VACCINATION AND HOST-TARGETED INTERVENTIONS FOR *STAPHYLOCOCCUS AUREUS* EXOTOXIN-MEDIATED DISEASES

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Abstract

Staphylococcus aureus is a major human pathogen capable of infecting nearly every tissue and causing a wide range of disease including skin and soft tissue infections, pneumonia, osteomyelitis, endocarditis, and toxic shock syndrome. *S. aureus* produces an array of exotoxins including superantigens and cytolysins, which contribute directly to disease by causing inflammation, immune evasion, and tissue damage. Recent evidence also suggests that superantigens and cytolysins modulate host epithelial cell signaling at mucosal surfaces and contribute directly to disease progression. Antibiotics can eliminate *S. aureus,* but have no effect on exotoxins already present within tissues. In addition, antibiotic resistance continues to increase in strains of *S. aureus*. Therefore, I hypothesize that anti-staphylococcal therapies, which target exotoxins or the down-stream host responses to exotoxins, should be explored to treat or prevent *S. aureus* infections.

In this study, I investigated the mechanism of action of the superantigen, Toxic Shock Syndrome Toxin (TSST)-1, and the cytolysins, alpha-toxin and gamma-toxin, at the vaginal mucosal surface. Historically, TSST-1 was determined to promote disease (toxic shock syndrome) by widespread activation of CD4+ T-cells and antigen-presenting cells. However, recently, TSST-1 was determined to have direct proinflammatory effects on vaginal epithelial cells through an epidermal growth factor receptor-dependent pathway. Alpha-toxin's mechanism of action and synergy with TSST-1 at the vaginal mucosa was characterized using *in vitro, ex vivo,* and *in vivo* models. Furthermore, novel actions of gamma-toxin at the vaginal mucosa were identified and the mechanism of action characterized. From this work, I proposed a model of cell-signaling by TSST-1,

alpha-toxin, and gamma-toxin at the vaginal mucosa, which demonstrates a conserved proinflammatory and immune-modulating effect by *S. aureus* exotoxins.

Proof of concept was established for two novel therapies for prevention and treatment of *S. aureus* infections. The tyrosine kinase inhibitor, AG1478, which inhibits the epidermal growth factor receptor signaling pathway, inhibited cytokine production induced by TSST-1, alpha-toxin, and gamma-toxin at the vaginal mucosa and prevented lethal menstrual toxic shock syndrome in rabbits challenged with live *S. aureus*. In addition, a vaccine targeting multiple exotoxins of *S. aureus* was highly effective in preventing lethal intrapulmonary challenge with a wide range of clinical strains and for treatment of the lethal effects of exotoxins in rabbits.

In summary, these studies describe the mechanism of action of multiple *S. aureus* exotoxins and provide proof of concept for targeting exotoxins for treatment or prevention of toxin-mediated diseases. These studies demonstrate the importance of local proinflammatory effects during *S. aureus* infections, which contribute directly to the initiation of clinical disease. The data further highlight the importance of exotoxins during disease and add both insight and complexity to the field of *S. aureus* pathogenesis.

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List of Abbreviations

ADAM - A disintegrin and metalloproteinase

AG1478 - 4-(3-chloroanilino)-6,7-dimethoxyquinazoline

Agr - Accessory gene regulator

APCs - Antigen presenting cells

AREG - Amphiregulin

AUC - Area under the curve

C5aR and C5L2 - Complement component 5 receptor

CA - Community-acquired

CDC - Centers for Disease Control

CXCR and CCR - Chemokine receptor type

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

FAK - Focal adhesion points

GML - Glycerol monolaurate

HA - Hospital-associated

HB-EGF - Heparin binding- epidermal growth factor

Hla / *hla* - Alpha-toxin

Hlg / *hlg* - Gamma-toxin

HO - Hospital-onset

HVECs - Human vaginal epithelial cells

IL-1β - Interleukin-1 beta

IL-6 - Interleukin 6

IL-8 - Interleukin 8

INF-γ - Interferon gamma

IVIG - Intravenous immunoglobulin class G

LD_{100} - Lethal dose 100%

LD_{50} - Lethal dose 50%

MHC-II - Major histocompatibility complex class 2

MIC - Minimum inhibitory concentration

MIP-3α (CCL20) - Macrophage inflammatory protein-3α

MMP - Matrix metalloproteinase

MRSA - Methicillin-resistant *Staphyloccus aureus*

MSSA - Methicillin-sensitive *Staphyloccus aureus*

mTSS - Menstrual toxic shock syndrome

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

PFGE - Pulsed-field gel electrophoresis

PVL / *lukF/S* - Panton-Valentine leukocidin

PVM - Porcine vaginal mucosa

SCC*mec* - Staphylococcal chromosomal cassette

SEB - Staphylococcal enterotoxin B

SEC - Staphylococcal enterotoxin C

SSTIs - Skin and soft tissue infections

TCR - T-cell receptor

TGF-α - Transforming growth factor-α

TKIs - Tyrosine kinase inhibitors

TMP-SMX - Trimethoprin-sulfamethoxazole

TNF-α - Tumor necrosis factor alpha,

TSS - Toxic shock syndrome

TSST-1 / *tst* - Toxic shock syndrome toxin 1

VISA - Vancomycin intermediate *S. aureus*

Chapter I

Introduction

1.1 Background

1.1.1 History of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive pathogen, which is capable of infecting and colonizing nearly every human tissue and causing a wide range of infections including skin and soft tissue infections, pneumonia, necrotizing fasciitis, endocarditis, and toxic shock syndrome (TSS) [1]. *S. aureus* was first identified by Alexander Ogston in 1880 as the primary microorganism associated with abscesses [2]. Over 100 years later, *S. aureus* remains the primary cause of abscesses and skin and soft tissue infections [1]. The organism owes its success as a human pathogen to numerous virulence factors, acquisition of antibiotic resistance genes, and the ability to survive by asymptomatic colonization of up to 30% of the population [3,4].

Shortly after the introduction of methicillin in 1961, the first pan-β-lactam resistant strains of *S. aureus* were identified from penicillin-resistant strains [5]. Subsequently, between 1975 and 1991 the prevalence of methicillin-resistant *S. aureus* (MRSA) increased from 2.4% to 29% [6]. Currently, >60% of all strains of *S. aureus* are methicillin-resistant and in certain populations, such as children, MRSA accounts for >75% of *S. aureus* isolates [7,8]. Prior to 1990, MRSA was primarily considered a hospital-associated (HA) infection [9]. However, by 2005 approximately 90% of MRSA infections were community-acquired (CA), with an incidence rate of 316 cases per 100,000 people [9]. Methicillin-resistance is encoded on the staphylococcal chromosomal cassette (SCC*mec*)[10]. This is a mobile genetic element containing multiple antibiotic resistance genes and *mecA,* which encodes an alternate penicillin-binding protein

(PBP2a) that provides resistance to almost every β-lactam antibiotic [10]. Historically, HA-MRSA strains contained SCC*mec* type I, II, or III and were often resistant to erythromycin, clindamycin, and fluoroquinolones, while CA-MRSA contained SCC*mec* type IV and V and were sensitive to most non-β-lactams [7]. Recently, the distinction between CA- and HA-MRSA has blurred and it is estimated that 20-65% of nosocomial infections classify as CA-MRSA isolates [7,11]. The same is true for infections of community origin, which are commonly classified as HA-MRSA isolates [7,11]. Changes in the distribution of HA- and CA-MRSA have led to the re-classification of MRSA isolates to hospital-onset (HO) if the infection occurred during hospitalization, and healthcare-associated community-onset (HACO) if the infection occurred ≤4 days following discharge from hospitalization [12]. Emergence of methicillin-resistant *S. aureus* (MRSA) has further complicated treatment and caused increased mortality, longer hospitalization, and overall increased medical costs [13]. MRSA is not inherently more virulent than methicillin-sensitive *S. aureus,* but has limited the availability of effective treatments [13].

1.1.2 Epidemiology

The virulence of *S. aureus* is remarkable considering the organism commonly colonizes the population without causing disease. *S. aureus* is a major cause of both hospital and community acquired infections. *S. aureus* causes approximately 500,000 outpatient infections per year and 80,000 invasive infections per year, which resulted in nearly 12,000 deaths in 2011in the United States [12,14]. Nosocomial infections by *S.*

aureus occur at rate of approximately 13.8 per 1000 hospitalizations, and are commonly associated with surgical site infections, catheter line infections, ventilator-associated pneumonia and dialysis [15]. Community-acquired infections are predominatly skin and soft tissue infections, abscesses and wound infections with the most common complications being bacteremia and sepsis [7,12]. *S. aureus* is also a common cause of osteomyelitis in children and endocarditis in the elderly or intravenous drug users, which can require valve-replacement surgery [1]. Infection by *S. aureus* can also cause TSS, which differs from other staphylococcal diseases in that it is mediated by the production of exotoxins rather than bacterial invasion [16]. Mortality caused by *S. aureus* is highest in metastatic infections (bacteremia and endocarditis) and pneumonia, which ranges from 11% to 43% [1]. TSS is associated with a mortality rate of 4% to 5% [16].

1.1.3 Current Therapy

In hospitals, vancomycin is the recommended therapy for treatment of invasive infections caused, or suspected to be caused, by MRSA with clindamycin, tetracycline or trimethoprim-sulfamethoxazole (TMP-SMX) recommended for outpatient treatment of uncomplicated infections [17]. Due to the high prevalence of MRSA, empiric therapy for *S. aureus* is always targeted for MRSA until susceptibility testing or, PCR analysis for the presence of the *mecA* gene, is completed [17]. Alternative therapy for the treatment of MRSA includes linezolid, TMP-SMX, daptomycin, telavancin, ceftaroline, quinupristin-dalfopristin, tetracyclines, dalbavancin or oritavancin [17–19]. If the infection is known to be MSSA then nafcillin, oxacillin or cefazolin are recommended due to more rapid

bactericidal activity of these antibiotics [1]. In the treatment of MRSA pneumonia, linezolid is more efficacious than vancomycin at microbial eradication (56.5% vs. 47.4%) and results in increased patient survival (86.7% vs. 70%) [20]. Treatment also includes, when applicable, drainage and irrigation of infected tissue or abscesses, debridement of necrotic tissue, supportive care and removal of tampons during mTSS [1,17,21].

For the past 30 years, vancomycin has been the primary therapy for the treatment of invasive MRSA infections. Currently, vancomycin accounts for nearly all empiric therapy for the treatment of invasive *S. aureus* infections due to the increase in prevalence of MRSA, [22]. Success of vancomycin therapy is highly predicted by the vancomycin pharmacodynamic parameter of the area under the curve (AUC) divided by the minimum inhibitory concentration (MIC) [22]. Based on animal and human data, a targeted AUC/MIC ratio of >400 has been established as the target for optimal therapy and failure rates are most commonly correlated with vancomycin-intermediate *S. aureus* (VISA) of MIC ≥2 μg/ml [22–24]. While the incidence of vancomycin resistance (*vanA,* median MIC of 512 μg/ml) has remained extremely rare with only 9 isolates reported prior to 2011, there has been a dramatic rise in VISA [23]. Between 2004 and 2009, the percentage of MRSA and MSSA isolates with susceptibility to vancomycin (MIC < 2 μg/ml) has decreased from ~100% for MRSA and MSSA to 95.77% and 91.07%, respectively [23]. With an increasing prevalence of higher vancomycin MICs in *S. aureus,* there is a pressing need for alternative therapies.

1.2 Toxic Shock Syndrome (TSS)

1.2.1 Epidemiology

Development of TSS can occur from either sites of pre-existing asymptomatic colonization or *de novo* infection, and is clinically divided into the two major categories of menstrual (mTSS) and non-menstrual TSS [1]. mTSS occurs within several days after onset of menstruation and is associated with tampon use and oxygenation of the vaginal cavity [16]. Non-menstrual TSS is caused by a number of clinical manifestations of *S. aureus* infections including but not limited to wound or surgical site infections, abscesses, sinusitis, osteomyelitis or respiratory infections including post-influenza pneumonia [16].

In the last century, the incidence of TSS in the United States peaked in 1981 reaching a rate of approximately 14 cases per 100,000 people with 74-90% of all cases being mTSS [25,26]. The increase in total cases of TSS was a result of increased incidence of mTSS, which was associated with the market introduction of high absorbency tampons in 1978 that contained carboxymethylcellulose and polyester [25,27,28]. Prior to high absorbency tampons, the incidence of TSS and mTSS was 0.3 per 100,000 people and 1 per 100,000 women, respectively [25]. The incidence of TSS in males is <0.5 per 100,000 [28]. Following the removal of high absorbency tampons and polyacrylate from tampons over the next 14 years, the incidence of TSS and mTSS decreased to the pre-1980 rate of ~1 per 100,000 people [25].

Between 2000 and 2006 the rates of mTSS peaked in 2003 at 3.4 cases per 100,000 in all women with an average incidence rate of 0.69 cases per 100,000 in all women and 1.41 cases per 100,000 in women 13-24 years old [25,26,29]. The rate of non-menstrual

TSS remained constant and similar to historical data throughout the same period at approximately 0.32 per 100,000 people [29]. While the cause of an increase in mTSS cases is unknown there has been a shift in prevalence of circulating *S. aureus* strains. Between 2000 and 2005, the incidence of community-associated MRSA infections increased from ~10% to 30% [30]. In the same period, a shift in the distribution of PFGE clonal types was observed in strains of *S. aureus*. USA200 strains, which are commonly associated with mTSS and produce TSST-1, were commonly considered hospital-associated [29,31]. However, a community surveillance of nasal colonization by *S. aureus,* between 2001 to 2004, determined that USA200 strains accounted for approximately 20% of all strains [31]. The USA200 strains are a common colonizer of mucosal surfaces and are the predominant producer of TSST-1[31]. Additionally, USA400 strains, which represented the dominant CA-MRSA strains decreased in prevalence from 63% in 2000 to 11% in 2005, while USA300 strains increased in prevalence from 6% to 68% in the same time period [30]. USA300 strains can encode the Panton-Valentine leukotoxin and are generally more dermonecrotic than USA400 strains [31,32]. Furthermore, the USA200 and USA300 strains are typically more resistant to macrolides, fluoroquinolones, mupirocin and tetracycline [31,32].

1.2.2 Clinical Manifestations

Staphylococcal TSS is a systemic multisystem and multiorgan disease with a broad range of clinical manifestations that complicate diagnosis. Clinically, TSS is defined as a capillary leakage syndrome resulting in severe hypotension (systolic pressure of <90 mm of Hg), hypoalbuminemia and edema, often in an absence of bacteremia [33,34]. Hypotension is the primary clinical concern for TSS patients, which is mediated by immune dysregulation (including a cytokine storm). The severe hypotension combined with edema often leads to coagulation within the extremities [21]. Other symptoms of TSS, which are present and required by the Centers for Disease Control (CDC) for a TSS diagnosis include fever ≥38.9° C, large full body rash defined as diffuse macular erythroderma, and desquamation particularly on extremities 1-2 weeks post onset of rash [34]. Multisystem involvement of three or more additional organ systems is also required for diagnosis of TSS including: gastrointestinal, muscular (myalgia), mucous membranes, renal or hepatic [34]. Additional clinical pathologies may include hypoalbuminemia, decreased platelet count, or central nervous system problems after abolishment of fever and hypotension [34]. However, in practice there is no single diagnostic test that can identify a positive case of TSS, thus most clinical diagnoses of TSS are probable cases. In a 2000-2006 survey in the United States, a majority of probable cases (80%) diagnosed and treated as staphylococcal TSS did not meet all the requirements of confirmed cases [29]. The primary symptoms of hypotension, fever, rash and multisystem involvement were present in 93-98%, but desquamation was only present in 33% cases with all isolates positive for superantigens [29].

1.2.3 Morbidity and Mortality

The overall mortality rate for mTSS peaked in 1980 at 5.5% and has since declined to an estimated 1.8%, with women <24 years old being at the highest risk of ~4% case mortality [16,29]. Meanwhile, the case mortality rate for non-menstrual TSS has remained relatively constant at ~5% [16]. While mortality in TSS cases remains relatively low, long term morbidity has been observed in up to 90% of cases [35]. The long term, and often life-long, morbidities are a result of severe manifestations of the disease and are primarily a result of prolonged hypotension, fever, mucosal edema, cerebral edema, profound myalgia or arthralgia [35]. The most common long-term morbidities, which occur in almost every patient typically include chronic diarrhea and anorexia, nail and hair loss, menstrual irregularity and delayed venous capillary filling (poor oxygenation of extremities) which may take months to a year to revert to normal [35]. The more severe morbidities are associated with abnormal electroencephalography readings and damage to the heart, with 23% of mTSS patients experiencing cardiomyopathy [35]. Greater than 50% of patients report long term memory loss and impaired concentration or apraxia [36]. Recurrence of TSS or mTSS is also a major concern, which generally occurs within 3 months, and has been reported to be as high as 22%, but is exclusively associated with patients who did not receive appropriate anti-staphylococcal therapy [35].

1.2.4 Treatment of Toxic Shock Syndrome

Current treatment for *S. aureus* TSS and mTSS are similar. First choice antibiotics for treatment of TSS include vancomycin for MRSA and oxacillin or other β-lactams for MSSA, with clindamycin or linezolid included as additional therapy for both MRSA and MSSA to inhibit protein synthesis and suppress toxin production [21,37]. Alternative antibiotics (linezolid, daptomycin, aminoglycosides, tetracyclines or TMP-SMX) are used when the patient is intolerant to first line therapy or other complications arise [37]. Supportive therapy is initiated to combat physiological effects of the disease and includes fluid resuscitation with saline, vasopressors and general supportive care (i.e. sedation and antipyretics) [21]. Removal of the bacterial source is done if possible, either through tampon removal, drainage or cleaning of abscesses or debridement of necrotic tissue [21].

1.3 Alternative Therapies for *S. aureus*

1.3.1 Intravenous Immunoglobulin

Pooled intravenous immunoglobulin class G (IVIG) collected from healthy populations is used as adjunct therapy in treatment of serious Gram-positive sepsis and toxic shock [21]. Much of the evidence suggests a benefit for treatment of streptococcal (GAS) infections in reduction of mortality with limited evidence for treatment of staphylococcal infections [16,38,39]. Despite limited clinical trial experience in treatment of *S. aureus* toxic shock, IVIG is often included as adjunct therapy in severe cases [21,40,41]. The suspected mechanism of action of IVIG is to provide broad-spectrum anti-bacterial and anti-toxin neutralizing antibodies and an anti-inflammatory effect

through Fc-receptor-mediated mechanisms [16]. While the evidence for treatment of serious *S. aureus* infections with IVIG is limited, pooled IgM-enriched IVIG has been shown to be beneficial in treatment of neonatal *Staphylococcal* sepsis, reducing mortality by more than 50% when included as adjunct therapy [42]. Laboratory assessments have also demonstrated that IVIG reduces the hemolytic activity and coagulation potential of *S. aureus* by 10-fold and 2-fold respectively, and IVIG resulted in reduced mortality in murine infection models [43]. However, outside of neonates the evidence is minimal to support the use of IVIG in the treatment of *S. aureus* septic or toxic shock.

1.3.2 Vaccination

Several phase II and III clinical trials have been conducted for active and passive immunization against a variety of *S. aureus* infections for either treatment of an active infection or prevention in high risk populations [44]. To date, all strategies have involved targeting of bacterial surface proteins. There have been 3 recent prototype vaccines that progressed to phase-III trials, which focused on the strategy of promoting antibody opsonization, phagocytosis, preventing bacterial clumping and nutrient acquisition. The first vaccine, StaphVAX® (Nabi), immunized against capsule proteins (CP5 and CP8) for prevention of *S. aureus* bacteremia in 1804 haemodialysis patients [45]. The targeting of capsule proteins for active vaccination is a strategy successfully employed for *Streptococcus pneumoniae* vaccination [46]. Unfortunately, the StaphVAX® failed to show efficacy at 54 weeks, but during post-hoc analysis it showed minor efficacy at 40 weeks (57%) [45]. Based on these findings, Nabi™ conducted a second trial for

StaphVAX® with double the population and a shorter (6 month) efficacy end point. This clinical study also failed and remains unpublished [44]. The second vaccine, Veronate® (Inhibitex), was a passive immunization consisting of pooled human IgG, which targeted clumping factor A for prevention of sepsis in nearly 2000 low birth weight infants. Inhibitex also failed to show any reduction in infection rate or mortality [47]. The third and most recent clinical trial was conducted by Merck to test an active vaccine directed at IsdB (V710®), a surface iron-sequestering protein, in over 8000 patients scheduled for full median sternotomy [48]. Not only did V710® fail to show efficacy as similar rates of *S. aureus* infection between vaccinated and placebo groups occurred, but patients who received V710® were 5 times more likely to die from the infection [48]. While speculative, the failure and increased lethality of the V710® vaccine may be a result of bacterial clumping through IgG cross-linking, a known mechanism for virulence and survival, or by promoting *agr*-induced toxin production through iron starvation [49–51]. In addition to the trials mentioned above, a number of phase-II vaccination trials have been conducted, which all targeted surface proteins and failed to show efficacy in humans (Table 1.1). Currently, no FDA approved *S. aureus* vaccine exists and efforts targeting surface proteins, which showed efficacy in animal models, consistently failed to translate to humans. However, more recent attempts to vaccinate against *S. aureus* have begun to focus on vaccination against single exotoxins and combinations of exotoxins and surface proteins [52].

TABLE 1.1: Clinical Trials for *S. aureus* Vaccines

Product	Components	Phase	Population	Result
StaphVax [45]	CP5 and CP8 Conjugated to exoprotein A	III	Prevention of *S. aureus* bacteremia in haemodialysis patients (n=1804)	No efficacy at 54 weeks; post hoc efficacy at 40 weeks of 57%
Veronate [47]	Pooled human IgG to *S. aureus* ClfA and *S. epidermidis* SdrG	III	Prevent of staphylococcal sepsis in low birth weight infants (n=1983)	No efficacy
V710 [48]	IsdB	III	Prevention of *S. aureus* infection following cardiac surgery (n=8031)	Study stopped due to increase in *S. aureus* infection and mortality in vaccinated group
Altastaph [53]	Pooled human anti-CP5 and-CP8	II	Treatment of *S. aureus* bacteremia (n=40)	No difference in mortality, shorter hospital stay (9 vs. 14 days)
Aurograb (NCT00217841)	FAb against ABC transporter component GrfA	II	Not posted	No efficacy
Aurexis Tefibazumab[44]	Humanized MCAb to ClfA	II	Treatment of *S. aureus* infection (n=63)	No efficacy
STEBVax/NIAID (NCT00974935)	SEB Toxoid	I	Safety and immunogenicity (n=29)	Results not posted
Nabi (NCT01011335)	Alpha-toxin and Luks-PV Toxoids	I	Dose escalation safety and immunogenicity (n=179)	Results not posted

1.3.3 Glycerol Monolaurate

Glycerol monolaurate (GML) is a naturally derived and a generally recognized as safe (GRAS) compound used as a food and cosmetic emulsifier and approved by the FDA. GML was discovered to have anti-staphylococcal and anti-inflammatory activity [54,55]. Both growth of *S. aureus* as well as exotoxin production (alpha-toxin, exfoliative toxin A, and TSST-1) are inhibited by GML *in vitro* [54]. Additionally, GML also inhibits *S. aureus* exotoxin activity, including TSST-1 induced proliferation in mononuclear cells and cytokine production in epithelial cells as well as stabilizes eukaryotic membranes to reduce hemolytic activity of *S. aureus* alpha- and beta-toxins [56]. Intravaginal administration of GML also protects rabbits against lethal challenge by TSST-1 in a model for mTSS [56]. Clinical trials of GML in people found that when GML was included in tampons the prevalence of *S. aureus* was reduced [57]. There was also a reduction in exotoxin production and vaginal IL-8 levels in women colonized with *S. aureus* [57]. GML is safe for long term use and does not significantly alter native vaginal flora [58]. Thus, the inclusion of GML in tampons may be effective in reducing the incidence of mTSS.

1.4 Exotoxins of *S. aureus*

1.4.1 Toxic Shock Syndrome Toxin

TSST-1 is encoded by the *tst* gene, which is present in a mobile genetic element on the staphylococcal pathogenicity island-1 [33]. TSST-1 is encoded as a 234 amino-acid precursor with a 40 amino acid signal sequence [59]. The mature form of TSST-1 is a

22kD protein with an isoelectric point of 7.2 [59]. The structure of TSST-1 is comprised of two adjacent domains. Domain A is comprised of amino acids 1-17 and 90-194 and is formed from 5 β-sheets and a long central alpha-helix containing multiple residues responsible for TCR binding [60]. Domain B is comprised of amino acids 18-89 and forms a cleft structure comprised of 5 β-sheets which bind MHC-II [60]. The toxin contains many hydrophobic amino acids and is soluble in lipid membranes, but retains high water solubility [33]. The folded structure of the toxin is very stable, which is attributed to a lack of cysteine residues [33]. For example, TSST-1 retains biological activity following 1 h of boiling and is resistant to proteolysis by many proteinases [33].

S. aureus controls expression of TSST-1 via the SarA promoter under control of the quorum-sensing *agr* regulatory system [49]. Induction of SarA and subsequently TSST-1 occurs in late log-phase growth in the presence of a stressful environment and host factors including low oxygen, glucose, blood, and immune factors [61]. In addition, Sar proteins amplify *agr* quorum-sensing responses, which induces TSST-1 expression dependent on the bacterial burden [61]. The multiple regulatory mechanism of TSST-1 production causes TSST-1 to be produced during an active *S. aureus* infection or colonization.

The primary pathogenesis of TSST-1 results from non-specific crosslinking of T-cell receptors with MHC-II on antigen-presenting cells resulting in aberrant activation of T-cells and cytokine production [62]. However, the possibility of alternate binding sites in TSST-1 is likely, mediating additional lethal properties separate from superantigenicity. Studies of TSST-1 mutants and animal strains of *S. aureus* have suggested that the

mitogenic, pyrogenic and enhancement of endotoxic shock are separate mechanisms of action. This was suggested when TSST-1 from sheep mastitis isolates of *S. aureus* was found to be lethal in rabbits but did not induce T-cell proliferation [63]. Additionally, when human TSST-1 is mutated (Gln136Ala) it retained superantigenicity, but lost lethality in rabbits even at 20 times the lethal dose [64]. Combined, this suggests that TSST-1 possesses additional receptors and mechanisms of action beyond MHC-II and TCR interactions in animals.

1.4.2 Toxic Shock Syndrome Toxin Additional Activity

In addition to superantigenicity, TSST-1 has been characterized to have additional effects at the vaginal epithelium. The vaginal epithelial response to TSST-1 is thought to be receptor-mediated with a potent induction of IL-8, TNF-α, MIP-3α, and IL-1β by epithelial keratinocytes [65]. Production of proinflammatory cytokines *in vitro* from a human vaginal epithelium cell line (HVEC) was dependent on the activation of (a) disintegrin and metalloproteinase (ADAM-10/17), that subsequently caused release of epidermal growth factor receptor (EGFR) ligands [66,67]. Specifically, ADAM17 mediates the shedding of amphiregulin and the subsequent activation of EGFR [66,67]. Signaling through EGFR is involved in numerous cellular processes including NF-κB activation of the innate immune response [68]. During most infections, activation of the innate immune response would be beneficial. However, TSST-1 lethality in rabbits was found to be dependent on mucosal epithelial activation of NF-κB, and could be attenuated through local vaginal mucosal inhibition of NF-κB with curcumin [66]. The cytokine

response induced by TSST-1 in vaginal epithelial cells is mediated by the ADAM, EGFR, and NF-κB pathways and can be blocked by inhibition at multiple steps within the cascade [66,67]. Furthermore, TSST-1 is synergistic with alpha-toxin, which is known to utilize ADAM10 as a receptor in the stimulation of IL-8 [69,70]. While the vaginal epithelial receptor is not known, evidence suggests a specific receptor-mediated inflammatory response over a general stress response by epithelial cells [67].

1.4.3 Alpha-Toxin

Alpha-toxin of *S. aureus* is a pore-forming cytolysin, predominantly characterized for its hemolytic and dermonecrotic effects [33,71]. In addition to lysis of epithelial cells and erythrocytes, alpha-toxin also shows high activity against monocytes and endothelial cells. The likely role of alpha-toxin in pathogenesis is that of promoting bacterial invasion. The combined actions of alpha-toxin in disruption of epithelial cells and erythrocytes directly under the skin produce the hallmarks of sepsis including inflammation, coagulopathy, decreased tissue oxygenation as well as increasing iron availability and killing of immune cells [33,72,73]. In addition to promoting an environment favorable for bacterial growth, alpha-toxin causes destruction of the endothelium, the last physical barrier preventing sepsis and bacteremia [69]. The destructive effects of alpha-toxin are mediated by pore formation in the target cell membrane and were recently discovered to occur through receptor-specific disruption of cell-cell tight junctions [72].

The alpha-toxin gene, *hla,* is present as a single copy on the staphylococcal chromosome and is highly conserved across strains of *S. aureus* [73]. Alpha-toxin is secreted as water-soluble monomers predominantly comprised of beta-barrel sheets (65%) of approximately 33kD in size [33,74]. Once secreted, the monomers self-assemble to form hexameric or heptameric pores in the membrane of target cells [71,75]. Binding of monomers appears to be both through receptor-specific interactions or non-specific interactions with cell membrane cholesterol at higher concentrations [69,76,77]. Receptor-specific interactions of alpha-toxin monomers appear to be mediated by the ADAM10 receptor or β1-integrin [69,78]. Once the monomers bind to the target cell they insert and distribute through the lipid bilayer where they oligomerize to form a pore with minimal structural change within each subunit. The pore formed by alpha-toxin is 1-2 nm in diameter allowing for rapid efflux of Ca+, K+, and Na+ and other small molecules of less than ~1000 molecular weight. The efflux of cations causes disruption of cell-membrane charge and creates an osmotic imbalance within the cell, ultimately leading to cell lysis.

Alpha-toxin is associated with skin and soft tissue infections and necrotizing pneumonia commonly caused by USA300 and USA400 strains. In USA200 clinical *S. aureus* isolates associated with mucosal infections and mTSS, the prevalence of alpha-toxin production is only 20% [79]. Lack of alpha-toxin (*hla*-) production is associated with a CAG to TAG stop codon mutation at position 113 [80]. In strains with the alpha-toxin stop mutation, there is still minor production of alpha- toxin (<10-50 of wild type) due to read through of the stop codon [80]. In clinical isolates, truncated alpha-toxin is

also produced in *hla*- strains consisting of 40%, 51%, 55%, and 70% of the wild-type sequence [80]. The truncated alpha-toxin is not hemolytic but it is unknown if it plays a role in cell signaling [80].

1.4.4 Alpha-toxin Cell Signaling

Alpha-toxin disrupts focal adhesion points within the target cell through an ADAM10-dependent mechanism which leads to a loss of epithelial integrity [72]. Binding of alpha-toxin to ADAM10 occurs in cholesterol caveolar rafts and the interaction likely promotes clustering of ADAM10 and oligomerization of alpha-toxin [72]. Association with and clustering of ADAM10 is speculated to promote activation of caveolae-associated proteins, activation of focal adhesion kinase (FAK) and Src kinase, and ultimately dephosphorylation of focal adhesion proteins and disruption of cell-cell junctions [69,72]. While alpha-toxin appears to work through multiple mechanisms, the requirement of ADAM10 for pathogenesis in alpha-toxin mediated disease has been established [81]. Across multiple *S. aureus* infection models in mice, inhibition of ADAM10 or ADAM10-knockouts display markedly decreased pathology. In epithelial models of *S. aureus* skin and lung infections, conditional ADAM10 knockout mice showed nearly complete remission of abscesses and preservation of alveolar structure, respectively, along with increased survival [81,82]. Additionally, *ex vivo* endothelial tissue was susceptible to focal adhesion disruption resulting in loss of barrier integrity when treated with sub-cytolytic doses of alpha-toxin, which could be attenuated with an ADAM10 specific inhibitor (GI254023X) [69].

1.4.5 Gamma-toxins

The gamma-toxins (Hlg) of *S. aureus* are encoded within 2 adjacent loci compromised of three genes with two promoters (Figure 1.1A) [83]. The *hlg* genes are orientated with *hlgA* under a unique promoter and *hlgB* and *hlgC* directly adjacent downstream under a shared promoter [83]. The gamma-toxin genes are highly prevalent, present in >99% of all *S. aureus* clinical isolates from any source of isolation, including colonizing and infectious isolates [84]. Transcription of the gamma-toxin genes occurs in response to multiple conditions. When *S. aureus is* exposed to blood, the *hlg* genes are the most up-regulated at the transcriptional level of all known virulence and defensive genes with *hlgA* increasing 145-fold and *hlgB* and *hlgC* increasing 34-fold after 90 min of exposure [85]. Additionally, when *S. aureus* is challenged with neutrophils or exposed to neutrophil azurophilic granule proteins, *hlg*A, *hlgB,* and *hlgC* are again the most up-regulated of the toxin or hemolysin genes [86].

Structurally, the gamma-toxins of *S. auerus* are β-barrel pore-forming toxins comprised of an S and F class subunit, corresponding to slow and fast elution from an ion exchange column. Gamma-toxins are atypical in that they are comprised of the F component (HlgB) with the S component either consisting of the A subunit (HlgA) or the C subunit (HlgC) that form octameric pores comprised of either HlgAB or HlgBC at 1:1 ratios, functionally creating two toxins (Figure 1.1B) [87,88]. The primary structures of HlgA and HlgC share 70% homology in amino acid sequence and approximately 30% homology with HlgB [83,89]. Interestingly, the HlgB subunit shares 27% homology with the single component cytolysin alpha-toxin (Hla) and is identical to the PVL F-subunit

[89,90]. The Hlg subunit secondary structures are predominatly comprised of beta-sheets and all contain a compacted beta-sheet stem with an alpha-helical hinge which reorients upon oligomerization to form a beta-barrel pore (Figure 1.1C) [91].

FIGURE 1.1: Gamma-toxin Locus and Toxin Structure

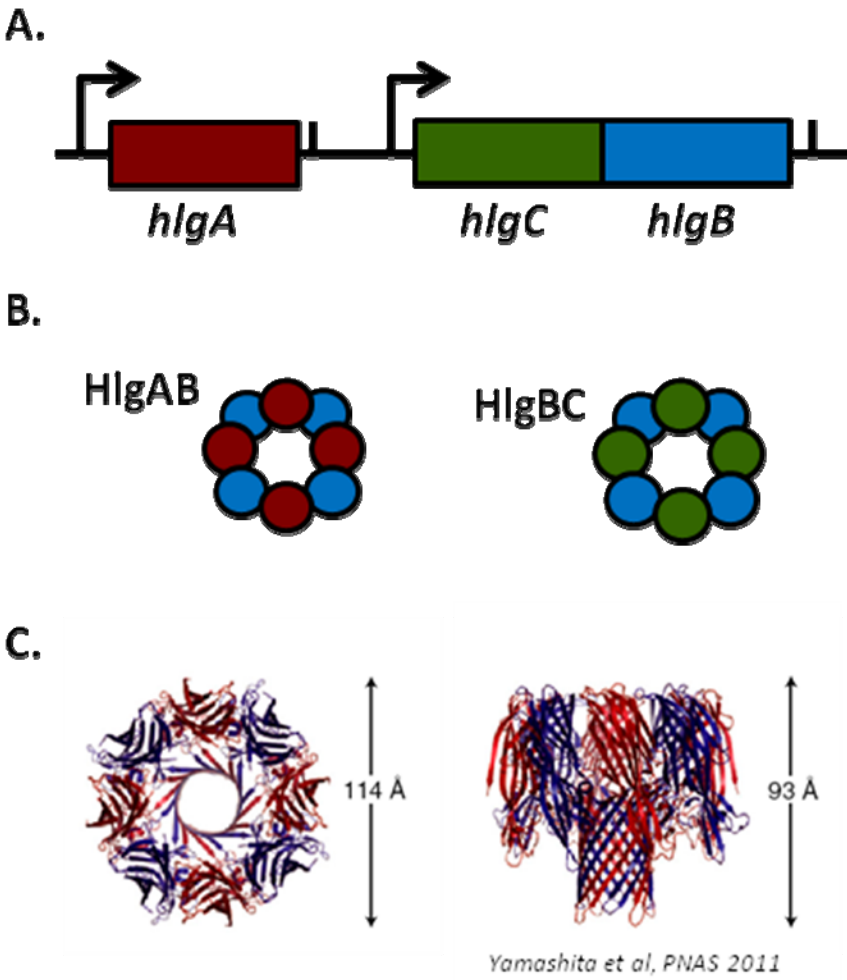


Figure 1.1: *(**A**) Gamma-toxin genes are located within two operons containing hlgA or hlgC and hlgB. The relative location of promoters (↱) and terminator sequences (ı) are indicated. The hlgA gene is transcribed independently while hlgC and hlgB are co-transcribed (**B**) Octameric orientation of gamma-toxins HlgAB and HlgBC formed at 1:1 molar ratios with alternating subunits. (**C**) Ribbon structure of gamma-toxin HlgAB from Yamashito et al* [91].

1.4.6 Gamma-toxin Binding

Pore formation in erythrocytes is initiated by HlgB binding to an unknown receptor or lipid rafts [91]. Upon binding of HlgB to erythrocytes, either HlgA or HlgC bind HlgB forming a dimer and reorienting a stem structure to insert into the lipid membrane [91]. Once HlgAB or HlgBC dimers form in the membrane they oligomerize to form hexameric, heptameric, or octameric pores [88]. While it is possible for gamma-toxins to form hexameric and heptameric pores or be comprised of a mix of HlgAB and HlgBC these forms represent a small proportion of total pores, are less stable, and do not have any known unique role [88,92]. The predominant octameric form creates a hydrophilic transmembrane pore within the target cell wall with an average diameter of 6.2 nm [88]. The size of the pore complex (114 Å diameter 93 Å depth) formed by gamma-toxins, HlgAB or HlgBC, is very similar in size to alpha-toxin [91]. However, the pore channel formed by gamma-toxin is three times the size of alpha-toxin, suggesting that efflux of cations through gamma-toxin pores is faster and less specific [71].

While it is thought the HlgB subunit initiates binding to cells, the HlgA and HlgC subunits are thought to confer specificity. Both HlgAB and HlgBC are strongly hemolytic to both human and rabbit erythrocytes as well as lytic to human neutrophils. HlgBC is capable of inducing cytokine production and causing lysis of human macrophages, whereas HlgAB shows no activity against human macrophages [93]. However, in murine macrophages the inverse is observed, with HlgAB showing strong pro-inflammatory and cytolytic activity while HlgBC is inert [93]. Furthermore, HlgAB has three times more cytolytic activity against human lymphocytes than against human neutrophils, whereas

HlgBC has no activity against human lymphocytes [96]. Differential effects of gamma-toxin on macrophages not only highlight toxin target specificity but also species differences. The differential activity of gamma-toxin HlgAB and HlgBC stipulates a requirement for multiple receptors. Interestingly, the target specificity for gamma-toxin appears to be dependent on HlgA, HlgB, or HlgC and the target cell type. In human erythrocytes, it is known that HlgB binds first, which would indicate that the specificity is determined by HlgB through an unknown receptor [91]. Neutrophils and macrophages each have multiple receptors that are specific for either HlgAB or HlgBC, and specificity appears to be dependent on HlgA and HlgC. The chemokine receptors, CXCR1, CXCR2 and CCR2, have been determined to bind HlgA and thereby facilitate lysis of neutrophils and monocytes [96]. The IL-8 receptors, CXCR1 and CXCR2, are expressed on neutrophils and monocytes as well as a variety of other cell types including lymphocytes, granulocytes, fibroblasts, endothelial cells, and epithelial cells [96,97]. The constitutive expression of CXCR1/2 is minimal in vaginal tissue, but can be induced in response to cellular injury [97,98].CCR2 expression is mostly limited to monocytes and lymphocytes [96,97]. For HlgC, the complement receptors, C5aR and C5L2, have been identified as binding targets and facilitate lysis of neutrophils, monocytes, and macrophages, where these receptors are expressed at high levels on the cell surface [96]. C5aR are constitutively expressed in cervix tissue but not vaginal tissue [99]. Currently there are no known receptors for HlgB.

