STAPHYLOCOCCAL TOXIC SHOCK SYNDROME TOXIN -1 INTERACTIONS WITH HUMAN VAGINAL EPITHILIAL CELLS AND NOVEL THERAPUTICS.

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

MATTHEW MICHAEL SCHAEFERS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

MARNIE L. PETERSON, ADVISOR

JANUARY, 2011

Acknowledgements

There are many people that have helped me throughout my time in graduate school. First of all I want to thank my advisor Dr. Marnie Peterson for giving me the many opportunities I have been given. She has given the support, encouragement, and guidance to complete my studies. She has pushed me to become a better scientist while insisting that I keep my work life in balance with my personal life. I also want to thank the other members of the lab; Drs. Michele Anderson, Ying-Chi Lin, and Laura Breshears**,** and Liz Horn, Laurie Baeker Hovde, and Aaron Gillman. Their assistance and guidance was invaluable in the completion of my project.

I would also like to acknowledgement the guidance from my committee members; Drs. Rory Remmel, Peter Southern, Tim Tracy and Mark Kirstein. I would also like to thank Dr. Remmel for giving me the opportunity to gain valuable teaching experience by allowing me to teach in his class. I would also want to thank our collaborator Dr. Patrick Schlievert (Department of Microbiology, University of Minnesota), and his former graduate students Drs. Kristi Strandberg and Amanda Brosnahan. Their assistance with experiments including the rabbit-curcumin experiments was critical for my project. I would like to thank Dr. Peter Southern again, for his assistance in tissue acquisition and his assistance staining of this tissue. I would also like the thank Dr. Jayanth Panyam (Department of Pharmaceutics, University of Minnesota) and his graduate student, Alex Grill for their assistance with determination of curcumin and their future collaboration working with curcumin coated microparticles. I would also like thank the Experimental and Clinical Pharmacology staff, faculty, and students for their guidance and assistance.

I also would like to thank my family for the support and understanding. They might not have always understood what I am doing or why I'm doing it but they always supported my decision to pursue my doctorate and understanding the increased demands on my schedule. Last but not least, I would like to thank my wife, Kristi, for her love, support, encouragement, and understanding throughout the whole graduate school process. Her support throughout the whole process allowed me to stay motivated to complete my project.

Abstract

Staphylococcus aureus is a significant human pathogen that causes a wide range of diseases from skin and soft tissue infections, pneumonia, osteomyelitis, to toxic shock syndrome (TSS). *S. aureus* initiates infections at skin and mucosal surfaces by producing a multitude of virulence factors, including superantigens (SAgs). SAgs are exotoxins that enhance the ability of *S. aureus* to cause infection by dysregulation of the host's immune system. Numerous Staphylococcal SAgs have been identified including the staphylococcal enterotoxins (SEA- SEU) and Toxic Shock Syndrome Toxin-1 (TSST-1). SAgs cause a wide range of diseases such as food-poisoning, atopic dermatitis, and TSS by relatively unknown mechanisms of action on mucosal surfaces. The ability of SAgs to non-specifically cross-link T-cells and antigen presenting cells (APC), which results in a cytokine storm and toxic shock syndrome have been studied extensively. However, interactions of SAgs with mucosal surfaces remain poorly understood.

TSST-1, the most common cause of menstrual TSS (mTSS), induces proinflammatory cytokines from vaginal epithelial cells. These proinflammatory effects are hypothesized to contribute to the progression of mTSS by disrupting the permeability barrier of the vaginal mucosa directly and by causing a migration of neutrophils, macrophages and lymphocytes to the site of infection.

The aims of this thesis were to 1) characterize TSST-1's mechanism of action on human vaginal epithelial cells (HVEC), leading to the induction of proinflammatory cytokines, including the identification of the TSST-1 HVEC receptor and residues of

TSST-1 critical for HVEC interactions, and to 2) evaluate curcumin, an anti-inflammatory compound, as an anti-TSS mucosal therapeutic.

HVEC were exposed to TSST-1, and cytokine expression levels were determined by real-time reverse transcription polymerase chain reaction (PCR) and multiplex cytokine assay. IL-6, IL-8, MIP-3α, and TNF- α transcripts were up-regulated (1.5- to 12-fold), with corresponding increases in protein expression. TSST-1 activated an NF-κB luciferase reporter in HVEC, suggesting that NF-κB is a downstream target of TSST-1 signaling. Previous studies have suggested that major histocompatibility complex class II molecules (MHC II) could serve as the epithelial receptors for SAgs, and when activated, induce cytokines. Flow cytometry and Western blotting of HVEC did not detect MHC II molecules. These data suggest that MHC II is not the HVEC surface receptor responsible for induction of cytokines by TSST-1 and another undefined SAg epithelial receptor present on the surface of HVEC is implicated.

A dodecapeptide region (TSST-1 amino acids F119 to D130) that is relatively conserved among SAgs has been implicated in SAg penetration of the epithelium. Single amino acid mutations were constructed in TSST-1 amino acids D120 to D130. All mutants maintained superantigenicity similar to wild type toxin. TSST-1 mutants induced IL-8 from HVEC; however, three toxin mutants (S127A, T128A, and D130A) induced lower levels of IL-8 compared to wild type TSST-1. These toxin mutants were administered intravenously to rabbits and all three were 100% lethal. When administered vaginally to rabbits, D130A toxin was nonlethal, while wild type TSST-1 was 100% lethal. Residue D130 may contribute to toxin binding to an epithelial receptor that allows

it to penetrate the vaginal mucosa, induce cytokines/chemokines from epithelial cells, and cause TSS.

CD40 was explored as a candidate HVEC receptor for TSST-1, based on a previous study which described synergistic activity of CD40 with MHC II for TSST-1 to induce cytokines from monocytes. HVEC (by flow cytometry) and *ex vivo* human ectocervical tissue (by immunohistochemistry) both expressed CD40. The biological role of CD40 in TSST-1-induced cytokine production was evaluated by reducing CD40 expression in HVEC by stable transfection with small hairpin RNA (shRNA) plasmids by 60% on the protein level. Surprisingly, CD40 shRNA-expressing HVEC produced more IL-8 in response to TSST-1 compared to irrelevant shRNA expressing HVEC. TSST-1 also bound to HVEC expressing shRNA more than HVEC expressing irrelevant shRNA. These data suggest that CD40 is not the HVEC receptor for TSST-1 that is responsible for induction of proinflammatory cytokines.

Curcumin, a component of the spice turmeric, is a compound that has been used in traditional medicine therapies for 4,000 years. Curcumin was evaluated as a potential anti-TSST-1 agent by targeting the host mucosal response to TSST-1 and *S. aureus*. Curcumin inhibited *S. aureus* exoprotein- or live *S. aureus*-induced IL-8 production in an *ex vivo* porcine vaginal tissue model. The importance of TSST-1-induced inflammation of the vaginal mucosa in TSS disease progression was tested by using curcumin to prevent TSS in a rabbit vaginal model. Curcumin co-administrated with TSST-1 intravaginally significantly reduced lethality of TSST-1 by 60% (5 of 8 rabbits survived whereas 0 of 8 rabbits survived in TSST-1 controls, $p < 0.05$). In addition, TNF-α was

undetectable from serum or vaginal tissue of curcumin-treated rabbits that survived. These data demonstrate the importance of local inflammation in the progression of TSS, and curcumin as a potential anti-TSS agent.

These studies describe the importance of TSST-1 interactions with vaginal epithelial cells in disease progression to TSS. The mechanism of TSST-1 activation of proinflammatory cytokines was determined to be through the NF-κB pathway with TSST-1 amino acid D130 being critical for induction of cytokines from epithelial cells. However, the NF-κB pathway was not induced by TSST-1 binding to MHC II molecules or CD40 suggesting the role of an unidentified epithelial receptor. These studies also determined that curcumin inhibits *S. aureus* exoprotein-induced cytokine response in *ex vivo* vaginal porcine tissue; and when administered intravaginally, curcumin partially prevents TSS in a rabbit model of TSS, demonstrating the importance of local inflammation caused by TSST-1 in progression of TSS. These studies were conducted to develop novel therapies for the prevention and treatment of superantigen-mediated diseases.

Table of Contents

List of Tables

List of Figures

List of Commonly Used Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cells
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CA-MRSA	Community-associated methicillin-resistant *S. aureus*
CDC	Center for Disease Control and Prevention
CFU	Colony forming units
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
Cur	Curcumin
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FtsZ	Filamenting temperature-sensitive mutant Z
GML	Glycerol monolaurate
hr	Hours
HA-MRSA	Healthcare-associated methicillin-resistant *S. aureus*
H&E	Hematoxylin and eosin stain
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase

HPV	Human papillomavirus
Hsp	Heat Shock Protein
HVEC	Human vaginal epithelial cells
IACUC	Institutional Animal Care and Use Committee
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IgG Fc	Immunoglobulin G fragment crystallizable region
IHC	Immunohistochemistry
IL	Interleukin
IRB	Institution Review Board
IU	International unit
IV	Intravenous
IVIG	Intravenous immunoglobulin
KSFM	Keratinocyte serum-free media
LC/MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
MHC I	Major histocompatibility complex class I molecules
MHC II	Major histocompatibility complex class II molecules
MIC	Minimum Inhibitory concentration
Min	Minute
MIP	Macrophage Inflammatory Protein

mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
mTSS	Menstrual Toxic Shock Syndrome
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
ND	Not Determined
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
non-mTSS	Non- Menstrual Toxic Shock Syndrome
Ova	Ovalbumin
PBS	Phosphate buffered saline
PBP	Penicillin Binding Protein
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PDG	Peptidoglycan
PE	Phycoerythrin
PK	Pharmacokinetics
PMBC	Peripheral blood mononuclear cells
PVDF	Polyvinylidene fluoride
PVL	Panton-Valentine leukocidin
RPMI	Roswell Park Memorial Institute medium
SAg	Superantigen
SAgs	Superantigens

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Staphylococcal enterotoxin
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SPE	Streptococcal pyrogenic exotoxins
TCR	T-cell receptor
TNF	Tumor necrosis factor
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin 1
UD	Undetected
WT	Wild-type

Chapter 1- Introduction

I. Overview

Staphylococcus aureus is a significant human pathogen that causes a plethora of diseases including skin and soft tissue infections, necrotizing pneumonia and toxic shock syndrome. Superantigens (SAgs) are proinflammatory exotoxins produced by *S. aureus* at mucosal surfaces that disrupt the immune response to infection by non-specifically cross-linking antigen presenting cells (APC) to T-cells. Staphylococcal SAgs include Staphylococcal enterotoxins (SEA- SEU) and Toxic Shock Syndrome Toxin-1 (TSST-1). SAgs have been implicated in a multitude of diseases such as food-poisoning, atopic dermatitis, and toxic shock syndrome (TSS) [1]. Interactions of SAg with professional immune cells (T-cells and APC) have been extensively studied while interactions of SAg with epithelial cells, the initial host cells exposed to toxin, remain poorly understood. Previous studies determined that TSST-1 and other SAgs (SEA, SEB and SEC) induce proinflammatory cytokine synthesis and release [i.e. interleukin (IL)-1β, macrophage inflammatory protein (MIP)-3α, IL-6, IL-8, and tumor necrosis factor (TNF)-α] from immortalized human vaginal epithelial cells (HVEC) and other epithelial cell types (respiratory and intestinal) [2-6]. The proinflammatory signaling pathways induced in epithelial cells by TSST-1 have not been investigated. Nuclear factor-kappa B (NF-κB) is an important transcription factor that regulates the expression of many genes involved with proinflammatory processes, and is likely involved in the TSST-1-induced response in HVEC. In addition to a lack of studies describing epithelial response mechanisms to SAgs, the epithelial receptors involved in directly binding SAgs and activating proinflammatory pathways have not been identified. Major histocompatibility complex

class II molecules (MHC II) have been suggested as epithelial receptors when up-regulated on the surface of epithelial cells by interferon (IFN)-γ. However, in epithelial cell populations < 5% of the cells are expressing detectable levels of MHC II on their surfaces. Therefore, studies have explored the possibility of a novel epithelial receptor [3, 7].

Therapies for superantigen (SAg)-mediated diseases primarily include antibiotics directed at the causative organism, including methicillin-sensitive or methicillin-resistant *S. aureus*, as well as supportive care such as vasopressors and fluids. Currently, intravenous immunoglobulin (IVIG) and corticosteroids are the only therapies in clinical practice that target the SAg (neutralization of the toxin) or SAg-induced effects on the host (suppression of the immune cells), respectively. Novel therapies that target the SAg mechanism of action on host cells are needed as SAgs have been directly implicated in the morbidity and mortality associated with *S. aureus* infections [8, 9].

Experimental anti-SAg therapeutic approaches include: prevention of the proinflammatory effects of SAgs on host cells (anti-inflammatory agents), prevention of SAg-binding to target cells (SAg antagonists), and prevention of SAg production by *S. aureus* (anti-toxin agents). Curcumin, a component of the Indian spice turmeric, has been used in traditional medicine to treat many diseases for over 4,000 years. Based on curcumin's anti-inflammatory, anti-tumorgenesis, and anti-microbial properties it was decided that curcumin is an ideal candidate for a novel anti-SAg therapeutic agent, which could block the proinflammatory effects of SAgs on epithelial cells. Characterization of the SAg-induced proinflammatory pathways and identification of the SAg epithelial

receptor involved in the activation of these pathways would lead to the generation of new targets for therapies in the treatment in SAg-mediated diseases; and therefore, is the focus of this thesis.

The studies contained in this thesis involve a comprehensive approach that includes molecular, *ex vivo* and *in vivo* experimentation for an understanding of the importance of the proinflammatory effects of TSST-1 on the vaginal mucosa in the progression of menstrual-associated TSS (mTSS). Specifically, these studies include a characterization of TSST-1's mechanism of action of inducing of proinflammatory cytokines from HVEC, which leads to the induction of proinflammatory cytokines, including the exploration of a postulated TSST-1 HVEC receptor and identification of TSST-1 residues critical for HVEC interactions; and secondly an evaluation of curcumin, an anti-inflammatory compound, as an anti-TSS mucosal therapeutic.

Studies described in this thesis focus specifically on TSST-1 interactions with vaginal epithelial cells and role in TSS, but I predict these findings are applicable to interactions of other SAg with epithelial cells derived from different anatomical sites.

II. Staphylococcus aureus and associated diseases

II. a. Background

Staphylococcus aureus, a Gram-positive coccus, is the causative agent of a wide range of human diseases ranging from skin infections to endocarditis, sepsis, pneumonia, and osteomyelitis, which have considerable impact on the population [10, 11]. Additionally, *S. aureus* produce a wide variety of exotoxins, which can cause clinical diseases including, TSS, atopic dermatitis, and food poisoning [12, 13]. In these diseases the toxins, not the bacteria, are primarily responsible for the pathogenic effects. Elucidating the role of toxins in the pathogenesis of *S. aureus* and research to develop anti-staphylococcal therapies to prevent toxin-mediated diseases is spurred on by the emergence of *S. aureus* resistance to antibiotics.

II. b. Colonization

Approximately thirty percent of the human population is permanently colonized in the nares with *S. aureus* that possess genes to numerous exotoxins (including superantigens and cytolysins), but suffer no ill effects [14, 15]. A greater fraction of the population is colonized intermediately with *S. aureus*, meaning that during a longitudinal study, *S. aureus* was not recovered from the nose at every sampling point. Other common sites of colonization include the intestine, skin, perineal regions, vagina, and pharynx [11, 16]. When someone is clinically infected with *S. aureus*, the infecting strain is often the same as the colonizing strain, demonstrating that colonization is an important step in the pathogenic cycle of *S. aureus* [17].

II. c. Virulence factors

A wide range of virulence factors enhance the pathogenicity of *S. aureus*. These virulence factors can be divided into two groups; cell-associated and secreted factors [18-20]. The cell-associated virulence factors include clumping factor, protein A, and fibronectin-binding proteins. Secreted virulence factors are toxins that are secreted during stationary phase growth include the hemolysins (alpha, beta, gamma, and delta toxin), exoenzymes (proteases, lipases and nucleases), and SAgs (described in detail in Introduction, Section III). All of these virulence factors allow the bacterium to persist in the host and cause disease. The antibiotic resistance mechanisms conferring resistance to methicillin increasingly have been found in *S. aureus* isolates that produce SAgs, including TSST-1, increasing the virulence of these strains [21-23]. Table 1 summarizes the major virulence factors produced by *S. aureus.*

Virulence Factor(s)	*Mechanism(s) of Action*
Capsular Polysaccharides	Thick polysaccharide protecting and preventing phagocytosis of *S. aureus*
Hemolysins (α ,β ,γ, and δ)	Forms pores on host cell membranes causing proinflammatory cytokine release and cell lysis
SAgs	Non-antigen specific cross linking of MHC II and TCR
Protein A	Binding of host Ig prevents opsonization and phagocytosis of *S. aureus*
CHIPS	Inhibition of complement, by binding to and blocking complement receptors
PVL	Forms pores in leukocytes and other cell types
Eap/Map	Binds to ICAM-1, which inhibits binding to LFA-1 and prevents leukocyte adhesion and attachment
MSCRAMM (elastin-, collagen-, and fibronectin-binding proteins, clumping factors)	Allows *S. aureus* binding to host cells and invasion of host cells
ica locus	Encodes for genes for biofilm formation
Exoenzymes [proteases (staphopain), lipases, coagulase, nuclease]	Destruction of host tissue, freeing nutrients

Table 1. Staphylococcal Virulence factors. Abbreviations; chemotaxis inhibitory protein of *S. aureus* (CHIPS), extracellular adherence protein (Eap), MHC II analogue protein (Map), Inter-Cellular Adhesion Molecule-1 (ICAM-1), Lymphocyte function-associated antigen 1(LFA-1), Microbial surface components recognizing adhesive matrix molecules (MSCRAMM), Panton-Valentine leukocidin (PVL), T-Cell receptor (TCR). Sources [18, 20, 24-26]

II. d. Methicillin-resistant *S. aureus (MRSA)*

Shortly after the introduction of penicillin in the 1940s, penicillin resistant *S. aureus* isolates were identified [27]. Methicillin and other semi-synthetic penicillins were introduced in the 1960's and resistance to these agents emerged by the late 1960's. Methicillin-resistant *S. aureus* (MRSA) have become a significant human health problem [28]. MRSA infections have become so widespread that >60% of *S. aureus* isolates in hospital intensive care units in the United States are MRSA [29]. Resistance to methicillin occurs by a change in the target site of β-lactam antibiotics, penicillin binding protein (PBP2a), which is encoded by the *mecA* gene [30]. *mecA* is carried by staphylococcal cassette chromosomes (SCC) which are mobile genetic elements [31].

II. e. Hospital- and community-associated MRSA

MRSA isolates are divided into two categories, healthcare-associated MRSA (HA-MRSA) or community-associated MRSA (CA-MRSA), which were originally grouped according to where the infection was acquired [32]. These two types of MRSA are now classified by the Center for Disease Control and Prevention (CDC) based on the staphylococcal cassette chromosomes mec (SCC*mec*) type which confers resistance to antibiotics [28, 30, 33]. These two categories of MRSA often differ by the population they infect. HA-MRSA often infect older people with co-morbid conditions such as chronic obstructive lung disease, diabetes, and end stage renal disease, while CA-MRSA infect younger, otherwise healthy people. In general, the secreted virulence factors produced by HA-MRSA differ from CA-MRSA. HA-MRSA express the SAgs; TSST-1, SEA, SEB, and SED while CA-MRSA expresses SEB and SEC. CA-MRSA also express

PVL, a leukocidin [19, 33-37]. Additionally, CA-MRSA infections often occur initially as skin and soft tissue infections as opposed to endocarditis, sepsis, and osteomyelitis, which are commonly caused by HA-MRSA [33]. Recent studies have suggested that CA-MRSA strains are more virulent than HA-MRSA strains, and HA-MRSA are more opportunistic than CA-MRSA strains [38].

MRSA cause an estimated 19,000 deaths each year in the United States, which is comparable to the combined deaths due to acquired immunodeficiency syndrome (AIDS), tuberculosis, and viral hepatitis [39, 40]. MRSA infections comprise only a portion of the total *S. aureus* infections, so the total deaths associated with *S. aureus* are higher than the 19,000 estimated for MRSA infections. The overall burden of disease associated with *S. aureus* infections is significant, with an estimated 500,000 inpatient hospital stays per year involving a *S. aureus* infection [41, 42]. The economic burden is dramatic as well. In 2004, the estimated cost of *S. aureus* infections was $14.5 billion per year in the United States [42].

II. f. Antimicrobial therapies

The choice of antibiotic therapy depends on the location of the infection and whether the infection is caused by methicillin-sensitive *S. aureus* (MSSA) or MRSA. Table 2 summarizes anti-staphylococcal therapies including the antibiotic name and/or antibiotic class, mechanisms of action and corresponding resistance mechanisms that *S. aureus* has developed or acquired. Beta lactams such as penicillinase-resistant penicillins (oxacillin, nafcillin, and dicloxacillin), amoxicillin with clavulanic acid, ampicillin with sulbactam, cephalosporins (i.e., cefazolin, ceftriaxone, and cefepime) and carbapenams (imipenem,

ertapenem, meropenem, doripenem) are effective if the causative agent is MSSA. If the infecting agent is MRSA the drug of choice is vancomycin [43, 44]. However, current treatment guidelines specify that vancomycin may not be the optimal treatment for MRSA infections if the organism has a minimum inhibitory concentration (MIC) to vancomycin of ≥2 mg/L (as treatment failures occur more frequently with higher MICs) [43, 44]. Linezolid, daptomycin, Synercid® (quinupristin and dalfopristin), ceftaroline, telavancin, minocycline, trimethoprim-sulfamethoxazole, and clindamycin are all alternative treatment options for MRSA. Recently, ceftaroline, a new cephalosporin has been approved for the treatment of MRSA. Ceftaroline binds to the altered PBP that is produced in MRSA, PBP2a. By binding to PBP2a ceftaroline is able to inhibit cell wall synthesis and bacterial growth [45]. β-lactam antibiotics and vancomycin can cause an increase in production of SAgs at sub-growth inhibitory concentrations making these agents less appropriate for use in cases where SAg-mediated disease is suspected [46, 47]. Other agents that inhibit protein expression such as gentamcin, clindamycin, or linezolid may be beneficial because these antibiotics reduce the amount of toxin being produced for treatment of SAg-mediated diseases [48].

Antibiotic	*Mechanism of Action*	*Mechanism of Resistance*
Penicillinase-resistant penicillins	Inhibits cross linking of PDG by binding to PBP	Altered PBP
Cephalosporins	Inhibits cross linking of PDG by binding to PBP	Altered PBP
Carbapenams	Inhibits cross linking of PDG by binding to PBP	Altered PBP
Macrolides	Inhibits protein translation by binding to 50S subunit	Change in rRNA, Efflux pumps, inactivation of drug
Trimethoprim-Sulfamethoxazole	Inhibits tetrahydrofolic acid production	Change in enzyme, over production of enzyme
Tetracyclines	Inhibits protein translation by binding to 30S subunit	Expression of TetO and TetM protect ribosome
Aminoglycosides	Inhibits protein translation by binding to 30S subunit	Acetylation and inactivation of antibiotic
Vancomycin	Binds to D-Ala-D-Ala tail on PDG preventing PPG extension	Modification to D-Ala-D-Lac on PDG, thickening of cell wall
Linezolid	Inhibits protein translation by binding to 50S subunit	Changes in target site on rRNA
Daptomycin	Binds to and disrupts bacterial membrane	Thicker cell wall, prevents drug from getting to active site
Quinupristin/ Dalfopristin (Synercid®)	Inhibits protein translation by binding to 50S subunit	Methylation on target site on rRNA
Telavancin	Binds to D-Ala-D-Ala tail on PDG preventing PDG extension, binds to and disrupts bacterial membrane	Unknown, possibly similar to Vancomycin
Clindamycin	Inhibits protein translation by binding to 50S subunit	Methylation on target site of rRNA
Rifampin	Inhibits transcription by inhibiting rpoB	Mutations in rpoB
Tigecycline	Inhibits protein translation by binding to 30S subunit	Expression of TetM, protect ribosome, efflux pumps

Table 2. Antibiotics for treatment of *S. aureus* infections. Abbreviations: Peptidoglycan (PDG), Ribosomal RNA (rRNA).

III. Superantigens and superantigen- mediated diseases

III. a. Superantigen mechanisms of action

Superantigens (SAgs) are 22-30 kDa proteins produced by multiple species of pathogenic microorganisms, including *S. aureus* that have profound effects on the host immune system. This effect is caused by the SAg's ability to crosslink the α- or β-chains of MHC II molecules on APCs with variable regions of the β-chain of T-cell receptors (TCR) [1, 26, 49-55]. SAg cross-linking occurs independent of antigen specific interactions of the TCR and antigen peptide (Figure 1). Non-specific receptor cross-linking by SAgs leads to massive cytokine release from T cells [IL-2, TNF-β, and IFN-γ] and macrophages (IL-1β and TNF-α) [1, 55, 56]. Cytokines are small proteins that are produced and released by cells that affect the immune system. Different cytokines can stimulate while others suppress the immune system. Chemokines are a class of cytokines that attract immune cells to the site. Table 3 describes cytokines that are commonly mentioned through this thesis. The massive cytokine release caused by the SAg leads to the progression of capillary leakage, fever, shock, and death that are associated with SAgs [57-64]. The magnitude of the SAg-induced T-cell activation is dramatic. A typical antigen can activate 1 in 10,000 T-cells while SAgs can activate up to 20% of the T-cell population [1, 50].

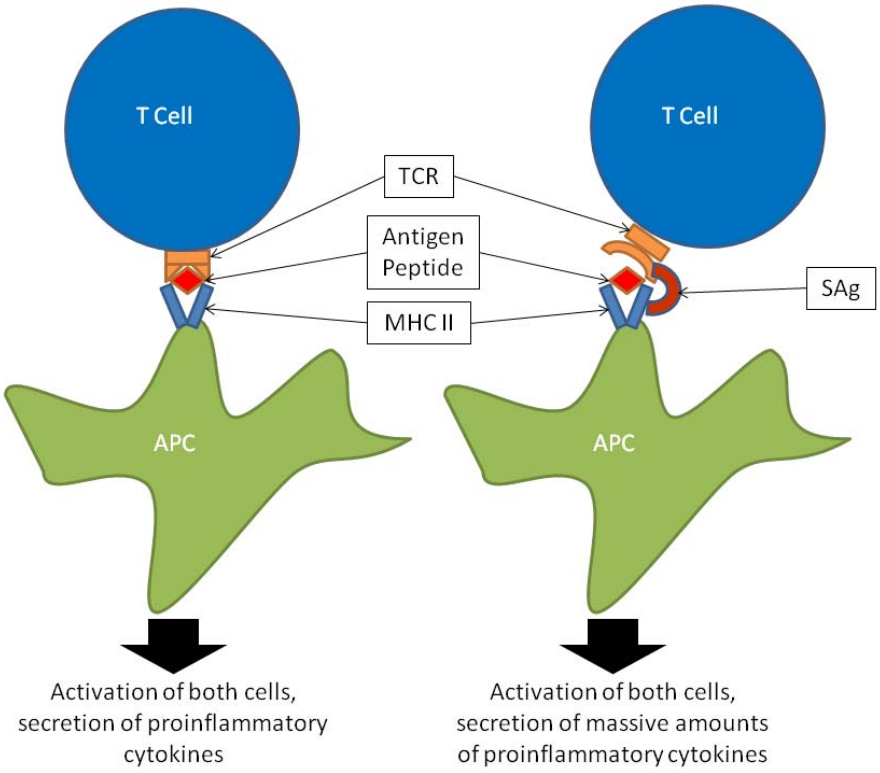

Figure 1. SAg mechanism of action. Conventional antigens (left) only activate a small fraction of CD^{4+} T-helper cells in an antigen specific manner (1 in 10,000 T-cells). SAgs (right) activate a large fraction of CD^{4+}T-cells (up to 20% of the population) and APCs in a non-antigen specific manner resulting in a cytokine storm.

Cytokine	*Function*
IL-1β	Induces fever, activates T-cells and macrophages, stimulates epithelial cells to produce other proinflammatory cytokines
IL-2	Induces T-cell proliferation
IL-6	Induces T-cell and B- cell growth and differentiation
IL-8	Chemokine that attracts neutrophils
TNF-α	Induces fever and causes local inflammation
MIP-3α (CCL20)	Chemokine that attracts lymphocytes and dendritic cells
IFN-γ	Induced antiviral state, activates macrophages, increases MHC II expression

Table 3. List of common cytokines and their function(s).

III. b. SAg distribution and Structure

SAgs have been identified in *S. aureus*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Yersinia enterocolitica*, Cytomegalovirus, Herpes Virus, Rabies Virus, and *Candida albicans* [1, 13, 55, 65, 66]. SAgs have been studied most extensively in *S. aureus* and *S. pyogenes* [66]. *S. pyogenes* produce streptococcal pyrogenic exotoxins (SPEs) with SPEA and SPEC most commonly associated with human disease [1]. The SAgs produced by *S. aureus* include SEA-SEU (with the exception of F) and TSST-1 [1, 13]. The staphylococcal SAgs have 15-90% amino acid homology [66]. The staphylococcal and streptococcal SAgs share a high degree of homology and were likely derived from a common ancestor and horizontally transferred between the two species. The SAgs are encoded on mobile genetic elements such as pathogenicity islands, which can be mobilized and transferred to other bacteria in the presence of phage [1]. SAgs are also encoded on and transferred between bacteria by phage, transposons, and plasmids [1, 66]. The staphylococcal and streptococcal SAgs can be divided into 5 groups based on protein sequence. Group I contains only TSST-1 as this toxin is so divergent; group II contains SEB, SEC3, SEG, and SPEA; group III contains SEA, SED, SEE, SEH, and SEJ; group IV contains SPEC, SPEG, and SPEJ; and group V contains SEI, SEK, SEL, and SEP[1].

The structure of TSST-1 (Figure 2) and its interactions with MHC II and TCR have been studied extensively [66, 67]. TSST-1 has one MHC II binding domain while the majority of the other SAgs have two MHC II binding domains [68, 69]. Studies have determined that G31 and S32 residues of TSST-1 interact with MHC II [67, 69, 70].

Human Leukocyte Antigen (HLA)-DR, DQ, and DP are the proteins that comprise MHC II molecules [71]. TSST-1 and other SEs bind HLA-DR with a higher affinity than HLA-DQ or DP [13, 72]. Residue Q136 is critical for TSST-1 to interact with Vβ-TCRs [67]. A twelve amino acid (dodecapeptide) region (TSST-1 position 119 to 130) is highlighted in Figure 2. This region is highly conserved between SAgs (33-91% homology) (Table 4) and is predicted to be important in interactions with epithelial cells based on studies described in Chapter 3 of this thesis.

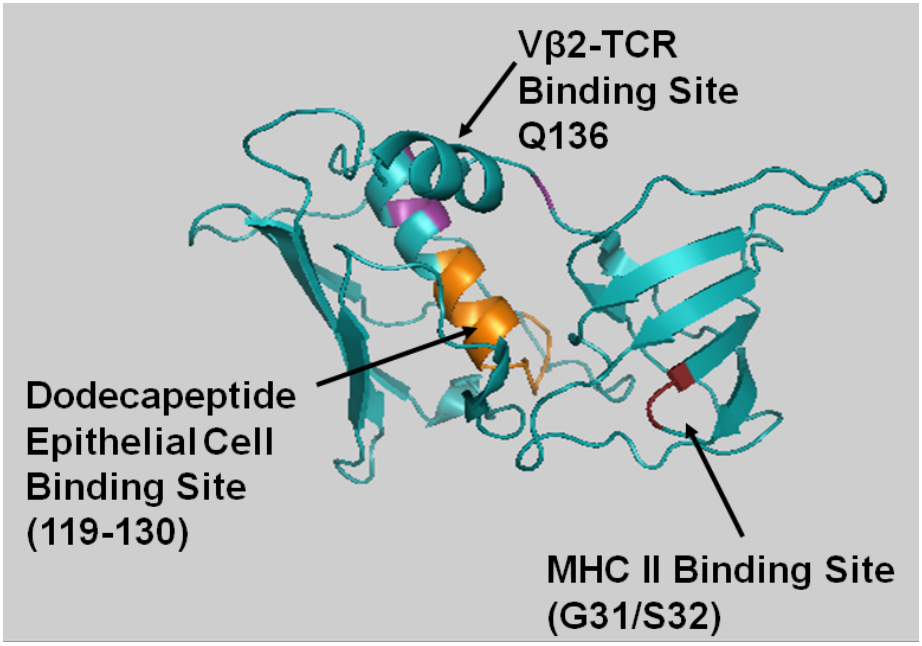

Figure 2. TSST-1 Structure. Schematic model of TSST-1 showing eukaryotic cell binding domains. Purple residues (H135/Q136) are involved in Vβ2-TCR binding; red residues (G31/S32) in α-chain MHC II molecule binding; and orange residues (dodecapeptide) are hypothesized to be involved in epithelial cell binding. Image was generated using PyMOL (www.pymol.org).

Superantigen	*Dodecapeptide Sequence*	*Homology to SEB*
SEB	T-N-K-K-K-V-T-A-Q-E-L-D	100%
SEC	T-**D**-K-K-**S**-V-T-A-Q-E-L-D	91.67%
SPEA	T-N-K-K-**M**-V-T-A-Q-E-L-D	91.67%
TSST-1	**F**-**D**-K-K-**Q**-**L**-**A**-**I**-**S**-**T**-L-D	33.33%

Table 4. Amino acid sequence of dodecapeptide. Non-conserved peptides are shown in red. (Table from Broshahan *et al.* [7])

III. c. Factors that affect SAg production

TSST-1 is produced by *S. aureus* during post-exponential phase growth under the control of the accessory gene regulator (*agr)* quorum sensing system. *Agr* is a two-component system that regulates the expression of multiple virulence factors [73, 74]. AgrD is constitutively produced and processed into an autoinducer peptide (AIP) which is secreted outside of the cell by AgrB. Once *S. aureus* reaches a high enough local concentration and thereby AIP reaches a threshold concentration, AIP is able to signal to the bacterial cell by AgrC. AgrC then phosphorylates AgrA which then induces transcription of target genes, including TSST-1 and other SAgs.

The conditions under which inserted tampons reside are ideal for *S. aureus* to synthesize TSST-1: temperatures of 35 $^{\circ}$C to 40°C, pH of 6.5 to 8.0 (pH of the vaginal canal during menstruation), protein-containing media, low glucose, and at least 2% oxygen balanced with 7% CO_2 [75, 76]. Oxygen concentrations $\geq$ 2% induce TSST-1 production [77], suggesting that the role tampons play in mTSS is to introduce oxygen into an otherwise anaerobic environment. Tampon insertion increases the oxygen content to levels where TSST-1 can be produced for 6 hours (hr) or more [78].

III. d. Toxic Shock Syndrome (TSS): description of disease and prevalence

TSS is an acute-onset, potentially fatal, multi-system illness characterized by fever, hypotension, sunburn-like rash, peeling of the skin upon recovery, and multi-organ dysfunction [79-81]. TSS most commonly occurs when *S. aureus* colonizes mucosal

surfaces and produces SAgs, which penetrate into the blood stream and activate APC and T-cells in a non-antigen specific manner. This immune cell over-activation leads to the massive expression of cytokines namely; IL-2, IFN-γ and TNF- α, which lead to the progression of clinical disease [56]. For TSS to occur there must be an absence of toxin neutralizing antibodies [82, 83]. It is estimated that 80-90% of healthy adult women have a sufficient titer (≥1:32) of TSST-1 neutralizing antibody to prevent TSS from occurring in the presence of TSST-1 [82].

TSS can be divided into two groups; mTSS and non-menstrual-associated TSS (non-mTSS) [12]. Non-mTSS often occurs when a surgical site becomes infected with *S. aureus* or when *S. aureus* infects pulmonary tissue following an influenza infection [84]. mTSS occurs in women exclusively while non-mTSS occurs equally in men and women. mTSS is almost always associated with TSST-1 while non-mTSS typically is caused by SEB, SEC, or TSST-1 [1, 85-88].

The most common symptoms associated with TSS include dizziness, myalgia, vomiting, headache, and diarrhea [89]. The CDC criteria for TSS are listed in Table 5. A patient is said to have TSS if they fulfill criteria 1-5. If 4 out of 5 criteria are met, they are categorized as probable TSS. The incidence of mTSS in the U.S. is 1.05 cases per 100,000 menstruating women [90]. Data on the prevalence of non-mTSS is not as clear. But a large study comprising 5,296 cases of TSS that were reported to the CDC between 1979-1996 estimated that 26% of all TSS cases are non-mTSS [91]. A recent French study determined the mortality was higher for non-mTSS compared to mTSS (22% versus 0%) [92]. In this study 50% of the non-mTSS cases had septicemia while none of

the mTSS cases had septicemia, additionally three of the seven fatal cases of non-mTSS had septicemia. This data suggests that the increase in mortality seen in non-mTSS is not solely due to septicemia.

Approximately 9-20% of women in the United States aged 13-40 years old are colonized with *S. aureus* vaginally. Up to 20% of these isolates carry the gene for TSST-1 [82, 93], which means approximately 2-4% of all women carry *S. aureus* vaginally that produce TSST-1. A study in Japan concluded that approximately 80% of MRSA strains isolated from hospital patients were positive for TSST-1, compared to 20% of MSSA isolates from the same population [94]. The expression of SAgs is prevalent among *S. aureus*, a study in Germany in 2003 determined that 50.8 % of clinical *S. aureus* isolates carried the gene for at least one SAg (SEA-SEE and TSST-1) [95]. Collectively, these epidemiological studies confirm the global distribution of SAg-producing *S. aureus* strains and emphasize the importance of SAg-mediated disease as a continuing global health problem in the 21st century.

Criteria 1. Temperature ≥38.9°C	
Criteria 2. Hypotension: systolic blood pressure ≤ 90 mm Hg (for adults)	
Criteria 3. Rash	
Criteria 4. Desquamation of the skin 1-2 weeks after onset of illness.	
Criteria 5. Abnormalities in 3 or more organ systems:	
Gastrointestinal	Vomiting or diarrhea at onset of illness
Muscular	Severe myalgia or creatine kinase level; at least twice the upper limit of normal
Mucous Membrane	Vaginal, oropharyngeal, or conjunctival hyperemia
Renal	Blood urea nitrogen or creatinine at least twice the upper limit of normal or urinary sediment with pyuria in the absence of urinary tract infection
Hepatic	Total bilirubin, ALT, AST levels at least twice the upper limit of normal
Hematological	Platelet count <100,00/mm^3
Central Nervous System	Disorientation or alterations in consciousness without focal neurological signs when fever and hypotension are absent.
Negative results for following lab tests: Blood, throat or CSF cultures (may be positive for *S. aureus*) Rise in titer to spotted fever group rickettsial infection, leptospirosis, or measles	

Table 5. **CDC criteria for TSS.** A patient is confirmed to have TSS if they fulfill criteria 1-5. Probable TSS is the diagnosis when the patient meets 4 out of the 5 criteria. Source (http://www.cdc.gov/ncphi/od/ai/casedef/toxicsscurrent.htm).Abbreveations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), Cerebrospinal fluid (CSF).

III. e. Other SAg-associated diseases

In addition to TSS, SAgs have been implicated in several other diseases (Table 6) [96]. *S. aureus* and their SAgs are the second most common cause of food poisoning. Staphylococcal food-borne disease is characterized by a rapid (less than 6 hr) onset of hypersalivation, nausea, vomiting, abdominal pain, and diarrhea, which normally occurs later. These symptoms are a result of the massive immune response to the SAgs (SEs) produced by *S. aureus*, which are ingested by eating contaminated food [97, 98]. Atopic dermatitis is a chronic, relapsing, allergic, inflammatory disorder of the skin that is also associated with SAgs [99]. Ninety percent of resident microflora in an atopic dermatitis lesion are *S. aureus* and 70% of these isolates produce one or more SAgs (commonly TSST-1, SEB and SEC) [100]. Atopic dermatitis occurs when SAgs and bacteria induce an inflammatory reaction at the site of dermatitis. SAgs have been implicated in exacerbation of airway diseases such as allergies, chronic rhinosinusitis, chronic obstructive pulmonary disease (COPD), asthma, and nasal polyps [101, 102]. Patients with severe asthma have elevated levels of SEA- and SEB-producing *S. aureus* and elevated titers of Immunoglobulin (Ig) E against these SAgs compared to healthy controls [103]. Anti-SAg titers have a positive correlation with the severity of asthma symptoms [104]. The hypothesis is that the production of SAgs causes chronic inflammation within the affected tissues [105, 106]. Table 6 summarizes these and several other Staphylococcal SAg-associated diseases.

Disease	***Pathogenesis***
TSS	SAgs are produced by *S. aureus* on the vaginal mucosa and penetrate through the mucosa into the bloodstream and non-specifically stimulate APCs and T-cells
Staphylococcal Food-Borne Disease	SAg or SAg-producing *S. aureus* are consumed with contaminated food. SAgs induce a local, massive cytokine response leading to symptoms including nausea, vomiting, abdominal pain, cramping, and diarrhea
Atopic Dermatitis	SAg-producing *S. aureus* on skin induce a massive cytokine response leading to symptoms including red, inflamed, and itchy rash
Respiratory Disorders *	Presence of SAgs induce a cytokine response and proliferation of T-cells causing or exacerbating these diseases
Purpura Fulminans	Purpura fulminans is characterized by cutaneous hemorrhage and necrosis caused by disseminated intravascular coagulation and dermal vascular thrombosis. Purpura fulminans is a complication after necrotizing pneumonia or TSS. The massive cytokine response can induce coagulation cascades which can cause the symptoms of purpura fulminans [107, 108]
Kawasaki's disease	Kawasaki's disease is characterized by symptoms similar to TSS with coronary artery aneurysms. Pathogenesis is unknown but with SAgs suspected [109, 110]

Table 6. Staphylococcal SAg-associated diseases.

*Respiratory Disorders including: asthma, COPD, nasal polyps, and chronic rhinosinusitis

III. f. Current therapies for SAg-associated diseases, including TSS

Current therapies for TSS are primarily supportive care (including maintenance of circulation and oxygenation) and antibiotics [12]. The source of infection should be removed if possible, e.g. removal of the tampon, wound debridement or abscess drainage. There is evidence that treatment with an antibiotic that inhibits protein (toxin) translation, such as gentamcin, clindamycin, tigecycline, or linezolid may be beneficial because these antibiotics reduce the amount of toxin being produced [48]. IVIG and corticosteroids can be used, although there is no data from controlled trials to support their use [12]. The logic supporting IVIG use is that pooled antibodies from multiple donors neutralize the toxin. One study has that shown dexamethasone, which binds to the corticosteroid receptor inhibiting the immune response, is able to inhibit SAg-induced cytokine production by peripheral blood mononuclear cells (PBMC) *in vitro* supporting its use as an anti-TSS agent [111]. To date, there has not been a controlled study to determine the most effective TSS therapy. A series of observational studies have demonstrated that IVIG is able to improve survival in Streptococcal TSS [112, 113].

