent 50 μ m. (D) Quantification of apoptotic MEFs (Caspase 3 positive) after infection with either WT or $\Delta slo sagI^-$ or no infection as control at an interval of every two hours in the initial 12 hpi. Data was collected from five discrete fields of view per time point for apoptotic positive MEF quantification.

4.6 Streptolysin-induced ER stress and apoptosis result in the release of a host-associated factor that is crucial for biofilm formation

Our collaborators have previously shown that GAS JS95 streptolysins induce ER stress in MEFs (177, 232). Another report also provides evidence for SLO-mediated ER stress that leads to apoptotic cell death in keratinocytes (228). Since either SLO or SLS expression could complement biofilm formation of the streptolysin-null mutant, we hypothesized that ER stress could be involved in host cell-associated biofilm formation. To test this, we supplemented $\Delta slo sagI^-$ grown on MEFs with filtered condition media (CM) from MEFs that were treated with thapsigargin (TG), an endoplasmic reticulum (ER) stress inducer (242). We observed a significant increase in MEF-associated biofilm formation for CM obtained from MEFs treated with TG for 36 or 48 h (**Fig 23A**). The CM obtained from untreated MEFs did not exert any effect on biofilm formation (**Fig 23G**). Prolonged unmitigated ER stress can trigger apoptosis (243). Indeed, after 24 h of TG treatment, apoptotic MEFs started to appear and their number increased considerably after 36 h of treatment (**Fig 25**). To examine if apoptosis of MEFs *per se* may be accountable for promoting MEFs-associated biofilm formation, we treated MEFs with the drug etoposide (ET) which inhibits DNA damage repair and consequently triggers apoptosis in an ER-stress independent manner (**Appendix, supplementary figure 7**) (244). Similar to the experiment described in Fig 22A, we supplemented $\Delta slo sagI^-$ grown on MEFs with filtered CM from MEFs treated with ET. In contrast to the CM of TG-treated MEFs, CM of ET-treated MEFs failed to promote biofilm formation (**Fig 23B**), even though after 36 h, a large population of MEFs was already apoptotic (**Fig 25**). Neither TG nor ET affected planktonic GAS growth (**Fig 24**). These results indicated that the host, during the ER stress-apoptosis cascade, secretes a biofilm-promoting factor that is able to complement the attenuated biofilm formation of the double mutant.

To test whether streptolysin-induced ER stress and apoptosis also elicited the release of a secreted biofilm-promoting factor, similar to TG treated MEFs, we examined the ability of CM from MEFs infected with WT GAS to restore biofilm formation of the attenuated $\Delta slo sagI^-$ mutant. Indeed, $\Delta slo sagI^-$ biofilm biomass accumulation was restored when complemented with CM from MEFs infected with WT GAS, but not CM from $\Delta slo sagI^-$ infected MEFs, corroborating the requirement of streptolysins in this process (**Fig 23C, E**). Similar to CM from TG-treated MEFs (**Fig 23A**), CM from WT-infected MEFs collected at

early times (3 hpi), prior to measurable cell death (**Fig 22A, C**) yielded only partial restoration of $\Delta slo sagI^-$ biofilms (**Fig 23C**). We only observed full complementation of the double mutant by CM from WT-infected MEFs at time points when substantial cell death was also observed, i.e 7 hpi (**Fig 23C**). Biofilm formation was not restored by CM from MEFs infected with WT at later time points (12 hpi and 24 hpi) (**Fig 23C**), due to significant acidification of the media by these time points as a result of metabolic byproduct accumulation (**Appendix, supplementary fig 6**), rendering it unfit for complementation.

Conditioned media from WT GAS grown on polystyrene alone in the absence of MEFs could partially complement the $\Delta slo sagI^-$ mutant biofilm defect (**Fig 23D**), likely due to the presence of the secreted streptolysins in the CM resulting in post-transfer cell death of MEFs. This partial complementation suggests that an additional secreted host-associated factor is essential for the full functional complementation of $\Delta slo sagI^-$ biofilm formation observed with WT-infected MEF CM or TG-treated MEF CM. CM collected from $\Delta slo sagI^-$ either on MEFs or on plastic failed to complement the double mutant (**Fig 23E, F**), indicating the direct role of GAS streptolysins in these events. Since asparagine was previously demonstrated to be released from MEFs by the upregulation of asparagine synthase (ASNS) as a result of streptolysin-induced UPR via the PERK-eIF2-ATF4-ASNS pathway, we tested whether the soluble biofilm-inducing factor was asparagine (61, 177). However, supplementation of the culture media with a range of asparagine concentrations failed to restore $\Delta slo sagI^-$ biofilms back to the WT levels (**Fig 23H**), showing that the presence of an additional host-associated secreted factor, distinct from asparagine, is required for GAS biofilm formation.

TG is a specific inhibitor of the sarcoplasmic reticulum calcium pump (SERCA), that causes calcium dysregulation leading to loss of calcium dependent chaperones like calnexin that triggers UPR (245). To further substantiate that the effects we observe are not solely attributed to calcium flux, we used two additional drugs to verify the role of UPR in GAS biofilm formation. DTT (dithiothreitol) is a strong reducing thiol known to induce ER stress by preventing di-sulphide bond formation in proteins and also doubles as a pro-apoptotic drug (77). CM generated by treating MEFs with DTT for even as short as 12 h was sufficient to restore the mutant biofilm phenotype to WT levels (**Appendix, supplementary fig 8A**). On the contrary, when the broad spectrum drug staurosporine (STS) that has been implicated in a variety of cellular responses (86, 246) was used in a similar manner, the effects were not restorative (**Appendix, supplementary fig 8B**). Taken together, there is strong evidence that

ER stress driven apoptosis is a key pathway that gets activated during GAS biofilm formation on host cells.