1.4.7 Gamma-toxin Roles in Infection

Despite being present and expressed in almost all strains of *S. aureus,* little is known about the role of gamma-toxin in disease [84,96]. Early studies on gamma-toxin responses were based on the observation that the purified toxin induced proinflammatory responses and caused cytotoxicity to the vitreous humor and conjunctiva of rabbit eye [100]. Ocular infection in rabbits by a gamma-toxin deletion mutant resulted in markedly decreased virulence and 4-5 log decrease in bacterial CFU in the eye versus wild type *S. aureus* [100]. These studies demonstrated a critical role of gamma-toxin in a mucosal and endophthalmitis rabbit eye model. Subsequent studies of gamma-toxin were focused in murine sepsis models, despite CCR2 being the only human analog of known gamma-toxin receptors in mice [96]. However, despite a lack of monocyte and neutrophils receptors present in mice, gamma-toxin has shown a critical role in both murine sepsis and septic arthritis [96,101]. In mice injected intravenously with alpha-toxin and gamma-toxin deficient *S. aureus* mutants, the incidence of septic arthritis was reduced from 50% to 15% with nearly complete prevention of bone destruction [101]. Recent studies evaluating gamma-toxin receptors have also identified a role of gamma-toxin in dissemination of *S. aureus* from the peritoneal cavity into the blood, with gamma-toxin deficient *S. aureus* mutants showing a greater than 100-fold decrease in blood CFU 24 h post infection [96]. Despite limited data for a role for gamma-toxins in pathogenesis, there is evidence from both mouse and rabbit studies that these toxins play a critical role in multiple diseases caused by *S. aureus*.

1.4.8 Other Exotoxins

S. aureus produces numerous superantigens and cytolysins with many redundancies (Table 1.2). Often the activity of one exotoxin can be subverted by another, which may utilize the same cellular pathways and receptors. With superantigens, this is demonstrated by the ability of all superantigens (>20) to simulate CD4+ T-cells and antigen presenting cells, although the specificity to a particular Vβ domain of the T-cell receptor differs [33,102]. The cytolysins often have multiple cellular targets with varying specificity and activity, which often overlap with the targets of other cytolysins. This is demonstrated with gamma-toxin HlgBC and Panton-Valentine Leukocidin sharing the same cellular receptors and targets but having differential lytic activity and clinical manifestations [1,93]. The redundancies in activity extend to most virulence factors of *S. aureus* included lipases, proteases, clotting factors, and superantigen-like proteins [1,33,41]. Overall, the activities of one exotoxin can be replaced by another, or combination of other, exotoxin(s).

TABLE 1.2: Key *S. aureus* Exotoxins and Known Receptors and Activity

Toxin	Cellular Targets	Known Receptors	Activity
Superantigens			
TSST-1 [62,67,103]	CD4+ T-cells and Antigen Presenting Cells	Vβ Region of TCR and MHC-II	Hyper-stimulation and T-cell proliferation
	Vaginal Epithelial Cells	Unknown GPRC	Cytotoxic and Proinflammatory
	Endothelial cells	Unknown	Cytotoxic
SEB and SEC [33]	CD4+ T-cells and Antigen Presenting Cells	Vβ Region of TCR and MHC-II	Hyper-stimulation and T-cell proliferation
	Possible Endothelial Cells	Unknown	Barrier Disruption
Cytolysins			
Alpha-toxin [33,69,81,104]	Many Cells Including Epithelial Cells	ADAM10, β1-Integrin	Cell lysis and Disruption of focal adhesion points
	Erythrocytes	ADAM10, Lipid Rafts	
	Endothelial Cells	ADAM10	
	Monocytes	Unknown	Caspase induced apoptosis
	T-cells	CD95	
Beta-toxin [33]	Many Cells, Primarily Erythrocytes and Fibroblasts	Sphingomyelin	Cell Lysis
Gamma-toxin HlgAB [95,96]	Neutrophils and Erythrocytes	CXCR1/2 CCr2	Cell Lysis
Gamma-toxin HlgBC [95,96]	Macrophages, Neutrophils, and Erythrocytes	C5aR C5L2	Cell Lysis
Panton-Valentine Leukocidin (PVL) [105]	Macrophages, Monocytes, and Neutrophils	C5aR C5L2	Cell Lysis

1.5 Mucosal Inflammation during *S. aureus* Infection

1.5.1 Mucosal Models

The epithelium is a primary organ of the body covering the external surfaces and acting as a first line barrier to physical, chemical and infectious insults. The dermal, oral, esophageal and vaginal surfaces are composed of stratified squamous epithelium tissue. The structure of stratified squamous epithelium tissue originates from a proliferating basal cell layer that increases in stratification while flattening as layers become more apical [106]. The typical membrane thickness is approximately 120-200 μM [106]. The epithelium can be tough and keratinized such as the case with skin or more flexible and non-keratinized such as the oral cavity, esophageal and vaginal surfaces. Non-keratinized epithelium also contains intercellular lipids which act as a hydrophobic barrier, or conversely a medium for lipophilic compounds [70,106].

The use of cell culture monolayers or stratified layers cannot elucidate the complex interactions of mixed cells and tissues in the vaginal epithelium. Ethical and practical implications make human vaginal tissue difficult to obtain for routine testing and screening. Beyond difficulty in obtaining large amounts of healthy tissue the inter-person variability in baseline characteristics makes mechanistic studies difficult to interpret. The structure and orientation of the vaginal tissue for most common laboratory animals differs substantially from humans with the exception of the pig [106]. The porcine vaginal mucosa is remarkably similar to human tissue in terms of pH, structure, histology, thickness and permeability. The tissue is nearly identical in terms of pH and lipid composition and only differs by ~40 μM in epithelial cell thickness [106].

Additionally, the flux (cpm/cm^2/min) through the tissue by water and numerous hydrophilic and hydrophobic compounds is nearly identical [106]. Porcine vaginal tissue is also similar with the presence of CD-1 and SLA-DR positive intra-epithelial dendritic cells, the majority of APCs present in human vaginal tissue [106,107]. The pig is a convenient model for studying the vaginal mucosal due to the ease of obtaining tissue from gilts that are derived from genetically similar populations and raised under identical conditions.

1.5.2 Proinflammatory Effects of Exotoxin at the Vaginal Mucosa

Epithelial cells are the first site of interaction of any *S. aureus* exotoxins with the vaginal mucosa. Immortalized human vaginal epithelial cells (HVECs) are an established model for simple vaginal keratinocytes that exhibit distinct phenotpyical characteristics of primary vaginal cell cultures [108]. The innate immune response in HVECs is activated by both superantigens and cytolysins. TSST-1 (50-100 μg/ml) exhibits saturable binding and is able to induce a proinflammatory response in cell cultures, but does not show significant lethality to cells even at high concentrations (500 μg/ml) [65,66]. Alpha-toxin (50 μg/ml) also induces a cytokine response similar to TSST-1, but causes cell lysis [70]. Additionally, TSST-1 at sub-stimulatory concentrations (10 μg/ml) in the presence of alpha-toxin (50 μg/ml) is synergistic in stimulating cytokine production in HVECs [70]. The concentrations of TSST-1 required to induce a proinflammatory effect in HVECs are similar to those found in the tampons of mTSS patients [109]. Furthermore, protein fractions from *S. aureus* supernatants containing combinations of exotoxins were

found to be very potent in stimulating cytokine production in HVECs. A protein fraction containing superantigens (TSST and SEC) as well as alpha-toxin and 6 other lipases, proteases, and kinases was found to stimulate a potent IL-8 response and cytotoxicity at concentrations as low as 2 μg/ml of total protein [110]. The synergy in response indicates there is likely a complex mechanism by which *S. aureus* exotoxins promote disease at the epithelium.

Mucosal tissue is not comprised of a homogenous population of cells. Epithelial cells and immune cells are routinely intermingled and both cell types can respond to *S. aureus* exotoxins. Within the vaginal epithelium, there are resident immune cells which primarily consist of Langerhans cells, dendritic cells, CD4+ T-cells and CD8+ T-cells [107]. Interestingly, the concentration of immune cells in the vaginal epithelium increases by 6-38% during menstruation, with Langerhans cells showing the largest increase of 38% [107]. The *lamina propria* lies below the epithelium and is comprised of loose connective tissue containing fibroblasts, immune cells, vasculature and lymphatic networks. TSST-1 is able to bind and penetrate into vaginal mucosal tissue and localizes in the epithelial layer and the *lamina propria* [65]. Penetration of TSST-1 into the tissue can occur through passive diffusion and endocytosis [111]. TSST-1 has been shown to independently cause pathology at the vaginal mucosa in porcine tissue similar to what is observed in clinical cases of mTSS, specifically vaginal mucosal sub-epithelial splitting and separation of the epithelial cells from the *lamina propria* [40,111]. The pathology induced by TSST-1 can be enhanced by damaging epithelial integrity, with either co-incubation with alpha-toxin or incision [70,111]. Alpha-toxin is also able to

independently cause sub-epithelial splitting and epithelial sloughing at higher concentrations (≥50 μg/ml) [70]. However, alpha-toxin is likely to play a role in only a subset of mTSS cases as it is produced in less than 20% of mTSS clinical isolates suggesting that other cytolysins such as the ubiquitous gamma-toxins may contribute to disease [112].

The role of gamma-toxin in mTSS has not previously been characterized despite the almost universal presence of the gamma-toxin genes in clinical isolates of *S. aureus*. However, there is some evidence to indicate that gamma-toxin may be of importance. In a USA200 strain (CDC587) isolated from patients with mTSS the exotoxins profiles were characterized and compared to a USA200 strain (MNPE) isolated from patients with pneumonia. Fractions from MNPE were found to be much more cytotoxic and proinflammatory to epithelial cells, which was attributed to high production of alpha-toxin [110]. This is consistent with epithelial necrosis observed in *S. aureus* pneumonia and skin infections [1]. MNPE does produce significant amounts of gamma-toxin [110]. In contrast, the mTSS isolate, CDC587, produced minimal amounts of alpha-toxin but produced large amounts of gamma-toxin [110]. Exoproteins from CDC587 were also cytotoxic and proinflammatory to vaginal epithelial cells, but less than high alpha-toxin-producing strains of *S. aureus* [110]. The decreased epithelial cell cytotoxicity caused by exotoxins from CDC587 is likely critical to the strain's ability to colonize the vaginal mucosa.

1.5.3 Mucosal Immunobiology

Superantigens modulate the local immune response at the vaginal epithelium to promote conditions favorable for *S. aureus* survival and promote systemic disease following colonization or initial infection. The uninfected vaginal epithelium contains resident CD4+ T cells and antigen-presenting cells, but at levels unlikely to cause significant pathology if exposed to superantigens. Superantigens and cytolysins stimulate potent IL-8, IL-6, and MIP-3α production in HVECs [65,66,70]. These chemokines are also produced by vaginal tissues and can promote recruitment of immune cells, such as neutrophils, although *S. aureus* is well adapted to survive and escape from these cells [113]. The cytokine response produced by the vaginal epithelial cells leads to activation of an acute response, induction of fever, and suppression of regulatory T-cells by IL-6 and activation of dendritic cells by MIP-3α [114]. Activated dendritic cells begin up-regulating antigen presentation, promoting T-cell recruitment, and trafficking to local lymph nodes [115]. Dendritic cells also effectively bind superantigen, but fail to process it within endosomes or lysosomes [115]. Unprocessed superantigen can then associate with MHC-II within the lysosome and, after display on the cell surface, can link MHC-II with TCRs nonspecifically activating T cells [115]. Activation of dendritic cells and T-cells by MHC-II and TCR or dendritic cells by MIP-3α produced from epithelial cells leads to further recruitment of APCs and T-cells as well as trafficking of dendritic cells to the lymph nodes, ultimately exposing superantigens to more of their target cells [115].

Local or compartmental effects of superantigens appear to be critical mediators of TSST-1. Because TSST-1 can cause lethal disease from the vaginal mucosa in rabbits,

the mechanism of pathogenesis may not be consistent with systemic infection models. The intravaginal LD_{100} of TSST-1 in an endotoxin-enhanced (0.1 μg/kg) rabbit model is ~5 μg/kg, which is less than the intravenous LD_{50} (5.9 μg/kg with 0.1 μg/kg LPS), with death in both cases occurring within 48 h [66,116]. The discrepancy in doses required for lethality in rabbits for an intravenous or vaginal administration of TSST-1 would suggest either TSST-1 can rapidly and completely penetrate into the systemic circulation from the vagina or it has lethal activity that originates from within vaginal tissue. Furthermore, rabbit models of mTSS demonstrate a critical role for local effects of TSST-1 at the vaginal mucosa. Curcumin, an inhibitor of NF-κB, when applied topically to the vagina in rabbits prevented death induced by lethal intravaginal challenge with TSST-1 [66]. The lethal effects of TSST-1 in rabbits from the vaginal mucosa are independent of TSST-1 penetration or superantigenicity. A single amino acid TSST-1 mutant (D130A), which does not stimulate cytokine production in HVECs, readily penetrates the vaginal mucosal tissue [117]. Additionally, the D130A TSST-1 mutant retains superantigenicity and lethality when injected intravenously but it loses lethality when administered intravaginally [117]. Combined these experiments highlight a critical role for vaginal inflammation in the progression of mTSS.

1.5.4 Toxic Shock Syndrome Toxin Distribution and Penetration

The distribution of TSST-1 indicates that it is actively sequestered in tissues. Experiments to determine TSST-1 clearance in pigs demonstrated that when injected as an IV bolus only 13% of the expected plasma concentration was observed (assuming

equal distribution among plasma and extracellular fluid) [118]. TSST-1 has a log P value of 2.5 and high solubility in water (~500 µg/ml), therefore the expected volume of distribution of TSST-1 should not be more than 2.5x the total body fluid of the pigs used for the experiment (~30 L) [119]. Extrapolating from the data, the calculated apparent volume of distribution for TSST-1 in pigs is ~230 L (blood volume of ~2 L, total fluid volume of ~30 L), which suggest sequestration of TSST-1 in tissue [118].

Experiments measuring TSST-1 penetration across *ex vivo* vaginal mucosa appear to support local actions of TSST-1 at the vaginal mucosa. When TSST-1 flux is measured across *ex vivo* vaginal mucosa in continuous flow chambers to simulate blood flow only 0.25% of the 10 µg applied dose was able to perfuse across the tissue over an 8 h period [70]. Furthermore, other experiments have demonstrated that ~98.5% of TSST-1, remains within the vaginal tissue with ~1.5% penetrating across over 8 h [111]. Combined, the data suggest that TSST-1 has a high affinity to enter the tissue but a low disassociation into systemic fluids supporting the findings that TSST-1 remains within the vaginal mucosal tissue.

1.6 Cellular Receptors

1.6.1 Epidermal Growth Factor Receptor

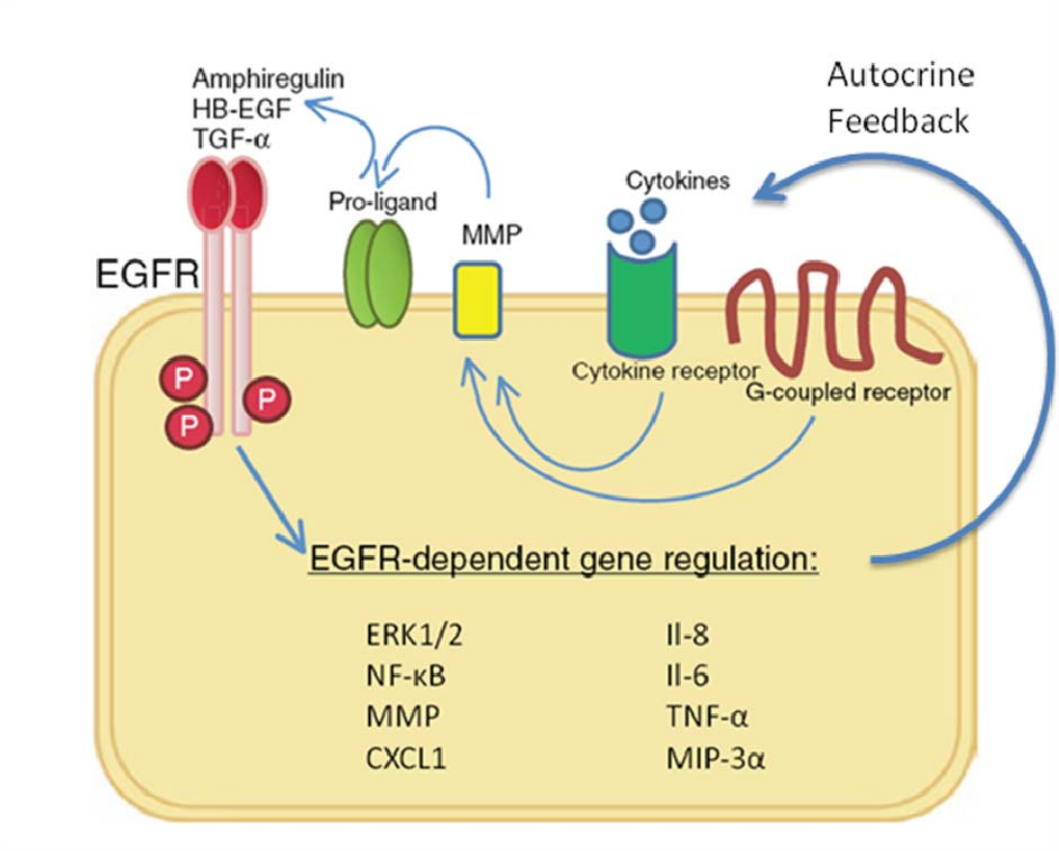
Exotoxins stimulate proinflammatory responses in vaginal epithelial cells through an EGFR-dependent pathway and vaginal epithelial proinflammatory responses are required for lethality in mTSS rabbit models [67,117]. These data suggest a critical role for EGFR in the pathogenesis of mTSS. Under homeostasis, EGFR signaling participates

in regulating core cellular functions including survival, migration, and cell division [120]. EGFR is a tyrosine kinase receptor, which is activated by a family of ligands, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, and heparin binding-EGF (HB-EGF) [68]. The type and degree of responses produced by EGFR engagements are dependent on numerous factors including the number of activated receptors, activation of co-factors and the ligand [68]. Certain ligands are also more potent stimulators of EGFR through endosome recycling, which inhibits down regulation of EGFR following activation. The ligands TGF-α and amphiregulin activate EGFR endocytosis, but inhibit trafficking to the lysosome, and cause recycling of the receptor and ligand back to the cell surface [121]. HB-EGF causes the opposite effect when it activates EGFR, promoting trafficking to the lysosome, and thus down regulating the EGFR response [121].

Activation of EGFR plays a role in regulation of the innate immune response through autocrine and paracrine feedback signaling (Figure 1.2). The primary response to EGFR includes TGF-α, which acts as a feedback loop in EGFR signaling [68]. TGF-α also activates local T-cells and induces IL-8 production, which mediates neutrophil chemotaxis [122,123]. Overall, the response induced by EGFR induces activation of local T-cells and macrophages via production of IL-6, IL-8, IL-1, INF-γ and TNF-α, while inducing chemotaxis and activation of dendritic cells and neutrophils via production of IL-8 and MIP-3α from epithelial cells [68,115,123,124]. Interestingly, activation of EGFR also down regulates the chemokines CCL2, CCL5 and CXCL1, which are mediators of systemic recruitment of T-cells, monocytes, macrophages, natural killer

cells and granulocytes [125–127]. In histological observations of vaginal tissue in mTSS patients, where potent EGFR signaling is expected to occur, the immune cells present represent the response described above, consisting of predominantly leukocytes, neutrophils and dendritic cells, while lacking macrophages and granulocytes [40]. Thus, over activation of EGFR likely produces an immune response rich in neutrophils and activated dendritic cells and T-cells, which is a population of cells *S. aureus* excels at surviving within and exploiting via superantigens to cause disease.

FIGURE 1.2: EGFR Activation in Epithelial Cells



Adapted from Büchau 2010 [122]

Figure 1.2: Epidermal growth factor receptor (EGFR) activation in epithelial cells. *Activation of proinflammatory EGFR signaling occurs as a result of cytokine signaling or other stimuli leading to activation of ADAMs or other matrix metalloproteinases (MMPs) which shed EGFR pro-ligands which activate EGFR-signaling. EGFR contributes to its own activation through autocrine and paracrine feedback mechanisms, which further enhance the cellular response.*

1.6.2 (A) Disintegrin and Metalloproteases

The (a) disintegrin and metalloproteases (ADAM) are multi-domain proteins that contain an inhibitory pro-peptide domain and active disintegrin and metalloprotease domains [128]. Once the inhibitory pro-peptide domain is cleaved by effector proteins or via autocatalysis, the activated ADAMs act as sheddases releasing growth factors, cytokines and other regulatory signals from the cell surface [129]. A total of 29 different ADAM genes are found in humans and each type is generally expressed on either somatic tissue, reproductive tissue or immune cells [130]. The general functions of ADAMs in homeostasis consist of cell matrix remodeling, cell adhesion, shedding of growth factors and immune surveillance [130]. Dysregulation of ADAMs has been associated with several diseases, most notably cancer by increased shedding of growth ligands by ADAM 9, 10, 12, and 15 and inflammatory diseases by dysregulation of TGF-α shedding by ADAM 17 [130].

ADAM10 and ADAM17 are critical mediators of *S. aureus* exotoxin activity. ADAM10 is required for cytolytic activity by alpha-toxin by acting as a cell receptor and scaffold for toxin binding, although it should be noted that other receptors and mechanisms of binding have been identified [72,78]. The requirement for ADAM10 extends to *S. aureus* infections in mice, where inhibition of ADAM10 directly or with tissue-specific knockdown attenuated disease in SSTI and pneumonia infection models, respectively [72,81,82]. Studies have also identified ADAM17, and to a lesser degree ADAM10, as critical upstream mediators of EGFR-signaling in HVECs [67]. This is likely a result of ADAM17-mediated shedding of TGF-α and amphiregulin, potent EGFR

ligands, whereas ADAM10 mediates shedding of the less potent HB-EGF [67,129]. Unlike alpha-toxin interactions with ADAM10, the interactions of TSST-1 and ADAMs are likely indirect and specific to vaginal epithelial cells [67]. While ADAM10 and ADAM17 are ubiquitously expressed on somatic cells they are activated by different stimuli, which is demonstrated, among numerous examples, by the inability of TSST-1 to stimulate cytokine production in immortalized lung epithelial cells [67,129,130].

1.7 Epidermal Growth Factor Receptor Inhibitors

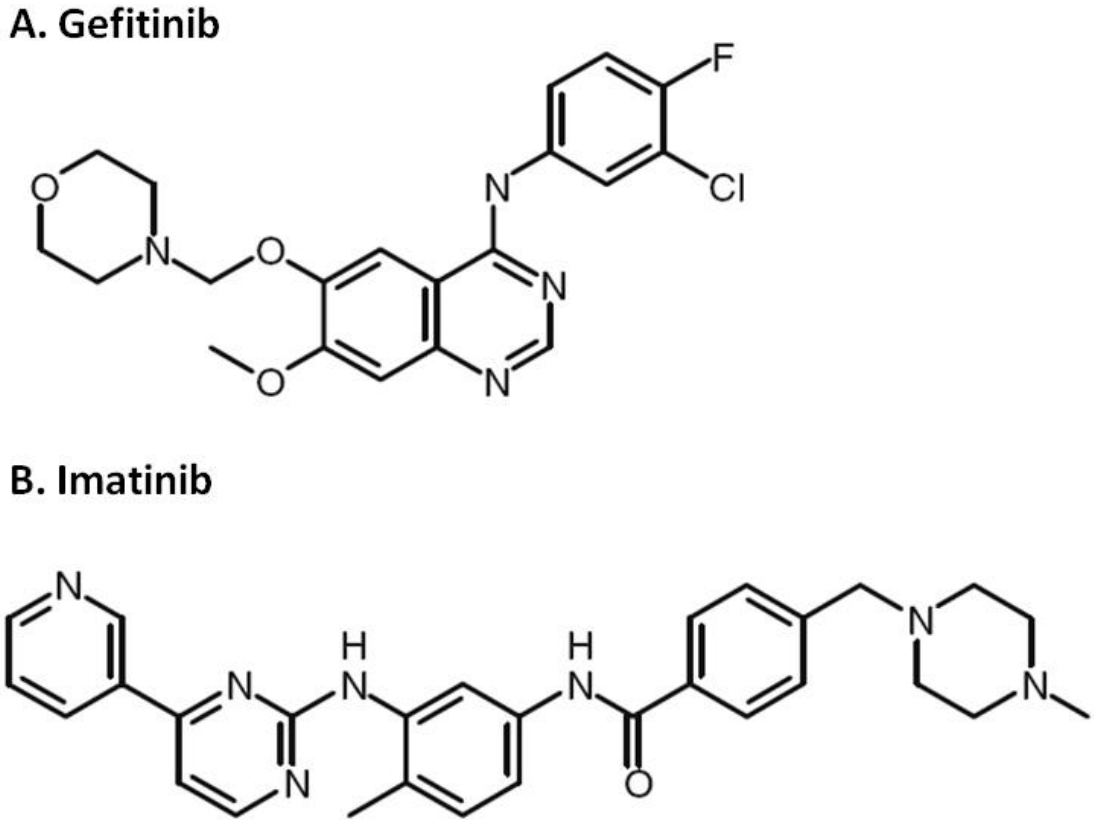
1.7.1 Tyrosine Kinase Inhibitors

Targeting EGFR through chemical or antibody inhibition has been used therapeutically for years in the treatment of cancer to inhibit cell growth and inflammation. Tyrosine kinase inhibitors (TKIs) are a recently discovered class of small molecule inhibitors with broad or sub-type selectivity for EGFRs [131]. Inhibition of EGFR by TKIs is accomplished through either competitive or non-competitive binding to the EGFR-ATP binding site [131]. The structures of TKIs fall into two general classes (Figure 1.3). The first class of TKIs is based off an anilinoquinazoline core modified for selectivity and pharmacokinetics, and includes the common clinical drugs, gefitinib and erlotinib [132–134]. Recently developed TKIs were based on crystal structures of their targets and generally are formed from chains of modified benzene rings [131]. Currently, TKIs are used primarily for the treatment of cancers, mostly non-small-cell lung and breast cancer. More recently, a novel TKI, Xeljanz®, which inhibits the downstream activity of EGFR at the JAK3/STAT pathway is being used to treat the inflammatory

disease rheumatoid arthritis [135]. TKI a re also being studied and show efficacy in treatment of other chronic inflammatory including asthma and as anti-viral agents against human papillomavirus [131,136,137].

S. aureus exotoxins are potent inducers of cytokine production through modulation of EGFR signaling pathways in epithelial cells, thus raising the possibility that inhibition of EGFR may attenuate toxin mediated disease. However, modulation of the inflammatory response during infection is a delicate process, over modulation may promote bacterial invasion through immunosuppression. Ideally, any modulation to suppress a hyper-inflammatory response would need prompt activation and would need to be easily reversed. AG1478 is an ideal candidate to study possible therapeutic applications of a TKI to inhibit *S. aureus* toxin-mediated effects in mucosal disease. AG1478 is rapidly absorbed and is a competitive inhibitor of EGFR (ErbB1) with a very short half-life, which allows for local application and quick reversal of therapeutic effects after dosing [138].

FIGURE 1.3: Structures of Tyrosine Kinase Inhibitor



Representative chemical structures of two main classes of tyrosine kinase inhibitors. *Chemical structures of (**A**) gefitinib comprised of an anilinoquinazoline core and (**B**) imatinib comprised of a chain of modified benzene rings.*

1.7.2 AG1478:

The compound 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is a small molecule (315.75 MW) inhibitor specific for EGFR tyrosine kinase activity. Traditionally, EGFR TKIs are used as anti-mitogenic agents for the treatment of cancer with clinical indications, including small lung cancer or pancreatic cancer [133,134]. The quinazoline class of TKIs are competitive or irreversible inhibitors of the ATP binding site within the kinase domain and highly potent for EGFR [132]. The compound AG1478 acts as a competitive inhibitor specific to EGFR (ErbB1) with ~24 min effective duration with a single non-sustained dose *in vitro*. The primary chemical features, which confer the compound's activity, are an electron donating group at position 6-7 within the quinazoline ring and the high lipophilic nature of the aniline allowing for rapid association across cell membranes [138].

1.7.3 AG1478 Pharmacokinetics

The pharmacokinetic profile (short half-life) of AG1478 is not suitable for chronic therapy due to the need for frequent dosing, and therefore the compound was mostly abandoned for the treatment of cancers (Table 1.3). AG1478 distributes to tissue within minutes following IV bolus and has a dose-dependent β-phase half-life in animal models of 30-48 min [138]. While no human pharmacokinetics studies have been conducted, the human pharmacokinetic values predicted through simple allometric scaling ((*Weight Human/Weight Mouse)*$^{3/4}$) predicts an estimated half-life and intrinsic clearance of 31 min and 355 L/h following IV dosing, respectively. However, the clearance of AG1478

appears to be the only negative aspect of the compound when it comes to chronic therapy. AG1478 is a strongly lipophilic compound displaying an estimated volume of distribution of ~250L in humans with preferential distribution to the skin and highly perfused tissues including the liver, kidneys and mucosal surfaces [138]. The compound also displays differential activity across a wide range of doses. At doses of 10-20 μM, AG1478 inhibits cell proliferation and enhances death from apoptotic stimuli by reduced production of caspase inhibitors [139]. At the lower dose of 1 μM, AG1478 attenuates TSST-1 induced cytokine production and shedding of EGFR ligands in HVECs [67]. In rat *in vivo* studies, anti-mitogenic concentrations were obtainable with an infusion of 24 μmol/kg/h of AG1478, with a steady-state concentration of ~10 μM [138]. Toxicities were observed in rats with an infusion of 161 μmol/kg/h of AG1478, or an approximate steady state concentration of 100 μM, when rats experienced lethargy [138]. While it is uncertain what the lethal dose of AG1478 is, the chemically similar drug gefitinib (Iressa®, AstraZeneca) does not cause lethality at dosages of <4.4 mmol/kg in murine models [133].

TABLE 1.3: Pharmacokinetic Parameters of AG1478 and Similar Compounds

Drug	Species	Bioavailability	Clearance	Volume of Distribution	Half Life	Protein Binding
AG1478 [138]	Mice Human *est.*	60-100 %	0.7 L/h 335 L/h	0.52 L 250 L	30-48 min 31 min	N/A
Gefitinib [133]	Human	60-100 %	35.7 L/h	1400 L	48 h	91 %
Erlotinib [134]	Human	60-100 %	5.3 L/h	232 L	36 h	93 %

Human estimates based on simple allometric scaling ((Weight Human/Weight Mouse)$^{3/4}$).

1.7.4 AG1478 Metabolism

Current metabolism of AG1478 is not known, but can be speculated based on the metabolites and excretion of chemically similar TKIs in clinical use including gefitinib, erlotinib, sunitinib and lapatinib. All TKIs are primarily metabolized by cytochrome p450s (CYP) with CYP3A4 and CYP3A5 being the dominating metabolizing enzymes and glucuronosyltransferases playing a minimal role initially [132]. Gefitinib, the most structurally similar clinical TKI to AG1478, is predominantly metabolized by CYP3A4 and CYP2D6. The primary site of metabolism in gefitinib is oxidation of the morpholine ring, a structure not present in AG1478 [140]. However, the second most abundant metabolites of gefitinib are generated by O-demethylation of the quinazoline-methoxy group and defluorination and oxidation of the chlorinated phenyl group [140]. In comparison to gefitinib, AG1478 has 2 methoxy group additions to the quinazoline ring, which would likely both undergo O-demethylation. Furthermore, gefitinib contains a fluorine atom at the para-position of the phenol ring, which was introduced to prevent para-hydroxylation, whereas AG1478 contains a hydrogen atom. Combined, the predicted oxidative metabolism would yield 7 possible metabolites (Figure 1.4) with possible biological activity, as the O-demethylation metabolites of gefitinib are biologically active [132,140]. Any resulting phenolic metabolites would be expected to undergo rapid glucuronidation or sulfation.

FIGURE 1.4: AG1478 Structure and Potential Sites of Metabolism

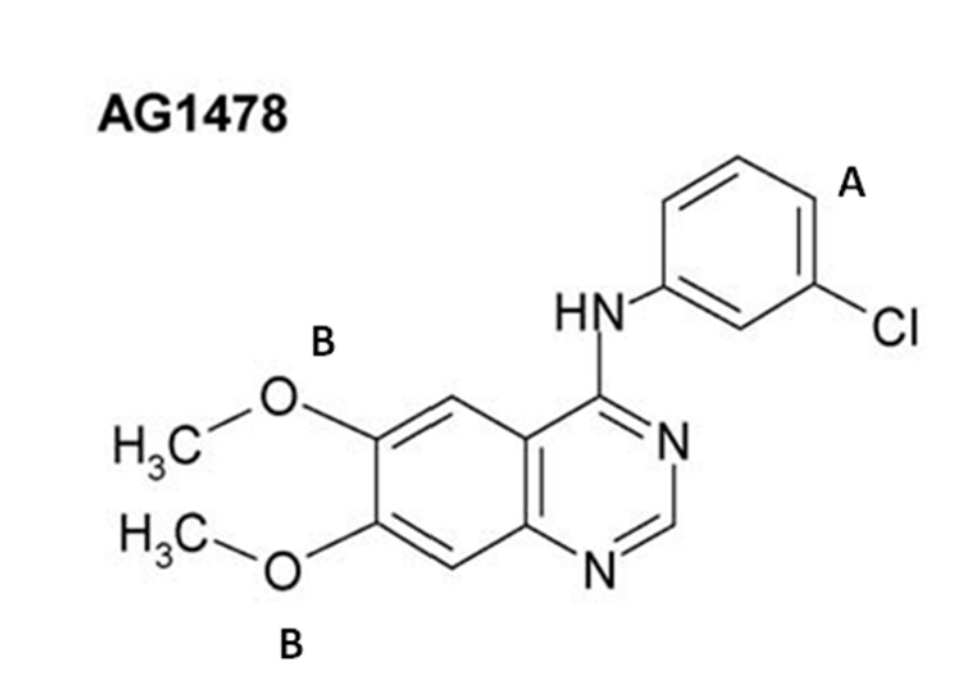


Figure 1.3: *Chemical structure of AG1478 showing likely (**A**) sites of oxidation and (**B**) O-demethylation during metabolism.*

ATP-binding cassette (ABC) transporters are commonly involved in multidrug resistance. AG1478 is known to interact with ABCB1 and ABCG2, which are present in vaginal epithelial cells [141]. In ABCB1 and ABCG2 over-expressing cells, AG1478 increased intracellular accumulation of the transporter probes mitoxantrone and paclitaxel, respectively [141]. While gefitinib is a known competitive inhibitor of ABCG2, AG1478 lacks the hydrophobic side chain, critical for gefitinib inhibition [138,141,142]. Therefore it is currently unknown if AG1478 acts as a competitive inhibitor or a substrate for ABC transporters. The transporters, ABCB1 and ABCG2 are expressed on most tissues of the body including epithelial and mucosal surfaces [143,144]. ABC transporters are often over-expressed in cancerous cells and play a significant role in drug resistance [145]. In healthy, non-cancerous cells, normal expression of these proteins does not have a significant effect on drug potency [141]. However, a number of conditions, specifically inflammation and hypoxia during infection, can induce over-expression of ABC transporters in non-cancerous cells, which could affect the activity of AG1478 during treatment. ABC transporters are regulated by the acute phase response, which is initiated during almost all physical or bacterial traumas [145]. The cytokine IL-6, commonly produced during bacterial infections, activates the enhancer-binding protein C/EBPβ which increases transcription by activating promoters of multiple ABC genes [145,146]. Hypoxia, which is common during bacterial infections, also induces expression of an array of drug-resistance proteins including ABC transporters through activation of the promoter hypoxia-inducible factor-1 [147]. If AG1478 is to be used in situations of inflammation or trauma, then further

studies should be conducted to determine if it is acting as a competitive inhibitor or a substrate for ABCB1 and ABCG2 because, if AG1478 is a substrate then dose adjustments may need to be considered.

1.8 Summary

S. aureus exotoxins are the primary virulence factors which cause damage to host tissue during infections [1,33,110]. While important in infections, the bacterial surface proteins primarily play a protective role by facilitating bacterial binding, clumping and immune evasion [1]. To date, all attempts at active or passive vaccination targeting *S.aureus* surface proteins have either failed to be protective or increased disease severity [44,148]. The rationale for targeting surface proteins was based on the success of previous vaccines for other bacteria. The surface proteins were well conserved, highly expressed, and in similar bacterial species (*Streptococcus pneumoniae*) opsonization has proven protective [149]. However, diseases caused by *S. aureus* infection are heavily associated with a subset of specific exotoxins for different types of infections, for example alpha-toxin facilitates SSTIs, TSST-1 causes (m)TSS, and PVL facilitates necrotizing pneumonia [33]. Different exotoxins are critical for specific diseases of *S. aureus* and thus targeting the actions of the exotoxins, rather than bacterial surface proteins, may provide efficacy for treatment and prevention of infections. However, redundancies in mechanism(s) of action for *S. aureus* exotoxins may result in the role of a neutralized toxin being replaced by another similar toxin, with the same cell targets. An example of this is observed with the superantigens TSST-1, SEC and SEB, which all

induce T-cell proliferation through nearly identical pathways [33,62]. To avoid this problem, any anti-toxin therapy should target the actions of multiple exotoxins or inhibit the conserved effects within the host.

TSS and mTSS are exotoxin mediated diseases that can occur with an absence of bacteremia or invasive infections, which is common in mTSS patients [1,40]. These diseases are facilitated by, and result from, a small subset of toxins with superantigen properties (TSST-1, SEC, and SEB) that drive the pathogenesis [21]. Other exotoxins, such as cytolysins, enhance superantigen pathogenesis [70]. Furthermore, the pathology and damage to the host during (m)TSS is a result of hyper-activation of the host immune response and not from direct tissue damage by superantigens [62]. During mTSS, the initial steps of the disease are likely localized within the vaginal mucosal tissue, with TSST-1 having high affinity for tissue, promoting a localized cytokine response, and promoting active trafficking of superantigens to the local lymph nodes by tissue resident dendritic cells [70,109,111,115]. The systemic pathologies of mTSS could thus be a result of the initial hyper-activation of T-cells within the mucosal immune system by superantigens localized in vaginal tissue and local mucosal immune compartments. Therefore, the possibility of inhibiting the local immune response transiently may be beneficial as adjunct therapy in treatment of mTSS.

Currently, prophylaxis for *S. aureus* infection is limited to hygiene in most populations and antibiotics in at risk groups prior to surgery, *i.e.* cardiac surgery patients [22]. Broad-coverage or universal *S. aureus* vaccines have failed and with redundancies in exotoxins produced and high community prevalence it may not be practical to

immunize everyone in the population against multiple exotoxins due to cost and most people, even those colonized, being at very low risk of serious infection [3]. However, certain groups are at high risk of *S. aureus* infections (cardiac surgery and chronic hemodialysis-patients) and some people experience high incidence of recurrent infections (abscesses in children). Thus, targeting certain groups to prevent specific infections using a cocktail of *S. aureus* toxoids unique to the infection type could prove beneficial and cost effective, especially to prevent hospital-acquired infections, which greatly increase the length of stay [13].

Despite mTSS pathology being mediated by a hyper-immune response, no current therapy targets this aspect [17]. While bacteremia or bacterial invasion of tissue is not present in most cases of mTSS, it would likely be unwise to inhibit T-cell function as this could promote bacterial spread. However, if the pathogenesis of mTSS is dependent on the cytokine-signaling cascade initiated by TSST-1 at the vaginal epithelium, it may be possible to block an innate immune response induced by and beneficial to *S. aureus* as adjunct therapy. With the innate immune response induced by TSST-1 being dependent on EGFR, it may be possible to attenuate disease using the EGFR TKI, AG1478, to improve clinical outcomes by reducing the intensity of the hyper-immune activation.