Other experimental therapies for the treatment of SAg-mediated diseases have been studied. Several novel therapies target either the production or the binding of SAgs. There have been a series of experiments that used soluble Vβ domains of the TCR to antagonize SAg-T-cell interactions. Vβ2 or Vβ28 domains with 3 million fold greater binding affinity than wild-type Vβ domains of the TCR have been generated. These Vβ domains prevented TSST-1 or SEB-induced disease in rabbit models of TSS [114, 115]. Another experimental therapy for SAg disease includes the use of the α and β chains of

hemoglobin [116]. This therapy is based on the observation that *S. aureus* does not produce TSST-1 in the presence of blood. The authors determined that α and β chains of hemoglobin are able to inhibit production of exoproteins in *S. aureus*. To date, this therapy has not been tested in an animal model, but shows promise as a novel therapy. Additionally, there are novel therapies that target the proinflammatory effects of SAg on host cells. These therapies include NF-κB inhibitors and glycerol monolaurate (GML). These therapies will be discussed in detail in Section V. of the Introduction.

IV. Superantigen interactions with mucosal surfaces

IV. a. Vaginal Epithelium

The human ectocervical and vaginal epithelium consist of stratified squamous epithelial cells, while the endocervical epithelium is comprised of columnar epithelial cells. These cells are primarily non-keratinized, though during mid-cycle the cells become keratinized [117]. The importance of vaginal and cervical epithelial cells to the immune system was realized only recently when epithelial cells were recognized as non-professional antigen presenting cells, which are capable of presenting antigen to T-cell even though that is not the cell's major function as are macrophages and dendritic cells [118, 119].

Several models have been developed to study interactions of microbes with vaginal epithelial cells. Immortalized human female reproductive epithelial cell lines have been used in multiple studies [120-122]. These cell lines have been transformed with the E6/E7 genes from Human Papillomavirus (HPV) 16, which allow the cells to be propagated over many generations. Figure 3 shows HVEC used by Peterson *et al.* [2] to determine morphological effects of TSST-1 on HVEC as well as studies outlined in this thesis. These morphological changes include a rounding of the cells and loss of cell junctions, which can be seen in Figure 3B. Advantages to this model include a human non-cancer derived immortalized vaginal epithelial cell model (unlike the commonly used Henrietta Lacks (HeLa) cells, which were derived from a cervical carcinoma), which is easy and inexpensive to maintain. However, transformation with E6/E7 is known to cause dysregulation of multiple pathways, so validation of results with other

model systems is required [123-125]. These cells grow as homogenous monolayer which makes conducting experiments on full thickness tissue impossible to do, which is another limitation of this model. Full thickness tissue contains cells at different stages of cell growth, fibroblasts and other cells of the immune system, including dendritic cells, macrophages and lymphocytes.

An *ex vivo* porcine vaginal mucosal model has been developed by our laboratory as well as others to overcome the limitations inherent in immortalized cell culture monolayers. Porcine vaginal tissue is very similar to human vaginal tissue in its cellular content and permeability. Both tissues consist of multi-cellular layer of non-keratinized stratified squamous epithelium with intracellular lipids providing a permeability barrier [126-130]. The use of *ex vivo* tissue allows the ability to conduct experiments on full thickness tissue. However, without vasculature there is no migration of lymphocytes, which limits the immune response. Figure 4 depicts the similarities between human and pig vaginal epithelium tissue when stained with hematoxylin and eosin (H & E). The similarities between porcine and human vaginal tissue make porcine tissue an ideal model for human vaginal tissue.

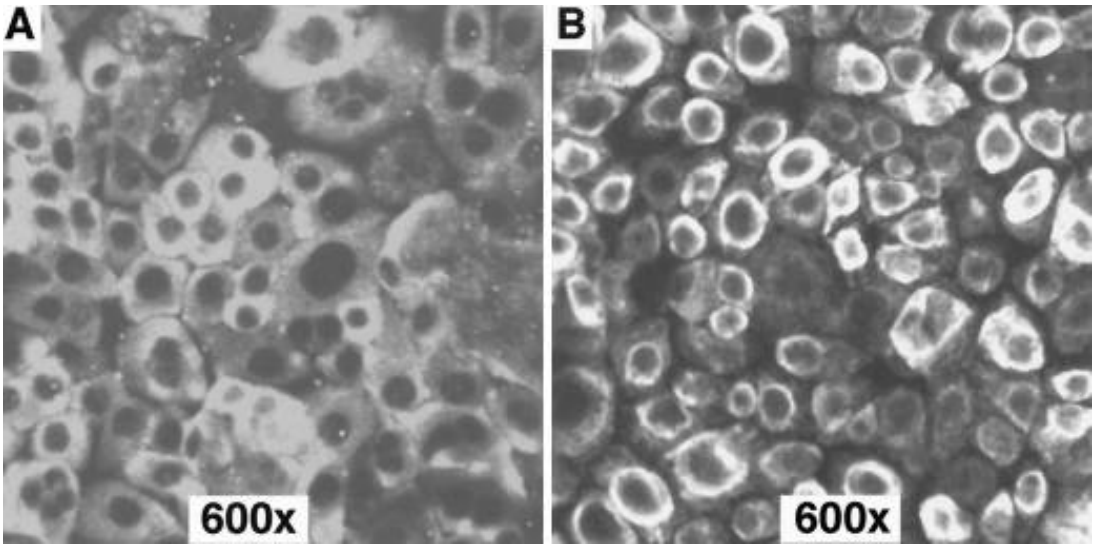

Figure 3. TSST-1 causes changes in HVEC morphology. A. HVEC grown to confluence, B. HVEC after exposure to TSST-1 100 μg/ml for 6 hr. Image from Peterson *et al.* [2]. Image used with permission.

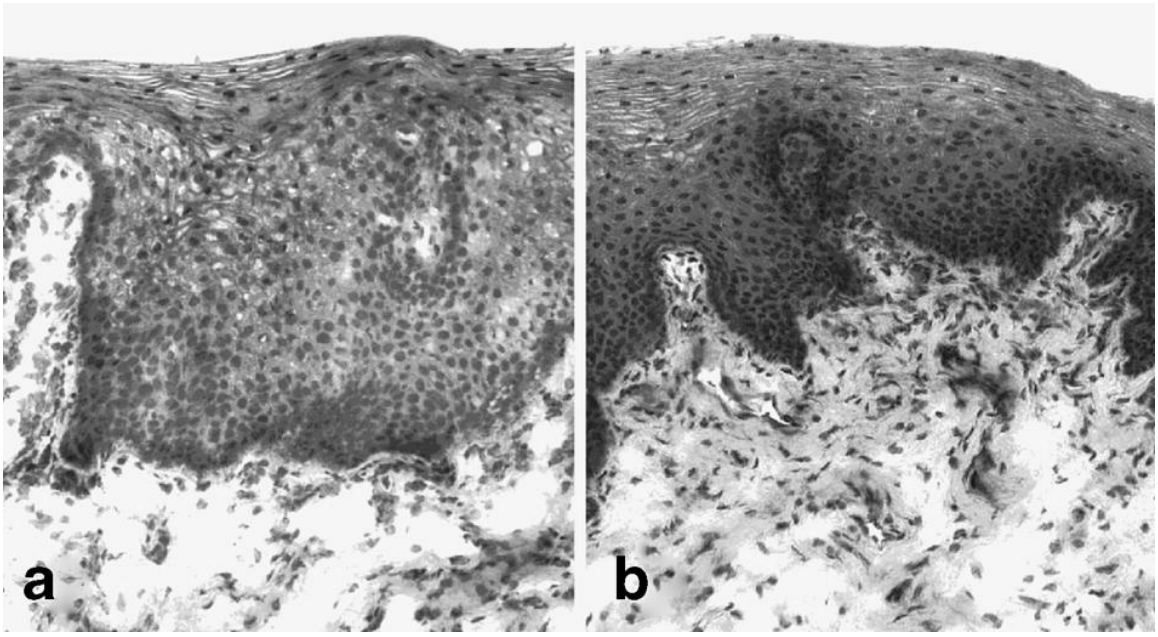

Figure 4. Comparison of Human and Porcine Vaginal Tissue. H & E staining of Human (A) and porcine (B) vaginal tissue shows similarities between the two species. Figure from Squier *et al.* [127]. Image used with permission.

IV. b. Superantigen proinflammatory effects on epithelial cells

Interactions between SAgs and epithelial cells are important as *S. aureus* infections are commonly initiated at mucosal surfaces. Therefore, epithelial cells are the first cells to be exposed to SAgs. mTSS is caused by SAgs penetrating into the host's circulatory system, while the bacteria remain on the mucosal surface [2]. SAgs penetrate epithelial layers to reach the underlying tissue and bloodstream to interact with the high amount of professional immune cells (APC and T-cells) residing there. The effects of *S. aureus* exotoxins, including TSST-1, on the vaginal mucosal tissue are dramatic. A disruption in the mucosal barrier and significant histological changes were observed in vaginal epithelium taken from a woman who died of mTSS, including separation and disruption of the epithelial basal-lateral layer also blood vessel dilation and immune cell migration can be seen within this tissue (Figure 5) [131].

Limited studies examining interactions between epithelial cells and SAgs have shown that SAgs can induce changes in epithelial cellular morphology and secretion of proinflammatory cytokines/chemokines from vaginal, bronchial, nasal, and intestinal cells (Table 7) [2-6]. Aubert *et al.* reported that TSST-1 induced TNF-α and IL-8 from primary bronchial epithelial cells when the cells were pre-treated with IFN-γ for 48 hr to up-regulate MHC II molecules [3]. O'Brien *et al.* determined that SEA and SEB induced IL-8 from primary nasal epithelial cells; however, they noted an increase in the amount of IL-8 secreted by the cells when they were pre-treated with IFN-γ. The authors did not detect any TNF-α produced by these cells following stimulation with SAgs [5]. Damm *et al.* also described an increase in IL-8 and IL-6 secretion from primary nasal epithelial

cells when stimulated with SEB without pre-treatment with IFN-γ [132]. Musch *et al.* determined that intestinal epithelial cells increase production of heat shock proteins (Hsp) 25 and 72 in response to stress induced by SEB. Hsp are important to protect critical cellular functions [4].

Peterson *et al.* showed a marked increase in expression of proinflammatory cytokines from HVEC in response to TSST-1 or live *S. aureus* [2]. In addition, disruption in the morphology of the HVEC monolayer after incubation with TSST-1 was noted (Figure 3B). The HVEC contracted and appeared to lose cell-to-cell contact. A wide range of proinflammatory cytokines (CXCL 1-3, IL-8, CCL20 [MIP-3α], IL-1α, Il-1β, and TNF-α) were significantly upregulated compared to unstimulated controls when measured by Affymetrix® Human GeneChip U133A microarray analysis. CCL20, IL-1β, TNF-α IL-6, and IL-8 were also detected at the protein level [2]. Recent studies suggest that cytokine production by vaginal epithelial cells in response to TSST-1 destabilizes the vaginal mucosal barrier, allowing TSST-1 to penetrate into the submucosa and access the immune system and the vasculature [2].

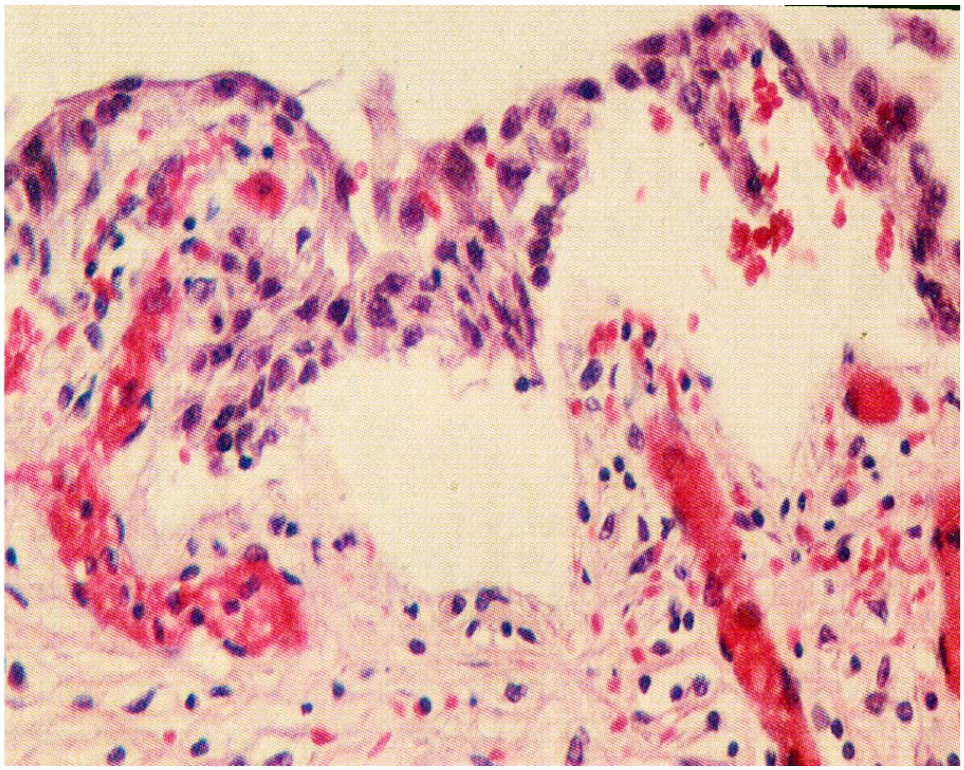

Figure 5. mTSS effects on vaginal mucosal tissue. Vaginal mucosa detached at basal layer from tissue of a woman that died of TSS. Figure from Larkin *et al.* [131]. Image used with permission.

Superantigen	*Tissue*	*Effects*	*Receptors*	*Reference*
SEB	Primary Human Nasal Epithelial Cells	Induction of IL-6 protein, without pre-treatment with IFN-γ	ND	[132]
SEA SEB	Primary Human Nasal Epithelial Cells	Induction of IL-8 protein, with and without pre-treatment with IFN-γ	ND	[5]
TSST-1	Primary Human Bronchial Epithelial Cells	Induction of TNF-α and IL-8 (mRNA), with and without pre-treatment with IFN-γ	MHC II	[3]
TSST	Immortalized Human Vaginal Epithelial Cells	Disruption of HVEC shape; induction of pro-inflammatory cytokines	Non-MHC II	[2]
SEB	Mouse Small Intestinal Epithelial Cells	Induction of Hsp dependent on ERK 1/2 and p38 MAPK pathways	ND	[4]
SEA	Immortalized Human Intestinal Epithelial Cells	Increase in intracellular Ca^{2+} showing direct effect on signaling pathways	ND	[6]

Table 7. **Studies examining SAg interactions with epithelial cells**.

Abbreviations: messenger RNA (mRNA) extracellular-signal-regulated kinases (ERK), Mitogen-activated protein kinases (MAPK), Not determined (ND)

IV. c. SAg epithelial receptors

The receptors responsible for the interaction of SAgs with epithelial cells have not been characterized. Previously, MHC II molecules were suggested as possible bronchial epithelial cell receptors for TSST-1 leading to cytokine induction [3]. MHC II molecules are important in presenting antigen peptides to T-cells, but also play an important role in activating the APC. MHC II molecules are able to stimulate cytokine production via NF-κB in the absence of T-cells [133, 134]. O'Brien *et al.* demonstrated that primary nasal epithelial cells produce proinflammatory cytokines in response to the SAgs, SEA and SEB [5]. However, cells were stimulated with IFN-γ to increase MHC II expression prior to SAg exposure to maximize cytokine production. In this system, MHC II antibodies reduced, but did not completely abrogate, cytokine production. These data suggest that MHC II molecules are not the sole receptor on epithelial cells for SAgs. Additionally, non-MHC II receptors for TSST-1 have been demonstrated on human conjunctival epithelial cells and the authors propose that these receptors also exist on the surface of endothelial cells [135-137]. TSST-1 binding to a non- MHC II receptor, which could induce cytokines and chemokines, was supported by a study which noted SEA and SEB were able to bind and induce cytokines from MHC II-deficient macrophages [138].

IV. d. Non-MHC II receptors

MHC II is probably not a major receptor on epithelial cells for TSST-1, as TSST-1 has only one MHC II binding domain and is not able to cause MHC II dimerization, which is needed for production of cytokines [1, 139]. Studies have suggested a non-

MHC II receptor for SAgs. O'Brien *et al.* demonstrated that nasal epithelial cells produced proinflammatory cytokines in response to TSST-1 despite minimal MHC II expression [5]. In addition, SEA-induced cytokine expression was only partially inhibited (40-70%) by blocking with anti-MHC II antibodies suggesting a non-MHC II receptor [5].

A series of studies suggest a relatively conserved twelve amino acid domain of SAgs is important for binding and initiating the proinflammatory response in epithelial cells [140-143]. This dodecapeptide region can be found in most SAgs and is relatively conserved among SAgs in sequence similarity (33-91% homology) and structure (Table 2). The dodecapeptide region in TSST-1 is highlighted in the orange color in Figure 2. This region was originally identified by Wang *et al.* which determined that peptides generated to a region of streptococcal pepsin-extracted type 5 M protein, which was similar in sequence to the dodecapeptide regions of multiple streptococcal and staphylococcal SAgs, was able to inhibit SAg-induced T-cell proliferation [144]. Synthetic SEB peptides corresponding to this region are able to inhibit SAg transcytosis (SEB, SEC and TSST-1 across intestinal epithelial cells) [2, 130, 142, 145]. It is important to note that this region is also distinct from those regions required for TCR and MHC II binding [69, 146].

Peptides generated against the dodecapeptide sequence in SEC are capable of neutralizing the ability of SAg to stimulate T-cells [147]. Arad *et al.* demonstrated that a dodecapeptide variant (YNKKKATVQELD) of an SEB region (original sequence TNKKKVTAQELD) could inhibit the SEB-induced expression of IL-2, IFN-γ, and TNF-

α mRNA from PBMC [140-142, 148]. These authors also demonstrated that the peptides protected the mice from developing lethal shock. Peterson *et al.* were able to block TSST-1-induced IL-8 production from HVEC with the same dodecapeptide described by Arad *et al.* [2]. These data highlight the importance of this region for interactions with epithelial cells.

V. Proinflammatory pathways and Anti-inflammatory agents

V. a. Nuclear factor-kappa B (NF-κB) and Novel Anti-inflammatory agents

NF-κB is an important transcription factor that regulates the expression of many genes including growth factors, cytokines, chemokines, and cell adhesion molecules [149-151]. When the cell is activated by cytokines, stress, DNA damage, or pathogens, NF-κB is activated by signal transduction and translocates to the nucleus [152]. NF-κB then binds to DNA and induces transcription of target genes [150]. Figure 6 shows the NF-κB pathway for activation of cytokines and chemokines. Several studies have demonstrated that SEA, SEB, and SEC1 induce cytokine production via an NF-κB-dependent mechanism in human PBMC, macrophages, and monocytes [134, 153, 154].

NF-κB inhibitors have been investigated for use as therapies in cancer and autoimmune diseases and, more recently, as an inhibitor of SAg-mediated diseases [155]. NF-κB inhibitors (pyrrolidine dithiocarbamate, curcumin, sodium pyrithione, and N-acetyl-L-cysteine) successfully blocked SEA-induced cytokine production in PBMC and prevented SEA-induced fever in a rabbit model [156]. The inhibitors used in this study likely inhibited multiple pathways in addition to NF- κB, but the authors only reported inhibition of NF- κB. Another study was able to inhibit the affects of SAgs with an inhibitor specific to NF- κB. Liu *et al.* were able to prevent SEB-induced cytokine production and migration of immune cells to the site of infection in a murine lung model of infection with an intracellular peptide that inhibits the translocation of NF- κB (p50/RelA) into the nuclease [157].

In addition to agents that specifically target the NF-κB pathway, other agents have been studied for their anti-inflammatory properties and ability to reduce the effects of SAg- or other pathogen-induced proinflammatory responses from epithelial cells. GML, a surfactant that is naturally present in human breast milk and that is widely used in foods and cosmetics [158], was able to inhibit the production of SAg-induced cytokines from epithelial cells, protect against TSS in a rabbit model, and inhibit production of SAgs from *S. aureus* [158, 159]. GML was also able to inhibit the growth of pathogenic organisms while not affecting the growth of the normal flora of the vagina [160-162]. Additionally, GML has been tested as an additive to tampons which decreased the amount of *S. aureus*, TSST-1, and IL-8 found in the vagina after tampon use [163].

Other studies explored novel compounds as potential anti-inflammatory agents against SAg-induced inflammation by targeting professional immune cells (T-cells and APC). Nakagawa *et al.* were able to block TSST-1-induced production of cytokines in PBMC using anisodamine (20 μg/ml), a naturally occurring tropane alkoid that has been used in Chinese herbal medicines. Anisodamine (50 mg/kg via intraperitoneal injection) was able to protect mice from TSST-1-induced lethality [164]. Watson *et al.* used epigallocatechin gallate (100 μM); a component of green tea, to inhibit SEB-induced cytokines from PBMC [153]. Several studies have used pyrrolidine dithiocarbamate, sodium pyrithione, N-acetyl-L-cysteine, and curcumin to inhibit SEA-, SEB-, and TSST-1-induced cytokine production from PBMCs [156, 165]. These studies focused on the activity of the inhibitors on professional immune cells (APCs and T cells) while the activity of inhibitors on epithelial cells remains to be investigated.

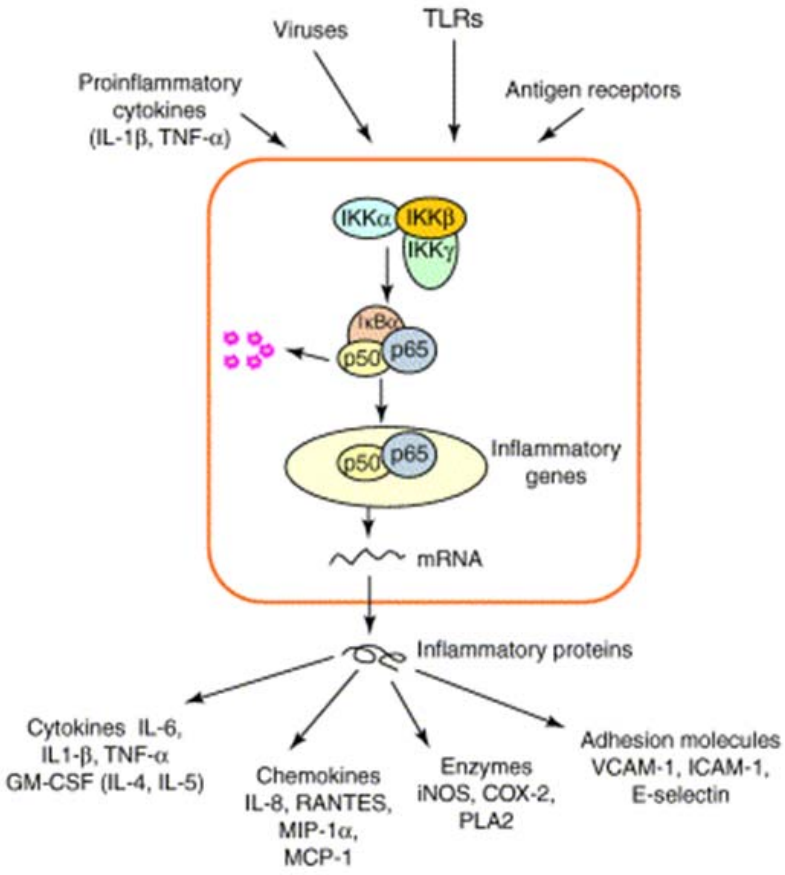

Figure 6. NF-κB Classical Pathway. NF-κB is activated by a wide range of signals. Through signaling cascades the IκB kinase (IKK) complex (consisting of IKK α, IKKβ, and IKKγ) becomes activated and phosphorylates the repressor IαBα. This phosphorylation results in ubiquitination and then degradation of IαBα freeing p50 and p65 (RelA). RelA and p50, duplexed together, then translocate to the nucleus where they bind to NF-κB specific regions on DNA and induce expression of NF- κB responsive genes. Figure from [152]. Image used with permission.

V. b. Curcumin

Curcumin, a diferuloylmethane, is a component of the root of the Indian spice Turmeric (*Curcuma longa*) that has anti-inflammatory, anti-angiogenesis, and antimicrobial properties [166-168]. Turmeric is a main component of the curries that have been used as a spice for thousands of years across Asia and India [169]. The structure of curcumin can be seen in Figure 7. Curcumin is a bis-α, β- unsaturated β-diketone that exhibits keto-enol tautomerism[170]. The aryl rings may be substituted by varying numbers of hydroxy or methoxy groups in a symmetrical or asymmetrical fashion to produce analogs of curcumin or curcuminoids. Curcumin is the most abundantly occurring natural analogue at 77% of all curcuminoids, followed by demethoxycurcumin (17%) in which one methoxy group is absent, and then bis-demethoxycurcumin (3%) in which the methoxy group is absent from both the aryl rings (Figure 7A) [170].

Based on curcumin's wide use in food, it has been designated as an international food additive E100 [171]. The FDA has classified curcumin as 'generally recognized as safe'. Curcumin has been used as a medicinal agent for 4,000 years across India for the treatment of rheumatism, skin diseases, intestinal worms, diarrhea, fevers, hepatic disorders, and inflammation [169]. During the past 10 years, Western medicine has taken notice of curcumin and a vast amount of research has been conducted to explore the medicinal properties of curcumin [166, 169, 171, 172].

Research with curcumin in the past ten years has focused primarily on its anti-tumor properties [171]. Curcumin is able to regulate a wide range of cellular pathways

including the activation and production of cytokines, cellular enzymes, growth factors, receptors, transcriptional factors, protein kinases, and adhesion molecules [167, 173]. There also has been research pertaining to the use of curcumin as an antimicrobial agent against bacteria, fungi, and viruses [174-176].

Oral doses of curcumin as high as 12 grams per day are extremely well tolerated in humans [177]. The oral bioavailability of curcumin is low because of its low (<1%) gastrointestinal absorption, efficient first pass metabolism, rapid elimination and poor aqueous solubility [178-181]. Additionally curcumin is highly protein bound (>99%) in serum by albumin [182]. The exposure of curcumin can be increased (~2000%) by co-administration with piperine (a component of black pepper, which inhibits 1st pass metabolism) [183]. Experiments in rats after an oral dose (2 g/kg) yielded half lifes ranging from 4 to 12 hours [183-185]. Curcumin is primarily eliminated through hepatic glucuronidation and sulphation, and then excreted in the stool after bilary excretion (Figure 7B) [186]. Peak serum concentrations after oral dosing occur 1-2 hr after dose and are undetectable 12 hr after dosing. There is some uncertainty about renal excretion as curcumin can sometimes be detected in the urine after an oral dose, as one clinical trial was able to detect curcumin in urine while others have not been able to [171, 187]. Curcumin pharmacokinetic and dynamic properties are summarized in Table 8.

Curcumin is able to inhibit a wide range of cellular processes that are critical in inflammation and tumorgenesis [167, 173]. These pathways include transcription factors; activator pathway (AP)-1, NF-κB, and Signal Transducers and Activators of Transcription (STAT). Many of these pathways are up-regulated in tumor cells. By

suppressing these transcription factors, primarily NF-κB, the production of many inflammatory cytokines, such as TNF-α and the interleukins is inhibited. Curcumin is also able to inhibit many enzymes such as lipoxygenase (LOX) and cyclooxygenase (COX), and inducible nitrogen oxide synthase (iNOS) which are reactive oxygen species generating enzymes and involved in inflammatory pathways. Curcumin also inhibits the activity of many receptors such as epidermal growth factor receptor-1 (EGFR1), human epidermal growth factor receptor-1(HER-2), vascular endothelial growth factor (VEGF), and androgen receptors that are over expressed in tumor cells [166, 167, 169, 173].

In addition to curcumin affecting eukaryotic cellular processes, curcumin also has effects on microbes. Curcumin is able to inhibit the growth of several bacteria including *Staphylococcus*, *Lactobacillus, Streptococcus, Enterococcus, Escherichia, and Bacillus* [174, 188-191] and several fungal species [175]. Several of these studies are up to 60 years old and details about concentrations of curcumin used and other methodological details are limited. These studies were conducted in broth culture, which does not mimic the natural state of most infections, i.e. a biofilm. During biofilm growth, the bacteria attach to a surface and produce a polysaccharide that can protect the bacteria from the host's immune system and antibiotics [192]. Bacteria in biofilms are more resistant to antibiotics compared to bacteria growing in a planktonic state [193].

There have been two mechanisms proposed for the antibacterial activity of curcumin. The first mechanism is a phototoxic effect, which occurs when both Gram positive and Gram negative bacteria are exposed to micromolar (1-10 μM) amounts of curcumin [176, 194]. The second mechanism is an inhibition of the protein; FtsZ observed in

Escherichia and *Bacillus* at higher curcumin concentrations (10-100 μM). FtsZ is a critical structural protein that is forms a ring in the middle of a dividing cell where the two daughter cells will separate. When FtsZ is inhibited the bacterial cell is unable to divide thereby inhibiting bacterial growth [189, 190]. Curcumin also inhibited the virulence of *Pseudomonas aeruginosa* by disrupting the quorum-sensing mechanisms thereby reducing expression of virulence factors at sub-inhibitory concentrations [195].

There have been several studies investigating the use of curcumin in bacterial infections at mucosal surfaces, focusing on the anti-inflammatory affects of curcumin. Lüer *et al.* determined that curcumin was able to inhibit *Moraxella catarrhalis* growth and attachment to a pharyngeal epithelial cell line. Additionally, curcumin inhibited *M. catarrhalis* invasion and induction of IL-8 [196]. Wessler *et al.* determined that curcumin inhibited *Neisseria gonorrhoeae*-induced cytokines by blocking NF-κB signaling in cervical epithelial cells [197]. Moon *et al.* determined that curcumin inhibited *E. coli*-produced shiga-like toxin from binding to intestinal epithelial cells. Curcumin protected the epithelial cells from a toxin-induced death and inhibited toxin-induced IL-8 production [198].

Results from experiments in animal models of infection and curcumin vary but suggest that curcumin reduces proinflammatory cytokines and inflammation. This data indicate that curcumin may be a feasible option as an anti-TSS agent. Bansal *et al.* investigated the role of curcumin alone and in combination with Augmentin (which contains amoxicillin and clavulanic acid) against *Klebsiella pneumoniae* infection in murine lung model. Curcumin alone or with Augmentin reduced neutrophil infiltration

into the lung tissue and diminished the production of proinflammatory proteins without an additional reduction in bacterial load due to treatment with Augmentin [199]. Marathe *et al.* investigated the effects of curcumin on *Salmonella enterica* serovar Typhimurium infection in a murine model. Curcumin increased the bacterial counts within several tissues within the mouse, likely due to a curcumin-induced increase expression in genes enabling intracellular survival. In this study, the bacteria were more resistant to anti-microbial proteins (polymyxin B and protamine) after treatment with curcumin than bacteria that were not treated. The authors did not determine if there was a reduction in inflammation at the site of infection [200].

There has been at least one clinical trial of curcumin for an infectious disease in humans [201]. Di Mario *et al.* investigated the use curcumin for treatment of *Helicobacter pylori*, a common cause of gastric ulcers. Curcumin (30 mg) was given two times daily for seven days in combination with several other non-antibiotic agents (zinc acetate, bovine lactoferrin, and lysolecithin). Twenty-five patients infected with *H. pylori* were assessed at baseline and two months after completion of the seven day course. These patients were not given any antibiotic treatment. The curcumin-based treatment did not result in eradication of *H. pylori*, although there was a significant decrease in the symptoms of gastric inflammation [201]. These data suggest that curcumin may be effective in treatment of gastritis caused by *H. pylori* in combination with other agents.

The use of a topical vaginal application of curcumin is currently being investigated as treatment for other diseases such as cancers of the female reproductive tract. Currently

there is a clinical study underway for patients diagnosed with uterine cervical dysplasia at Emory University (www.clinicaltrials.gov Trial #NCT01035580). In this dose escalation study, patients insert a 500 mg capsule vaginally (up to 2 g per day) to determine the maximum tolerated dose. Results from this study are not yet available.

Curcumin possess anti-inflammatory properties, which makes curcumin a potential anti-TSS agent. Because many of the symptoms of TSS are due to the massive release of cytokines blocking downstream effects of TSS, anti-inflammatory agents may be successful treatment options. A vaginal application of curcumin may reduce the amount of cytokines that are produced in response to the SAg, reducing trafficking of immune cells (i.e. macrophages, dendritic cells, T-cells and neutrophils) to the site and preventing progression to TSS. The use of curcumin to inhibit the proinflammatory effect of SAgs from PBMC has been studied (introduction section V.a.), but no study has investigated the use of curcumin on mucosal surfaces to inhibit cytokine production [156, 165].

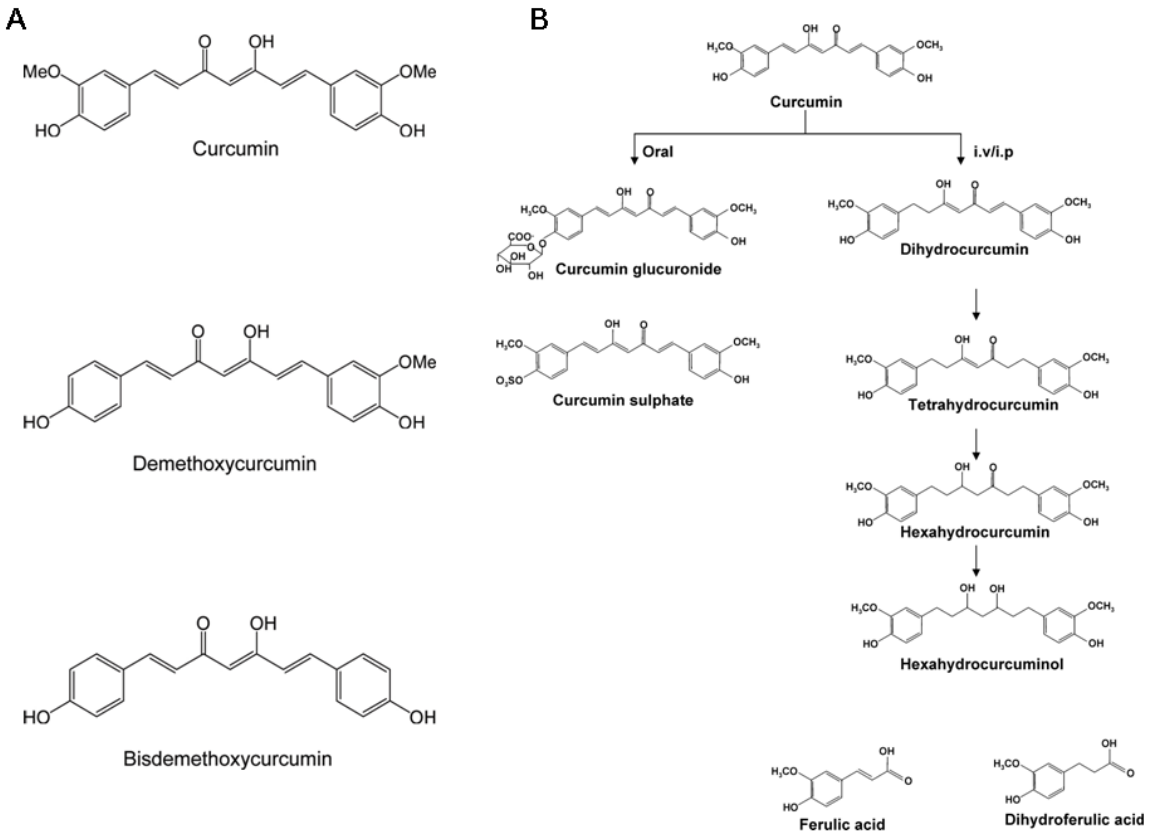

Figure 7. Structure of curcumin, natural analogues and primary metabolites.

Curcumin has the International Union of Pure and Applied Chemistry (IUPAC) name; (1*E*, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-dione. Figure 7A shows structures of curcumin natural analogues from [202] used with permission under free access agreement. Figure 7B shows structure of metabolites of curcumin from [178] used with permission. Abbreviation: intraperitoneal (i.p.)

Pharmacokinetic properties	Absorption	Poor oral absorption (<1%), rapid fast pass metabolism
	Distribution	Peak serum concentrations after oral dosing occur 1-2 hr after dose and are undetectable 12 hr after dosing, volume of distribution in rat 1360 L/kg after oral dose, highly protein bound (>99%), half life 4-12 hours after oral dose
	Metabolism	Hepatic glucuronidation and sulphation, and then excreted in the stool
	Excretion	Uncertainty about renal excretion as curcumin can sometimes be detected in the urine after an oral dose in clinical study, as one study was able to detect curcumin in urine while others have not been able to. In animal studies no curcumin detected in urine post oral dose.
Pharmacodynamic properties	Molecular Targets	Inhibition of AP-1, NF-κB, STAT; and the production and activity of many inflammatory cytokines (TNF α and IL), LOX, COX, iNOS, EGFR1, HER-2, VEGF, and androgen receptors
	Safety	Oral doses of curcumin as high as 12 grams per day are extremely well tolerated in humans (no events greater than Grade 1)

Table 8. **Pharmacokinetic (PK)/Pharmacodynamic (PD) properties of curcumin**.

VI. Aims of this work

Central hypothesis: TSST-1 binds to specific receptors on vaginal epithelial cells and triggers a proinflammatory response that recruits immune cells to the area. This proinflammatory effect disrupts the mucosal layer and allows progression to TSS. Prevention of the proinflammatory response is a proposed mechanism for therapeutics to treat superantigen-mediated diseases. (A model to describe this overall approach is presented in Figure 8.)

Specific aim 1: Characterization of the interaction and outcomes of TSST-1 exposure to HVEC.

Rationale: Limited studies of SAg interactions with epithelial cells have described a cytokine response that is generated in response to the toxin. This aim was investigated by: 1) measuring the dose-dependent cytokine response from HVEC in response to TSST-1; 2) identifying the HVEC cell signaling pathways that are activated in response to TSST-1; 3) identifying the critical amino acids on TSST-1 for this interaction with HVEC; and 4) exploring potential TSST-1 receptors on HVEC (MHC II and CD40) as described in Chapters 2 through 4.

Specific aim 2: Evaluation of curcumin as a therapeutic agent to prevent TSS.

Rationale: By using curcumin, a known inhibitor of multiple cell-signaling pathways, the production of TSST-1-induced cytokines from epithelial cells is hypothesized to be reduced or eliminated. By reducing the cytokines produced by the epithelial cells, it is hypothesized that there would be fewer immune cells (i.e. macrophages, dendritic cells,

T-cells and neutrophils) trafficking to the site, thereby preventing progression to TSS. In Chapter 5, curcumin was evaluated as a novel agent to prevent TSS.

Data from this work will enhance our understanding of SAg-mediated diseases with the goal of developing novel treatment for SAg-mediated diseases. By characterizing the effects of TSST-1 on vaginal epithelial cells and identification of signaling pathways involved in SAg-induced cytokine production novel drug targets will be identified. Identification of the epithelial receptor will provide insights into the mechanism of SAg-induced cytokine production and also identify novel drug targets. Finally, work with curcumin will highlight the importance of the proinflammatory response induced at epithelial surfaces by SAgs and demonstrate the use of curcumin as an anti-TSS agent.

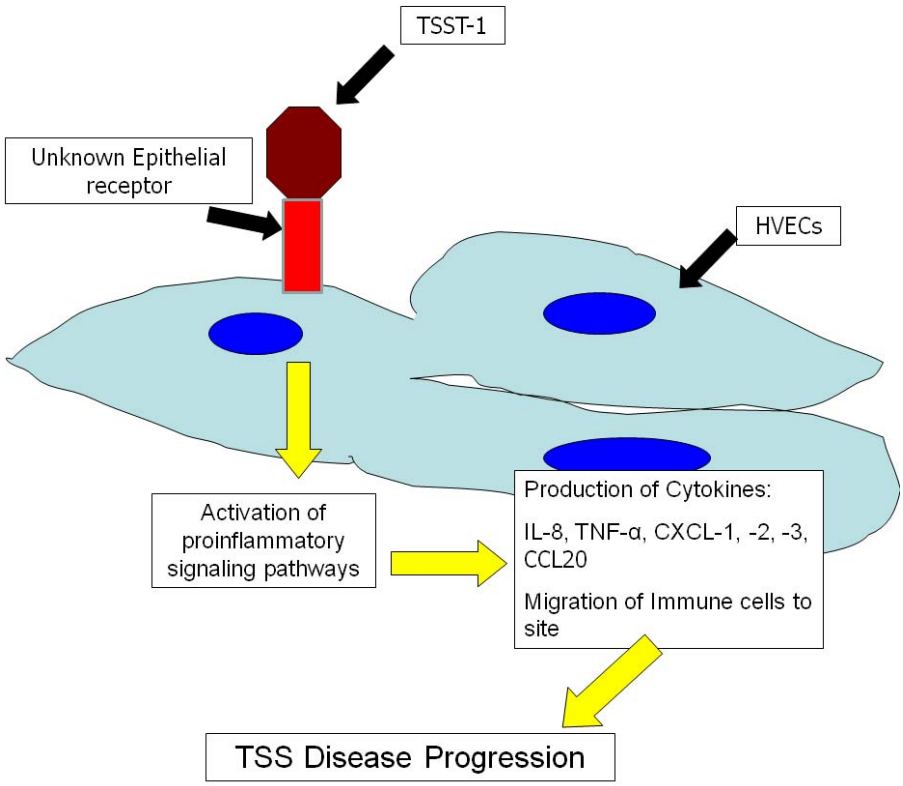

Figure 8. Proposed Model of TSST-1 effects on vaginal epithelium. *S. aureus* produces exotoxins including TSST-1 within the vaginal lumen. It is hypothesized TSST-1 binds to an unknown receptor on HVEC, which leads to the induction of NF-κB and possibly other proinflammatory pathways. This activation of NF-κB leads to the production of cytokines by epithelial cells, which leads to the recruitment of immune cells (including macrophages and T cells) to the site of infection. The immune cells cause a disruption in the epithelial layer leading to the penetration of the toxin, not the bacteria, into the bloodstream where the toxin systemically cross links APCs and T cells leading to TSS.

Chapter 2- TSST-1 Induces Cytokines from HVEC Independent of MHC II

Studies part of a manuscript under review at the *Journal of Immunology* (January 2011).

In these studies purification of TSST-1 was done by members of Patrick Schlievert's laboratory (Department of Microbiology, School of Medicine, University of Minnesota). Several real-time RT-PCR assays were completed by Liz Horn (Peterson laboratory), cytokine multiplex assays were done by the Cytokine Reference Lab (School of Medicine, University of Minnesota), and NF-κB luciferase reporter experiments were performed by Laura Breshears (Peterson laboratory). I analyzed all the data and conducted the TSST-1 and HVEC exposure and viability assays; and flow cytometry and histology studies with the assistance of Michele Anderson (Peterson laboratory).

I. Introduction

S. aureus is a significant human pathogen that causes a wide range of diseases such as mTSS [79-81]. mTSS most commonly occurs when *S. aureus* colonizes the vaginal mucosa and produces the SAg, TSST-1. TSST-1 and other SAgs have the ability to crosslink MHC II molecules on APCs to the TCR leading to activation of both cells and a massive cytokine release. Interactions between SAg and epithelial cells are poorly understood, despite epithelial cells being the first cell type that encounters SAgs during disease progression.