Figure 23: Streptolysin-induced ER stress and apoptosis result in the release of a host-associated factor that is crucial for biofilm formation

Conditioned media (CM) generated by treating uninfected MEFs with either ER-stress inducing drug thapsigargin (TG) (A), or non-ER stress mediated apoptosis-inducing drug etoposide (ET) (B), or no drug/untreated (G) and collected at the indicated times were used as infection medium for the $\Delta slo sagI^-$ double mutant biofilms on MEFs compared to biofilm biomass of WT GAS grown on MEFs in fresh media (FM). Fresh media alone (FM) or drug supplemented fresh media (FM+TG/ET) serve as negative controls. Similarly, CM generated by growing WT GAS or the $\Delta slo sagI^-$ double mutant on MEFs or on polystyrene plates (C, D, E, F) were collected at the indicated times and used as growth medium for the $\Delta slo sagI^-$ double mutant in comparison to WT GAS biofilms on MEFs in FM, with the same controls mentioned above. The resulting biofilms were quantified by CV assay. (H) Biofilm biomass of WT GAS and the $\Delta slo sagI^-$ double mutant on MEFs with increasing concentrations of asparagine quantified by CV assay. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by one-way Anova using the Dunnett's multiple comparison test (A-G) or two-way Anova using the uncorrected Fisher's LSD test (H). p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***) ; p < 0.0001 (****)).

Figure 24: GAS shows no growth defects in the presence of TG and ET

Enumeration of viable GAS CFU after treatment with drugs TG and ET for 24 h and the untreated and DMSO (buffer) controls. Data show the mean $\pm$ SEM values of 2 biological replicates. Statistical significance was calculated by multiple T tests using Sidak Bonferroni test. $p < 0.05$ was considered significant; “ns” denotes no significance.

Figure 25: TG and ET treatment induces apoptotic cell death in mammalian cells

Representative epifluorescence images (x20) of MEFs treated with thapsigargin (TG) or etoposide (ET) for indicated time points and subsequently stained with the Apoptosis & Necrosis Kit. Green indicates cleavage of Caspase3 substrate in apoptotic MEFs and the Red Dot2 nucleic acid dye stains the DNA of necrotic cells with compromised membranes red. Scale bars represent 50µm.

4.7 GAS requires streptolysins for *in vivo* microcolony formation

The *in vivo* roles of SLO and SLS have been explored previously, mostly in a well-established dermonecrotic skin model of mice (27, 36, 204) and murine models of subcutaneous soft tissue infections (205, 206, 241). However, these studies were restricted to phenotypic analyses, largely deriving conclusions based on gross readouts like changes in body weight, lesion formation, dissemination of bacteria to internal organs and mouse mortality, without any analysis of tissue-associated GAS aggregates during this infection. A very recent study describing biofilm formation by clinical strains of GAS from NSTI patients for the first time validates the presence of *in vivo* biofilms that were analysed based on parameters similar to the work presented in this thesis (33).

To assess the contribution of the streptolysins towards GAS microcolony/biofilm formation *in vivo*, we challenged inbred female BALB/c mice with subcutaneous injections of GAS strains in a well-established murine necrotic skin infection model described previously (79, 172). In order to characterize the *in vivo* GAS aggregates to be biofilm-like “microcolonies”, we followed the guidelines that were followed in previous studies (33, 63-65). GAS virulence and microcolony formation were assessed and ascertained by multiple methods, including macroscopic analysis of lesion development, histological analysis of lesion biopsy, CFU determination of viable bacteria from the lesion biopsy, immunostaining for GAS microcolonies in lesion biopsies and subsequent qualitative and quantitative analyses of fluorescence signals to estimate the aggregation and spread of GAS microcolonies in the fascia and a preliminary microscopic analysis of microcolony matrix and local host inflammatory responses within the fascia.

4.7.1 GAS requires streptolysins for lesion formation in a murine model of subcutaneous infection

An inoculum of 1×10^8 CFU of logarithmic phase WT GAS were injected subcutaneously in the shaved flank of the mice. The same inoculum of the mutant $\Delta slo sagI^-$ strain was injected in the contralateral flank of the same animal. The single mutants Δslo and $sagI^-$ were also injected to observe gross anatomical effects like lesion formation by GAS strains. We observed that at 24 hpi, WT GAS, as well as the single mutants Δslo and $sagI^-$ were able to form macroscopic lesions on mouse flanks at the site of injection, but the double mutant failed to induce any visible lesion (**Fig 26A**). We next tested whether pre-induction of ER stress at

the injection site would complement the attenuated streptolysin null strain, as we observed *in vitro*. We injected the ER stressor TG or the non-ER mediated apoptosis inducer ET at standardized doses in the mouse flanks 12 h prior to bacterial infection. The drug treatment was followed by GAS infection at the same region of the flank for an additional 12 h, and the injected areas were observed for lesion formation. Similar to the previous observation, whereas the WT infection resulted in the formation of a visibly severe lesion after 12 hpi, the double mutant lesion was not apparent, underscoring the role of these potent cytolysins in host damage and virulence (**Fig 26A**). Lesion formation by the single mutants Δslo and $sagI^-$ was comparable to the WT phenotype (**Fig 26A**) thereby substantiating previous observation of functional redundancy between SLO and SLS. Upon pre-induction of ER stress by TG, the double mutant was rescued from its attenuation in lesion formation which was not observed upon pre-treatment with ET (**Fig 26B**).

