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The role of superantigens during Staphylococcus aureus nasal colonization and infection

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

THE ROLE OF SUPERANTIGENS DURING *STAPHYLOCOCCUS AUREUS* NASAL COLONIZATION AND INFECTION

(Thesis format: Integrated Article)

by

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Abstract

Superantigens (SAgs) are potent toxins produced by bacteria such as *Staphylococcus aureus* that function to overactivate T cells resulting in massive cytokine production and immune activation. Despite decades of research on the structure and function of these proteins, as well as their role in severe diseases such as toxic shock syndrome, the question as to why strains of *S. aureus* produce SAgs and the role that they play in the life cycle of these bacteria remains unanswered. The contribution of SAgs towards pathogenicity and bacterial survival *in vivo* were assessed using isogenic SAg deletion knockouts in conjunction with SAg-sensitive humanized transgenic HLA-DR4 mice. Since *S. aureus* are able to successfully colonize human nares in addition to causing infections, the bacterial strains were assessed in a model of nasal colonization as well as a model of bacteremia. Compared to wild-type *S. aureus* COL and Newman, the SAg-deletion mutants COL Δ*seb* and Newman Δ*sea* were able to establish higher bacterial loads in the nose, suggesting that SAgs are involved in regulating bacterial densities during colonization. Thus, SAgs may act as 'checkpoints' of dissemination from the nose. In contrast, Newman Δ*sea* had reduced counts during bacteremia compared to the wild-type strain in a liver-specific phenotype. Staphylococcal enterotoxin A (SEA)-expressing *S. aureus* Newman induced IFN-γ, IL-12 and chemokine responses which resulted in increased trafficking of $CD11b^{+}Ly6G^{+}$ neutrophils into the liver. Additionally, wild-type infection resulted in higher numbers of hepatic abscesses containing viable bacteria compared to Newman Δ*sea* representing a specialized *in vivo* niche for *S. aureus*. Thus, the mechanism of pathogenicity was due to increased neutrophil infiltration and abscess formation in the liver, as a result of SEA-mediated cytokine and chemokine release. Although staphylococcal SAgs appear to play opposing roles in the different models, the overall function of these toxins appears to be manipulation of the immune system to maintain a niche environment in order to persist and survive.

Keywords

Superantigen, *Staphylococcus aureus*, nasal colonization, bacteremia, infection, neutrophil, T cell, abscess, transgenic mouse, enterotoxin, toxic shock, infection

Co-Authorship Statement

All studies presented in this thesis were completed by Stacey Xu in the laboratory of Dr. John McCormick with assistance from co-authors as listed below. John McCormick contributed to conception, design, data analysis, interpretation, and manuscript preparation for all experiments.

Chapter 1: Sections including figures from the introduction have been previously published in a review co-authored by John McCormick:

Xu, S.X. and J.K. McCormick. (2012) Staphylococcal superantigens in colonization and disease. *Frontiers in Cellular and Infection Microbiology*. **2**:52.

Chapter 2: The construction of *S. aureus* Newman Δ*sea* and sensitivity of HLA-DR4 mice to SAgs has been previously published in :

Xu, S.X., Gilmore K.J., Szabo P.A., Zeppa J.J., Baroja M.L., Haeryfar S.M. and J.K. McCormick. (2014) Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival *in vivo*. Infection and Immunity. 82(9):3588-98.

The rest of the chapter is being prepared for submission.

Katherine Kasper assisted with the set-up of the nasal colonization model and original infection experiments. Joseph Zeppa also assisted with infection experiments and antibody detection assays.

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Kevin Gilmore assisted with histological preparations of tissue sections and staining procedures. Peter Szabo assisted with some flow cytometry techniques, Joseph Zeppa assisted with infection experiments and injections. Lorea Baroja taught me flow cytometry procedures and analysis, as well as providing expertise on immunological assays. Mansour Haeryfar provided reagents and critical reading of the manuscript.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