Limited studies looking into the effect of SAgs on the target epithelial cells have described changes in epithelial cellular morphology and secretion of proinflammatory cytokines/chemokines from vaginal, bronchial, nasal, and intestinal cells [2-5]. Recent studies suggest that cytokine production by vaginal epithelial cells in response to TSST-1 destabilizes the vaginal mucosal barrier, allowing TSST-1 to penetrate into the submucosa and access the immune system and the vasculature [2]. The receptors responsible for SAg interaction with epithelial cells are not well characterized. Previously, MHC II molecules were suggested as epithelial receptors for SAg that could lead to induction of cytokine synthesis [3, 5]. However, in these published studies, the epithelial cells were stimulated with IFN-γ to increase MHC II expression prior to SAg exposure to maximize cytokine production. Non-MHC II epithelial receptors for TSST-1 were demonstrated on human conjunctival epithelial cells, and these receptors were also suggested to exist on the surface of endothelial cells [135-137]. Non–MHC II receptors for TSST-1 have not been identified.

The signaling pathways activated in epithelial cells in response to SAgs have not been investigated. One likely downstream target of SAgs is the transcription factor NF-κB. NF-κB regulates the expression of many genes, including growth factors, cytokines, chemokines, and cell adhesion molecules [149-151] in response to external stimuli. Many of the proteins that are up-regulated in response to TSST-1 and other SAgs are known NF-κB regulated genes [2, 3, 5].

This study was conducted to characterize TSST-1 induction of proinflammatory cytokines from HVEC; and to evaluate the role of NF-κB and MHC II in TSST-1 induced cytokine production. This work confirms previous results demonstrating cytokine responses from a HVEC line in response to TSST-1 and demonstrates that the HVEC response is dose-dependent, with limited cell death. This study also establishes that NF-κB is activated in response to TSST-1, suggesting that NF-κB is a component of the TSST-1 induced signaling pathway. Lastly, our data show that MHC II is not associated with TSST-1 induced cytokine production by HVEC.

II. Materials and Methods

Culture of HVEC

The HVEC line used in this study was generated by transforming primary vaginal epithelial cells from premenopausal woman with the E6/E7 genes of HPV 16. This line (CRL-2616) was obtained from American Type Culture Collection (ATCC, Manassas, VA) [122, 203]. HVEC were maintained in keratinocyte serum-free medium (KSFM) in 75-cm^2 flasks (BD Falcon, Bedford, MA), 6-well, or 96-well microtiter plates (Corning, Corning, NY) at 37 °C in 7% CO_2, and supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor as provided by the manufacturer, 0.4 mM calcium chloride, and 50 international units (IU)/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Fungizone; Gibco, Invitrogen). On the day before and day of experimentation, antimicrobials were not used since it has been observed that amphotericin B reduces cytokine production by HVEC.

Toxin purification

S. aureus RN4220, containing pCE107, was for the production of wild type TSST-1; strain RN4220 does not produce SAgs [204, 205]. The plasmid pCE107 is a high-copy-number plasmid containing *tstH,* the gene for TSST-1 [205]. All media, reagents, and glassware used for toxin production were maintained pyrogen free. *S. aureus* RN4220 was grown up in beef heart media, cultures were treated with 80% ethanol (final concentration) at 4°C, and the precipitates resolubilized in water. TSST-1 was purified by two rounds of isoelectric focusing (IEF). IEF was carried out by initial separation first using a pH gradient of 3.5 to 10, followed by a second pH gradient of 6 to 8. The

isoelectric point of TSST-1 is 7.2 [86]. TSST-1 was identified by double immunodiffusion based on reactivity with a specific polyclonal antibody generated against wild type toxin [206]. Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which demonstrated a single protein band at a molecular weight of 22 kDa. Protein content of purified toxins were quantified by the Bio-Rad protein assay (Bio-Rad Corporation, Hercules, CA) with the superantigen SEB used for standard curve generation. TSST-1, thus prepared, contained no detectable lipopolysaccharide (LPS), as tested by *Limulus* assay (Sigma, St. Louis, Mo.); nor were peptidoglycan, lipoteichoic acid, hemolysin, protease, or lipase (<1 part per 10^6) detectable by combinations of bioassay and SDS-PAGE. Comparable results were obtained with multiple batches of TSST-1.

Toxin was also verified as homogeneous when samples were subjected to reverse-phase high performance liquid chromatography (HPLC) [Protein C4 column from Vydac (Hesperia, CA) 0 to 60% gradient of acetonitrile with 0.1% trifluoroacetic acid over a 30 minutes (min) time period]; proteins eluted as sharp single peaks.

Cytokine assays

HVEC were grown to confluence and exposed to TSST-1 for the indicated time in KSFM without antibiotics. Control wells of HVEC contained KSFM without antibiotics and without stimuli. Supernates were removed and stored at -20°C until secreted cytokines were measured by the Luminex (Austin, TX) multiplex assay, with Fluorokine MAP multiplex assay kits (R & D Systems, Minneapolis, MN). The HVEC were removed from the flasks by trypsinization (5 to 8 min at 37 °C) and collected by

centrifugation (250 g for 5 min). Total RNA was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA) per kit directions. Complementary DNA (cDNA) was generated by reverse transcription (iScript, Bio-Rad Corporation) following kit directions. Expression of cytokine genes was measured by real time reverse transcription polymerase chain reaction (PCR) with SYBR® Advantage® Premix (Clonetech, Mountain View, CA) on a MYiQ™ iCycler (Bio-Rad Corporation, Hercules, CA). Expression of cytokine genes was normalized to expression human hypoxanthine phosphoribosyltransferase (HPRT) in unstimulated cells by delta delta CT method with use of primers listed in Table 9. The delta delta CT method determines expression of experimental genes in an treatment by normalizing to a housekeeping gene in a control treatment [207]. For some experiments, IL-8 was measured as a marker cytokine; cell culture supernates were collected and tested for IL-8 by enzyme linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, MN) according to manufacturer directions. Unless otherwise specified, all experiments were conducted in triplicate, and graphs represent means ± standard deviation (SD).

Cell viability assay

CellTiter 96®Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) measured cell survival per manufacturer protocol after 6 hr exposure to TSST-1. Briefly, after 6 hr exposure to TSST-1 and removal of supernates, 100 µl of fresh KSFM and 20 µl of reagent were added. Cells were incubated for 2-3 hr at 37 ^{O}C in 7% CO_2, and absorbance at 490 nm wavelength was determined.

NF-κB Activation Assay

HVEC were seeded in KSFM with antibiotics and growth factors into 96-well plates at 15,000 cells/well. After 24 hr, the medium was replaced with KFSM free of antibiotics or growth factors and the cells were transfected with NF-κB reporter, negative control, or positive control plasmids (SABiosciences, Frederick, MD) with the Genejuice transfection reagent (EMD4 Biosciences, Gibbstown, NJ). The NF-κB reporter plasmid contains an NF-κB binding domain sequence which controls the expression of the firefly luciferase gene. The negative contains a basal promoter (NF-κB binding domain sequence) controlling the firefly luciferase gene. The positive control plasmid contained a constitutively active firefly luciferase gene. All plasmids were mixed with constitutively active renilla luciferase to control for transfection. Forty-eight hr after transfection the medium was changed and the cells were stimulated with 50 ng/ml IL-1β (Biolegend, San Diego, CA), 250 μg/ml TSST-1, or were left unstimulated. After 4 hr, the level of NF-κB activation was determined by the Dual-Glo luciferase assay (Promega, Madison, WI) and a Beckman Coulter LD400 luminometer.

Western Blotting

HVEC were grown in 225-cm^2 flasks (BD Falcon, Bedford, MA) and stimulated with IFN-γ (200 IU/ml) for 24 hr or left untreated. HVEC were lysed for 5 min at 4°C with lysis buffer [50mM Tris, 0.25M NaCl, 1% NP-40, complete protease inhibitors without Ethylenediaminetetraacetic acid (EDTA) (Roche, Indianapolis, IN)]. Lysates were centrifuged for 5 min at 16,000 g at 4°C. Proteins were separated by SDS-PAGE on 4-20% gradient acrylamide Mini-Protean® TGX™ (Bio-Rad Corporation, Hercules, CA)

and transferred to a polyvinylidene fluoride (PVDF) membrane. MHC II was detected with an anti-HLA DR + DP + DQ antibody [clone CR3/43] (Abcam, Cambridge, MA) with anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Cell Signaling, Danvers, MA) and SuperSignal ® West Dura substrate (Pierce, Rockford, IL). Blots were stripped and actin was detected with anti-actin ACTN05 (C4) (Abcam, Cambridge, MA) and a Fast Western blot kit (Pierce, Rockford, IL).

Flow cytometry

HVEC were grown to confluence and removed from plastic with trypsin. As a positive control, cells were stimulated with 200 IU/ml IFN- γ (Leinco, St. Louis, MO) for 48 hr to increase surface MHC II expression. The cells were washed with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 5mM $NaPO_4$, 0.15M NaCl) three times and suspended at $5.0x10^5$ cells/tube. Cells were blocked with 1 μg of an antibody with irrelevant specificity to control for non-specific binding (eBioscience, San Diego, CA), for 20 min. Cells were washed and incubated with 0.5 μg of anti-MHC II monoclonal antibody (anti-DR+DP+DQ) conjugated to phycoerythrin (PE) (Abcam, Cambridge, MA). Alternatively, PE-conjugated isotype control antibody (eBioscience, San Diego, CA) was added to 0.5 ml of cell suspension and incubated for 30 min at room temperature in the dark. Live cells were selectively analyzed based on forward and side scatter. 10,000 cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

Cervical tissue and Immunohistochemistry

Cervical tissue was obtained by standard surgical procedures from three premenopausal women with pathological conditions not involving the cervix. Experimental use of human tissue for these studies was conducted with Institutional Review Board (IRB) approval. Tissue samples spanning the cervical transformation zone were immediately fixed in Streck Tissue Fixative (Streck Laboratories, La Vista, NE) and then processed by standard methods for paraffin embedding and microtome sectioning [208]. For signal detection by colorimetric immunohistochemistry (IHC), primary anti-MHC II antibodies (final concentration 2 μg/mL) (anti-HLA-DR, DQ, DP, Dako, Carpinteria, CA), followed by biotinylated secondary antibodies and streptavidin-peroxidase conjugates (ABC System; Vector Laboratories, Burlingame, CA) were utilized. Cervical tissues were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO) and mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

Statistical analysis

Unless otherwise specified, significance was determined by one way analysis of variance (ANOVA) followed by Dunnett's Multiple comparison test, comparing experimental group to control group (GraphPad Prism, La Jolla, CA).

IL-8	Forward- 5’ AGCCTTCCTGATTTCTGCAGCTCT Reverse- 5’ AATTTCTGTGTTGGCGCAGTGTGG
IL-6	Forward- 5’ CATGTGTGAAAGCAGCAAGAGGC Reverse- 5’ CACCAGGCAAGTCTCCTCATTGAA
TNF-α	Forward- 5’ TGCCTGCTGCACTTTGGAGTGAT Reverse- 5’ GGTTCGAGAAGATGATCTGACTGCCT
MIP-3 α	Forward- 5’ TTGCTCCTGGCTGCTTTGATGT Reverse- 5’ TGCCGTGTGAAGCCCACAATAA
HPRT	Forward- 5’ GGTGAAAAGGACCCCACGAA Reverse- 5’ AGTCAAGGGCATATCCTACA

Table 9. Primers used in real-time reverse transcription PCR.

III. Results

TSST-1 induces cytokine/chemokines release from HVEC.

Our previous studies determined that HVEC incubated with TSST-1 (100 μg/ml) increased production of chemokines and cytokines, including CCL20 (MIP-3α), CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), IL-1β, IL-6, IL-8, and TNF-α [2]. These previous studies characterized a HVEC line obtained as a gift from the University of Iowa. In those prior studies it was suggested that a concentration of TSST-1 100 μg/ml was physiologically relevant, as this concentration of TSST-1 could be present in proximity to mucosal surfaces [7, 209, 210]. The studies presented in this manuscript utilized an unrelated, immortalized HVEC line derived from another woman and obtained from ATCC [203]. HVEC were exposed to increasing concentrations of TSST-1 (10-500 μg/ml) for 6 hr, and toxicity for HVEC was measured (Figure 9). There was little difference in HVEC viability over the entire range of TSST-1 concentrations (75% of HVEC remaining viable in the presence of up to 500 μg/ml TSST-1). The ability of TSST-1 to induce multiple proinflammatory cytokines and chemokines from this HVEC line was characterized by exposing confluent HVEC to TSST-1 (50 or 100 μg/ml) compared to media controls for 6 hr. Previous studies had determined that 6 hr was an optimal time for measuring cytokine release in response to TSST-1 [2]. HVEC gene transcriptional changes were measured by collecting total RNA and conducting real time-reverse transcription PCR. Transcription of cytokines and chemokines was up-regulated 1.5 to 12 fold over 6 hr in response to TSST-1 (Figure 10A). Multiplex cytokine array measured IL-6, IL-8, MIP-3α, and TNF-α and all were significantly up-regulated in a

dose dependent manner in response to TSST-1 (Figure 10B-E). mRNA and protein expression correlated well, as IL-6, IL-8, MIP-3α, and TNF-α were all up-regulated in both assays. These results correlated with our previous studies on the University of Iowa HVEC line [2].

TSST-1 stimulates NF-κB in HVEC.

The NF-κB pathway was investigated as a possible target of TSST-1 signaling based on the cytokines that were upregulated in response to TSST-1. NF-κB responsive luciferase reporters were used to measure NF-κB activation in response to TSST-1 or IL-1β (as a positive control, data not shown) [120]. TSST-1 induced a statistically significant (p value <0.001) increase of 50% in NF-κB activity compared to media only at 4 hr exposure (Figure 11). IL-1β was used as a positive control and induced a significant increase of 80% in NF-κB activity compared to media only (data not shown). The difference in luciferase activity between stimulated and unstimulated was fairly small, though statistically significant [120]. This small difference is likely due to the background stimulation of the NF-κB pathway by HPV 16 E6/E7 used to immortalize the cell line [123-125]. This NF-κB activation in unstimulated HVEC can be also be seen in the high level of IL-8 made in the absence of TSST-1 (Figure 11) or in the high level of phosphorylated (active) NF-κB detected by Western blots (data not shown). There was no luciferase activation in cells transfected with a negative control plasmid with a minimal promoter, indicating that the observed luciferase activity was specific for NF-κB. These results demonstrate that NF- κB was activated in HVEC in response to TSST-1.

HVEC do not constitutively express MHC II, and TSST-1 does not induce MHC II expression.

It has been suggested that MHC II molecules may act as receptors for SAgs on epithelial cells [3, 5]. The potential role of MHC II molecules as HVEC surface receptors for TSST-1 was explored by Western blotting and flow cytometry. Western blots of whole cell lysates demonstrated that HVEC do not express detectable quantities of MHC II unless stimulated with IFN-γ, a known inducer of MHC II expression (Figure 12A). Lack of MHC II expression was further investigated by flow cytometry analyses, which confirmed HVEC do not express MHC II molecules unless stimulated with IFN-γ (Figure 12 B and C, dotted black line). When stimulated with IFN-γ, MHC II expression increased 60-80% (200 IU/ml IFN-γ for 48 hr, dashed line). The effect of TSST-1 on MHC II expression was determined via flow cytometry to address the possibility that TSST-1 may induce the expression of MHC II molecules, which could then serve as a TSST-1 receptor. HVEC were exposed to TSST-1 (50 μg/ml) or left untreated for 5 or 24 hr. No change in the surface expression of MHC II molecules by HVEC after exposure to TSST-1 (gray line) compared to untreated (dotted black line) was noted (Figure 12 B and C). There was no significant difference between TSST-1 stimulated and unstimulated HVEC at either time point by comparing mean fluorescence. These data indicate the MHC II cannot serve as a major receptor for TSST-1 on HVEC.

MHC II expression at the basal layer of human ectocervical tissue.

The expression pattern of MHC II in normal, uninfected, ectocervical tissue (identical histology to vaginal epithelium) was examined by immunohistochemistry (IHC) (Figure 13, positive cells indicated by arrows). Expression of MHC II was low and the MHC II-positive cells are likely to be migratory immune cells that are naturally present within the tissue [127]. Tissues obtained from three additional women similarly showed minimal MHC II positive cells at the basal layer. Negative controls, not treated with primary anti-HLA antibody, were negative for staining (data not shown). H & E staining demonstrated that lymphocyte influx was minimal, which was expected, as the tissues appeared otherwise normal. These data indicate that MHC II is an unlikely candidate for the TSST-1 receptor on epithelial cells in stratified squamous epithelium in the ectocervix.

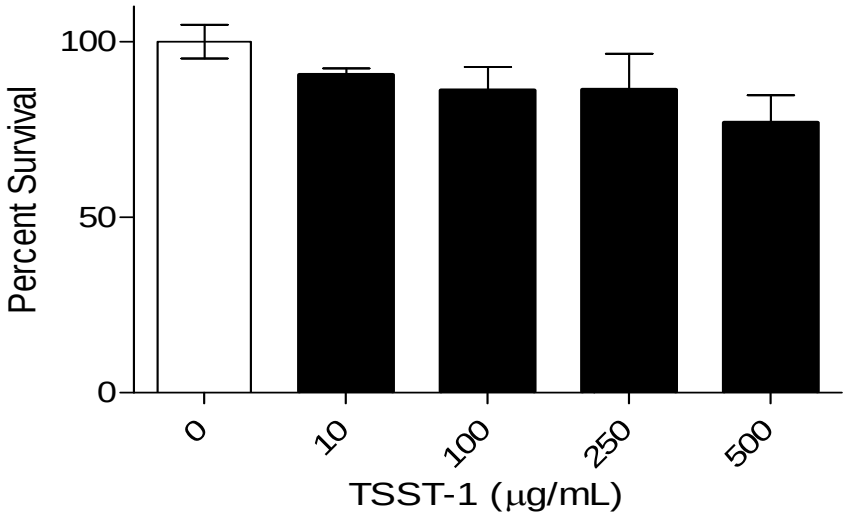

Figure 9. **TSST-1 is not toxic to HVEC.** HVEC were exposed to TSST-1 (0-500 μg/ml) for 6 hr and the viability was determined by the Celltiter Assay. Data presented are representative of three independent experiments done in triplicate, mean ± SD.

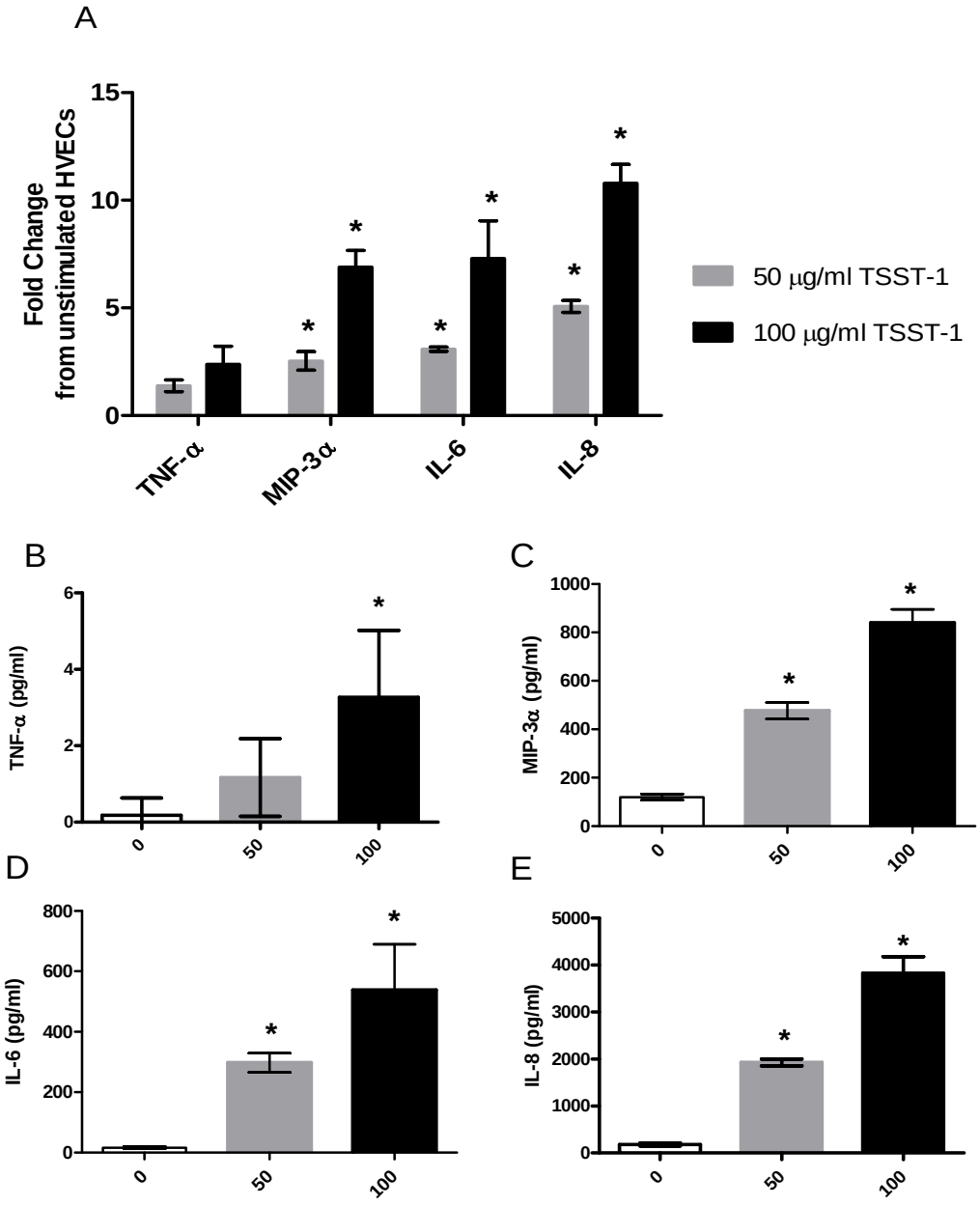

Figure 10. TSST-1 induces dose-dependent cytokine response from immortalized HVEC. HVEC were exposed to TSST-1 (0, 50, and 100 μg/ml) for 6 hr, then RNA was harvested and supernatants collected. Changes in expression of proinflammatory genes were measured by (A) quantitative reverse transcription PCR and (B-E) secreted

cytokines were measured via multiplex. Data presented in (A) are representative of three independent experiments done in triplicate, data presented in (B-E) are triplicates from one experiment, mean ± SD. * denotes $p < 0.05$ compared to unstimulated control.

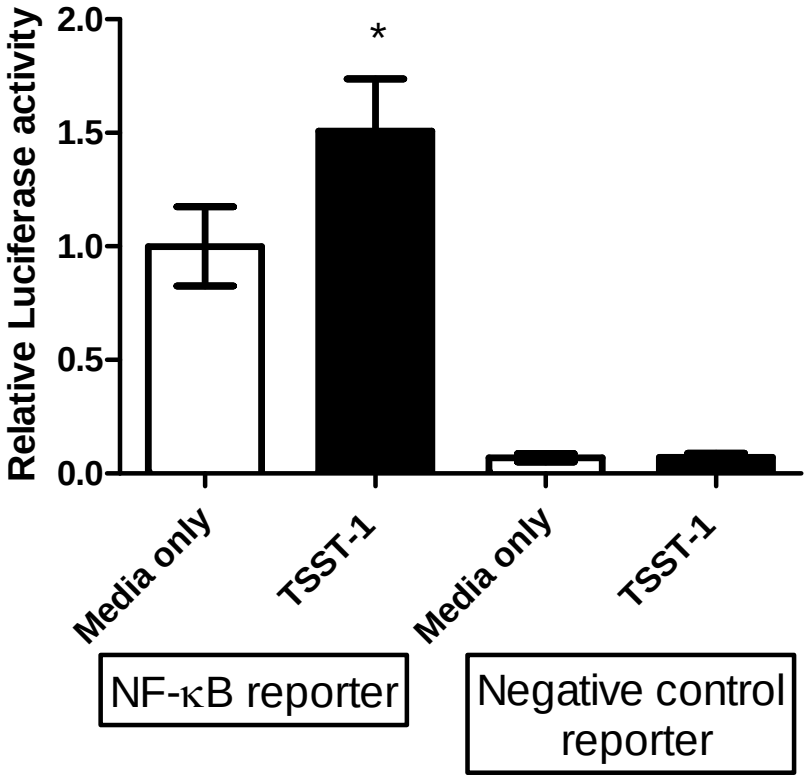

Figure 11. TSST-1 stimulates NF-κB in HVEC. HVEC were transfected with plasmids with NF-κB response elements or a basal promoter (as a negative control) controlling expression of luciferase. Transfected cells were then treated with TSST-1 (250 μg/ml) or left untreated for 4 hr, when luciferase activity was measured. Data presented in this figure are from 15 replicates done in three independent experiments, mean ± SD. * denotes $p < 0.001$ compared to media only control transfected with NF-κB reporter.

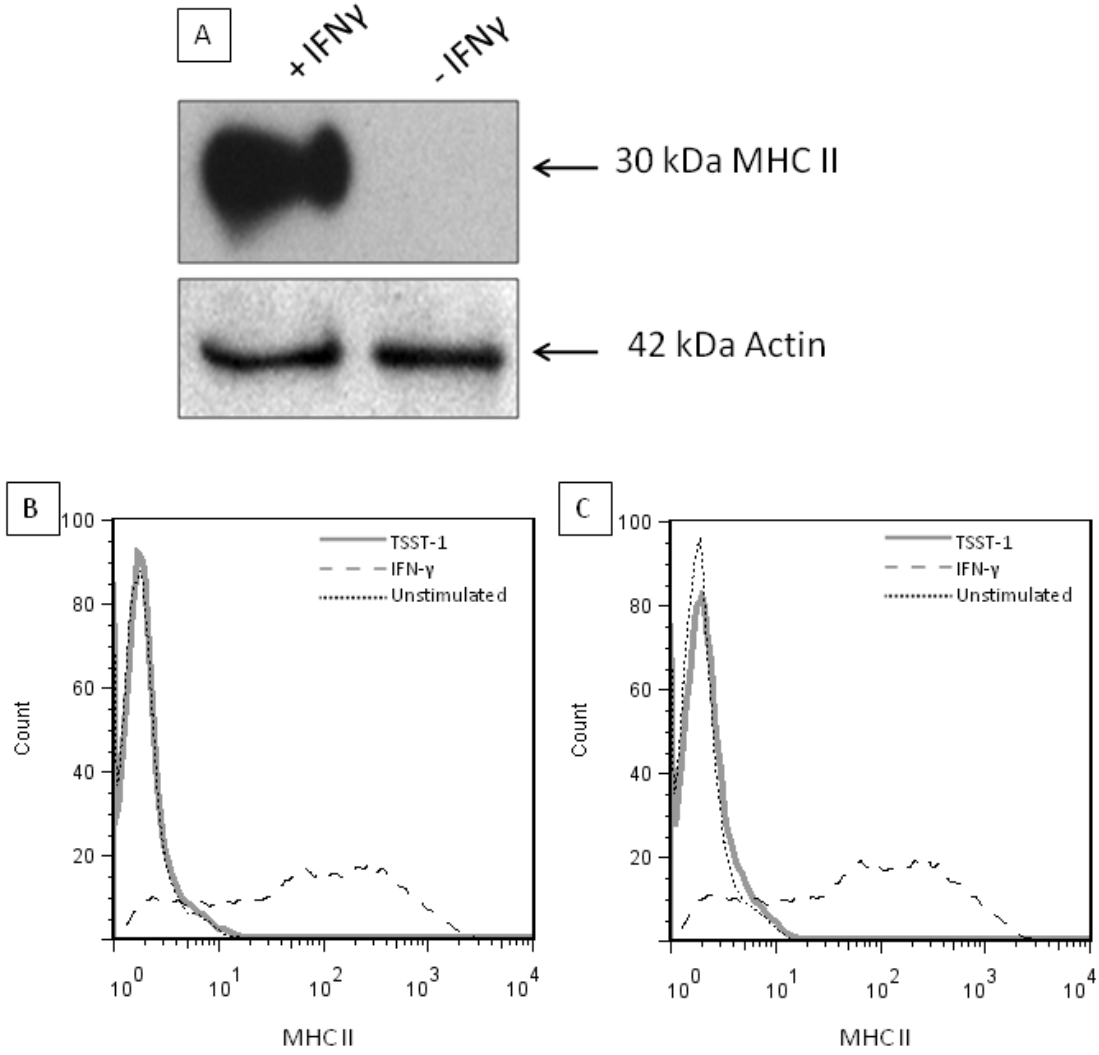

Figure 12. HVEC do not express MHC II nor does TSST-1 affect MHC II expression on the surface of HVEC. HVEC were treated with IFN-γ or left untreated, lysed and the amount of MHC II was measured by Western blotting (A). HVEC were exposed to TSST-1 (50 µg/ml, gray, solid line) or left untreated (black dotted line) for 5 hr (B) or 24 hr (C). As a positive control for MHC II expression, HVEC were stimulated with IFN-γ (Dashed line). When unstimulated none of the HVEC expressed, but when stimulated with IFN- γ 60-80% of HVEC were MHC II positive. The mean fluorescence

± SEM for unstimulated and TSST-1 stimulated was 2.28 ± 0.10 and 2.31 ±0.03 respectively at 5 hr and 2.28± 0.05 and 2.49 ±0.07 at 24 hr. There was no significant difference between TSST-1 stimulated and unstimulated at either time point. Plots are representative of three independent experiments done in triplicate.

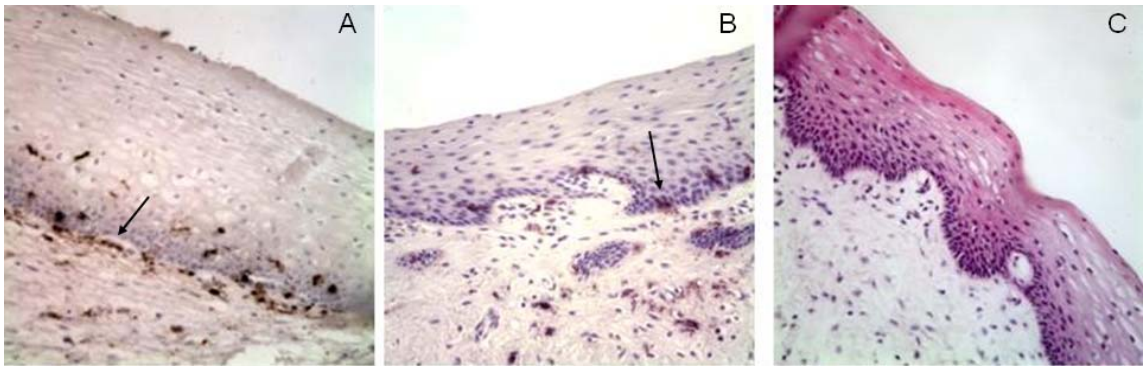

Figure 13. Sparse MHC II positive cells in human ectocervix tissue. A,B: IHC staining of MHC II (arrow, brown stain) in normal stratified squamous ectocervical epithelium. Tissue was taken from two different women in proximity to the basal epithelial cell layer, 400X. C: H & E staining of ectocervix.

IV. Discussion

Two distinct HVEC lines, the ATCC line used in the current study and a cell line obtained from the University of Iowa examined previously [2], had a robust cytokine response to TSST-1. The high HVEC survival rate after high exposure to TSST-1 demonstrates that the increase in IL-8 release from HVEC is not a function of cell death. This cytokine production occurs independent of immune cells and is hypothesized to be critical for disease progression [209]. It has been suggested that MHC II molecules may mediate the response of epithelial cells to SAgs. HVEC respond to TSST-1 despite the lack of MHC II on the surface of the cells indicating the presence of an unidentified receptor for TSST-1. IHC performed *ex vivo* shows that MHC II are present only at low levels in normal cervical tissue [121, 122]. The expression of MHC II was localized to the basal epithelium not on the outer surface of the epithelial layer where the first SAg-epithelial interactions are likely to occur. Low levels of MHC II expression in normal tissue and on the surface of HVEC suggest that HVEC are a valid *in vitro* model to study TSST-1 interactions with epithelial cell receptors.

mTSS typically occurs when the SAg, TSST-1, is produced vaginally by *S. aureus* in the presence of tampons [79, 80]. *S. aureus* normally remains localized on the vaginal mucosa, whereas TSST-1 penetrates mucosal surfaces to become systemic [211, 212]. After penetrating the mucosal surface TSST-1 acts as a bridge between CD4+ T cells and macrophages thus stimulating cytokine release [1].

SAgs such as TSST-1 induce cytokine secretion from a variety of epithelial cells in the absence of T cells, including vaginal, bronchial, intestinal, and nasal epithelial cells

[2-5]. We hypothesize that TSST-1 binds an epithelial cell receptor and induces proinflammatory cytokines, which leads to an influx of inflammatory cells. The cells, in turn causes disruption of the vaginal mucosa and increase the ability of the toxin to penetrate into the bloodstream and induce TSS.

The mechanism(s) of TSST-1 induction of cytokines from epithelial cells has not been elucidated. The ability of TSST-1 to induce TNF-α and IL-8 production in primary bronchial epithelial cells was reported by Aubert *et al.* to be meditated by at least in part MHC II [3]. To achieve IL-8 gene induction up to 14.8-fold greater than TSST-1 unstimulated cells, the primary bronchial epithelial cells required pre-incubation with IFN-γ thus increasing MHC II expression [213]. Flow cytometry showed that < 5% of the bronchial epithelial cells expressed MHC II in the absence of IFN-γ, whereas, 48 hr incubation with 200 IU/ml of IFN-γ resulted in MHC II expression up to 92%. Of note, a small induction (approximately 2-fold) of IL-8 was detectable upon TSST-1 stimulation (10 μg/ml) even in the absence of IFN-γ. O'Brien *et al.* reported similar results for primary nasal epithelial cells, where blocking antibodies against MHC II added to cultures with SAgs decreased IL-8 secretion by just 40% in IFN-γ unstimulated and 75% in IFN-γ stimulated cells [5] suggesting the presence of another SAg receptor. These findings support our observation that TSST-1 can induce cytokine responses from epithelial cells without MHC II.

Downstream signaling events in superantigen-stimulated epithelial cells have not been investigated. Previous studies have demonstrated that NF-κB is activated in human PBMCs in response to superantigens [154, 156]. The cytokine production and NF-κB

activation observed in PBMC was likely due to the superantigen cross-linking MHC II and TCR. The current study shows that the transcription factor NF-κB is activated in HVEC in response to TSST-1 in an immune-cell independent manner. These data suggest the presence of a novel TSST-1 receptor on HVEC that signals through NF- κB upon stimulation.

Our data, which suggest the existence of an unknown HVEC receptor, are supported by several studies, including those that explored cellular binding domains of SAgs that were independent of MHC II and TCR binding domains [140-143, 145, 204]. A conserved dodecapeptide region of SAgs that is independent of MHC II and T-cell receptor binding domains [140-142], is important for SAg transcytosis across human colonic epithelial cell monolayers [145]. A synthetic dodecapeptide peptide derived from SEB and corresponding to TSST-1 position 119 to 130 is able to antagonize IL-8 induction in HVEC by TSST-1 [2]. These data suggest that this dodecapeptide region is important for the interactions of SAgs with epithelial cells both *in vitro* and *in vivo*. Beharka *et al.* determined that MHC II deficient macrophages produce IL-6 in response to stimulation with SEA and SEB in equal or greater amounts than MHC II positive macrophages [138, 214]. Collectively, these studies and the HVEC data presented here suggest the presence of a third mammalian receptor for SAgs (in addition to MHC II and T cell receptors) which is necessary for the induction of cytokines from epithelial cells. Experiments to identify this additional receptor and downstream components of the signaling pathway are ongoing. Identification of this receptor and its major signaling pathways will likely be useful in developing preventative therapies for TSS as well as

other SAg-induced diseases.

Chapter 3- Novel Toxic Shock Syndrome Toxin-1

Amino Acids Required for Biological Activity

Studies published in: ***Biochemistry*** 47:1 2995-3003. 2008. Novel toxic shock syndrome toxin-1 amino acids required for biological activity.

In these studies, I was involved design of the experiments, generation of mutant plasmids, screening of mutant toxins on ATCC HVEC, analysis of data, and drafting of the manuscript. Purification of toxins, screening of mutants on Iowa HVEC, penetration studies, and animal experiments were completed by members of Patrick Schlievert's laboratory (Department of Microbiology, School of Medicine, University of Minnesota).

I. Introduction

The SAg, TSST-1 is responsible for the majority of mTSS cases and half of all non-mTSS [85, 86]. Previous studies have demonstrated that TSST-1 causes an increase in production of proinflammatory cytokines and chemokines, such as TNF-α, IL-8, and MIP-3α, from multiple epithelial cell types [2, 3, 5]. This induction of proinflammatory cytokines and subsequent recruitment of inflammatory cells are predicted to be critical in disease progression. A 12-amino-acid (dodecapeptide) region can be found in most superantigens and is relatively conserved among superantigens in sequence similarity and structure. It is important to note that this region is also distinct from those regions required for Vβ-TCR and MHC II binding [69, 146]. Peptides generated against the dodecapeptide sequence in SEC are capable of neutralizing SEC's ability to stimulate T cells [147]. Arad *et al.* demonstrated that a dodecapeptide variant (YNKKKATVQELD) of this SEB region (original sequence **T**NKKK**V**T**A**QELD) could inhibit the SEB-induced expression of IL-2, IFN-γ, and TNF-α mRNA from PBMC [140-142, 148]. Another study determined that the variant SEB dodecapeptide antagonist is capable of competitively inhibiting TSST-1-induced chemokine production from HVEC [2].

Based on these findings we sought to identify the role of the dodecapeptide sequence in interactions with the vaginal mucosa. To evaluate the role of the dodecapeptide we generated single amino acids mutants at every residue in the region. The purpose of this study was to identify critical amino acids within the dodecapeptide of TSST-1 that are required to induce HVEC cytokine production and ultimately those that are involved in mTSS when the superantigen is administered vaginally.

II. Methods and Materials

Generation of TSST-1 mutants

The plasmid pCE107 is a shuttle vector, consisting of pUC18 (origin of replication for *Escherichia coli*), pE194 (origin of replication for *S. aureus*) and *tstH*, which was mutated by the Stratagene Quick Change II site directed mutagenesis kit (La Jolla, CA). Primers were generated to change each amino acid in the TSST-1 dodecapeptide region (120-DKKQLAISTLD-130) to alanine (A125 was changed to serine; Table 10). The T128A mutant was generated previously [215]. The primers were used in site-directed PCR per manufacturer protocol. Each PCR product was transformed into *E. coli* XL1-Blue supercompetent cells by heat shock and grown overnight in LB with 50 μg/ml ampicillin. Plasmids were collected from transformants with the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Plasmid preparations were electoporated into *S. aureus* RN4420 (200 ohms, 1.9 kV), and *S. aureus* were grown overnight in Bacto Todd Hewitt (Becton Dickinson and Company, Sparks, MD) broth with 5 μg/ml erythromycin. Mutations in the dodecapeptide region were confirmed by DNA sequencing.

Toxin purification

S. aureus RN4220, containing pCE107, was used in the production of WT TSST-1 and TSST-1 mutants; strain RN4220 does not produce SAgs [204, 205]. The plasmid pCE107 is a high-copy-number plasmid containing *tstH,* the gene for TSST-1 [205]. TSST-1 mutants were purified by the same method as WT TSST-1. All medium, reagents, and glassware used for toxin production were maintained pyrogen free. *S. aureus* RN4220 was grown up in beef heart media, cultures were treated with 80%

ethanol (final concentration) at 4°C, and the precipitates were resolubilized in water. TSST-1 was purified by two rounds of IEF. IEF was carried out by first using a pH gradient of 3.5 to 10, followed by a pH gradient of 6 to 8. The isoelectric point of TSST-1 is 7.2 [86]. WT TSST-1 and mutant proteins were identified by double immunodiffusion based on reactivity with a specific polyclonal antibody generated against WT toxin [206]. Purity was confirmed by SDS-PAGE, which demonstrated single protein bands at a molecular weight of 22 kDa. Purified toxins were quantified by the Bio-Rad protein assay (Bio-Rad Co., Hercules, CA) with the superantigen SEB used for standard curve generation. TSST-1, thus prepared, contained no detectable LPS, as tested by *Limulus* assay (Sigma, St. Louis, Mo.); nor were peptidoglycan, lipoteichoic acid, hemolysin, protease, or lipase (<1 part per 10^6) detectable by combinations of bioassay and SDS-PAGE. Comparable results were obtained with multiple batches of TSST-1.

WT and mutant toxins were also verified as homogeneous when samples were subjected to reversed-phase HPLC (Protein C4 column from Vydac [Hesperia, CA] 0 to 60% gradient of acetonitrile with 0.1% trifluoroacetic acid over a 30 min time period); proteins eluted as sharp single peaks.

WT TSST-1 and mutants A125S, S127A, and D130A were internally labeled with ^{35}S-methionine for porcine mucosa penetration studies. In previous labeling studies with TSST-1, approximately 10^7 disintegrations per minute/μg (dpm/μg) protein may be achieved. The protein purification is the same as that described above, except bacterial strains were cultured in 50 ml media containing 10 mCi ^{35}S-methionine.

Culture of HVEC

Two immortalized HVEC lines were used in this study; both were generated by transforming primary HVEC from premenopausal woman with the E6/E7 genes of HPV 16. One HVEC line is described by Peterson *et al.*, [2, 216, 217]. Another vaginal epithelial cell line (CRL-2616) was obtained from ATCC (Manassas, VA) [122, 203]. Both cell lines were maintained in KSFM in 75-cm^2 flasks (BD Falcon, Bedford, MA), 6-well, or 96-well microtiter plates (Corning, Corning, NY) at 37°C in 7% CO_2, and supplemented with bovine pituitary extract and epidermal growth factor as provided by the manufacturer, 0.4 mM calcium chloride, and 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Fungizone; Gibco Invitrogen, Carlsbad, CA). On days of experimentation, antimicrobials were not used since it has been observed that amphotericin B reduces cytokine production by HVEC.

IL-8 Assay

HVEC were grown to confluence in 96 well plates and exposed to purified TSST-1 or mutants (10-200 μg/ml) for 6 or 24 hr in KSFM without antibiotics. Control wells of HVEC contained KSFM without antibiotics and without TSST-1. Cell culture supernates were collected and tested for IL-8 by ELISA (R & D Systems, Minneapolis, MN) according to manufacturer directions.

Superantigenicity assay

WT and mutant toxins were tested for superantigenicity in a four day assay [218]. Cellular proliferation was measured based on DNA uptake of ^{3}H-thymidine. Briefly, PBMC were isolated from heparinized (100 units/ml) human blood by Ficoll-Hypaque

sedimentation. Human blood was drawn in accordance with an approved University of Minnesota IRB protocol. PBMC were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium (Lonza, Walkersville, MD) with 2% fetal calf serum (JRH Biosciences, Inc., Lenexa, KA), 200 μM L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were incubated with toxin (1 μg-0.00001 μg per well) for three days. 18 hr prior to the completion of the experiment each well received 1 μCi of ^{3}H-thymidine in 20 μl of medium. Cellular DNA was collected on glass-fiber filters using a MASH II® apparatus (Microbiological Associates, Bethesda, MD). A liquid scintillation counter (model LS, Beckman Instruments, Fullerton, CA) was used to measure thymidine uptake. Data were reported as the percent of WT stimulation, based on average counts per min (cpm) of three or four replicate samples.