Figure 26: GAS requires streptolysins for lesion formation in a murine model of subcutaneous infection

(A) Gross anatomical changes and lesion formation in mouse flank tissue 12 hpi with GAS strains; left and right indicate mouse flanks. (B) Lesion formation observed after PBS or drug treatment for pre-induction of either ER stress or non-ER stress mediated apoptosis by thapsigargin (TG) or etoposide (ET) for 12 h respectively followed by infection with GAS WT or the double mutant at the same site as the drug treatment for an additional 12 h.

4.7.2 GAS streptolysins enhance *in vivo* bacterial survival and proliferation

An initial inoculum of 1×10^8 CFU of GAS (WT or double mutant) was injected subcutaneously into contralateral murine flanks 12 h after injection with either PBS or drugs. Viable bacteria were isolated from the site of infection 12 hpi and quantified to assess the role of streptolysins in *in vivo* GAS survival and proliferation. The double mutant was significantly attenuated in CFU compared to the WT, which was restored to WT levels upon pre-treatment with TG, but not when pre-treated with ET (Fig 27). These results indicate that streptolysins are essential to *in vivo* survival in the host and provide an essential link between virulence and microcolony formation in the host.

Figure 27: GAS streptolysins enhance *in vivo* bacterial survival and proliferation

Enumeration of viable bacteria retrieved from mouse tissue after 12 h pre-treatment of site of infection with ER stressor (TG), or apoptosis inducer (ET), followed by infection with either WT or the double mutant GAS strain for 12 hpi. CFU values were normalized to biopsy weights. Dotted line represents initial inoculum of infection (1×10^8 CFU). Data show the mean $\pm$ SEM values of 10 biological replicates. Statistical significance was calculated by two-way Anova using Sidak's multiple comparisons test. p value < 0.05 was considered significant ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****)).

4.7.3 GAS requires streptolysins for *in vivo* microcolony formation

Histopathological analysis of hematoxylin and eosin (H&E) stained sections from biopsies of mouse flanks infected with GAS strains showed aggregation of WT GAS clusters within regions of damaged dermal and fascial tissue, along with extensive PMN infiltration (**Fig 28A**). By contrast, the sections from tissues infected with the $\Delta slo sagI^-$ mutant showed significantly less tissue damage, with almost no sign of inflammation and with GAS aggregation in only discrete regions of the biopsy (**Fig 28A**). Immunostaining of tissue sections from across the lateral plane of the biopsy corroborated the histological observations, wherein intense clusters of WT GAS microcolonies appeared to be growing within damaged fascia whereas the $\Delta slo sagI^-$ mutant was severely attenuated in microcolony aggregation, with most tissue regions showing almost no signs of GAS aggregation or tissue damage (**Fig 28B**).

A)

WT infection

4X

10X

100X

Δ *slo sagI*- infection

4X

10X

100X

Figure 3 displays histological analysis of skin layers in PBS, WT, and $\Delta slo sagl^-$ mice. The figure is organized into three vertical panels corresponding to the three mouse genotypes. Each panel shows a cross-section of skin with four distinct layers labeled on the right: epidermis, dermis, fascia, and muscle. The staining highlights nuclei (blue), collagen (red), and elastin (green). Scale bars at the bottom of each panel indicate 50 μm .

Figure 28: GAS requires streptolysins for *in vivo* microcolony formation

(A) H&E stained sections of WT or $\Delta slo sagI^-$ infected mouse flank tissue at x4, x10 and x100 magnification under light microscope. WT infected tissue shows dense GAS clusters (black arrows) in damaged fascia with enhanced PMN infiltration as compared to $\Delta slo sagI^-$ infected tissue where tissue damage, GAS aggregation and PMN infiltration are attenuated.

(B) Distribution of GAS microcolonies across the transverse plane of the infected tissue biopsy represented by tiled x20 CLSM images of tissue sections immunostained by anti-GAS antibody (green), phalloidin AF 594 (red) and DAPI (blue).

4.7.4 Streptolysin- induced ER stress drives *in vivo* GAS microcolony formation

Since pre-treatment of host cells with TG, but not with ET, restored the ability of $\Delta slo sagI^-$ to form biofilms on host cells (**Fig 23A, B**), we tested whether TG also rescued the attenuated microcolony formation of the double mutant in *in vivo*. We subcutaneously injected PBS (as untreated control), TG (to induce ER stress-mediated apoptosis) or ET (to induce non-ER stress-mediated apoptosis) at the infection site for 12 h followed by GAS infection as described earlier. To determine the drug dosage and to ascertain that the two drugs did indeed cause *in vivo* ER stress or apoptosis, respectively, within 12 hpi, we immunostained sections of only drug treated tissue biopsies with CHOP antibody (an ER stress marker) or performed the TUNEL assay to demarcate apoptotic cells (**Fig 29A**). Streptolysin induced ER stress was similarly monitored by staining tissue sections injected with GAS strains either with PBS or drug pretreatment (**Fig 29B**). Pre-treatment of mice with TG restored the ability of $\Delta slo sagI^-$ to form microcolonies. These microcolonies spread extensively, from the fascia into the deep muscle region, which was not the case for ET pretreated mutant microcolonies (**Fig 29C**). There also seems to be an additive effect of TG on the WT infection, as observed by the excessive CHOP positive staining of the same (**Fig 29B**), causing WT colonies to disseminate deeper from dermis towards fascia (**Fig 29C**). Taken together, the restoration of mutant microcolony aggregation by pre-induction of ER stress underscores the importance of the role of streptolysins in these processes.