Herein are studies on the characterization of *S. aureus* exotoxins mechanism(s) of pathogenesis and novel strategies in treatment and prevention of toxin-mediated diseases. The primary undertaking investigates the mechanism of pathogenesis and proinflammatory effects of the *S. aureus* cytolysins, gamma- and alpha-toxins, and their contribution and synergy with TSST-1 during mTSS. In addition, prevention of infection

was investigated by vaccination solely targeting exotoxins for protection against *S. aureus* pneumonia. Lastly, a novel treatment strategy was developed and investigated for prevention of mTSS by targeting EGFR with the TKI AG1478. Combined, this work identifies the mechanism of action of critical toxins and introduces novel approaches for treatment of *S. aureus* disease.

1.9 Aims of this work

The central hypothesis of my research is that local proinflammatory effects of the *S. aureus* exotoxins TSST-1, alpha-toxin, and gamma-toxin are critical mediators of systemic disease including TSS.

Specific aim 1: Characterization of the cytotoxic and proinflammatory effects of gamma-toxins on the vaginal epithelium, including the role of EGFR.

Rationale: Gamma-toxins are among the most ubiquitous and highly expressed toxins of *S. aureus,* but little is known about their effects at the vaginal mucosa. Recent evidence suggests that cytolysins modulate cell signaling independent of their cytotoxic activity. Furthermore, proinflammatory and cytotoxic effects of cytolysins are known to potentiate actions of superantigens. This study investigates the effects and mechanisms of action (including the role of EGFR) of gamma-toxins on the vaginal mucosa.

Specific aim 2: Characterize the mechanism of action for alpha-toxin's proinflammatory effects and pathogenesis at the vaginal mucosa.

Rationale: Alpha-toxin is cytotoxic and a potent stimulatory of cytokine production at the vaginal mucosa. Additionally, alpha-toxin is known to enhance TSST-1 induced cytokine production in HVECs and disrupt cell-cell junctions independent of cytolytic activity. This study investigates the mechanism of alpha-toxin-induced cytokine production at the vaginal mucosa and the potentiating of proinflammatory and lethal effects of TSST-1.

Specific aim 3: Investigate the potential of the EGFR inhibitor, AG1478, to attenuate disease *in vivo* in a rabbit mTSS model.

Rationale: The proinflammatory effects of the superantigen TSST-1 and cytolysin alpha-toxin are both mediated through EGFR. Inhibition of EGFR by AG1478 attenuated cytokine production *in vivo* and *ex vivo*. Pathogenic and lethal effects of superantigens are mediated by hyper-stimulation of cytokine production. This study investigates the safety and efficacy of AG1478 in prevention of *S. aureus* mTSS in rabbits.

Specific aim 4: Evaluate the potential of vaccination against exotoxins for prevention of *S. aureus* pneumonia in rabbits.

Rationale: All recent attempts to develop a *S. aureus* vaccine have targeted surface proteins and all failed to provide protection, with the most recent attempts enhancing lethality in clinical trials. Exotoxins of *S. aureus* are the dominant mediators of tissue destruction and invasion in developing and progressing infections. This study aims to investigate the potential of active and passive immunization for protection against *S. aureus* fatal pneumonia in rabbits.

Chapter II.

Gamma-toxin of *Staphylococcus aureus* Induces Proinflammatory Effects at the Mucosa through Epidermal Growth Factor Receptor Signaling

Sections of this study will be part of a future manuscript with me as the primary author. All studies in the section were performed by me.

2.1 Introduction

S. aureus is able to cause the systemic disease toxic shock syndrome (TSS) through the activity of superantigens, toxic shock syndrome toxin-1 (TSST-1) [29]. Most toxic shock cases are associated with menstruation (menstrual, mTSS) and occur in the absence of significant bacteremia [29,40]. *S. aureus* produces an arsenal of secreted toxins, including superantigens such as TSST-1, as well as an array of cytolysins. The cytolysins of *S. aureus* have primarily been characterized as hemolysins and leukocidins [95]. However, there is evidence that these secreted cytolysins may contribute to mTSS through enhancement of local inflammation and disruption of the epithelial barrier thus increasing superantigen penetration into the mucosa [66,70]. In this study, we examined the binding and signaling capabilities of the gamma-toxin cytolysins in female reproductive tract cells and tissues.

The *S. aureus* cytolysins are pore-forming toxins that are initially secreted as monomers [150]. The monomer subunits insert into target cell membranes, oligomerize and form pores thereby causing cation efflux, osmotic imbalance and cell lysis [150]. Previous studies have linked gamma-toxins to pathogenesis of *S. aureus* in murine bacteremia and septic arthritis and demonstrated lysis of human neutrophils, macrophages, and red blood cells [85,87,93,101]. The gamma-toxin genes (*hlgA, hlgB,* and *hlgC)* are the most up-regulated cytolysins transcribed when *S. aureus* is exposed to blood and are present in nearly all strains (99%) [84,85]. However, despite widespread prevalence and induction after exposure to blood, little is known about gamma-toxin interactions with the

vaginal epithelium, a mucosal surface commonly colonized by *S. aureus* and regularly exposed to blood.

The primary mediator of mTSS is TSST-1 which is responsible for nearly 100% of cases and the only superantigen capable of causing disease from the vaginal mucosa in animal models [33]. However, TSST-1 must penetrate the epithelium to gain access to its primary targets, T-cells and antigen presenting cells to cause mTSS. While TSST-1 can flux across the vaginal mucosa independently, penetration through tissue is enhanced when epithelial integrity is compromised [70]. Disruption of epithelial integrity can result from direct injury through cell lysis or as a result of inflammation. Independently, TSST-1 can induce proinflammatory cytokines in immortalized human vaginal epithelial cells (HVECs) through activation of (a) disintegrin and metalloproteinases (ADAM-10 and-17) resulting in shedding of epidermal growth factor (EGFR) ligands and subsequent feedback activation of EGFR [67,122]. While EGFR-signaling is strongly associated with homeostasis and growth of epithelial cells, the EGFR-signaling pathway is also a component of an innate immune response to injury [122]. Another *S. aureus*-encoded cytolysin, alpha-toxin, which is structurally and functionally similar to gamma-toxin, increases TSST-1 penetration through disruption of the vaginal mucosa and is also synergistic with TSST-1 in stimulating cytokine production [70]. Synergy between alpha-toxin and TSST-1 may result from co-stimulation of ADAM10, an epithelial receptor for alpha-toxin, leading to increased shedding of EGFR ligands [72]. However, alpha-toxin is likely to play a role in only a subset of mTSS cases as it is produced by less than 20% of

mTSS clinical isolates, whereas gamma-toxins are almost ubiquitously produced and thus likely contribute to pathogenesis across a range of *S. aureus* infections [112].

In this study, the effects of the gamma-toxins, HlgAB and HlgBC, on the vaginal epithelium were investigated. We examined the cytotoxicity of gamma-toxins and their ability to induce proinflammatory cytokines in *in vitro* and *ex vivo* human and porcine vaginal mucosa models. We found that gamma-toxins (HlgAB and HlgBC) were cytotoxic to HVECs and induced production of proinflammatory cytokines at sub-cytolytic doses. Additionally, the pathway by which gamma-toxins stimulate proinflammatory effects in vaginal epithelial cells was partially characterized with respect to known interactions of the *S. aureus* toxins TSST-1 and alpha-toxin. Gamma-toxins were determined to induce cytokine production in HVECs, which was dependent on EGFR-signaling. In *ex vivo* full thickness porcine vaginal mucosa, a model for human vaginal mucosa, and *ex vivo* human ectocervical tissues, gamma-toxins stimulated a potent dose-dependent IL-8 response, which was attenuated through inhibition of EGFR with a tyrosine kinase inhibitor (TKI), AG1478. Binding of gamma-toxin to HVECs was also characterized by flow cytometry and potential receptors identified through co-precipitation of gamma-toxin subunits and HVEC proteins followed by LC-MS/MS analysis. We hypothesized that gamma-toxins play a role in disease at the vaginal mucosa through direct cytotoxic and proinflammatory actions through the EGFR signaling pathway. Overall, our data support this hypothesis and denote novel roles for gamma-toxins in *S. aureus*-induced diseases.

2.2 Materials and Methods

Gamma-Toxin Purification

Gamma-toxin HlgB was cloned from *S. aureus* strain Newman (Primers: GGCCGT-CGACAAAGAAACTGAAAACAATAAATAGCTA, GGCCGGAT-CCGGGTATAGGGGTTTTAGTATGACATC) into the pET30a plasmid incorporating an N-terminal histidine tag then transformed into chemically competent *E. coli* (BL21, Invitrogen). Expression was induced in Luria broth (kanamycin 30 μg/ml) containing 0.3 mM IPTG. Bacterial pellets were lysed using lysozyme (100 μg/ml) and sonication. The supernatant was sterile filtered and incubated with Ni-NTA resin (Invitrogen) to bind the HlgB subunit. Due to poor expression in *E. coli*, gamma-toxin HlgA and HlgC were cloned into a plasmid (pOS1) and transformed into *S. aureus* strain Newman (Obtained from Dr. Victor Torres, NYU). Expression was induced in Todd-Hewitt Broth containing chloramphenicol (10 μg/ml). The bacteria supernatants were sterile filtered and incubated with Ni-NTA resin (Invitrogen) to bind the subunits. The proteins were eluted from the resin with an imidazole gradient. Endotoxin from HlgB preparation was removed using Pierce High Capacity Endotoxin Removal Resin per manufacture's protocol (PI88267). Final endotoxin concentration was calculated by LAL assay and determined to be non-stimulatory in any of our models (<0.125 EU/ml, Data not shown). Biotinylated protein was produced by using an EZ-Link NHS-PEG_4-Biotinylation Kit (Thermo, 21455) per manufacturer's instructions. Briefly, protein was dialyzed into PBS overnight at 4°C then incubated with a 20mM excess of NHS-PEG_4-Biotin for 30 minutes at room temperature. Excess biotin was removed by dialyzing into PBS overnight at 4°C.

Hemolytic Assay

Rabbit erythrocytes were harvested fresh, pelleted, and washed 3x with PBS (Sigma) then resuspended to a final volume of 5% (v/v) in PBS 0.4% agar (Mercury) autoclaved and cooled to 45°C. Agar slides were prepared with 0.3 mm wells. Gamma-toxin HlgAB or HlgBC was incubated in PBS, 10 µl per well, diluted 1:1 from 2.5-640 ng per well, for 4 h at 37°C with 7 % CO_2. Individual subunits HlgA, HlgB, and HlgC were incubated at 640 ng as a negative control. Zones of hemolysis were calculated as the area of the outer zone minus area of the well.

Porcine Tissue

Porcine tissue was collected and processed prior to treatment as previously described [151]. Briefly, porcine vaginal tissue was collected at time of slaughter in RPMI Media 1640 (Gibco, 11875-093) supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), amphotericin B (2.5 µg/ml) and gentamicin (40 µg/ml). Antibiotics were included to eliminate native flora. Within 3 h of slaughter 5 mm biopsies were taken and excess muscle tissue trimmed. The biopsies were washed three times in then incubated half-submerged for 30 min at 37^{O}C antibiotic-free RPMI. Following washing, the biopsies were placed mucosal side up on PET track-etched 0.4 µm cell culture inserts (BD Biosciences) in 6-well plates above fresh antibiotic- and serum-free media.

Cytokine Assay: Porcine Tissue

Processed porcine vaginal tissue biopsies were treated topically with gamma-toxin HlgAB or HlgBC, at 1:1 molar ratios and incubated for 6 h at 37 ^{O}C and 7% CO_2. After

treatment with gamma-toxin, biopsies were washed three times with PBS and each biopsy homogenized in 250 µl PBS. Homogenates were centrifuged at 13,000 rpm for 1 min and the supernatant assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8 (DY535).

Inhibitors: Porcine

Porcine vaginal tissue biopsies were prepared as described earlier. Prior to treatment with gamma-toxin (HlgAB or HlgBC at 1:1 molar ratio), the explants were pre-treated at -30 and -10 min with 8 µl of 4mM AG1478 (Tocris Bioscience) in 10 % DMSO (aq). The explants were incubated for 6 h and assayed for IL-8 as described earlier.

Cell Cultures

HVECs (ATCC CRL-2616 [108]) were grown in tissue culture-treated flasks in keratinocyte serum-free media (KSFM) (Gibco, 17005-042) supplemented with bovine pituitary extract (50 µg/ml), recombinant human epidermal growth factor (0.2 ng/ml), $CaCl_2$ (0.4mM), penicillin (25 IU/ml), streptomycin (25 µg/ml), gentamicin (40 µg/ml), and amphotericin B (2.5 µg/ml); referred to as complete media. Cells were maintained at $37^{O}C$ and 7% CO_2. For all assays, 96-well plates were seeded with HVECs at 50,000 cells/well and grown for 24 h in complete media, then the media was replaced with KSFM supplemented with $CaCl_2$ (0.4mM), referred to as minimal media, for 24 h prior to treatment.

Cytokine, Shedding, and Cytotoxicity Assay: Cell Culture

Cells were treated in 96 well plates with 100 µl minimal media containing gamma-toxin subunits HlgAB or HlgBC at 1:1 molar ratios for 6 h at $37^{O}C$ and 7% CO_2. After

incubation, the media was collected and assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8, IL-6, TNF-α, or MIP-3α (DY208, DY206, DY210, DY360) or the EGFR ligands AREG, TGF-α, and HB-EGF (DY262, DY240, DY259). Following removal of the supernatant for cytokine assays, the media was replaced with 100 µl minimal media supplemented with 20 µl MTS tetrazolium (Promega, CellTiter-96 G3582) and incubated for 2 h at 37^{O}C and 7% CO_2. The optical density was measured and viability calculated as (*(treated – 100% lysis) / (Untreated – 100% Lysis)*).

Inhibitors: Cell Culture

Inhibitors were added at twice the final concentration in 50 µl of minimal KSFM 30 minutes prior to the addition of gamma-toxin, HlgAB or HlgBC containing 1:1 molar ratio of individual subunits, in a final volume of 100 µl for 6 h at 37 ^{O}C in 7% CO_2. AG1478 (Tocris Bioscience) was used at a final concentration of 1 µM, TAPI (Enzo Life Sciences) at 50 µM, and UO126 (Tocris Biosciences) at 10 µM. Inhibitor concentrations for HVECs were chosen based on previous studies [67].

Flow Cytometry

HVECs were grown as described earlier, trypsinized, and resuspended at 5x10^5 cells/ml. Cells were treated with 25 µg/ml of the biotinylated (*b*-) *b*-HlgA, *b*-HlgB, or *b*-HlgC for 30 mins on ice, the equivalent dose used for cytokine and shedding assays. Non-biotinylated HlgB at 25 µg/ml was also incubated with biotinylated HlgA or HlgC. Cells were washed three times with PBS and incubated with 250 ng streptavidin r-phycoerythrin conjugated (Strep-PE, Peirce, 21627) at 5 µg/ml in PBS for 20 min. The cells were fixed in 2% paraformaldehyde for 15 mins on ice, washed with PBS, and analyzed by flow

cytometry (FACSCalibur, BD Biosciences) for 20,000 events. Cells were gated based on forward and side scatter area. Positive selection gate for Strep-PE was defined to exclude 99% of the Strep-PE control subset.

Human Tissue

Human vaginal ectocervix was obtained from UMN BioNet Tissue Procurement post-surgery. 5mm biopsies were taken from normal tissue, trimmed of excess connective tissue, washed three times in minimal KSFM, and incubated half-submerged in minimal KSFM for 30 min at 37 ^{O}C in 7% CO2. Following washing, the biopsies were placed mucosal side up on PET track-etched 0.4 μm cell culture inserts (BD Biosciences) in 6-well plates above minimal KSFM. The explants were treated at -30 and -10 mins with 8 μl of 4 mM AG1478 (Tocris Bioscience) in 10 % DMSO (aq) prior to the addition of gamma-toxin (HlgAB or HlgBC 1:1) for 6 h at 37 ^{O}C in 7% CO2. Following treatment, the biopsies were washed three times in PBS then homogenized in 250 μl PBS. Homogenates were centrifuged at 13,000 rpm for 1 min and the supernatant assayed by ELISA for IL-8 using manufactures protocol (R&D, DY208).

Synergy

HVECs were seeded at 50,000 cells per well in a 96-well plate with complete KSFM for 24 h, then the media removed and replaced with minimal KSFM for 24 h at 37^{O}C at 7% CO_2. The media was replaced with 100 μl of minimal KSFM containing 50 μg/ml TSST-1, the maximum dose which does not elicit a significant response, or minimal KSFM. Gamma-toxin HlgAB or HlgBC was diluted 1:1 from 6.25 – 25 μg/ml with or without TSST-1at 50 μg/ml and incubated for 6 h at 37^{O}C at 7% CO_2. After

incubation, the media was collected and assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8 (DY208).

Pull Downs

HVECs were grown to confluence ($2.25x10^7$ cells) in a T225 flask (Corning, 431082) in complete KSFM at 37 ^{O}C in 7% CO_2. Cells were lysed with 4 ml of Nonidet P-40 lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40) with a Roche complete protease inhibitor tablet for 5 mins on ice. Lysates were pelleted at 14,000 rpms for 5 min at 4 ^{O}C and the soluble fraction transferred to equilibrated streptavidin agarose resin (Pierce, 20357) at 1ml lysate per 50 µl resin bed volume and incubate for 30 min at 4 °C to remove non-specific binding. Lysates were removed and in biotinylated HlgB was added at a concentration of 100 µg/ml and incubated for 1 h at 4 ^{O}C. After 1 h, 50 µl bed volume of fresh equilibrated streptavidin agarose resin were added and incubated for an additional 1 h at 4 °C. The lysate was removed and the resin was washed 3x in 1 ml of PBS at 4°C and the resin resuspended in 60 µl Laemmli sample buffer (Bio-Rad, 161-0737EDU). The sample was run on 4-20% mini-protean TGX gel (Bio-Rad, 456-1096) and imaged with Bio-Rad Silver Stain (161-0443) following the manufacture's protocol. Following staining, a gel band present at ~130 kD present in the *b*-HlgB treated cells and not present in untreated cells or *b*-HlgB preparation was excised, destained, and sent to the University of Minnesota Center for Mass Spectrometry and Proteomics for sequencing. Briefly the gel was destained with 30 mM potassium ferricyanide with 100 mM sodium thiosulphate, and then washed with water. The proteins in-gel were reduced and alkylated with 75 ul 10 mM DTT in 10 mM ammonium bicarbonate followed by 5ul

of 55 mM iodoacetamide in 100 mM NH_4HCO_3 then washed with 1:1 acentonitrile:100mM ammonium bicarbonate followed by 100% acetonitrile . The proteins were rehydrated in digestion buffer containing trypsin 12.5 ng/µl 50 mM NH_4HCO_3 5 mM $CaCl_2$ (aq) and terminated with 0.1% formic acid. The peptides were analyzed in 0.1% formic acid 5:95 H_2O:acetonitrile using a LTQ ion trap mass spectrometer (Thermo Finnigan), a linear ion trap by MS scan and MS/MS. Protein fragments were analyzed by Scaffold software (Proteome Software, v 4.4.1) and sequences compared by BLAST (NCBI, BLASTP Suite) to Pubmed database.

2.3 Results

2.3.1 Gamma-toxin Hemolytic Activity

Gamma-toxins have been previously reported to be hemolytic to rabbit erythrocytes [87]. Hemolysis was observed as low as 25 ng/ml and showed dose dependent lysis of rabbit erythrocytes in soft agar in the nanogram range (Figure 2.1). The biological activity of our purified recombinant gamma-toxins was comparable to previously reported studies. Gamma-toxin HlgAB or HlgBC at 250 ng/ml generated a large zone of hemolytic activity in soft agar and this correlated with 250 ng/ml of gamma-toxin showing near complete lysis for erythrocyte suspensions in PBS [87]. This assay confirmed the biological activity of our gamma-toxin preparation was similar to those used in previous studies.

FIGURE 2.1: Validation of Gamma-toxin Hemolytic Activity

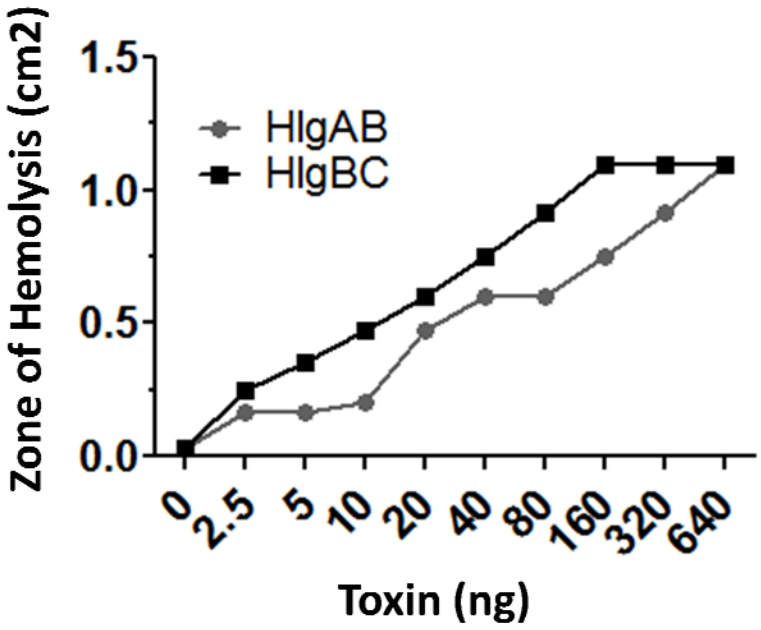


Figure 2.3.1: Gamma-toxin is hemolytic to rabbit erythrocytes. *Gamma-toxin was incubated in 100 µl PBS at listed dose at a 1:1 molar ratio of gamma-toxin HlgAB or HlgBC in 0.3 mm diameter wells within 0.4% agar containing 5% rabbit erythrocytes for 4 h and the zone of hemolysis measured. Data represent areas of clearance (hemolysins) from individual wells.*

2.3.2 Gamma-Toxins are Cytotoxic and Proinflammatory to Vaginal Epithelial Cells

The potential of gamma-toxin to induce cytokine production in tissue was investigated using *ex vivo* PVM. Both HlgAB and HlgBC were proinflammatory to *ex vivo* porcine vaginal mucosa explants, stimulating IL-8 production in a dose-dependent fashion (Figure 2.2). HlgAB and HlgBC significantly stimulated IL-8 production over untreated tissue at nanogram amounts of toxin (125-250 ng/biopsies). HlgBC stimulated a more potent IL-8 response in porcine tissue than HlgAB, generating greater than 2-fold IL-8 production at proinflammatory doses. The data suggest that gamma-toxins may play a role in outside in signaling at the vaginal mucosa. However, due to the potential presence of tissue resident immune cells, known targets of gamma-toxins and sources of IL-8 production, we investigated the actions of gamma-toxins in epithelial cell cultures.

Both gamma-toxin HlgAB and HlgBC stimulated a dose dependent IL-8 response in HVECs at >50 μg/ml (Figure 2.3). Additionally, HlgAB and HlgBC were both found to be cytotoxic at higher concentrations (>100-200 μg/ml). In contrast to *ex vivo* porcine vaginal mucosa, HlgAB stimulated a greater IL-8 response and enhanced cytotoxicity over HlgBC in HVECs. Furthermore, gamma-toxin HlgAB and HlgBC at sub-cytotoxic doses (50 μg/ml) stimulated production of IL-6, TNF-α and MIP-3α, with gamma-toxin HlgAB being more potent than HlgBC at inducing cytokine production (Figure 2.4). These data suggest that gamma-toxins disrupt the vaginal mucosa through direct cytotoxic and proinflammatory effects on the vaginal epithelium.

FIGURE 2.2: Gamma-toxins Stimulate IL-8 Production in Porcine Vaginal Mucosa

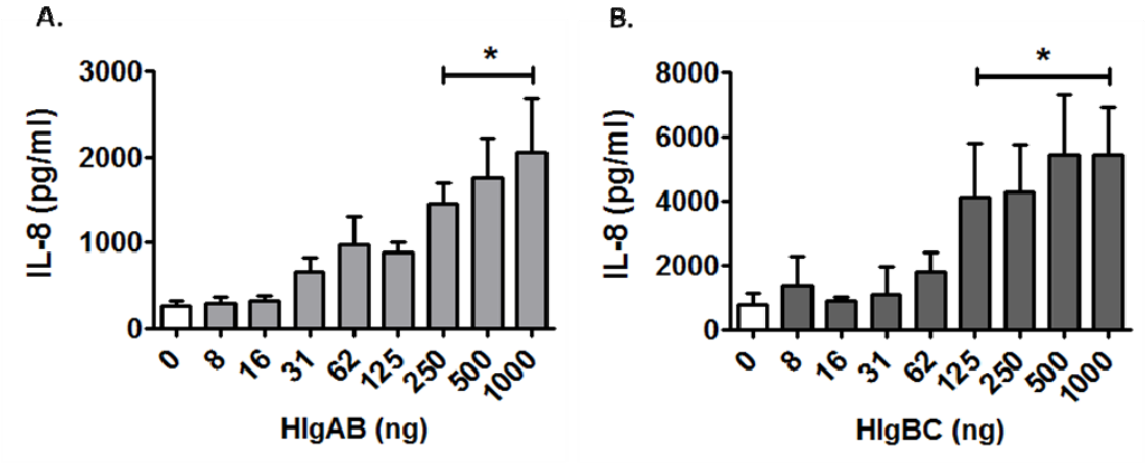


Figure 2.2: Gamma-toxin stimulates IL-8 production in porcine vaginal mucosa biopsies. *Tissue was treated topically with (**A**) HlgAB or (**B**) HlgBC at listed doses at a 1:1 molar ratio in 4 µl TBS. Data represent IL-8 concentrations +/- SEM from homogenized biopsies 6 h after treatment. Gamma-toxin significantly induced (*, p value <0.05) IL-8 production over control, analyzed by one way ANOVA with Dunnet's correction. Data are combined averages from 3 experiments each with n=3.*

FIGURE 2.3: Gamma-toxin Induced Cytotoxicity and IL-8 Production in HVECs

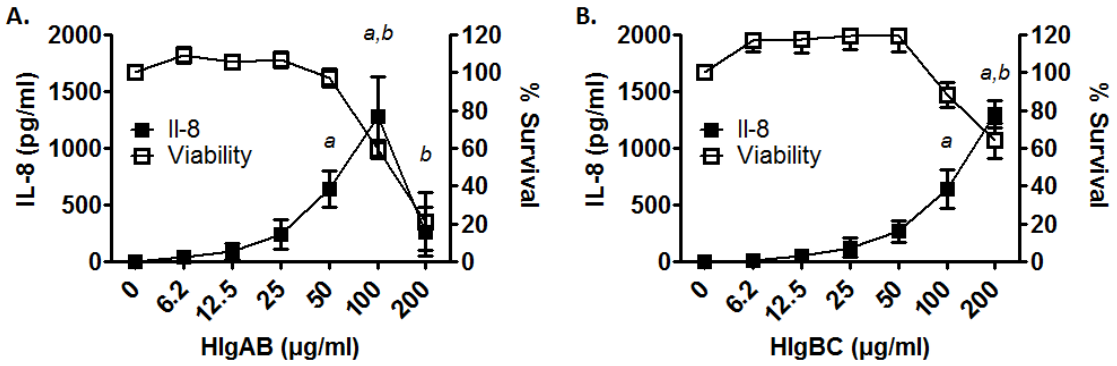


Figure 2.3: Gamma-toxins induce (■) IL-8 production and (□) cytotoxicity in HVECs. *Cells were incubated with (**A**) HlgAB or (**B**) HlgBC at indicated doses at a 1:1 molar ratio in 100 µl minimal keratinocyte serum free media. Data show IL-8 concentration +/- SEM in the media and cell viability as compared to untreated controls after 6 h. Gamma-toxin significantly induced (p value <0.05) (**a**) IL-8 production and (**b**) cell death over untreated control, analyzed by one-way ANOVA with Dunnet's correction. Data are combined averages from 3 experiments each with n=3.*

FIGURE 2.4: Gamma-toxins Induce Cytokine Production in HVECs

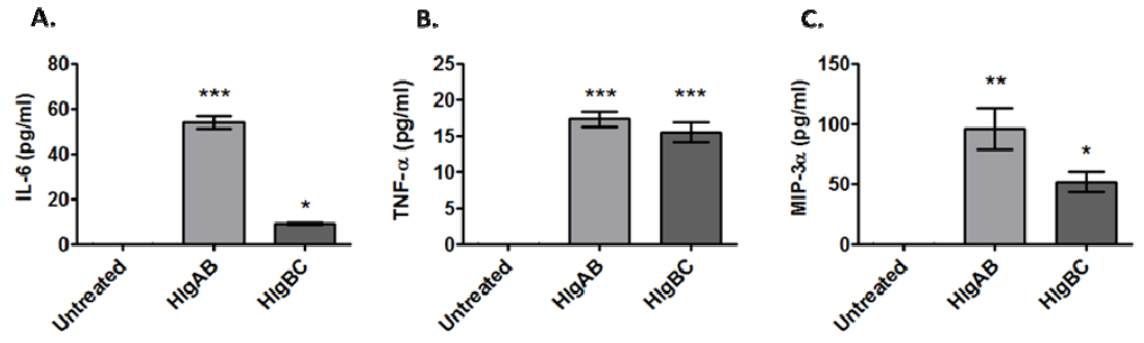


Figure 2.4: Gamma-toxins induce production of proinflammatory cytokines in HVECs. *Cells were incubated with 50 µg/ml HlgAB or HlgBC at a 1:1 molar ratio in 100 µl minimal keratinocyte serum free media. Data show (**A**) IL-6, (**B**) TNF-α, and (**C**) MIP-3α concentrations +/- SEM in the media following 6 h post treatment. Gamma-toxin significantly induced cytokine production *,**,*** (p value < 0.05, 0.01, 0.001) over untreated controls, analyzed by one-way ANOVA with Bonferroni's correction. Data are combined averages from 3 experiments each with n=3.*

2.3.3 Activity of Gamma-toxin is Dependent on Both Subunits

The pro-inflammatory effects of gamma-toxin required the formation of heterodimeric aggregates of either HlgAB or HlgBC. HVEC or porcine tissue treated with individual subunits of gamma-toxin HlgA, HlgB, and HlgC at 50 μg/ml or 1000 ng/explant respectively did not induce cytokine production in either model when compared to HlgAB or HlgBC at 50 μg/ml or 1000 ng/explant gamma-toxin at 1:1 molar ratio (Figure 2.5). The lack of IL-8 production in either HVECs or porcine vaginal mucosa by individual gamma-toxin subunits also confirms that the pro-inflammatory effects are not a result of a contaminant or non-specific interactions. Minor production of IL-8 was observed in porcine tissue treated with HlgB at 1000 ng, but never reached significance when compared to untreated controls. The minor effect by HlgB is not attributed to LPS contamination. Tissue treated with HlgB was exposed to <0.01 EU per explant of exogenous endotoxin, and the minimum dose of endotoxin required to induce IL-8 production in porcine vaginal mucosa was determined to be ~12.5 EU per explant (Data not shown). Combined these results demonstrate that cytokine production induced by gamma-toxin at the vaginal epithelial surface is dependent on HlgAB or HlgBC heterodimer formation. Furthermore, if the individual subunits of gamma-toxin bind to a HVECs receptor, it is likely that the activation of the receptor is dependent on the HlgAB or HlgBC heterodimer as well.

FIGURE 2.5: Activity of Individual Gamma-toxin Subunits

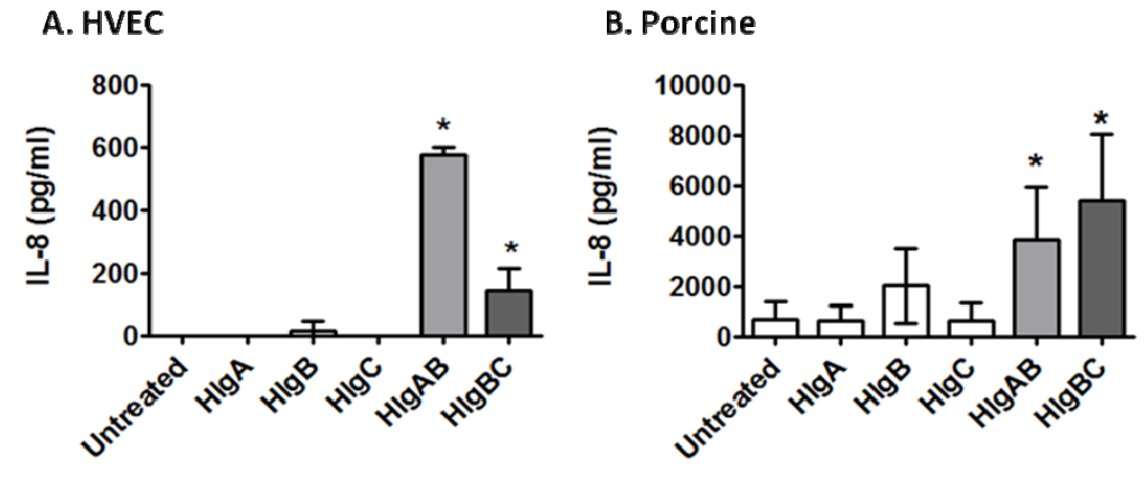


Figure 2.5: Individual subunits of gamma-toxin do not induce IL-8 production. *(A) HVECS treated with HlgAB or HlgBC at 50 µg/ml at a 1:1 molar ratio or 50 µg/ml of the individual subunits in 100 µl minimal keratinocyte serum free media. (**B**) Ex vivo porcine vaginal explants treated with HlgAB or HlgBC at 1000 ng/explant at a 1:1 molar ratio or 1000 ng/explant of the individual subunits in TBS. Data show IL-8 concentration +/- SEM in the media as compared to untreated controls after 6 h. HlgAB and HlgBC significantly induced IL-8 production over untreated controls while individual subunits did not (*, p value <0.05, Students t test). Data are combined averages from 3 experiments each with n=3.*

2.3.4 Gamma-Toxin Stimulates Shedding of EGFR Ligands in HVECs

Because exotoxins like TSST-1 and α-toxin stimulate HVEC cytokine production through EGFR signaling, we examined the role of the EGFR in the response to gamma-toxin [67]. The IL-8 response to gamma-toxins in HVECs was dependent on ADAMs and EGFR. Through inhibition of EGFR-signaling via AG1478, the IL-8 response of HVECs to gamma-toxin HlgAB and HlgBC at 50 μg/ml or 100 μg/ml, respectively, was attenuated greater than 2-fold (Figure 2.6A, 2.6B). Inhibition of MAP kinase via UO126, also attenuated the IL-8 response to 50 μg/ml HlgAB or 100 μg/ml HlgBC by >2 fold (Figure 2.6C, 2.6D). MAP kinase is a downstream signaling kinase of EGFR-dependent NF-κB activation. Peculiarly, the HVEC IL-8 response to 50 μg/ml HlgAB could be attenuated through inhibition of ADAMs via TAPI-1, whereas the IL-8 response to 100 μg/ml HlgBC was not affected (Figure 2.6A, 2.6B). This differential inhibition of the HVEC IL-8 response to gamma-toxins suggests that each toxin stimulates an EGFR-dependent cytokine response through a different mechanism.

Shedding of EGFR ligands in HVECs was stimulated by gamma-toxins. Differential shedding of EGFR ligands in HVECs was observed in response to gamma-toxin HlgAB or HlgBC. Gamma-toxin HlgAB at 50 μg/ml stimulated shedding of amphiregulin and TGF-α, known to be shed by ADAM-17 (Figure 2.7A&B). Interestingly, HlgBC did not significantly stimulate shedding of amphiregulin and TGF-α, but did stimulate shedding of HB-EGF (Figure 2.7C). These data help explain the differential attenuation of gamma-toxin induced IL-8 production in HVECs by TAPI-1 and

suggest that gamma-toxin induced cytokine production is facilitated through shedding of EGFR ligands.

FIGURE 2.6: Inhibition of Gamma-toxin Induced IL-8 Production in HVECS

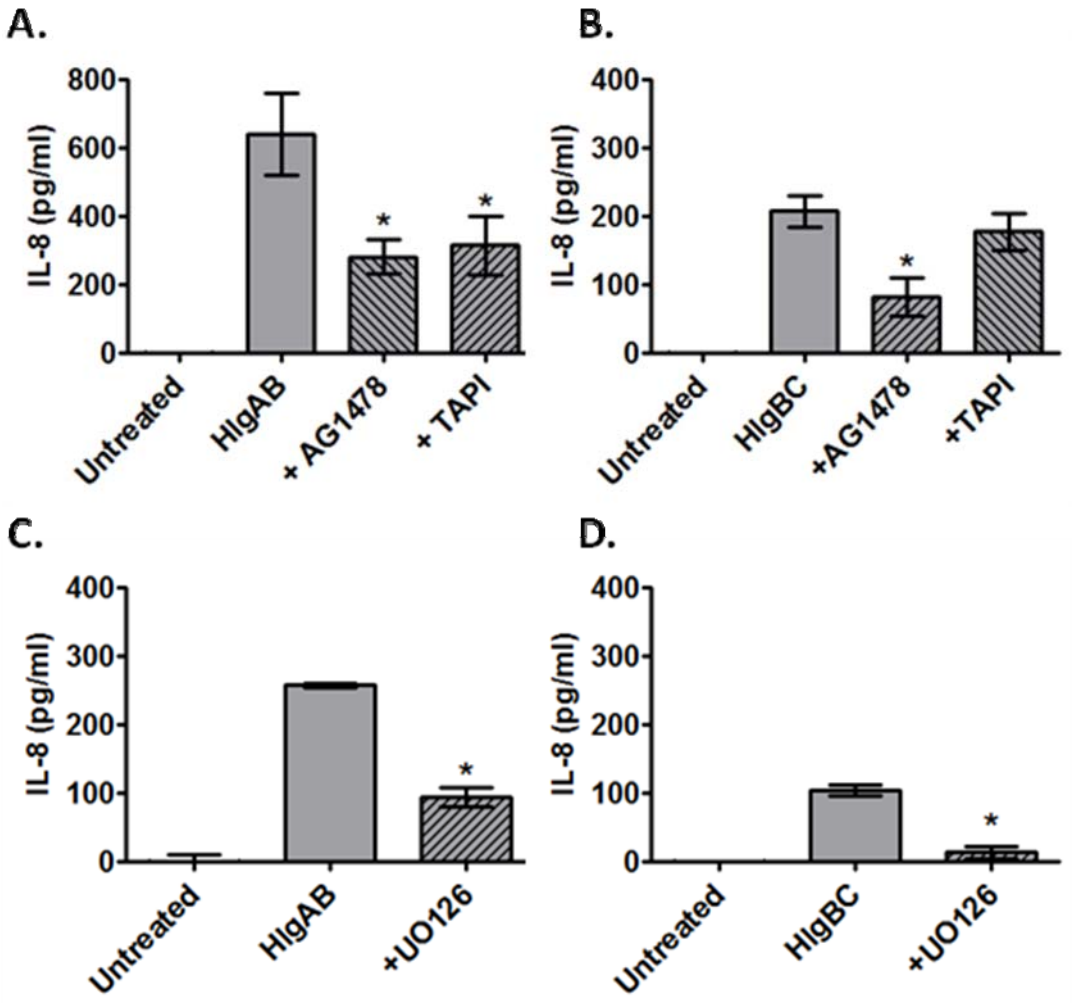


Figure 2.6: EGFR and ADAM inhibition attenuate gamma-toxin induced IL-8 production in HVECs. *Cells were incubated with the lowest dose of gamma-toxin required for cytokine production, either 50 µg/ml HlgAB or 100 µg/ml HlgBC at a 1:1 molar ratio in 100 µl minimal keratinocyte serum free media (**A, B**) with the EGFR inhibitor AG1478 at 1 µM or the ADAM inhibitor TAPI-1 at 50 µM. Data show IL-8 concentrations +/- SEM in the media 6 h after treatment. HlgAB activity was attenuated by both EGFR and ADAM*

*inhibition, while HlgBC was only affected by EGFR inhibition. EGFR dependent IL-8 production was confirmed through inhibition of MAP kinase, a downstream target of EGFR NF-κB signaling, with UO126 at 10 μM (**C, D**). The inhibitors significantly reduced gamma-toxin-induced IL-8 production (*, p value <0.05) analyzed by one way ANOVA with Bonferroni's correction. Data are combined averages from 3 experiments each with n=3.*

FIGURE 2.7: Gamma-toxin Induced shedding of EGFR Ligands in HVECs

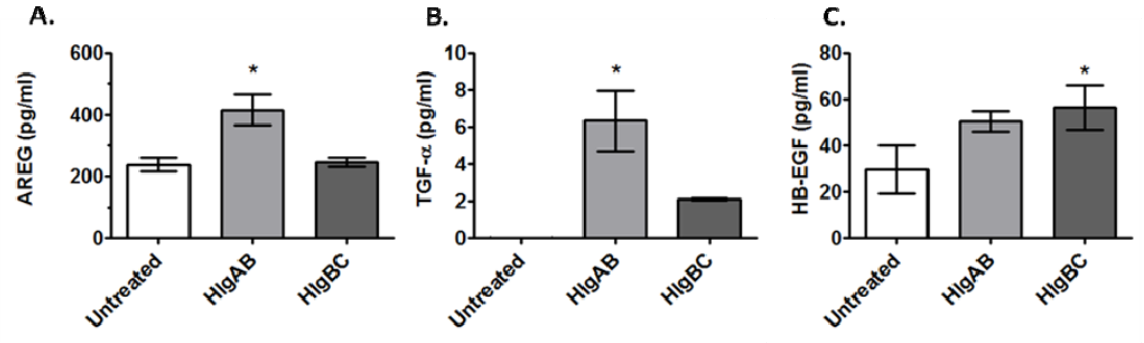


Figure 2.7: Gamma-toxin induces shedding of EGFR ligands in HVECs. *Cells were incubated with 50 µg/ml HlgAB or 100 µg/ml HlgBC at a 1:1 molar ratio in 100 µl minimal keratinocyte serum free media. Data show (**A**) amphiregulin, (**B**) tumor growth factor-α, and (**C**) heparin-binding EGF concentrations +/- SEM in the media 6 h after treatment. Gamma-toxin significantly induced EGFR ligand shedding (*, p value < 0.05) over untreated controls as determined by one way ANOVA with Bonferroni's correction. Data are combined averages from 3 experiments each with n=3.*

2.3.5 Gamma-toxin Induced IL-8 Production in *Ex Vivo* Tissue is Attenuated through Inhibition of EGFR

Inhibition of EGFR-signaling attenuated the IL-8 response to gamma-toxins in *ex vivo* porcine vaginal mucosa. Since both gamma-toxin HlgAB and HlgBC act through an EGFR-dependent mechanism, we investigated the ability of AG1478 to inhibit the IL-8 response in an *ex vivo* model. To determine the ability of AG1478 to inhibit the gamma-toxin-induced IL-8 response in full-thickness porcine vaginal mucosa we performed a dose escalation study with AG1478, starting at 1 nmol, on tissue exposed to 500 ng HlgBC per explant (Figure 2.8A). AG1478 inhibited the porcine vaginal mucosal IL-8 response to gamma-toxin HlgBC at nanomolar doses (8-12 nmol per 5 mm biopsy). Additionally, AG1478 (8 nmol per explant) attenuated the IL-8 response in porcine vaginal mucosa from 500 ng per explant of HlgAB or HlgBC (Figure 2.8B). The data demonstrate that even in a tissue model, which contains multiple cell-types and tissue-resident immune cells, the gamma-toxin-induced IL-8 response is dependent on EGFR-signaling.