Rabbit model experiments

Selected toxin mutants were tested *in vivo* in the rabbit model as originally described by Kim and Watson [219] and Schlievert [220]. This model tests the ability of superantigens to enhance shock caused by LPS from *Salmonella enterica* serovar Typhimurium. Toxins, dissolved in PBS (pH 7.2), were initially given to young adult American Dutch-belted rabbits (1.0-2.0 kg) as an intravenous (IV) dose (10 μg/kg) or an intravaginal dose (10 μg/animal). Toxins given IV were administered in the marginal ear veins. Intravaginal dosing was done as previously described [221]. Briefly, rabbits were anesthetized with ketamine (25 mg/kg; Phoenix Pharmaceuticals, Inc., St. Joseph, MO) and xylazine (20 mg/kg; Phoenix Pharmaceuticals, Inc.) prior to insertion of catheters into rabbit vaginas. Toxins were delivered in 0.1 ml volumes. For all conditions, LPS (5

μg/kg; 1/100th lethal dose 50% endpoint of LPS alone) was administered IV in the marginal ear veins 4 hr after the initial superantigen doses, and the rabbits were monitored for 48 hr. Rabbits displaying signs of severe illness (failure to right themselves and exhibit escape behavior), and those remaining healthy at the conclusion of the experiment, were euthanized with Beuthanasia D (1 ml/kg, Schering-Plough Animal Health Corp., Union, NJ) according to the University of Minnesota Institutional Animal Care and Use Committee (IACUC) requirement.

Penetration studies

An *ex vivo* porcine vaginal permeability model for superantigens has been previously described [2, 127, 130]. Vaginal mucosal tissue was isolated from pigs at slaughter and used within 3 hours. Perfusion chambers were used to mount pieces of tissue (8-10 mm in diameter), with an exposed surface of 0.2 cm^2. PBS was continuously pumped through the lower chamber so that hourly cell-free samples of TSST-1 penetrating completely through the mucosa (up to 12 hr), could be taken. Seven replicates were used for each condition. Radiolabeled ^{35}S-methionine TSST-1 (WT or mutants, 15 μg/ml) was added to the upper chamber in PBS. Flux was calculated from the relationship flux = Q/At, where Q is the quantity of radiolabel traversing the tissue (disintegrations per minute) in time t (minutes) and A is the exposed epithelial surface area in square centimeters. Units of flux are therefore disintegrations per minute per square centimeter per minute. Flux units were then converted to nanograms of toxin per square centimeter per minute based on the disintegrations per minute per nanogram of toxin.

Statistics

Significance of differences in two means were computed through determination of standard deviations and through Student's *t* test analysis of unpaired, normally distributed data. In studies of radiolabeled TSST-1 and selected mutants to penetrate intact *ex vivo* porcine vaginal tissue, the total amount of toxin (in ng) to penetrate through the tissue was calculated based on the quantity of radiolabel traversing the tissue (determined as disintegrations per minute) and corrected for using the disintegrations per minute of the original toxin solution. Values at each of the hourly sampling intervals were plotted against time and when there was no increase in value the chamber was assumed to have reached a steady state; 3-4 subsequent readings were then taken to calculate the mean for that chamber. For the seven replicate perfusion chambers a mean value and a measure of dispersion (standard error of the mean) was calculated. Different treatments were compared using a one-way analysis of variance and Duncan's multiple range test. Pairwise differences are considered significant if $p \leq 0.05$

D120A	
Forward	5’-A AAG TAT TGG CCA AAG TTC G**C**T AAA AAA CAA TTA GC
Reverse	5’- AT AGC TAA TTG TTT TTT A**G**C GAA CTT TGG CCA ATA C
K121A	
Forward	5’-TGG CCA AAG TTC GAT **GC**A AAA CAA TTA GCT ATA
Reverse	5’-TAT AGC TAA TTG TTT T**GC** ATC GAA CTT TGG CCA
K122A	
Forward	5’- GG CCA AAG TTC GAT AAA **GC**A CAA TTA GCT ATA TCA AC
Reverse	5’- AA AGT TGA TAT AGC TAA TTG T**GC** TTT ATC GAA CTT TG
Q123A	
Forward	5’- CA AAG TTC GAT AAA AAA **GC**A TTA GCT ATA TCA ACT TT
Reverse	5’- TC TAA AGT TGA TAT AGC TAA T**GC** TTT TTT ATC GAA CT-
L124A	
Forward	5’- AG TTC GAT AAA AAA CAA **GC**A GCT ATA TCA ACT TTA GA
Reverse	5’-AA GTC TAA AGT TGA TAT AGC T**GC** TTG TTTTTT ATC GA
A125S	
Forward	5’- TC GAT AAA AAA CAA TTA **AG**T ATA TCA ACT TTA GAC TT
Reverse	5’- TC AAA GTC TAA AGT TGA TAT A**CT** TAA TTG TTT TTT AT
I126A	
Forward	5’- AT AAA AAA CAA TTA GCT **GC**A TCA ACT TTA GAC TTT GA
Reverse	5’ AT TTC AAA GTC TAA AGT TGA T**GC** AGC TTA TTG TTT TT
S127A	
Forward	5’-AAA CAA TTA GCT ATA **G**CA ACT TTA GAC TTT GAA
Reverse	5’-TTC AAA GTC TAA AGT TG**C** TAT AGC TAA TTG TTT-3’
L129A	
Forward	5’-AA TTA GCT ATA TCA ACT **GC**A GAC TTT GAA ATT CGT CA
Reverse	5’- GT ATG ACG AAT TTC AAA GTC T**GC** AGT TGA TAT AGC TA
D130A	
Forward	5’-GCT ATA TCA ACT TTA G**C**C TTT GAA ATT CGT CAT
Reverse	5’-ATG ACG AAT TTC AAA G**G**C TAA AGT TGA TAT AGC

Table 10. Primers used for site directed mutagenesis of *tstH*. Mutagenic bases are bolded.

III. Results

Cytokine production in response to TSST-1 mutants

Two different immortalized HVEC lines were used to test the ability of TSST-1 mutants to stimulate production of IL-8. HVEC were incubated with toxins, supernates were collected, and IL-8 was measured by ELISA. Previous studies showed that wild type TSST-1 elicits strong IL-8 responses from HVEC [2]. Over a range of toxin doses (10 μg/ml to 200 μg/ml), most mutants exhibited activity similar to wild type TSST-1 (Figure 14). Three of the mutants (Q123A, L124A and A125S) displayed significantly increased IL-8 production from HVEC (at one dose each) compared to WT TSST-1 ($p<0.05$). Two mutants (D120A and L124A) did not show expected dose response curves within the initial range of concentrations tested (10 μg/ml to 200 μg/ml). These mutants were further diluted (1 μg/ml and 0.1 μg/ml) in order to ascertain the fall-off of responsiveness to the toxins. TSST-1 mutations at the carboxyl end of the dodecapeptide sequence (S127A, T128A, and D130A) resulted in greatly reduced IL-8 stimulating activity. S127A at 200, 100, and 20 μg/ml and T128A at 200 and 100 μg/ml were statistically significantly different compared to wild type TSST-1 ($p<0.05$). The last amino acid in the sequence, D130A, exhibited the most drastic decrease in IL-8 production as compared to WT toxin; all concentrations were significantly lower than WT TSST-1 ($p<0.05$). D130A was verified to be non-toxic to the cells by trypan blue dye exclusion. A mutant towards the amino-terminus of the dodecapeptide, K121A, induced significantly lower levels of IL-8 from HVEC at low concentrations (20 and 10 μg/ml) compared to WT TSST-1 ($p<0.05$). The second cell line, CRL-2616, demonstrated a

similar response to the mutants, however mutant S127A showed anomalous activity in this line (Figure 15). Even though a high dose of S127A (100 μg/ml) demonstrated WT activity on these cells, a lower concentration of 10 μg/ml was shown to have no activity compared to WT toxin at this concentration. This confirmed that two separate lines of HVEC respond to TSST-1 by the production of IL-8 and that key residues within the dodecapeptide region may be responsible for inducing this action. A cartoon of the structure of TSST-1 is shown in Figure 16A. The dodecapeptide region is highlighted in yellow. Important residues in the dodecapeptide region are shown in Figure 16B (red residues are changes that led to decreased IL-8 production, whereas violet residues are changes that led to increased IL-8 production; not all residues are visible in this image).

Superantigenicity of TSST-1 mutants

Although the dodecapeptide sequence is distinct from both the Vβ-TCR and MHC II binding sites [69, 222], the superantigenicity of the toxins was analyzed with a ^{3}H-thymidine assay, and each toxin was added to the wells at an initial concentration of 1 μg per well with 10-fold dilutions ranging down to 0.00001 μg per well. All mutants exhibited WT activity, shown as a percentage of the WT TSST-1 average cpm (Figure 17), with the exception of three surface exposed mutants, Q123A, L124A, and A125S, which had significantly higher activity at the 0.1 μg/ml dose (all $p<0.01$). Changes in these residues may act to enhance affinity for either the TCR or the MHC II receptor.

Effects of TSST-1 mutants *in vivo*

The four amino acids at the carboxy-terminal end of the dodecapeptide sequence were further examined since three of the four residues in this region were unable to induce WT

TSST-1 levels of IL-8 production from HVEC. In order to test the ability of the mutants to induce TSS *in vivo*, American Dutch-belted rabbits were used in two separate models of endotoxin enhancement (Table 11). Initially, each toxin was administered IV at 10 μg/kg to two rabbits. Four hours later, LPS derived from *S. enterica* serovar Typhimurium was administered via the marginal ear vein at 5 μg/kg. Since this model delivers the toxin directly into the bloodstream, it tests solely the superantigenic activity of the mutants *in vivo*. Of the three mutants tested in this model (S127A, L129A, and D130A) all were determined to be lethal after 24 hr. The T128A mutant was tested originally by Murray *et al.* in a similar model and was also shown to be lethal [204]. This again confirms that the carboxyl end of the dodecapeptide sequence is not required for superantigenicity. The second model of endotoxin enhancement involved giving young adult female rabbits the toxin intravaginally as a bolus dose of 10 μg. This was accomplished by inserting a catheter into the vaginal tract of each rabbit and administering the toxin in a 0.1ml volume. LPS was given four hours later as described above. Of the four mutants (S127A, T128A, L129A, and D130A), only L129A was fully lethal in this model as compared to WT. This corresponded with the ability of L129A to elicit a WT IL-8 response from HVEC. The D130A mutant, however, was incapable of causing any illness or lethality when given intravaginally to rabbits even though it maintained lethality when given IV. Mutants S127A and T128A were administered as a 10 μg dose and caused illness or lethality after 24 hr, however, the progression of disease was delayed compared to that caused by WT TSST-1.

Penetration of porcine vaginal mucosa by TSST-1 mutants

The inability of D130A to cause TSS from the rabbit vaginal tract led us to believe that this TSST-1 mutant may be incapable of penetrating the mucosa in order to interact with underlying immune cells. To test this possibility, we used an *ex vivo* model of superantigen penetration that has been previously described [2, 127, 130]. This model uses an automated perfusion chamber mounted with freshly harvested porcine vaginal mucosal tissue to assess the ability of superantigens to penetrate vaginal mucosa. WT TSST-1 and mutants D130A, S127A, and A125S were internally labeled with ^{35}S-methionine and added to the upper chambers (in replicates of 7) at a concentration of 15 μg/ml in PBS. Hourly samples (up to 12 hr) were collected from the lower compartment and tested for radioactivity in order to determine the amount of superantigen penetrating the mucosa. Surprisingly, D130A and S127A penetrated the mucosa faster than both wild type toxin and A125S (Figure 18). There was a statistically significant difference in flux (ng/sq. cm/min) between both D130A and S127A and other toxins (p value <0.05); there was no significant difference between wild type TSST-1 and A125S flux. The mean steady state fluxes for D130A and S127A were 4.11 ng/sq. cm/min and 3.35 ng/sq. cm/min, respectively, compared to 2.27 ng/sq. cm/min for A125S and 2.15 ng/sq. cm/min for WT TSST-1. After 12 hr, only an average of 237 ng total had accumulated in the lower chamber for WT TSST-1. A125S was similar to WT toxin in total accumulation of 239 ng, whereas S127A and D130A accumulated 394 ng and 488 ng total, respectively.

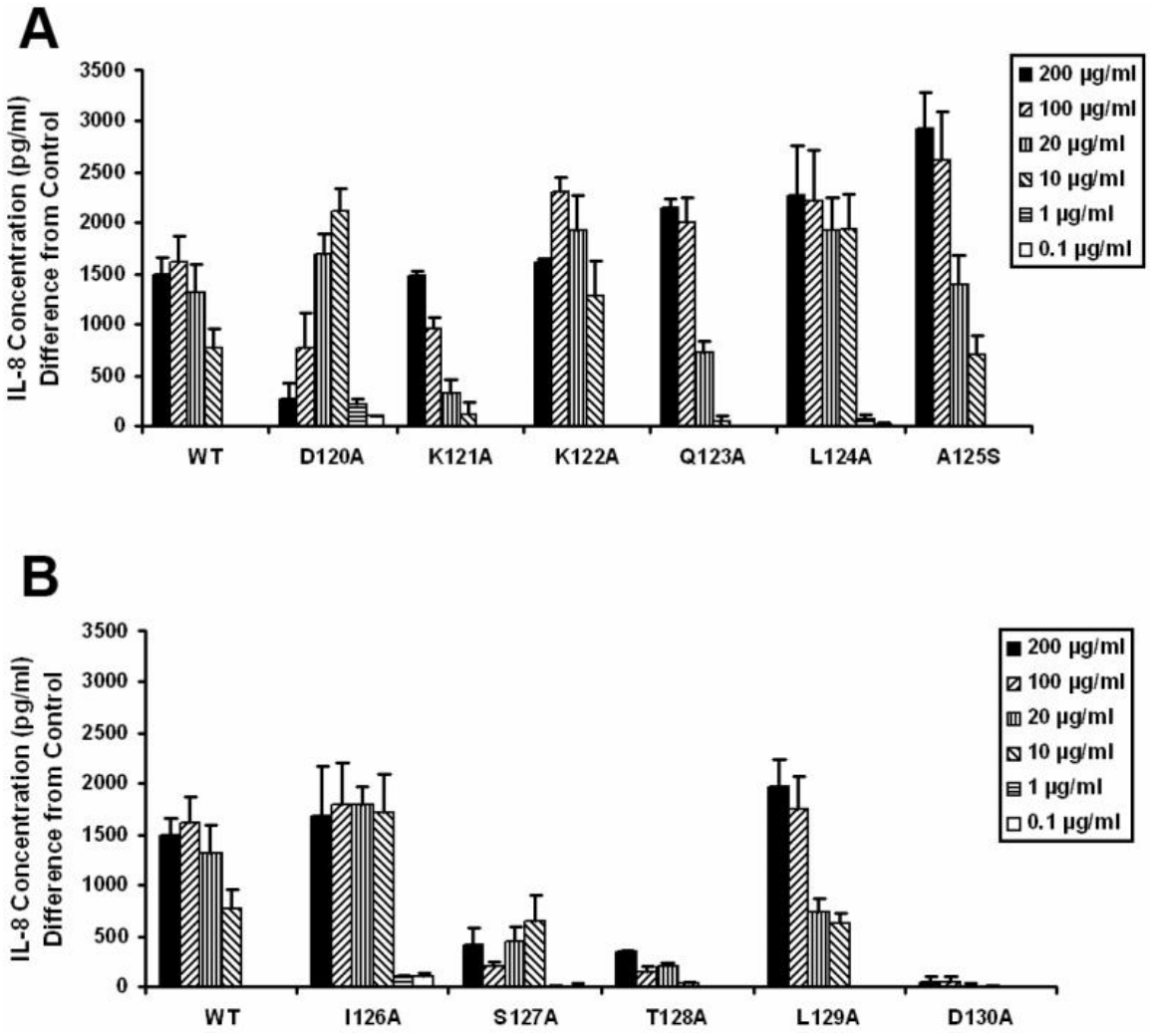

Figure 14. IL-8 Dose-Dependent Response of HVEC to TSST-1 Mutants. Cells were incubated with toxin for 6 hr at concentrations of 200 µg/ml, 100 µg/ml, 20 µg/ml, and 10 µg/ml. Additional concentrations of 1 µg/ml and 0.1 µg/ml are shown for D120A, L124A, A125S, I126A, S127A. HVEC supernates were collected and assayed by ELISA for IL-8 production. (A) First six amino-terminal mutants. (B) Last five carboxy-terminal mutants. Results are reported as the differences from media only control ± Standard error of the means (SEM).

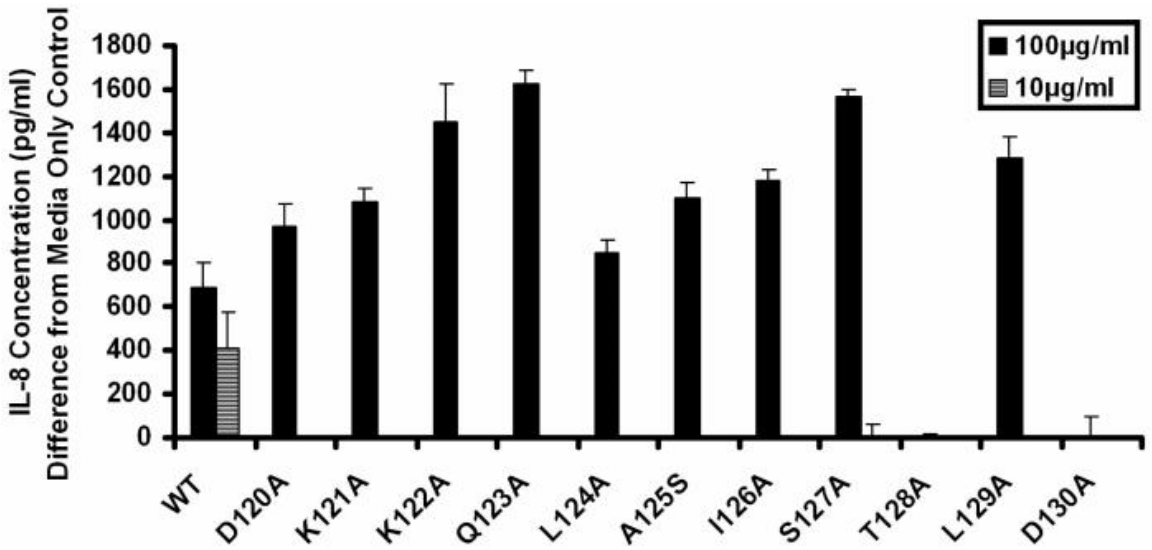

Figure 15. IL-8 Response of ATCC HVEC to TSST-1 Mutants. Cells were incubated with WT or mutant TSST-1 at 100 µg/ml for 24 hr. Cell supernates were then collected and analyzed for IL-8 production by ELISA. All results are reported as difference from a media only control ± SEM. Carboxyl terminal mutants T128A and D130A show no reactivity from the cells, similar to that seen from the HVEC. A lower dose (10 µg/ml) is shown for WT and S127A toxins only to demonstrate that even though S127A at 100 µg/ml is highly reactive; its reactivity is quickly lost at 10 µg/ml.

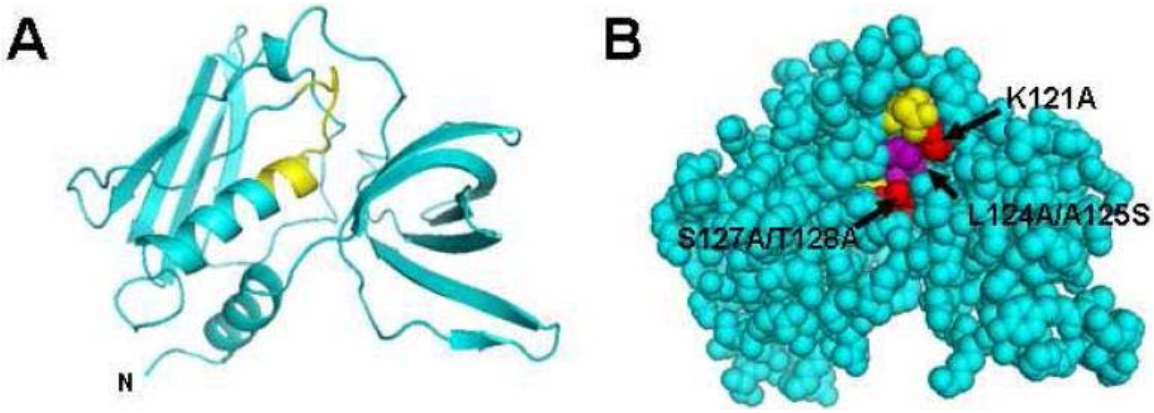

Figure 16. Structure of TSST-1. **(**A) Cartoon of TSST-1. The dodecapeptide region is shown in yellow. (B) Filled in structure of TSST-1. Amino acid residue changes that resulted in decreased IL-8 production from HVEC are shown in red, changes that maintained WT IL-8 production are shown in yellow, and changes that caused an increase in IL-8 production are shown in violet (some residues are not visible in this image). Structures generated using PyMOL (DeLano Scientific LLC, South San Francisco, CA).

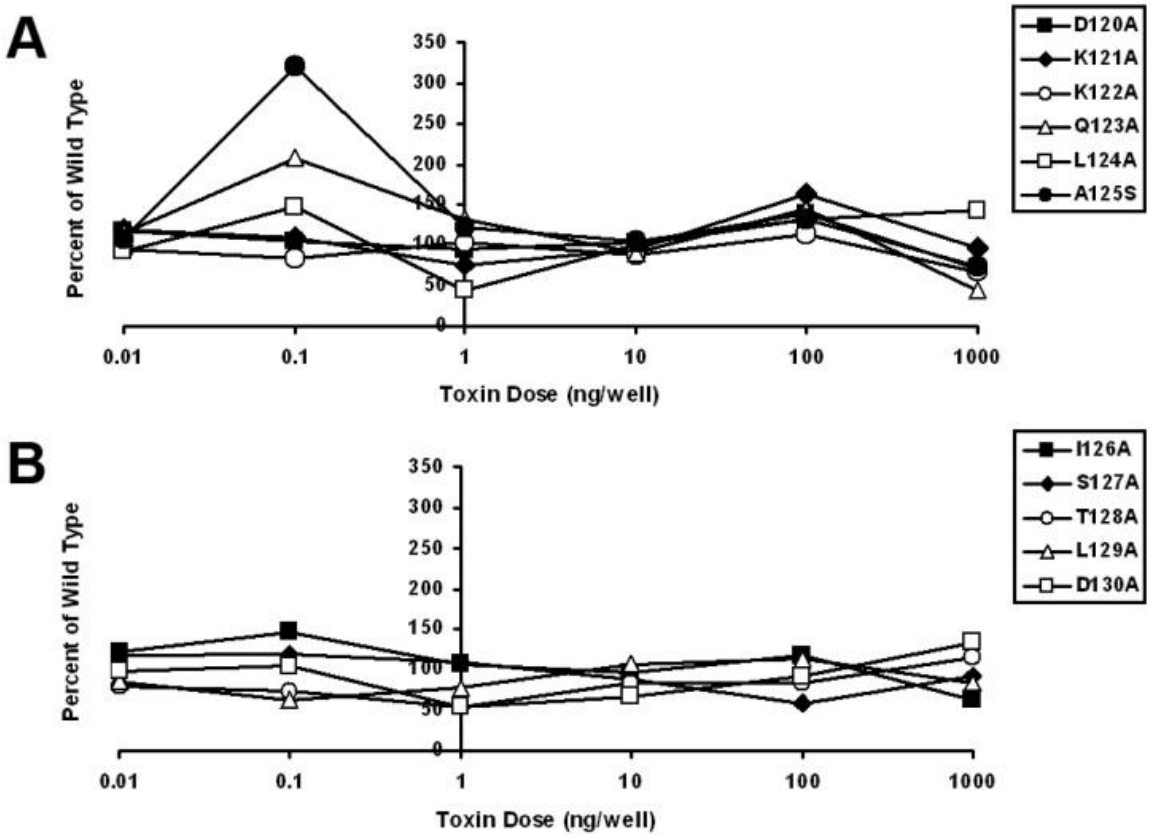

Figure 17. Superantigenicity of TSST-1 Mutants. Toxins were incubated with PBMC for 4 days; ^{3}H-thymidine was added 18 hr prior to the conclusion of the experiment. Cellular DNA was harvested, and thymidine uptake was measured using a liquid scintillation counter. The average counts per minute were converted to percent of WT TSST-1. (A) Mutants D120A through A125S, (B) Mutants I126A through D130A.

Mutant	*Route*	*Dose*	*Survival at:*		
			3 hr	**24 hr**	**48 hr**
D130A	IV	10 μg/kg	2/2	0/2	0/2
	Intravaginal	10 μg	3/3	3/3	3/3
L129A	IV	10 μg/kg	2/2	0/2	0/2
	Intravaginal	10 μg	1/3	0/3	0/3
T128A	Intravaginal	10 μg	3/3	1/3*	0/3
S127A	IV	10 μg/kg	2/2	0/2	0/2
	Intravaginal	10 μg	3/3	3/3*	1/3*
Wild type	Intravaginal	10 μg	1/3	0/3	0/3

Table 11. Rabbit models of TSS. Rabbits were given TSST-1 (WT or mutant) either IV or intravaginally. For the IV condition, toxin (10 μg/kg) was administered via the marginal ear vein. For the intravaginal condition, toxin (10 μg bolus dose) was given via a catheter inserted in the vaginal tract. For both conditions, rabbits were given LPS (5 μg/kg) through the marginal ear vein 4 hr later. All toxins administered IV were lethal (T128A was tested by Murray *et al.*[204]; WT TSST-1 was not used for this set of experiments since all mutants were found to be lethal). Only the D130A mutant was found to be nonlethal in the intravaginal model. *Animals remaining displayed symptoms of TSS and were euthanized prior to the end of the experiment.

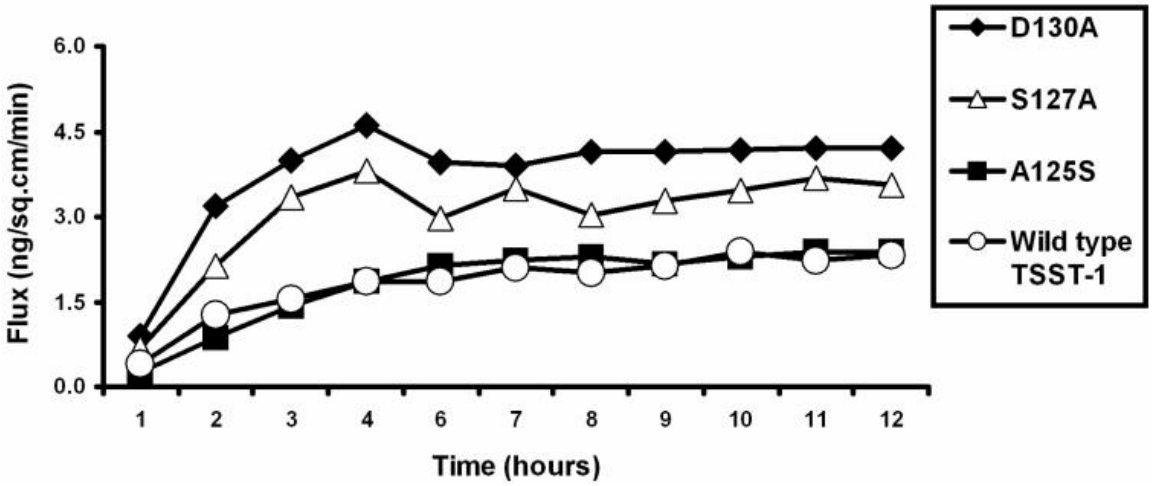

Figure 18. Penetration of TSST-1 Mutants Through Porcine Vaginal Mucosa. WT TSST-1 and mutants A125S, S127A, and D130A (15 μg/ml each) were internally labeled with ^{35}S-methionine and added to the upper compartment of a perfusion chamber holding an 8-10mm piece of freshly harvested porcine vaginal tissue. PBS was continuously pumped through the lower compartment and collected hourly for 12 hr. Flow through was analyzed for radioactivity to determine the amount of penetrating toxin. Units of flux are in nanograms of toxin per square centimeter per minute and are an average of 7 replicates per condition.

IV. Discussion

The purpose of this study was to examine the role of a dodecapeptide region of TSST-1 (F119-D130), which is structurally conserved and variably conserved in primary amino acid sequence among superantigens (primary TSST-1 sequence is most different from other superantigens), in superantigenicity, vaginal mucosal penetration, and stimulation of IL-8 production from HVEC. Previous studies demonstrated that TSST-1 induces proinflammatory cytokines and chemokines from HVEC [2]. In addition, a SEB-like dodecapeptide antagonist (YNKKKATVQELD), in 10-fold excess amounts, competitively inhibited TSST-1-induced production of chemokines MIP-3α and IL-8 from the same HVEC [2]. These studies led us to hypothesize that residues within the dodecapeptide region are required for superantigen-induced cytokine production from the vaginal mucosal epithelium.

In order to address this possibility, we generated single-site alanine mutants along the TSST-1 dodecapeptide region, except changing Ala 125 to Ser, and analyzed the ability of the resultant mutants to induce IL-8 responses from HVEC. Alanine scanning, a technique often employed to change charged, potentially surface-exposed, amino acids to alanine, was used for mutant production. Charged residues are hydrophilic, are able to form ion pairs and hydrogen bonds, and tend to be located on protein surfaces, and therefore may interact directly with host receptors. Additionally, alteration of non-exposed (buried) amino acid residues to alanine is likely to create a "hole" which is more easily tolerated structurally than the presence of a larger or charged buried amino acid. In this regard, all TSST-1 mutants retained approximately equal abilities to react with

polyclonal antibodies raised against WT TSST-1, indicating sufficient structural integrity was maintained for reactivity with highly specific immunoglobulins.

Most mutants induced IL-8 responses from two HVEC lines similarly to WT TSST-1; however three mutants (S127A, T128A, and D130A) at the carboxy-terminal end of the dodecapeptide exhibited decreases in IL-8 production from the cells. We hypothesize that residues in this region are important for interactions with an undescribed epithelial cell receptor that leads to cytokine production; this receptor is distinct from the two known TSST-1 receptors, Vβ2-TCR [146] and MHC II [69]. Residue T128 is surface exposed, seven amino acids away from residues important in TCR interaction with TSST-1 (H135 and Q136), at the base of a diagonal α-helix on the back of TSST-1 (in the standard view shown in Figure 15). This residue may directly contact the HVEC receptor. In contrast, S127 and D130 are non-exposed residues, and these may cause conformational changes that alter receptor interaction.

Three mutants in the central region of the dodecapeptide, Q123A, L124A and A125S, exhibited increased IL-8 production from HVEC. These same three mutants were also more superantigenic at the 0.1 μg/well dose, compared to WT TSST-1. These amino acid residues appear to cause TSST-1 structural alterations that improve receptor interaction, possibly affecting both the epithelial cell receptor and one or both of Vβ2-TCR or MHC II. All three of these residues are surface exposed and may directly contact the epithelial cell receptor.

Although the dodecapeptide region is distinct from the TSST-1 Vβ2-TCR and MHC II binding sites [69, 146], we tested the superantigenicity of the mutants through assaying

PBMC proliferation. All mutants exhibited WT or greater (Q123A, L124A, and A125S) activity, which confirms that the dodecapeptide region is not required for superantigenicity, but may positively influence activity. It is potentially important that the HVEC response to TSST-1 requires greater amounts of superantigen (typically ≥10 μg/ml) than superantigenicity ($\geq 10^{-3}$ μg/ml) for responsiveness.

We used two rabbit models of TSS development to assess the role of dodecapeptide mutants *in vivo*. The IV TSS model tests TSST-1 induction of proinflammatory cytokines due to the interaction of Vβ2-TCR^+ T cells with macrophages, whereas the intravaginal model tests first the ability of TSST-1 to penetrate intact stratified mucosa, and subsequently superantigenicity. Four mutants at the carboxy-terminus of the dodecapeptide (S127A, T128A, L129A, and D130A) were tested in these two rabbit models of TSS. When given IV, all mutants displayed lethality comparable to WT TSST-1 (T128A was tested previously and also found to be lethal [204]), consistent with their observed *in vitro* WT superantigenicity. However, a differential lethal effect was seen when the mutants were administered intravaginally; L129A, which also maintained WT cytokine-inducing activity on HVEC, maintained WT lethality after intravaginal administration. Both S127A and T128A, two mutants that showed decreased ability to induce IL-8 from HVEC caused lethality when given intravaginally; however the progression of disease was delayed compared to WT TSST-1. D130A, which did not induce IL-8 from HVEC, was completely nonlethal when given intravaginally.

We hypothesized that the mutation at D130 altered the ability of TSST-1 to penetrate the vaginal mucosa. To examine penetration of mutants, we used an *ex vivo* porcine

model that has been used previously to assess superantigen penetration of vaginal tissue [2, 127, 130]. This model tests the ability of superantigens to penetrate vaginal stratified, squamous epithelial layers that lack tight junctions, in contrast to the single cell layer with tight junctions found in the intestinal tract. Shupp *et al.* previously showed that the dodecapeptide region is important for transcytosis across intestinal epithelial cells; however our studies demonstrate that this region is also important for interactions with stratified, squamous mucosa [145]. Our collective studies suggest that superantigen transcytosis of the vaginal stratified, squamous epithelium is less important in superantigen penetration than superantigen induction of inflammation by causing cytokine production from epithelial cells to increase permeability, and resulting in superantigen movement around rather than through epithelial cells. We chose to study three mutants, D130A (no IL-8 from HVEC), S127A (low IL-8 from HVEC), A125S (high IL-8 from HVEC), along with WT TSST-1. Unexpectedly, both D130A and S127A penetrated the porcine vaginal mucosa more quickly than either WT TSST-1 or the mutant A125S ($p<0.05$). Because this assay is based on counting radiolabeled TSST-1 as it moves through the tissue, it is possible that D130A and S127A were being degraded by mucosal proteases, and therefore, we detected labeled amino acids. WT TSST-1 is resistant to trypsin degradation; however some residue changes within the toxin are capable of rendering the protein sensitive to trypsin. In fact, Murray *et al.* demonstrated that one mutant within the dodecapeptide, T128A, is partially sensitive to trypsin degradation [204]. This is interesting because this mutant also had reduced IL-8 activity when incubated with HVEC in our study. We analyzed the trypsin sensitivity of three

mutants (D130A, S127A, A125S) as previously described [204], but did not see reduction in intact TSST-1 over 4 hr (data not shown), indicating trypsin degradation is not responsible for lack of activity at the epithelium.

It is unclear why the D130A mutant lacks toxicity when applied vaginally in the rabbit model when the same mutant penetrates porcine tissue faster than WT TSST-1. If altering D130 results in a lower binding affinity of TSST-1 for epithelial cells, the mutant may simply move through the mucosa more quickly, consistent with our findings. However, the data suggest D130 must not be able access the circulation subsequent to penetration, since the mutant exhibits WT lethality when administered IV. Further studies are needed to ascertain what role TSST-1 persistence in vaginal epithelium through interaction with the epithelial cell receptor plays in the development of mTSS.

Chapter 4- Role of CD40 as the Receptor for TSST-1 on Vaginal Epithelial cells

These experiments will be included in a future manuscript.

In these studies, purification of TSST-1 was completed by members of Patrick Schlievert's laboratory (Department of Microbiology, School of Medicine, University of Minnesota). Cervical tissue acquisition and IHC was performed in Dr. Peter Southern's laboratory (Department of Microbiology, School of Medicine, University of Minnesota). TSST-1 ligand overlay assays were performed in Dr. Patrick Schleivert's laboratory (Department of Microbiology, School of Medicine, University of Minnesota).

I. Introduction

S. aureus is a significant human pathogen that causes a wide range of diseases such as mTSS [79-81]. mTSS occurs when *S. aureus* colonizes the vaginal mucosa and produces the SAg, TSST-1. SAg interactions with professional immune cells (i.e. T cells and macrophages) have been extensively studied, while studies looking at SAg interactions with epithelial cells are limited. Previous studies of SAg interactions with epithelial cells describe the induction of a proinflammatory state in epithelial cells by SAgs, which includes altered cellular morphology and increased secretion of proinflammatory cytokines [2-4]. The epithelial receptor(s) responsible for this proinflammatory state following binding to SAg is not known. MHC II molecules have been suggested as the epithelial SAg receptor, despite MHC II being present on only ~0 to 10% of primary epithelial cells [135-137]. Non-MHC II SAg receptors have been suggested and were supported by a study, which noted SAgs, SEA and SEB, were able to bind and induce cytokines from MHC II-deficient macrophages [138]. These studies did not attempt to isolate or identify the epithelial and/or non-MHC II SAg receptors. In addition, studies described in Chapter 2 conclude that MHC II is unlikely to be the primary receptor for TSST-1 on the surface of vaginal epithelial cells.

SAgs possess a relatively conserved domain that has been identified as important for initiation of the proinflammatory state in epithelial cells [140-143]. This domain of the SAg structure is separate from the binding domains for MHC II and Vβ-TCR and includes a region spanning 12 amino acids; positions 119 to 130 of TSST-1 [204] (see Chapter 1, Figure 2 and Chapter 3). We determined several residues in this region were

critical in TSST-1 interactions with vaginal mucosal surfaces. When these residues were mutated the ability of TSST-1 to induce IL-8 from HVEC was lost as was the ability to induce TSS in a rabbit vaginal exposure model (see Chapter 3, Results) [7].

Collectively, these studies suggest the presence of a third mammalian epithelial receptor for SAgs, in addition to MHC II and TCR, which is necessary for the induction of cytokines in epithelial cells. This receptor is also believed to be present on APCs [142]; however, there are only a small number of known molecules on APCs, including CD40, that transduce signals. CD40, a member of the tumor necrosis factor receptor family, was identified originally on B lymphocytes and found to interact with CD40L on activated T cells to regulate humoral and cell-mediated immunity [223]. CD40 has also be characterized on APC such as macrophages and dendritic cells, and found to interact with CD40L, which causes the induction of cytokines [224]. CD40 is recognized as a particularly significant receptor on APCs and epithelial cells for its direct interaction with T cells through interaction with CD40L [225-227].

A previous study described synergistic activity of CD40 with MHC II for TSST-1 to induce cytokines from monocytes, suggesting a role for CD40 in the induction of TSST-1 induced cytokines [139]. CD40 was selected for analysis as a possible epithelial surface receptor for TSST-1 based on its ability to induce the NF-κB pathway [228]. NF- κB activation leads to expression of many genes that are upregulated in HVEC is response to TSST-1 [2, 149]. Additionally NF- κB was shown to be activated in HVEC in response to TSST-1 (Chapter 2). The goal of this study was to determine the role of CD40 as a possible TSST-1 receptor on HVEC.

II. Materials and methods

Culture of HVEC

Immortalized HVEC used in these studies were obtained from ATCC (Manassas, VA) (CRL-2616). The cells were generated by transforming primary HVEC from a premenopausal woman with the E6/E7 genes of HPV 16 [122, 203]. HVEC were maintained in KSFM in 75-cm^2 flasks (BD Falcon, Bedford, MA), 6-well, or 96-well microtiter plates (Corning, Corning, NY) at 37 ^{O}C in 7% CO_2, and supplemented with bovine pituitary extract and epidermal growth factor as provided by the manufacturer, 0.4 mM calcium chloride, and 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Fungizone; Gibco, Invitrogen). On days of experimentation, antimicrobials were not used since it has been observed that amphotericin B reduces cytokine production by HVEC.

Flow Cytometry

HVEC were grown to confluence and harvested by trypsinization (5 to 8 min at 37 ^{O}C) and collected by centrifugation (250 x g for 5 min). The cells were washed with flow cytometry staining buffer (1% BSA in PBS) three times and suspended at 10^6 cells/ml. Ten μl of anti-CD40 conjugated to R-PE (BD Pharmingen, 555590, San Jose, CA) were added to 0.5 ml of cell suspension and incubated for 30 min at room temperature in the dark. Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) until 10,000 cells were counted. Forward and side scatter and fluorescence were normalized with isotype controls.

Normal human cervical tissue

Cervical tissue was obtained by standard surgical procedures from premenopausal women with pathological conditions not involving the cervix. Experimental use of human tissue for these studies was conducted with IRB approval. Any tissue with gross macroscopic abnormality was excluded from this study. Tissue processing was initiated in the laboratory within 3 hr of completion of the surgery. Tissue samples spanning the cervical transformation zone were immediately fixed in Streck Tissue Fixative (Streck Laboratories, La Vista, NE) and then processed by standard methods for paraffin embedding and microtome sectioning [208].

Immunohistochemistry

For signal detection by colorimetric IHC, primary anti-CD40 antibodies (1:50 dilution) (CBL486, Chemicon, Temecula, CA), followed by biotinylated secondary antibodies and streptavidin-peroxidase conjugates (ABC System; Vector Laboratories, Burlingame, CA) were utilized. Cervical and colonic tissues were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO) and mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

Toxin purification

S. aureus RN4220, containing pCE107, was used in the production of wild type TSST-1; strain RN4220 does not produce SAgs [204, 205]. The plasmid pCE107 is a high-copy-number plasmid containing *tstH,* the gene for TSST-1 [205]. All media, reagents, and glassware used for toxin production were maintained pyrogen free. *S. aureus* RN4220 were grown up in beef heart media, cultures were treated with 80%

ethanol (final concentration) at 4°C, and the precipitates resolubilized in water. TSST-1 was purified by two sets of IEF. IEF was carried out by first with a pH gradient of 3.5 to 10, followed by a second separation with a pH gradient of 6 to 8. The isoelectric point of TSST-1 is 7.2 [86]. TSST-1 and mutant proteins were identified by double immunodiffusion based on reactivity with a specific polyclonal antibody generated against wild type toxin [206]. Purified toxins were quantified with the Bio-Rad protein assay (Bio-Rad Corporation, Hercules, CA) with the superantigen SEB used for standard curve generation. TSST-1, thus prepared, contained no detectable LPS, as tested by *Limulus* assay (Sigma, St. Louis, Mo.); nor were peptidoglycan, lipoteichoic acid, hemolysin, protease, or lipase (<1 part per 10^6) detectable by combinations of bioassay and SDS-PAGE. Comparable results were obtained with multiple batches of TSST-1.

TSST-1 was also verified as homogeneous when samples were subjected to reversed-phase HPLC [Protein C4 column from Vydac (Hesperia, CA) 0 to 60% gradient of acetonitrile with 0.1% trifluoroacetic acid over a 30 min time period]; proteins eluted as sharp single peaks. TSST-1 was labeled with biotin for use in TSST-1 binding experiments. Biotin labeling was done with an EZ-Link Sulfo NHS-SS Biotin Kit (Pierce, Rockford, IL) per manufacturer's directions. Concentration of labeled TSST-1 was determined using the Bio-Rad protein assay (Bio-Rad Corporation, Hercules, CA) with unlabeled TSST-1 used for standard curve generation.