Figure 29: Streptolysin induced ER stress drives *in vivo* GAS microcolony formation

(A) x20 CLSM images of tissue sections after treatment with PBS, TG or ET for 12 h and immunostaining with anti-CHOP antibody (red, for ER stress positive cells), TUNEL staining (green, for apoptosis positive cells) or DAPI (blue, all nuclei). (B) Tiled x20 CLSM images of tissue sections after pre-treatment with PBS, TG or ET, followed by GAS infection in mouse flank tissue and CHOP/TUNEL co-immunostaining. (C) Distribution of GAS microcolonies after pretreatment with PBS or drugs, across the transverse plane of the tissue biopsy represented by tiled x20 CLSM images of tissue sections immunostained by anti-GAS antibody (green), phalloidin AF 594 (red) and DAPI (blue). Scale bars represent 50 μ m.

4.7.5 ER stress promotes the spread and dissemination of *in vivo* GAS microcolonies

The double mutant, although originally observed to form no microcolonies in the initial biopsy sections analyzed (**Fig 28A, B, 29C**), displayed very densely packed, discrete microcolonies when additional sections of the biopsies were scrutinized (**Fig 30A, Appendix, supplementary fig 9**). However, these microcolonies were localized to a limited zone of the biopsy and became apparent only when additional serial sections of different zones of the biopsy were analyzed and compared by immunostaining and confocal microscopy (**Fig 30A, Appendix, supplementary fig 9**).

CFU quantification merely served to determine the number of viable bacteria colonizing the mouse flanks. For a more detailed examination of these biofilm-like microcolonies, CLSM based analysis was done by immunostaining GAS aggregates in multiple sections (represented in Fig 30A as sections 1-4) from across the lateral plane of the biopsies (**Fig 30A**). The images were then quantified for mean fluorescence intensity (MFI) values that represented the biomass of these microcolonies per section.

In order to quantify the degree of spread of the microcolonies beyond the site of bacterial infection, the MFI values of immunostained GAS microcolonies from 40 serial sections per biopsy (spaced at 30 μm) were quantified, and the trend of aggregate distribution laterally across the biopsy was plotted for each type of infection from 5 different mice (**Fig 30B-G**). From this figure, it is clear that WT microcolonies are spread throughout the breadth of the biopsy, and TG or ET administration does not significantly alter the microcolony distribution of WT microcolonies (**Fig 30 A, B, F, G**). On the other hand, the MFI distribution pattern observed for the double mutant is significantly altered, with MFI values spiking only towards the center region of the biopsy (**Fig 30 A, C**). This distribution pattern indicates that mutant microcolonies relegate themselves to a discrete region of the infected area, possibly at the original site of bacterial injection. Strikingly, upon pre-treatment with TG, these sequestered microcolonies begin to spread to other regions of the biopsy (**Fig 30 A, D, Appendix, supplementary fig 9**) which does not replicate upon treatment with ET (**Fig 30E**). Hence it can be concluded that the double mutant is attenuated not so much in microcolony *formation* as much as microcolony *spread*.

Additionally, to gauge the “size” of these microcolonies per biopsy, the cumulative sum of MFI from all 40 sections quantified per biopsy from each experimental condition (performed

on 5 mice) were plotted and compared (**Fig 30H**). These results indicate that the WT microcolonies are significantly greater in “size” (total MFI/biopsy) as compared to the mutant microcolonies, and upon TG pre-treatment, there is restoration of mutant microcolonies to WT levels.

* 20x images of fascia only

** Section 1-4 represent discrete regions of fascia along the lateral plane of a biopsy to compare microcolony aggregation across the breadth of the biopsy

Figure 30: ER stress promotes the spread and dissemination of *in vivo* GAS microcolonies

(A) x20 tiled CLSM images of tissue sections showing only the fascia from infected mouse biopsies immunostained with anti-GAS antibody (green), phalloidin AF 594 (red) and DAPI (blue) representing distribution of GAS microcolonies from discrete regions spanning the lateral plane of the biopsy, represented as sections 1-4. (B-G) Quantitative analysis of GAS spread within the biopsy represented by plotting the mean fluorescence intensity (MFI) from x20 CLSM images of immunostained serial sections. 40 serial sections (spaced 30 μ m apart) per biopsy spanning the lateral plane of the biopsies from 5 mice were analyzed and their MFI/section plotted. (H) Total mean fluorescence intensity (MFI) of GAS microcolonies per biopsy from x20 CLSM images of 40 immunostained serial sections as a measure of the total microcolony aggregation per biopsy. Data represents the total MFI/biopsy from 5 mice per condition. p value < 0.05 was considered significant (p < 0.05 (*); p < 0.01 (**); p < 0.001 (***); p < 0.0001 (****)).