^{o}C	degrees Celcius
µL	microlitre
µg	microgram
Δ	delta/deletion
Δ*sea*	*sea* deletion
Δ*seb*	*seb* deletion
× *g*	times gravity
ACK	ammonium-chloride-potassium
AD	atopic dermatitis
ADAM-10	a disintegrin and metalloproteinase domain-containing protein 10
agr	accessory gene regulator
Amp	ampicillin
AMP	antimicrobial peptide
APC	antigen presenting cell
B6	C56Bl/6 mice
BCA	bicinchoninic acid
bp	base pair
BCR	B cell receptor
BSA	bovine serum albumin
CA-MRSA	community-acquired methicillin-resistant *Staphylococcus aureus*
CD	Crohn's disease
CFU	colony forming unit
CHIPS	chemotaxis inhibitory protein of *Staphylococcus aureus*
CLA	cutaneous lymphocyte-associated antigen
Clf	clumping factor
Cm	chloramphenicol
Coa	coagulase
CoNS	coagulase-negative staphylococci
CPM	counts per minute
CRS	chronic rhinosinusitis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DR4-B6	C56Bl/6 mice expressing human transgenic HLA-DR4
Eap	extracellular adherence protein
ECM	extracellular matrix
Efb	extracellular fibrinogen binding
egc	enterotoxin gene cluster
ELISA	enzyme-linked immunosorbent assay
Erm	erythromycin
FBS	fetal bovine serum
FLIPr	formyl peptide receptor-like 1 inhibitor
FnBP	fibronectin-binding protein
FPR	formylated peptide receptor
FR	framework region
g	gram

GAS	Group A *Streptococcus*
h	human
HBD	human beta defensin
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leukocyte antigen
HNP	human neutrophil peptide
HRP	horseradish peroxidase
IE	infectious endocarditis
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon-induced protein 10
IPTG	isopropyl β-D-1-thiogalactopyranoside
IV	intravenous
IVIG	intravenous immunoglobulin
K	cytokeratin
Kan	kanamycin
kb	kilobase
KD	Kawasaki disease
kDa	kiloDalton
L	litre
LB	Luria Bertani
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
MAM	*Mycoplasma arthritidis* T cell mitogen
MCP-1	monocyte chemotactic protein 1
MHC	major histocompatibility complex
mg	milligram
MIP-2	macrophage inflammatory protein 2
mL	millilitre
MRSA	methicillin-resistant *Staphylococcus aureus*
MSA	mannitol salt agar
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
mTOR	mammalian target of rapamycin
M.W.	molecular weight
NALT	nasal-associated lymphoid tissue
ng	nanogram
OD	optical density
PAI	pathogenicity island
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween-20
pg	picogram
pMHC	peptide antigen presented within major histocompatibility complex
PSM	phenol soluble modulin
PVDF	polyvinylidene difluoride

PVL	Panton-Valentine leukocidin
RPM	revolutions per minute
rSAg	recombinant superantigen
SAB	*Staphylococcus aureus* bacteremia
SAg	superantigen
SAK	staphylokinase
SCIN	staphylococcal complement inhibitor
SdrC/SdrD	serine-aspartic acid repeat protein C/D
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	staphylococcal enterotoxin
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEl	staphylococcal enterotoxin-like
SFP	staphylococcal food poisoning
SIRS	systemic inflammatory response syndrome
Sm	streptomycin sulfate
SMEZ	streptococcal mitogenic exotoxin Z
SmR	streptomycin sulfate resistant
Spe	streptococcal pyrogenic exotoxins
SSA	streptococcal superantigen
SSL	staphylococcal superantigen-like
SSTI	skin and soft tissue infection
TCA	trichloroacetic acid
TCR	t cell receptor
TEMED	tetramethylethylenediamine
Tet	tetracycline
tetR	tetracycline resistant
TEV	tobacco etch virus
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
Treg	regulatory T cell
TSA	tryptic soy agar
TSB	tryptic soy broth
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin 1
V	volts
Vβ	variable beta chain
v/v	volume per volume
vWbp	von Willebrand binding protein
w/v	weight per volume
WTA	wall teichoic acid
YPM	*Yersinia pseudotuberculosis*-derived mitogen

Chapter 1 Introduction[1]

[1] Parts of this chapter have been previously published and are adapted from:

Xu, S.X. and J.K. McCormick. (2012) Staphylococcal superantigens in colonization and disease. *Frontiers in Cellular and Infection Microbiology*. **2**:52. doi: 10.3389/fcimb.2012.00052