TSST-1 and CD40 Ligand Overlay

A ligand overlay assay was conducted to determine direct binding of TSST-1 to CD40. Purified CD40/IgG Fc chimera (1 μg) (R&D systems, Minneapolis, MN), IgG Fc

(0.6 μg) (R&D systems, Minneapolis, MN) and ovalbumin (ova) (1 μg) were subjected to non-denaturing PAGE and transferred to PVDF membranes. Ovalbumin was used as an irrelevant protein control and IgG Fc (0.6μg) was used as a control because the CD40/IgG Fc chimera purified protein (R&D systems) contains the IgG Fc fragment. Approximately equimolar concentrations of IgG Fc to the CD40/IgG Fc chimera were run on protein gels and then transferred to PVDF membranes. PVDF membranes were incubated with Tris buffered saline+ 0.05% Tween 20 (TBST) +1% bovine serum albumin containing TSST-1 (500 μg/50 ml) for 24 hr at room temperature. Membranes were washed gently 3 times with TBST, and then incubated with anti-TSST-1 rabbit IgG (Toxin Technology, Sarasota, FL) for 2 hr, washed as above, and incubated with goat anti-rabbit IgG, conjugated to alkaline phosphatase (Sigma, St. Louis, MO) for 2 hr. After a final series of 3 washes, blots were developed with 20 ml substrate (Nitroblue tetrazolium and Chloro,bromo-indolyl phosphate) in Tris-magnesium chloride buffer (0.1 M Tris, 100 mM sodium chloride, 5 mM MgCl2, pH) 9.5 for approximately 15 min.

Generation of CD40 knockdown HVEC

HVEC CD40 knockdown lines were generated with CD40 shRNA producing plasmids from SABiosciences (KH00296P, SABiosciences, Frederick, MD). Four plasmids with insert sequences predicted to disrupt CD40 were provided with one irrelevant sequence control plasmid that was not homologous to any human or mouse sequence (Table 12). All plasmids contained a puromycin-resistance gene allowing for stable transfection. HVEC were seeded on to a 6 well plate ($2x10^5$ cells/well) and were grown to ~50% confluence. HVEC were transfected with Genejuice (EMD4

Biosciences, Gibbstown, NJ) at 6 µl transfection reagent per 1 µg of DNA according to the manufacturer's protocol. Two ml of fresh KSFM were added to each well of HVEC. For each plasmid 100 µl of KSFM was mixed with 6 µl of transfection agent and incubated for 5 min. One µg of endotoxin-free plasmid DNA was added to KSFM with transfection agent and allowed to incubate for 10 min. The DNA- transfection mix was then added dropwise to the 2.0 ml KSFM in each well. One well of HVEC served as the transfection negative control and was treated the same way as the other wells with the exception that no DNA was added. Two days later the medium was changed to KSFM containing 0.5 µg/ml puromycin. Medium was changed every 2-3 days, when HVEC were washed with 2 ml PBS to remove dead or dying cells. At day 7, 12, and 22 puromycin concentration was changed to 0.25, 0.1 and 0.2 µg/ml, respectively. At this time no transfection negative control HVEC were viable, while all transfected HVEC were proliferating and assumed to all be carrying and expressing the shRNA producing plasmid. Transfected HVEC were maintained the same way as untransfected HVEC with the exception that 0.2 µg/ml puromycin was added to the KSFM. Description of the mechanism of RNA interference (RNAi) can be found in the appendix.

Real-time PCR

CD40 shRNA expressing HVEC were grown to confluence in 25 cm^2 flasks in KSFM with puromycin. The HVEC were removed from the flasks by trypsinization (5 to 8 min at 37 ^{O}C) and collected by centrifugation (250 x g for 5 min). Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) per kit directions. cDNA was generated using reverse transcription (iScript, Bio-Rad Corporation) following kit directions.

Expression of CD40 was measured by quantitative PCR with SYBR® Advantage® Premix (Clonetech, Mountain View, CA) on a MYiQ™ iCycler instrument (Bio-Rad Corporation). CD40 primer sequence; forward CAGCCAGGACAGGAAAGTGGTGAGT, reverse CTTCTTCACAGGTGCAGATGGTGTC. Expression of CD40 was normalized to expression of human HPRT as a control primer with sequence listed in Table 9

IL-8 Assay

Transfected HVEC were grown to confluence in 96 well-plates and exposed to purified TSST-1 (100-250 μg/ml) or IL-1β (50 ng/ml) (Biolegend, San Diego, CA), for 6 hr in KSFM without antibiotics. Control wells of HVEC contained KSFM without antibiotics and without TSST-1. Cell culture supernates were collected and tested for IL-8 by ELISA (R & D Systems, Minneapolis, MN) according to manufacturer directions.

TSST-1 Binding Assay

CD40 or irrelevant shRNA expressing HVEC were grown to confluence and harvested by trypsinization. The cells were washed with flow cytometry-staining buffer three times and suspended at $3x10^5$ cells/tube. Five μg of biotin labeled TSST-1 were added to HVEC for 1 hour at 4°C. Unbound TSST-1 was washed away by washing 3 times with staining buffer. HVEC were then exposed to 1/100 diluted Streptavidin conjugated to PE (Pierce, Rockford, IL) for 30 min at 4°C in the dark. HVEC were washed three times with staining buffer. Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) until 20,000 cells were counted. Forward and side scatters and fluorescence were normalized with unstained controls.

Statistical analysis

Unless otherwise specified, significance was determined by one-way ANOVA followed by Dunnett's Multiple comparison test, comparing experimental columns to the control column (GraphPad Prism, La Jolla, CA).

Plasmid	Insert Sequence
Blue	CCTGGAACAGAGAGACACACT
Yellow	ACCATCTGCACCTGTGAAGAA
Red	TCTCCAATGTGTCATCTGCTT
Green	GCTTCTTCTCCAATGTGTCAT
Irrelevant Control	GGAATCTCATTCGATGCATAC

Table 12. Insert sequences of shRNA producing plasmids.

III. Results

CD40 is expressed by HVEC

Multiple studies have determined that epithelial cells produce proinflammatory cytokines in response to SAgs [2, 3, 5]. Some of these studies have suggested MHC II as a possible epithelial receptor, although there is evidence of a non-MHC II receptor (Chapter 2). CD40 was evaluated as a possible epithelial surface receptor for TSST-1 in part due its ability to induce the NF-κB pathway. To function as a receptor for TSST-1, CD40 needs to be expressed on the surface of HVEC. Flow cytometry analysis with fluorescently labeled antibodies to CD40 demonstrated that CD40 is present on the surface of more than 95% of the HVEC without prior stimulation with TSST-1 (Figure. 19).

CD40 is expressed at the epithelial basolateral layer of primary human cervical tissue

For CD40 to function as the epithelial SAg receptor, CD40 needs to be expressed by epithelial cells *in vivo*. CD40 expression *in vivo* was assessed by determining the distribution of CD40 positive cells in the human ectocervix by IHC in tissue from 10 donors. CD40 was localized to the basal layers of epithelial cells in primary cervical tissue (Figure 20), as has been described previously [229]. The images, Figure 20A and Figure 20B, are representative of tissue biopsies that were positive for CD40. CD40 staining was observed in 50% of these tissues. This data shows that CD40 is expressed *in vivo* in tissue where the epithelial receptor for SAg would be expected to be expressed.

TSST-1 binds purified CD40/IgG Fc chimera

To determine direct binding of TSST-1 to CD40, a Western immunoblot ligand overlay assay was conducted (Figure 21). TSST-1 bound to the CD40/IgG Fc chimera (lane 2). No TSST-1 binding to an irrelevant protein, Ova, (lane 4) was observed suggesting that TSST-1 binding to CD40/IgG Fc was specific. TSST-1 also bound to an equamolar amount of IgG Fc (0.6μg) (lane 3) as expected due to the presence of a β-grasp (Ig Fc binding) domain on the toxin. The apparent intensity of the band was greater in the CD40/IgG Fc chimera compared to IgG Fc alone, suggesting that more TSST-1 bound to *in vitro* CD40/IgG Fc than IgG Fc alone. Controls were run that demonstrated CD40/IgG Fc did not react with primary or secondary antibodies (data not shown).

Knocking down CD40

We hypothesized that CD40 mediates TSST-1 induced cytokine production, at least partially. Therefore, CD40 was knocked down with shRNA to determine the role of CD40 in TSST-1 induced cytokine production in HVEC. HVEC were transfected with one of five shRNA plasmids, four contained shRNA predicted to knockdown CD40 (designated: blue, green, red, yellow) and one with a shRNA sequence that did not interfere with any gene in humans or mice (irrelevant shRNA control). After stable transfection of HVEC, CD40 expression was measured by real time PCR (Figure 22A) and flow cytometry (Figure 22B). The blue vector had the greatest efficiency in knocking down CD40 expression (~55% reduction) compared to the other vectors. These results were consistent with what was expected for knockdown from the manufacturer.

The CD40 (blue) shRNA transfected HVEC were chosen for further investigation into the role of CD40 in TSST-1 induced cytokine expression. The green vector also knocked down CD40, but produced two sub-populations that expressed different amounts of CD40 (Figure 22C, two peaks on green histogram). Transfection with the irrelevant shRNA control plasmid did not affect CD40 expression compared to untransfected HVEC (Figure 22C, solid black line vs. dotted black line).

Role of CD40 in TSST-1 induction of IL-8 production by HVEC

HVEC transfected with the blue shRNA (55% CD40 knock down) or irrelevant shRNA control plasmids were exposed to TSST-1 (100 and 250 μg/ml) for 6 hr and IL-8 was measured in the supernate. Interestingly, high TSST-1 concentrations (250 μg/ml) caused an increase in the amount of IL-8 produced in the CD40 shRNA=expressing HVEC (Figure 23). There was no significant difference in IL-8 between CD40 shRNA and irrelevant shRNA expressing HVEC when exposed to TSST-1 100 μg/ml, IL-1β (a positive inducer of IL-8), or left unstimulated (media control). The amount of IL-8 measured in response to TSST-1 by untransfected HVEC was slightly lower than what was previously determined (data not shown), possibly due to the extra metabolic constraints of carrying the plasmid and growth under selection agent. To further investigate the role of CD40 as the TSST-1 receptor, TSST-1 (5 μg TSST-1/ $3x10^5$ cells) binding to CD40 shRNA-expressing HVEC was measured. The CD40 shRNA expressing HVEC bound more TSST-1 than irrelevant shRNA-expressing HVEC (Figure 24), suggesting CD40 is not the TSST-1 receptor on HVEC responsible for inducing cytokines.

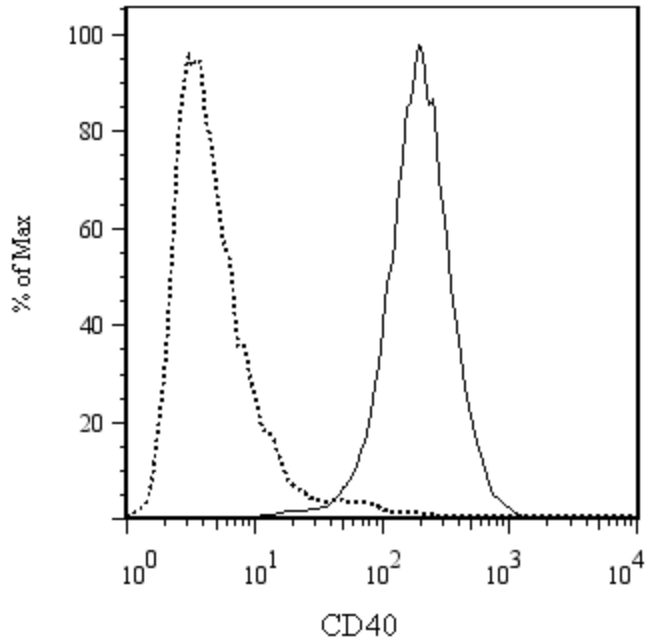

Figure 19. HVEC expression of CD40. HVEC were grown to confluence, harvested and prepared for analysis by flow cytometry. CD40 was measured by staining with CD40 specific antibody (solid line); control cells were stained with isotype matched irrelevant antibodies (dotted line). Data presented is representative of duplicate experiments done in triplicate.

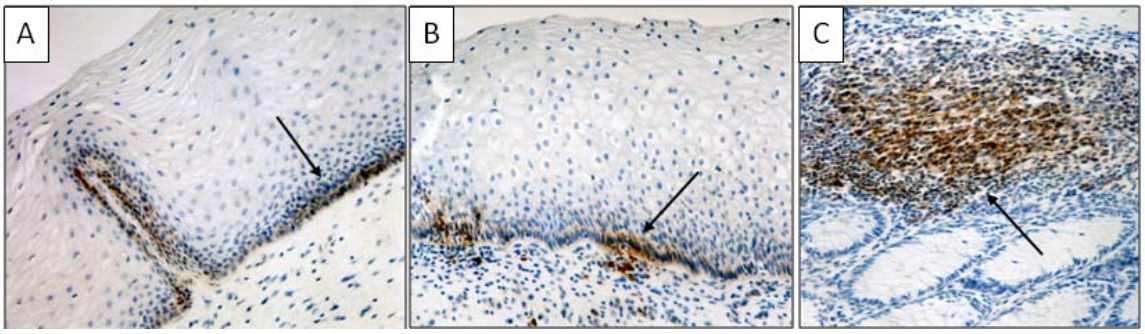

Figure 20. CD40 at basolateral layer of human epithelia (A, B) IHC of CD40 (brown stain) in normal squamous ectocervix. (C) Intestinal epithelium, which was used as a positive control. The arrows note CD40 at the basal layer of the normal squamous epithelium. Image taken at 200X magnification.

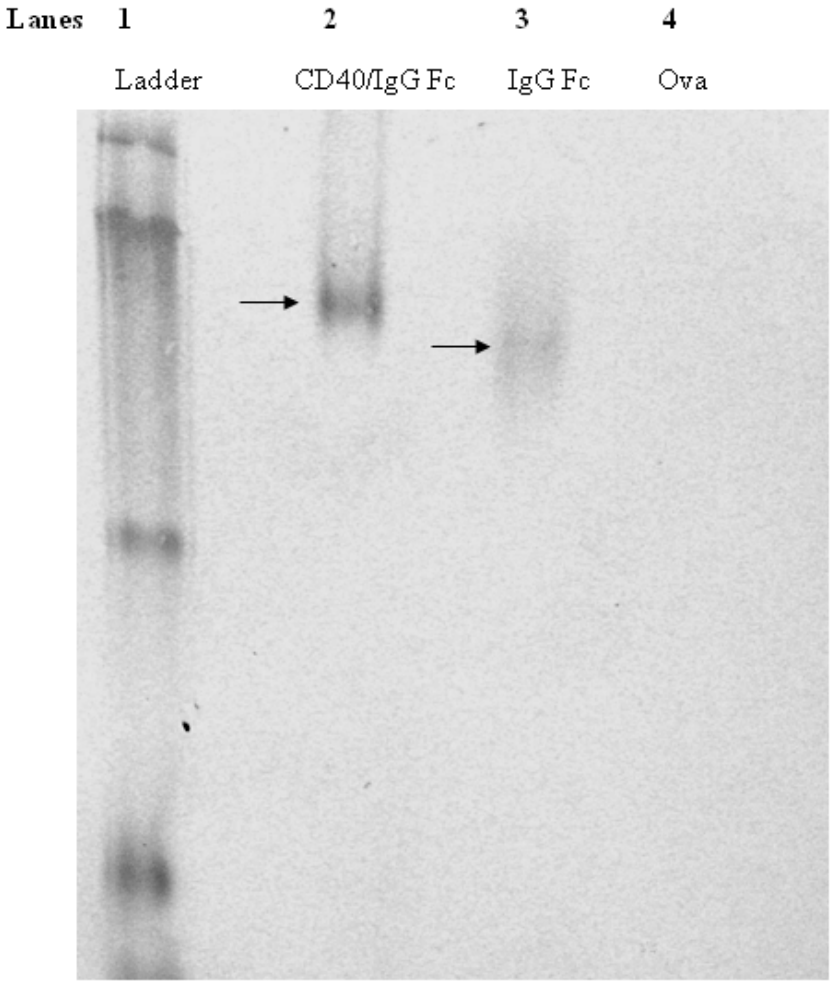

Figure 21. Ligand-overlay assay of TSST-1 binding CD40. Equimolar amounts of CD40/ IgG Fc chimera (1 μg) lane 2, IgG Fc (0.6 μg) lane 3, and Ovalbumin (1 μg) lane 4 were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. PVDF membranes were incubated with TSST-1 (500 μg/50 ml) and blotted with antibodies against TSST-1, followed by the secondary antibody. Figure is representative of experiment done 4 separate times.

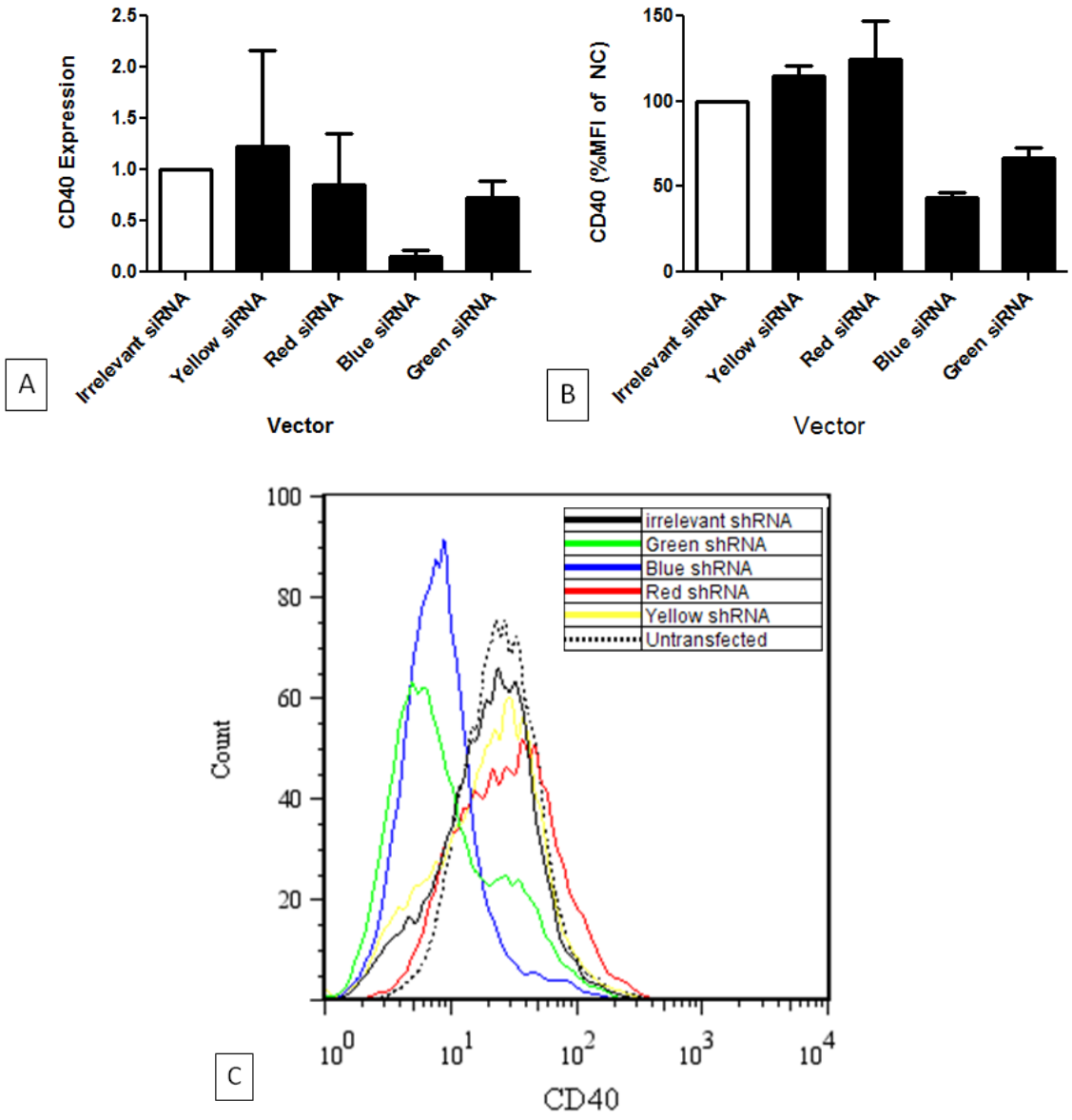

Figure 22. Knockdown of CD40 in HVEC. HVEC were transfected with CD40 shRNA plasmids (yellow, red, blue and green) and exposed to puromycin to ensure stable transfection. (A.) CD40 expression was measured by quantitative real-time PCR with CD40 specific primers. CD40 expression was normalized to HVEC carrying the plasmid with an irrelevant shRNA sequence (NC). Data presented are from 3 separate

experiments done in triplicate. (B, C) CD40 on HVEC was measured with flow cytometry with CD40 specific antibodies. Data in (B) is from 3 separate experiments done in triplicate, while data in (C) is representative of one of the experiments.

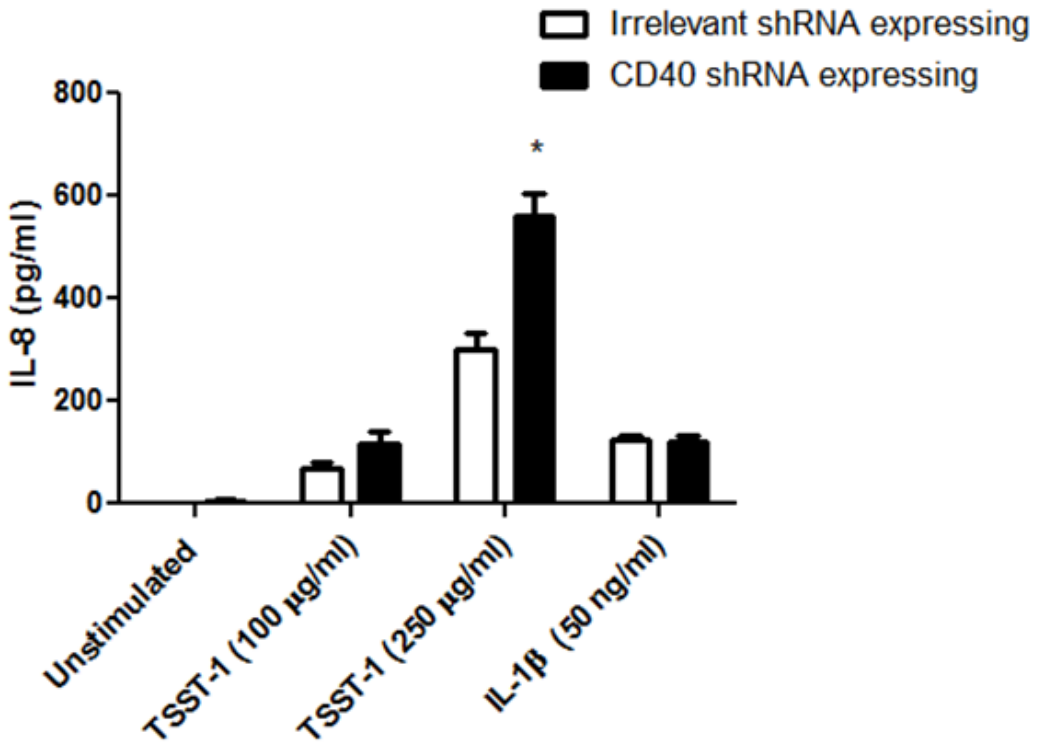

Figure 23. IL-8 produced by CD40 shRNA expressing HVEC in response to TSST-1. Transfected HVEC were exposed to TSST-1 (100 and 250 µg/ml), IL-1β (50 ng/ml), or left untreated for 6 hr, and IL-8 was measured using ELISA. (*) denotes p value < 0.05 compared to irrelevant shRNA-expressing HVEC. Data is representative of two independent experiments done in triplicate.

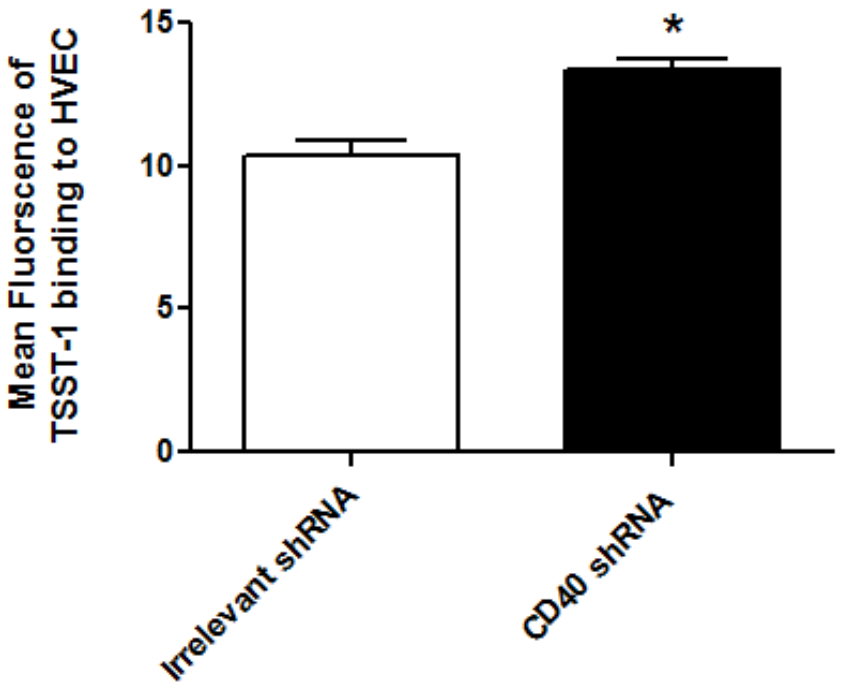

Figure 24. Increased TSST-1 binding to CD40 shRNA-expressing HVEC.

Transfected HVEC were exposed to 5 µg biotin labeled TSST-1 at 4°C, washed, exposed to streptavidin-PE, washed, and analyzed by flow cytometry. TSST-1 binding was measured by determining the mean fluorescence of PE on the HVEC. (*) denotes p value < 0.05 compared to irrelevant shRNA-expressing HVEC. Similar amounts of TSST-1 binding to irrelevant shRNA-expressing HVEC as untransfected HVEC was seen (Data not shown). Data is from one experiment done in triplicate.

IV. Discussion

mTSS occurs most often when the superantigen, TSST-1, is produced vaginally by *S. aureus* in the presence of tampons. *S. aureus* often remains localized on the vaginal mucosa; however, TSST-1 penetrates mucosal surfaces and has been isolated from the serum and urine of patients with TSS [211, 212]. To cause TSS, TSST-1 must penetrate the mucosal surface and subsequently cross-bridge CD4+ T cells and macrophages to release a cytokine storm [1]. The interactions between TSST-1 and SAg with epithelial cells remain poorly understood. Several studies have described a proinflammatory response induced by SAg from epithelial cells [2-5, 213]. MHC II has been suggested as a possible receptor responsible for this proinflammatory response [3]. However, there is evidence for another, non-MHC II receptor (Chapter 2).

In this study, we evaluated CD40 as a candidate for the vaginal epithelial receptor for TSST-1. The ability of CD40 to serve as a receptor for bacterial products has been described previously. Wang *et al.* determined CD40 on the surface of monocytes bound *Mycobacterium tuberculosis* Hsp 70, which caused these cells to produce cytokines [230]. Co-immunoprecipitation of *M. tuberculosis* heat shock protein (Hsp) 70 and CD40 indicated a physical association between these molecules. Studies have also suggested that CD40 is able to bind human Hsp 70 [231].

Our experiments determined that CD40 was expressed on the surface of HVEC and was present in ~50% of ectocervical tissue obtained from otherwise healthy women. This data matches with Hill *et al.* [228] who observed CD40 expression at the basal epithelial layer of ectocervical tissue in 40% to 60% of otherwise healthy women via IHC. The

high expression of CD40 on the HVEC may be due to the immortalization process the cells have undergone with transfection of HPV 16 E6/E7 genes. Researchers also noted that HPV-positive cervical tissue had a dramatic increase in CD40 expression throughout the epithelium [228]. However, our theory of CD40 functioning as a HVEC receptor for TSST-1 is not believed to be an artifact of the immortalization process, as CD40 expression has been noted on multiple different types of epithelial surfaces of airway, intestinal, keratinocytes, and renal tubular cells [232-236].

CD40 needs to be able to bind to TSST-1 to be the SAg receptor, so TSST-1-CD40 interactions were assessed by a ligand-overlay assay. TSST-1 was able to bind a CD40/IgG Fc chimera which suggested that CD40 bound TSST-1. However, there also was binding of TSST-1 to IgG Fc alone, which was predicted based on the presence of a β-grasp (Ig Fc binding) domain on the toxin. The increase in intensity of the band in the CD40/IgG Fc lane compared to the IgG Fc lane led us to believe that TSST-1 was interacting with the CD40 portion of the chimera in addition to the IgG Fc. This data suggested that CD40 may serve as the epithelial receptor for TSST-1 *in vitro*.

We investigated the possible role of CD40 as an epithelial cells receptor for TSST-1 by knocking down CD40 expression in HVEC by shRNA technology. Four commercially available shRNA plasmids were evaluated for their ability to knockdown CD40. The blue vector was chosen based on its ability to knockdown CD40 by 55% on the protein level compared to HVEC transfected with an irrelevant sequence control plasmid. Interestingly, CD40 shRNA-expressing HVEC made more IL-8 in response to TSST-1 and bound more TSST-1 than irrelevant shRNA-expressing HVEC. CD40 and

irrelevant shRNA-expressing HVEC made similar amounts of IL-8 when left unstimulated or stimulated with IL-1β suggesting that there were no effects on IL-8 production by knocking down CD40. Additionally, based on the TSST-1 binding assay of the CD40 shRNA-expressing HVEC, it does not appear TSST-1 interacts with CD40. This data show that it is highly unlikely that CD40 is the HVEC receptor for TSST-1.

This increase in TSST-1 binding and IL-8 production may be a result of an increase in the expression of the unknown receptor on the surface of CD40 shRNA-expressing HVEC. It is possible that other surface proteins are expressed more to fill the void created by knocking down CD40. Since TSST-1 does not appear to bind CD40 on the surface of HVEC the binding between the two proteins seen in the ligand overlay assay is likely due to an artifact of the use of the IgG Fc chimera. The presence of CD40 conjugated to IgG Fc may have exposed residues causing TSST-1 to bind more than TSST-1 bound IgG Fc. Use of a non- chimera CD40 molecule in a ligand overlay assay may be used to overcome this artifact.

CD40 was also explored as a candidate receptor for TSST-1 based on Mehindate *et al.* where SEB and TSST-1 induced cytokine gene expression (IL-1β and TNF-α) from a human monocyte cell line (THP-1) pre-treated with anti-CD40 monoclonal antibody [139]. The authors attributed this phenomenon to the anti-CD40 antibody being able to overcome the requirement for MHC II to dimerize and induce cytokines [139]. With the findings from our study, this phenomenon, seen in Mehindate *et al.*, is possibly due to an artifact of CD40 and MHC II being in close proximity to each other on the cell [237] and

not CD40 serving as a receptor or co-receptor. Studies to identify the HVEC receptor for TSST-1 are ongoing.

Chapter 5- Curcumin as an anti-TSS agent

Studies part of a manuscript under review at the *Journal of Immunology* (January 2011).

Rabbit experiments were done with the assistance and guidance of Dr. Patrick Schlievert (Department of Microbiology, School of Medicine, University of Minnesota) and Ying-Chi Lin (Peterson laboratory). Curcumin analyses were conducted with the assistance of Alex E. Grill and Dr. Jayanth Panyam (Department of Pharmaceutics, College of Pharmacy, University of Minnesota).

I. Introduction

Curcumin is a component of the Indian spice Turmeric (*Curcuma longa*) that has anti-inflammatory, anti-angiogenic, and antimicrobial properties [166-168]. The structure of curcumin, a diferuloylmethane, can be seen in Chapter 1, Figure 7. Turmeric is a main component of curries, and has been used as spice for thousands of years across Asia and India [169]. Curcumin has been used as a medicinal agent for over 4000 years across India for the treatment of rheumatism, skin diseases, intestinal worms, diarrhea, fevers, hepatic disorders, and inflammation [169]. Recently, Western medicine has taken notice of curcumin and conducted a vast amount of research with curcumin during the past 10 years [166, 172, 175, 187, 201, 238].

Curcumin regulates a wide range of cellular pathways, including the activation and production of cytokines, cellular enzymes, growth factors, receptors, transcriptional factors, protein kinases, and adhesion molecules [167, 173]. Curcumin inhibits a wide range of cellular factors that are critical in inflammation and tumorgenesis such as NF-κB [167, 173]. Curcumin inhibits the induction of proinflammatory pathways in several epithelial cell types in response to several different stimuli including microbes [196-198]. Curcumin has also been studied as an antimicrobial agent against bacteria, fungi, and viruses [174-176]. Curcumin has mixed results with infectious diseases in animal models, suggesting the effectiveness of curcumin is affected by the causative organism and location of infection [199, 200]. However, curcumin, in combination with other non-antibiotic agents, given orally was able to reduce the symptoms of *Helicobacter pylori*-induced gastric ulcers [201].

Our studies indicate the superantigen proinflammatory effects on vaginal mucosa are critical for progression to TSS and mortality [2, 7, 209]. A strategy for prevention of staphylococcal TSS is to block or prevent the proinflammatory effects of TSST-1 on the vaginal mucosa. This theory is supported by a study that used an inhibitor specific to NF- κB, an intracellular peptide that inhibited the translocation of NF- κB into the nuclease, to prevent SEB-induced cytokine production and migration of immune cells to the site of infection in a murine lung model of infection [157]. The effects of curcumin on SAg-induced inflammation or *S. aureus* infections on a mucosal surface have not been investigated previously. Therefore, the aims of this study were: 1) to evaluate the effects curcumin on vaginal mucosa and *S. aureus* growth, including its ability to reduce *S. aureus* exotoxin induction of IL-8 at non-toxic doses in an *ex vivo* porcine vaginal mucosal model; and 2) to determine effects on mortality and TNF-α in a rabbit vaginal TSS model. Because of similarities in structure and permeability, porcine vaginal mucosa was used as a model for human vaginal mucosa for evaluation of the proinflammatory effects of *S. aureus* and exotoxins and the cytotoxicity, antimicrobial, and anti-IL-8 dose-ranging effects of curcumin [127, 239]

II. Materials and Methods

Ex vivo porcine vaginal mucosa culture

An *ex vivo* porcine vaginal tissue model was used to determine the feasibility for curcumin as a topical anti-TSS agent. This *ex vivo* model was developed in our lab to evaluate the activity of potential antibacterial and antiseptic agents [240]. This model is an effective model for the initial events of infection because the full thickness porcine vaginal explants mimic human tissue [127].

Specimens of normal porcine vaginal mucosa were excised from animals at slaughter and transported to the laboratory in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Tissue was utilized within 3 hr of excision. Tissue samples of uniform size were obtained from the porcine vagina with a 5mm biopsy punch. Excess muscle tissue was trimmed away with a scalpel. Explants were washed in medium without serum and antibiotic-free 3 times. The plugs were then placed mucosal side up on a 0.4 μm cell culture insert in 6-well plates containing fresh RPMI 1640 without serum and antibiotics. The mucosal surface was continually exposed to air.

Evaluation of curcumin cytotoxicity

Viability of the *ex vivo* porcine vaginal tissue explants was quantified by a Cell Growth Determination Kit (Sigma, St. Louis, MO). This assay is based by the reduction of the tetrazolium salt 1-4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT) by live tissue into an acidified isopropanol soluble product (Sigma, St. Louis, MO). For this assay explants were incubated with various amounts of curcumin (Sigma, St. Louis, MO)

in 100% dimethyl sulfoxide (DMSO) at 37°C, 7% CO_2 for 24 hr. Explants were washed 3 times and incubated with MTT substrate for an additional 3 hr at 37°C, 7% CO_2. Reduced MTT was extracted in acidified isopropanol at room temperature for 2 hr. Optical density of the formazan end product was measured spectrophotometrically (570 nm). The effect of each formulation on tissue viability is expressed relative to untreated tissue control. An iodine-based 3M proprietary antiseptic that has been determined previously by our lab to be toxic to *ex vivo* porcine tissue (reduced viability by 90% after 24 hr exposures) was used as a positive control.

Inhibition of IL-8 and inhibition of *S. aureus* growth

Two representative *S. aureus* USA200 TSS isolates were used in this study. MNPE (α-toxin positive) was isolated from pulmonary post-influenza TSS strain and the strain, MN8 (α-toxin mutant) was isolated from menstrual vaginal TSS. *S. aureus* MNPE exotoxins were used as they induced more IL-8 from the vaginal explants than *S. aureus* MN8, which produces minimal amounts of α-toxin due to a mutation/stop codon at position 113 [241, 242]. The ability of curcumin to inhibit *S. aureus* growth and *S. aureus*-induced IL-8 was assessed. 5mm porcine vaginal explants, in triplicate per group, were exposed to filtered (0.2 μm filter size) overnight *S. aureus* MNPE culture supernate (containing SEC, TSST-1, α-toxin [19]) or live *S. aureus* MN8 for 1 hr, when curcumin (1.375-135 nmoles/explant), in 10% DMSO, was added to the explants for an additional 6 hr. Tissue explants were then placed into 0.25 ml PBS and vortexed at maximum speed for 2 min. Bacterial load was measured by serial plating of the explant lysates onto sheep blood agar plates, and colonies were counted the following day and colony forming units

(CFU)/ml was determined. Explant lysates were cleared of debris by centrifugation (14,000 g for 2 min); supernate was removed and saved for IL-8 measurement. IL-8 was measured by a pig-specific ELISA kit (R & D Systems, Minneapolis, MN) following the manufacturer's protocol.

Rabbit vaginal TSS model

A rabbit vaginal mTSS model was used to determine the ability of curcumin to inhibit TSST-1-induced TSS and lethality in rabbits, which was described elsewhere [219-221]. It is important to mention that rabbit vaginal epithelial morphology is different than that of humans and porcine. The rabbit vaginal epithelial is composed on a single layer or columnar epithelial cells as opposed to the multilayer squamous epithelium seen in porcine and human tissue [243]. Despite these morphological differences rabbits are commonly used as a model for vaginal microbicides [244]This model tests the ability of superantigens to enhance shock caused by LPS from *Salmonella enterica* serovar Typhimurium. Rabbits were anesthetized with ketamine (25 mg/kg; Phoenix Pharmaceuticals, Inc., St. Joseph, MO) and xylazine (20 mg/kg; Phoenix Pharmaceuticals, Inc.) prior to insertion of catheters into the vaginal vault. TSST-1 (10 μg) was co-administered vaginally in 0.2 ml in 3% DMSO with or without curcumin (1.62 μmoles). LPS (0.1 μg/kg) was administered IV in the marginal ear veins 4 hr after instillation of TSST-1, and the rabbits were monitored for an additional 66 hr. Rabbits displaying signs of severe illness (failure to right themselves and exhibit escape behavior), or those remaining healthy at the conclusion of the experiment, were euthanized with Beuthanasia D (1 ml/kg, Schering-Plough Animal Health Corp., Union,

NJ) according to the University of Minnesota IACUC requirement. Blood was collected via cardiac puncture and allowed to clot in serum collection tube (BD Vacutainer, Franklin Lakes, NJ), serum was separated by centrifugation (1,000g for 10 min) and serum was stored at -20°C for TNF-α and curcumin determination. Vaginal tissue was removed and split; one-half stored at -20°C for TNF-α and curcumin determination and half fixed in 10% formalin for histology. Liver samples were also removed and stored at -20°C for curcumin determination.

TNF-α determination in rabbit serum and vaginal tissue

TNF-α was measured in this study as this assay was previously used in our lab and had been previously published in this model [64, 159]. TNF-α was measured in serum and vaginal tissue by a rabbit-specific ELISA (BD Bioscience, Franklin Lakes, NJ). Vaginal tissue was placed in 0.25 ml ELISA reagent diluent (PBS with 1% BSA and 0.05% Tween 20) and homogenized (three 20 second bursts at max power). Debris was removed by centrifugation (14,000 g for 2 min); supernate was removed and stored (-20°C) for TNF-α measurement. For serum samples the lower limit of detection for the assay was 0.5 ng/ml, with the lower limit of detection in vaginal tissue being 0.125 ng/ vaginal tissue section.

Histology of rabbit vaginal tissue

Selected rabbit vaginal tissue samples were fixed in formalin for histological examination. Following embedding in paraffin, six-micron sections were cut and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO) and mounted in Vectamount (Vector Laboratories, Burlingame, CA). The slides were examined microscopically to

determine the condition of the tissue. The original magnification was 200X. Histological findings were determined by a veterinary pathologist at the University of Minnesota Cancer Center.

Curcumin Determination

Curcumin was assayed using a previously established liquid chromatography-mass spectrometry (LC-MS) assay [245]. Tissues were homogenized and lyophilized. Curcumin was extracted from dry tissue using ethyl acetate (2 mL) overnight. Extracts were evaporated under nitrogen stream and then reconstituted in methanol. Chromatography was performed on an Agilent Technologies (1200 series) system with negative ESI connected to a TSQ Quantum system. An Agilent XDB-C18 1.8 □m, 4.6 x 50 mm column was used for separation. Mobile phase consisted of (A) 10 mM ammonium acetate and (B) acetonitrile. Gradient flow (0.5 mL/min) with a total run time of 10 min was used: 0-4.5 min: 45-100% B, 4.5-5.5 min: 100% B, 5.5-6 min: 100-45% B, 6-10 min: 45% B. The following mass transitions were monitored: curcumin - 367→ 216, hydroxybenzophenone 197 → 92. The lower limit of quantification was 0.15 nmoles/tissue section or 0.15 nmoles/ml serum. To estimate the total amount of curcumin within the tissue at late (70 h post curcumin dose) time point, the following calculation was done: Average curcumin concentration (nmoles/mg dry tissue weight) x 9.1 mg tissue weight (average tissue section weight) x 4 (approximately ¼ of total vaginal tissue was used for curcumin determination) = estimated total vaginal curcumin.

Statistical analysis

Each experiment was repeated a minimum of 3 times. Data presented are mean of triplicates ± SEM. ANOVA followed by Bonferroni's post-test were performed using the GraphPad PRISM software (GraphPad Software, Inc., California). Significance between curcumin- and untreated mortality in rabbits was determined by Log-rank test.

III. Results

Curcumin is non-toxic to vaginal mucosa

Curcumin was evaluated as a possible therapeutic agent for SAg-mediated diseases. *In vitro* studies determined that curcumin (30 μM) inhibited IL-8 induced from HVECs by *S. aureus* MNPE filtered overnight supernates, which contained SAgs (TSST-1 and SEC), cytolysins, and proteases (see appendix). However, the LD_{50} of curcumin on the HVECs was approximately 30 μM; therefore, it was difficult to separate the reduction in IL-8 production from toxic effects. So, a previously developed full thickness *ex vivo* porcine vaginal mucosal model was used to evaluate curcumin's toxicity and potential to reduce proinflammatory cytokines. The toxicity of curcumin was assessed by exposing *ex vivo* porcine vaginal epithelial explants to curcumin overnight. Curcumin (60-1,350 nmol) was applied to 5 mm tissue explants for 24 hr and then the viability of tissue explants were measured with an MTT assay. There was no reduction viability at any dose that was tested, including the highest dose studied, 1,350 nmol (Figure 25).