4.7.6 *In vivo* GAS microcolonies are encased in bacterial exopolysaccharides

In order to substantiate the biofilm phenotype of *in vivo* microcolonies formed by both WT and mutant strains in the fascia, tissue sections of biopsies from infected mice were co-stained with exopolysaccharide specific lectin WGA AF 555 and GAS specific antibody and analyzed by CLSM imaging as done in previous studies (33). The WT microcolonies display a robust WGA positive staining that co-localizes with GAS positive staining. The double mutant is attenuated in both microcolony aggregation as well as exopolysaccharide specific staining (Fig 31).

Figure 31: GAS microcolonies are encased in bacterial exopolysaccharides *in vivo*

x20 tiled CLSM images of tissue sections from infected mouse biopsies immunostained with anti-GAS antibody (green), WGA AF 555 (red) and DAPI (blue) against GAS microcolonies, exopolysaccharides and all nuclei. Inset shows the split view of fascia colonized by GAS microcolonies. Scale bars represent 50 μm .

4.8 Conclusion:

In this study, I have characterized dense tissue-associated aggregates formed by GAS in the fascia of mice flanks to be biofilm-like “microcolonies” that stain positive for EPS specific marker WGA, hinting that these aggregates form a “matrix”. Histopathological analysis further supported the formation of dense GAS aggregates that were spread throughout the fascia. The physical dimensions of the microcolonies was analysed by staining serial sections of tissue biopsies and quantifying the fluorescence intensities of these microcolonies. From this data, it can be said that whereas the WT microcolony is spread across the biopsy, the mutant microcolonies are restricted to a discrete region that might be the original site of infection. Most importantly, I have demonstrated that these microcolonies are formed exclusively when GAS streptolysins induce ER stress in the tissue, culminating in host apoptotic cell death that lies in concordance to previous histopathology of NF in the host (80). We have also shown that ER stress allows GAS to survive and proliferate better in the host, while concurrently helping in the spread and dissemination of the microcolonies beyond the site of infection. These microcolonies also elicit an inflammatory response from the host and preliminary results indicate that there is extensive PMN infiltration to the site of GAS aggregation as compared to the PBS injected tissue (**Fig 28A, Appendix, supplementary fig10**). Additional analysis of this event may be done in the future, including examination of local cytokine levels and other inflammatory markers.

Taken together, these results establish a newly defined role of streptolysin-induced ER stress in GAS lesion formation, microcolony aggregation, distribution and spread during the course of soft tissue infection in the host. This establishes a strong link between pathogenesis and biofilm formation in acute GAS infections.

CHAPTER 5

Discussion and future perspectives

Discussion and future perspectives

Biofilm formation is a potent strategy employed by pathogenic bacteria to cause disease in the host (247). The contribution of biofilms to GAS pathogenesis and disease has only recently been appreciated, and largely as a counterpoint to GAS internalization as the predominant mechanism of long term persistence and carriage in disease states (7, 248). The majority of GAS biofilm determinants examined to date have been studied on abiotic substrates, which may fail to mimic the physiological microenvironment encountered by GAS during infection (42, 108, 124) or in tissue culture systems that model host-pathogen interaction driven biofilm formation (29, 131, 249). However, it is accepted that biofilm formation mechanisms identified during *in vitro* studies do not always have clinical relevance (39). Some studies have employed animal based models to decipher the role of biofilms (42, 83, 110, 120, 128), but have largely focused on chronic infections because it is challenging to replicate acute infections *in vivo* (39). Further, most studies published to date on GAS biofilms have focused on pharyngeal- and skin-associated GAS strains (106, 108, 119, 123), so very little is known about the contribution of biofilm formation to soft tissue infections like necrotizing fasciitis (NF) where the bacteria grow rapidly to form high-density bacterial communities between the severely damaged surrounding tissue (80, 250). A very recent study validated the presence of dense GAS tissue-associated biofilms from patients of necrotizing soft tissue infections (NSTI) (33). Clinical GAS strains were observed to form biofilm or non-biofilm aggregates that differed in ultrastructure and staining for extracellular polysaccharides. In another comprehensive study that aimed to delineate the global transcriptomic profile of GAS MGAS5005 strain during murine invasive soft tissue infection, histopathological analysis of debrided tissue from mice infected with MGAS5005 identified dense GAS aggregates nestled within regions of multifocal necrosis and acute inflammation (250). Although much was discussed about the various virulence factors upregulated during GAS soft tissue infection, the specific molecular determinants that contribute to the formation of these GAS tissue-associated aggregates was not addressed. In fact, to date, very few genetic analyses have been performed to examine the genes that may be involved in biofilm or microcolony formation in association with host cells (42, 52, 118). However, these studies reported contradictory results, possibly due to differences in the model of study, GAS strain, and/or stage of infection. Therefore, a general GAS biofilm-specific transcriptome profile has been difficult

to establish. It is therefore important that relevant models suitable to the type and stage of infection be developed to deepen our understanding of biofilm-mediated virulence mechanisms that cause persistent or acute GAS infections.

In the present study, I established a robust acute infection-associated GAS biofilm model using the necrotizing GAS strain JS95 on mouse embryonic fibroblasts (MEFs). I demonstrated that host-associated biofilms have distinct molecular requirements compared to biofilms grown on an abiotic surface. I also showed that GAS forms biofilms both *in vitro* and *in vivo*, via a process that requires rapid streptolysin-mediated ER-stress and apoptosis, leading to the release of a host factor from the host that promotes subsequent biomass accumulation. These results define a new role for GAS streptolysins in acute infection-associated biofilm formation.