1.1 Superantigen overview

The term ‘superantigen’ (SAg) was originally coined by Marrack and Kappler to describe a group of molecules that target T cell receptors (TCR), resulting in massive immune activation (1). This mechanism differs from non-specific T cell mitogens (such as Concanavalin A), as SAgs are dependent on interactions with the TCR β-chain variable domain (Vβ) on T cells, and major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APCs) (1-3). The family of SAgs originally included the endogenous mouse Minor lymphocyte stimulating antigens and was later expanded to include pyrogenic bacterial factors such as staphylococcal enterotoxin B (SEB) (1, 4). Microbial genome sequencing projects over the last decade have led to the characterization of a large and expanding family of exotoxins that includes many genetically and antigenically distinct proteins that possess similar function. SAgs are found primarily in the Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes*, but are also found in a few other species of β-hemolytic streptococci, coagulase negative staphylococci, *Mycoplasma arthritidis*, *Yersinia pseudotuberculosis*, and *Pseudomonas fluorescens*.

1.2 Bacterial superantigens

1.2.1 *Staphylococcus aureus* superantigens

The staphylococcal SAgs include the staphylococcal enterotoxins (SEs), the staphylococcal enterotoxin-like (SEls) proteins, and toxic shock syndrome toxin-1 (TSST-1) (5). Before the discovery of their superantigenic function, the SEs were originally defined by their ability to cause staphylococcal food poisoning (SFP) including emesis (vomiting), and currently include the SEs A, B, C, D, E, G, H, I, R, and T. The SEl toxins, although both homologous and structurally similar to the SEs, either do not induce emesis, or have not been formally demonstrated to induce emesis, and include the SEls J, K, L, M, N, O, P, Q, S, U, V, and X. It is important to note that although designated as a “SEl” toxin, some of these may possess undemonstrated emetic activity and be reclassified in the future as bona fide enterotoxins.

All staphylococcal SAgs are encoded on mobile genetic elements such as pathogenicity islands (PAIs), plasmids or phage with the exception of the newly discovered *sel-x*, which is genome-encoded (6, 7). This novel SAg has been found in all strains except for those in clonal complex 30, which is thought to be a result of deletion event in an ancestral strain (6). Previous epidemiological studies suggest that approximately 80% of *S. aureus* strains harbour SAg genes, with an average of 5-6 per strain (8-10).

1.2.1.1 Enterotoxin gene cluster

An operon of SAgs known as the enterotoxin gene cluster (*egc*) is comprised of the SAgs *seg*, *sel-i*, *sel-m*, *sel-n*, *sel-o* and sometimes *sel-u* (11, 12). This cluster is commonly found in clinical *S. aureus* strains and it has been proposed that this 'nursery' of SAgs may not be linked to toxemia, but colonization (9, 11). Supporting this notion is the finding that *egc*-encoded SAgs do not induce neutralizing antibodies despite high prevalence and superantigenic activity *in vitro* (13, 14). Conversely, non-*egc* SAgs are highly immunogenic and the majority of the population has neutralizing antibodies against one or more of these SAgs. It is thought that differential expression patterns may explain some of these divergent findings, as *egc* SAgs are produced during early exponential growth whereas most classic SAgs are produced during late-exponential and stationary phase (9).

1.2.1.2 Staphylococcal superantigen-like proteins

Originally named the staphylococcal exotoxin-like proteins (SETs) (15), this group of toxins has been renamed the staphylococcal superantigen-like proteins (SSLs) although these toxins do not possess SAg activity (5). This family of proteins are structurally similar to the staphylococcal SAgs, hence the name, and appear to be primarily involved in immune evasion including prevention of bacterial clearance by neutrophils (16). To date, all sequenced staphylococcal strains carry *ssl* genes and humans have developed neutralizing antibodies against SSLs, implying a role in bacterial fitness (16).