Curcumin prevents *S. aureus*-induced IL-8

Curcumin was tested for the ability to inhibit *S. aureus* induced IL-8 release in the *ex vivo* porcine tissue model. Explants were exposed for 1 hr to a live menstrual TSS *S. aureus* isolate (MN8) (Figure 26) or a pulmonary post-influenza TSS *S. aureus* isolate (MNPE) filtered supernates (Figure 28), (only contains only secreted proteins). Explants were then treated with curcumin for an additional 6 hr. Tissue was then disrupted by vortexing and IL-8 release was measured by ELISA. Curcumin significantly inhibited IL-8 production ($p < 0.05$ compared to vehicle control) with doses as low as 13 nmoles of

curcumin per tissue explant. Interestingly when stimulated with 10^7 CFU of live *S. aureus* 67.5 nmol of curcumin inhibit Il-8 release more than 135 nmol of curcumin. This difference was not significant (p value 0.122, t test) but was seen in multiple independent experiments. Bacterial counts were measured after exposure to curcumin and no reduction in bacterial counts after exposure with curcumin (67 to 135 nmoles) was noted compared to DMSO vehicle control (Figure 27).

Curcumin protects rabbits in a lethal TSS model

Since curcumin inhibited *S. aureus* (MNPE) exoprotein-induced IL-8 from *ex vivo* porcine vaginal mucosa, the ability of the compound to prevent TSS in a rabbit vaginal TSS model following vaginal application of TSST-1 was explored. TSST-1 proinflammatory effects on the vaginal epithelial cells were hypothesized to contribute to TSS. The rabbit TSS model is based on studies described previously where TSST-1 potentiated LPS toxicity [219-221]. In this model, sub-lethal doses of TSST-1 and LPS were used, when administered together the combination caused a lethal shock within a short period of time. TSST-1 (10 μg) was administered vaginally via a catheter with or without curcumin (1.62 μmoles) (8 rabbits per group). 1.62 μmoles of curcumin was chosen as the dose because this was the largest dose that could be delivered with the following constraints; the maximum amount of DMSO (3%) and the maximum volume (200 μl) that was predicted for delivery in this model. 1.62 μmoles (0.53 μmoles/cm^2 vaginal tissue) was higher than the minimum effective dose in the porcine tissue model (13.5 nmoles or 0.17 μmoles/cm^2).

After curcumin application, rabbits were allowed to recover for 4 hr, when LPS (0.1 μg/kg) was given via a marginal ear vein. Rabbits were then monitored for survival, and were euthanatized on failure to exhibit escape behavior and to right themselves. All rabbits, curcumin treated and untreated, showed mild symptoms of TSS, including lethargy and diarrhea, immediately post LPS administration, which subsided in rabbits treated with curcumin that survived. Curcumin significantly reduced lethality and TSS caused by TSST-1 (5 of 8 rabbits survived, whereas 0 of 8 control rabbits survived; p value 0.0028 Figure 29). The difference in survival rates between curcumin treated and the untreated controls was significantly different at all assessed time points (5, 18, 42, and 66 hr). All surviving curcumin treated rabbits were euthanized at the end of experiment (66 hr after LPS dose).

TNF-α was detected in the serum of 8 of 11 rabbits (curcumin-treated or untreated) that died during experimentation, while none of the rabbits that were treated with curcumin and survived had detectable TNF-α in the serum or vaginal tissue (Figure 30A & Table 13). TNF-α was detected in vaginal tissue from 5 of 11 rabbits that died during experimentation and all five animals had detectable amounts of TNF-α in the serum (Figure 30B & Table 13). There was no significant difference in TNF-α in the serum or vaginal tissue between the groups [untreated and died (n=8), curcumin-treated and died (n=3), and curcumin-treated and survived (n=5)] as there was a wide variation in values and small sample size. However, TNF-α was undetectable from serum or vaginal tissue of all curcumin-treated rabbits that survived at the end of the experiment.

Vaginal tissue morphology and disruption was assessed by H & E staining of *ex vivo* vaginal tissue sections from 11 of 16 rabbit vaginal tissues, which were selected randomly post-experimentation and were analyzed by a veterinary pathologist. Rabbit vaginal mucosa, consists of a single layer of columnar epithelial cells [244]. Most of the tissue sections signs of inflammation including blood vessel dilation and congestion and cell fragmentation. Lymphocytes and heterophils (rabbit neutrophil) were seen within the half of the tissue samples. In rare samples edema was seen in addition, cell fractioning was seen which is consistent with cellular death. This inflammation was independent of treatment, time of death, or TNF-α in the tissue (Table 13). Representative pictures of the tissue can be seen Figure 31 and a full copy of the pathologist's report can be seen in Table 15 in the appendix.

Curcumin was quantified in serum, liver, and vaginal tissue samples to determine the resident levels in the tissue and systemic exposure to the compound. Curcumin was undetectable in all serum or liver tissue samples. Curcumin was detected in 7 of 8 rabbits that were administered intravaginal curcumin (Figure 32 and Table 14). At ~12 hr post curcumin application there was ~12% of the original dose remaining within the vaginal tissue, while 0.8% of the original dose remained at 70 hrs post curcumin application.

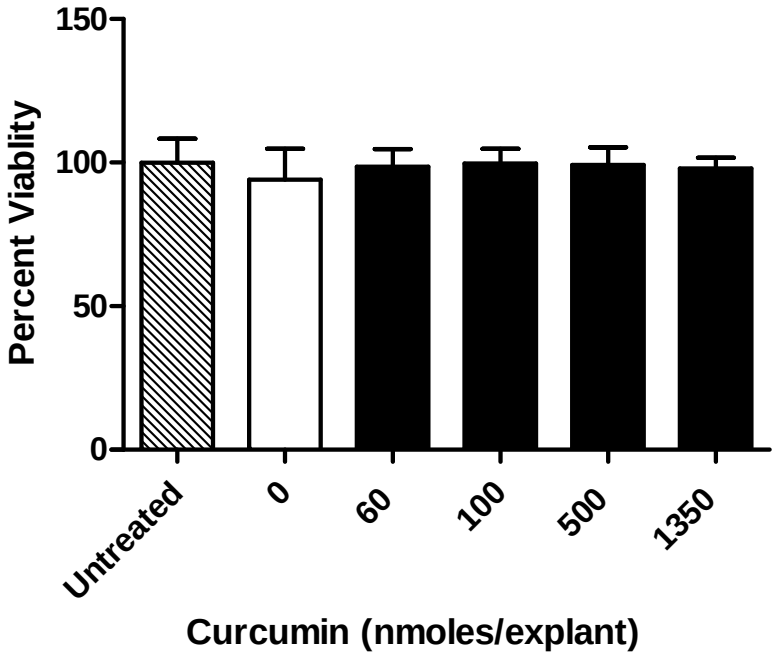

Figure 25. Curcumin is non-toxic to porcine vaginal explants. Porcine vaginal explants were exposed mucosal side to the indicated amount of curcumin in 5 µl of 100% DMSO and incubated for 24 hr. Tissue viability was measured by a MTT assay and normalized to tissue left untreated (no DMSO or curcumin). Data presented as mean ±SD. Experiments were performed in triplicate three independent times.* denotes $p < 0.05$ compared to vehicle control (0 nmoles curcumin) by ANOVA.

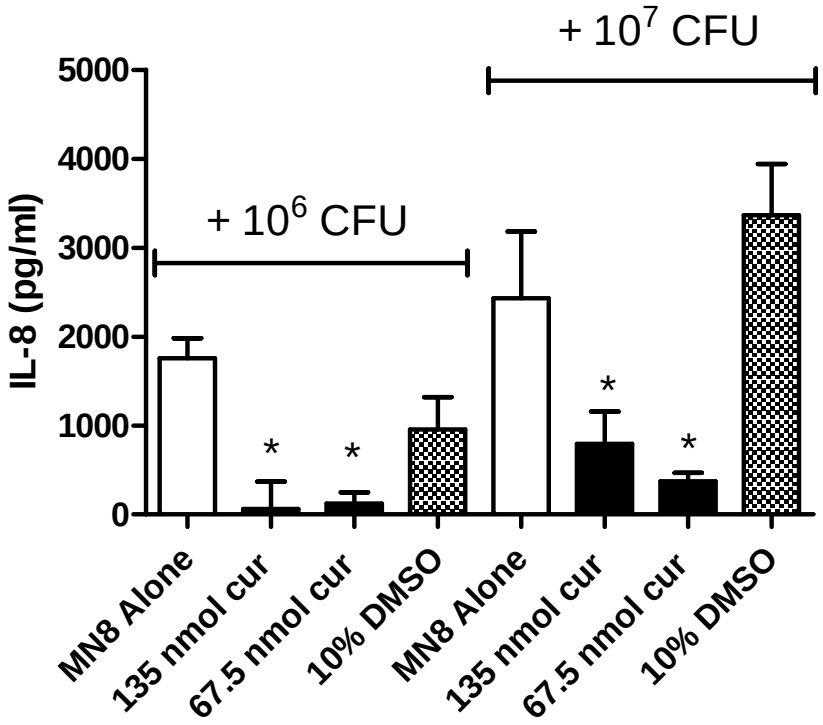

Figure 26. Curcumin inhibits *S. aureus* (MN8)-induced IL-8 production. *Ex vivo* porcine vaginal mucosal explants were inoculated with live *S. aureus* (MN8; mTSS isolate) at 1 x 10^6 or 1x10^7 CFU for 1 hr, when curcumin at the indicated dose in 10% DMSO was added for an additional 6 hrs. Tissue was disrupted by vortexing and IL-8 was measured via ELISA. Data presented as mean ±SD. Data are representative of an experiment done in triplicate three independent times. * denotes $p < 0.05$ compared to corresponding DMSO control by ANOVA. Abbreviations curcumin (cur).

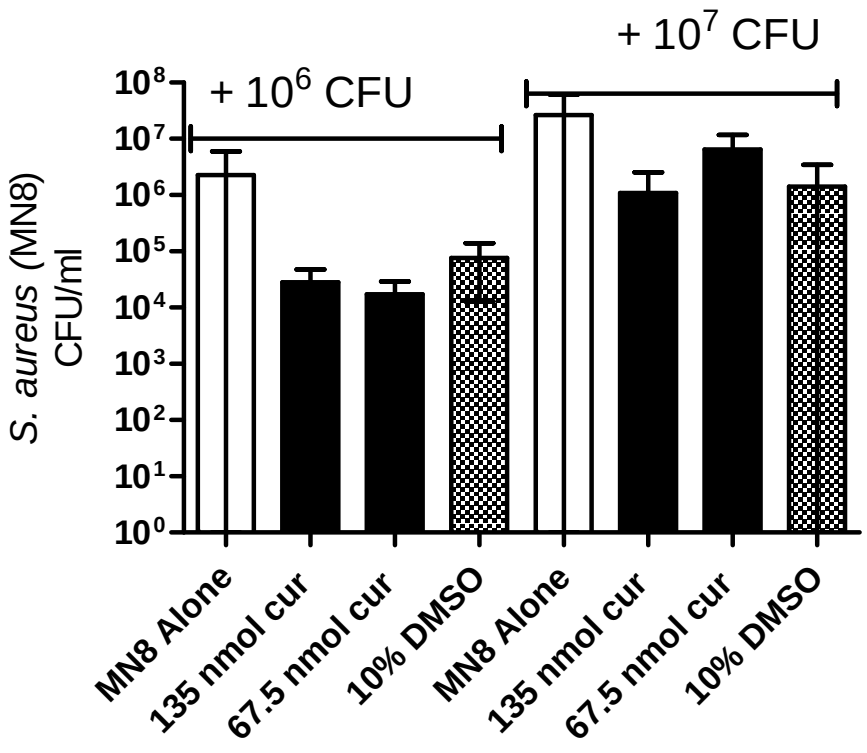

Figure 27. Curcumin minimally inhibits *S. aureus* growth on vaginal mucosal explants. *Ex vivo* porcine vaginal mucosal explants were inoculated with live *S. aureus* (MN8; mTSS isolate) at 1 x 10^6 or 1x10^7 CFU for 1 hr, then curcumin at the indicted dose in 10% DMSO was added for an additional 6 hr. Tissue was disrupted via vortexing and viable *S. aureus* were counted. These samples are the same samples from which IL-8 release was measured in figure 26. Data presented as mean ±SD Data are representative of an experiment done in triplicate three independent times.* denotes $p < 0.05$ compared to corresponding DMSO control by ANOVA.

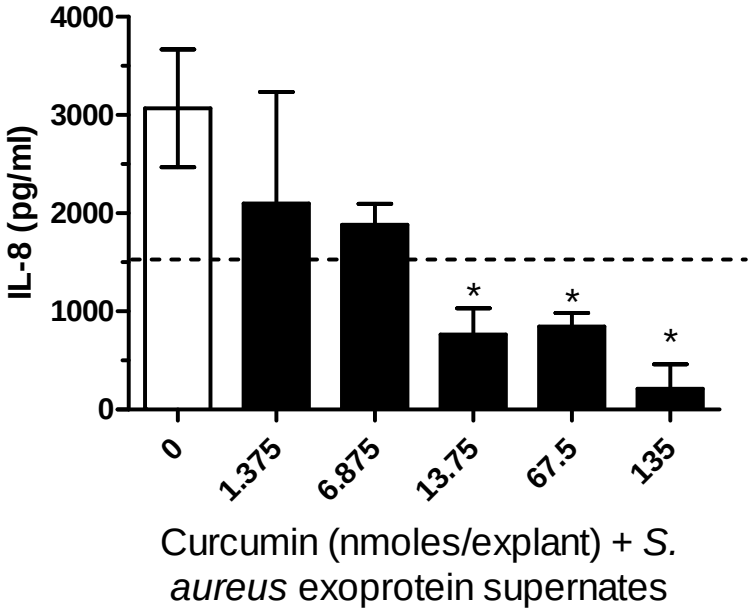

Figure 28. Curcumin inhibits *S. aureus* exoprotein-induced IL-8 release. Porcine vaginal explants were exposed to filtered *S. aureus* (MNPE) overnight culture supernatants for 1 hr, when the indicated amount of curcumin was added to explants in 5 µl of 10% DMSO and incubated for an additional 6 hr. Tissue was disrupted via vortexing and IL-8 was measured by ELISA. Dotted line indicates amount of IL-8 produced by unstimulated, untreated tissue explants. Data are presented as mean ±SD. Data are representative of an experiment done in triplicate three independent times.* denotes $p < 0.05$ compared to vehicle control (0 nmoles curcumin) by ANOVA.

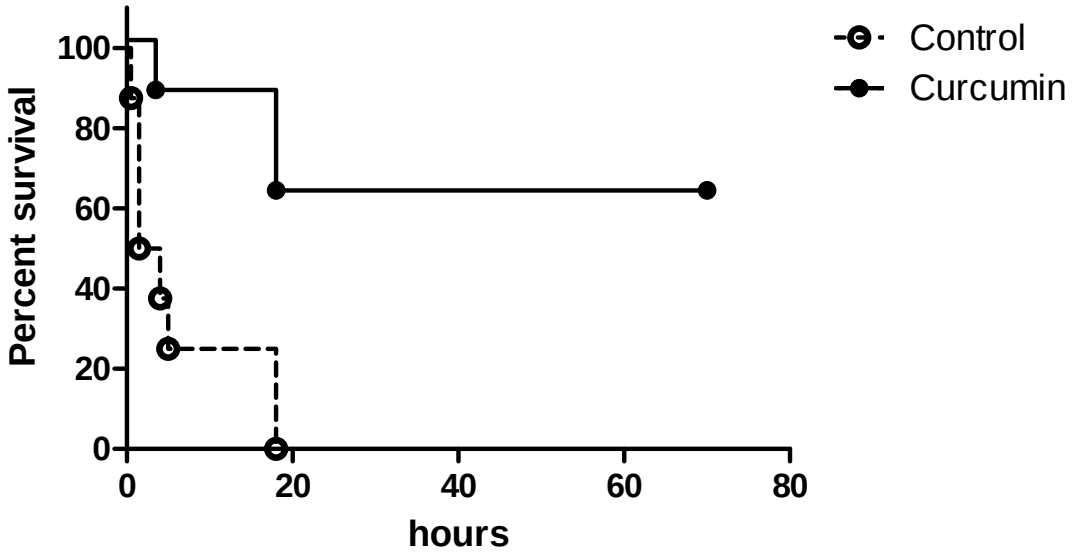

Figure 29. Curcumin protects rabbit from a lethal TSS model. TSST-1 (10 μg) was administered intravaginally with or without curcumin (1.62 μmoles) in 3% DMSO (n= 8 rabbits per group). LPS (0.1 μg/kg) was administered 4 hr later via ear marginal vein. The rabbits were monitored for survival. Curcumin significantly reduced TSS-induced mortality compared to untreated ($p<0.003$, Log-rank test).

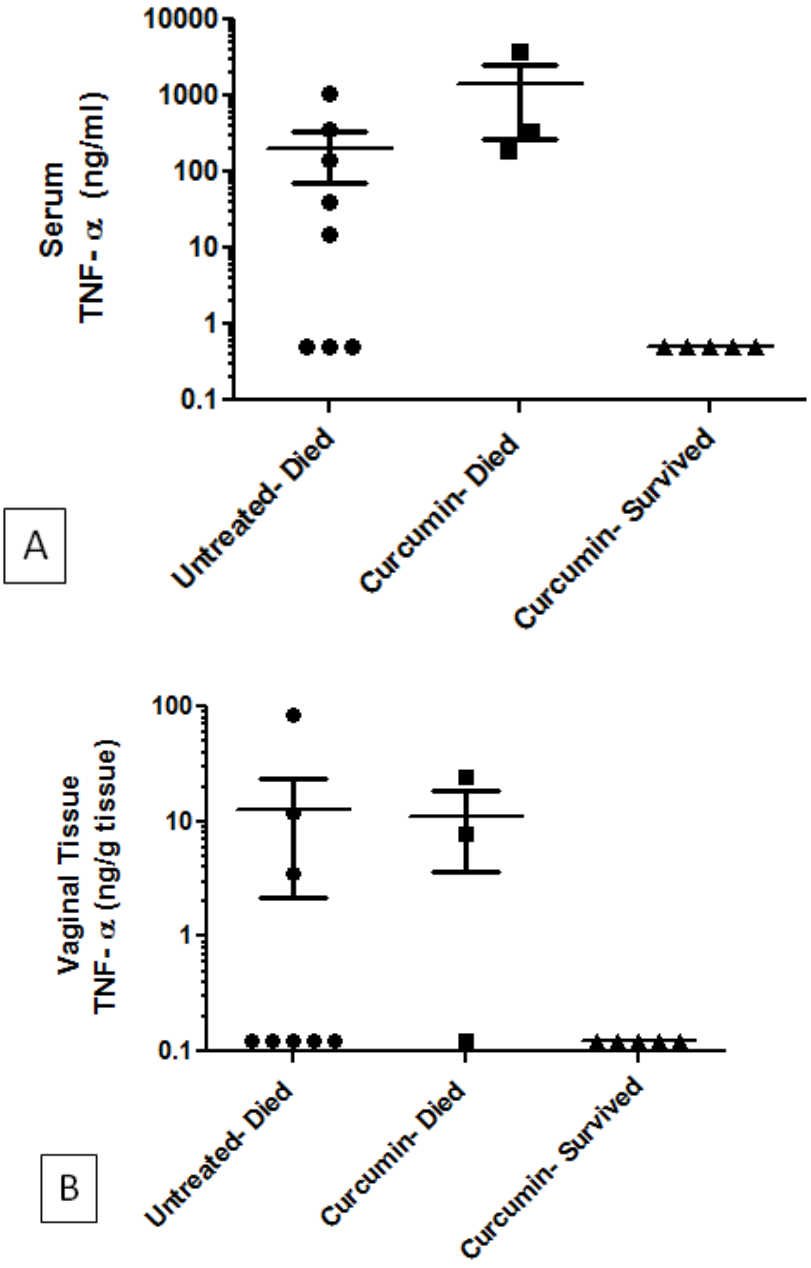

Figure 30. TNF-α was undetectable in serum (A) and vaginal tissue (B) in rabbits that survived the lethal model of TSS. TNF-α in serum and homogenized vaginal tissue was determined by ELISA. Each data point corresponds to data from 1 rabbit measured in duplicate. Data presented as mean ±SEM. Lower limits of detection were; serum 0.5 ng/ml and vaginal tissue 0.125 ng/ vaginal tissue section.

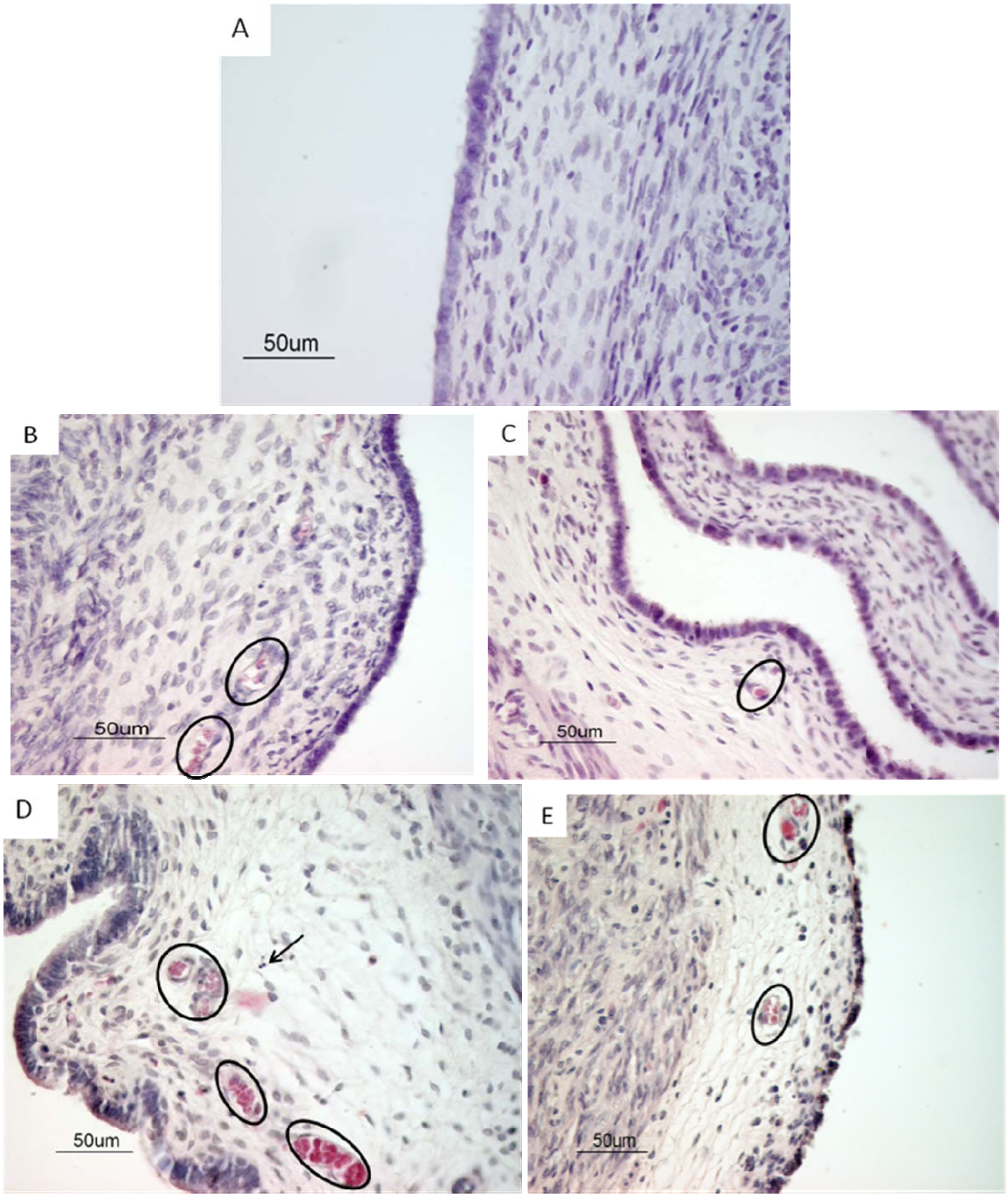

Figure 31. TSST-1 induces vaginal tissue disruption, including blood vessel dilatation, in rabbit model of TSS. H & E staining of rabbit vaginal columnar epithelial and mucosal tissue. Circles indicate blood vessel dilatation and congestion. Arrows indicate cell fractioning and cell death. All images 200X. A. Healthy, normal tissue;

picture from. B. Rabbit #1, untreated, died. C. Rabbit #8, untreated, died. D. Rabbit #14, curcumin treated, died. E. Rabbit #13, curcumin treated, survived.

Rabbit	*Treatment*	*Time of Death (hrs. post-LPS)*	*Serum TNF-α (ng/ml)*	*Vaginal Tissue TNF-α (ng/g)*
1	Control	4 hr	1090	11.6
2	Control	4-18 hr	UD	UD
3	Control	1.5 hr	353	3.58
4	Curcumin	Survived	UD	UD
5	Curcumin	4-18 hr	342	24.8
6	Curcumin	Survived	UD	UD
7	Control	1.5 hr	144	86.0
8	Control	1.5 hr	40.3	UD
9	Control	0.5 hr	15.5	UD
10	Control	5-18 hr	UD	UD
11	Control	4-5 hr	UD	UD
12	Curcumin	Survived	UD	UD
13	Curcumin	Survived	UD	UD
14	Curcumin	3.5 hr	3730	7.89
15	Curcumin	Survived	UD	UD
16	Curcumin	5-18 hr	187	UD

Table 13. Rabbit treatment, survival, and TNF-α concentrations. Time of death was the number of hours from LPS dose. When the exact time was not known as the rabbit succumbed overnight, a range of times was given. Abbreviation; Undetected (UD). Lower limit of detection for serum: 0.5 ng/ml, vaginal tissue: 0.125 ng/ tissue section.

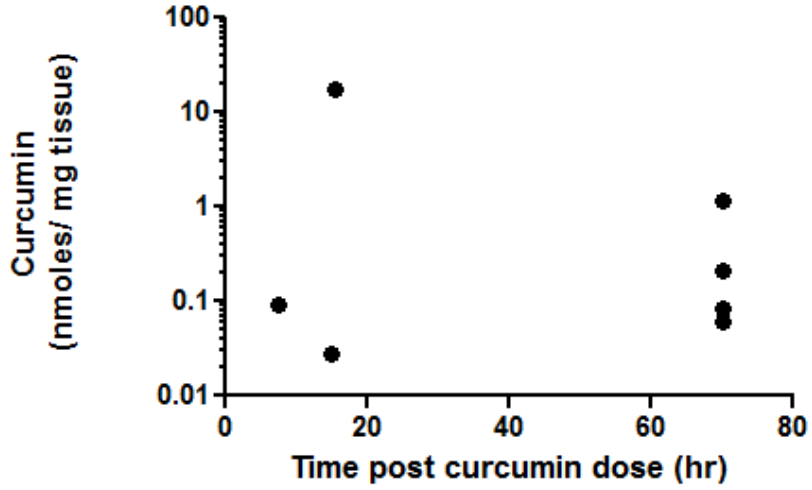

Figure 32. Curcumin concentrations from vaginal tissue. Curcumin levels were determined in vaginal tissue samples by LC/MS and the results expressed in terms of nmoles per mg of dry tissue weight. Each point corresponds to 1 rabbit. Curcumin values were plotted according to time of sampling post curcumin application. For rabbits that succumbed overnight, the median time between time left and time found was plotted. The lower limit of quantification was 0.15 nmoles/tissue section.

Rabbit	*Treatment*	*Time of Sample (hrs. post-Curcumin)*	*Vaginal Tissue Curcumin (nmol/mg)*
4	Curcumin	70 hr	0.210
5	Curcumin	8-22 hr	0.027
6	Curcumin	70 hr	0.083
12	Curcumin	70 hr	1.15
13	Curcumin	70 hr	UD
14	Curcumin	7.5 hr	0.090
15	Curcumin	70 hr	0.062
16	Curcumin	9-22 hr	17.6

Table 14. Curcumin concentrations on and within rabbit vaginal tissue. Time of sample is hrs post curcumin. When the exact time was not known due to the rabbits dying overnight, a range of time is given. Weight of tissue is dry weight of tissue measured after lyophilization. LC/MS assay, with lower limit of quantification of 0.15 nmoles/ tissue section, was used to determine curcumin amounts.

IV. Discussion

Curcumin is a component of the Indian spice Turmeric (*Curcuma longa*) that has anti-inflammatory, anti-angiogenesis, and antimicrobial properties [166-168]. In this study, curcumin was evaluated for its potential to prevent TSS. TSST-1 up-regulates the NF-κB pathway, induces cytokines from epithelial cells, and is responsible for TSS. Curcumin was chosen as an anti-TSS agent because it regulates a wide range of cellular pathways, including the activation and production of cytokines, cellular enzymes, growth factors, receptors, transcriptional factors, protein kinases, and adhesion molecules [167, 173]. The inhibitory effects of curcumin on SAg-induced changes on immune cells have been described, where SEA- and SEB-induced cytokines from PBMC and SE-induced fever in rabbits were inhibited by curcumin [156].

In this study, curcumin inhibited *S. aureus* induced IL-8 from porcine vaginal explants. Curcumin reduced IL-8 concentrations to below background when explants were stimulated with live bacteria or an exoprotein mixture from an overnight *S. aureus* culture, which contained SAgs (SEC and TSST-1) and the hemolysin, α-toxin. The doses of curcumin that were effective in reducing IL-8 were at least 100-fold lower than doses that were toxic to the tissue. The maximum possible dose of curcumin per explant due to solubility limits was 1340 nmol, which was not toxic. A vaginal application of curcumin significantly protected rabbits from TSS. The dose of curcumin used in the rabbit model (0.53 μmoles/cm^2) was higher than the minimum effective dose to inhibit *S. aureus* exotoxin induction of IL-8 in the porcine tissue model (13.5 nmoles or 0.17 μmoles/cm^2). The dose administered in the rabbit model was also much lower (97% lower) than the

highest dose tested in the porcine tissue toxicity model (1350 nmoles or 17 μmoles/cm^2) suggesting that doses of curcumin used in the animal experiment are safe for vaginal application.

Curcumin did not consistently or significantly reduce bacterial load in the porcine tissue model compared to vehicle controls that contained DMSO (10%). Doses of 135 nmoles (1.7 μmoles/cm^2) and 67.5 nmoles (0.85 μmoles/cm^2) minimally and non-significantly reduced bacterial load in the porcine tissue model. These doses were much higher (~2 fold) than the dose used in the rabbit TSS experiments suggesting that the amount of curcumin within the rabbit vaginal lumen is unlikely to have antibacterial effects. Previous studies describe the ability of curcumin to inhibit the growth of several bacteria including *Staphylococcus*, *Lactobacillus, Streptococcus, Enterococcus, Escherichia, and Bacillus* [174, 188-191]. These studies were conducted in broth culture, which does not mimic the natural state of most infections, i.e. a biofilm. During biofilm growth, the bacteria attach to a surface and produce a polysaccharide that can protect the bacteria from the host's immune system and antibiotics [192]. Bacteria in biofilms are more resistant to antibiotics compared to bacteria growing in a planktonic state [193].

Normal flora of the vagina such as *Lactobacillus* plays an important role in the health of the host and prevents the growth of pathogenic organisms. It is important that a vaginal microbicide or anti-inflammatory therapy have minimal effects on the normal flora as well as be non-irritating or toxic to the vaginal mucosa [246]. Based on the findings of this study, I predict that curcumin in amounts that are therapeutic in inhibiting

the proinflammatory response would be too low to have an effect on the vaginal flora, but further experimentation is needed.

Previous studies have demonstrated that TNF-α can be detected in the serum of rabbits that were tested in a TSST-1 potentiated TSS model [64]. The amounts of TNF-α in these rabbits varied widely in these studies, between 700-7,000 ng/ml, depending on the dose of LPS and TSST-1. These studies likely had a wide variation between animals as reported SEM were as large as 50% of the mean value. This variation was also observed in the current study where not all rabbits that died had detectable levels of TNF-α while others had values as high as 3,700 ng/ml. One possible reason for the variation was that TNF-α degraded between the time of death and time of serum/tissue processing. Some rabbits died overnight and were not processed until the following morning. All animals that succumbed to TSS and had undetectable TNF-α had died overnight, though not all animals that died overnight had undetectable TNF-α. Since the rabbits were not monitored throughout the night it is impossible if degradation occurred.

Unlike previous studies with this model, TNF-α was measured in vaginal tissue as a potential biomarker of the proinflammatory effects of TSST-1 and the anti-inflammatory effects of curcumin. In these studies, a similar variation in vaginal tissue TNF-α levels was also observed. TNF-α was only detected in vaginal tissue in rabbits that had detectable levels in serum. There did not appear to be a correlation between TNF-α levels within the serum and vaginal tissue (r^2 0.0181, Figure 36 appendix). Due to the large variation in TNF-α levels and the small sample size, it was difficult to draw any definitive conclusions about TNF-α levels in curcumin-treated versus untreated rabbits.

However, TNF-α was undetectable from serum or vaginal tissue of all curcumin-treated rabbits that survived at the time of testing.

The ability of curcumin to prevent tissue disruption due to effects of TSST-1 was also assessed. There have been no other published reports describing the effects of TSST-1 in this model on rabbit vaginal tissue. Histological reports indicated that inflammation was seen equally throughout the samples. There was no apparent relationship between tissue disruption between treatments, rabbit survival, tissue TNF-α levels, or time of death on tissue disruption. All rabbits that succumb to TSS died within 20 hr, which may be insufficient time to cause significant tissue disruption. Other markers such as staining for immune cell markers will be done in the future to determine if there is any difference in tissue inflammation and disruption between treatment groups.

Curcumin was measured in serum, vaginal, and liver tissues to determine the concentrations of curcumin that remained in the tissue as well as potential systemic exposures. There was no detectable curcumin in serum or liver, which was expected based on the rapid metabolism of curcumin [186]. Curcumin was detectable on and within the vaginal tissue in 7 of 8 rabbits that were given curcumin (13.2 to 0.8% of the original dose at 12.6 to 70 hr, respectively). There was wide variation in curcumin levels between animals. It is unknown why vaginal curcumin was lower in 2 of 3 rabbits that succumbed to TSS at the early time points compared to those that survived. It is possible that the low vaginal curcumin levels were the reason these animals died while other curcumin-treated rabbits survived. In the animals that succumbed the curcumin levels

may have been below an effective concentration to reduce local inflammation, which resulted in progression of TSS.

The observed amounts of curcumin in tissue were higher than that was seen in other studies. In one study, ~1% and ~16% of administered curcumin dose was seen in liver and intestinal tissue, respectively, 1 hr after an intraperitoneal curcumin application [247]. Even lower amount of oral curcumin dose was seen in another study, ~0.1% and 3% in liver and intestinal tissue, respectively, 24 hr after dosing [184]. A pharmacokinetic (PK) study needs to be conducted to completely understand the PK profile of curcumin after an intravaginal application and determine the minimal effective amount of curcumin within vaginal tissue to prevent TSS.

Conclusions from the secondary endpoints (TNF-α, curcumin levels, and histological changes) are limited by the study design. All tissue samples from these assays were taken when the animals died or were euthanized. Samples from rabbits that survived were taken when the animals were euthanized at the end of the experiment, which was 66 hr after LPS treatment. This long delay could allow TNF-α levels to return to normal (if they were elevated) and curcumin to be cleared from the body. In this study, we were limited in blood draws we were able to take by IACUC protocols to prevent unnecessary stress to the animals. In future studies, curcumin-treated rabbits will be euthanized at similar time points when untreated rabbits die, to allow between group comparisons. An additional limitation of this study is the histological difference between rabbit and human vaginal mucosa. The rabbit vagina is lined with a singular layer of columnar epithelial cells while the human and porcine vagina is lined with multiple layers of squamous

epithelial cells [243]. The rabbit model may not be the perfect model for interactions with human vaginal epithelial cells, but still has been used in multiple studies for preliminary studies before expensive primate studies or clinical trials [158, 244].

The findings from this study correlate well with the findings other studies which showed that curcumin was able to reduce the proinflammatory response to infections. Di Mario *et al.*, which investigated the use curcumin for treatment of *H. pylori*, a common cause of gastric ulcers [201]. Curcumin (30 mg) was given two times daily for seven days in combination with several other non-antibiotic agents (zinc acetate, bovine lactoferrin, and lysolecithin). Twenty-five *H. pylori* infected patients were assessed at baseline and two months after completion of the seven day therapy for eradication of *H. pylori* infections and symptoms of gastric inflammation. Curcumin did not significantly reduce *H. pylori* infections, although there was a significant decrease in the symptoms of gastric inflammation [201].

Similar results were also observed in a recent study that demonstrated oral administration of curcumin was able to inhibit *Klebsiella pneumonia*-induced local TNF-α release, neutrophil infiltration into lung tissue, and diminished the production of proinflammatory proteins in a murine lung infection model. The reduction in tissue inflammation and related proinflammatory biomarkers were observed histologically and biochemically, and without reductions in bacterial loads. However, when curcumin was co-administered with Augmentin (which contains amoxicillin and clavulanic acid) a reduction in bacterial counts and inflammation was seen [199]. In this study, there was no mention of outcomes of the infection, so it is not known whether curcumin reduced

lethality in this model. This study and our study suggest that curcumin is able to inhibit the proinflammatory effects of an infectious agent and reduce or prevent clinical disease with or without co-administration of antimicrobials.

Chapter 6- Final Conclusions and Future Directions

I. Final Conclusions

S. aureus is a significant human pathogen that causes a wide range of diseases including TSS. SAgs, including TSST-1, are proinflammatory exotoxins that disrupt the host's immune response to *S. aureus* infections. TSST-1 acts as a bridge to cross-link CD4+ T cells and macrophages to each other non-antigen specifically thus stimulating them to cause a cytokine storm [1]. Epithelial cells are the first cell type exposed to TSST-1 during TSS disease progression, so understanding the interactions between TSST-1 and the vaginal mucosa are critical to the development of anti-TSS therapeutic strategies. Previous studies described proinflammatory responses that included increases in cytokines and chemokines by epithelial cells derived from different anatomical sites in response to SAg [2, 3, 5]. The proinflammatory response generated by TSST-1 within the epithelial tissue was hypothesized to be critical in disease progression. The effects of TSST-1 on vaginal epithelial cells are poorly understood and were the focus of my thesis.

The studies in this thesis describe the ability of TSST-1 to up-regulate HVEC cytokines and chemokines via the NF-κB pathway independent of MHC II (Chapter 2). Several studies have demonstrated that SAg induce NF-κB in PBMC, but the ability of SAg to up-regulate the NF-κB pathway in epithelial cells had not been studied previously [154, 156].

The role of NF-κB and other proinflammatory pathways in SAg-mediated disease was investigated by inhibiting the NF-κB pathway in HVEC with curcumin (Chapter 5). Curcumin is a component of the Indian spice Turmeric that has anti-inflammatory, anti-angiogenesis, and anti-microbial properties [166-168]. Previously, curcumin was

determined to inhibit SEA- and SEB- induced cytokines from PBMC and reduce SE-induced fever in rabbits [156]. In an *ex vivo* porcine vaginal tissue model, curcumin inhibited *S. aureus* and corresponding exoproteins abilities to increase IL-8 secretion from the vaginal mucosal. This inhibition of IL-8 from vaginal tissue was independent of curcumin's ability to inhibit bacterial growth. Furthermore, curcumin inhibited IL-8 to levels below background regardless of being applied to the mucosal surface before or after *S. aureus*. The importance of the epithelial proinflammatory response induced by SAg was tested *in vivo* in a rabbit model of TSS. TSST-1 was administered intravaginally followed by LPS administered IV 4 hr later. TSST-1 potentates the toxicity of LPS the combination and is 100% fatal if left untreated [219-221]. Curcumin co-administered with TSST-1 intravaginally significantly reduced the lethality of TSST-1 (60% survival) and all surviving, curcumin treated animals had no detectable TNF-α in the vaginal mucosa or in the bloodstream suggesting that the proinflammatory response induced by TSST-1 from the epithelial cells is critical for disease progression. These findings are supported by another study where GML was able to inhibit cytokines induced from HVEC by SAg and protected rabbits from TSS using the same animal model [158, 160, 162, 163].

The epithelial receptors responsible for SAg-induced cytokine production remain to be identified. Several studies have suggested MHC II as the epithelial receptor for SAg. Primary bronchial epithelial cell induction of cytokines following exposure to TSST-1 was MHC II dependent, where antibodies against MHC II prevented TSST-1 binding to bronchial epithelial cells [3]. Studies described in Chapter 2 demonstrate that HVEC

make IL-8 in response to TSST-1 without MHC II being expressed on HVEC, suggesting that MHC II is unlikely to be the receptor on HVEC.

The presence of a non-MHC II epithelial receptor for SAg has also been proposed in other studies [7, 140-143, 145, 204]. A conserved dodecapeptide region of SAgs, independent of MHC II and T-cell receptor binding domains [140-142], has been suggested as an epithelial binding domain. This conserved dodecapeptide region was important for SAg transcytosis across human colonic epithelial cell monolayers [145]; and a synthetic dodecapeptide peptide derived from SEB and corresponding to TSST-1 position 119 to 130 was able to antagonize IL-8 induction in HVEC by TSST-1 [2]. We explored the role of this region in TSST-1-epithelial interactions by mutating residues within this region [positions 119 to 130] (Chapter 3). Importantly, a single amino acid mutation within this domain (D130A) significantly reduced the ability of TSST-1 to induce IL-8 from HVEC and to cause TSS when administered vaginally in a rabbit model. These data suggested that the TSST-1 dodecapeptide region is important for interactions with epithelial cells both *in vitro* and *in vivo*.

These studies identified residues on TSST-1 that are needed for the induction of proinflammatory cytokines from HVEC that are independent of MHC II, and strongly support the presence of a non-TCR or MHC II receptor for SAg on epithelial cells. CD40 was chosen as a possible HVEC receptor for TSST-1 on HVEC based on a study that found CD40 worked synergistically with MHC II to induce cytokines in monocytes in response to SAgs [139]. CD40's role was investigated by knocking down CD40 in HVEC by shRNA plasmids (Chapter 4). The CD40 knocked down HVEC (~50% knock

down) produced more IL-8 in response to TSST-1 and bound more TSST-1 than control HVEC suggesting that CD40 is not the epithelial receptor for SAgs.