Recent studies aimed at establishing GAS biofilms on mammalian cells were optimized for later stage biofilms, whereby GAS isolates from different sources (strains JRS4 (M6; rheumatic fever), GAS771 (M3; NF) and MGAS315 (M3; STSS)) were first allowed to grow on fixed keratinocytes, and then the pre-formed biofilms were transplanted to live keratinocytes (42). This allowed the authors to bypass early infection events during which the host cells were rapidly killed and detached from the substratum in the first 4 hpi, and instead study factors that are important when pre-formed biofilms encountered live host cells (42). In contrast to the high toxicity induced by MGAS315 on epithelial cells that caused them to detach in 4 hpi, as reported by Marks *et al*, we show that NF GAS strain JS95 grows robust biofilms on top of MEFs, and the MEFs stay adherent for at least 72 hpi, allowing us to study the initial interactions that are important for subsequent biofilm formation on the same substratum, akin to a physiological set up where GAS encounters healthy tissue that progressively turns necrotic. This model was not only applicable to JS95-MEF interactions, since it could be extended to other murine and human cell lines, as well as to other GAS strains.

Using this mammalian cell-based biofilm model, I showed that host-associated biofilm formation was abrogated in a mutant of WT JS95 lacking both SLO and SLS, whereas biofilm formation by this mutant was not affected on polystyrene. Single mutants lacking either SLO or SLS did not show any attenuation, indicating that the two cytolysins harbour functional redundancy. Streptolysins O and S are involved in a number of host cellular responses, including oncosis and apoptosis of a variety of cell types (182, 197, 200, 226, 228), although

a role of these streptolysins in biofilm formation has not been previously examined. We show here that SLO/SLS-mediated ER stress (as previously shown by our collaborators) or thapsigargin-mediated ER stress, both of which result in host apoptosis, promote GAS biofilm formation on MEFs. Killing MEFs by other means such as etoposide-induced apoptosis (in the absence of ER stress) did not support biofilm formation. And while eventually apoptosis in MEFs infected with the SLO/SLS double mutant was observed at 24 hpi, it was much delayed as compared to WT GAS infection. To support this possibility, I showed that the delayed cell death is due to the pH changes that occur as a consequence of accumulation of toxic metabolic byproducts. Regardless, delayed apoptosis was not sufficient to support biofilm formation in a strain lacking both streptolysins, suggesting that this cell death is not ER stress-mediated.

The data suggests two, non-mutually exclusive possibilities: 1) that ER stress-induced apoptotic cell death in the initial stages of GAS infection gives rise to dead cells that serve as better substrates for GAS adherence and subsequent biofilm proliferation, and 2) ER-stress mediated apoptotic cell death results in the release of a factor that promotes biofilm formation. In support of the latter hypothesis, biofilm formation was restored in the biofilm deficient $\Delta slo sagI^-$ strain with the addition of conditioned media (CM) from MEFs infected with WT GAS or treated with the ER-stressor thapsigargin in the absence of bacteria, indicating that the secreted biofilm-promoting factor is host-derived. CM from GAS grown on polystyrene surfaces could partially restore $\Delta slo sagI^-$ biofilm formation, presumably due to post-transfer cell death via SLO/SLS secreted into the CM. CM from MEFs infected with the streptolysin null strain also failed to complement biofilm formation in the absence of streptolysins, indicating that the host biofilm-promoting factor is not released in the absence of streptolysin-mediated MEF cell death. Moreover, $\Delta slo sagI^-$ biofilm formation was only complemented with supernatants collected from MEFs treated with the ER stress inducing drug thapsigargin (TG), but not etoposide (ET), a topoisomerase inhibitor that causes ER stress-independent apoptosis. Notably, in all cases, complementation was seen only with CM from cells exhibiting markers for apoptosis suggesting that the biofilm-promoting factor is released at the onset of ER stress-induced apoptosis. Additional supporting data where DTT (ER stressor and pro-apoptotic thiol) was used also showed a similar complementary effect like TG, thereby proving that these effects were not due to a mere dysregulation of calcium levels induced by TG, but rather a general phenomenon that also worked when DTT inhibited di-sulphide bond formation in proteins thereby causing UPR. Supplementation of

the media with calcium may be done as a future work to further substantiate the significance of induction of ER stress by TG via calcium dysregulation and its role in complementation of the mutant. Going forward, it will be important to further characterize the specific role of ER stress in biofilm formation, decoupled from the resulting apoptosis, either by delaying or blocking cell death by using specific UPR inhibitors and blockers (230) to fully appreciate the emerging significance of ER stress as a host defence strategy (230).

The *in vivo* roles of SLO and SLS have been largely examined in a mouse dermonecrotic skin model (27, 36, 204) and mouse models of subcutaneous soft tissue infections (205, 206, 241), where only gross histopathological analyses and mouse lethality were reported. We extend these reports to show that GAS forms dense tissue-associated aggregates or microcolonies spread across the fascia, and give rise to extensive inflammation and tissue damage with bacterial spread, all within the first 12 h of infection, in contrast to other *in vivo* models where the period of infection lasted anywhere between two days to a month, possibly due to differential GAS traits arising from other strains (110, 120, 127, 128). Extensive microscopic analysis of the *in situ* architecture of tissue-associated microcolonies, their localization and spread showed that all these were attenuated in the absence of streptolysins. Most importantly, we demonstrated that the bacterial microcolonies are formed exclusively when GAS streptolysins induce ER stress in the tissue, culminating in host apoptotic cell death which recapitulates previous histopathology of NF in the host (80). Furthermore, we have shown that ER stress also allows GAS to survive and proliferate better in the host, while concurrently helping in the spread and dissemination of the microcolonies beyond the site of infection. To our knowledge, this is the first report that implicates SLO and SLS with these events. A schematic of the proposed mechanism of GAS biofilm formation in the host has been described in **Figure 32**.