1.2.2 Streptococcal superantigens

Historically known as scarlet fever toxins, SAgs encoded by *Streptococcus pyogenes* [group A *Streptococcus* (GAS)] are now known as streptococcal pyrogenic exotoxins (Spes). The characteristic clinical rash of scarlet fever is caused by Spes, also formerly known as the erythrogenic toxins (17). A similar rash also develops during streptococcal toxic shock syndrome (TSS), for which the causative agents are also streptococcal SAgs. Streptococcal TSS, which occurs typically during invasive GAS infections, differs from staphylococcal TSS (discussed below), and is accompanied by bacteremia in more than 60% of cases and results in high mortality rates (18). In addition to severe streptococcal infections, streptococcal SAgs have recently been shown by our group to be critical for GAS nasopharyngeal colonization (19).

To date, fourteen streptococcal SAgs have been identified: SpeA, SpeC, SpeG-P, streptococcal superantigen (SSA), and streptococcal mitogenic exotoxin Z (SMEZ). The streptococcal SAgs are primarily found in GAS, but have also been identified in group C and G streptococci (20). Like their staphylococcal counterparts, streptococcal SAgs are commonly found on mobile genetic elements. Together, this suggests that these SAgs either shared a common ancestor or resulted from interspecies horizontal gene transfer (18).

1.2.3 Coagulase-negative staphylococcal superantigens

Coagulase-negative staphylococci (CoNS) are generally considered to be commensals, especially in comparison to the highly pathogenic coagulase-positive *S. aureus,* which often colonize common mucosal surfaces of humans and animals. However, there is an increased prevalence of CoNS strains in human disease, particularly bacteremia associated with indwelling medical devices such as catheters (21). Staphylococcal SAgs have been found in CoNS species from both humans and livestock (22, 23). Recently, a PAI encoding *sec* and *sel-l* was discovered in a clinical strain of *S. epidermidis*, most likely a result of horizontal gene transfer from *S. aureus* since PAIs have never been identified in *S. epidermis* previously (24).

1.2.4 Gram negative SAgs

Yersinia pseudotuberculosis is an enteric pathogen that induces a scarlet fever-like rash, and produces a SAg known as *Yersinia pseudotuberculosis*-derived mitogen (YPM) (25). YPM has been shown to induce shock in experimental models (26) and exacerbates mortality during systemic infections (27). Additionally, there is evidence of Vβ-skewing and anti-YPM antibodies in patients with *Y. pseudotuberculosis* infections (28).

Lacking a cell wall, the bacterium *Mycoplasma arthritidis* causes acute arthritis in rodents and was found to be mitogenic for T cells (29). The factor responsible was determined to be *Mycoplasma arthritidis* T cell mitogen (MAM), and was classified as a SAg since it specifically stimulated Vβ6 and Vβ8 mouse T cells (30). Although MAM can result in shock, MAM does not appear to play a role in the induction of arthritis in mice (31).