Gamma-toxins stimulated IL-8 production in *ex vivo* human tissue through an EGFR dependent mechanism. Human ectocervix tissue was used to validate HVEC and porcine results instead of vaginal tissue due to availability following hysterectomies. Fresh premenopausal human vaginal tissue could not be obtained in a reasonable time-frame due to vaginectomies being rare and the lack post-mortem donors. Tissue treated with gamma-toxin HlgAB (1000 ng) or HlgBC (125-1000 ng) showed a dose dependent increase in IL-8 production. Significant IL-8 production was observed with HlgBC at 1000 ng per explant, and could be attenuated to baseline through inhibition of EGFR with 8 nmol of AG1478

(Figure 2.9). Human tissue showed high inter-person variability in background IL-8 levels, ranging from 500-3000 pg/ml, which proportionally affected the tissue response to gamma-toxin (data not shown). However, the IL-8 response of the tissue to gamma-toxins was consistent when normalized to fold change in IL-8 over baseline. These data demonstrate that gamma-toxins stimulate IL-8 production in human vaginal mucosal tissue which is dependent on EGFR signaling.

FIGURE 2.8: Inhibition of Gamma-toxin Induced IL-8 Production in Porcine Vaginal Mucosa

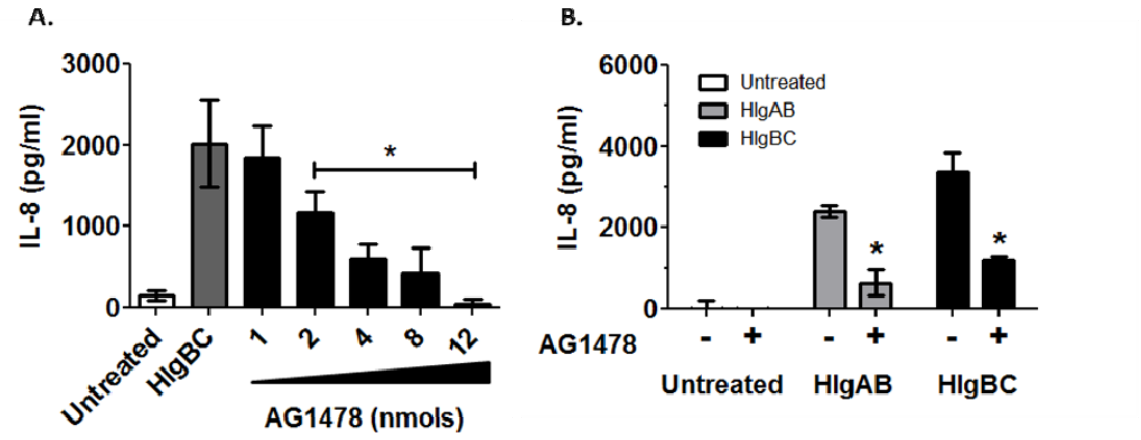


Figure 2.8: EGFR inhibition attenuates gamma-toxin induced IL-8 production in ex vivo porcine vaginal mucosa. *(**A**) Tissue was treated topically with 500 ng/explant of HlgBC and AG1478 at listed dose or (**B**) 500 ng/explant of HlgAB or HlgBC and 8 nmol AG1478 in 16 µl 10% DMSO. Data represent IL-8 concentrations +/- SEM from homogenized biopsies 6 h after treatment. AG1478 significantly reduced gamma-toxin induced IL-8 production (*, p value <0.05) as analyzed by (**A**) one-way ANOVA with Bonferroni's correction compared to HlgBC treated or (**B**) Students t-test as compared to stimulated. Data are combined averages from 3 experiments each with n=3.*

FIGURE 2.9: Inhibition of Gamma-toxin Induced IL-8 Production in Human Tissue

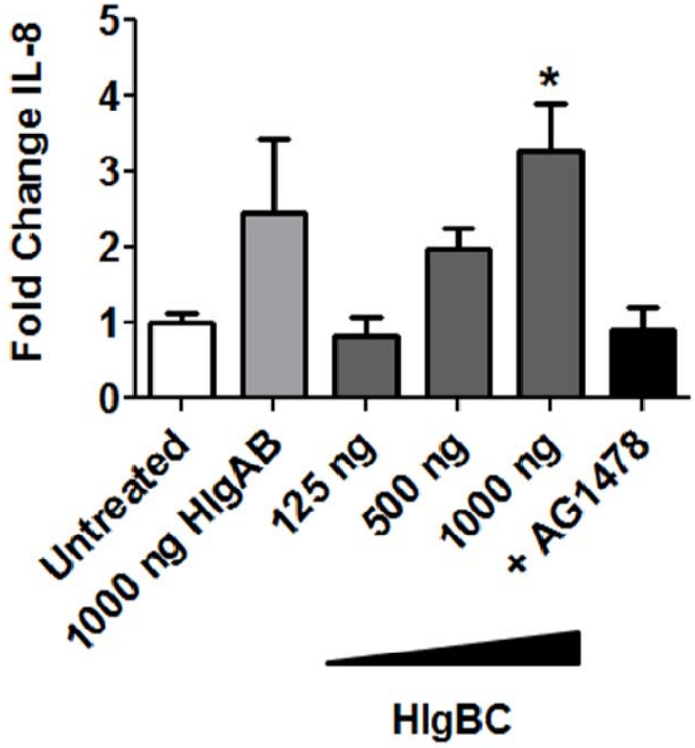


Figure 2.9: Inhibition of gamma-toxin induced IL-8 in ex vivo human ectocervix tissue. *Tissue was treated topically with HlgAB or HlgBC at listed dose at a 1:1 molar ratio in 16 µl TBS or HlgBC at 1000 ng/explant with 8 nmol AG1478. Data represent fold change in IL-8 concentrations +/- SEM from homogenized biopsies 6 h after treatment. Fold change in IL-8 over baseline was used due to high inter-person variability in background IL-8. HlgBC significantly stimulated IL-8 production compared to untreated or in combination with AG1478 (*, p value <0.05 for both), and HlgAB did not significantly increase IL-8 concentrations (-0.66 to 3.6 95% CI) as analyzed by one-way ANOVA with Bonferroni's correction. Data represent 3 combined experiments with n=3 for each.*

FIGURE 2.10: Gamma-toxin Induced IL-8 Production in Human Tissue is not affected by DMSO

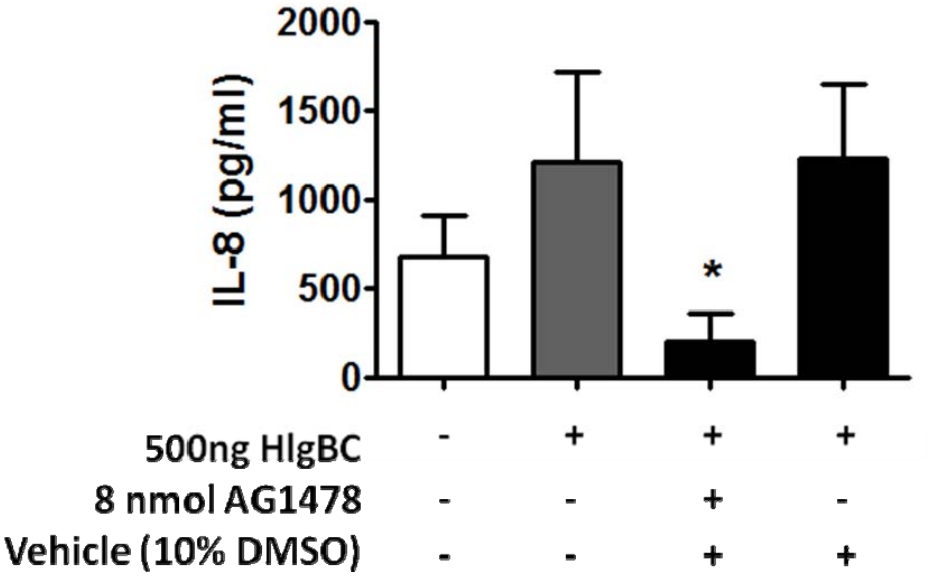


Figure 2.10: Delivery vehicle does not reduce inflammation. *Ex vivo human ectocervix was incubated with HlgBC 500 ng/explant at a 1:1 molar ratio with 8 nmol AG1478 in 10% DMSO. Data show IL-8 concentrations +/- SEM. AG1478 significantly reduced IL-8 from treated tissue with no effect observed from 10% DMSO (*, p value <0.05). Data are representative of 3 independent experiments with n=3.*

2.3.6 Gamma-toxins Enhance Proinflammatory Effects of TSST-1 in HVECs

Since the cytolysin alpha-toxin is known to enhance TSST-1 induced IL-8 production in HVECs, the ability of gamma-toxins HlgAB and HlgBC to enhance TSST-1 induced IL-8 production in HVECs was investigated [67,70]. Cells incubated with a minimal proinflammatory dose of TSST-1 (50 μg/ml) showed enhanced IL-8 production when incubated with gamma-toxin HlgAB or HlgBC (Figure 2.11). The increase in IL-8 production in HVECs upon co-incubation of HlgAB and TSST-1 was approximately twice the sum of the response to each individual toxin across all doses tested (6.25 – 25 μg/ml). The combined effect of TSST-1 and HlgBC in HVECs was similar but less pronounced. These data suggest that the gamma-toxins HlgAB and HlgBC may potentiate the proinflammatory effects of TSST-1 at the vaginal mucosa by enhancing cytokine production.

FIGURE 2.11: Synergistic Interactions between Gamma-toxins and TSST-1 Induce IL-8 Production in HVECs

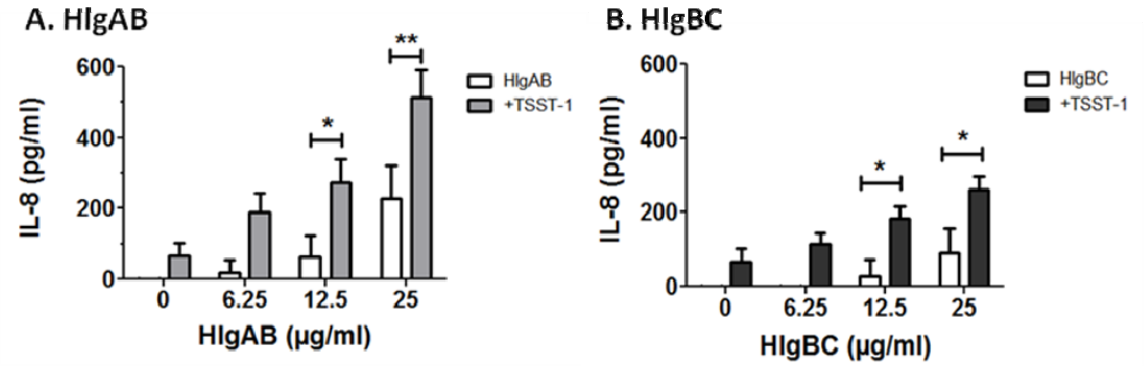
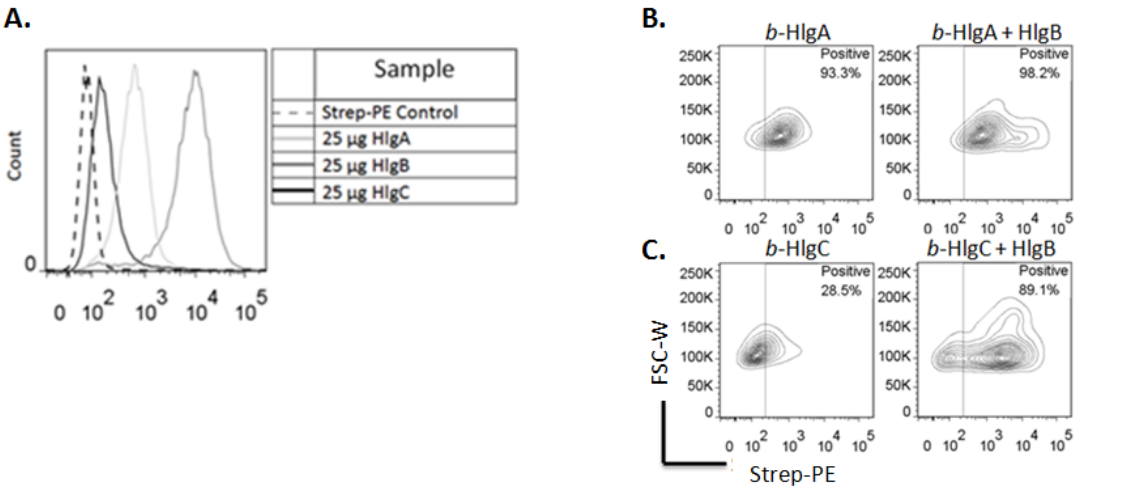


Figure 2.11: Gamma-toxin enhances TSST-1 stimulation of IL-8 production in HVECs. *Cells treated with a subinflammatory dose of TSST-1(50 µg/ml) and a minimal inflammatory dose range of gamma-toxins HlgAB and HlgBC at 1:1 molar ratio in minimal keratinocyte serum free media for 6 h produced significantly more IL-8 than either individual toxin. Data are shown as the mean ± SD. Synergy was defined as a significant difference between the observed IL-8 production from gamma-toxin + TSST-1 and the null-hypothetical sum of the individual response (*, p value <0.05, student's t-test) Data are the combined means of 3 experiments each with n=3.*

2.3.7 HlgB Facilitates Binding of HlgA and HlgC to HVECs

Gamma-toxin subunits exhibit differential binding to HVECs (Figure 2.12A). The biotinylated *b*-HlgA and *b*-HlgB subunits both bound HVECs with high affinity at ~93% and ~97% positive cells, respectively. The *b*-HlgB exhibited higher intensity with ~$1x10^4$ events per cell and *b*-HlgA with ~$6x10^2$ events per cell. The *b*-HlgC bound poorly with 29% cells positive with an intensity of ~$1.5x10^2$ events per cell. With *b*-HlgB binding with the greatest affinity, we sought to determine if HlgB could facilitate the binding of *b*-HlgA or *b*-HlgC to HVECs. Upon co-incubation of non-biotinylated HlgB with either *b*-HlgA (Figure 2.12B) or *b*-HlgC (Figure 2.12C) there was a shift in the percent positive cells and signal intensity. The percent of *b*-HlgA positive cells increased to ~98% and more notably *b*-HlgC positive cells increased to 89%, indicating that HlgB, and heterodimer formation, drives binding of gamma-toxins to epithelial cells. These data suggest that both HlgA and HlgB bind HVECs and HlgC binding is dependent on HlgB, whereas HlgA binding is only slightly enhanced. Additionally, these data support the enhanced IL-8 production observed by HlgAB over HlgBC in HVECs, by increased binding intensity of HlgA and HlgB.

FIGURE 2.12: HlgB Component Facilitates Binding of Heterodimeric Gamma-toxins to HVECs



Subunit	% Bound	Intensity	%Bound + HlgB	Intensity + HlgB
HlgB	99%	++++	-	-
HlgA	93%	++	**98%**	**++++**
HlgC	28%	+	**89%**	**+++**

Figure 2.12: Gamma-toxin HlgB facilitates binding of HlgA and HlgC to HVECs. *HVEC suspensions were incubated with 25 µg/ml of each biotinylated gamma-toxin subunit for 30 min prior to fixation and staining. (**A**) Data show relative binding of individual subunits on HVECs. Biotinylated HlgA and HlgC were incubated with non-biotinylated HlgB at 50 µg/ml total toxin at a 1:1 molar ratio for 30 min prior to fixation and staining. HlgB increased the percentage of cells bound and the intensity of binding for both biotinylated (**B**) HlgA and (**C**) HlgC. The table indicates percent-positive cells for each subunit with or without non-biotinylated HlgB and the relative signal intensity. Data are representative of 3 experiments.*

2.3.8 Protein Identification

Laminin subunits co-precipitate with gamma-toxin HlgB upon incubation in HVEC lysate (Table 2.1). Laminin-γ2 (rf15741138) and laminin-β3 (rf189083719) subunits were identified by LC-MS/MS within a ~130 kDa band present in HVEC lysates treated with *b*-HlgB after incubation in streptavidin agarose resin (Figure 2.13). The molecular weight of laminin-γ2 and laminin-β3 are 131 and 130 kDa respectively and are components of Laminin-332.While the percentage coverage of laminin-γ2 and laminin-β3 was low (~10%) the analysis identified 10 and 5 unique peptide fragments, respectively. The peptides masses ranged from 932-1691 Da. A Pubmed BLAST analysis against all known proteins of humans, *S. aureus* and *E. coli* relieved that ~25% of the total peptides present in the 130 kDa band were unique to laminin-γ2 and laminin-β3. The laminin-γ2 and laminin-β3 were present at approximately 2:1 ratio, respectively. An intracellular mitochondrial pyruvate carboxylase of 130 kDa was also identified, but was excluded as a possible gamma-toxin target due to a lack of surface exposure when cells are intact. Laminin-332 is expressed at the basal epithelial surface and constitutes a major component of the extracellular matrix, playing an important role in cell adhesion and modulation of EGFR-signaling through co-localization [152,153]. The data suggest that the Laminin-332 may represent an extra-cellular binding protein for gamma-toxin, or play a role in gamma-toxin modulation of EGFR signaling.

FIGURE 2.13: Co- Precipitation of HVEC Lysate and Gamma-toxin HlgB

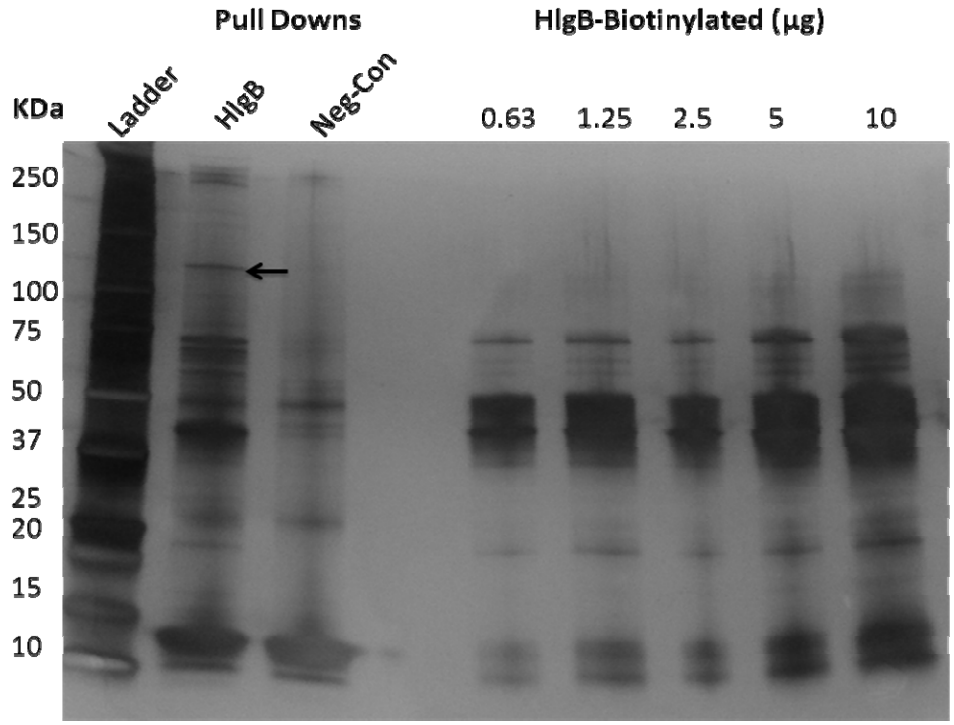


Figure 2.13: A single ~130KDa band is present in HVEC lysates treated with biotinylated gamma-toxin subunit HlgB following precipitation from streptavidin resin. *The 130KDa band (denoted with an arrow) was the only visible band observed across 3 independent experiments that was not present in HVEC lysates treated with biotin or the gamma-toxin HlgB preparation. Image shows cell lysates separated on a 4-20% Mini-PROTEAN TGX gel followed by silver staining.*

TABLE 2.1: Protein Identification

Protein		Ref. ID.	Size	Unique Peptides*
Laminin-β3	Structural Laminin-332 Component	189083719	130 kDa	10
Laminin-γ3	Structural Laminin-332 Component	157419138	131 kDa	5

Table 2.1: Proteins identified by co-precipitation which may interact with gamma-toxin subunit HlgB. *Laminin-332 is a known modulator of EGFR. A mitochondrial pyruvate carboxylases was also present in the fraction but was considered unlikely to interact with gamma-toxin when cells are intact due to intracellular localization. *Number of peptide fragments identified which were unique to respective protein.*

2.4 Discussion

S. aureus colonization is prevalent in the community with >30% of the population asymptomatically colonized at any time, with mucosal surfaces being the primary sites of colonization [3]. In this study, a potential novel pathway, EGFR-signaling, was determined to be induced by gamma-toxins, which produced a pro-inflammatory response in vaginal epithelial cells and disrupted the vaginal mucosa. These findings describe a new means by which *S. aureus* may gain access to the host. These novel actions of gamma-toxins highlight the importance of this under-studied toxin, and further elucidate the complex mechanisms by which *S. aureus* utilizes an arsenal of toxins to promote disease and invasion from mucosal sites.

Gamma-toxins were independently cytotoxic and stimulated production of proinflammatory cytokines (IL-8, IL-6, TNFα, and MIP-3α) by vaginal epithelial cells. The cytokine profile induced by gamma-toxins closely mimics the cytokine profile induced by other *S. aureus* toxins, including TSST-1 [65]. Based on other studies, the chemo-attractant response elicited from vaginal epithelial cells by gamma-toxins is suspected to promote recruitment of neutrophils, leukocytes, and dendritic cells, which may create a favorable environment for disease [154,155]. *S. aureus* is well adapted to resist killing by neutrophils, and multiple toxins have been implicated in promoting their recruitment including TSST-1, Panton-Valentine leukocidin and phenol-soluble modulins [86,113]. Furthermore, it is speculated that over-recruitment of neutrophils during *S. aureus* infection promotes tissue damage through increased inflammation and aids in *S. aureus* dissemination within the host [155,156]. Additionally, the cytokines produced in response

to gamma-toxins promote recruitment and activation of leukocytes and dendritic cells, which are known to traffic superantigens to draining lymph nodes where non-specific activation and expansion of T-cells can occur, potentiating TSS [115].

Redundancies in *S. aureus* virulence are common with production of numerous leukocidins with considerable homology and overlap in targets [95,157]. However, it would appear that these redundancies extend across toxin subsets in their mechanisms to induce inflammation. TSST-1 and alpha-toxin have been classically characterized as a superantigen and a cytolysin, respectively [33]. Recently, these toxins have been identified to be disruptive to the epithelium through receptor specific interactions. Alpha-toxin is known to interact with ADAMs, resulting in disruption of cell-cell junctions and ADAM expression is required for fatal disease in mice [69]. Additionally, TSST-1 was recently shown to stimulate a cytokine response in HVECs through an ADAM and EGFR-dependent pathway [67]. Our findings that gamma-toxins also interacts with this pathway suggests that EGFR-signaling may be commonly exploited by *S. aureus* to gain access to the host through inflammation and disruption of membrane integrity or to induce a favorable innate immune response.

Activation of EGFR in HVECs by gamma-toxins appears to be through stimulation of shedding of EGFR ligands, specifically AREG and TGF-α by HlgAB and HB-EGF by HlgBC. Interestingly, TSST-1 also induces shedding of AREG and TGF-α in HVECs [67]. Shedding of EGFR ligands in response to TSST-1 and gamma-toxins in HVECs appears to be mediated by ADAMs, which are known to facilitate this process [129]. ADAM activation, either directly or upstream, subsequently leads to autocrine and paracrine

feedback activation of EGFR, phosphorylation of MAP kinase and activation of NF-κB, which ultimately results in induction of the innate immune response [66,68,122]. The activation of EGFR mediated cytokine production in HVECs by multiple *S. aureus* exotoxins highlights the importance of this process during pathogenesis, and presents a potential host-target in attenuating progression of mTSS.

Inhibition of EGFR is a proven mechanism for prevention of cell division, and this strategy is used in treatment of EGFR over-expressing tumors [132]. However, we observed that AG1478 can act as a pathway-dependent anti-inflammatory agent. Attenuation of cytokine production induced by gamma-toxins at the vaginal mucosa via inhibition of EGFR-signaling raises potential clinical implications. Through the use of a TKI (AG1478) we blocked the IL-8 response to gamma-toxins in HVECs, *ex vivo* porcine vaginal mucosa and *ex vivo* human ectocervical tissue. Other studies have shown that AG1478 can inhibit the IL-8 response in HVECs to TSST-1 and reduce shedding of EGFR ligands, likely due to inhibition of feedback signaling [15]. Currently there are FDA approved TKIs that target EGFR and the inhibitor included in these studies, AG1478, is a derivative of the approved drug gefitinib [138]. This raises the possibility of local inhibition of EGFR as adjunct therapy for treatment of mTSS or other mucosal infections as a means to shut down a proinflammatory response induced by, and beneficial to, *S. aureus*.

Binding of gamma-toxin to cells is both subunit and cell-type specific. In phagocytes, gamma-toxin HlgAB binds to neutrophils and monocytes and HlgBC binds to monocytes, suggesting that HlgA and HlgC mediate target cell specificity [96]. However, binding of

gamma-toxin to erythrocytes is initiated by HlgB through an unknown receptor [91]. We demonstrated that HlgB binds with high affinity to HVECs and facilitates the binding of HlgA and HlgC. Furthermore, we observed a possible interaction of HlgB with the laminin-γ2 and laminin-β3 subunits. The laminin-γ2 and laminin-β3 combined with laminin-α3 make up laminin-332, which is a major component of the ECM and mediator of cell migration, which is highly expressed on epithelial basement membranes cells [152,158,159]. The laminin-γ2 also modulates EGFR signaling by co-localization resulting in disruption of tight-junctions and downstream activation of ERK1/2 signaling, and the subsequent up-regulation of MMPs [152]. While laminin-332 is not membrane bound, it directly binds to numerous membrane bound proteins, including integrins [153] Combined, this suggests that gamma-toxin may interact with laminin-332 to modulate EGFR signaling in HVECs, and possibly utilize laminin-332 as an extracellular binding protein to associate gamma-toxin with epithelial cells. Alternatively, binding to laminin-332 by HlgB may be a separate activity of gamma-toxin, independent of epithelial cell effects, where HlgB binding to laminin-332 disrupts the extracellular matrix. However, further studies are needed to confirm any interactions of gamma-toxins with laminin-332.

Differential effects of gamma-toxin HlgAB and HlgBC have been observed both across cell-types and across species. The two forms of gamma-toxin exhibit overlap in their specificity with both being able to lyse neutrophils, but only HlgBC has lytic and proinflammatory activity against human macrophages [93,95]. In our study, HlgAB was more cytotoxic and induced greater IL-8 production in HVECs than HlgBC. The HlgA and HlgB subunits also exhibited high binding affinity to HVECs, with HlgB minimally

increasing binding of HlgA. This is in contrast to HlgBC, which was less cytotoxic and proinflammatory in HVECs with HlgC requiring HlgB to associate with HVECs. However, in *ex vivo* tissue HlgBC was notably more proinflammatory than HlgAB at equivalent doses. The enhanced potency in *ex vivo* tissue may be due to cellular differences of the epithelial cells, or HlgBC stimulating tissue-resident macrophages, which are known to synergize with the epithelial response to enhance cytokine production [93].

From our observations, I propose the following mechanism of action by gamma-toxins HlgAB and HlgBC (Figure 2.14). HlgAB binding begins with lamin-332 or an unknown G-protein coupled receptor. This leads to downstream activation of ADAM17 through an unknown mechanism and modulation of the EGFR proinflammatory signaling by gamma-toxin [160]. ADAM17 induces shedding of AREG and TGF-α, which are potent EGFR ligands that undergo endocytic recycling [121]. Activation of EGFR-signaling induces downstream activation of ERK1/2, which phosphorylates ADAM17 further inducing shedding of EGFR ligands resulting in NF-κB induced IL-8 production [68,130]. I propose that HlgBC first interacts with laminin-332 or an unknown G-protein coupled receptor. Possible activation of ADAM10, or another sheddase, then occurs through an unknown mechanism which induces shedding of HB-EGF, and subsequent activation of EGFR-signaling resulting in NF-κB induced IL-8 production [68]. HB-EGF does not undergo endocytotic recycling, and is a less potent EGFR ligand than AREG and TGF-α [121]. Additionally, ERK1/2 is not known to interact with ADAM10, which may explain the less potent response of HlgBC in HVECs [121]. Combined, these data

suggest a differential mechanism of action by HlgAB and HlgBC on HVECs, which produce overlapping inflammatory effect.

This study demonstrates a pathogenic activity of gamma-toxins on the vaginal mucosa. Furthermore, it highlights the importance of ADAMs and EGFR in *S. aureus* mucosal pathogenesis, a pathway shared by multiple *S. aureus* exotoxins. Redundancies in *S. aureus* virulence factors have hindered therapeutic development. However, if the redundant exotoxins promote disease through a shared host pathway, this raises the potential of host-targeted interventions as therapy for *S. aureus* infections.

Figure 2.14: Proposed Proinflammatory Signaling Pathway activated by Gamma-toxins in HVECs

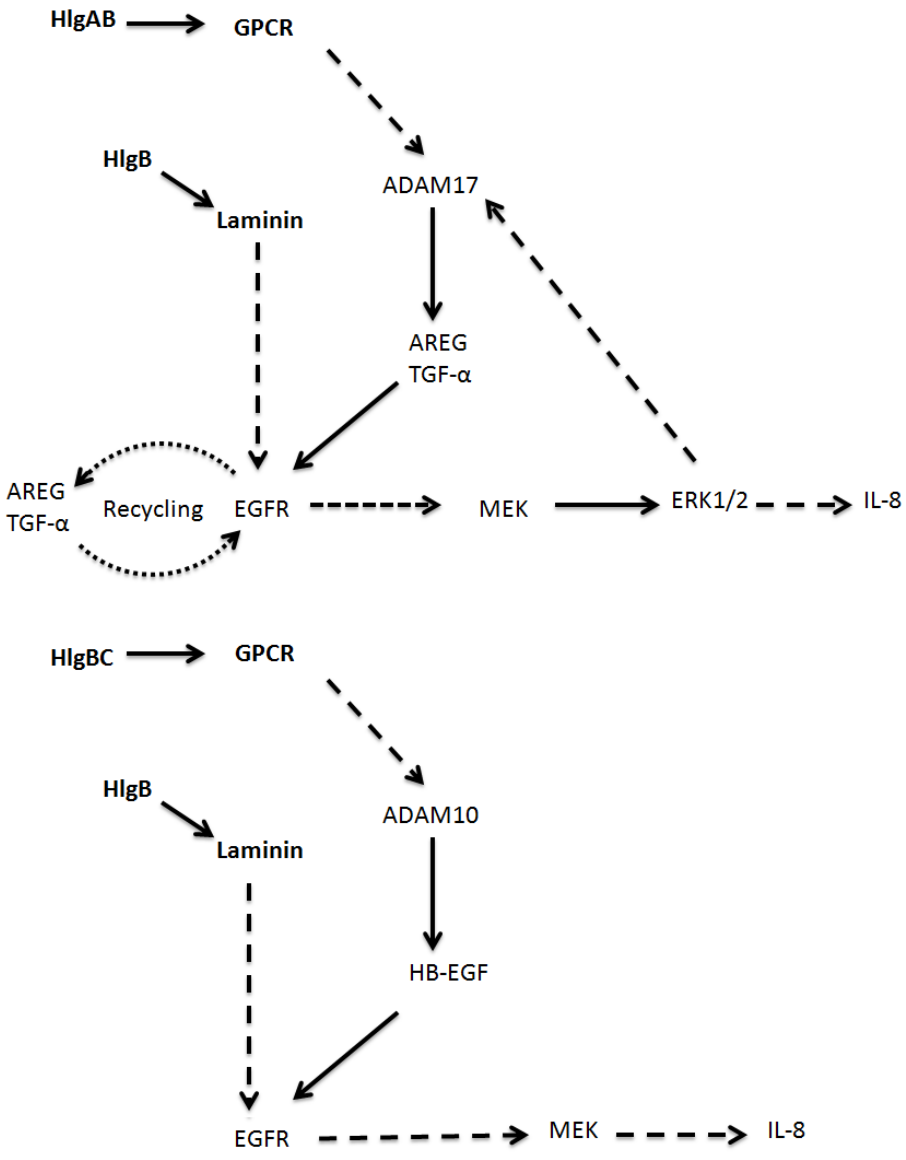


Figure 2.14: Proposed signaling pathways induced by gamma-toxins in HVECs. *The possible interactions of (**A**) HlgAB or (**B**) HlgBC with known or possible receptors and the signaling pathway activated. Solid line denotes direct interactions and dashed for indirect activation.*

CHAPTER III.

Alpha-toxin of *S. aureus* enhances the Lethal Effects of TSST-1 at the Vaginal Mucosa

Sections of this study have been submitted for publication.
Non-animal experiments were performed by Dr. Laura Breshears and me in collaboration. Animal studies were performed by Dr. Schlievert in collaboration with us.

3.1 Introduction

Alpha-toxin of *S. aureus* is primarily characterized as a cytolysin and associated with invasive tissue infections, but this important exotoxin has recently been implicated in cell signaling. In immortalized human vaginal epithelial cells (HVECs), alpha-toxin enhances TSST-1 induced cytokine production at subcytolytic doses [70]. Cytokine production induced by TSST-1 in HVECs is dependent on epidermal growth factor receptor (EGFR) signaling [67]. Furthermore, alpha-toxin is known to interact with sheddases of EGFR ligands in immortalized human-lung cells [72]. Combined, this supports the possibility of both alpha-toxin and TSST-1 activating cell-signaling through a converging pathway. Thus, we investigated the ability of alpha-toxin to activate EGFR-signaling and enhance TSST-1 pathogenesis.

EGFR is a critical receptor with numerous ligands, co-factors and feedback loops, which regulates homeostasis and the innate immune response in mammalian cells [68]. In epithelial cells, EGFR plays a primary role in wound healing, inflammatory responses and tight junction integrity [122,161,162]. Dysregulation of EGFR contributes to a variety of disease conditions including cancer, pulmonary diseases and folliculitis [163–165]. The role of EGFR for each disease varies significantly and is dependent on a plethora of co-factors, which modulate the intensity of each potential EGFR signaling cascade [68]. Clinically, targeting EGFR with chemotherapeutics or antibodies is already standard of care for several types of cancer [133,134].

Modulation of EGFR-signaling has already been implicated in the pathogenesis of *S. aureus* infections. In *S. aureus* pneumonia, lipoteichoic acid stimulates shedding of

heparin-binding epidermal growth factor (HB-EGF) by ADAM-10, independent of toll-like receptors, which activates EGFR in lung epithelial cells and promotes hyper-secretion of mucin [166]. *S. aureus* protein A also activates EGFR and TNF-α signaling pathways during early stages of infection, which promotes actin depolymerization causing loss of tight junction integrity in epithelial cells [167]. Shedding of EGFR ligands induced by TSST-1 and the subsequent activation of NF-κB plays a critical role in lethality of mTSS in rabbit infection models [66,67]. Modulation of EGFR signaling, either directly or indirectly, appears to play a critical role in the pathogenesis of *S. aureus*.

Alpha-toxin contributes significantly to diseases of *S. aureus* and is being pursued as a therapeutic target by multiple companies. Traditionally, alpha-toxin has been characterized as a pore-forming hemolytic and dermonecrotic toxin [33]. Recently, alpha-toxin was identified to modulate mucosal epithelial-cell signaling by binding to ADAM10 leading to intracellular dephosphorylation of focal adhesion points, which results in loss of tight junction integrity [72]. Furthermore, alpha-toxin is cytotoxic and acts synergistically with TSST-1 to induce IL-8 production in HVECs [70]. This may result from ADAM10 shedding of HB-EGF and subsequent activation of EGFR and the downstream effectors ERK1/2, which subsequently activates ADAM17 [128,130]. TSST-1 also stimulates ADAM10 and ADAM17 dependent shedding of EGFR ligands in HVECs [67]. Thus, the enhanced IL-8 production from the combination TSST-1 and alpha-toxin in HVECs may result from increased shedding of EGFR ligands by ADAM10/17.

Activation of EGFR-signaling is a major inducer of cell proliferation. Alpha-toxin has been previously characterized to induce cell proliferation in immortalized human skin keratinocytes (HaCaTs) at sublytic doses [168]. The mitogenic effects of alpha-toxin are evident only 72 h after a short 2 h application and subsequent removal of alpha-toxin [168]. Notably, the cell proliferation could be inhibited upon co-incubation with anti-EGFR antibodies or EGFR-specific inhibitors [168]. However, inhibition of EGFR-signaling would cause general inhibition of cell growth and survival, independent of any mitogens [161]. Furthermore, the contribution of alpha-toxin in disease may lead to abscess formation, but it is unlikely that mitogenic activity plays a role in the pathogenesis of mTSS as the mucosal epithelium is completely destroyed in patients who have succumbed to the disease [40]. Overall, this suggests that EGFR may be directly or indirectly modulated by alpha-toxin, but further investigations are needed to confirm any biologically relevant interactions.