Virulence is classically defined as bacteria induced host-damage and can be substantiated with parameters like high bacterial burden and bacterial dissemination resulting in tissue and cellular damage, enhanced inflammatory response and macroscopic observations like lesion or wound formation (69). From my data, the streptolysin null mutant is attenuated in CFU counts, microcolony aggregation and spread and therefore has less biomass as compared to the WT microcolonies. The mutant also inflicts lesser tissue damage as compared to WT, based on the absence of lesion formation and histological analysis. Induction of ER stress and apoptosis is significantly higher for WT infected tissues, causing pronounced tissue damage

as seen by histology as well as CLSM imaging. Preliminary examination of WT infected tissue for inflammation depicted a massive recruitment of PMNs around the bacterial aggregates that indicate the induction of an immune response (**Appendix fig10**). Further analysis to measure local cytokine levels will be done as part of the future work and has been discussed in the Section 4.8 of Chapter 4. All these observations imply that lesser virulence leads to abrogated microcolonies, thereby linking pathogenesis to microcolony formation.

Work from our collaborator's lab showed that SLO and SLS from GAS strain JS95 growing under planktonic conditions also induces ER stress in MEFs, resulting in asparagine release from mammalian cells, which in turn promotes GAS growth (177). However, the biofilm-promoting factor that we have discovered in this study is distinct from asparagine because media supplemented with asparagine failed to restore $\Delta slo sagI^-$ biofilm formation. The identity of this factor is currently under investigation. A recent study of *Pseudomonas aeruginosa* aggregate formation on mammalian cells identified a factor in the supernatants of infected epithelial cells that could rescue aggregate formation in a Type III secretion mutant that otherwise did not form aggregates on polarized epithelial cells. Interestingly, they used the pore-forming toxin streptolysin O as a positive control for cytotoxicity in these assays (251), suggesting that the host-derived biofilm-promoting factor that we have identified may be a broad spectrum signal for pathogenic biofilm formation, making it a potentially attractive point of intervention for a variety of biofilm-associated diseases. To that end, the identification of the ER stress-mediated host secreted factor that augments biofilm formation is an important future direction of this project. This factor could take any number of forms, including a peptide, small molecule, protein, or even eDNA. Preliminary tests should be done to test whether this factor is heat labile, or degraded by addition of enzymes like proteinase K or DNase to initially characterize the biochemical nature of the factor. Mass spectrometric or metabolomics analysis of biologically active fractions might give a better insight to the identity of this factor.

In this study, we have shown that SLS and SLO are important factors for host-associated biofilm. However, residual biofilm is still formed even in the absence of these streptolysins. Going forward, it will be important to identify what additional GAS factors are involved in host-associated biofilms. Although transcriptomics analysis provides an overall picture of the differentially expressed genes, it fails to directly establish which regulated genes are important for bacterial survival in that particular environment. To address this caveat, we are

also pursuing a parallel non-targeted approach to delineate GAS factors with a direct role in biofilm formation on host cells by screening a Tn mutant library in the JS95 background that has been developed by our collaborators using a mariner-based transposon, osKaR, that enables Transposon-Site Hybridization (TraSH) in GAS (252). Mutant bacterial strains from the TraSH library will be analysed for their ability to mediate biofilm formation on cells. This analysis will allow for screening and rapid detection of genes functionally important for biofilm formation by GAS on MEFs on a genome-wide scale. Additionally, creation and screening a Tn mutant library in the $\Delta slo sagI^-$ background to identify additional biofilm-promoting factors that may otherwise be masked by the strong phenotype displayed by the streptolysins, and testing them in animal models to validate their *in vivo* roles, may identify additional infection-associated biofilm factors.

To complement the Tn library screen, future work should also include transcriptome profiling of bacterial and host genes in GAS biofilms grown on MEFs using both WT and double mutant strains by Illumina based RNA-seq in comparison to GAS biofilms on abiotic surfaces. It would also be interesting to observe the host-associated transcriptional profile upon infection of these two strains, to appreciate the precise roles of the streptolysins in inducing ER stress and downstream signalling that results in the release of the biofilm-promoting factor.

Our future aims serve to provide a comprehensive analysis of biofilm/microcolony formation mediated GAS pathogenesis in soft tissue infection. Comparison of the GAS and host expression profiles generated *in vitro* and *in vivo* will allow us to define biofilm-specific signatures that come into play during GAS infections. The characterization of the *in situ* spatial and temporal development of host-associated GAS biofilms will also shed light on events like microcolony development and dispersal and how it contributes to GAS dissemination and disease progression. Genes or pathways of interest from the Tn mutant library screen will unearth new targets on which to base future biofilm studies. On the other hand, understanding the host response to GAS biofilms can provide a holistic insight into the pathophysiology of GAS disease driven by varied host-pathogen interactions. Such insights could contribute to the development of better biomarkers, therapeutics and treatment strategies to combat this serious human pathogen.