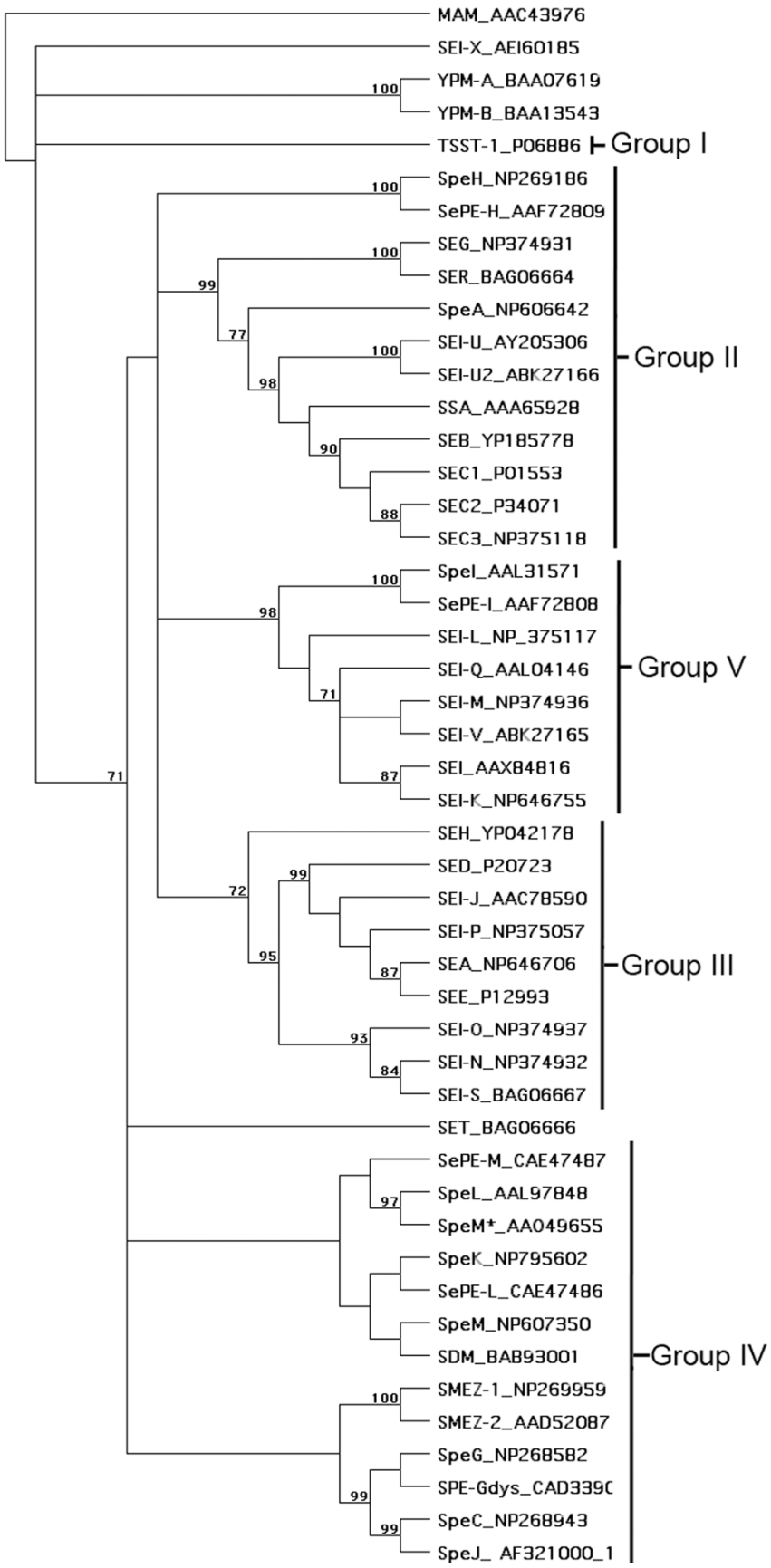
A Crohn's disease (CD)-associated bacterial protein, known as I2, has also been identified as a structurally-distinct SAg (32) encoded by *pilT* from *Pseudomonas fluorescens* (33, 34). It is thought that the ability to affect T cells contributes to the development of inflammatory bowel diseases and that *P. fluorescens* could be an important bacterial pathogen involved in the pathogenesis of CD (32, 34).

1.3 Superantigen genetics and classification

A phylogenetic classification scheme of the SAg exotoxins based on amino acid alignments is shown in Fig. 1.1, where SAgs from staphylococci and streptococci are placed into five evolutionary groups (18). Within this classification, TSST-1 sits as an evolutionarily distinct SAg that does not induce emesis (35) and is the only member of the Group I SAgs. TSST-1 is believed to be the major, if not sole cause of the menstrual form of TSS (36, 37). The Group II SAgs contain both staphylococcal and streptococcal SAgs including SEB, SEC, and SpeA. After TSST-1, SEB has been historically most commonly linked with non-menstrual-associated cases of staphylococcal TSS (48), while SpeA has been historically most commonly linked with streptococcal TSS (49). The Group III SAgs include only staphylococcal SAgs, and in general terms, this group contains SAgs most commonly associated with SFP such as SEA, SED, and SEE,

Figure 1.1. Phylogenetic tree of known bacterial SAgs.

The unrooted tree was based on the alignment of amino acid sequences constructed with the unweighted pair group method using arithmetic averages (UPGMA) in MacVector 7.2.3. The SAg abbreviations are indicated followed by the relevant accession number. As previously proposed (18), the five main groups of SAgs belonging to the pyrogenic toxin class are indicated. MAM, YPM, and non-Group A streptococcal SAgs are also included in the analysis. The number of times each branch was supported from 1000 bootstraps is shown as a percentage.