The local pro-inflammatory effects caused by TSST-1 and alpha-toxin at the vaginal mucosa likely play a critical role in pathogenesis during mTSS. Notably, when porcine vaginal mucosa is exposed to TSST-1, a majority of the toxin localizes in the *lamina propria*, suggesting the toxin has an affinity to be retained within the tissue [111]. Additionally, TSST-1 is actively transported by dendritic cells to local lymph nodes, suggesting that the proinflammatory actions of TSST-1 may be localized to the mucosa and local lymphatic systems [115]. Further evidence to support local pro-inflammatory effects include a TSST-1 mutant (D130A), which is not proinflammatory to HVECs but retains superantigenicity, vaginal permeability and is lethal when administered

intravenously to rabbits, but loses lethality when administered intravaginally [117]. Furthermore, the D130A TSST-1 mutant penetrates across the vaginal mucosa at a rate nearly twice the wild type, which further suggests that toxin penetration does not play a significant role during mTSS [117].

The goal of this study is to examine the synergistic interactions between alpha-toxin and TSST-1 in promoting disease by *S. aureus* at the vaginal mucosa. We hypothesize that like TSST-1, alpha-toxin stimulates a pro-inflammatory response at the vaginal mucosa mediated through an ADAM- and EGFR-dependent mechanism. Furthermore, we hypothesize that the combined pro-inflammatory effects of TSST-1 and alpha-toxin at the vaginal mucosa are critical for menstrual toxic shock progression. The data presented support these hypotheses, revealing the importance of local EGFR signaling during staphylococcal mucosal infections. Additionally, the data demonstrate a rationale for pursuing inhibition of EGFR as a possible therapeutic target.

3.2 Methods

Toxin Purification

Alpha-toxin was purified as previously described [70]. Briefly, *S. aureus* strain MNJA was grown in beef heart media and the supernatant precipitated with ethanol at 4 °C and resolublized in water. Two step isoelectric focusing was used. The first phase used a pH gradient from 3.5 to 10 followed by a second phase using a pH gradient from 7 to 9. The isoelectric point of alpha-toxin is 8.5. Purity was confirmed by SDS-PAGE and the yield of purified toxin was quantified using a Bio-Rad protein assay (Bio-Rad, 500-0006).

TSST-1 was purified from *S. aureus* by an established method using successive thin layer isoelectric focusing [64]. TSST-1 (1 mg/ml), thus prepared, contained no detectable LPS, as tested by Limulus assay (Sigma). Peptidoglycan, lipoteichoic acid, hemolysin, protease, or lipase (<1 part/10^6) were not detectable by combinations of bioassay and SDS-PAGE. Comparable results were obtained with multiple batches of TSST-1.

Cell Culture

Human Vaginal Epithelial Cells (HVECs) (ATCC CRL-2616) were grown in tissue culture-treated flasks using keratinocyte serum-free media (KSFM) (Gibco) supplemented with bovine pituitary extract (50 µg/ml), recombinant human epiderminal growth factor (0.2 ng/ml), $CaCl_2$ (0.4mM), penicillin (25 IU/ml), streptomycin (25 µg/ml), gentamicin (40 µg/ml), and amphotericin B (2.5 µg/ml); referred to as complete media. Cells were maintained at 37^O C and 7% CO_2. For all assays, 96-well plates were

seeded with HVECs at 50,000 cells/well and grown for 24 h in complete media then the media replaced with KSFM supplemented with $CaCL_2$ (0.4mM), referred to as minimal media, for 24 h prior to treatment.

Cytokine, Shedding, and Cytotoxicity Assay: Cell Culture

Cells were treated in 96 well plates with 100 µl minimal media with toxin for 6 h at 37^{O}C and 7% CO_2. After incubation, the media was collected and assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8, (DY208) or the EGFR ligands AREG, TGF-α, and HB-EGF (DY262, DY240, DY259). Following removal of the supernatant for cytokine assays, the media was replaced with 100 µl minimal media supplemented with 20 µl MTS tetrazolium (Promega, CellTiter-96 G3582) and incubated for 2 h at 37^{O}C and 7% CO_2. The optical density was measured and viability calculated as (*(treated – 100% lysis) / (Untreated – 100% Lysis)*).

Inhibitors: Cell Culture

Inhibitors were added at twice the final concentration in 50 µl of minimal KSFM 30 minutes prior to the addition of gamma-toxin, HlgAB or HlgBC at 1:1 molar ratio, in a final volume of 100 µl for 6 h at 37 ^{O}C in 7% CO_2. AG1478 (Tocris Bioscience) was used at a final concentration of 1 µM, TAPI (Enzo Life Sciences) at 50 µM, and UO126 (Tocris Biosciences) at 10 µM. Inhibitor concentrations for HVECs were chosen based on previous studies [67].

Synergy Experiments

Cells were treated with minimal media with or without 50 µg/ml TSST-1, a minimally inflammatory concentration, and increasing doses of alpha-toxin in a total

volume of 100 µl per well for 6 h at 37^{O} C 5% CO_2. Following incubation, supernatants were assayed for IL-8 via ELISA (R&D, DouSet ELISA DY208) and cell viability by MTS as described previously (Promega, CellTiter-96 G3582) [Chapter II]. The toxins were considered synergistic when the combined effect of toxins was greater than the hypothetical sum of the effects of each toxin individually.

Porcine Tissue

Porcine tissue was collected and processed prior to treatment as previously described (17). Briefly, porcine vaginal tissue was collected at time of slaughter in RPMI Media 1640 (Gibco, 11875-093) supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), amphotericin B (2.5 µg/ml) and gentamicin (40 µg/ml). Antibiotics were included to eliminate native flora. Within 3 h of slaughter 5 mm biopsies were taken and excess muscle tissue trimmed. The biopsies were washed three times then incubated half-submerged for 30 min at 37^{O} C in antibiotic-free RPMI. Following washing, the biopsies were placed mucosal side up on PET track-etched 0.4 µm cell culture inserts (BD Biosciences) in 6-well plates above fresh antibiotic- and serum-free media. The biopsies were then treated topically with toxin, TSST-1 or alpha-toxin, at listed concentrations in 2 µl PBS for 6 h. The inhibitor AG1478 (Tocris 1276) was applied 30 min and 10 min prior to toxin at 8 nmol per application. For IL-8 quantification, biopsies were homogenized in 250 µl PBS. Homogenates were centrifuged at 13,000 rpm for 1 min and the supernatant assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8 (DY535).

Penetration Studies

Porcine tissue, collected as described previously, was trimmed to 2 cm wide squares and excess muscle tissue trimmed. Tissue sections were placed in a Neoflon Dual Flow In-Line Cell (PermeGear 1KM21-11.28-VD) with the mucosal surface exposed to air and the basal surface exposed to a reservoir of PBS recycled at a flow of 1 ml/min. The exposed mucosa (1 cm diameter) was treated with TSST-1 (40 µg) with or without alpha-toxin (5 µg) and AG1478 (11.2 µg) in 100 µl PBS for 8 h at 37 °C. After incubation, the tissue is biopsied (5 mm) and washed 3x in PBS to remove external unbound toxin then homogenized in 250 µl PBS and assayed for TSST-1 to estimate tissue concentrations. The reservoir media was collected and assayed for TSST-1 to determine total penetration.

TSST-1 ELISA

The TSST-1 ELISA was developed in our laboratory. Polyclonal capture antibodies (Toxin Tech LTI101) were bound in PBS at 0.5 µg/ml to 96-well ELISA plates (Thermo YO-01928-08) overnight at room temperature. Samples were diluted appropriately in PBS 1% BSA (Sigma A7030) and incubated for 2 h at room temperature. Biotinylated detection antibodies (Toxin Tech SBTC101) were used at a concentration of 30 ng/ml in PBS 1% BSA for 2h at room temperature. Streptavidin-HRP (R&D DY998) was added for 20 min at room temperature. The plates were developed with TMB Solution (Invitrogen 00-2023) for 15 min at room temperature and stopped with 2N HCl. Wells were washed three times in PBS 0.05% Tween 20 between each step.

Animal Studies

Young adult Dutch belted rabbits weighing ~2 kg were obtained from Bakkom Rabbitry (Red Wing, MN). The rabbits were anesthetized with ketamine and xylazine for insertion of an intravaginal catheter. Alpha-toxin and TSST-1 were administered intravaginally in 0.2 ml PBS. In rabbits that received TSST-1, endotoxin from *Salmonella typhimurium* was administered intravenously 4 h after TSST-1. Surviving animals at the end of the experiment were euthanized with Euthasol. All animal experiments were performed according to established guidelines and a protocol approved by the University of Iowa Institutional Animal Care and Use Committee.

3.3 Results

3.31 Alpha-toxin Induces IL-8 in HVECs through ADAM and EGFR Signaling

Alpha-toxin was a potent inducer of IL-8 production in HVECs, stimulating a maximal response at a dose between 1.25-2.5 μg/ml after 6 h of treatment (Figure 3.1A). A decline in IL-8 production was observed at doses ≥5 μg/ml, which corresponded to ~70% reduction in cell viability measured by MTS (Figure 3.1B) (Cell Titer-96, Promega). A dose of 1 μg/ml consistently induced production of IL-8 while exhibiting minimal toxicity and was used to explore the mechanism of action. Since gamma-toxins stimulated shedding of AREG, TGF-α and HB-EGF, the ability of alpha-toxin to induce shedding of EGFR ligands was investigated [67]. Alpha-toxin was found to induce shedding of AREG (Figure 3.1D) but not TGF-α or HB-EGF (Data not shown). Alpha-toxin induced production of IL-8 and AREG shedding in HVECs was attenuated in the presence of AG1478 and TAPI-1, an EGFR and pan-ADAM inhibitor respectively (Figure 3.1C&D). To investigate the role of ADAMs in alpha-toxin signaling, ADAM10 and ADAM17 knockdown mutants were generated. ADAM10 and ADAM17 were chosen to investigate because alpha-toxin binds ADAM10 in lung epithelial cells and ADAM17 is the primary mediator of AREG shedding [72,128,130]. Shedding of AREG was reduced slightly (~18%) in ADAM10 HVEC knockdowns and nearly completely (~89%) in ADAM17 HVEC knockdowns (Figure 3.1F). Reduction in IL-8 production was observed in both ADAM10 HVEC knockdowns (~39%) and ADAM17 knockdowns (~60%) (Figure 3.1E). This suggests that both sheddases play a moderate role in the IL-8 response induced by alpha-toxin in HVECs with the dominant mediator being ADAM17.

In shedding of AREG, which is a potent EGFR ligand, it appears that ADAM17 is the mediator of shedding and ADAM10 only plays a minor role.

FIGURE 3.1: Alpha-toxin Cytotoxicity and IL-8 Production in HVECs

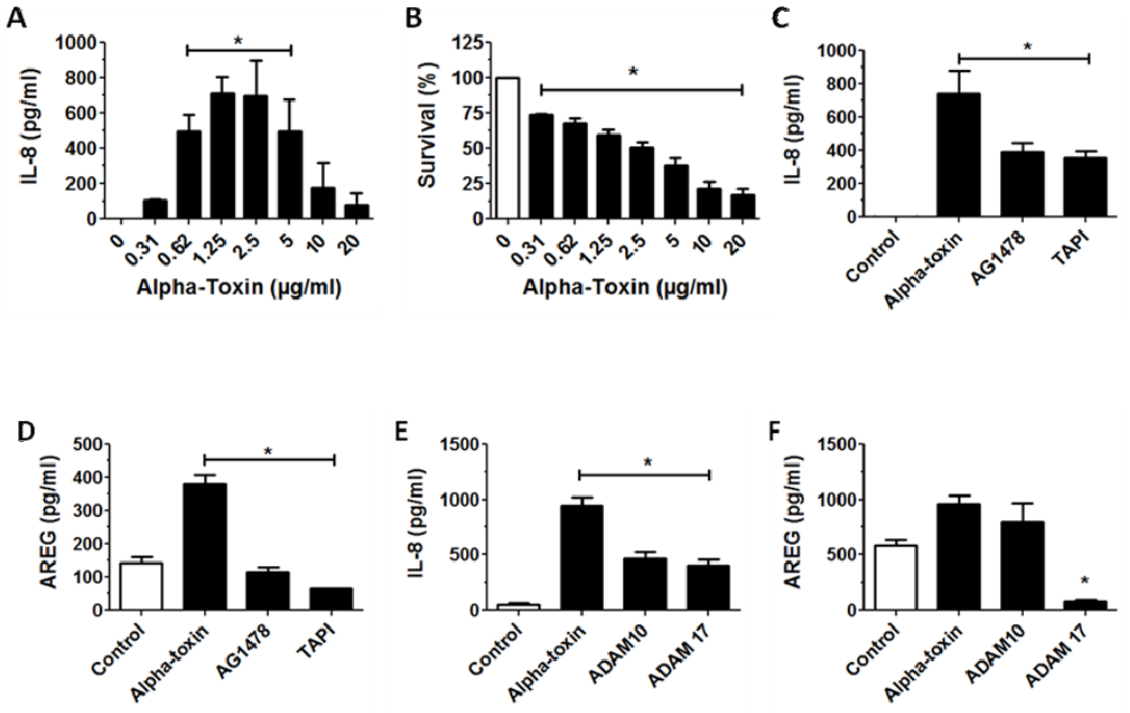


Figure 3.1: Alpha-toxin is proinflammatory to HVECs through ADAM dependent shedding of the EGFR ligand AREG*. (**A**) Cells treated with alpha-toxin exhibited a dose dependent IL-8 response over 6h. (**B**) Viability decreased at concentrations ≥5 µg/ml corresponding to a >50% decrease in cell viability. (**C, D**) Alpha-toxin (1 µg/ml) stimulated IL-8 production dependent on shedding of amphiregulin (AREG) by ADAMs and activation of EGFR. (**E, F**) Using siRNA, stimulation of IL-8 was confirmed to be dependent on ADAM10&17 while shedding of AREG was mostly dependent on ADAM17. Data are shown as the mean ± SD. Significant differences between untreated (**A, B**) or alpha-toxin treated (**C-F**) are denoted (*, p value <0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3.*

3.3.2 TSST-1 and Alpha-toxin are Synergistic in Stimulating IL-8 in HVECS

Superantigens and cytolysins are both proinflammatory to the vaginal mucosa and are known to act through overlapping pathways. We hypothesized that alpha-toxin and TSST-1 synergy in stimulating cytokine production was a result of cell signaling and independent of cytolytic activity. Synergy of TSST-1 and alpha-toxin in stimulating cytokine production is already known [70]. However, previous investigations utilized high doses of alpha-toxin >5 μg/ml and did not investigate cytotoxicity or the mechanism. We investigated the combined ability of TSST-1 and alpha-toxin at sub-stimulatory doses to enhance the HVEC cytokine response. Cells were treated with a non-proinflammatory to minimally proinflammatory dose of alpha-toxin (0.125-0.5 μg/ml) with or without 50 μg/ml of TSST-1, a minimally proinflammatory dose [66]. The HVEC response to TSST-1 combined with alpha-toxin was synergistic, with the combined response being significantly greater than the hypothetical sum of the individual responses (AB > A+B) (Figure 3.2A). However, the combined response was solely limited to cytokine production, with the cytotoxicity of alpha-toxin unaffected by incubation with 50 μg/ml of TSST-1 (Figure 3.2B). Alpha-toxin and TSST-1 both independently act through EGFR, so we investigated the ability of EGFR inhibition to attenuate the synergistic response of the combined toxins. Following pre-treatment with the EGFR inhibitor AG1478, the HVEC IL-8 response to the combined toxins was reduced 80-95% (Figure 3.2B). The data show that the combined synergy in cytokine response to TSST-1 and alpha-toxin is dependent on the activity of EGFR and independent of cytolytic activity.

FIGURE3.2: Alpha-toxin Synergy with TSST-1 is EGFR Dependent

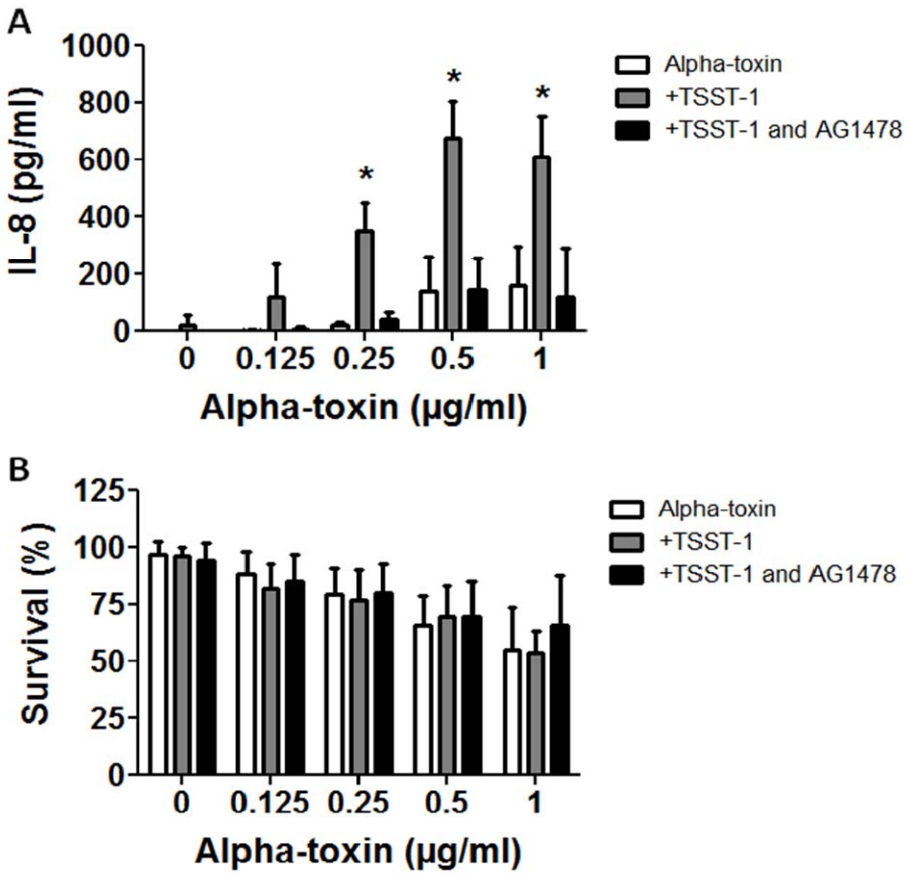


Figure 3.2: Alpha-toxin is synergistic with TSST-1 in stimulating IL-8 production in HVECs. *Cells treated with a sub-proinflammatory dose of TSST-1 and a minimal proinflammatory dose of alpha-toxin for 6 h (**A**) produced significantly more IL-8 (**B**) without affecting cell viability in response to alpha-toxin alone. Inhibition of EGFR via AG1478 attenuated the synergistic IL-8 response but had no effect on cell viability. Data are shown as the mean ± SD. Synergy was defined as a significant difference between the observed alpha-toxin + TSST-1 and the null hypothetical sum of each individual response (*, p value <0.05, Student's t-test) Data are the combined means of 3 experiments each with n=3.*

3.3.3 EGFR Signaling is required for TSST-1 and Alpha-toxin Induced IL-8 Production in *ex vivo* Tissue

TSST-1 and alpha-toxin are both known to stimulate IL-8 production *in vitro,* therefore we hypothesized the effect would be preserved in tissue. Porcine vaginal mucosa was used to investigate the mechanism of cytokine stimulation in *ex vivo* tissue. Porcine vaginal tissue is an established model used to investigate bacterial, toxin and inflammatory interactions, as it exhibits remarkable similarity to human tissue in structure, histology and permeability [66,70,106,169]. We hypothesized that TSST-1 would independently bind to, and be retained, within the vaginal mucosa and stimulate production of proinflammatory cytokines. TSST-1 was applied topically to mucosal explants from 0.1-40 μg/ml for 8 h, and then the explants were washed thoroughly to remove any unbound toxin. We observed that TSST-1 was retained within the mucosa in a dose dependent fashion, with an average retention of between 10-20% of the applied toxin (Figure 3.3). TSST-1 also stimulated production of IL-8 in a dose dependent fashion with a saturable response at ≥10 μg (Figure 3.4A). The *ex vivo* tissue response to TSST-1 appears more potent than the HVEC response, which requires ≥100 μg/ml to reach a maximum response [66]. This may result from TSST-1 accumulating in *ex vivo* tissue, increasing localized concentrations or stimulation of other cell types in the *ex vivo* tissue. IL-8 production to a dose response of alpha-toxin (0.05-5 μg/explant) on porcine vaginal mucosa was also characterized. The potency of alpha-toxin in porcine vaginal mucosa was similar to HVECs with a maximal IL-8 response peaking between 1-2 μg/explant, and a decrease in response at ≥5 μg/explant (Figure 3.4B). Also similar to the

in vitro results, alpha-toxin was cytotoxic to the porcine vaginal mucosa, reducing viability by ~31%, which corresponded to the decrease in the IL-8 response (Figure 3.5). Because EGFR inhibition in HVECs attenuated IL-8 production in response to both TSST-1 and alpha-toxin, we investigated the EGFR pathway in porcine tissue. The EGFR inhibitor, AG1478, attenuated the IL-8 response to both TSST-1 at 20 μg/explant and alpha-toxin 100ng/explant (Figure 3.4C). The doses used to investigate the IL-8 response were chosen because they lacked significant cytotoxicity (Figure 3.5). Additionally, the combined response in *ex vivo* tissue to both toxins was amplified and could be completely blocked by EGFR inhibition (Figure 3.4D). Combined, the data demonstrate that TSST-1 and alpha-toxin induced IL-8 production in *ex vivo* tissue is dependent on EGFR-signaling, and not a result of cellular damage.

FIGURE 3.3: TSST-1 Retention in Porcine Vaginal Mucosa

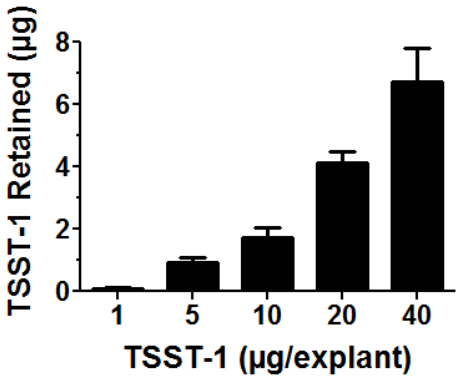


Figure 3.3: TSST-1 is retained in a dose dependent fashion in porcine vaginal mucosa. *Tissue treated with TSST-1 for 6h was assayed for toxin retention following washing. Data are shown as the mean ± SD. Retention was between 10-20% of the applied dose and retention was concentration dependent. Data are the combined means of 3 experiments each with n=3.*

FIGURE 3.4: Alpha-toxin and TSST-1 Cytokine Production in Porcine Vaginal Mucosa

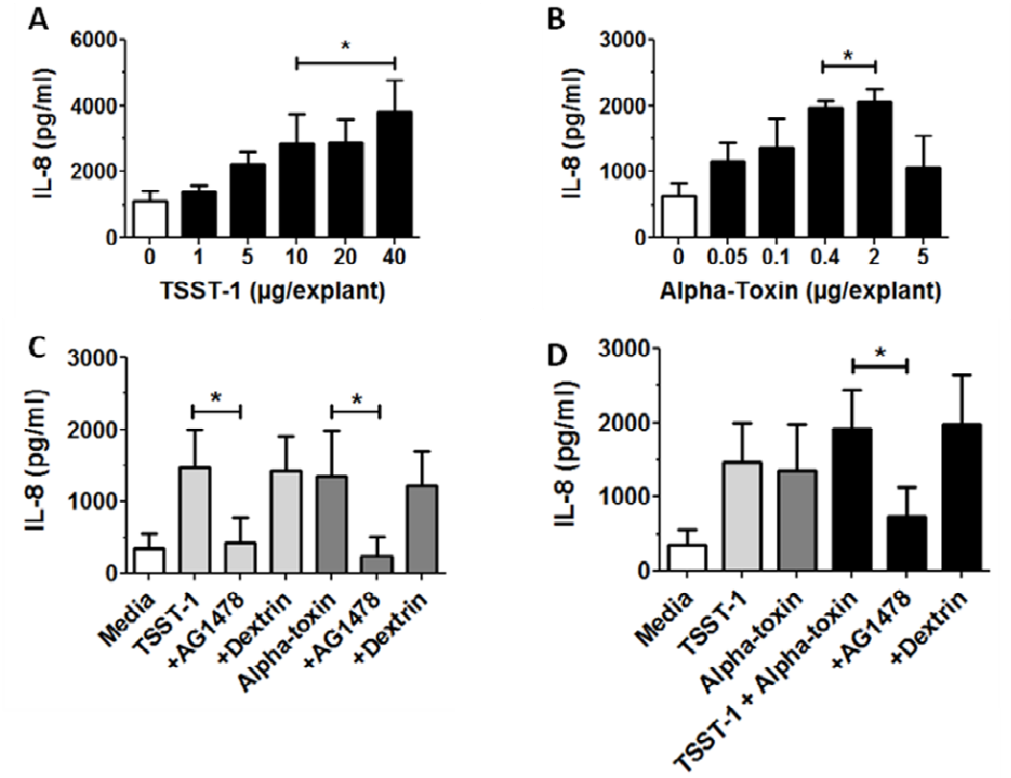


Figure 3.4: Alpha-toxin and TSST-1 stimulation of IL-8 production in ex vivo tissue depends on EGFR signaling. *Porcine vaginal mucosa treated with TSST-1(**A**) or alpha-toxin (**B**) for 6h showed a dose dependent increase in IL-8 production. IL-8 production decreased in response to alpha-toxin at 5 µg/ml, corresponding to a decrease in tissue viability (**Fig 3.5B**). Inhibition of EGFR by AG1478 attenuated the IL-8 response to both TSST-1 and alpha-toxin (**C**) as well as the response to both toxins (**D**). Data are shown as the mean ± SD. Significant differences between untreated (**A&B**) or toxin treated (**C&D**) are denoted (*, p value <0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3.*

FIGURE 3.5: Alpha-toxin and TSST-1 Cytotoxicity in Porcine Vaginal Mucosa

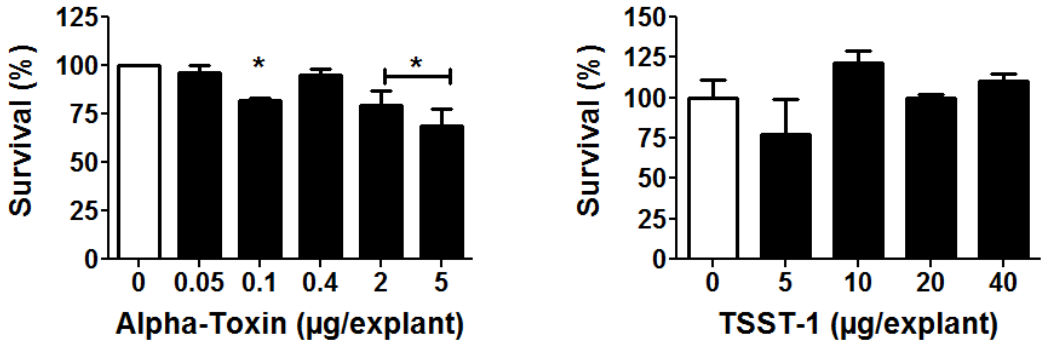


Figure 3.5: Effects of TSST-1 and alpha-toxin on the viability of porcine vaginal mucosa. *Tissue treated with a dose range of TSST-1 or alpha-toxin was assayed for viability by MTT assay. No effect on viability was observed in response to TSST-1 (**A**). Tissue treated with alpha-toxin showed a 31% decrease in viability at 5 µg/explant (**B**). Data are shown as the mean ± SD. Significant differences from untreated tissue pieces are denoted (*, p value <0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3.*

3.3.4 EGFR Signaling is not required for TSST-1 Retention or Penetration at the Vaginal Mucosa

Flux of TSST-1 across the vaginal mucosa has been suggested as a mechanism of pathogenesis during progression of mTSS [33,65,70]. Alpha-toxin increases the amount of TSST-1 that penetrates across the vaginal mucosa [70]. It has been postulated that the cytotoxic and proinflammatory effects of alpha-toxin are responsible for the increased toxin penetration [70]. To investigate the role of proinflammatory cytokines in TSST-1 penetration across vaginal tissue, TSST-1 40 μg and alpha-toxin 5 μg were added topically to 1 cm^2 of porcine vaginal mucosa with or without the EGFR inhibitor AG1478. A Neoflon Dual Flow In-Line chamber was used to determine both TSST-1 retention and penetration. The chamber simulates tissue blood flow by allowing a topical treatment to the apical surface to be applied while a reservoir of media circulates below the basal surface. When applied independently, after 8 h 4.1% of the applied TSST-1 was retained in the tissue while 0.062% penetrated (Table 3.1). When alpha-toxin was applied 3.1% of the applied TSST-1 was retained and 0.1% penetrated. This was a 1.6 fold increase and although not significant it corresponds to previous experiments [70]. When the EGFR inhibitor AG1478 was included 2.5% of the applied TSST-1 was retained in the tissue and 0.15% penetrated through. This was a 2.4 fold increase when compared to TSST-1 alone, again significance was not reached. However, this suggests that proinflammatory effects of IL-8 do not play a major role, due to the trend of increased TSST-1 in the presence of alpha-toxin not abating in the presence of EGFR

inhibition. The data suggest that TSST-1 induction of EGFR-signaling plays a role in disease which is independent of TSST-1 penetration across the vaginal mucosa.

TABLE 3.1: Effects of Inflammation on TSST-1 Penetration and Retention in Porcine Vaginal Mucosa

Treatment	TSST-1 Retention	TSST-1 Penetration	Penetration Fold-change
40 µg TSST-1	1653 ± 523 ng (4.1 %)	24.8 ± 13.72 ng (0.062 %)	
40 µg TSST-1 + 5 µg Alpha-toxin	1258 ± 486 ng (3.1 %)	41.37 ± 33.43 ng (0.10 %)	1.67
40 µg TSST-1 + 5 µg Alpha-toxin + 11.2 µg AG1478	996 ± 393 ng (2.5 %)	59.88 ± 59.19 ng (0.15%)	2.41

Table 3.1: Inhibition of EGFR-signaling does not reduce TSST-1 penetration across porcine vaginal mucosal. *Porcine tissue was treated with TSST-1 ± alpha-toxin ± AG1478 for 8 h and the TSST-1 retained in the tissue after washing and penetration into the reservoir was assessed. Data show mean ± SD and the percent of the applied in parentheses. Fold change is relative to TSST-1 alone treatment. Data are the combined means of 3 experiments each with n=2.*

3.3.5 Alpha-toxin Increases Lethality of TSST-1 in mTSS

To investigate the combined enhanced effects of TSST-1 and alpha-toxin *in vivo,* a rabbit endotoxin-enhanced mTSS model was used [170]. Young adult Dutch belted rabbits (~2kg) were treated intravaginally with alpha-toxin, TSST-1, or the two toxins combined. To investigate if TSST-1 mediated disease can be enhanced in the presence of alpha-toxin, the maximal sub-lethal dose of each toxin was determined. All 3 rabbits treated intravaginally with 5 and 50 μg of alpha-toxin died within 48 and 24 h, respectively, while those treated with 0.5 μg of alpha-toxin survived (Table 3.2). The lethal dose of TSST-1 in rabbits has already been well characterized, and ~10 μg per rabbit is the minimal dose required for lethality in an endotoxin enhanced model. Since endotoxin is a major factor for TSST-1 lethality in rabbits, it was titrated to determine the maximal sub-lethal dose. The mechanism of endotoxin enhancement of TSS is thought to be a result of priming of the immune response [116]. All 3 rabbits treated intravaginally with 10 μg of TSST-1 and 1 or 0.1 μg endotoxin succumbed within 48 h, while those treated with 0.01 μg of endotoxin and 10 μg of TSST-1 survived (Table 3.2). Thus, to investigate the combined lethal effects of superantigens and cytolysins, a dosing regimen of 0.5 μg alpha-toxin and 10 μg TSST-1 with 0.01 μg LPS was chosen, due to the lack of lethality of the individual toxins. All 5 rabbits which were given both toxins succumbed within 24 h (Table 3.2). As expected, all 5 rabbits in each group treated with individual toxins survived the duration of the experiment. The data show that alpha-toxin enhances the lethality of TSST-1 during mTSS, and indicate that the combined effects of multiple toxins may play a critical role during pathogenesis.

TABLE 3.2: Alpha-toxin Enhances TSST-1 Lethality in Rabbit mTSS

Dose of Alpha-toxin	Dead within 48 h
50 µg/rabbit	3/3
5 µg/rabbit	3/3
0.5 µg/rabbit	0/3

Dose of TSST-1	Dose of LPS	Dead within 48 h
10 µg/rabbit	1 µg/rabbit	3/3
10 µg/rabbit	0.1 µg/rabbit	3/3
10 µg/rabbit	0.01 µg/rabbit	0/3

Dose of Alpha-toxin	Dose of TSST-1	Dose of LPS	Dead within 48 h	P-Value
0.5 µg/rabbit	10 µg/rabbit	0.01 µg/rabbit	5/5	---
0.5 µg/rabbit	---	---	0/5	0.008
---	10 µg/rabbit	0.01 µg/rabbit	0/5	0.008

Table 3.2: Sub-lethal doses of TSST-1 and alpha-toxin combined restore lethality in a rabbit mTSS model. *Rabbits were challenged intravaginally with alpha-toxin or TSST-1 with endotoxin to determine a minimal sub-lethal dose. Rabbits challenged with sub-lethal doses of both toxins succumbed within 48 h. All animals which did not die within 48 h survived for the 7 day course of the experiment. P-value denotes significances vs. the combined toxins as determined by Fisher's exact test.*

3.4 Discussion

The innate immune response in skin and mucosal epithelial cells is heavily modulated by EGFR-signaling in response to injury, allergens or infection [68,122,163]. In a normal immune response to an insult, the epithelial surface would initiate recruitment and activation of immune cells inducing monocyte differentiation, neutrophil recruitment and dendritic cell trafficking [171]. The immune response is designed to protect the host and restore homeostasis, but dysregulation can promote pathogenesis. Hyper activation of an inflammatory response can promote edema, capillary leakage, disruption of cell junctions and prevent tissue repair, which can provide an opportunity for a pathogen to invade tissue or cause disease.

TSST-1 induces ADAM17 dependent shedding of the EGFR-ligands AREG and TGF-α in HVECs [67]. Herein, we demonstrated that alpha-toxin also induces shedding of AREG through an ADAM17 dependent pathway. AREG and TGF-α are potent EGFR ligands that undergo both receptor and ligand intracellular recycling, which may explain the synergistic response observed in cytokine production when alpha-toxin and TSST-1 are combined [121]. Additionally, activation of EGFR-signaling results in phosphorylation of the downstream effectors ERK1/2 which phosphorylates, thus activating, ADAM17, to cause additional shedding of EGFR ligands [130]. The converging cell signaling through ADAM17 and EGFR induced by TSST-1 and alpha-toxin in HVECs may mediate the enhanced cytokine production we, and others, have observed.

TSST-1 and alpha-toxin are known to stimulate production IL-8, IL-6, MIP-3α and TNF-α in HVECs, but synergy in toxin activity is only observed in IL-8 and IL-6 production [65,67,70]. The specific combination of cytokines induced by TSST-1 and alpha-toxin may promote pathogenesis by causing inflammation or modulating the immune response to create a more favorable environment for *S. aureus*. Enhanced IL-8 production would likely lead to increased neutrophil migration, a cell which *S. aureus* is well adapted to survive within and grow [86,172–174]. Thus, it is possible that hyper-recruitment of neutrophils, which is observed in fatal cases of mTSS, may be detrimental to the patient. Additionally, IL-6 amplifies MIP-3α expression which would promote additional recruitment of neutrophils, dendritic cells and T-cells[175,176]. Recruited dendritic cells can be activated by TNF-α, inducing MHC-II expression and migration to lymph nodes [177].TSST-1 is known to survive dendritic cell phagocytosis and traffic to the lymph nodes, where it is presented to CD4+ T-cells [115]. The combined information suggests a mechanism of mTSS pathogenesis, which propagates from the vaginal mucosa and mucosal immune compartment, rather than from systemic interactions.

Generally it has been thought that superantigen penetration across the vaginal mucosa is the mediator of mTSS [21,70,178]. Alpha-toxin is known to disrupt the integrity of the vaginal mucosal, promoting toxin penetration into the host. This is supported by the destructive properties of *S. aureus* exotoxins that increase superantigen penetration across the mucosa and into the vasculature. Both TSST-1 and alpha-toxin are able to lyse HVECs *in vitro* and induce cytokine production, and major histological changes resembling edema and capillary dilation in *ex vivo* vaginal tissue [66,70,106]. The

individual effects of TSST-1 and alpha-toxin on vaginal epithelium differ, with alpha-toxin causing general lysis and destruction from the outside in while TSST-1 causes exfoliation and separation of the epithelial cell layers from the *lamina propria* [70,111]. TSST-1 is also able to independently penetrate across and into the tissue, however, in experimental system the majority of the toxin remains localized at the epithelium and in the superficial *lamina propria* [111]. Independently, and at biologically relevant concentrations, TSST-1 (50 μg/ml) can cross the vaginal mucosa at a rate of 25 ng per 0.2 cm^2 per 8 h, which is increased to 55 ng per 0.2 cm^2 per 8 h in the presence of alpha-toxin (50 μg/ml) [70,109]. However, extrapolating from the rates of penetration, TSST-1 efflux across the vaginal mucosa would be unlikely to play a major role in mTSS.

In order for penetrating superantigen to cause toxic shock the concentration would need to reach a threshold capable of causing hyper T-cell proliferation and activation. Extrapolating from *ex vivo* porcine studies, and adjusting for the surface area of the average human vagina (87.46 cm^2, [179]), the total estimated penetration of TSST-1 into systemic circulation in humans over 8 h is estimated at ~11 μg and ~24 μg total, with or without alpha-toxin, respectively [70]. The rate of flux of superantigens across the porcine vaginal mucosa is nearly identical to that in human tissue [106]. Assuming no clearance of TSST-1 and localization only in the serum, the concentration of TSST-1 after 8 h of accumulation would only be 1.6 ng/ml or 3.4 ng/ml with alpha-toxin present (7 L blood volume), which is at the threshold of inducing T-cell stimulation by superantigens (1-10 ng/ml) [180]. However, superantigens undergo rapid renal and hepatic clearance from the serum with an estimated half-life of ~2.4 h, which when

modeled using a constant infusion yields a steady state concentration of 200 pg/ml, using the above assumptions, or 6 pg/ml when using the volume of distribution estimated from pigs of 230 L [118,181]. Interestingly, this prediction is similar to what is observed clinically in mTSS patients who have an average serum concentration of 30 pg/ml (93% of patients between 10-300 pg/ml) at the time of hospitalization, commonly ~2 days after onset of symptoms and the apex of clinical disease [182,183]. Thus, unless TSST-1 is nearly exclusively sequestered to the lymphatic system, it is unlikely that circulating superantigens play a major role in disease progression. Combined, this suggests a mechanism by which TSS is propagated from the mucosa, possibly through active trafficking by immune cells or diffusion into the local lymphatic systems.

The importance of local effects at the vaginal mucosa of TSST-1 and alpha toxin is further exemplified by our rabbit mTSS studies. Symptoms of TSS in rabbits can be induced by intravenous or intravaginal administration of superantigens [Table 3.2,6]. The intravenous lethality of TSST-1 is well characterized and is determined to be 5.9 μg/kg with 0.1 μg/kg of endotoxin and 17.8 μg/kg with 0.01μg/kg of endotoxin [116]. When we treated ~2 kg rabbits with 10 μg (~5 μg/kg) of TSST-1 and 0.1 μg (~0.05 μg/kg) of endotoxin intravaginally, a dose less than the intravenous LD50, we saw 100% lethality. Therefore, in order for systemic effects of TSST-1 to mediate mTSS, the penetration of the toxin, at minimum, would need to be almost instant and complete. Currently all experimental evidence shows that TSST-1 penetration across and out of the vaginal mucosa is <1% of the applied dose over 6-8 h [Table 3.1,8,19]. Local proinflammatory effects are further supported by the restored TSS lethality when sub-lethal doses of

TSST-1 with endotoxin and alpha-toxin are administered intravaginally. The lethality of endotoxin in the rabbit TSS model is thought to be enhanced by TSST-1, and be a significant factor in pathogenesis [33,116]. However, exogenous endotoxin is not an essential mediator of TSS in rabbits, and can be circumvented through continuous infusion of TSST-1 [184]. Additionally, neutralizing endotoxin by antibodies or polymyxin B had no effect on TSST-1 lethality in rabbits during continuous infusion [184]. However, chronic inflammation from continuous infusion of TSST-1 may enhance absorption of endotoxin at the gut, or other mucosal surfaces, from native flora. Thus, even though TSST-1 may enhance endotoxin lethality the inverse may be true as well, with endotoxin priming the immune response to be more sensitive to TSST-1. Although not determined, the restored lethality when alpha-toxin and TSST-1 are co-administered may be a result of the synergy in cytokine production, which could restore the priming lost by decreased endotoxin.