These studies focused on SAg interactions with epithelial cells and therapies targeted to prevent the effects of these interactions. As I and others have demonstrated, SAgs induce a proinflammatory response from epithelial cells [2, 3, 5]. This proinflammatory response is critical for TSS disease progression as when this local response is inhibited progression to clinical disease is inhibited. The local proinflammatory response can be inhibited by mutations in the toxin (toxoids), which render the toxin (D130A TSST-1, Chapter 3) unable to induced cytokines from epithelial cells, or the inhibition of cytokine production by epithelial cells using anti-inflammatory therapeutic agents (curcumin and GML, Chapter 5 [158]). The importance of local vaginal inflammation in disease progression was reported recently in a study where a GML-containing gel inhibited Simian immunodeficiency virus (SIV) (a model for HIV) vaginal transmission in a rhesus macaque model by blocking the proinflammatory response of the cervical and vaginal mucosa to SIV infection [160].

Local inflammation and tissue disruption by TSST-1 was part of my central hypothesis. In Chapter 5 all 10 rabbits that were evaluated histologically had equal amount of tissue disruption and inflammation within the tissue after exposure to TSST-1. This inflammation was equally seen rabbits that were treated with curcumin and left and untreated. Initially this data may not support my central hypothesis, as it would be expected that all rabbits that succumb to TSS would have tissue disruption and curcumin-treated rabbits that survived would not have signs of inflammation. One possible

explanation for this finding is the H & E staining is not sensitive enough to determine relative amounts of tissue disruption and inflammation. In future studies, other techniques such as immunohistochemical staining of immune cells markers will be used to differentiate the amount of inflammation in different tissues.

The epithelial receptor(s) for SAg, which lead to proinflammatory effects in vaginal epithelial cells, remain to be identified. MHC II has been suggested as a possible epithelial receptor for SAgs [3, 5]. Multiple findings in this thesis suggest that MHC II is not responsible for TSST-1 induced cytokine production from HVEC. Studies in Chapter 2 demonstrated that HVEC do not express MHC II unless stimulated with IFN-γ. If MHC II was the HVEC receptor, I would expect HVEC to express MHC II in the absence of IFN-γ. Studies in Chapter 3 further suggested that MHC II is not the HVEC receptor. By mutating a single residue within TSST-1 (D130A) the HVEC IL-8 response was significantly reduced compared to WT TSST-1. This region is known to be independent of regions that interact with MHC II suggesting a non-MHC II receptor for TSST-1 on HVEC.

Limitations to these studies have been outlined in previous chapters. These limitations include the use of an HVEC immortalized cell line containing HPV genes, which could affect the overall findings as viral onco-genes (E6 and E7) can disrupt the normal response of cells to stimuli. Another specific limitation in Chapter 5 was the inability to compare the secondary endpoints (i.e., TNF-α and curcumin in vaginal mucosa and serum) between curcumin-treated rabbits that survived versus controls since samples were obtained at different time points. Samples from rabbits that survived the

experiment were taken at the end of the study when the rabbits were euthanized (66 hr post LPS) versus rabbits that succumbed to TSS (controls and curcumin treated) were obtained at ~3 to 24 hr post LPS.

One global limitation to this thesis is that the proinflammatory response of TSST-1 was investigated independent of other *S. aureus* exotoxins or live *S. aureus* as *in vivo S. aureus* produces multiple proteins (exotoxins) that induce proinflammatory responses from epithelial cells. These proinflammatory proteins, especially alpha toxin, function in concert with TSST-1 to induce a proinflammatory response that is critical in TSS disease progression [209]. This limitation was addressed in Chapter 5, when *S. aureus* exoproteins were used to stimulate *ex vivo* porcine vaginal tissue. This exoprotein mixture contained multiple proinflammatory proteins, including cytotoxins and superantigens [19], which caused an increase in IL-8 secretion from vaginal tissue that was inhibited by curcumin.

The studies completed as part of my thesis are important for the understanding of SAg interactions with epithelial cells. Little is known about the interactions of SAg and epithelial cells despite epithelial cells being the first cell type that is exposed to TSST-1. The SAg-epithelial interactions explored in my thesis include the identification of signaling pathways induced in response to TSST-1, epithelial receptor(s) for TSST-1, and residues of TSST-1 that interact with epithelial cells. Studies to identify these components were completed with the goal of generating new insights for the development of new therapies to prevent SAg-mediated diseases. These treatments will most likely be used in combination with anti-bacterial agents to counteract the bacteria on

several fronts. One route of novel therapies will focus on inhibiting the host response to SAg. Curcumin, which was investigated in this thesis, is one example of a therapy that targets the host response to SAg-mediated diseases. Identification of the epithelial receptor and residues on the SAg that interact with the receptor will lead to novel therapies that can prevent (antagonize) SAg interactions with epithelial cells. By preventing SAg interactions with the epithelial cell, I predicate that disease progression to TSS will be inhibited.

My central hypothesis described TSST-1 binding to specific receptors on vaginal epithelial cells and triggering a proinflammatory response that recruits immune cells to the area. This proinflammatory effect disrupts the mucosal layer and allows progression to TSS. Prevention of the proinflammatory response is a proposed mechanism for therapeutics to treat SAg-mediated diseases. (A model to describe this overall approach is presented in Figure 8.) My findings have ruled out MHC II and CD40 as epithelial receptors for TSST-1. My findings have determined that NF-κB is activated in response to TSST-1 and is likely responsible for cytokine production. Curcumin, an inhibitor of proinflammatory pathways, including NF-κB, was able to inhibit exoprotein-induced cytokine production. Curcumin also prevented TSS in a rabbit model potentially highlighting the importance of local inflammation in progression to TSS, supporting my central hypothesis. Inhibiting this local inflammation is a promising new approach of therapy for treatment of SAg-mediated diseases. An updated model of my central hypothesis can be seen in Figure 33.

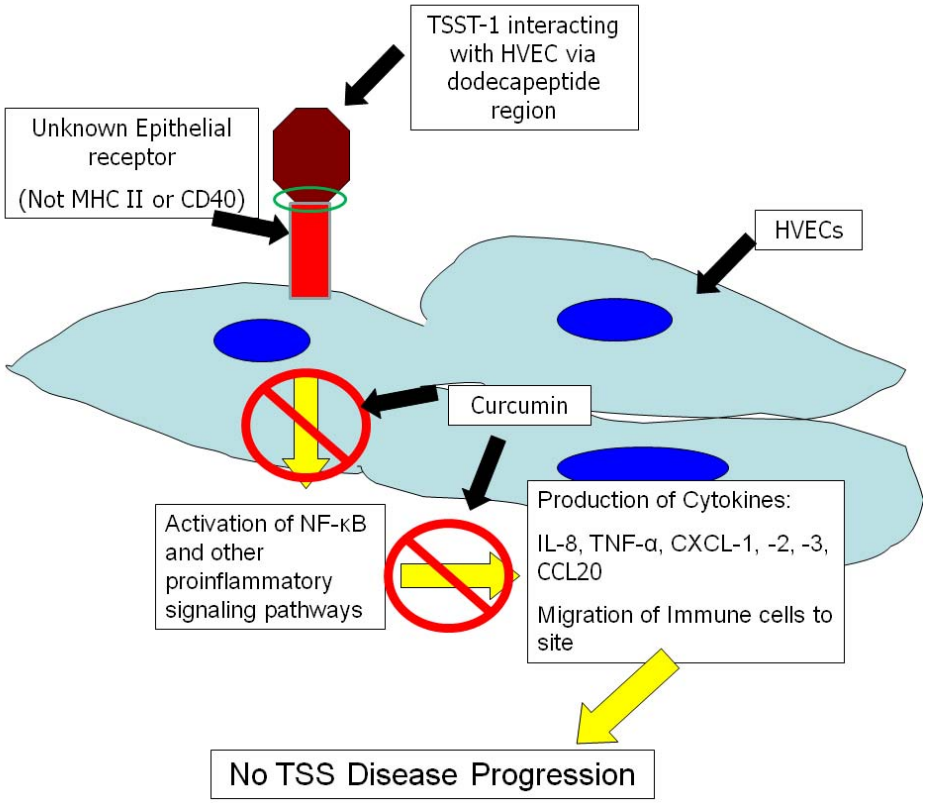

Figure 33. Updated Model of TSST-1 effects on vaginal epithelium. *S. aureus* produces exotoxins including TSST-1 within the vaginal lumen. TSST-1 binds to an unknown receptor on HVEC (not MHC II or CD40) via the dodecapeptide region on TSST-1, which leads to the activation of NF-κB and possibly other proinflammatory pathways. These pathways induce the expression of cytokines, which lead to the migration of immune cells (including macrophages and T cells) to the site of infection, which leads to progression of TSS. Curcumin is able to inhibit cytokine production and prevent disease likely by inhibiting proinflammatory pathways including NF-κB. Without local proinflammatory effects of TSST-1 on vaginal epithelial cells progression to TSS is inhibited.

II. Future Experiments

The results described in this thesis are the first steps in a full understanding of SAg interactions with epithelial cells. More studies are needed to fully understand SAg-epithelial interactions for novel therapies to be developed. This future work includes further characterization of TSST-1 induced proinflammatory pathways and identification of the HVEC receptor for TSST-1. Additional work will also include further development of curcumin as an anti-SAg therapy.

a. Characterization of TSST-1 induced proinflammatory pathways

These studies identified NF-κB involvement in the TSST-1-induced cytokine response from HVEC; however, upstream elements remain to be elucidated. There are multiple different adaptor proteins that are upstream of NF-κB. Identification of these proteins can lead to discovery of drug targets and identification of the epithelial receptor for SAg. Small interfering RNA (siRNA) NF- κB pathway gene arrays and an NF- κB luciferase reporter will be used to identify HVEC-signaling proteins that are important in TSST-1 induced NF-κB activation. The NF- κB reporter described in Chapter 2 is an effective tool for measuring NF- κB activation in response to stimuli. In these experiments, signaling proteins would be knocked down with siRNA and potential proteins of interest will be identified as they will have loss of NF-κB activation in response to TSST-1. The role of these proteins will be confirmed by Western blotting for activated (phosphorylated) proteins in response to TSST-1 exposure.

b. Identification of HVEC receptor for TSST-1

Identification of the HVEC receptor for TSST-1 remains an important aim that warrants future study. Potential receptors will be pulled down by use of His-Tagged TSST-1. Vectors for expression of a His-tagged TSST-1 have been constructed and recombinant protein expressed and purified. His-TSST-1 will be exposed to HVEC or HVEC lysate, and His-TSST-1/receptor complexes will be pulled down with nickel columns. Identification of the isolated epithelial receptors will be completed using mass spectrometry (time of flight). Candidate receptors will be evaluated in a similar manner to the approaches used with CD40 (Chapter 4) with studies to knock down candidate receptors and studies to determine corresponding proinflammatory responses to TSST-1.

Major histocompatibility complex class I molecules (MHC I), is a receptor that should be further investigated. MHC I was identified as a SAg receptor on MHC II deficient macrophages with anti-MHC I monoclonal antibodies that caused a reduction in IL-6 secretion in response to SEA and SEB [248], and later studies demonstrated SEA and SEC bound directly to MHC I [249, 250]. However a limited role of MHC I in TSS was supported by Stiles *et al.*, where MHC I knockout mice showed intermediate susceptibility to SEA (30% lethality) compared to wild type mice (93% lethality) and MHC II knock-out mice (resistant) [251]. Based on these findings MHC I is worth evaluating despite Aubert *et al.* findings that antibodies against MHC I did not block TSST-1 induced cytokine production in IFN-γ pretreated bronchial cells [3]. Of interest, MHC I was noted to be up-regulated (>2-fold) in both HVEC lines (ATCC and

University of Iowa) in response to TSST-1, as measured by microarray (unpublished data)[2].

c. Development of curcumin as a host-targeted anti-TSS therapeutic agent

Curcumin showed promise as a novel therapeutic agent by preventing TSS in a rabbit vaginal TSS model. In this study, DMSO (3% in water) was used as a vehicle for curcumin, since curcumin is not soluble in water alone. Future studies will evaluate other vehicles for their ability to deliver an increased amount of curcumin to the mucosal epithelial tissue. One vehicle that will be tested is the gel, KY® Warming™, which is comprised of propylene glycol, polyethylene glycol, and cellulose. This product does not contain water and curcumin should be soluble in this gel allowing a higher dose of curcumin to be delivered in a safe manner.

Another method of curcumin delivery that will be tested will be microparticles embedded with curcumin. Microparticles are small (μmeters in diameter) biodegradable molecules that are able to persist in the body for days to years and are able to elute drug reducing the need for multiple dosing [252]. There have been several studies, conducted by our collaborator Dr. Panyam, Department of Pharmaceutics, University of Minnesota, where microparticles were used to increase the exposure of curcumin in animal models [245, 253]. These studies have focused on applications of curcumin to treat cancer or inflammatory bowel disease. After curcumin administered by application with microparticles, curcumin was detected within the blood of a mouse multiple days after IV administration. A similar application of free curcumin resulted in curcumin

concentrations falling to undetectable levels in the serum several hr after application. A vaginal application of curcumin coated particles should include immediate- and controlled-released curcumin microparticles to have immediate and sustained effects on the vaginal mucosa after a single dose, which might be administered once or twice per week.

The experiments described in Chapter 5 were not a PK study, so a PK study needs to be completed to understand the kinetics of a vaginal dose of curcumin and determine the minimal effective of curcumin to prevent TSS. Once a formulation of curcumin has been chosen, a PK study can be conducted. In these experiments multiple rabbits will be given curcumin vaginally without TSST-1. At various time points multiple rabbits will be sacrificed and tissue (vaginal and liver) and serum will be collected. Curcumin levels will be measured by LC /MS, from which a complete PK profile can be determined. As part of the PK studies, the maximum tolerated dose of curcumin needs to be determined by dose escalation studies. Curcumin coated microparticles can be administered IV or subcutaneously for a more systemic exposure to curcumin, which may be beneficial for treatment of other infectious and SAg-mediated diseases. Other infectious diseases that curcumin can be evaluated for treatment include: pneumonia, respiratory TSS, skin infections, and *Staphylococcal* food-borne poisoning. Treatments in humans with curcumin will likely include treatment with antibiotics, so these studies should include a treatment arm that receives curcumin with appropriate antibiotics for that disease.

Chapter 7- Other Publications and Presentations Related to this Work

I. Manuscripts

Glycerol monolaurate and dodecylglycerol effects on *Staphylococcus aureus* and toxic shock syndrome toxin-1 *in vitro* and *in vivo*. Lin YC, Schlievert PM, Anderson MJ, Fair CL, **Schaefers MM**, Muthyala R, Peterson ML. *PLoS One.* 2009 Oct 19;4(10):e7499.

Abstract.

Background: Glycerol monolaurate (GML), a 12 carbon fatty acid monoester, inhibits *Staphylococcus aureus* growth and exotoxin production, but is degraded by *S. aureus* lipase. Therefore, dodecylglycerol (DDG), a 12 carbon fatty acid monoether, was compared *in vitro* and *in vivo* to GML for its effects on *S. aureus* growth, exotoxin production, and stability.

Methods: Antimicrobial effects of GML and DDG (0 to 500 μg/ml) on 54 clinical isolates of *S. aureus*, including pulsed-field gel electrophoresis (PFGE) types USA200, USA300, and USA400, were determined *in vitro*. A rabbit Wiffle ball infection model assessed GML and DDG (1 mg/ml instilled into the Wiffle ball every other day) effects on *S. aureus* (MN8) growth (inoculum $3x10^8$ CFU/ml), TSST-1 production, TNF-α concentrations and mortality over 7 days.

Results: DDG (50 and 100 μg/ml) inhibited *S. aureus* growth *in vitro* more effectively than GML ($p<0.01$) and was stable to lipase degradation. Unlike GML, DDG inhibition of TSST-1 was dependent on *S. aureus* growth. GML-treated (4 of 5; 80%) and DDG-treated rabbits (2 of 5; 40%) survived after 7 days. Control rabbits (5 of 5; 100%)

succumbed by day 4. GML suppressed TNF-α at the infection site on day 7; however, DDG did not (<10 ng/ml versus 80 ng/ml, respectively).

Conclusions: These data suggest that DDG was stable to *S. aureus* lipase and inhibited *S. aureus* growth at lower concentrations than GML *in vitro*. However, *in vivo*, GML was more effective than DDG by reducing mortality, and suppressing TNF-alpha, *S. aureus* growth and exotoxin production, which may reduce toxic shock syndrome. GML is proposed as a more effective anti-staphylococcal topical anti-infective candidate than DDG, despite its potential degradation by *S. aureus* lipase.

Reduction in *Staphylococcus aureus* growth and exotoxin production and in vaginal interleukin 8 levels due to glycerol monolaurate in tampons. Strandberg KL, Peterson ML, **Schaefers MM,** Case LC, Pack MC, Chase DJ, Schlievert PM. *Clin Infect Dis.* 2009 Dec 1;49(11):1711-7.

Abstract.

Background: Staphylococcal menstrual toxic shock syndrome depends on vaginal production of exotoxins. Glycerol monolaurate (GML) inhibits *Staphylococcus aureus* exotoxin production *in vitro*. The purpose of this study was to determine whether GML, as a tampon fiber finish, inhibits production of exotoxins and the cytokine interleukin 8 (IL-8) during normal tampon use.

Methods: On day 2 of menstruation, when vaginal *S. aureus* counts are high in colonized women, study participants exchanged their own preferred tampons, after wearing them for 2-6 hr, for study tampons with or without GML (assigned randomly and blindly), which they then wore for 4-6 hr. The women's own tampons and the study tampons with or without GML were assayed for *S. aureus*, the exotoxins TSST-1 and alpha-toxin, and IL-8.

Results: A total of 225 women completed the study. *S. aureus* was present in the tampons of 41 women (18%). Lower numbers of *S. aureus* and the exotoxins were detected in study tampons with or without GML than in women's own tampons; lower amounts of the exotoxins were present in study tampons with GML than study tampons without GML. The IL-8 level was lower in tampons from women without vaginal *S.*

aureus compared with women with *S. aureus* and was lower in study tampons with GML than in study tampons without GML.

Conclusions: Tampons that contain GML reduce *S. aureus* exotoxin production. *S. aureus* increases vaginal IL-8 levels, and GML reduces production of this proinflammatory cytokine. These results suggest that GML added to tampons provides additional safety relative to mTSS as well as benefits for vaginal health generally, thus supporting the addition of GML to tampons.

II. Posters and Oral Presentations

Role of Major Histocompatibility Complex Class II in Toxic Shock Syndrome Toxin-1 Induced Cytokine Production from Human Vaginal Epithelial Cells. **Schaefers, M.M.**, Breshears, L.M., Schlievert, P.M., Peterson,M.L. American Society for Microbiology, 120th General Meeting. San Diego, CA. May 23-27, 2010. [Poster]

Staphylococcal Superantigen Interactions with Human Vaginal Epithelial Cells. Tech Forum Seminar. 3M in St. Paul MN. October 27, 2009. [Podium].

NF-κB pathway Involved in Staphylococcal Toxic Shock Syndrome Toxin-1 Induction of Cytokines from Vaginal Epithelium. Peterson, M.L., **Schaefers,M.M.,** Schlievert, P.M. Women's Health Research Conference 2009. University of Minnesota. September 21, 2009. [Poster].

Superantigens Induce MHC class II Independent, NF-κB Dependent Cytokine Production in Human Vaginal Epithelial Cells. **Schaefers, M.M**., Anderson, M.J.., Schlievert, P.M., Peterson, M.L**.** 14th International Congress of Mucosal Immunology, Boston, MA. July 5-9, 2009. [Poster].

Staphylococcal Infections at Mucosal Surfaces. Experimental & Clinical Pharmacology Seminar. University of Minnesota, March 11, 2009. [Podium].

Toxic Shock Syndrome Toxin-1 Amino Acid Residues Required for Interaction with Human Vaginal Epithelial Cells. A.J. Brosnahan, P.M. Schlievert, **M.M. Schaefers**, M.L. Peterson. American Society for Microbiology, 108th General Meeting. Boston, MA. June 1-5, 2008. [Poster]

Novel approaches to Staphylococcal Vaccination. Experimental & Clinical Pharmacology Seminar. University of Minnesota, April 30, 2008. [Podium]

Toxic Shock Syndrome Toxin-1 binding to CD40 Induces Cytokine Production by Vaginal Epithelial Cells. **M.M. Schaefers**, P.M. Schlievert, M. L. Peterson**.** Gordon Research Conference, Staphylococcal Diseases. Les Diablerets, Switzerland. September 2-7, 2007. [Poster].

Exploration for Epithelial Receptor(s) that Bind *Staphylococcal* Superantigens & Induce Cytokines. **M.M. Schaefers**, P.M. Schlievert, P. Southern, M. L. Peterson.3M Science and Engineering Faculty Day event. 3M Center in St. Paul, MN. June 20, 2007. [Poster]

The Role of CD40 in *Staphylococcal* Superantigen Induced Inflammation of the Vaginal Epithelium. Center for Infectious Diseases and Microbiology Translational Research Seminar. University of Minnesota. November 15, 2006. [Podium]

Novel Receptors & Drug Targets for *Staphylococcus aureus* Toxin Shock Syndrome Toxin. Experimental &Clinical Pharmacology Seminar. University of Minnesota. October 18, 2006. [Podium]

Effects of Dodecylglycerol Against Methicillin-Sensitive and Methicillin-Resistant. C.Fair, **M.M. Schaefers**, D. Simonsen, R. Muthylala, P.M. Schlievert, ML Peterson. *Staphylococcus aureus*. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA. Sept. 27-30, 2006. [Poster]

Studies to Explore Human Vaginal Epithelial Cell Receptors that Bind *Staphylococcal* Toxic Shock Syndrome Toxin-1 and Induce Inflammation. M.L. Peterson, P.M. Schlievert, **M.M. Schaefers**. 12th International Symposium on Staphylococci & Staphylococcal Infections. Maastricht, The Netherlands. Sept. 3 to 6, 2006. [Poster]

Effects of Toxic Shock Syndrome Toxin-1 on Human Vaginal and Cervical Epithelial Cells and the Role of Major Histocompatibility Complex Class II Molecules. **M.M. Schaefers**, A.M. Walz, P. Southern, P.M. Schlievert**,** M.L. Peterson. American Society for Microbiology, 106th General Meeting. Orlando, FL. May 21-25, 2006 [Poster]

Staphylococcus aureus and Toxic Shock Syndrome Toxin Interactions with Mucosal Surfaces of the Female Reproductive Tract. Walz, P. Southern, **M. Schaefers,** M.

Peterson**.** Women's Health Research Conference 2005. University of Minnesota. September 26, 2005. [Poster]

#

References

1. McCormick JK, Yarwood JM, Schlievert PM: **Toxic shock syndrome and bacterial superantigens: an update.** *Annu Rev Microbiol* 2001, **55:**77-104.
2. Peterson ML, Ault K, Kremer MJ, Klingelhutz AJ, Davis CC, Squier CA, Schlievert PM: **The innate immune system is activated by stimulation of vaginal epithelial cells with *Staphylococcus aureus* and toxic shock syndrome toxin 1.** *Infect Immun* 2005, **73:**2164-2174.
3. Aubert V, Schneeberger D, Sauty A, Winter J, Sperisen P, Aubert JD, Spertini F: **Induction of tumor necrosis factor alpha and interleukin-8 gene expression in bronchial epithelial cells by toxic shock syndrome toxin 1.** *Infect Immun* 2000, **68:**120-124.
4. Musch MW, Petrof EO, Kojima K, Ren H, McKay DM, Chang EB: **Bacterial superantigen-treated intestinal epithelial cells upregulate heat shock proteins 25 and 72 and are resistant to oxidant cytotoxicity.** *Infect Immun* 2004, **72:**3187-3194.
5. O'Brien GJ, Riddell G, Elborn JS, Ennis M, Skibinski G: ***Staphylococcus aureus* enterotoxins induce IL-8 secretion by human nasal epithelial cells.** *Respir Res* 2006, **7:**115.
6. Hu DL, Suga S, Omoe K, Abe Y, Shinagawa K, Wakui M, Nakane A: **Staphylococcal enterotoxin A modulates intracellular Ca2+ signal pathway in human intestinal epithelial cells.** *FEBS Lett* 2005, **579:**4407-4412.
7. Brosnahan AJ, Schaefers MM, Amundson WH, Mantz MJ, Squier CA, Peterson ML, Schlievert PM: **Novel toxic shock syndrome toxin-1 amino acids required for biological activity.** *Biochemistry* 2008, **47:**12995-13003.
8. Mushtaq F, Hildrew S, Okugbeni G, Ellis RW, Deshpande S: **Necrotizing haemorrhagic pneumonia proves fatal in an immunocompetent child due to Panton-Valentine Leucocidin, toxic shock syndrome toxins 1 and 2 and enterotoxin C-producing *Staphylococcus aureus*.** *Acta Paediatr* 2008, **97:**985-987.
9. Assimacopoulos AP, Strandberg KL, Rotschafer JH, Schlievert PM: **Extreme pyrexia and rapid death due to *Staphylococcus aureus* infection: analysis of 2 cases.** *Clin Infect Dis* 2009, **48:**612-614.
10. Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, Beach M: **Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999.** *Clin Infect Dis* 2001, **32 Suppl 2:**S114-132.
11. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL: **The role of nasal carriage in *Staphylococcus aureus* infections.** *Lancet Infect Dis* 2005, **5:**751-762.

12. Murray RJ: **Recognition and management of *Staphylococcus aureus* toxin-mediated disease.** *Intern Med J* 2005, **35 Suppl 2:**S106-119.
13. Larkin EA, Carman RJ, Krakauer T, Stiles BG: ***Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire.** *Curr Med Chem* 2009, **16:**4003-4019.
14. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, Jensen BJ, Killgore G, Tenover FC, Kuehnert MJ: **Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004.** *J Infect Dis* 2008, **197:**1226-1234.
15. Miller M, Cook HA, Furuya EY, Bhat M, Lee MH, Vavagiakis P, Visintainer P, Vasquez G, Larson E, Lowy FD: ***Staphylococcus aureus* in the community: colonization versus infection.** *PLoS One* 2009, **4:**e6708.
16. Acton DS, Plat-Sinnige MJ, van Wamel W, de Groot N, van Belkum A: **Intestinal carriage of *Staphylococcus aureus*: how does its frequency compare with that of nasal carriage and what is its clinical impact?** *Eur J Clin Microbiol Infect Dis* 2009, **28:**115-127.
17. Safdar N, Bradley EA: **The risk of infection after nasal colonization with *Staphylococcus aureus*.** *Am J Med* 2008, **121:**310-315.
18. Foster TJ: **Immune evasion by staphylococci.** *Nat Rev Microbiol* 2005, **3:**948-958.
19. Schlievert PM, Strandberg KL, Lin YC, Peterson ML, Leung DY: **Secreted virulence factor comparison between methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, and its relevance to atopic dermatitis.** *J Allergy Clin Immunol* 2010, **125:**39-49.
20. Nizet V: **Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets.** *J Allergy Clin Immunol* 2007, **120:**13-22.
21. Fey PD, Said-Salim B, Rupp ME, Hinrichs SH, Boxrud DJ, Davis CC, Kreiswirth BN, Schlievert PM: **Comparative molecular analysis of community- or hospital-acquired methicillin-resistant *Staphylococcus aureus*.** *Antimicrob Agents Chemother* 2003, **47:**196-203.
22. Durand G, Bes M, Meugnier H, Enright MC, Forey F, Liassine N, Wenger A, Kikuchi K, Lina G, Vandenesch F, Etienne J: **Detection of new methicillin-resistant *Staphylococcus aureus* clones containing the toxic shock syndrome toxin 1 gene responsible for hospital- and community-acquired infections in France.** *J Clin Microbiol* 2006, **44:**847-853.
23. Diep BA, Carleton HA, Chang RF, Sensabaugh GF, Perdreau-Remington F: **Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*.** *J Infect Dis* 2006, **193:**1495-1503.
24. Gordon RJ, Lowy FD: **Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection.** *Clin Infect Dis* 2008, **46 Suppl 5:**S350-359.
25. DeLeo FR, Diep BA, Otto M: **Host defense and pathogenesis in *Staphylococcus aureus* infections.** *Infect Dis Clin North Am* 2009, **23:**17-34.

26. Dinges MM, Orwin PM, Schlievert PM: **Exotoxins of *Staphylococcus aureus*.** *Clin Microbiol Rev* 2000, **13:**16-34.
27. Shinefield HR, Ruff NL: **Staphylococcal infections: a historical perspective.** *Infect Dis Clin North Am* 2009, **23:**1-15.
28. Chambers HF: **The changing epidemiology of *Staphylococcus aureus*?** *Emerg Infect Dis* 2001, **7:**178-182.
29. **National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004.** *Am J Infect Control* 2004, **32:**470-485.
30. Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K: **Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*.** *Antimicrob Agents Chemother* 2001, **45:**1323-1336.
31. Malachowa N, DeLeo FR: **Mobile genetic elements of *Staphylococcus aureus*.** *Cell Mol Life Sci* 2010, **67:**3057-3071.
32. Miller LG, Kaplan SL: ***Staphylococcus aureus*: a community pathogen.** *Infect Dis Clin North Am* 2009, **23:**35-52.
33. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, Johnson SK, Vandenesch F, Fridkin S, O'Boyle C, et al: **Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection.** *JAMA* 2003, **290:**2976-2984.
34. Limbago B, Fosheim GE, Schoonover V, Crane CE, Nadle J, Petit S, Heltzel D, Ray SM, Harrison LH, Lynfield R, et al: **Characterization of methicillin-resistant *Staphylococcus aureus* isolates collected in 2005 and 2006 from patients with invasive disease: a population-based analysis.** *J Clin Microbiol* 2009, **47:**1344-1351.
35. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC: **Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database.** *J Clin Microbiol* 2003, **41:**5113-5120.
36. Said-Salim B, Mathema B, Kreiswirth BN: **Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen.** *Infect Control Hosp Epidemiol* 2003, **24:**451-455.
37. Rybak MJ, LaPlante KL: **Community-associated methicillin-resistant *Staphylococcus aureus*: a review.** *Pharmacotherapy* 2005, **25:**74-85.
38. Li M, Cheung GY, Hu J, Wang D, Joo HS, Deleo FR, Otto M: **Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains.** *J Infect Dis* 2010, **202:**1866-1876.
39. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, et al: **Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States.** *JAMA* 2007, **298:**1763-1771.

40. Boucher HW, Corey GR: **Epidemiology of methicillin-resistant *Staphylococcus aureus*.** *Clin Infect Dis* 2008, **46 Suppl 5:**S344-349.
41. Klein E, Smith DL, Laxminarayan R: **Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005.** *Emerg Infect Dis* 2007, **13:**1840-1846.
42. Noskin GA, Rubin RJ, Schentag JJ, Kluytmans J, Hedblom EC, Jacobson C, Smulders M, Gemmen E, Bharmal M: **National trends in *Staphylococcus aureus* infection rates: impact on economic burden and mortality over a 6-year period (1998-2003).** *Clin Infect Dis* 2007, **45:**1132-1140.
43. Rybak M, Lomaestro B, Rotschafer JC, Moellering R, Jr., Craig W, Billeter M, Dalovisio JR, Levine DP: **Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists.** *Am J Health Syst Pharm* 2009, **66:**82-98.
44. Rybak MJ, Lomaestro BM, Rotschafer JC, Moellering RC, Jr., Craig WA, Billeter M, Dalovisio JR, Levine DP: **Therapeutic monitoring of vancomycin in adults summary of consensus recommendations from the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists.** *Pharmacotherapy* 2009, **29:**1275-1279.
45. Steed ME, Rybak MJ: **Ceftaroline: a new cephalosporin with activity against resistant gram-positive pathogens.** *Pharmacotherapy* 2010, **30:**375-389.
46. Stevens DL, Ma Y, Salmi DB, McIndoo E, Wallace RJ, Bryant AE: **Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*.** *J Infect Dis* 2007, **195:**202-211.
47. Stevens DL RW, Hamilton SM, and Bryant A E. : **Successful Treatment of Staphylococcal Toxic Shock Syndrome with Linezolid: A Case Report and In Vitro Evaluation of the Production of Toxic Shock Syndrome Toxin Type 1 in the Presence of Antibiotics.** *Clinical Infectious Disease* 2006, **42:**729-730.
48. Gemmell CG: **Antibiotics and the expression of staphylococcal virulence.** *J Antimicrob Chemother* 1995, **36:**283-291.
49. Li H, Llera A, Malchiodi EL, Mariuzza RA: **The structural basis of T cell activation by superantigens.** *Annu Rev Immunol* 1999, **17:**435-466.
50. Choi Y, Lafferty JA, Clements JR, Todd JK, Gelfand EW, Kappler J, Marrack P, Kotzin BL: **Selective expansion of T cells expressing V beta 2 in toxic shock syndrome.** *J Exp Med* 1990, **172:**981-984.
51. Fraser JD: **High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR.** 1989, **339:**221-223.
52. Scholl P, Diez A, Geha R: **Staphylococcal enterotoxin B and toxic shock syndrome toxin-1 bind to distinct sites on HLA-DR and HLA-DQ molecules.** *J Immunol* 1989, **143:**2583-2588.

53. White J, Herman A, Pullen AM, Kubo R, Kappler JW, Marrack P: **The V[beta]-specific superantigen staphylococcal enterotoxin B: Stimulation of mature T cells and clonal deletion in neonatal mice.** *Cell* 1989, **56:**27-35.
54. Callahan JE, Herman A, Kappler JW, Marrack P: **Stimulation of B10.BR T cells with superantigenic staphylococcal toxins.** *J Immunol* 1990, **144:**2473-2479.
55. Marrack P, Kappler J: **The staphylococcal enterotoxins and their relatives.** *Science* 1990, **248:**705-711.
56. Jupin C, Anderson S, Damais C, Alouf JE, Parant M: **Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and gamma interferon.** *J Exp Med* 1988, **167:**752-761.
57. Lappin E, Ferguson AJ: **Gram-positive toxic shock syndromes.** *Lancet Infect Dis* 2009, **9:**281-290.
58. Alouf JE, Muller-Alouf H: **Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects.** *Int J Med Microbiol* 2003, **292:**429-440.
59. Schlievert PM, Bettin KM, Watson DW: **Effect of antipyretics on group A streptococcal pyrogenic exotoxin fever production and ability to enhance lethal endotoxin shock.** *Proc Soc Exp Biol Med* 1978, **157:**472-475.
60. Dinges MM, J. Jessurun, and P.M. Schlievert: **Comparisons of mouse and rabbit models of toxic shock syndrome.** *Int Congr Symp Ser* 1998, **229:**167-168.
61. Schlievert PM, Bettin KM, Watson DW: **Reinterpretation of the Dick test: role of group A streptococcal pyrogenic exotoxin.** *Infect Immun* 1979, **26:**467-472.
62. Schlievert PM, Watson DW: **Biogenic amine involvement in pyrogenicity and enhancement of lethal endotoxin shock by group A streptococcal pyrogenic exotoxin.** *Proc Soc Exp Biol Med* 1979, **162:**269-274.
63. Kotzin BL, Leung DY, Kappler J, Marrack P: **Superantigens and their potential role in human disease.** *Adv Immunol* 1993, **54:**99-166.
64. Dinges MM, Schlievert PM: **Comparative analysis of lipopolysaccharide-induced tumor necrosis factor alpha activity in serum and lethality in mice and rabbits pretreated with the staphylococcal superantigen toxic shock syndrome toxin 1.** *Infect Immun* 2001, **69:**7169-7172.
65. Huber BT, Hsu PN, Sutkowski N: **Virus-encoded superantigens.** *Microbiol Rev* 1996, **60:**473-482.
66. Fraser JD, Proft T: **The bacterial superantigen and superantigen-like proteins.** *Immunol Rev* 2008, **225:**226-243.
67. Hurley JM, Shimonkevitz R, Hanagan A, Enney K, Boen E, Malmstrom S, Kotzin BL, Matsumura M: **Identification of class II major histocompatibility complex and T cell receptor binding sites in the superantigen toxic shock syndrome toxin 1.** *J Exp Med* 1995, **181:**2229-2235.
68. Jardetzky TS, Brown JH, Gorga JC, Stern LJ, Urban RG, Chi YI, Stauffacher C, Strominger JL, Wiley DC: **Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen.** *Nature* 1994, **368:**711-718.

69. Kim J, Urban RG, Strominger JL, Wiley DC: **Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1.** *Science* 1994, **266:**1870-1874.
70. Wahlsten JL, Ramakrishnan S: **Separation of function between the domains of toxic shock syndrome toxin-1.** *J Immunol* 1998, **160:**854-859.
71. Altomonte M, Pucillo C, Maio M: **The overlooked "nonclassical" functions of major histocompatibility complex (MHC) class II antigens in immune and nonimmune cells.** *J Cell Physiol* 1999, **179:**251-256.
72. Scholl P, Diez A, Mourad W, Parsonnet J, Geha RS, Chatila T: **Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules.** *Proc Natl Acad Sci U S A* 1989, **86:**4210-4214.
73. Pragman AA, Yarwood JM, Tripp TJ, Schlievert PM: **Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*.** *J Bacteriol* 2004, **186:**2430-2438.
74. Yarwood JM, Schlievert PM: **Quorum sensing in *Staphylococcus* infections.** *J Clin Invest* 2003, **112:**1620-1625.
75. Kass EH, Kendrick MI, Tsai YC, Parsonnet J: **Interaction of magnesium ion, oxygen tension, and temperature in the production of toxic-shock-syndrome toxin-1 by *Staphylococcus aureus*.** *J Infect Dis* 1987, **155:**812-815.
76. Schlievert PM, Blomster DA: **Production of staphylococcal pyrogenic exotoxin type C: influence of physical and chemical factors.** *J Infect Dis* 1983, **147:**236-242.
77. Yarwood JM, Schlievert PM: **Oxygen and carbon dioxide regulation of toxic shock syndrome toxin 1 production by *Staphylococcus aureus* MN8.** *J Clin Microbiol* 2000, **38:**1797-1803.
78. Hill DR, Brunner ME, Schmitz DC, Davis CC, Flood JA, Schlievert PM, Wang-Weigand SZ, Osborn TW: **In vivo assessment of human vaginal oxygen and carbon dioxide levels during and post menses.** *J Appl Physiol* 2005, **99:**1582-1591.
79. Davis JP, Chesney PJ, Wand PJ, LaVenture M: **Toxic-shock syndrome: epidemiologic features, recurrence, risk factors, and prevention.** *N Engl J Med* 1980, **303:**1429-1435.
80. Shands KN, Schmid GP, Dan BB, Blum D, Guidotti RJ, Hargrett NT, Anderson RL, Hill DL, Broome CV, Band JD, Fraser DW: **Toxic-shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases.** *N Engl J Med* 1980, **303:**1436-1442.
81. Todd JK, Kapral FA, Fishaut M, Welch TR: **Toxic shock syndrome associated with phage group 1 staphylococci.** *Lancet* 1978, **2:**1116-1118.
82. Parsonnet J, Hansmann MA, Delaney ML, Modern PA, Dubois AM, Wieland-Alter W, Wissemann KW, Wild JE, Jones MB, Seymour JL, Onderdonk AB: **Prevalence of toxic shock syndrome toxin 1-producing *Staphylococcus aureus* and the presence of antibodies to this superantigen in menstruating women.** *J Clin Microbiol* 2005, **43:**4628-4634.

83. Parsonnet J, Goering RV, Hansmann MA, Jones MB, Ohtagaki K, Davis CC, Totsuka K: **Prevalence of toxic shock syndrome toxin 1 (TSST-1)-producing strains of *Staphylococcus aureus* and antibody to TSST-1 among healthy Japanese women.** *J Clin Microbiol* 2008, **46:**2731-2738.
84. MacDonald KL, Osterholm MT, Hedberg CW, Schrock CG, Peterson GF, Jentzen JM, Leonard SA, Schlievert PM: **Toxic shock syndrome. A newly recognized complication of influenza and influenzalike illness.** *Jama* 1987, **257:**1053-1058.
85. Bergdoll MS, Crass BA, Reiser RF, Robbins RN, Davis JP: **A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome *Staphylococcus aureus* isolates.** *Lancet* 1981, **1:**1017-1021.
86. Schlievert PM, Shands KN, Dan BB, Schmid GP, Nishimura RD: **Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome.** *J Infect Dis* 1981, **143:**509-516.
87. Schlievert PM: **Staphylococcal enterotoxin B and toxic-shock syndrome toxin-1 are significantly associated with non-menstrual TSS.** *Lancet* 1986, **1:**1149-1150.
88. Schlievert PM, Tripp TJ, Peterson ML: **Reemergence of staphylococcal toxic shock syndrome in Minneapolis-St. Paul, Minnesota, during the 2000-2003 surveillance period.** *J Clin Microbiol* 2004, **42:**2875-2876.
89. Osterholm MT, Davis JP, Gibson RW, Mandel JS, Wintermeyer LA, Helms CM, Forfang JC, Rondeau J, Vergeront JM: **Tri-state toxic-state syndrome study. I. Epidemiologic findings.** *J Infect Dis* 1982, **145:**431-440.
90. Gaventa S, Reingold AL, Hightower AW, Broome CV, Schwartz B, Hoppe C, Harwell J, Lefkowitz LK, Makintubee S, Cundiff DR, et al.: **Active surveillance for toxic shock syndrome in the United States, 1986.** *Rev Infect Dis* 1989, **11 Suppl 1:**S28-34.
91. Hajjeh RA, Reingold A, Weil A, Shutt K, Schuchat A, Perkins BA: **Toxic shock syndrome in the United States: surveillance update, 1979 1996.** *Emerg Infect Dis* 1999, **5:**807-810.
92. Descloux E, Perpoint T, Ferry T, Lina G, Bes M, Vandenesch F, Mohammedi I, Etienne J: **One in five mortality in non-menstrual toxic shock syndrome versus no mortality in menstrual cases in a balanced French series of 55 cases.** *Eur J Clin Microbiol Infect Dis* 2008, **27:**37-43.
93. Schlievert PM, Case LC, Strandberg KL, Tripp TJ, Lin YC, Peterson ML: **Vaginal *Staphylococcus aureus* superantigen profile shift from 1980 and 1981 to 2003, 2004, and 2005.** *J Clin Microbiol* 2007, **45:**2704-2707.
94. Hu DL, Omoe K, Inoue F, Kasai T, Yasujima M, Shinagawa K, Nakane A: **Comparative prevalence of superantigenic toxin genes in meticillin-resistant and meticillin-susceptible *Staphylococcus aureus* isolates.** *J Med Microbiol* 2008, **57:**1106-1112.
95. Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, Von Eiff C: **Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins**

among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J Clin Microbiol* 2003, **41:**1434-1439.
96. Baker MD, Acharya KR: **Superantigens: structure-function relationships.** *Int J Med Microbiol* 2004, **293:**529-537.
97. Balaban N, Rasooly A: **Staphylococcal enterotoxins.** *Int J Food Microbiol* 2000, **61:**1-10.
98. Le Loir Y, Baron F, Gautier M: ***Staphylococcus aureus* and food poisoning.** *Genet Mol Res* 2003, **2:**63-76.
99. Cardona ID, Cho SH, Leung DY: **Role of bacterial superantigens in atopic dermatitis : implications for future therapeutic strategies.** *Am J Clin Dermatol* 2006, **7:**273-279.
100. Schlievert PM, Case LC, Strandberg KL, Abrams BB, Leung DY: **Superantigen profile of *Staphylococcus aureus* isolates from patients with steroid-resistant atopic dermatitis.** *Clin Infect Dis* 2008, **46:**1562-1567.
101. Seiberling KA, Grammer L, Kern RC: **Chronic rhinosinusitis and superantigens.** *Otolaryngol Clin North Am* 2005, **38:**1215-1236, ix.
102. Hauk PJ, Wenzel SE, Trumble AE, Szefler SJ, Leung DY: **Increased T-cell receptor vbeta8+ T cells in bronchoalveolar lavage fluid of subjects with poorly controlled asthma: a potential role for microbial superantigens.** *J Allergy Clin Immunol* 1999, **104:**37-45.
103. Muralimohan G, Rossi RJ, Guernsey LA, Thrall RS, Vella AT: **Inhalation of *Staphylococcus aureus* enterotoxin A induces IFN-gamma and CD8 T cell-dependent airway and interstitial lung pathology in mice.** *J Immunol* 2008, **181:**3698-3705.
104. Bachert C, Gevaert P, Howarth P, Holtappels G, van Cauwenberge P, Johansson SG: **IgE to *Staphylococcus aureus* enterotoxins in serum is related to severity of asthma.** *J Allergy Clin Immunol* 2003, **111:**1131-1132.
105. Bachert C, van Zele T, Gevaert P, De Schrijver L, Van Cauwenberge P: **Superantigens and nasal polyps.** *Curr Allergy Asthma Rep* 2003, **3:**523-531.
106. Bachert C, Gevaert P, van Cauwenberge P: ***Staphylococcus aureus* superantigens and airway disease.** *Curr Allergy Asthma Rep* 2002, **2:**252-258.
107. Nolan J, Sinclair R: **Review of management of purpura fulminans and two case reports.** *Br J Anaesth* 2001, **86:**581-586.
108. Kravitz GR, Dries DJ, Peterson ML, Schlievert PM: **Purpura fulminans due to *Staphylococcus aureus*.** *Clin Infect Dis* 2005, **40:**941-947.
109. Matsubara K, Fukaya T: **The role of superantigens of group A *Streptococcus* and *Staphylococcus aureus* in Kawasaki disease.** *Curr Opin Infect Dis* 2007, **20:**298-303.
110. Pinna GS, Kafetzis DA, Tselkas OI, Skevaki CL: **Kawasaki disease: an overview.** *Curr Opin Infect Dis* 2008, **21:**263-270.
111. Krakauer T: **Inhibition of toxic shock syndrome toxin-1-induced cytokine production and T cell activation by interleukin-10, interleukin-4, and dexamethasone.** *J Infect Dis* 1995, **172:**988-992.