MAM_AAC43976
SEI-X_AEI60185
YPM-A_BAA07619
YPM-B_BAA13543
TSST-1_P06886
Group I
SpeH_NP269186
SePE-H_AAF72809
SEG_NP374931
SER_BAG06664
SpeA_NP606642
SEI-U_AY205306
SEI-U2_ABK27166
Group II
SSA_AAA65928
SEB_YP185778
SEC1_P01553
SEC2_P34071
SEC3_NP375118
SpeI_AAL31571
SePE-I_AAF72808
SEI-L_NP_375117
SEI-Q_AAL04146
SEI-M_NP374936
SEI-V_ABK27165
SEI_AAX84816
SEI-K_NP646755
Group V
SEH_YP042178
SED_P20723
SEI-J_AAC78590
SEI-P_NP375057
SEA_NP646706
SEE_P12993
SEI-O_NP374937
SEI-N_NP374932
SEI-S_BAG06667
Group III
SET_BAG06666
SePE-M_CAE47487
SpeL_AAL97848
SpeM*_AA049655
SpeK_NP795602
SePE-L_CAE47486
SpeM_NP607350
SDM_BAB93001
SMEZ-1_NP269959
SMEZ-2_AAD52087
SpeG_NP268582
SPE-Gdys_CAD3390
SpeC_NP268943
SpeJ_ AF321000_1
Group IV

although the Group II SAgs SEB and SEC are often implicated as well (50). Both Group II and III SAgs contain a unique "cysteine-loop structure" that is thought to be important for emetic activity (51). The Group IV SAgs are only populated by streptococcal SAgs and have both low and high-affinity binding sites for MHC II but do not induce emesis (52). The Group V SAgs, contain mostly staphylococcal SAgs (except SpeI and related orthologues), and other than SEI which has weak emetic activity, consists of only SEl toxins. In fact, SEI is the only SAg outside of the Group II and III SAgs demonstrated to have emetic activity, although this only occurred in one of four animals tested (53). SEl-X does not align well within the current classification system, but is encoded within the core chromosome of most *S. aureus* strains (6).