Currently, the data support a critical role of local EGFR signaling in the proinflammatory response of the vaginal mucosal epithelial surface to *S. aureus* exotoxins. Specifically, EGFR-signaling is absolutely required for the induction of cytokine responses to TSST-1 and alpha-toxin, and is independent of toxin-mediated cytolytic activity. Additionally, when multiple toxins, which act indirectly to stimulate EGFR, are combined the response is synergistic *in vitro, ex vivo* and *in vivo* in stimulating cytokine production or lethality. Combined, these data present an interesting therapeutic target. Could local inhibition of EGFR be used to treat TSS or mTSS? While long term inhibition of EGFR can inhibit wound healing, local transient inhibition could

restore EGFR-signaling homeostasis. Currently, multiple EGFR inhibitors are available clinically that could be used to investigate the novel application for treatment of infectious disease.

CHAPTER IV.

Local Epidermal Growth Factor Receptor Signaling Mediates the Systemic Pathogenic Effects of *Staphylococcus aureus* Exotoxins

Sections of this study have been submitted for publication.
Non-animal experiments were performed by Dr. Laura Breshears and me in collaboration. Animal studies were performed by Dr. Schlievert in collaboration with us.

4.1 Introduction

TSS and mTSS result from hyper-stimulation of CD4+ T-cells and antigen presenting cells by TSST-1, resulting in a cytokine storm causing the life-threatening shock [21]. Propagation of mTSS occurs from the vaginal mucosa and is not dependent on bacterial invasion [21,40]. Evidence suggests that local proinflammatory effects and activation of the innate immune response are required for systemic disease [Chapter III, 65]. During homeostasis, the resident adaptive immune cells and antigen presenting cells in vaginal mucosal tissue are unprimed in a sentinel state, and likely too low in number to account for the systemic effects of mTSS [107]. Furthermore, toxin penetration into the circulation is likely too low to account for disease [Chapter III.]. Thus, it is possible that disease results from either, or a combination of, recruitment of antigen presenting cells and CD4+ T-cells to the vaginal mucosa, or active trafficking of TSST-1 to the mucosal lymph nodes or transfer via lymphatic drainage system. The importance of local effects is supported by data from a TSST-1 single amino-acid substitution mutant (D130A). The TSST-1 mutant retains superantigenicity and intravenous lethality, but does not stimulate a cytokine response in HVECs and loses lethality when administered intravaginally in rabbits [117]. Furthermore, the intravenous LD_{50} of TSST-1 is very similar to the intravaginal LD_{100} in rabbits, suggesting a critical role of localized inflammation in disease [Chapter III., 5]. The epithelium is the first tissue encountered by *S. aureus* toxins and TSST-1, along with other *S. aureus* toxins, also activates the innate immune response at the epithelial surface through modulation of EGFR-signaling. Therefore, cytokine

production by the vaginal epithelium may be essential in the signaling cascade which results in mTSS.

Multiple toxins of *S. aureus,* including surface proteins, cell wall components and exotoxins, are known to modulate EGFR-signaling, which is thought to play a role in pathogenesis. Protein A binds to the TNF receptor-1 and EGFR to perturbed cytoskeleton arrangement and facilitate penetration of *S. aureus* across lung epithelial cells *in vivo* [167]. Additionally in lung cells, the gram-positive cell wall component lipoteichoic acid from *S. aureus* induces shedding of HB-EGF by ADAM10, independent of Toll-like receptors, promoting hyper-secretion of mucin [166]. Mitogenic effects are observed by the cytolysin alpha-toxin in epithelial cells, which are thought to be EGFR dependent [168]. In HVECs, the cytolysins, alpha- and gamma-toxins, as well as the superantigen TSST-1, all hyper-stimulate the innate immune response through ADAM10/17 dependent shedding of EGFR ligands [Chapter II., Chapter III., 65]. Over stimulation of cytokine production is known to promote disease by *S. aureus* through disruption of epithelial cell tight-junctions and progression to TSS [185]. Combined, this suggests that activation of EGFR-signaling is exploited by *S. aureus* during infection of multiple tissues.

Inhibition of EGFR-signaling is known to inhibit the pathogenic effects of several *S. aureus* toxins. *In vitro* inhibition of EGFR with the inhibitor AG1478 blocks both the mitogenic effects of alpha-toxin in lung cells and the proinflammatory effects of alpha-toxin, gamma-toxin and TSST-1 in HVECs [Chapter II., Chapter III., 3]. Furthermore, AG1478 inhibits the combined effects of alpha-toxin and TSST-1 in HVECs without affecting cell viability [Chapter III.]. Most notably, AG1478 inhibits the combined

action of alpha-toxin and TSST-1 in *ex vivo* porcine vaginal tissue [Chapter III.]. The efficacy of AG1478 in *ex vivo* tissue suggests that even in a complex system there is the potential to inhibit the mechanism of action of multiple toxins through inhibition of EGFR signaling. Ultimately, (m)TSS relies on hyper stimulation of CD4+ T-cells and antigen presenting cells to cause a cytokine storm [62]. If the hyper T-cell activation is dependent on priming by the innate immune response, then inhibiting EGFR may be utilized to slow or decrease the progression of (m)TSS. However, a model which represents the disease progression in humans along with a drug and delivery system is needed to test this hypothesis.

Tyrosine kinase inhibitors (TKIs) are a class of small-molecule competitive-inhibitors with specificities for the different classes of EGFR [131]. The first class of TKIs was based on anilinoquinazoline molecules modified for receptor-selectivity and pharmacokinetics-profiles for chronic treatment of cancers, which include the clinically used drug gefitinib and erlotinib [133,134]. The inhibitor AG1478 is a derivative of gefitinib, and is only used experimentally due to pharmacokinetic parameters which make it unsuitable for chronic therapy [138]. Most notably, the half-life of AG1478 is estimated to be ~30 min in humans [138]. However, the short half-life is ideal for acute treatment that can be quickly reversed upon discontinuation of administration. Ideally, if EGFR-signaling is critical for mTSS progression the goal for therapy would be to treat just long enough to shunt the inflammatory response but not inhibit wound healing, a side effect of chronic TKI therapy. AG1478 is a highly lipophilic compound which readily distributes to mucosal tissues when administered intravenously in mice [15]. Combined

with efficacy of the inhibitor in *ex vivo* tissue, this suggests the compound is readily absorbed when applied topically to mucosa. The water solubility of AG1478 is low (0.225 mM, ~79 μg/ml), and inorganic solvents are not ideal for topical mucosal administration [138]. However, in 30 % 2-hydroxypropyl-β-cyclodextrin (β-cyclodextrin) (aq) AG1478 at 8 mg/ml (22.7 mM) forms a stable viscous colloidal solution, which is suitable for topical administration.

TKIs and β-cyclodextrin are known to be safe in animal models, with high LD_{50} and are very inert, respectively. Data from rat studies show that the first signs of acute toxicity (lethargy) require serum concentrations of 100 μM (~35 μg/ml) [138]. Within 30 mins following IV infusion of AG1478 approximately 4% of the drug remains in the serum, thus in ~300g rats (blood volume ~ 60ml/kg) the intravenous toxic dose is ~5.3 mg/kg [138]. While the lethal dose of AG1478 is not known, the oral LD_{50} of the derivative drug gefitinib is 2000 mg/kg (4.4 mmol/kg) in mice [133]. β-cyclodextrins are extremely safe and poorly absorbed by mucosal surfaces [186]. In rat studies, β-cyclodextrin can be administered orally as 2.5% of the rat's total diet by weight for 3 generations with no systemic or mucosal adverse events [187]. However, cyclodextrins are known to modify the pharmacokinetic and dynamic properties of drugs [186]. Thus, it needs to be determined if the combined effects of AG1478 and β-cyclodextrin are still efficacious or toxic to the vaginal mucosa.

Current animal models for TSS rely on the phenomena of TSST-1 enhancing the lethal effects of endotoxin to produce TSS-like symptoms, despite endotoxin not being required if TSST-1 is administered continuously [184]. While endotoxin may play a role

in (m)TSS, the source would likely be from endogenous (intestinal or vaginal) sources. Preliminary models for mTSS in rabbits currently rely on endotoxin and acute onset of symptoms. Thus, there is a need for a progressive model of mTSS, which more closely resembles disease in humans, with symptom and disease onset occurring over a several day period in the presence of live *S. aureus*.

The goals of this study are three-part: 1) Determine the safety and efficacy of a colloidal suspension of AG1478 in β-cyclodextrin in *ex vivo* porcine vaginal mucosa: 2) Develop a progressive model of mTSS, which does not rely on exogenous endotoxin: and 3) Test the ability of EGFR inhibition to prevent disease in a rabbit mTSS model following challenge by live *S. aureus*. We hypothesize that inhibition of EGFR-signaling can attenuate disease in a rabbit model for mTSS. The combined data from studies presented here support our hypothesis and suggests that inhibition of EGFR-signaling may be a successful adjunct therapy for treatment of mTSS.

4.2 Methods

Toxin Purification

Alpha-toxin was purified as previously described [70]. Briefly, *S. aureus* strain MNJA was grown in beef heart media and the supernatant precipitated with ethanol at 4 °C and resolublized in water. Two step isoelectric focusing was used. The first phase used a pH gradient from 3.5 to 10 followed by a second phase using a pH gradient from 7 to 9. The isoelectric point of alpha-toxin is 8.5. Purity was confirmed by SDS-PAGE and the yield of purified toxin was quantified using a Bio-Rad protein assay (Bio-Rad, 500-0006).

TSST-1 was purified from *S. aureus* by an established method using successive thin layer isoelectric focusing [64]. TSST-1 (1 mg/ml), thus prepared, contained no detectable LPS, as tested by Limulus assay (Sigma). Peptidoglycan, lipoteichoic acid, hemolysin, protease, or lipase (<1 part/10^6) were not detectable by combinations of bioassay and SDS-PAGE. Comparable results were obtained with multiple batches of TSST-1.

Porcine Tissue

Porcine tissue was collected and processed prior to treatment as previously described (17). Briefly, porcine vaginal tissue was collected at time of slaughter in RPMI Media 1640 (Gibco, 11875-093) supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), amphotericin B (2.5 µg/ml) and gentamicin (40 µg/ml). Antibiotics were included to eliminate native flora. Within 3 h of slaughter 5 mm biopsies were taken and excess muscle tissue trimmed. The biopsies were washed three

times then incubated half-submerged for 30 min at 37^{O} C in antibiotic-free RPMI. Following washing, the biopsies were placed mucosal side up on PET track-etched 0.4 μm cell culture inserts (BD Biosciences) in 6-well plates above fresh antibiotic- and serum-free media. The biopsies were then treated topically with toxin, TSST-1 or alpha-toxin, at listed concentrations in 2 μl PBS for 6 h. Where the inhibitor AG1478 (Tocris 1276) or vehicle was used, it was applied at 30 mins and 10 mins prior to addition of toxin. For IL-8 quantification, biopsies were homogenized in 250 μl PBS. Homogenates were centrifuged at 13,000 rpm for 1 min and the supernatant assayed by ELISAs purchased from R&D Systems per manufacturer's protocol for IL-8 (DY535). The viability of explants was determined with a Cell Growth Determination kit (Sigma, CDG1) and is expressed relative to untreated tissue.

For bacterial infection of PVM, explants were exposed to MNPE (~10^6 CFU/explant) with or without 30 min prior exposure to AG1478 (1.4 μg/explant) or 10% DMSO vehicle (8 μl/explant) for 6 h at 37℃ with 7% CO_2. Explants were vortexed in 250 μl PBS for 4 min at 32000 rpm to release IL-8 for quantification via ELISA or bacteria for analysis of CFU/explant. Bacteria were plated on tryptic soy agar containing 5% sheep's blood (BD Diagnostics, 221261) on a spiral plater (Biotek, Microbiology International).

To estimate AG1478 penetration into vaginal tissue porcine vaginal mucosa was treated topically with AG1478 in 30% β-cyclodextrin (aq). Every 2 h, tissue explants were washed 3x with 1 ml PBS and homogenized in 500 μl methanol and incubated overnight at 4℃. Following overnight incubation, suspensions were pelleted and the

absorption peak of AG1478 was measured by spectrophotometer (330 nm). No detectable absorption was observed in controls at 330 nm. Tissue concentrations were calculated with a standard curve of AG1478 in methanol.

Animal Studies

Young adult female New Zealand white rabbits weighing 2-4kg kg were obtained from Bakkom Rabbitry (Red Wing, MN). The rabbits were anesthetized with IV ketamine (25 mg/kg) and xylazine (25 mg/kg) for insertion of an intravaginal catheter. Surviving animals at the end of the experiment were euthanized with Beuthanasia-D (1mL/kg) or when they failed to stand upright and not exhibit flight behavior. All animal experiments were performed according to established guidelines and a protocol approved by the University of Iowa Institutional Animal Care and Use Committee (protocols 1106140 and 4071100).

For animals treated with purified toxin, rabbits were dosed once daily by intravaginal catheter. Rabbits were treated every 12 h with TSST-1 at 30 µg/kg in 250 µl PBS with 250 µl 30% β-cyclodextrin (aq) or 250 µl 8 mg/ml AG1478 in 30% β-cyclodextrin (aq) for a total volume of 500 µl. Rabbits were monitored for death. For animals treated with live organisms, rabbits were dosed twice daily by intravaginal catheter. Treatment was 250 µl of 30% beta cyclodextrin or 250 µl of AG1478 at 8 mg/ml in 30% beta cyclodextrin administered vaginally and allowed to absorb for 5 minutes prior to addition of ~ 10^{10} MN8 in 500 µl THB. A cotton plug from a 1 ml pipette was inserted as a tampon and left in the vagina. Fevers were determined at each time point using rectal thermometers.

4.3 Results

4.3.1 *Ex Vivo* Efficacy of AG1478 in 2-Hydroxypropyl-β-Cyclodextrin (aq)

2-hydroxypropyl-β-cyclodextrin (aq) is an effective vehicle for delivery of AG1478. Because previous studies utilized DMSO (aq) as a delivery vehicle, the effectiveness of β-cyclodextrin (aq) for drug delivery needed to be determined. Porcine vaginal tissue treated with TSST-1 (20 μg/explant) or alpha-toxin (2 μg/explant) exhibited a similar response in IL-8 production as compared to previously observed data (Figure 4.1) [Chapter III.]. Application of 10 μl AG1478 4 mg/ml (40 μg/explant) in 15% β-cyclodextrin (aq) completely abrogated the IL-8 response to individual toxins (Figure 4.1A). The dose and concentration of AG1478 represents the maximal exposure achievable in rabbits utilizing our 8mg/ml AG1478 preparation in 30% β-cyclodextrin (aq), assuming 1:1 mixture of an aqueous treatment and drug. Lower doses of TSST-1 (5 μg/explant) and alpha-toxin (25 ng/explant) when combined did not demonstrate an additive effect, possibly due to the response being saturated, but the combined effects of the toxins were attenuated by AG1478 in 15% β-cyclodextrin (aq) (Figure 4.1B). Combined this demonstrates that AG1478 in β-cyclodextrin (aq) can inhibit cytokine production induced by TSST-1 and alpha-toxin.

FIGURE 4.1: Efficacy of AG1478 in Cyclodextrin in Porcine Vaginal Mucosa

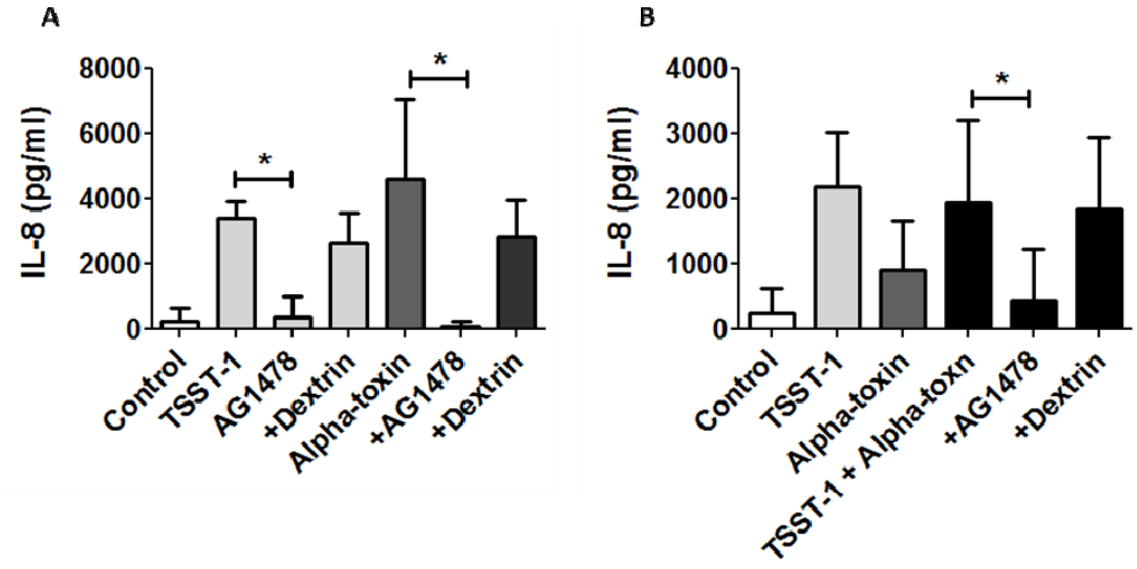


Figure 4.1: 30% 2-hydroxypropyl-β-cyclodextrin is an effective vehicle for delivery of AG1478. *(**A**) Inhibition of EGFR by AG1478 in 30% β-cyclodextrin attenuated the IL-8 response in porcine vaginal mucosa to high doses of either TSST-1 (20 µg/explant) and alpha-toxin (2 µg/explant) with no effect from β-cyclodextrin. (**B**) The IL-8 response to lower doses of TSST-1 (5 µg/explant) and alpha-toxin (25 ng/explant) and the combination was also reduced by AG1478 in 30% β-cyclodextrin. Significant differences between toxin treated and AG1478 treated are denoted (*, p value <0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3*

4.3.2 Safety of AG1478 in 2-Hydroxypropyl-β-Cyclodextrin (aq) in Porcine Vaginal Mucosa

AG1478 and 2-hydroxypropyl-β-cyclodextrin (aq) are not toxic to *ex vivo* porcine vaginal mucosa. To determine if AG1478 or the vehicle are toxic during acute treatment a dose response and time course for each was assayed in *ex vivo* porcine vaginal mucosa for effects on tissue viability. *Ex vivo* biopsies of porcine vaginal mucosa were treated for 6 h with β-cyclodextrin (aq) then assessed for acute toxicity up to a maximum solubility ~50% w/v. All concentrations of β-cyclodextrin (aq) were found to be non-toxic by MTT assay (Sigma) (Figure 4.2A). AG1478 acute effects on viability were assessed in 30% β-cyclodextrin (aq), a concentration which forms a stable colloidal solution. *Ex vivo* porcine vaginal mucosa treated with AG1478, up to 40 µg/explant, showed no acute toxicity over 6 h (Figure 4.2B). To assess short term toxicity of AG1478 in 30% β-cyclodextrin (aq), *ex vivo* porcine vaginal mucosa was treated daily for 3 days. Tissue treated daily with 16 µl of 30% β-cyclodextrin (aq) with or without 22 µg/explant of AG1478 showed no decrease in viability over 24 or 72 hours when assayed by MTT (Figure 4.2C&D). The dose and concentration approximate the maximum intravaginal exposure of AG1478 when diluted by other experimental treatment (purified toxin or bacteria) in rabbits (500 µl total volume at 1:1 ratio). The data confirm that AG1478 in β-cyclodextrin (aq) is not toxic to porcine vaginal mucosa and would likely be safe for short term application in humans, as human vaginal mucosa is markedly similar to porcine [106].

FIGURE 4.2: Safety of AG1478 in β-Cyclodextrin (aq) in Porcine Vaginal Mucosa

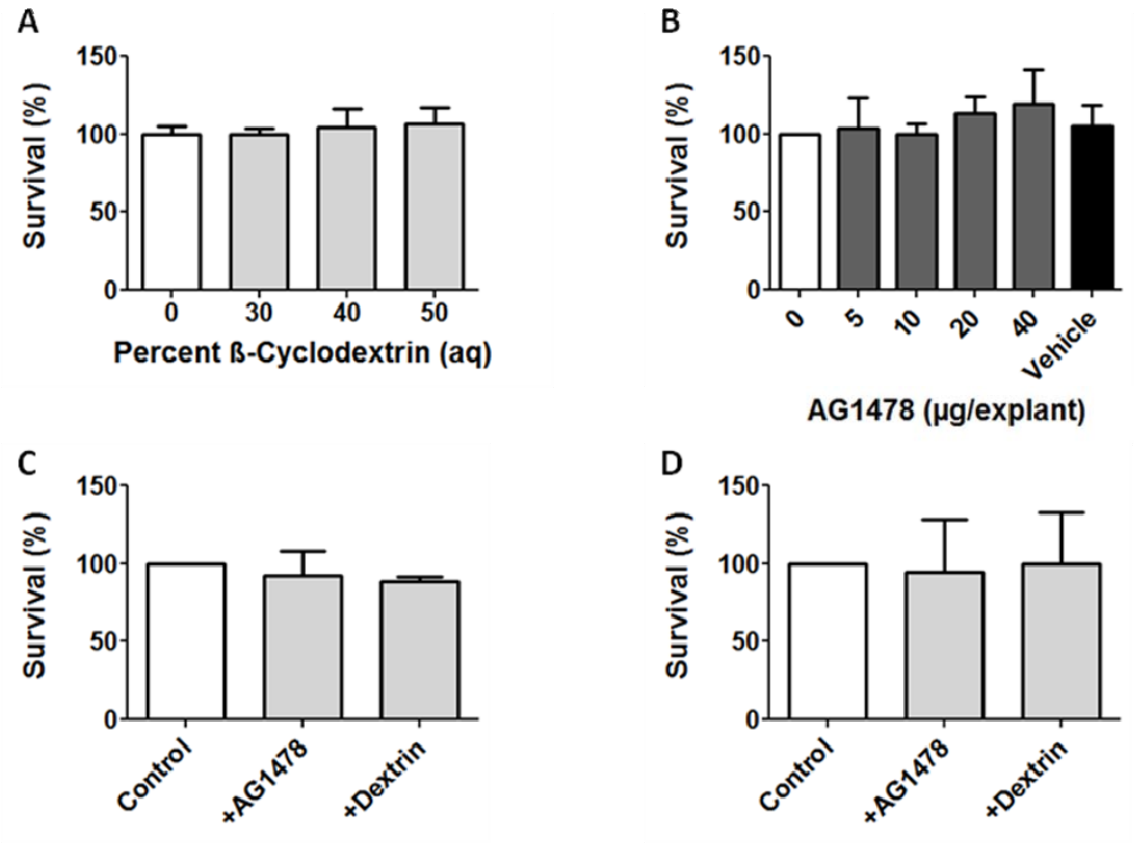


Figure 4.2: AG1478 and 2-hydroxypropyl-β-cyclodextrin (aq) are non-toxin to porcine vaginal mucosa. *(**A**) Porcine vaginal mucosa exposed to β-cyclodextrin (aq) (**B**) or AG1478 in 30% β-cyclodextrin (aq) at listed concentrations for 6 h showed no decrease in viability relative to control. (**C**) Tissue treated daily with AG1478 (22 µg/explant) (**D**) or 30% β-cyclodextrin (aq) (8 µl) showed no decrease in viability relative to control over 3 days. Significant difference was not observed relative to control (p value >0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3.*

4.3.3 Absorption of AG1478 in Porcine Vaginal Mucosa

AG1478 is effectively absorbed into porcine vaginal mucosa following topical administration. To determine if AG1478 is absorbed when applied topically and to estimate tissue concentrations, *ex vivo* porcine vaginal mucosa biopsies were treated with AG1478 (2.8 μg/explant) in 30% β-cyclodextrin (aq), and assayed following washing in PBS by spectrophotometry. At 2 h, 54% of the applied dose was retained in the tissue and by 8 h 41% of the dose remained (Figure 4.3). The data suggest that the vaginal clearance of AG1478 is low, as the estimated serum half-life is 30-41 min [138]. Furthermore, the data demonstrate that >50% of the applied dose of AG1478 is absorbed in < 2h.

FIGURE 4.3: AG1478 Absorption in Porcine Vaginal Mucosa

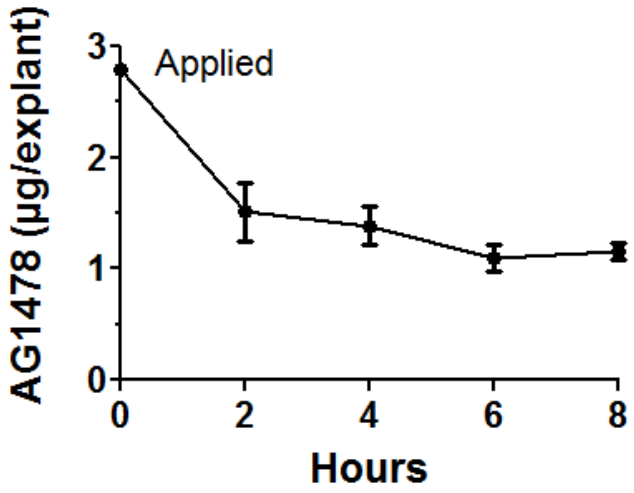


Figure 4.3: AG1478 is absorbed into porcine vaginal mucosa. *Ex vivo porcine vaginal mucosa biopsies treated with AG1478 in 30% β-cyclodextrin (aq) retained drug after washing in PBS for 8 h. Data are the combined means of 3 experiments, each with n=3.*

4.3.4 Non-Endotoxin Enhanced Rabbit Model for mTSS

Daily vaginal administration of TSST-1 can induce mTSS symptoms in rabbits in the absence of endogenous endotoxin. New Zealand white rabbits (~2kg, n=3) were treated daily with 30 μg/kg of TSST-1 in 0.5ml 15% 2-hydroxypropyl-β-cyclodextrin (aq) with or without AG1478 at 4mg/ml. All rabbits treated with TSST-1 succumbed within 3-4 days from mTSS-like symptoms (Figure 4.4). Rabbits treated with AG1478 survived for a longer period of 4-5 days, although the results were not significant ($p=0.06$, Mantlel-Cox test). The data show that in rabbits, mTSS-like disease that progresses over days can be achieved by continuous intravaginal exposure to TSST-1. This finding is important as this model system more closely resembles the progression of mTSS in humans. The data also provide evidence that chronic exposure can compensate for the acute effects of endotoxin. With the small number of animals the difference in outcomes was not significant ($p=0.06$, Mantel-Cox test), but the data strongly suggest that treatment of mTSS with AG1478 may slow disease progression. Any delay in disease progression would be clinically significant as by providing more time for other treatments to be effective.

FIGURE 4.4: AG1478 Inhibition of TSST-1 Lethality in Rabbits

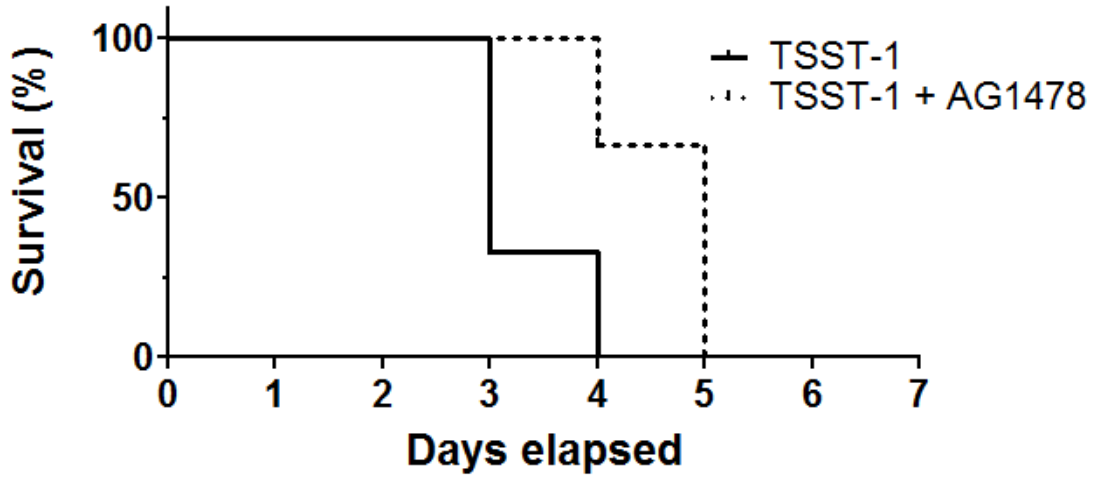


Figure 4.4: Daily intravaginal challenge with TSST-1 is lethal in rabbits. *Rabbits (n=3) challenged daily with TSST-1 (30 µg/kg) showed disease progression and lethality over 3-4 days. Daily intravaginal administration of AG1478 (2 mg/rabbit) delayed death by one day. With small numbers of animals per group, this finding is not statistically significant (p=0.06, Mantel-Cox test).*

4.3.5 Inhibition of *Ex Vivo* Proinflammatory Response to Live *S. aureus*

TSST-1 and alpha-toxin are highly produced in TSS^{+} USA200 strains in both broth culture and during infection of porcine vaginal mucosa [110,169]. To determine if EGFR-signaling is required for the IL-8 response to a TSS+ *S. aureus* strain in porcine vaginal mucosa, AG1478 (1.4 µg/explant) was applied prior to infection with ~10^6 CFU of *S. aureus* USA200 strain MNPE for 6 h. Production of IL-8 was reduced 2-fold, almost to background levels, with treatment of AG1478 (Figure 4.5A). The growth of MNPE on porcine vaginal mucosal tissue was unaffected by treatment with AG1478, indicating that reduction in IL-8 was not a result of reduced bacteria (Figure 4.5B). These data show that EGFR inhibition via AG1478 can inhibit the pro-inflammatory effects from live *S. aureus* on vaginal tissue.

FIGURE 4.5: Inhibition of the *S. aureus* Induced IL-8 Response in Porcine Vaginal Mucosa

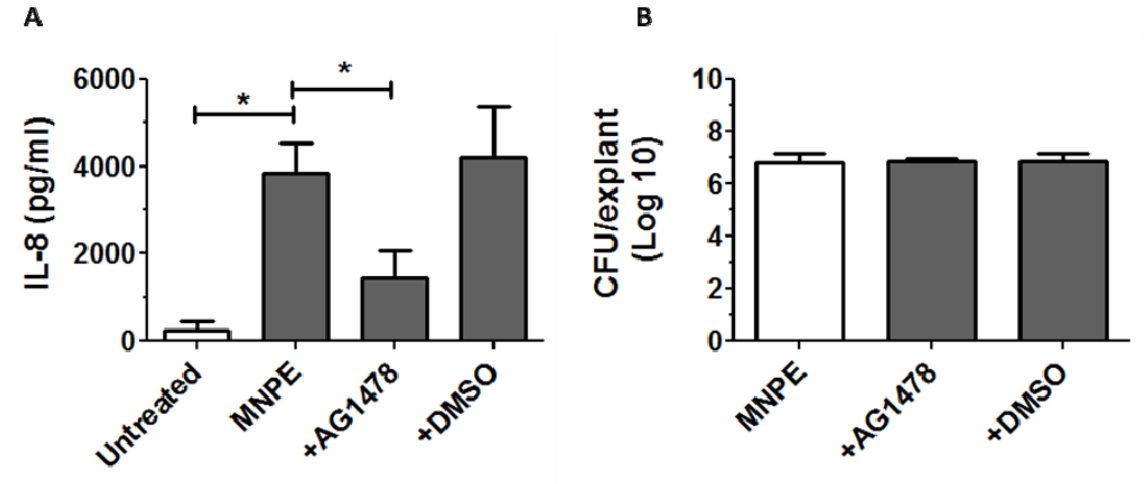


Figure 4.5: EGFR signaling is required for the porcine vaginal mucosa IL-8 response to live S. aureus. *Porcine vaginal mucosa biopsies were inoculated with 10^6 CFU of MPNE with or without AG1478 (1.4 µg/explant) for 6 h. (**A**) Tissue treated with AG1478 prior to infection showed significant reduction in IL-8. (**B**) AG1478 had no effect on bacterial growth or survival. Significant differences between bacteria treated and AG1478 treated tissue are denoted (*, p value <0.05, One way ANOVA with Dunnet's Correction). Data are the combined means of 3 experiments each with n=3.*

4.3.6 Efficacy of AG1478 in Rabbit mTSS with Live *S. aureus*

AG1478 prevents lethality against challenge by live *S. aureus* in a rabbit model of mTSS. New Zealand white rabbits (2-4 kg) were challenged twice daily with an mTSS strain of *S. aureus* MN8 (~10^{10} CFU in 250 µl PBS). Four out of five rabbits challenged intravaginally with *S. aureus* succumbed within 3 days. Vaginal administration of 250 µl AG1478 (4 mg/ml) in 30% β-Cyclodextrin (aq) 5 mins prior to challenged with *S. aureus* MN8 prevented lethality in four out of five rabbits (Figure 4.6A). The difference in survival between the groups was significant (p=0.03, Mantlel-Cox test). Additionally, rabbits treated with AG1478 showed a significant reduction in fever; a primary indicator of mTSS symptoms (Figure 4.6B). The data demonstrate that inhibition of EGFR via AG1478 reduces symptoms of mTSS and prevents lethality in rabbits against intravaginal challenge by live *S. aureus*.

FIGURE 4.6: EGFR Inhibition Prevents mTSS Lethality in Rabbits

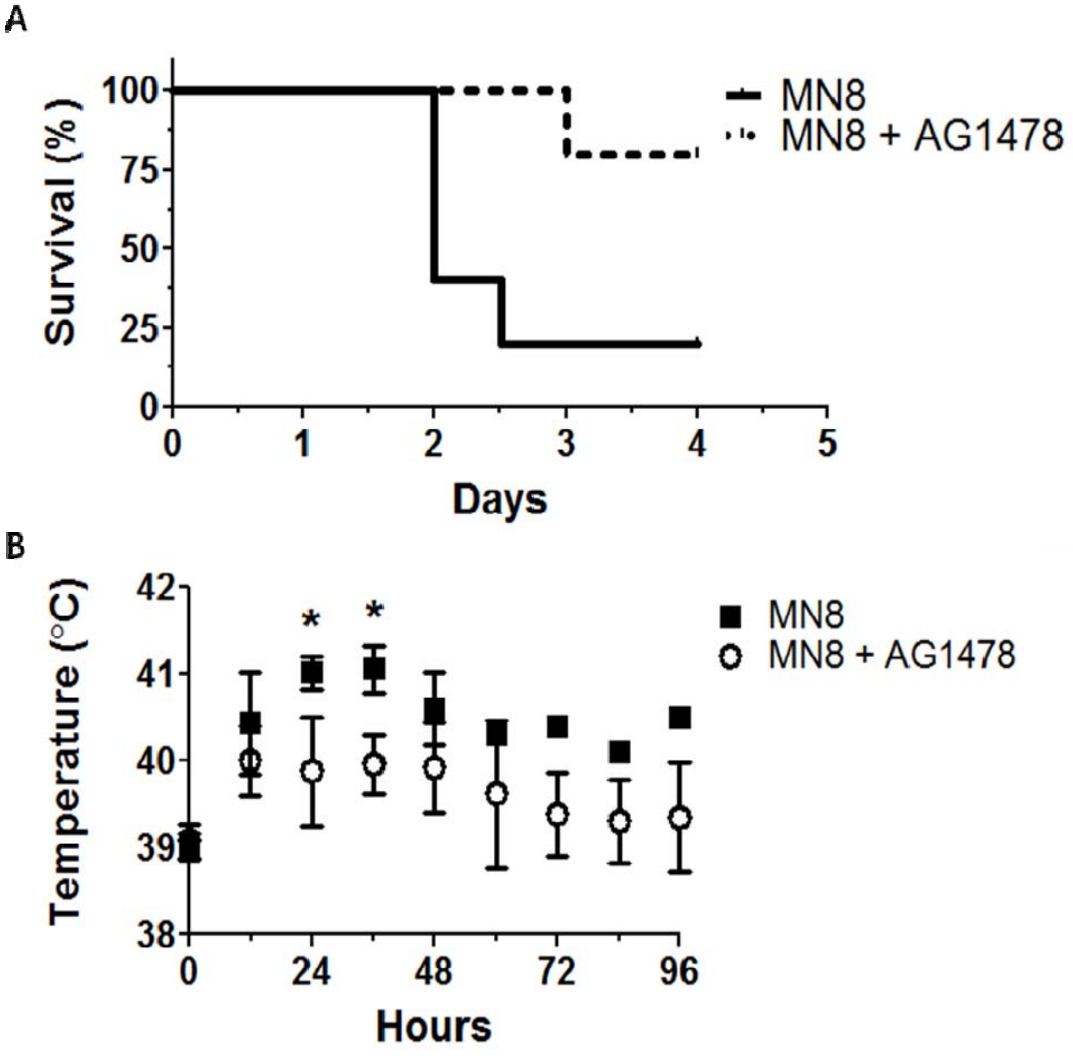


Figure 4.6: Inhibition of EGFR-signaling reduces mTSS lethality from live S. aureus in rabbits. *Rabbits (n=5) were intravaginally challenged with 10^{10} CFU of MN8 with AG1478 (2 mg/rabbit) or vehicle twice daily for 4 days or until death. (**A**) Treatment with AG1478 significantly reduced lethality ($p<0.05$, Mantel-Cox test). (**B**) Treatment with AG1478 also generally decreased fever, reaching statistical significance at 24 and 36 h ($p <0.05$, Student's t-test).*

4.4 Discussion

The primary role of EGFR in homeostasis is regulation of cell growth, division, differentiation and survival [120]. When tissue is challenged by injury or infection, EGFR-signaling plays a major role in wound healing and the innate immune response [68,120,163]. During infection by *S. aureus*, multiple virulence factors and exotoxins are known to exploit EGFR-signaling to modulate inflammation, tight junction integrity and promote mitogenicity [67,166–168]. Inhibition of EGFR-signaling prevents the cytokine response induced by *S. aureus* exotoxins including TSST-1, alpha-toxin and gamma-toxin [Chapter II., Chapter III.,4]. Additionally the combined pro-inflammatory effects of TSST-1 and alpha-toxin can be blocked through EGFR inhibition without affecting the cytotoxicity, which suggests the inflammatory response is specifically exploited during disease [Chapter III.]. To cause mTSS, *S. aureus* exploits the host immune response to create a cytokine storm, and it is possible that an epithelial immune response could begin or facilitate this process [180]. Inhibition of EGFR is already used therapeutically, making it an attractive target for inhibition of the pathogenesis of multiple *S. aureus* exotoxins.

Inhibiting EGFR-signaling is not without toxicities. Most toxicities of TKIs are associated with long term therapy, with the most common being cutaneous and including folliculitis, rashes and impaired wound healing [164,188]. TKIs are not used for short term therapy and acute toxicities are not well studied, but impairment of wound healing would likely occur immediately following treatment [171,188]. Any application of TKIs for treatment of mTSS would need to carefully consider effects on wound healing in

infected tissue. Prior to initiation of any therapy for mTSS, the bacterial infection and toxins are very disruptive to tissue. Until antibiotic therapy can reduce the bacterial burden, and the toxins are cleared from the tissue, any effect of TKIs on wound healing would likely be trivial compared to the destructive effects of the bacteria and toxins. Thus, there is likely a 24 to 72 h window where inhibition of EGFR-signaling could be used to inhibit cytokine production prior to antibiotics taking effect. Therefore, the primary safety concerns for using TKIs to treat mTSS would be to insure the preparation does not damage the epithelial surface or tissue, as short term effects on wound healing would likely be minimal.