Figure 32: Proposed model to demonstrate GAS biofilm formation in the host

Schematic representation of the proposed model to demonstrate the mechanism of GAS biofilm formation in the host. GAS secretes streptolysins that induce ER stress- mediated apoptosis in the host that is essential for biofilm formation. A host-specific biofilm-promoting factor is secreted during this cell death cascade that augments this event. These biofilms/microcolonies further disseminate to sterile tissue, thus assisting in disease progression during acute GAS infections like NF.

Appendix

Supplementary figure 1: GAS strain JS95 biofilms on different mammalian cell lines

Representative CLSM images of 3D orthogonal and 2D views of GAS biofilms growing on mouse embryonic fibroblasts (MEFs), human foreskin fibroblasts (HFFs) and human keratinocytes (HaCaTs) and uninfected cells acquired at x63 magnification. GAS is stained with anti-GAS antibody (green), MEFs with ConA AF 594 (red) and nuclei of MEFs with DAPI (blue). Scale bars represent 20 μm.

Supplementary figure 2: Time course analysis of uninfected MEFs by CLSM

Representative CLSM images of uninfected MEFs, originally seeded at a density of 50,000/well of 24 well tissue culture plates. MEFs were stained using ConA AF 488, and images were acquired at x20 magnification. Scale bars represent 50 μm.

Supplementary figure 3: Enzymatic treatment of uninfected MEFs

Uninfected MEFs were treated similar to GAS biofilms i.e either untreated (buffer control), or treated for 2 hrs with Proteinase K, or DNase I or Sodium metaperiodate at 37°C. MEFs were stained with ConA AF 488 (green). CLSM images are acquired at x20 magnification and scale bars represent 50μm.

Supplementary figure 4: WGA staining of planktonic GAS

Representative CLSM images of planktonic GAS stained with anti-GAS AF 488 (green) and WGA AF 555 (red) specific for Group A carbohydrate and the GlcNAc and sialic acid residues of the GAS capsule respectively. CLSM images are acquired at x63 magnification and scale bars represent 20μm.

Supplementary figure 5: GAS streptolysins induce early cell death in the host

Epifluorescence images (x20) of MEFs infected with either GAS WT or $\Delta s_{lo} sagI^-$ double mutant and stained with the LIVE/DEAD® Viability/Cytotoxicity Kit. Nuclei of all MEFs stain positive for Syto9 dye (green) whereas only dead MEFs stain positive for propidium iodide (PI) (red), with uninfected MEFs as controls. Scale bars represent 100 μm .

Supplementary figure 6: Media acidification leads to MEF cell death

(A) MEFs treated for 24 h with either fresh media, or CM (conditioned media) from uninfected MEFs or MEFs infected with the WT or double mutant for 12 h and stained with Syto 9 (green) or propidium iodide (PI; red) staining nuclei of total or only dead MEFs respectively by epifluorescence microscopy at x20 magnification. Scale bars represent 100 μ m. **(B)** Quantification of percentage of dead MEFs after treatment with 4 h, 7 h and 12 h CM from uninfected MEFs or MEFs infected with the WT or double mutant for 8 h, 12 h or 24 h. Data is represented by MEFs quantified from 20 fields of view per condition.

Supplementary figure 7: ER stress induction by drugs on MEFs

x20 CLSM images of MEFs untreated or treated for 12 h with TG and ET for induction of ER stress by staining for ER stress marker protein CHOP. MEFs are stained with anti-CHOP antibody (red, for ER stress positive cells) and DAPI (blue, all nuclei). Scale bares represent 50µm.

Supplementary figure 8: Complementation of double mutant biofilms using additional ER stressors

Conditioned media (CM) generated by treating uninfected MEFs with either ER-stress inducing drug dithiothreitol (DTT) (**A**), or broad spectrum drug staurosporine (STS) (**B**) collected at the indicated times were used as infection medium for the $\Delta slo sagI^-$ double mutant biofilms on MEFs compared to biofilm biomass of WT GAS grown on MEFs in fresh media (FM). Fresh media alone (FM) or drug supplemented fresh media (FM+drug) serve as negative controls. The resulting biofilms were quantified by CV assay. Data show the mean $\pm$ SEM values of 3 biological replicates. Statistical significance was calculated by one-way Anova using the Dunnet's multiple comparison test. $p < 0.05$ was considered significant. ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) ; $p < 0.0001$ (****)).

Supplementary figure 9: GAS streptolysins are essential for *in vivo* dissemination of GAS microcolonies

x20 tiled CLSM images of tissue sections from infected mouse biopsies immunostained by anti-GAS antibody (green), phalloidin AF 594 (red) and DAPI (blue). Sections 1-4 represent GAS microcolonies from randomly chosen discrete regions serially spanning the lateral plane of the biopsy. Double mutant microcolonies (white asterisks) exhibit attenuation in spread and distribution, which gets rescued upon pre-induction of ER stress by TG. Scale bars represent 50 μ m.

Supplementary figure 10: Inflammatory response triggered by GAS microcolony formation in host fascia

x20 CLSM images of the fascia region in tissue sections after injection with either PBS or WT for 12 h and immunostaining with anti-GAS antibody (GAS, green), phalloidin AF 594 (red, surrounding tissue) or DAPI (blue, all nuclei). White asterisks represent areas that show an increase in PMNs and other immune cells represented by sequestration of dense host nuclei (blue) at the site of GAS microcolony aggregation. Scale bars represent 50µm.

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