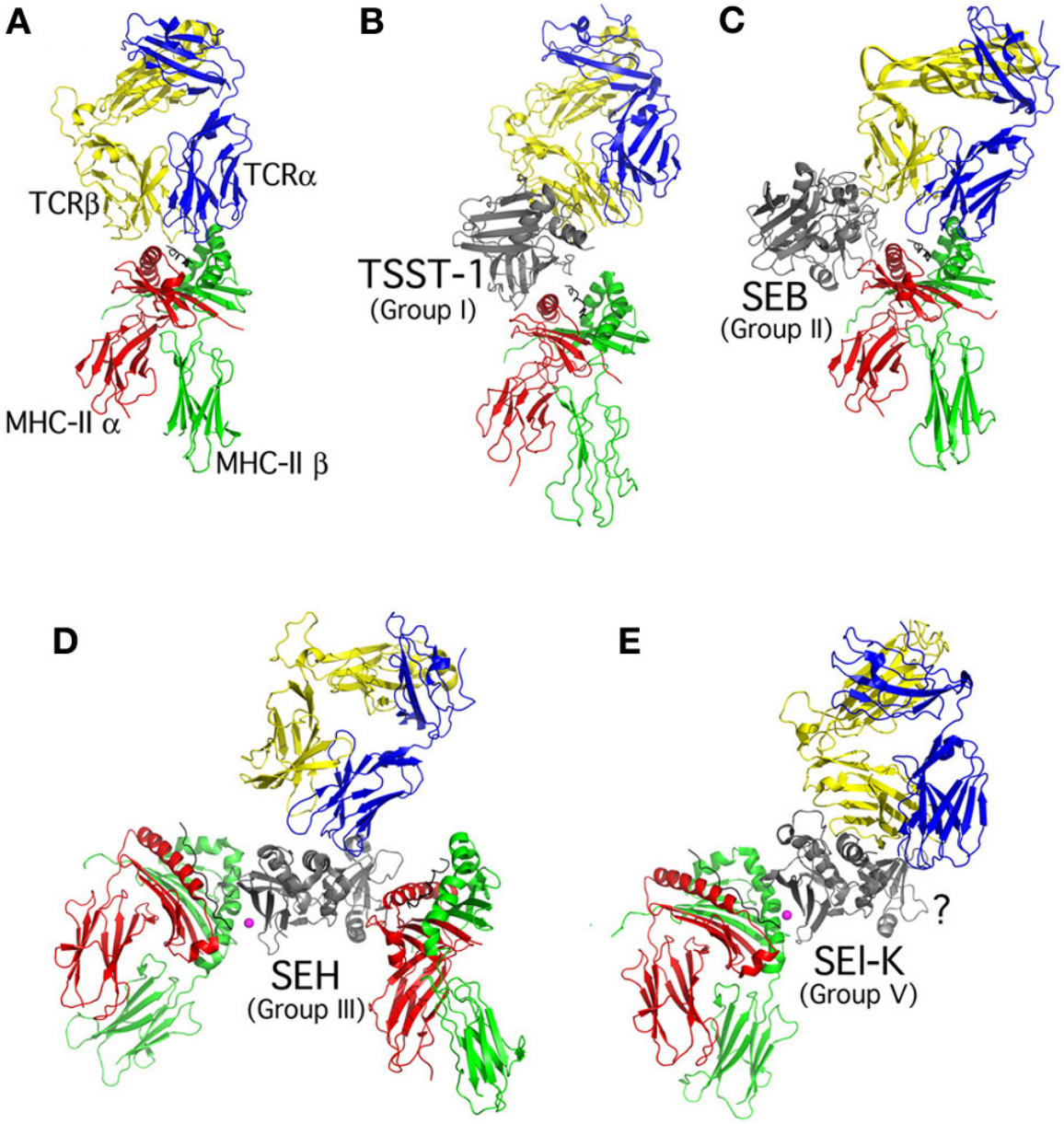
1.4 Superantigen structure

SAg pro-toxins include a secretion signal that is cleaved from the N-terminus upon export via the general Sec-dependent secretion pathway. SAgs are released as non-enzymatic, relatively small proteins, with the final toxin product ranging in size from ~22 to 29kDa. All SAgs are made of two structurally similar domains, linked through a central α-helix. The larger N-terminal domain contains a β-barrel motif similar to an OB-fold, while the smaller C-terminal domain contains the β-grasp motif, which is similar to immunoglobulin-binding domains (54).

Pioneering crystallographic studies with SEB in complex with human leukocyte antigen (HLA)-DR1 (42), and SEC3 in complex with the mouse TCR Vβ8.2-chain (41), established a molecular framework of how SAgs can activate so many T cells (55). These studies demonstrated that SAgs bind to lateral surfaces of both TCRs and processed peptide antigen presented within self-major histocompatibility (pMHC) complexes to "distort" the normal TCR-pMHC II interaction, such that the CDR3 loops of both TCR α- and β-chains (which are key for antigen recognition) are wedged away from the antigenic peptide (Fig. 1.2B–E). Through this mechanism, activation of the T cell is no longer antigen specific but dependent upon which Vβs can be bound by that particular SAg, explaining how SAgs are Vβ-specific (55). Large numbers of SAg-activated T cells can then release a multitude of pro-inflammatory cytokines which in severe cases may lead to

Figure 1.2 Structural overview of the SAg-mediated T cell activation complexes

Ribbon diagram models show (A) conventional T cell activation (38), and SAg-mediated T cell activation complexes for (B) Group I (e.g., TSST-1) (C) Group II (e.g., SEB) (D) Group III (e.g., SEH) and (E) Group V (e.g., SEl-K). Colors for TCR and MHC class II chains are labeled in Panel (A). The SAg activation complex models were generated by superposition of the TSST-Vβ (39) and TSST-MHC class II (40) structures, the SEC-Vβ (41) and SEB-MHC class II (42) structures, the SEH-VαVβ (43), SEH-MHC class II β-chain (44), and the SEA-MHC class II α-chain (45) structures, and the SEK-Vβ (46) and SEI-MHC II (47) structures. The TCR α-chain was modeled for clarity in each case from the conventional complex (38). The "?" in Panel (E) indicates that there is no current information regarding the presence, or absence, of the generic low-affinity MHC class II binding domain for Group V SAgs.