112. Kaul R, McGeer A, Norrby-Teglund A, Kotb M, Schwartz B, O'Rourke K, Talbot J, Low DE: **Intravenous immunoglobulin therapy for streptococcal toxic shock syndrome--a comparative observational study. The Canadian Streptococcal Study Group.** *Clin Infect Dis* 1999, **28:**800-807.
113. Valiquette L, Low DE, McGeer AJ: **Assessing the impact of intravenous immunoglobulin in the management of streptococcal toxic shock syndrome: a noble but difficult quest.** *Clin Infect Dis* 2009, **49:**1377-1379.
114. Buonpane RA, Churchill HR, Moza B, Sundberg EJ, Peterson ML, Schlievert PM, Kranz DM: **Neutralization of staphylococcal enterotoxin B by soluble, high-affinity receptor antagonists.** *Nat Med* 2007, **13:**725-729.
115. Yang X, Buonpane RA, Moza B, Rahman AK, Wang N, Schlievert PM, McCormick JK, Sundberg EJ, Kranz DM: **Neutralization of multiple staphylococcal superantigens by a single-chain protein consisting of affinity-matured, variable domain repeats.** *J Infect Dis* 2008, **198:**344-348.
116. Schlievert PM, Case LC, Nemeth KA, Davis CC, Sun Y, Qin W, Wang F, Brosnahan AJ, Mleziva JA, Peterson ML, Jones BE: **Alpha and beta chains of hemoglobin inhibit production of *Staphylococcus aureus* exotoxins.** *Biochemistry* 2007, **46:**14349-14358.
117. Owen JA, Jr.: **Physiology of the menstrual cycle.** *Am J Clin Nutr* 1975, **28:**333-338.
118. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L: **Innate and adaptive immunity in female genital tract: cellular responses and interactions.** *Immunol Rev* 2005, **206:**306-335.
119. Wira CR, Grant-Tschudy KS, Crane-Godreau MA: **Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection.** *Am J Reprod Immunol* 2005, **53:**65-76.
120. Fichorova RN, Trifonova RT, Gilbert RO, Costello CE, Hayes GR, Lucas JJ, Singh BN: ***Trichomonas vaginalis* lipophosphoglycan triggers a selective upregulation of cytokines by human female reproductive tract epithelial cells.** *Infect Immun* 2006, **74:**5773-5779.
121. Fichorova RN, Desai PJ, Gibson FC, 3rd, Genco CA: **Distinct pro-inflammatory host responses to *Neisseria gonorrhoeae* infection in immortalized human cervical and vaginal epithelial cells.** *Infect Immun* 2001, **69:**5840-5848.
122. Fichorova RN, Anderson DJ: **Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells.** *Biol Reprod* 1999, **60:**508-514.
123. Nees M, Geoghegan JM, Hyman T, Frank S, Miller L, Woodworth CD: **Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes.** *J Virol* 2001, **75:**4283-4296.
124. Spitkovsky D, Hehner SP, Hofmann TG, Moller A, Schmitz ML: **The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex.** *J Biol Chem* 2002, **277:**25576-25582.

125. James MA, Lee JH, Klingelhutz AJ: **Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner.** *J Virol* 2006, **80:**5301-5307.
126. van der Bijl P, Thompson IO, Squier CA: **Comparative permeability of human vaginal and buccal mucosa to water.** *Eur J Oral Sci* 1997, **105:**571-575.
127. Squier CA, Mantz MJ, Schlievert PM, Davis CC: **Porcine vagina Ex Vivo as a model for studying permeability and pathogenesis in mucosa.** *J Pharm Sci* 2008, **97:**9-21.
128. Law S, Wertz PW, Swartzendruber DC, Squier CA: **Regional variation in content, composition and organization of porcine epithelial barrier lipids revealed by thin-layer chromatography and transmission electron microscopy.** *Arch Oral Biol* 1995, **40:**1085-1091.
129. Wertz PW, van den Bergh B: **The physical, chemical and functional properties of lipids in the skin and other biological barriers.** *Chem Phys Lipids* 1998, **91:**85-96.
130. Davis CC, Kremer MJ, Schlievert PM, Squier CA: **Penetration of toxic shock syndrome toxin-1 across porcine vaginal mucosa *ex vivo*: permeability characteristics, toxin distribution, and tissue damage.** *Am J Obstet Gynecol* 2003, **189:**1785-1791.
131. Larkin SM, Williams DN, Osterholm MT, Tofte RW, Posalaky Z: **Toxic shock syndrome: clinical, laboratory, and pathologic findings in nine fatal cases.** *Ann Intern Med* 1982, **96:**858-864.
132. Damm M, Quante G, Rosenbohm J, Rieckmann R: **Pro-inflammatory effects of *Staphylococcus aureus* exotoxin B on nasal epithelial cells.** *Otolaryngol Head Neck Surg* 2006, **134:**245-249.
133. Trede NS, Tsytsykova AV, Chatila T, Goldfeld AE, Geha RS: **Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B.** *J Immunol* 1995, **155:**902-908.
134. Trede NS, Castigli E, Geha RS, Chatila T: **Microbial superantigens induce NF-kappa B in the human monocytic cell line THP-1.** *J Immunol* 1993, **150:**5604-5613.
135. Kushnaryov VM, Bergdoll MS, MacDonald HS, Vellinga J, Reiser R: **Study of staphylococcal toxic shock syndrome toxin in human epithelial cell culture.** *J Infect Dis* 1984, **150:**535-545.
136. Kushnaryov VM, MacDonald HS, Reiser R, Bergdoll MS: **Staphylococcal toxic shock toxin specifically binds to cultured human epithelial cells and is rapidly internalized.** *Infect Immun* 1984, **45:**566-571.
137. Kushnaryov VM, MacDonald HS, Reiser RF, Bergdoll MS: **Reaction of toxic shock syndrome toxin 1 with endothelium of human umbilical cord vein.** *Rev Infect Dis* 1989, **11 Suppl 1:**S282-287; discussion S287-288.
138. Beharka AA, Armstrong JW, Iandolo JJ, Chapes SK: **Binding and activation of major histocompatibility complex class II-deficient macrophages by staphylococcal exotoxins.** *Infect Immun* 1994, **62:**3907-3915.

139. Mehindate K, al-Daccak R, Damdoumi F, Mourad W: **Synergistic effect between CD40 and class II signals overcome the requirement for class II dimerization in superantigen-induced cytokine gene expression.** *Eur J Immunol* 1996, **26:**2075-2080.
140. Arad G, Hillman D, Levy R, Kaempfer R: **Superantigen antagonist blocks Th1 cytokine gene induction and lethal shock.** *J Leukoc Biol* 2001, **69:**921-927.
141. Arad G, Hillman D, Levy R, Kaempfer R: **Broad-spectrum immunity against superantigens is elicited in mice protected from lethal shock by a superantigen antagonist peptide.** *Immunol Lett* 2004, **91:**141-145.
142. Arad G, Levy R, Hillman D, Kaempfer R: **Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation.** *Nat Med* 2000, **6:**414-421.
143. Kaempfer R, Arad G, Levy R, Hillman D: **Defense against biologic warfare with superantigen toxins.** *Isr Med Assoc J* 2002, **4:**520-523.
144. Wang B, Schlievert PM, Gaber AO, Kotb M: **Localization of an immunologically functional region of the streptococcal superantigen pepsin-extracted fragment of type 5 M protein.** *J Immunol* 1993, **151:**1419-1429.
145. Shupp JW, Jett M, Pontzer CH: **Identification of a transcytosis epitope on staphylococcal enterotoxins.** *Infect Immun* 2002, **70:**2178-2186.
146. McCormick JK, Tripp TJ, Llera AS, Sundberg EJ, Dinges MM, Mariuzza RA, Schlievert PM: **Functional analysis of the TCR binding domain of toxic shock syndrome toxin-1 predicts further diversity in MHC class II/superantigen/TCR ternary complexes.** *J Immunol* 2003, **171:**1385-1392.
147. Hoffmann ML, Jablonski LM, Crum KK, Hackett SP, Chi YI, Stauffacher CV, Stevens DL, Bohach GA: **Predictions of T-cell receptor- and major histocompatibility complex-binding sites on staphylococcal enterotoxin C1.** *Infect Immun* 1994, **62:**3396-3407.
148. Arad G, Levy R, Kaempfer R: **Superantigen concomitantly induces Th1 cytokine genes and the ability to shut off their expression on re-exposure to superantigen.** *Immunol Lett* 2002, **82:**75-78.
149. Hayden MS, West AP, Ghosh S: **NF-kappaB and the immune response.** *Oncogene* 2006, **25:**6758-6780.
150. Hayden MS, Ghosh S: **Signaling to NF-kappaB.** *Genes Dev* 2004, **18:**2195-2224.
151. Perkins ND: **Integrating cell-signalling pathways with NF-kappaB and IKK function.** *Nat Rev Mol Cell Biol* 2007, **8:**49-62.
152. Bonizzi G, Karin M: **The two NF-kappaB activation pathways and their role in innate and adaptive immunity.** *Trends Immunol* 2004, **25:**280-288.
153. Watson JL, Vicario M, Wang A, Moreto M, McKay DM: **Immune cell activation and subsequent epithelial dysfunction by *Staphylococcus* enterotoxin B is attenuated by the green tea polyphenol (-)-epigallocatechin gallate.** *Cell Immunol* 2005, **237:**7-16.
154. Su CL, Cheng CC, Lin MT, Yeh HC, Lee MC, Lee JC, Won SJ: **Staphylococcal enterotoxin C1-induced pyrogenic cytokine production in human peripheral**

blood mononuclear cells is mediated by NADPH oxidase and nuclear factor-kappa B. *FEBS J* 2007, **274:**3633-3645.
155. Sethi G, Sung B, Aggarwal BB: **Nuclear factor-kappaB activation: from bench to bedside.** *Exp Biol Med (Maywood)* 2008, **233:**21-31.
156. Shao DZ, Lee JJ, Huang WT, Liao JF, Lin MT: **Inhibition of nuclear factor-kappa B prevents staphylococcal enterotoxin A-induced fever.** *Mol Cell Biochem* 2004, **262:**177-185.
157. Liu D, Zienkiewicz J, DiGiandomenico A, Hawiger J: **Suppression of acute lung inflammation by intracellular peptide delivery of a nuclear import inhibitor.** *Mol Ther* 2009, **17:**796-802.
158. Peterson ML, Schlievert PM: **Glycerol monolaurate inhibits the effects of Gram-positive select agents on eukaryotic cells.** *Biochemistry* 2006, **45:**2387-2397.
159. Lin YC, Schlievert PM, Anderson MJ, Fair CL, Schaefers MM, Muthyala R, Peterson ML: **Glycerol monolaurate and dodecylglycerol effects on *Staphylococcus aureus* and toxic shock syndrome toxin-1 *in vitro* and *in vivo*.** *PLoS One* 2009, **4:**e7499.
160. Li Q, Estes JD, Schlievert PM, Duan L, Brosnahan AJ, Southern PJ, Reilly CS, Peterson ML, Schultz-Darken N, Brunner KG, et al: **Glycerol monolaurate prevents mucosal SIV transmission.** *Nature* 2009, **458:**1034-1038.
161. Schlievert PM, Strandberg KL, Brosnahan AJ, Peterson ML, Pambuccian SE, Nephew KR, Brunner KG, Schultz-Darken NJ, Haase AT: **Glycerol monolaurate does not alter rhesus macaque (*Macaca mulatta*) vaginal lactobacilli and is safe for chronic use.** *Antimicrob Agents Chemother* 2008, **52:**4448-4454.
162. Strandberg KL, Peterson ML, Lin YC, Pack MC, Chase DJ, Schlievert PM: **Glycerol monolaurate inhibits *Candida* and *Gardnerella vaginalis in vitro* and *in vivo* but not *Lactobacillus*.** *Antimicrob Agents Chemother* 2010, **54:**597-601.
163. Strandberg KL, Peterson ML, Schaefers MM, Case LC, Pack MC, Chase DJ, Schlievert PM: **Reduction in *Staphylococcus aureus* growth and exotoxin production and in vaginal interleukin 8 levels due to glycerol monolaurate in tampons.** *Clin Infect Dis* 2009, **49:**1711-1717.
164. Nakagawa S, Kushiya K, Taneike I, Imanishi K, Uchiyama T, Yamamoto T: **Specific inhibitory action of anisodamine against a staphylococcal superantigenic toxin, toxic shock syndrome toxin 1 (TSST-1), leading to down-regulation of cytokine production and blocking of TSST-1 toxicity in mice.** *Clin Diagn Lab Immunol* 2005, **12:**399-408.
165. Krakauer T, Buckley M: **The potency of anti-oxidants in attenuating superantigen-induced pro-inflammatory cytokines correlates with inactivation of NF-kappaB.** *Immunopharmacol Immunotoxicol* 2008, **30:**163-179.
166. Jurenka JS: **Anti-inflammatory properties of curcumin, a major constituent of *Curcuma longa*: a review of preclinical and clinical research.** *Altern Med Rev* 2009, **14:**141-153.

167. Kunnumakkara AB, Anand P, Aggarwal BB: **Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins.** *Cancer Lett* 2008, **269:**199-225.
168. Aggarwal BB, Sundaram C, Malani N, Ichikawa H: **Curcumin: the Indian solid gold.** *Adv Exp Med Biol* 2007, **595:**1-75.
169. Jagetia GC, Aggarwal BB: **"Spicing up" of the immune system by curcumin.** *J Clin Immunol* 2007, **27:**19-35.
170. Anand P, Thomas SG, Kunnumakkara AB, Sundaram C, Harikumar KB, Sung B, Tharakan ST, Misra K, Priyadarsini IK, Rajasekharan KN, Aggarwal BB: **Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature.** *Biochem Pharmacol* 2008, **76:**1590-1611.
171. Epstein J, Sanderson IR, Macdonald TT: **Curcumin as a therapeutic agent: the evidence from *in vitro*, animal and human studies.** *Br J Nutr* 2010, **103:**1545-1557.
172. Hsu CH, Cheng AL: **Clinical studies with curcumin.** *Adv Exp Med Biol* 2007, **595:**471-480.
173. Lin JK: **Molecular targets of curcumin.** *Adv Exp Med Biol* 2007, **595:**227-243.
174. Singh RK, Rai D, Yadav D, Bhargava A, Balzarini J, De Clercq E: **Synthesis, antibacterial and antiviral properties of curcumin bioconjugates bearing dipeptide, fatty acids and folic acid.** *Eur J Med Chem* 2010, **45:**1078-1086.
175. Martins CV, da Silva DL, Neres AT, Magalhaes TF, Watanabe GA, Modolo LV, Sabino AA, de Fatima A, de Resende MA: **Curcumin as a promising antifungal of clinical interest.** *J Antimicrob Chemother* 2009, **63:**337-339.
176. Dahl TA, McGowan WM, Shand MA, Srinivasan VS: **Photokilling of bacteria by the natural dye curcumin.** *Arch Microbiol* 1989, **151:**183-185.
177. Lao CD, Ruffin MTt, Normolle D, Heath DD, Murray SI, Bailey JM, Boggs ME, Crowell J, Rock CL, Brenner DE: **Dose escalation of a curcuminoid formulation.** *BMC Complement Altern Med* 2006, **6:**10.
178. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB: **Bioavailability of curcumin: problems and promises.** *Mol Pharm* 2007, **4:**807-818.
179. Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, Farmer PB, Steward WP, Gescher AJ: **Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine.** *Cancer Epidemiol Biomarkers Prev* 2002, **11:**105-111.
180. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, Brenner DE: **Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects.** *Cancer Epidemiol Biomarkers Prev* 2008, **17:**1411-1417.
181. Yang KY, Lin LC, Tseng TY, Wang SC, Tsai TH: **Oral bioavailability of curcumin in rat and the herbal analysis from Curcuma longa by LC-MS/MS.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2007, **853:**183-189.
182. Barik A, Priyadarsini KI, Mohan H: **Photophysical studies on binding of curcumin to bovine serum albumins.** *Photochem Photobiol* 2003, **77:**597-603.

183. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS: **Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers.** *Planta Med* 1998, **64:**353-356.
184. Suresh D, Srinivasan K: **Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats.** *Indian J Med Res* 2010, **131:**682-691.
185. Li J, Jiang Y, Wen J, Fan G, Wu Y, Zhang C: **A rapid and simple HPLC method for the determination of curcumin in rat plasma: assay development, validation and application to a pharmacokinetic study of curcumin liposome.** *Biomed Chromatogr* 2009, **23:**1201-1207.
186. Sharma RA, Steward WP, Gescher AJ: **Pharmacokinetics and pharmacodynamics of curcumin.** *Adv Exp Med Biol* 2007, **595:**453-470.
187. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, et al: **Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance.** *Clin Cancer Res* 2004, **10:**6847-6854.
188. Schraufstatter E, Bernt H: **Antibacterial action of curcumin and related compounds.** *Nature* 1949, **164:**456.
189. Kaur S, Modi NH, Panda D, Roy N: **Probing the binding site of curcumin in *Escherichia coli* and *Bacillus subtilis* FtsZ--a structural insight to unveil antibacterial activity of curcumin.** *Eur J Med Chem* 2010, **45:**4209-4214.
190. Rai D, Singh JK, Roy N, Panda D: **Curcumin inhibits FtsZ assembly: an attractive mechanism for its antibacterial activity.** *Biochem J* 2008, **410:**147-155.
191. Bhavani Shankar TN, Sreenivasa Murthy V: **Effect of turmeric (Curcuma longa) fractions on the growth of some intestinal & pathogenic bacteria in vitro.** *Indian J Exp Biol* 1979, **17:**1363-1366.
192. Otto M: **Staphylococcal biofilms.** *Curr Top Microbiol Immunol* 2008, **322:**207-228.
193. Mah TF, O'Toole GA: **Mechanisms of biofilm resistance to antimicrobial agents.** *Trends Microbiol* 2001, **9:**34-39.
194. Hegge AB, Andersen T, Melvik JE, Bruzell E, Kristensen S, Tonnesen HH: **Formulation and bacterial phototoxicity of curcumin loaded alginate foams for wound treatment applications: Studies on curcumin and curcuminoides XLII.** *J Pharm Sci* 2010.
195. Rudrappa T, Bais HP: **Curcumin, a known phenolic from *Curcuma longa*, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models.** *J Agric Food Chem* 2008, **56:**1955-1962.
196. Luer S, Troller R, Jetter M, Spaniol V, Aebi C: **Topical curcumin can inhibit deleterious effects of upper respiratory tract bacteria on human oropharyngeal cells in vitro: potential role for patients with cancer therapy induced mucositis?** *Support Care Cancer* 2010.
197. Wessler S, Muenzner P, Meyer TF, Naumann M: **The anti-inflammatory compound curcumin inhibits *Neisseria gonorrhoeae*-induced NF-kappaB**

signaling, release of pro-inflammatory cytokines/chemokines and attenuates adhesion in late infection. *Biol Chem* 2005, **386:**481-490.
198. Moon DO, Jin CY, Lee JD, Choi YH, Ahn SC, Lee CM, Jeong SC, Park YM, Kim GY: **Curcumin decreases binding of Shiga-like toxin-1B on human intestinal epithelial cell line HT29 stimulated with TNF-alpha and IL-1beta: suppression of p38, JNK and NF-kappaB p65 as potential targets.** *Biol Pharm Bull* 2006, **29:**1470-1475.
199. Bansal S, Chhibber S: **Curcumin alone and in combination with augmentin protects against pulmonary inflammation and acute lung injury generated during *Klebsiella pneumoniae* B5055-induced lung infection in BALB/c mice.** *J Med Microbiol* 2010, **59:**429-437.
200. Marathe SA, Ray S, Chakravortty D: **Curcumin increases the pathogenicity of *Salmonella enterica* serovar Typhimurium in murine model.** *PLoS One* 2010, **5:**e11511.
201. Di Mario F, Cavallaro LG, Nouvenne A, Stefani N, Cavestro GM, Iori V, Maino M, Comparato G, Fanigliulo L, Morana E, et al: **A curcumin-based 1-week triple therapy for eradication of *Helicobacter pylori* infection: something to learn from failure?** *Helicobacter* 2007, **12:**238-243.
202. Cashman JR, Ghirmai S, Abel KJ, Fiala M: **Immune defects in Alzheimer's disease: new medications development.** *BMC Neurosci* 2008, **9 Suppl 2:**S13.
203. Fichorova RN, Rheinwald JG, Anderson DJ: **Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins.** *Biol Reprod* 1997, **57:**847-855.
204. Murray DL, Earhart CA, Mitchell DT, Ohlendorf DH, Novick RP, Schlievert PM: **Localization of biologically important regions on toxic shock syndrome toxin 1.** *Infect Immun* 1996, **64:**371-374.
205. Blomster-Hautamaa DA, Schlievert PM: **Preparation of toxic shock syndrome toxin-1.** *Methods Enzymol* 1988, **165:**37-43.
206. Schlievert PM: **Immunochemical Assays for Toxic Shock Syndrome Toxin-1.** *Methods Enzymol* 1988, **165:**339-344.
207. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001, **25:**402-408.
208. Kumar RB, Maher DM, Herzberg MC, Southern PJ: **Expression of HIV receptors, alternate receptors and co-receptors on tonsillar epithelium: implications for HIV binding and primary oral infection.** *Virol J* 2006, **3:**25.
209. Brosnahan AJ, Mantz MJ, Squier CA, Peterson ML, Schlievert PM: **Cytolysins augment superantigen penetration of stratified mucosa.** *J Immunol* 2009, **182:**2364-2373.
210. Schlievert PM, Nemeth KA, Davis CC, Peterson ML, Jones BE: ***Staphylococcus aureus* exotoxins are present in vivo in tampons.** *Clin Vaccine Immunol* 2010, **17:**722-727.

211. Wells DE, Reeves MW, McKinney RM, Graves LM, Olsvik O, Bergan T, Feeley JC: **Production and characterization of monoclonal antibodies to toxic shock syndrome toxin 1 and use of a monoclonal antibody in a rapid, one-step enzyme-linked immunosorbent assay for detection of picogram quantities of toxic shock syndrome toxin 1.** *J Clin Microbiol* 1987, **25:**516-521.
212. Miwa K, Fukuyama M, Kunitomo T, Igarashi H: **Rapid assay for detection of toxic shock syndrome toxin 1 from human sera.** *J Clin Microbiol* 1994, **32:**539-542.
213. Saunders NA, Smith RJ, Jetten AM: **Differential responsiveness of human bronchial epithelial cells, lung carcinoma cells, and bronchial fibroblasts to interferon-gamma in vitro.** *Am J Respir Cell Mol Biol* 1994, **11:**147-152.
214. Chapes SK, Hoynowski SM, Woods KM, Armstrong JW, Beharka AA, Iandolo JJ: ***Staphylococcus*-mediated T-cell activation and spontaneous natural killer cell activity in the absence of major histocompatibility complex class II molecules.** *Infect Immun* 1993, **61:**4013-4016.
215. Earhart CA, Mitchell DT, Murray DL, Pinheiro DM, Matsumura M, Schlievert PM, Ohlendorf DH: **Structures of five mutants of toxic shock syndrome toxin-1 with reduced biological activity.** *Biochemistry* 1998, **37:**7194-7202.
216. Halbert CL, Demers GW, Galloway DA: **The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells.** *J Virol* 1992, **66:**2125-2134.
217. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ: **Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells.** *Nature* 1998, **396:**84-88.
218. Poindexter NJ, Schlievert PM: **Toxic-shock-syndrome toxin 1-induced proliferation of lymphocytes: comparison of the mitogenic response of human, murine, and rabbit lymphocytes.** *J Infect Dis* 1985, **151:**65-72.
219. Kim YB, Watson DW: **A purified group A streptococcal pyrogenic exotoxin. Physiochemical and biological properties including the enhancement of susceptibility to endotoxin lethal shock.** *J Exp Med* 1970, **131:**611-622.
220. Schlievert PM: **Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C.** *Infect Immun* 1982, **36:**123-128.
221. Schlievert PM, Jablonski LM, Roggiani M, Sadler I, Callantine S, Mitchell DT, Ohlendorf DH, Bohach GA: **Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals.** *Infect Immun* 2000, **68:**3630-3634.
222. Moza B, Varma AK, Buonpane RA, Zhu P, Herfst CA, Nicholson MJ, Wilbuer AK, Seth NP, Wucherpfennig KW, McCormick JK, et al: **Structural basis of T-cell specificity and activation by the bacterial superantigen TSST-1.** *Embo J* 2007, **26:**1187-1197.
223. Grewal IS, Flavell RA: **CD40 and CD154 in cell-mediated immunity.** *Annu Rev Immunol* 1998, **16:**111-135.
224. van Kooten C, Banchereau J: **Functions of CD40 on B cells, dendritic cells and other cells.** *Curr Opin Immunol* 1997, **9:**330-337.

225. Galy AH, Spits H: **CD40 is functionally expressed on human thymic epithelial cells.** *J Immunol* 1992, **149:**775-782.
226. Foy TM, Durie FH, Noelle RJ: **The expansive role of CD40 and its ligand, gp39, in immunity.** *Semin Immunol* 1994, **6:**259-266.
227. Durie FH, Foy TM, Masters SR, Laman JD, Noelle RJ: **The role of CD40 in the regulation of humoral and cell-mediated immunity.** *Immunol Today* 1994, **15:**406-411.
228. Hill SC, Youde SJ, Man S, Teale GR, Baxendale AJ, Hislop A, Davies CC, Luesley DM, Blom AM, Rickinson AB, et al: **Activation of CD40 in cervical carcinoma cells facilitates CTL responses and augments chemotherapy-induced apoptosis.** *J Immunol* 2005, **174:**41-50.
229. King AE, Kelly RW, Critchley HO, Malmstrom A, Sennstrom M, Phipps RP: **Cd40 expression in uterine tissues: a key regulator of cytokine expression by fibroblasts.** *J Clin Endocrinol Metab* 2001, **86:**405-412.
230. Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, Duncan L, MacAry P, Younson JS, Singh M, Oehlmann W, et al: **CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines.** *Immunity* 2001, **15:**971-983.
231. Calderwood SK, Mambula SS, Gray PJ, Jr., Theriault JR: **Extracellular heat shock proteins in cell signaling.** *FEBS Lett* 2007.
232. Gaspari AA, Sempowski GD, Chess P, Gish J, Phipps RP: **Human epidermal keratinocytes are induced to secrete interleukin-6 and co-stimulate T lymphocyte proliferation by a CD40-dependent mechanism.** *Eur J Immunol* 1996, **26:**1371-1377.
233. Laxmanan S, Datta D, Geehan C, Briscoe DM, Pal S: **CD40: a mediator of pro- and anti-inflammatory signals in renal tubular epithelial cells.** *J Am Soc Nephrol* 2005, **16:**2714-2723.
234. Cagnoni F, Oddera S, Giron-Michel J, Riccio AM, Olsson S, Dellacasa P, Melioli G, Canonica GW, Azzarone B: **CD40 on adult human airway epithelial cells: expression and proinflammatory effects.** *J Immunol* 2004, **172:**3205-3214.
235. Gelbmann CM, Leeb SN, Vogl D, Maendel M, Herfarth H, Scholmerich J, Falk W, Rogler G: **Inducible CD40 expression mediates NFkappaB activation and cytokine secretion in human colonic fibroblasts.** *Gut* 2003, **52:**1448-1456.
236. Propst SM, Denson R, Rothstein E, Estell K, Schwiebert LM: **Proinflammatory and Th2-derived cytokines modulate CD40-mediated expression of inflammatory mediators in airway epithelia: implications for the role of epithelial CD40 in airway inflammation.** *J Immunol* 2000, **165:**2214-2221.
237. Leveille C, Chandad F, Al-Daccak R, Mourad W: **CD40 associates with the MHC class II molecules on human B cells.** *Eur J Immunol* 1999, **29:**3516-3526.
238. Gautam SC, Gao X, Dulchavsky S: **Immunomodulation by curcumin.** *Adv Exp Med Biol* 2007, **595:**321-341.
239. D'Cruz OJ, Erbeck D, Uckun FM: **A study of the potential of the pig as a model for the vaginal irritancy of benzalkonium chloride in comparison to the**

nonirritant microbicide PHI-443 and the spermicide vanadocene dithiocarbamate. *Toxicol Pathol* 2005, **33:**465-476.
240. Anderson MJ, Horn ME, Lin YC, Parks PJ, Peterson ML: **Efficacy of concurrent application of chlorhexidine gluconate and povidone iodine against six nosocomial pathogens.** *Am J Infect Control* 2010, **38:**826-831.
241. Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP: **Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction.** *J Bacteriol* 1994, **176:**4204-4209.
242. Schlievert PM, Deringer JR, Kim MH, Projan SJ, Novick RP: **Effect of glycerol monolaurate on bacterial growth and toxin production.** *Antimicrob Agents Chemother* 1992, **36:**626-631.
243. Rodriguez-Antolin J, Xelhuantzi N, Garcia-Lorenzana M, Cuevas E, Hudson R, Martinez-Gomez M: **General tissue characteristics of the lower urethral and vaginal walls in the domestic rabbit.** *Int Urogynecol J Pelvic Floor Dysfunct* 2009, **20:**53-60.
244. Fichorova RN, Bajpai M, Chandra N, Hsiu JG, Spangler M, Ratnam V, Doncel GF: **Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives.** *Biol Reprod* 2004, **71:**761-769.
245. Shahani K, Swaminathan SK, Freeman D, Blum A, Ma L, Panyam J: **Injectable sustained release microparticles of curcumin: a new concept for cancer chemoprevention.** *Cancer Res* 2010, **70:**4443-4452.
246. Sullivan A, Edlund C, Nord CE: **Effect of antimicrobial agents on the ecological balance of human microflora.** *Lancet Infect Dis* 2001, **1:**101-114.
247. Pan MH, Huang TM, Lin JK: **Biotransformation of curcumin through reduction and glucuronidation in mice.** *Drug Metab Dispos* 1999, **27:**486-494.
248. Beharka AA, Iandolo JJ, Chapes SK: **Staphylococcal enterotoxins bind H-2Db molecules on macrophages.** *Proc Natl Acad Sci U S A* 1995, **92:**6294-6298.
249. Wright AD, Chapes SK: **Cross-linking staphylococcal enterotoxin A bound to major histocompatibility complex class I is required for TNF-alpha secretion.** *Cell Immunol* 1999, **197:**129-135.
250. Chapes SK, Herpich AR: **Complex high affinity interactions occur between MHCI and superantigens.** *J Leukoc Biol* 1998, **64:**587-594.
251. Stiles BG, Bavari S, Krakauer T, Ulrich RG: **Toxicity of staphylococcal enterotoxins potentiated by lipopolysaccharide: major histocompatibility complex class II molecule dependency and cytokine release.** *Infect Immun* 1993, **61:**5333-5338.
252. Kohane DS: **Microparticles and nanoparticles for drug delivery.** *Biotechnol Bioeng* 2007, **96:**203-209.
253. Yadav VR, Suresh S, Devi K, Yadav S: **Novel formulation of solid lipid microparticles of curcumin for anti-angiogenic and anti-inflammatory activity for optimization of therapy of inflammatory bowel disease.** *J Pharm Pharmacol* 2009, **61:**311-321.

254. Hamilton AJ, Baulcombe DC: **A species of small antisense RNA in posttranscriptional gene silencing in plants.** *Science* 1999, **286:**950-952.
255. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: **Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.** *Nature* 2001, **411:**494-498.
256. Sandy P, Ventura A, Jacks T: **Mammalian RNAi: a practical guide.** *Biotechniques* 2005, **39:**215-224.

Appendix

Mechanism of RNAi

shRNA producing plasmids were used to knockdown CD40 in HVEC. shRNA uses RNA interference (RNAi) that knock down expression of a target gene by using siRNA molecules, which form double stranded RNA (dsRNA) molecules with target mRNA. This dsRNA molecule is quickly degraded by cellular mechanisms thereby reducing the amount of mRNA specific for the gene of interest [254, 255]. In this study, plasmids that produce shRNA were used. These plasmids express shRNA which form hairpins that are cleaved by cellular processes into siRNA (small interfering RNA) which can then knockdown expression of the target gene [256].

Experiments with curcumin and HVEC

Preliminary experiments were done to measure the toxicity of curcumin to HVEC and the ability of curcumin to inhibit IL-8 production. HVEC were exposed to curcumin (1-30 μM) in KSFM with 1% DMSO for 6 hr when cell survival was determined using CellTiter 96®Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Figure 34 shows increasing toxicity in HVEC as curcumin increases compared to vehicle control (1% DMSO). The LD_{50} for curcumin was estimated to be 30 μM.

The ability of curcumin to inhibit *S. aureus* exoprotein-induced IL-8 production from HVEC was also assessed. HVEC were exposed to curcumin and filtered (0.2 μm filter size) overnight *S. aureus* MNPE culture supernate (containing SEC, TSST-1, α-toxin [19] for 6 hr when secreted IL-8 was measured using ELISA (R & D Systems).

Figure 35 shows that curcumin at the LD_{50} (30 μM) is able to reduce the amount of IL-8 that is made in response to stimulation with *S. aureus* exoprotein. Curcumin at doses less than the LD_{50} had no effect on IL-8 production (data not shown). With this data it was difficult to separate the reduction in IL-8 production from toxic effects. So we decided to evaluate curcumin's properties on full-thickness porcine tissue, as we predicated full-thickness tissue would be more resistant to curcumin's toxicity.

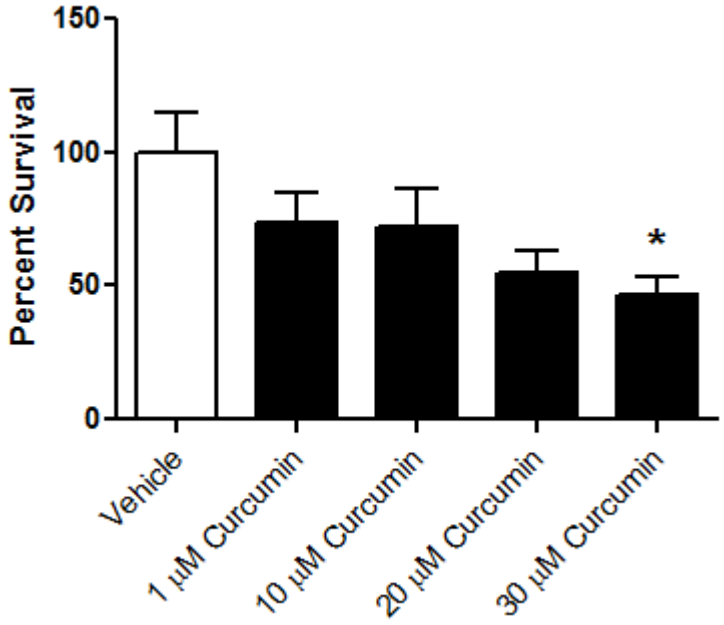

Figure 34 . Curcumin causes toxicity to HVEC. HVEC were exposed to curcumin (1-30 μM) in KSFM with 1% DMSO for 6 hr when cell survival was determined. * denotes p value < 0.05 compared to vehicle control (1% DMSO). Data is representative from two independent experiments done in triplicate. The LD_{50} for curcumin was estimated to be 30 μM.

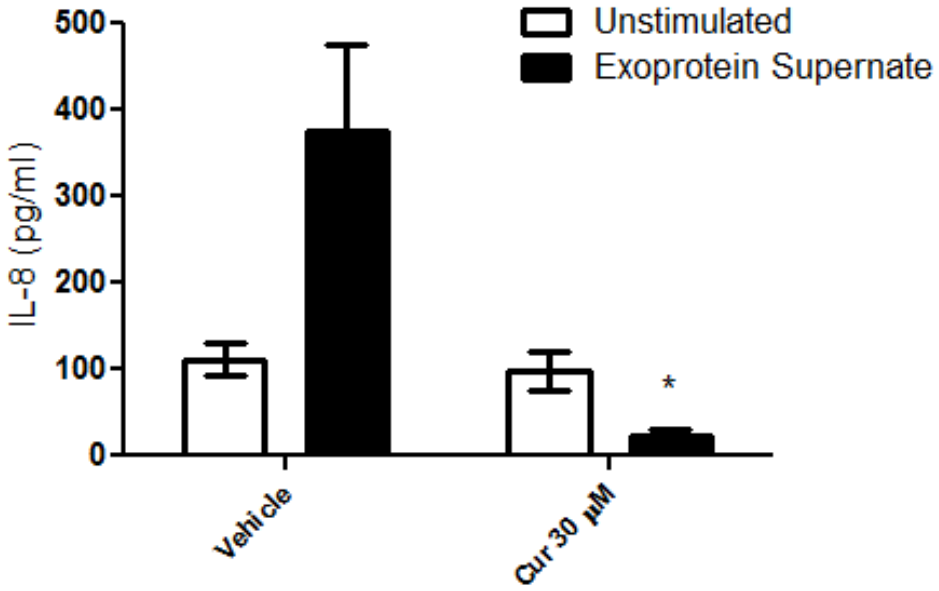

Figure 35. Curcumin inhibits *S. aureus* exoprotein-induced IL-8 in HVEC. HVEC were exposed to curcumin and filtered overnight *S. aureus* MNPE culture supernate (containing SEC, TSST-1, α-toxin) for 6 hr when secreted IL-8 was measured using ELISA. * denotes p value < 0.05 compared to vehicle control (1% DMSO). Data is representative from two independent experiments done in triplicate.

Rabbit #	*Treatment*	*Fate*	*Pathologist Findings*
1	Untreated	Died	5 sections examined. Mild diffuse vascular dilation and congestion in the lamina propria/submucosa.
7	Untreated	Died	5 sections examined. Moderate diffuse vascular dilation and congestion in the lamina propria/submucosa with moderate edema and rare heterophils, plasma cells and lymphocytes.
8	Untreated	Died	5 sections examined. Mild diffuse vascular dilation and congestion in the lamina propria/submucosa with rare heterophils and lymphocytes.
9	Untreated	Died	5 sections examined. Moderate diffuse vascular dilation and congestion in the lamina propria/submucosa with moderate edema and rare heterophils.
11	Untreated	Died	5 sections examined. Normal vaginal tissue.
6	Curcumin-treated	Survived	5 sections examined. Mild diffuse vascular dilation in the lamina propria/submucosa
13	Curcumin-treated	Survived	5 sections examined. Moderate-marked diffuse vascular dilation and congestion in the lamina propria/submucosa with moderate edema and rare heterophils and plasma cells. Few scattered apoptotic cells within submucosa.
14	Curcumin-treated	Died	5 sections examined. Moderate-marked diffuse vascular dilation and congestion in the lamina propria/submucosa with moderate edema and rare heterophils and plasma cells. Few scattered apoptotic cells within submucosa.
15	Curcumin-treated	Survived	5 sections examined. Mild-moderate diffuse vascular dilation in the lamina propria/submucosa Rare epithelium present, poor staining.
16	Curcumin-treated	Died	5 sections examined. Diffuse vascular dilation in the lamina propria/submucosa Epithelium is sparse in the sections (artifact).

Table 15. Pathologist report for H & E stained rabbit vaginal tissue slides from curcumin experiment.

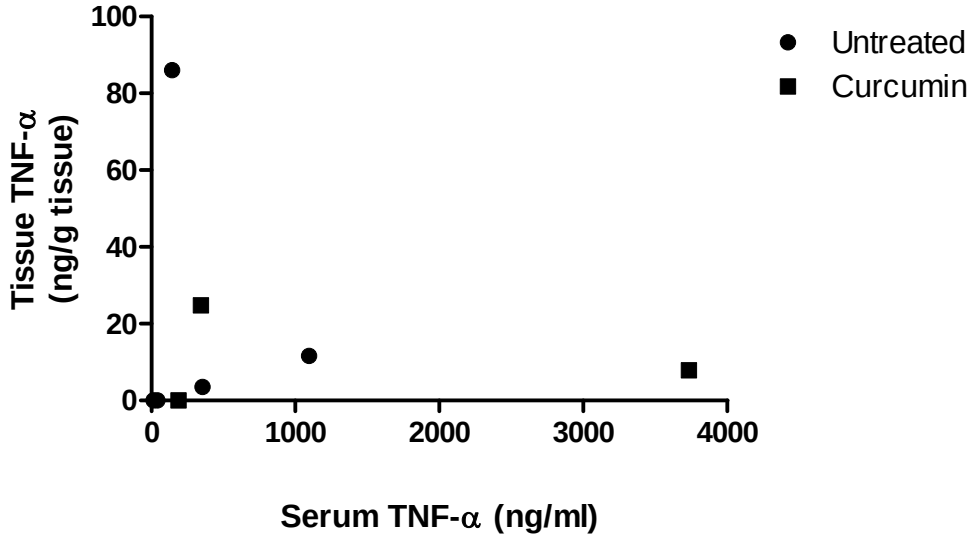

Figure 36. No correlation between serum and vaginal tissue TNF-α levels from rabbit TSS model. The r^2 value by linear regression is 0.0181.