Of the class, AG1478 has the lowest molecular weight and is highly lipophilic while lacking large hydrophobic side chains present in other TKIs [131,189]. Thus, the absorption across the vaginal mucosa of AG1478 is likely to be superior to other drugs within the class. We estimated the topical bioavailability in the tissue of AG1478 in 30% β-cyclodextrin (aq) was 54% after 2 h, which is adequate for drug delivery (Figure 4.3). TKIs as a class have low water solubility and organic or inorganic solvents are often irritants or facilitate penetration of small molecules, which could have an unknown effect of toxin penetration [131]. While minimally soluble in aqueous solutions, AG1478 forms a colloidal suspension in both 15% and 30% β-cyclodextrin (aq), which allows for even distribution across mucosal surfaces. There are many modifications to cyclodextrins to improve drug solubility, which have been proven safe on mucosal surfaces for long term use [186]. While we were limited in the scope of delivery vehicles we could test, it would

be beneficial going forward to determine a non-toxic vehicle for AG1478 to improve tissue absorption.

The lethality of TSS and mTSS have been thought to be partially a result of enhancement of the lethal effects of endotoxin from endogenous sources [116,190,191]. As a result, most models for TSS or mTSS rely on a combination of TSST-1 and endotoxin to produce acute onset of symptoms [66,116,170,190,191]. However, in humans the disease progresses over the course of days, first presenting as flu-like symptoms for 24-48 h that rapidly progress to shock and multi-organ failure within 8 h [21,183]. We observed similar pathologies in both our toxin and live organism rabbit models for mTSS. The onset of symptoms progressed over 48 h with fevers peaking between 24-48 hours followed by rapid progression of disease and death within 48-72 h. Our model provides a more clinically relevant method for studying mTSS in rabbits and developing potential therapeutics, as the progression of disease and the timeframe overlap with what is seen in humans.

Herein, we demonstrated that inhibition of EGFR-signaling via topical AG1478 can prevent lethality in a live *S. aureus* rabbit model for mTSS. Furthermore, AG1478 treatment reduced fever in rabbits, which is a primary symptom and indicator of disease severity. We speculate this is due to preventing activation of the innate immune response and subsequent *S. aureus* induced production of the cytokines IL-8, MIP-3α and TNF-α, which modify the host immune response to promote pathogenesis [Chapter II, Chapter III, 4]. IL-8 production would lead to an increased neutrophil migration which *S. aureus* is well adapted to survive in and lyse, which can cause further tissue damage and

inflammation [86,172–174]. MIP-3α is a key chemokine in attracting immature dendritic cells to the site of infection in addition to also promoting neutrophil migration [175]. TNF-α is a key factor in dendritic cell activation, inducing MHC-II expression and migration to lymph nodes [177].TSST-1 survives dendritic cell phagocytosis, and subsequently trafficked to the lymph nodes where it is presented to CD4+ T-cells [115]. Thus, it is possible that inhibiting EGFR-induced production of cytokines subsequently limits superantigen trafficking out of the tissue reducing exposure to T-cells.

Current clinical therapy for TSS and mTSS are highly successful at preventing lethality, with mortality between 1.8-4% following appropriate therapy [16,29]. However, it is concerning that >70% of patients suffer at least one major long-term morbidity including cognitive impairment, apraxia, cardiomyopathy or recurrence of disease [35,36]. The cognitive and cardio complications result from the prolong stress of shock and fever and can be life-long, creating a substantial burden for the patient [21,35,36]. If inhibition of EGFR-signaling via TKIs can reduce the severity of disease while antibiotic therapies take effect, then it will likely result in a reduction in mTSS morbidities. It is possible that this could be accomplished easily, and at low cost, through 1 or 2 intravaginal administrations of a gel or suspension upon hospitalization. This proposed TKI therapy would be combined with current standard of care treatments, including intravenous antibiotic treatment and life support. Clearly, more research is needed before any human use can be considered.

We demonstrated proof of concept for TKIs for the prevention of mTSS. A possible mechanism of action is also proposed, but further research tracking antigen presenting

cells, T-cells and TSST-1 migration and distribution through mucosal tissue is needed to confirm the hypothesis. Future experiments should be conducted to determine if AG1478 or other TKIs could be used as adjunct therapy for treatment of *S. aureus* TSS or mTSS. Furthermore, the drug and delivery method we used for our experiments were designed to test our hypothesis and would need to be optimized for therapeutic adaptation. It would be beneficial to test other TKIs and delivery vehicles for efficacy in animals to find an optimal combination. A possible delivery vehicle could include a glycerol monolaurate (GML) containing gel with a propylene glycol base, because GML has anti-bacterial and anti-inflammatory effects on the vaginal mucosa [54,56]. In conclusion, we believe TKIs are worth pursuing as an adjunct therapy for treatment of mTSS caused by *S. aureus.*

CHAPTER V.

Vaccination against *Staphylococcus aureus* Pneumonia

This chapter was published in The Journal of Infectious Diseases 2014;209:1955–62.
This study was performed by Dr. Spaulding in Dr. Schlievert's laboratory, in collaboration with Dr. Peterson and myself. I assisted with MN8 infections and surgeries, hemolytic assays, ELISA design, and provided gamma-toxin.

5.1 Introduction

Staphylococcus aureus is capable of infecting nearly every tissue of the body, and utilizes a variety of exotoxins and surface factors to cause disease [33]. The most prevalent conditions caused by *S. aureus* are uncomplicated skin and soft tissue infections (SSTIs) which are generally not life-threatening with appropriate care [1]. Uncomplicated SSTIs can progress to complicated infections such as bacteremia, endocarditis, osteomyelitis or necrotizing pneumonia which have much higher rates of mortality and severe morbidities [13]. Treatment of complicated infections is costly, regularly requiring longer hospital stays and sometimes surgery [13].

To date, no vaccine exists for prevention of *S. aureus* infection. Despite many vaccine candidates reaching phase-II or phase-III clinical trials they have universally failed. Primarily, attempts have focused on surface factors with clumping factor-A and the polysaccharide capsule being the most investigated [44,148]. A capsular vaccine showed minor efficacy in preventing *S. aureus* bacteremia in dialysis patients out to 40 weeks, but was ineffective by 57 weeks [44]. Passive immunization against capsule for treatment of *S. aureus* infection reduced the duration of hospital stay, but had no effect on mortality [44]. The most recent attempt targeted an iron surface determinant (IsdB) for prevention of infection post-cardiac surgery, but was ended prematurely due to an increase in all-cause mortality and infections in the vaccinated group [48]. These failed attempts represent a substantial cost in effort, money and time.

The precise reason for failure in developing a *S. aureus* vaccine is unknown, but evidence suggests that use of cell-surface factors may be to blame. The organisms *S. aureus* and *Enterococcus spp.* are known to aggregate during infection to promote

virulence [50,51,192]. Aggregation in these organisms is thought to promote biofilm formation, which enhances defense against the host and induces quorum-sensing regulated virulence factors [49–51,192]. Active vaccination generates immunoglobulin-G (IgG), which in addition to opsonization also aggregates bacteria. In rabbits challenged with *E. faecalis* in an endocarditis model, vaccination against an aggregation factor increased lethality while passive immunization with Fab fragments was protective [50]. Additionally, rabbits vaccinated with *S. aureus* surface proteins enriched for iron surface determinants showed complete lethality when challenged in an endocarditis model, while unvaccinated animals recovered completely [193]. *S. aureus* also produces numerous aggregation factors. Thus it is likely that IgG promotes clumping and biofilm formation which enhances *S. aureus* virulence [51]. In addition, it is also possible the increased lethality is a result of enhanced toxin production, resulting from limiting iron acquisition [49]. The surface proteins of *S. aureus* are primarily involved in host defense and nutrient acquisition, whereas secreted virulence factors cause tissue destruction, promote invasive infection, lyse immune cells and enhance biofilm formation [33,169]. Hence, we propose that a vaccination strategy targeting exotoxins could be protective against infection by *S. aureus*.

Exotoxins of *S. aureus* are critical determinants for both the location of infection and severity of disease. The *S. aureus* USA300 and USA400 strains are the most common causes of SSTIs and community-acquired pneumonia and commonly possess the staphylococcal chromosomal cassette (SCC), which produces high levels of the cytotoxin α-toxin and superantigens SEB and SEC [194]. In contrast, USA200 strains are

commonly associated with mTSS and endocarditis, and typically produce the exotoxins TSST-1, SEC and low levels of alpha-toxin [169,193,194]. The cytolysins, beta-toxin and gamma-toxin are produced by nearly all strains of *S. aureus,* and are known to play critical roles in tissue destruction, nutrient acquisition and lysis of immune cells but are not directly associated with specific diseases [33,84]. However, the cytotoxin Panton-Valentine leukocidin (PVL), present in ~5% of USA300 strains, shares the gamma-toxin B subunit and is associated with invasive necrotizing pneumonia [33,89]. The toxins produced by *S. aureus* are dependent on the strain and SCC type, which results in specific strains being commonly associated with infections of specific tissues.

Previous clinical trials of *S. aureus* vaccines have targeted one or two surface proteins for prevention of a single condition, and have failed to provide efficacy or actually increased lethality [44,48]. There has been limited investigation using exotoxins for a vaccine, but they have demonstrated efficacy in animal models against single strains of *S. aureus*. In a murine SSTI, a monoclonal antibody directed at alpha-toxin reduced abscess formation from USA300 strain Newman [195]. Additionally, rabbits vaccinated with a cocktail of exotoxins were protected from lethal pneumonia or sepsis when challenged with the USA200 strain MNPE [196].

The goal of this study is to investigate the ability of a multi-component vaccine consisting of biologically inactive or sub-lethal amounts of TSST-1, SEB, SEC, alpha-toxin, beta-toxin and the gamma-toxin B subunit in prevention of lethal pneumonia in rabbits against multiple *S. aureus* strains. The *S. aureus* strains USA100, 200, 300 and 400 all cause infectious pneumonia, thus providing an infectious model which can be

used to test vaccine efficacy against different strains. Herein, we investigated vaccination against exotoxins to protect rabbits from *S. aureus* pneumonia following intrapulmonary challenge. Furthermore, we investigated the antibody response to vaccination, and the ability of passive immunization to protect rabbits from *S. aureus* pneumonia. Lastly, we determined the ability of commercially available human IVIG to prevent and treat toxin-mediated disease. In conclusion, we demonstrated efficacy of a multi-component exotoxin vaccine for prevention of pneumonia and toxin-mediated disease induced by diverse strains of *S. aureus*.

5.2 Methods

Bacterial Strains

The toxins TSST-1 and TSST-1 toxoid with G31S and S32P mutations (disrupted binding to MHC-II), SEB and SEB toxoid Q210A (lacks T-cell receptor binding), SEC and SEC toxoid N23A (lacks T-cell receptor binding), Sel-X, and alpha-toxin toxoid H35A (lacks oligermization) were purified from attenuated laboratory *S. aureus* strain RN4220 expressing individual genes on plasmids [196]. RN4220 was also the source for wild type beta-toxin. *S. aureus* MNJA and MNPE were the sources of wild type alpha-toxin. *E. coli* with pET-30(a)$^{+}$ was the source of gamma-toxin B subunit.

S. aureus strains used in pneumonia challenge experiments are listed (Table 5.1) with their PFGE clonal group and toxin genes present that were relevant to the study. The USA 200 strains MNPA, MN8, and CDC587 contain the alpha-toxin gene with a stop codon mutation at position 113 which produces truncated alpha-toxin and full length alpha-toxin by read through at 1/50 the level of the wild type gene [169].

Inoculation for intrapulmonary infection was prepared from overnight cultures grown to stationary phase in Todd Hewitt broth (Difco Laboratories) at 37°C with aeration. The cultures were washed in PBS following centrifugation at 20,800xg for 5 mins and resuspended in Todd-Hewitt broth at ~2.5-4 x 10^{9} CFU/0.4 ml for infection.

TABLE 5.1: *S. aureus* Strains Used for Vaccination Studies

Strain	Clonal Group	Superantigens	Cytolysins	Source
IA209 (MSSA)	USA100	SE*l*-X	α-, β-, γ-toxin	Dr. Horswill, Uni. of Iowa
MNPE (MSSA)	USA200	TSST-1, SEC	α-, β-, γ-toxin	Postinfluenza pneumonia
MNPA (CA-MRSA)	USA200	TSST-1	α-, β-, γ-toxin[a]	Menstrual TSS
MN8 (MSSA)	USA200	TSST-1, SEC	α-, β-, γ-toxin[a]	Menstrual TSS
CDC587 (MSSA)	USA200	TSST-1, SEC	α-, β-, γ-toxin[a]	Menstrual TSS
LAC (CA-MRSA)	USA300	SE*l*-X	α-, β-, γ-toxin	F. R. Deleo, NIH
MNLe (MSSA)	USA300	SE*l*-X	α-, β-, γ-toxin	Necrotizing pneumonia
MW2 (CA-MRSA)	USA400	SEC, SE*l*-X	α-, β-, γ-toxin	Necrotizing pneumonia
C99-529 (MRSA)	USA400	SEB, SE*l*-X	α-, β-, γ-toxin	Necrotizing pneumonia

Clonal type is determined by PFGE and methicillin-resistance by the presence of SCCmec DNA.

Production of wild-type gamma-toxin among strains is similar. Beta-toxin production in USA200 is consistent but varies among other clonal groups with all strains producing some beta-toxin.

[a] *These strains contain an alpha-toxin gene with a stop codon mutation and produce 1/50 the amount of wild-type alpha-toxin.*

Toxin Purification

All reagents used were pyrogen-free. Purification of the toxins or toxoids of TSST-1, SEB, SEC, SEl-X and alpha-toxin was done from RN4220 grown overnight in dialyzable beef-heart medium. The exoproteins were harvested from overnight cultures by ethanol precipitation (80% aq v/v) and resolubilized in water. Purification was done by thin-layer isoelectric focusing and confirmed by SDS page. Purified products were then assayed for hemolysins, lipases, nucleases and proteases [79,197]. Wild-type alpha-toxin was prepared similarly except the initial toxin precipitation utilized 80% ammonium sulfate (aq). All proteins electrophoresed as a single band in Western immunoblots with hyperimmune antisera raised against the purified toxins. Proteins were quantified by the Bio-Rad protein assay.

The gamma-toxin nontoxic B chain was produced in *E. coli*, where expression was induced in Luria broth (kanamycin 30 μg/mL) containing 0.3 mM isopropyl β-D-1-thiogalactopyranoside. Bacterial pellets were lysed with lysozyme (100 μg/mL) and sonication. The lysate supernatant was sterile-filtered and incubated with Ni- NTA resin (Invitrogen) to bind the B chain. The protein was eluted from the resin with an imidazole gradient. Endotoxin from HlgB preparation was removed using Pierce High Capacity Endotoxin Removal Resin per the manufacturer's protocol (PI88267). Final endotoxin concentration was calculated by LAL assay and determined to be between 0.0125-0.125 EU/ml (~1.25-12.5 pg/ml, data not shown).

Rabbits

Young adult New Zealand White rabbits male and female weighing ~2-3 kg were purchased from Bakkom Rabbitry. Rabbit use adhered to established protocols of the Universities of Iowa and Minnesota Institutional Animal Care and Use Committees.

Vaccination Schedule

Rabbits were vaccinated subcutaneously in the nap against biologically active and inactive toxins. For all toxoids, beta-toxin or gamma-toxin B subunit each vaccination contained 25 μg of each protein in Freund's incomplete adjuvant. For vaccination against biologically active toxins, rabbits received sub-lethal doses of 10 μg of each toxin in Freund's incomplete adjuvant. Rabbits received a total of 3 vaccine immunizations, administered once a week for 3 weeks.

Antibody Titer

One week after the final vaccination blood was drawn and centrifuged to collect serum. Serum antibodies to wild-type toxins were determined by ELISA. In brief, 96-well maxisorp plates (NUNC) were coated with 1.0 μg/well with wild-type protein or 10 μg/well of SEl-X. Rabbit serum was serially diluted 2-fold and incubated for 1.5 h at room temperature and washed. Wells were incubated with anti-rabbit horseradish peroxidase conjugated antibodies (Sigma) for 1.5 h then washed. Wells were then incubated with O-phenylenediamine and hydrogen peroxide. Reactions were stopped with 0.16 M sulfuric acid and IgG levels determined by absorbance at 490 nm. Relative antibody titers were expressed as the reciprocal of the last dilution to give a positive reaction.

Rabbit Infection Model

One week after final vaccination, rabbits were challenged by intrapulmonary infection with 2.0-4.0x10^9 CFUs in 0.4 ml Todd-Hewitt broth. For inoculation, rabbits were anesthetized intravenously with ketamine (25 mg/kg) and xylazine (25 mg/kg) (Phoenix Pharmaceuticals). The rabbit necks were shaved and a small incision was made to expose the trachea. An incision was made in the trachea and a 1 mm diameter polyethylene catheter (Becton Dickinson) was threaded to the left bronchi. Bacteria were administered through the catheter and the incision sutured. Rabbits were monitored for 7 days for development of pneumonia, death and TSS symptoms, including fever, difficulty breathing, diarrhea, reddening of eyes and hypotension. Rabbits were euthanized when they simultaneously could not remain upright and failed to exhibit flight response. Surviving rabbits were euthanized at the end of the 7 day experiment. Euthanasia was performed with 1 ml/kg Beuthansia-D (Schering-Plough).

For passive immunization studies against live organisms, rabbits were administered 3 ml of pooled serum from vaccinated animals and challenged as described above with USA200 strain MNPE. To evaluate passive protection against toxin-mediated disease, rabbits were administered 12,000 μg anti-SEB IgG (ZLB Bioplasma AG) intravenously at the time of infection or 24 and 48 h post challenge. Rabbits were anesthetized as described above and challenged with 200 μg SEB by intratracheal injection. Animals were monitored for survival and symptoms of TSS for 4 days as described above.

5.3 Results

5.3.1 Seven-Component Vaccine

Vaccination against *S. aureus* with a cocktail of 7 exotoxins protected rabbits from lethal intrapulmonary challenge. USA100 strains are common colonizers of the nares and a cause of hospital acquired-pneumonia. The primary toxins produced by USA100 strains are alpha-toxin, beta-toxin, gamma-toxin and SEl-X, with the SEC gene present, although production varies among strains [198]. Five of the six vaccinated rabbits survived, and cleared the infection completely by 7 days. The only recognizable adverse event in the vaccinated animals was development of fever at 24-48 hours post challenge. In contrast, none of the non-vaccinated rabbits survived, and all developed pulmonary disease and TSS symptoms including fever, diarrhea, weight loss and hypotension (Table 5.2). Postmortem examination of the lungs showed severe hemorrhagic and necrotic tissue in non-vaccinated rabbits compared to minor pulmonary damage with sterile granulomas in vaccinated rabbits (Figure 5.1). Prior to vaccination all rabbits had ≤1:32 antibody titers which significantly increased post vaccination (Figure 5.2).

Passive immunization with serum protected rabbits from lethal intrapulmonary challenge by *S. aureus*. To evaluate if protection was antibody mediated, serum from rabbits immunized with the 7 exotoxin vaccine was injected 15 min prior to infection in naïve rabbits. Rabbits were challenged by intrapulmonary infection with MNPE, a USA200 strain that is a cause of post-influenza pneumonia and produces TSST-1, SEC, alpha-toxin, beta-toxin and gamma-toxin [110]. All 6 rabbits receiving serum from

vaccinated animals survived for the course of the 7 day experiment, and their lungs appeared similar to rabbits which received active vaccination. In contrast 5 of the 6 rabbits which received control serum died before 7 days and all developed pulmonary illness (Table 5.3). All rabbits in both groups showed symptoms of TSS.

5.3.2 Monovalent and Multivalent Vaccines

Superantigens are critical for *S. aureus* pneumonia. To evaluate protection from vaccination against challenge from USA200 strains a total of 35 rabbits were vaccinated with a trivalent vaccine (n=27) against TSST-1, SEC and alpha-toxin, or a monovalent vaccine (n=8) against TSST-1. The rabbits were challenged with 4 strains of USA200, 3 of which were from mTSS patients who expired and 1 from post-influenza pneumonia [79,199–201]. None of the 35 vaccinated animals succumbed to intrapulmonary challenge by any of the USA200 strains and remained healthy for the 7 day period. In contrast, 34 of 35 nonvaccinated animals succumbed to pulmonary infection and TSS within 7 days (Table 5.2). As observed earlier, the lungs of vaccinated animals only had minor damage with sterile granulomas, whereas nonvaccinated animals had severe necrosis (Figure 5.1). Additionally, protection against challenge by MNPA was achieved with only vaccination against TSST-1.

Immunity to TSST-1 and alpha-toxin protected rabbits from pneumonia by USA300 strains of *S. aureus*. A total of 24 rabbits were immunized with a bivalent vaccine of TSST-1 and alpha-toxin, or a monovalent vaccine of TSST-1. The rabbits were challenged with 2 different USA300 strains, including a CA-MRSA strain and a

MSSA strain from patients with necrotizing pneumonia [202,203]. As observed previously, all vaccinated rabbits survived challenged from intrapulmonary infection, and cleared the infection with minimal tissue damage in the lungs. Again, all non-vaccinated animals succumbed to intrapulmonary challenge within 7 days with severe necrosis of the lungs (Table 5.2). Furthermore, rabbits vaccinated against TSST-1 and alpha-toxin, or just TSST-1, survived challenge against CA-MRSA, indicating that TSST-1 is a critical mediator of *S. aureus* pneumonia in rabbits.

Immunity to SEC and alpha-toxin protected rabbits from pneumonia by USA400 strains of *S. aureus*. To investigate protection in USA400 strains a total of 23 rabbits were vaccinated and challenged with 2 clinical isolates from patients with necrotizing pneumonia. The first group of 8 rabbits was immunized with a bivalent vaccine against SEB and alpha-toxin then challenged with MRSA strain 99-529 (SEB^+, alpha-$toxin^+$). In the second group, 8 rabbits were immunized with a bivalent vaccine against SEC and alpha-toxin, and 7 were immunized with a monovalent vaccine against SEC then challenged with CA-MRSA strain MW2 (SEC^+, alpha-$toxin^+$). All rabbits that received a bivalent vaccine and 6 out of 7 that received the monovalent vaccine survived intrapulmonary challenge from *S. aureus* while all non-vaccinated animals succumbed within 7 days (Table 5.2). As observed before, all surviving animals cleared the infection with minor lung tissue damage, while those that died had severe necrosis in the lungs (Figure 5.1). These data further support a critical role of superantigens in *S. aureus* pneumonia, with vaccination against either TSST-1 or SEC being sufficient to provide protection.

TABLE 5.2: Rabbit Survival following Intrapulmonary Challenge by *S. aureus*

Clonal Group	Strain	Vaccination Against	Vaccinated Survival	Nonvaccinated Survival
USA100	IA209	TSST-1, SEB, SEC, SEl-X, α-toxin, β-toxin, γ-toxin	5/6[a]	0/6
USA200	MNPE	TSST-1, SEC, α-toxin	11/11[a]	0/11
	CDC587	TSST-1, SEC, α-toxin	8/8	0/8
	MNPA	TSST-1	8/8	1/8
	MN8	TSST-1, SEC, α-toxin	8/8[a]	0/8
USA300	LAC	TSST-1, α-toxin	8/8	0/8
	LAC	TSST-1	8/8	0/8
	MNLevy	TSST-1, α-toxin	8/8[a]	0/8
USA400	C99-529	SEB, α-toxin	8/8[a]	0/8
	MW2	SEC, α-toxin	8/8	0/8
	MW2	SEC	6/7	0/7
Total Rabbits [b]			**86/88**	**1/88**

[a] *Animals in these groups were evaluated for CFUs. All surviving animals had no detectable CFU in their lungs at 7 days compared to $>10^9$ CFU/lung in animals that died.*

[b] *P value = $1x10^{-46}$ (Total Rabbits, Vaccinated vs. Nonvaccinated, Fisher's exact test)*

FIGURE 5.1: Rabbit Lungs in Nonvaccinated vs. Vaccinated

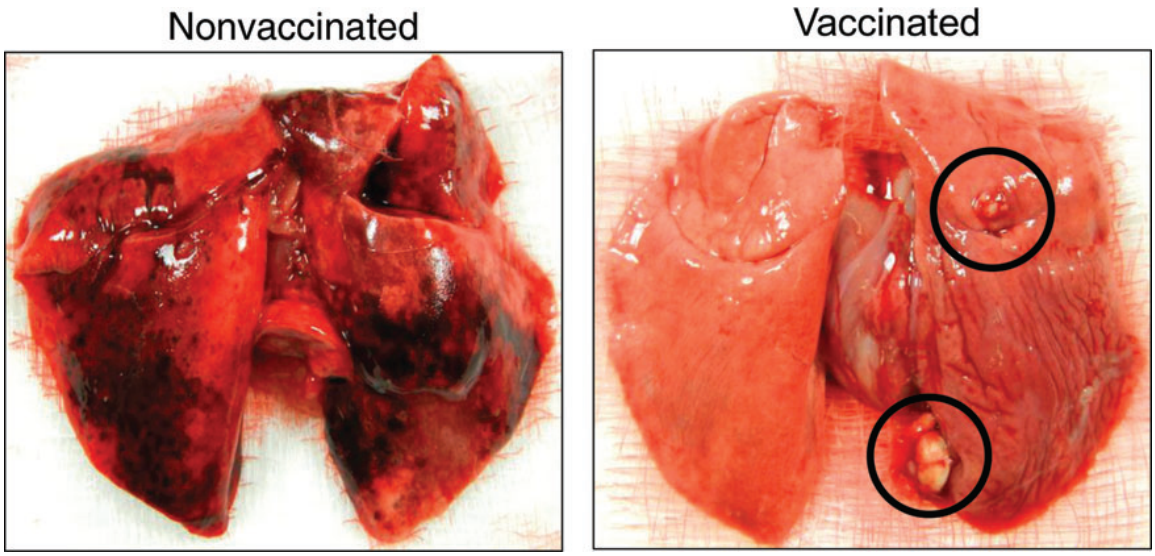


Figure 5.1: Representative images of lungs from nonvaccinated and vaccinated animals. *Images are from animals challenged with 2.0-4.0x109 CFU of IA209 with or without 7-component vaccine. Lungs from nonvaccinated animals at the time of death had severe hemorrhage and necrosis. Vaccinated animals that survived for the course of the experiment showed minimal tissue damage with sterile granulomas (Circled). Lungs from all animals in each group, nonvaccinated or vaccinated, were similar.*

FIGURE 5.2: Antibody Titers following Vaccination in Rabbits

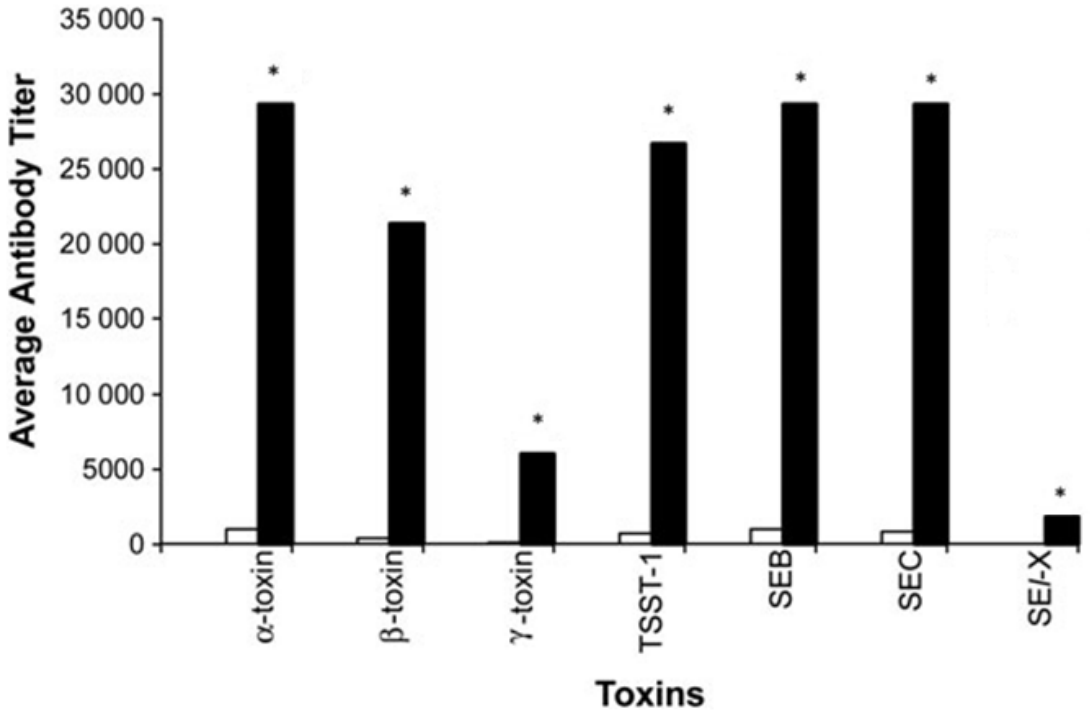


Figure 5.2: Antibody titers after immunization with 7-component vaccine. *Antibody titers were measured after 1 week following 3 weekly immunizations in rabbits. Data represent the reciprocal of the last dilution of serum to produce positive response in an ELISA. Significance between control and vaccinated serum within groups is denoted (*, P<0.05, Student's t test).*

FIGURE 5.3: Rabbit Survival Following Passive Immunization

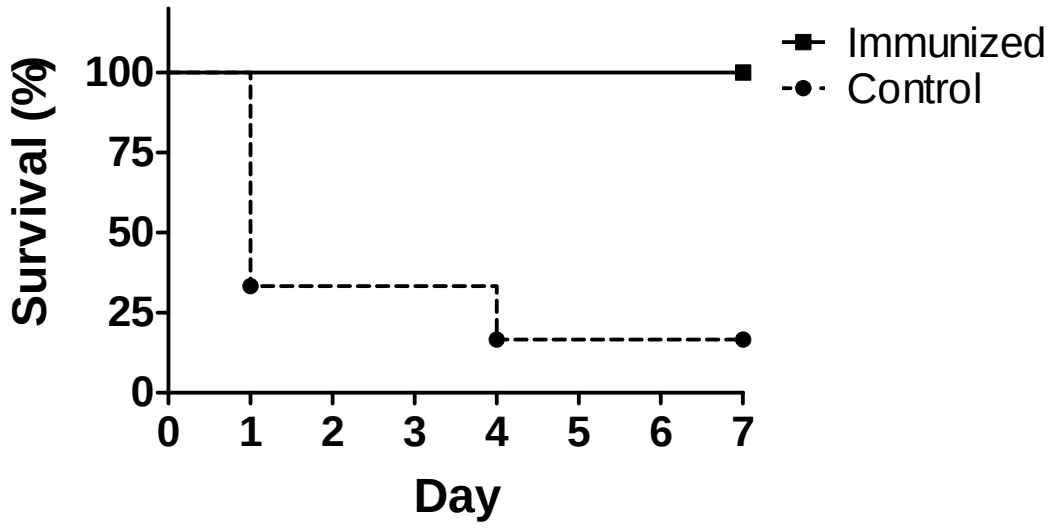


Figure 5.3: Passive immunization with serum protected rabbits from intrapulmonary challenge. *Rabbits given serum (6 per group) from rabbits immunized with 7-component vaccine or control serum and challenged intrapulmonary with 2.0-4.0x10^{9} CFU of S. aureus USA200 strain MNPE. Significant differences in survival over 7 days were observed between groups (P=0.004, Log-rank Mantel-Cox test).*

5.3.3 Treatment of Lethal Superantigen Intrapulmonary Disease in Rabbits

Passive immunization against SEB protected rabbits from toxin-mediated disease. Since rabbits were protected from both active vaccination and passive vaccination with serum, the ability of commercial human IVIG to protect rabbits from intrapulmonary challenge with SEB was investigated (Figure 5.4). When rabbits, 5 per group, were administered IVIG simultaneously with SEB, or 24 h following challenge, all rabbits survived for 4 days compared to none in the control group. When treatment was delayed for 48 h, 4 out of 5 animals survived the duration of the experiment. The typical disease progression in rabbits was a high fever developing during the first day, reduced temperature and shock by day 2, and death by day 3. Rabbits treated immediately with IVIG did not develop symptoms, and when treatment was delayed symptoms resolved within 24 h.

FIGURE 5.4: Treatment of Exotoxin Mediated Disease by IVIG in Rabbits

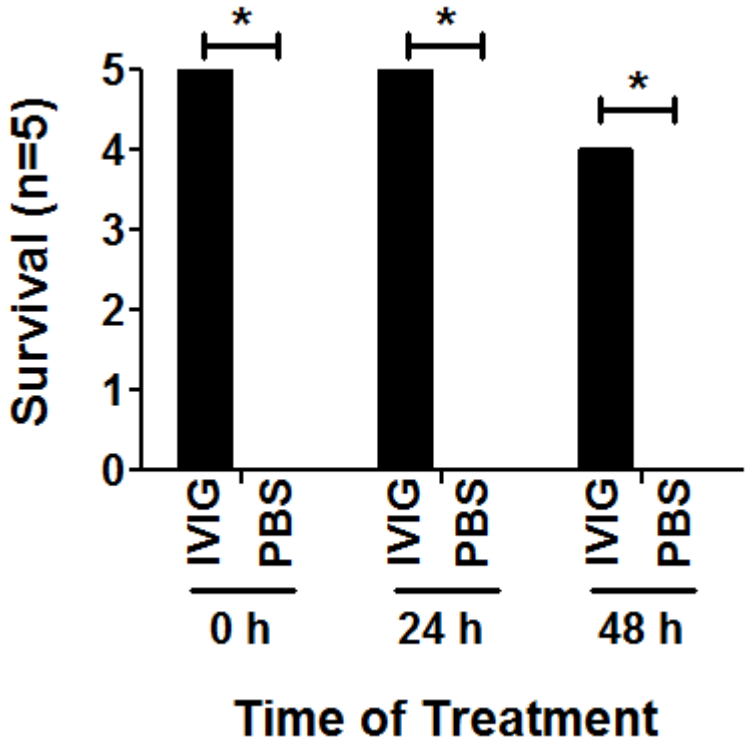


Figure 5.4: Human IVIG protects rabbits from lethal challenge with superantigens. *Rabbits passively immunized (5 per group) with human anti-SEB at time of challenge, or at delayed times after challenge, were protected from lethal intrapulmonary challenge with 200 μg SEB. Data represent survival over 4 day period and significant differences within groups are denoted (*, P<0.05, Student's t test).*

5.4 Discussion

Herein, we demonstrated that vaccination against *S. aureus* exotoxins is efficacious in preventing lethal pneumonia in rabbits challenged with multiple clinical isolates. Passive or active immunization with the 7 component exotoxins vaccine was safe and effective against USA100 and USA200 strains. Active vaccination against superantigens, TSST-1 or SEC, was also found to be sufficient for protection in USA200, USA300 and USA400 strains. Furthermore, protection was observed for both MRSA and MSSA strains.

We evaluated protection for pneumonia, but the strains used are associated with other infection types. The USA100 IA209 and USA200 MNPE strains represent common sources of hospital-acquired and post-influenza pneumonia, respectively [198,200]. However, the USA200 strains are commonly associated with mTSS, which is mediated by the superantigen TSST-1 [35]. While we did not evaluate vaccination against mTSS, high levels of antibodies against superantigens or TSST-1 are considered protective for both TSS and mTSS [33,204]. The USA300 strains are typically associated with both community- and hospital-acquired SSTIs and generally produce low levels of superantigens, except for SE*l*-X, and high levels of core virulence factors including, alpha-toxin, beta-toxin and gamma-toxin [32,205]. Interestingly, vaccination solely against TSST-1 was protective in preventing pneumonia in rabbits, which may be partially attributed to anti-TSST-1 antibody cross-reactivity with SE*l*-X [206,207]. Additionally, USA300 strains are the predominant carriers of the PVL toxin, which is associated with invasive necrotizing infections [32,33]. While we did not evaluate the

role of PVL in this study, the gamma-toxin B subunit is homologous to the PVL-F subunit and vaccination against this single subunit would likely provide protection against both toxins [87]. The USA400 strains we used were typical for CA-MRSA necrotizing pneumonia, but USA400 are common causes of SSTIs [8]. As observed in other strains, vaccination against only superantigens provided ~85% protection from lethal *S. aureus* pneumonia by USA400 strains. However, when alpha-toxin was included protection was complete, which may be due to alpha-toxin's critical role in pneumonia [195].

Superantigens have typically been associated with TSS following development of infection [206]. However, we demonstrated that vaccination against only superantigens prevents lethal pneumonia, indicating a critical role in development of disease. In contrast, alpha-toxin is clinically and experimentally linked to pneumonia, and vaccination against alpha-toxin is protective in murine *S. aureus* pneumonia [14,32,195,208]. However, mice are resistant to the lethal effects of superantigens, and in mice cytolysins likely play a more critical role [208]. Even though we evaluated protection against pneumonia, the 7-compenent vaccination would likely provide protection against a large range of infections. Superantigens are already known to play a critical role in bacteremia, lethal endocarditis and kidney infection, demonstrated in rabbits with infection attenuated by deletion mutants [209]. Additionally, vaccination against alpha-toxin is protective against *S. aureus* SSTIs in mice, preventing and reducing abscesses [210]. Furthermore, both alpha- and gamma-toxin are required for *S. aureus* induced rheumatoid arthritis in mice [101]. Combined, the individual toxins of the 7-

component vaccine are known, or are likely to protect against a majority of *S. aureus* pathologies including pneumonia, SSTIs, endocarditis, bacteremia and auto-immune diseases.

Our model to develop a *S. aureus* vaccine is different from most previous attempts. Nearly every attempt to date has targeted surface proteins in an effort to promote clearance of the *S. aureus*. Targeting exotoxins should not directly facilitate clearance, but pulmonary infection in rabbits was cleared within 7 days with vaccination. Clearance of *S. aureus* by targeting exotoxins may be a result of preventing the immune modulation and destructive effects from exotoxins, allowing for the immune system to function effectively [33,157]. Furthermore, we demonstrated a therapeutic application of targeting superantigens for treatment of toxin mediated disease.

A second major difference in our approach to develop a *S. aureus* vaccine is the use of rabbits as a model. Previous attempts commonly used murine or non-human primates, which are both highly resistant to superantigens and show moderate resistance to alpha-toxin [208,211]. Nearly all clinical isolates produce large amounts of at least one superantigen and one cytolysin, which is commonly alpha-toxin [33]. Thus, studies of *S. aureus* infections in mice and non-human primates disregard major virulence factors. Rabbits, however, are susceptible to the lethal effects of superantigens and cytolysins, and progression of disease is more similar to humans [116,209,212]. We believe our rabbit model is more likely to represent clinical conditions in humans.

We showed that protection from *S. aureus* pneumonia was antibody-mediated, likely not requiring specific CD4+ or CD8+ T-cells. This was demonstrated by passive

immunization protecting naïve rabbits against lethal intrapulmonary challenge from *S. aureus*. However in this experiment, we did not evaluate CD4+ T-cell or macrophages in clearance of *S. aureus*. Furthermore, protection and treatment of toxin-mediated disease in rabbits could be accomplished using human derived antibodies. This supports the hypothesis that toxin-neutralizing effects dominate, and that phagocytosis or activation of complement is less important for protection.

To develop a vaccine for use in humans several issues need to be considered. Any vaccination for human use could not include any active toxin. Currently, toxoids are available for most of the 7-component vaccine, and the gamma-toxin B subunit is not biologically active [83,196]. Furthermore, since the neutralizing effects mediate protection, administering humanized monoclonal FAb therapeutically could subvert the need to vaccinate. It also may not be financially or logistically reasonable to vaccinate the entire population, considering that nearly one-third the population are colonized with *S. aureus* and the vast majority do not develop infection [3]. Furthermore, the targets of the 7-component vaccine are minimally expressed during colonization, thus vaccination against exotoxins would likely have little impact on colonization [85]. However, there are groups which are at higher risk for infection. The most predictable groups at high-risk for infection are intensive care unit patients, people on ventilators, dialysis patients or surgical patients [213]. Other high-risk groups include people who are often in close proximity with shared facilities including athletes, prisoners and soldiers, for which an active vaccination strategy could be employed [14]. In these high-risk groups, the rate of *S. aureus* infection can be up to >10-fold more than the rest of the population [14].

Therefore, there is the potential to greatly reduce the burden of *S. aureus* infections by vaccinating a relatively small subset of the population.

The need for *S. aureus* vaccine is great, as the organism causes a substantial amount of both community and hospital infections. The incidence of just hospital-acquired infections is ~500,000 a year in the United States at a total cost of >6 billion dollars, or $3,000 - $35,000 per case [15]. We demonstrated that vaccination against superantigens and cytolysins is protective against lethal intrapulmonary pneumonia in rabbits from a broad set of clinical isolates. Furthermore, vaccination against exotoxins allowed rabbits to clear the infection. Combined, the data further our understanding of *S. aureus* infections and highlight new methods for preventing or treating disease.

Chapter VI

Final Conclusions

6.1 Toxins during Disease

This thesis examines novel effects of exotoxins and their potential as therapeutic targets in *S. aureus* infections. Historically, each toxin of *S. aureus* has been characterized to perform a single action; cytolysins form pores and superantigens over-stimulate antigen presenting cells and T-cells. However, in the last several years important immune and cellular modulating effects of TSST-1 and alpha-toxin have been characterized on mucosal surfaces [67,82]. For this thesis, the novel effects of alpha-toxin and gamma-toxin on vaginal mucosa in regards to TSST-1 and mTSS were investigated. Characterizing exotoxin effects on the vaginal mucosa led to the exploration of host-targeted treatment for prevention of mTSS in animal models, which targets the host response to the immune-modulating effects of the toxin. Furthermore, an immunization strategy against exotoxins to prevent *S. aureus* pneumonia was characterized. Combined this work demonstrates the potential of targeting and inhibiting exotoxins of *S. aureus* to prevent lethal disease.

Most attempts at developing therapeutics for *S. aureus* have historically targeted surface proteins [44,148]. The predominant roles of the surface proteins of *S. aureus* involve defense and nutrient acquisition. For example, surface proteins can promote clotting, bind antibodies (protein A), facilitate biofilm formation via clumping factors, and bind iron to support bacterial transcription and replication [1]. Strategies to develop a *S. aureus* vaccine were based on the assumption that inhibiting iron uptake and preventing clumping would both block cell division and promote phagocytosis of *S. aureus*. This strategy resulted in either no protection or increased lethality in clinical

trials [44,48].The failure of these trials is thought to be attributed to IgG mediated aggregation or enhanced exotoxin production due to nutrient starvation [193]. These past failures of targeting surface proteins demonstrate the need for a new approach. Since the destructive, invasive and immune-modulating effects of *S. aureus* are heavily dependent on exotoxins our focus is on inhibiting exotoxin activity [33].

The contribution of exotoxins to *S. aureus* virulence is not limited to a single destructive action per toxin, and numerous actions and modulatory effects are now known to occur in a variety of tissues. The historical roles of superantigens, specifically TSST-1, were thought to be primarily in hyper-stimulating CD4+ T-cells and antigen presenting cells [180]. Recently, studies have shown that TSST-1 is involved in and required for other pathogenic processes. Independently, TSST-1 is observed to be destructive and proinflammatory to vaginal epithelial cells through an EGFR-dependent pathway. [67]. Furthermore, alpha-toxin, which was thought to only be a cytolysin, was recently identified to modulate ADAM10 in lung epithelial cells to disrupt focal adhesion points [72]. The studies described in this thesis identified novel cellular targets and mechanisms of action that elucidate a complex pathway, by which superantigens and cytolysins promote pathogenesis at the vaginal mucosa.

Despite gamma-toxins being encoded by almost every strain of *S. aureus,* their contributions to disease processes are not well characterized. Gamma-toxins have primarily been characterized as leukocidins, involved in defense against immune cells [96]. However, I found gamma-toxins are cytolytic to vaginal epithelial cells at concentrations similar to the concentration of alpha-toxin and TSST-1 found in mTSS

patients [109]. Additionally, gamma-toxins induce pro-inflammatory signals in exposed vaginal epithelial cells and full thickness porcine vaginal tissue. Differential effects of the gamma-toxins were observed on epithelial cells or whole thickness tissue through an ADAM and EGFR dependent pathway. The more potent response by HlgBC in *ex vivo* tissue could result from stimulation of tissue-resident macrophages, since HlgAB lacks any activity against human macrophages [93]. In contrast, the enhanced cytokine production and cytotoxicity by HlgAB on HVECs suggests that while both mediate destructive effects on tissue, the gamma-toxin HlgAB dominates initial interactions with the intact epithelial surface. Interestingly, the proinflammatory effects of TSST-1 and gamma-toxins share a common signaling mechanism, and the two toxins are synergistic in stimulating IL-8 production in HVECs. The importance of these novel actions are strengthened with gamma-toxin genes being the most up-regulated, by a factor of >50 fold, over any other exotoxins when *S. aureus* is exposed to blood, which is present during menstruation [85]. Combined this suggests that gamma-toxins have additional roles in disease beyond lysis of immune cells, and may play a critical role in development of mTSS.

For nearly a century, alpha-toxin has been considered a dominant toxin in the mechanisms driving invasive and tissue-destructive *S. aureus* infections. Alpha-toxin has varying cytolytic activity across a large variety of cells, and several receptors have been proposed including lipid rafts, β1-integrin and ADAM10 [72,77,78]. The destructive effects of alpha-toxin on the vaginal mucosa were already known, but the role in pathogenesis remained unclear [70]. Initially, it was suspected that alpha-toxin

destruction of tissue promotes TSST-1 penetration into the systemic circulation [70]. However, based on penetration estimates and animal observations, I believe this to be an unlikely mechanism. Specifically, the intravenous LD_{50} of TSST-1 (5.9 μg/kg) is greater than the intravaginal LD_{100} of TSST-1 (5 μg/kg) in rabbits [Chapter III., 22]. Additionally, TSST-1 penetration across the vaginal mucosa is <1% of the total applied dose over 8 h , and even by generous estimates would not reach the minimal concentrations to cause T-cell proliferation [Chapter III., 21,23]. Instead, I believe that the local proinflammatory actions of superantigens and cytolysins are critical mediators of mTSS.

In addition to lysing vaginal epithelial cells, alpha-toxin also induces a potent cytokine response that is dependent on ADAMs and EGFR. The cell-signaling induced in response to alpha-toxin in HVECs converges with cell-signaling induced by TSST-1 to produce a synergistic cytokine response. Both TSST-1 and alpha-toxin predominantly activate ADAM17, leading to shedding of EGFR ligands responsible for stimulating cytokine production. This suggests that the response induced by superantigens and cytolysins at the epithelial surface is a specific modulation of the host immune response. Alpha-toxin also plays a role in *S. aureus* colonization of the vaginal mucosa as a critical component for biofilm formation, suggesting an additional role of alpha-toxin [Chapter III., 26]. Despite alpha-toxin having potent effects at the vaginal mucosa, only ~20% of mTSS associated strains produce full-length alpha-toxin, due to a stop codon mutation [79]. However, strains with the stop codon mutation still produce 1/50 the wild-type amounts by a read through mechanism, as well as truncated forms of alpha-toxin 40-70%

the size of wild-type toxin, which may still activate cell signaling [110]. It is likely that low level alpha-toxin production and truncated forms play a role in cell signaling, but this has not been investigated.

6.2 Mucosal Immunobiology and mTSS

The traditional pathways for TSS and mTSS pathogenesis were thought to be similar. The predominant mediator of shock was thought to be the systemic effects of TSST-1 on immune cells, increased endotoxin penetration in the gut, hepatotoxicity and enhanced endotoxin lethality [116,178,180,190,214]. Cytolysins were thought to enhance superantigen penetration into the systemic circulation by disrupting epithelial surface integrity [70]. However, I believe mTSS results from specific modulation of the host immune response at the vaginal mucosa, which produces an environment favorable to *S. aureus* causing the classic cytokine storm to propagate from the mucosal immune compartment.

Superantigens and cytolysins produce potent proinflammatory responses in HVECs through overlapping cell-signaling pathways. TSST-1 is known to stimulate ADAM10/17 shedding of EGFR ligands at the vaginal epithelium [67]. The primary ligands shed by HVECs in response to TSST-1 are AREG and TGF-α, which are potent stimulators of EGFR-signaling due to endocytic recycling [67,121]. We determined that alpha-toxin also stimulates a cytokine response in HVECs through a related pathway. Alpha-toxin has been previously reported to interact with ADAM10 in lung epithelial cells [72]. While we did observe a minimal effect by ADAM10 in HVECs, the potent

effects of alpha-toxin in HVECs were dominated by ADAM17. Upon activation, ADAM17 mediates shedding of the potent EGFR ligand AREG, which undergoes receptor-ligand endocytic recycling and also activates ERK1/2 that subsequently activates ADAM17 shedding activity resulting in a feed-back loop [128,130]. Interestingly, the gamma-toxins, HlgAB and HlgBC were found to activate EGFR-signaling, but through different pathways. Both gamma-toxins induced shedding of EGFR ligands. HlgAB stimulated shedding of AREG and TGF-α, which are shed only by ADAM17 of the ADAM family of sheddases [128]. The shedding of AREG and TGF-α in HVECs from HlgAB likely explains the more potent cytokine response and the enhanced synergy in IL-8 production with TSST-1. In contrast, HlgBC only stimulates shedding of the less potent HB-EGF, likely by ADAM10 [128]. However, the specific sheddases activated by gamma-toxins were not confirmed. The activation of EGFR-signaling by these toxins leads to NF-κB activation and potent production of IL-8, IL-6, TNF-α or MIP-3α by vaginal epithelial cells in response to TSST-1, alpha-toxin or gamma-toxin [Chapter II., Chapter III.,7,33].

FIGURE 6.1: Proposed Signaling Pathway ofTSST-1, Alpha-toxin, and Gamma-toxins

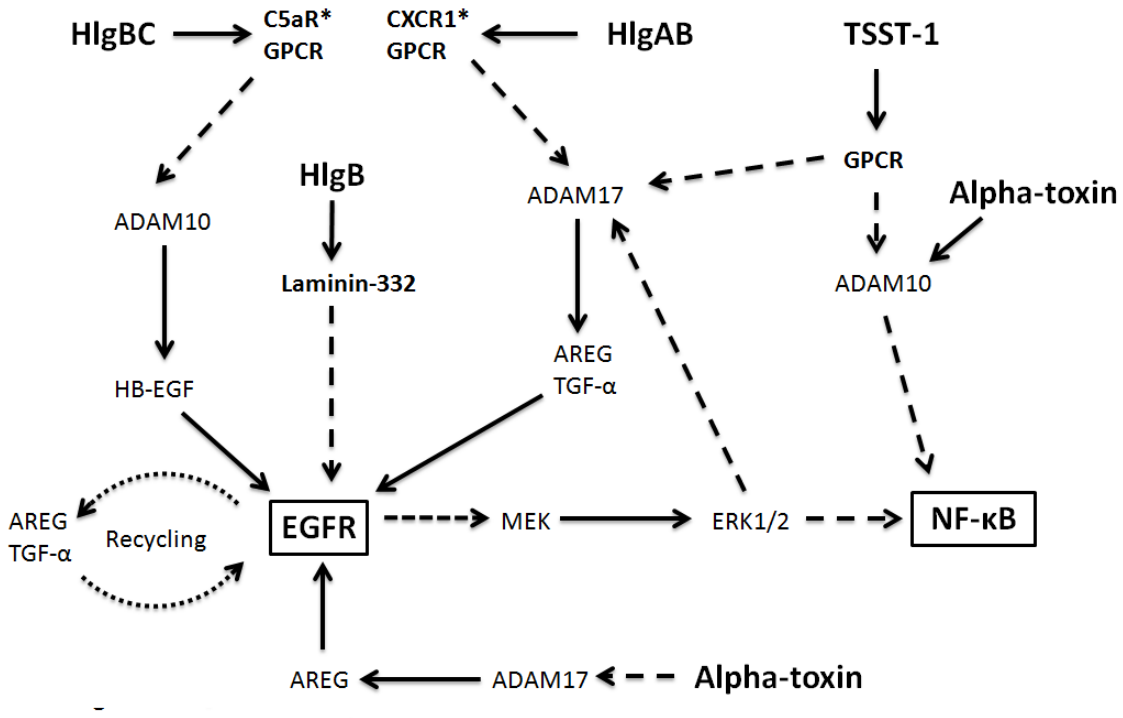


Figure 6.1: Model of potential mechanisms of action that activate EGFR signaling by TSST-1, alpha-toxin, or gamma-toxins in HVECs. *Solid lines represent direct interactions and dashed lines represent indirect interactions. TSST-1 is known to interact with an unknown G-protein coupled receptor (GPCR) and induce ADAM17 dependent shedding of amphiregulin (AREG) and TGF-α. ADAM10 is also influential in stimulating a cytokine response to TSST-1 but through an unknown mechanism. Alpha-toxin stimulates shedding of AREG by ADAM17. The HVEC IL-8 response to alpha-toxin is partially dependent on ADAM10, but the mechanism is unknown as alpha-toxin did not stimulate shedding of HB-EGF. HlgAB is known to interact with the G-protein coupled IL-8 receptors (CXCR1) in leukocytes and these receptors are minimally expressed but*

inducible in HVECs. HlgAB may also interact with an unknown G-protein coupled receptor. Either activation of CXCR1 or unknown receptors leads to shedding of AREG and TGF-α by ADAM 17. HlgBC is known to interact with the G-protein coupled C5aR in leukocytes and these receptors are present in HVECs. HlgBC may also interact with an unknown G-protein coupled receptor. HlgBC then induces shedding of HB-EGF by ADAM10. The HlgB subunit of HlgAB and HlgBC binds laminin-332 which disturbs normal laminin-332 binding and allows laminin-332 to bind and modulate EGFR signaling to enhance proinflammatory responses and ERK activation of ADAMs. Activation of EGFR by AREG, TGF-α, or HB-EGF leads to downstream activation of MEK, ERK1/2, and ultimately induces the NF-κB pro-inflammatory transcriptional activation cascade. When AREG and TGF-α bind EGFR the ligand-receptor complex undergoes endocytic recycling. Activated ERK1/2 leads to phosphorylation and activation of ADAM17.

** CXCR1and C5aR are known receptors for gamma-toxin in leukocytes but the constitutive expression in epithelial cells is minimal, and thus likely do not play a role in the initial interactions of gamma-toxins with epithelial cells* [96,99,215].

The cytokine response induced by superantigens and cytolysins likely produces an environment favorable for *S. aureus* pathogenesis. IL-8 is a potent chemoattractant for neutrophils [113]. Neutrophils are observed at high concentrations in patients who succumb to mTSS [40]. *S. aureus* is well adapted to not only survive but grow within and utilize neutrophils for dissemination [86,113]. Furthermore, hyper-recruitment or rapid lysis of neutrophils results in tissue damage, capillary leakage and edema, which are pathologies observed in mTSS patients [40,113,155]. MIP-3α is also a potent chemoattractant for dendritic cells and T-cells, and production of MIP-3α is further amplified by IL-6 in non-immune cells [114,176]. The cytokine TNF-α further amplifies NF-κB signaling as well as activating dendritic cells and promoting trafficking to local mucosal lymph nodes [177]. Activated dendritic cells then express MHC-II and are able to interact with tissue resident, or recruited, CD4+ T-cells, that are activated by TSST-1 [62]. Furthermore, TSST-1 survives endocytosis by dendritic cells and is actively trafficked to the lymph nodes, where it is exposed to CD4+ T-cells [115]. This culminates in activated dendritic cells trafficking to local lymph nodes where they bring TSST-1 to high concentrations of T-cells and induce T-cell proliferation [115]. Combined, I believe this represents how local proinflammatory effects at the vaginal mucosa create the disease defining “cytokine storm” and propagate the systemic symptoms of mTSS.

6.3 EGFR Inhibition as a Therapeutic Target

Inhibition of EGFR signaling prevents the pathogenic effects of *S. aureus* exotoxins. TSST-1, alpha-toxin, and gamma-toxins all induce activation of the innate immune response and cytokine production in vaginal epithelial cells or vaginal mucosa through EGFR-signaling. Inhibition of EGFR, or upstream and downstream signaling of EGFR, attenuates both cellular and tissue proinflammatory responses to superantigens and cytolysins. The stimulation of EGFR-signaling by cytolysins is independent of their cytolytic activity with inhibition of EGFR having no effect on cell lysis. This suggests a dual function of cytolysins on the vaginal epithelium, consisting of barrier destruction and modulation of the innate response. Modulation of the host innate immune response at the vaginal mucosa through EGFR appears to be a critical mediator for progression to mTSS.

During homeostasis, EGFR-signaling predominantly regulates cell survival, growth and division to maintain epithelial barrier integrity [120]. When tissue is damaged either through trauma of infection, EGFR-signaling promotes wound healing, production of anti-microbial peptides and IL-8 for rapid recruitment of neutrophils [122]. For most bacterial infections, this response is beneficial to the host, and either eliminates or suppresses the infection until the adaptive immune response is activated. However, *S. aureus* is heavily adapted both to kill neutrophils and survive within neutrophils to disseminate to other tissues [86,113]. It would appear that during mTSS, *S. aureus* specifically activates and modulates EGFR-signaling to saturate the tissue with both neutrophils and lymphocytes, which may contribute to the cytokine storm. This is

supported by clinical observations, where patients who succumb to mTSS show high concentrations of neutrophils and leukocytes within vaginal tissue, with an absence of macrophages [40].

Inhibiting EGFR-signaling with AG1478 attenuates toxin-mediated disease, and prevents mTSS lethality in rabbits, challenged with live organisms. While AG1478 was investigated and found to be effective as a mono-therapy, any adaptation of EGFR inhibition for clinical use would be as adjunct therapy with current standard of care antibiotic therapy and life-support systems. Currently, therapy for mTSS consists of vasopressors, fluid replacement, antibiotics and irrigation of the site of infection. This results in a fairly large window of time where antibiotics are eradicating the bacteria, but high concentrations of active toxin remain present in the tissue. While broad immune suppression would likely be detrimental to a patient with an active infection, targeting a single pathway of the innate immune response which is actively stimulated by toxins may improve patient outcomes. Current therapies for treatment of mTSS are highly effective at preventing mortality, with death occurring in 1-4% of patients [29]. However, more than 70% of mTSS patients suffer significant life-long morbidities, which are most commonly cardiovascular or cognitive resulting from prolonged fever, hypotension and shock [35,36]. In a rabbit model for mTSS, treatment with AG1478 significantly reduced fever at the apex of disease. In general, when mTSS patients are admitted to the hospital they are at, or close, to the peak of symptoms. Including an EGFR inhibitor early in treatment could reduce fever and shock in patients while antibiotics take effect, which could reduce both mortality and morbidity.

The biggest potential concerns of inhibiting EGFR-signaling during infection in patients would result from immune inhibition and inhibiting tissue repair. While inhibition of EGFR prevented mTSS lethality in rabbits challenged with *S. aureus,* the human response may be more dependent of the effects of EGFR-signaling to clear the infection. Inhibition of EGFR-signaling would certainly inhibit epithelial tissue regeneration and repair [171,216]. However, the consequence of short term EGFR inhibition on wound healing would likely be minimal and easily reversible by removing treatment. Furthermore, in our models for mTSS treatment, AG1478 showed no observable effect on epithelial barrier integrity, suggesting that short term AG1478 treatment neither promotes nor prevents significant tissue damage. Other toxicities of AG1478, and TKIs in general, are predominantly cutaneous and involve rashes and dehydration of mucosal surfaces (xerosis), but these are associated with long term therapy (6 to 9 months) [216].

Of the available TKIs, I believe that AG1478 would be the most useful to develop into a therapy for treatment of (m)TSS. This is due to the chemical and pharmacokinetic properties of AG1478, which made it clinically irrelevant for the common uses of TKIs in treatment of cancer. AG1478 is smaller and more lipophilic than any of the clinically approved TKIs, which suggests it would be better at penetrating into mucosal membranes when applied topically [131,138]. AG1478 also readily distributes to mucosal tissues when given systemically, which implies the drug would remain at high concentrations in the vaginal mucosa when applied topically if dosed every 1-2 h [138].The most beneficial aspect of AG1478, over other TKIs, for our proposed application is the very short half-

life. Based on allometric scaling from murine studies, the half life of AG1478 in humans is estimated to be approximately 30 min [138]. AG1478 is also a competitive inhibitor and the effects are reversible upon removal of the drug [138]. Combined, this would allow for treatment to be instilled for several hours, or a day and then the effects would be rapidly reversed when treatment was discontinued.

6.4 Vaccination against *S. aureus*

Targeting the effects of exotoxins with vaccination or antibodies prevents disease induction during *S. aureus* infections. The exotoxins, particularly superantigens and cytolysins, are the predominant mediators of the invasive and destructive pathologies that occur during *S. aureus* infection [33,208]. In contrast, the surface proteins of *S. aureus* are mostly involved in bacterial survival through clotting, clumping, nutrient acquisition, and binding the FC-region of IgG [1]. Additionally, antibodies directed at the surface of *S. aureus* may aid in pathogenesis by promoting bacterial clumping and inducing toxin production [48,61,193]. We showed that active or passive vaccination against exotoxins prevented lethality in rabbits against intrapulmonary challenge from a broad selection of clinical strains of *S. aureus*. Unexpectedly, targeting exotoxins also promoted clearance of the *S. aureus* in the rabbit pneumonia model. This suggests that the destructive effects of superantigens and cytolysins are critical in bacterial defense against the host immune response or that exotoxins cause tissue damage more rapidly than the infection can be contained by the innate and adaptive immune systems. Historically, superantigens of *S. aureus* were not generally considered to play a role in infection of tissue [33].

Interestingly, vaccination against superantigens alone was sufficient to prevent lethality and clear infection of *S. aureus* pneumonia in rabbits. While vaccination based exclusively on superantigens and cytolysins was investigated for intrapulmonary infection, it is likely the same approach would be successfully in prevention of other infections. In *S. aureus* SSTIs, vaccination against alpha-toxin significantly reduced abscesses in mice [217]. Furthermore, a critical role for superantigens in *S. aureus* infective endocarditis in a rabbit model was recently identified [209]. This suggests that vaccination against exotoxins could be used to prevent SSTIs and endocarditis. However, targeting single exotoxins for protection would likely result in vaccine failure over time due to redundancies in *S. aureus* virulence factors, which is the rationale for the 7-component vaccine development [Chapter 5].

6.5 Summary

S. aureus utilizes an array of exotoxins with overlapping and redundant activities to cause disease [33,95]. Inhibition of any single activity, or vaccination against a single exotoxin of *S. aureus* could become ineffective quickly, as the organism readily acquires new genetic information [14,218]. Furthermore, increased production of one toxin can substitute for the activity of another [33,95]. Inhibition of EGFR-signaling blocks the action of TSST-1, alpha-toxin and gamma-toxin, thus making it unlikely for other toxins to supplement the effects of 3 toxins simultaneously. While we did show that vaccination against either TSST-1 or SEC was sufficient to prevent *S. aureus* pneumonia in a rabbit model, any vaccine developed for human use should include multiple superantigens and

cytolysins, as multiple pneumonia-causing strains have different toxin profiles [110,193,203]. Employing a multiple target (>2) approach for treatment and prevention of *S. aureus* is a strategy I believe could be developed into clinical therapy.

In this work, we demonstrated novel cytotoxic and proinflammatory effects of gamma-toxins at the vaginal mucosa. Furthermore, these studies identified novel immune-modulating effects of cytolysins with superantigens at the vaginal mucosa. A model of disease progression is proposed for mTSS based on these and others findings, that supports local proinflammatory effects as critical mediators for mTSS. Lastly, a potential adjunct therapy for treatment of mTSS, and a new vaccine for treatment and prevention of *S. aureus* infections were proposed. Combined, the results of this work further our understanding of *S. aureus* virulence and pathogenesis.

CHAPTER VII

Future Directions

7.1 Future Directions

7.1.1 Gamma-toxin Interactions with Mucosal Surfaces

In this study, I characterized the novel actions of gamma-toxins HlgAB and HlgBC, as mediated through ADAMs and EGFR on vaginal mucosa. My findings highlight a role for gamma-toxins in eliciting pro-inflammatory signals during tissue invasion in sharp contrast to the classic roles of hemolysin and leukocidin generally attributed to gamma-toxins. It is reasonable to assume that gamma-toxins may play a role in tissue invasion at other mucosal sites of colonization or infection as all mucosal surfaces express EGFR and ADAMs [123,130]. The role of gamma-toxins during tissue invasion is likely limited to mucosal surfaces, or in post-tissue invasion as gamma-toxins are not known to be dermonecrotic [87].

S. aureus regularly colonizes mucosal surfaces e.g. nasal or vaginal mucosa [3,219]. ADAM10/17 and EGFR are expressed on all mucosal epithelial cells, and thus gamma-toxins have the potential to interact with any mucosal surfaces, assuming that other unknown receptors are also present [120,130,220]. Initial investigations would characterize the effects of purified gamma-toxins on an array of mucosal-derived cells from a variety of tissues to identify potential interactions. Interestingly, preliminary studies I conducted showed no effect of gamma-toxins on lung-derived A549 cells (Data not shown), which are potent producers of ADAM10 [72]. However, it would be wise to investigate the effects of gamma-toxins in other lung epithelial cell lines to confirm that the apparent phenomenon of lung epithelial cell non-responsiveness is not cell-line specific. Due to the prevalence of *S. aureus* nasal colonization it would also be beneficial

to investigate the effects of purified gamma-toxins on oropharyngeal, nasal and olfactory epithelial cells. The primary goal would be to determine if the cytotoxic and cell-signaling effects observed in HVECs are conserved in other mucosal epithelial cells. Additionally, since a majority of people colonized with *S. aureus* do not develop infections, the role of gamma-toxins in colonization should be investigated.

The gamma-toxin receptor on mucosal epithelial cells needs to be identified. I observed that HlgB enhanced binding of HlgA and HlgC to HVECs, and that HlgB apparently binds to laminin-332 in HVEC cell extracts. Since laminin-332 is bound to multiple integrins, it would be sensible to determine if antibodies to laminin-332 or the known gamma-toxin receptors (CXCR1, CXCR2, C5aR) can block binding of individual subunits or gamma-toxins to HVECs. Since laminin-332 is expressed on the basement membrane of cells, it is possible that HlgB binding to laminin-332 may be a secondary activity, or non-specific binding, unrelated to interactions of gamma-toxin at epithelial surfaces. Thus, further pull-down studies utilizing histidine tagged vs. biotinylated subunits with a variety of detergents (nonionic, anionic or zwitterionic) should be done to confirm interactions of HlgB with laminin-332 or identify other interactions in HVECs. Pull-downs would include comparisons between whole cell lysates and plasma membranes. Whole cell lysates would be prepared from cells treated with or without trypsin that have been washed in PBS and lysed by a variety of detergents. Plasma membrane fractions would be prepared from cells lysed with B-PER and centrifuged at 12,000 rpm to separate soluble cytosolic proteins. The recovered plasma membrane pellet would be resuspended in detergent and assayed for gamma-toxin binding.

7.1.2 Investigate AG1478 Pharmacokinetics and Pharmacodynamics in Treatment of *S. aureus* Infections

We demonstrated that topical application of AG1478 prevented lethality in a chronic rabbit mTSS model. However, our model was limited to preventing development of symptoms and not therapy. Additionally, our drug delivery system was designed to safely administer as much AG1478 as possible, which was not optimized. The first steps in pursuing AG1478 or TKIs for treatment of *S. aureus* (m)TSS would be to optimize our drug administration.

AG1478 is minimally soluble in 30% 2-hydroxy-beta-cyclodextrin (aq) at ~1 µg/ml, but forms a colloidal solution. The first step would be to determine AG1478 solubility in a variety of gels or topical suspensions including other cyclodextrins and glycerol monolaurate, which is known to have anti-staphylococcal activities [56]. The activity of soluble or other colloidal solutions of AG1478 would need to be tested to determine efficacy.

The pharmacokinetics of AG1478 are also not well characterized outside of intravenous murine models, and would need to be determined in other animals to estimate pharmacokinetic parameters in humans. The systemic absorption of AG1478 from topical application and the tissue concentrations over time would need to be determined across several animal models including rabbits for use in our model, porcine due to similarity with human tissue, and non-human primates. This would then allow development of a dosing strategy in humans.

The first pharmacokinetic studies that need to be done are in rabbits, since they are the primarily model for (m)TSS. To evaluate the pharmacokinetics of AG1478 in rabbits, ≥3 New Zealand White rabbits would be used for the intravenous study and ≥9 rabbits for the intravaginal absorption study. For the initial intravenous pharmacokinetic studies, each rabbit would receive a dose 2 mg/kg (toxicity in mice is 275 mg/kg) intravenously through ear vein injection [138]. A total of 1 ml of blood would be drawn every 15 min for the first hour and every 30 min for up to 3 hours. Total blood draw from a 2 kg rabbit would not exceed 10 ml in 1 week, <0.5% of the rabbit's body weight as required by University of Minnesota Research Animal Resources. Serum would be separated, and AG1478 extracted following the protocol in Chapter 8.2. A dose of 2 mg/kg should allow for detection of AG1478 for up to 5 half-lives if at least 10% of the drug is distributed to the serum. Assuming 25-fold concentration following extraction from serum, the minimum detectable serum concentration of AG1478 is ~6.5 ng/ml. The serum concentrations of AG1478 would be modeled to estimate the pharmacokinetic parameters. For intravaginal absorption studies, the rabbits would be dosed intravaginally with 4 mg/kg of AG1478 in 30% beta-cyclodextrin (8 mg/ml stock). Rabbits (≥3 per group) would have blood draw every 15 min for the first hour then every 30 min for up to 3 h. A group of rabbits would be sacrificed at 45 min, 1.5 h and 3 h and vaginal tissue excised. Vaginal tissue would be homogenized and diluted 1:4 (weight by volume) in methanol and incubated overnight at 4° C to extract AG1478. Using reverse-phase high pressure liquid chromatography, AG1478 tissue concentrations would be determined from the methanol extracts by comparison to a standard curve prepared from spiked

tissue. The serum taken from rabbits would be assayed as described in Chapter 8.2 to determine absorption. At an intravaginal dose of 4 mg/kg AG1478, the drug should be detectable in serum for at least 3 half-lives if absorption is >5%. To ensure that AG1478 can be detected and not waste animals, a small number of rabbits, for both intravenous and intravaginal studies, should be done first and the results determined.

While our model investigated prevention of mTSS, I believe TKIs could be used to treat mTSS or other *S. aureus* infections. Since AG1478 reduced symptoms of mTSS during early infection in experimental infections that used very high bacterial inocula, I believe that application early in disease may reduce symptoms, and lessen morbidity and mortality. The first step would be to determine if AG1478 applied after development of symptoms in rabbits could reduce disease symptoms and lethality during mTSS. This would also allow investigating AG1478 in combination with other therapies including fluid replacement, antibiotics, or vasopressors in rabbit mTSS. Furthermore, I believe that AG1478, or other TKIs, may be therapeutic in treatment of *S. aureus* TSS originating from other mucosal or epithelial surfaces. If TSS from all epithelial surfaces progresses in a similar mechanism then inhibition of EGFR could be utilized as adjunct therapy for prevention and treatment of TSS.

7.1.3 Exotoxin Vaccine for Prevention of *S. aureus* Infection

Vaccination against *S. aureus* exotoxins and toxoids prevented lethality in a rabbit pneumonia model. It would be worth investigating if the same vaccine could prevent disease in other *S. aureus* infection models. Initially, the 7-component vaccine should be

tested in prevention and treatment of SSTI, including both abscesses and surgical-site infections in rabbit models. Prevention and treatment of *S. aureus* bacteremia and endocarditis should also be investigated, as current models already exist which demonstrate a requirement for superantigens [209]. If the 7-component vaccine is successful in treatment and prevention of all or most of these infection types, then demonstrated efficacy in animal models would be expanded to cover the most predictable and common hospital-acquired infections. This would raise the possibility of using the exotoxin vaccine or antibodies as prophylaxis in intensive care units, or before surgery, in addition to antimicrobial therapy.

The 7-component vaccine needs to be further refined before use in humans. The current vaccine uses both toxin and toxoids, which would not be usable in humans. Toxoids would need to be generated for β-toxin and possibly gamma-toxin HlgB subunit, since the HlgB subunit may have independent binding activities. The superantigen toxoids were generated to prevent MHC-II or T-cell binding and the alpha-toxoid designed to prevent oligomerization [193]. However, both superantigens and alpha-toxin appear to have biological activity independent of superantigenicity and cytotoxicity [72,117]. Thus, the safety of these toxoids would need to be investigated, and possibly further altered or denatured to prevent their secondary activities. The safety and immunogenicity of the final vaccine would need to be further studied in multiple animals to ensure that it elicits a potent and safe antibody response. Lastly, even though non-human primates are resistant to lethal effects of superantigens, the safety and efficacy of any vaccine would need to be studied in them prior to advancing to phase-I clinical trials

to identify any primate-specific risks. The nearly-complete efficacy from vacation against exotoxins in preventing lethal pneumonia in rabbits supports pursuing further development of a *S. aureus* exotoxin vaccine.

Chapter VIII

Related Work

8.1 Manuscripts

Alpha-toxin promotes Staphylococcus aureus mucosal biofilm formation
Michele J. Anderson, Ying-Chi Lin, Aaron N. Gillman, Patrick J. Parks, Patrick M. Schlievert, and Marnie L. Peterson
Cellular and Infection Microbiology, May, 2012
Vol. 2, Article 64
Modified Abstract.

Background: *Staphylococcus aureus* causes many diseases in humans, ranging from mild skin infections to serious, life-threatening, superantigen-mediated TSS. *S. aureus* may be asymptomatically carried in the anterior nares or vagina or on the skin, serving as a reservoir for infection. Pulsed-field gel electrophoresis clonal type USA200 is the most widely disseminated colonizer and the leading cause of TSS. The cytolysin α-toxin (also known as α-hemolysin or Hla) is the major epithelial proinflammatory exotoxin produced by TSS *S. aureus* USA200 isolates. The current study aims to characterize the differences between TSS USA200 strains [high (hla(+)) and low (hla(-)) α-toxin producers] in their ability to disrupt vaginal mucosal tissue and to characterize the subsequent infection.

Methods: Tissue viability post-infection and biofilm formation of TSS USA200 isolates CDC587 and MN8, which contain the α-toxin pseudogene (hla(-)), MNPE (hla(+)), and MNPE isogenic hla knockout (hlaKO), were observed via LIVE/DEAD® staining and confocal microscopy. Bacterial growth in tissue was quantified by CFU and α-toxin production in wild-type strains compared by Western immunoblot. The ability of exogenous α-toxin to restore the biofilm phenotype in MNPE (Δhla) was also investigated.

Results: All TSS strains grew to similar bacterial densities (1-5 × 10(8) CFU) on the mucosa and were proinflammatory over 3 days. However, MNPE formed biofilms with significant reductions in the mucosal viability whereas neither CDC587 (hla(-)), MN8 (hla(-)), nor MNPE hlaKO formed biofilms. The latter strains were also less cytotoxic than wild-type MNPE. The addition of exogenous, purified α-toxin to MNPE hlaKO restored the biofilm phenotype.

Conclusion: We speculate that α-toxin affects *S. aureus* phenotypic growth on vaginal mucosa by promoting tissue disruption and biofilm formation. Further, α-toxin mutants (hla(-)) are not benign colonizers, but rather form a different type of infection, which we have termed high density pathogenic variants (HDPV).

8.2 Extraction of AG1478 from Human Sera

Unpublished Methods.

8.2.1 Introduction

In order to perform pharmacokinetic studies on AG1478 an extraction method for separating and measuring AG1478 in human serum is needed. A simple column-extraction method and reverse-phase high pressure liquid chromatography (R-HPLC) protocol for detecting AG1478 in human serum was developed.

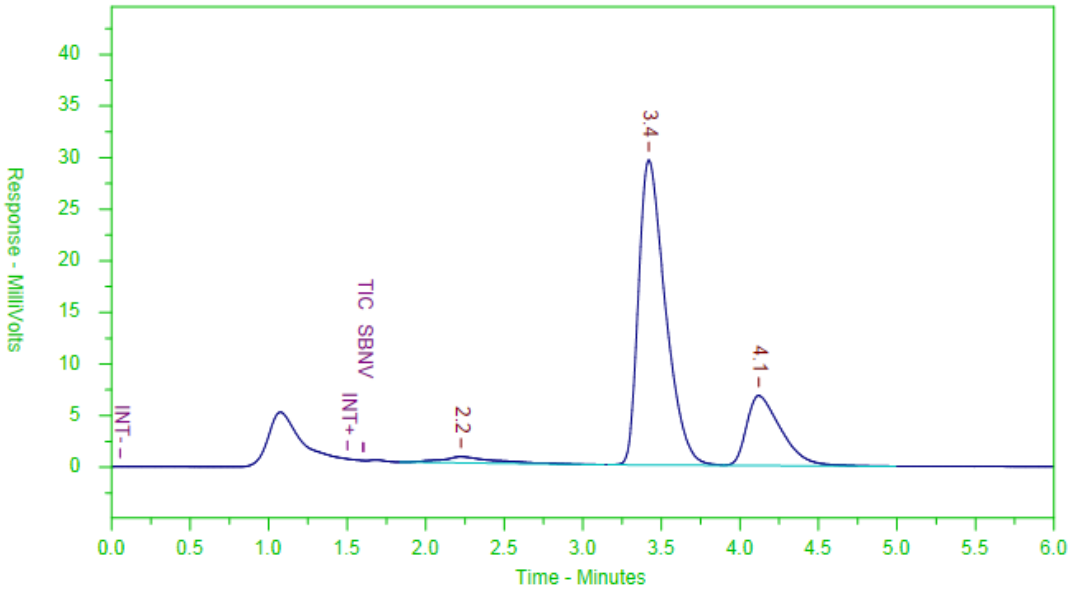
8.2.2 Methods

Extraction was performed using strong cation-exchange columns (Strata, 8B-S029-TAK) with a 200 rpm centrifugation for 2 minutes between each step. Columns were pretreated with 1 ml methanol then 1 ml 0.1 N HCl (aq) prior to human plasma. Columns were treated with 1 ml human plasma 1ml containing 25 µg/ml AG1478 and 10 µg/ml gefitinib (Standard) and followed by 2 washes of 1 ml 0.1 N HCL (aq). The columns were eluted in 2-steps of 250 µl and 750 µl 5% NH_4OH in methanol. Eluted samples were evaporated at 37° C for 30 min under nitrogen then resuspended in 250 µl of R-HPLC buffer (45% 25mM acetate pH 4.5 20mM triethylamine (aq) and 55% acetonitrile by volume). In triplicate, 25 µl of the sample was loaded for R-HPLC at 45°C for 6 min and absorbance read at 330 nm. The peak absorption of AG1478 is 330 nm and gefitinib is 340 nm [138,221].

8.2.3 Result and Conclusions

AG1478 can be successfully extracted from human serum and analyzed by R-HPLC under the conditions above (Fig. 8.1). Further experiments are needed to determine the range of quantification.

FIGURE 8.1: Representative Chromatograph of AG1478 Serum Extraction



Peak #	Ret. Time	Name	Amount	Amt %	Area	Area %	Type	Width
1	2.22		0.00	N/A	14670	3.127	BB	0.46
2	3.42		0.00	N/A	351269	74.866	BV	0.18
3	4.12		0.00	N/A	103256	22.007	VB	0.23

Total Area = 469195.6 Total Height = 36949.25 Total Amount = 0

Figure 8.1: Chromatograph of AG1478 and gefitinib. *AG1478 is symmetric with a peak retention time of 3.4 min. Gefitinib has slight right skewed symmetry with a peak retention time of 4.1. The extraction protocol yields a sample with ~97.9% purity for AG1478 and gefitinib (area 2 + area 3)/ (Total Area). Data are representative of the extraction protocol performed in triplicate.*

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