A
TCRβ
TCRα
MHC-II α
MHC-II β
B
TSST-1
(Group I)
C
SEB
(Group II)
D
SEH
(Group III)
E
?
SEI-K
(Group V)

the “cytokine storm” phenomenon characteristic of TSS (18). Stimulation of APCs by SAgs also contributes to cytokine release due to the involvement of MyD88, which activates NF-κB, leading to production of pro-inflammatory molecules (56).

1.4.1 Structural and molecular mapping of superantigen targets

Recent years have seen a number of further advances in the structural characterization of the staphylococcal SAgs, and there is now a broader picture as to how SAgs from the different evolutionary groups **(**Fig. 1.1**)** function to distort the normal process of T cell activation (Fig. 1.2). For example, the Group I SAg TSST-1 (Fig. 1.2B), which is extremely specific for the human Vβ2^{+} T cells (2), forms a unique T cell activation complex by binding the MHC II α-chain through a relatively low-affinity interface that is highly influenced by different antigenic peptides within MHC II (40, 57). Also, TSST-1 recognizes unique amino acid insertions from Vβ2 within both CDR2 and framework region (FR) 3, explaining the extreme Vβ-specificity of this SAg (39, 58, 59). There are no direct TCR-MHC II contacts in this T cell activation complex. Group II SAgs (Fig. 1.2C) such as SEB, SEC3, and SpeA, are more “promiscuous” in their Vβ-targets, and engage TCR Vβ through “conformation-dependent” mechanisms that are thought to be less dependent on specific Vβ amino acid side-chains (41, 60, 61). These SAgs bind the MHC II α-chain through an N-terminal, low-affinity binding domain, yet in contrast to TSST-1, this binding is antigenic peptide-independent (42). Group III SAgs (Fig. 1.2D) consist of only staphylococcal SAgs, and these toxins are thought to be able to cross-link MHC II molecules (62, 63) through both a low-affinity site similar to Group II, (45) as well as a high-affinity, zinc-dependent MHC II β-chain interface located within the β-grasp domain of the SAg (44). The only structural information for how Group III SAgs engage TCR is for SEH (43), which represents somewhat of an outlier within Group III, and is the only known Vα-specific SAg (64, 65). Group IV SAgs are restricted to only streptococcal members, and these toxins bind Vβ similar to the Group II SAgs, although with a larger footprint (61), and contain a high-affinity MHC II β-chain binding domain similar to Group III (66). Considerable evidence indicates the presence of a low-affinity MHC II α-chain interaction, likely similar to Group II (67-69), although this interaction has not been characterized structurally. The Group V SAgs contain a high-affinity MHC

II β-chain binding domain (70) similar to Group III, and bind the TCR Vβ with a more "lateral" position extending into FR4 (Fig. 1.2E) (46). There is currently no information relating to the presence, or absence, of the generic low-affinity MHC II interface with Group V SAgs.

Within the SAg family of toxins, each member is able to efficiently activate large numbers of T cells, regardless of subtle or dramatic differences within the different SAg-mediated T cell activation complexes. However, the one common structural feature of all characterized SAgs, with the exception of the Vα-specific SEH, is the engagement of the Vβ CDR2 loop, and this loop appears to be the critical determinant for Vβ-specificity (59).

1.5 Superantigen-host receptor interactions

1.5.1 T cell activation

Normal T cell-mediated immunity is initiated through the interaction of an αβ TCR and pMHC complexes **(**Fig. 1.2A**)** (71, 72)**.** If the TCR specifically recognizes the antigen as foreign, these interactions will activate the tyrosine kinase Lck (associated with co-receptors CD4 and CD8), which in turn will activate downstream cell signaling resulting in activation of transcription factors to induce T cell proliferation and differentiation (73). As TCRs are extraordinarily diverse molecules, only ~0.01% of naïve T cells will recognize a given antigen (74).

SAg-mediated T cell activation is both quantitatively and qualitatively distinct from conventional T cell activation (75). As the defining feature of the SAg toxin is the ability to activate T lymphocytes in a Vβ-dependent manner (3), very large numbers of T cells can be activated upon SAg exposure. TCR diversity is concentrated within the CDR3 loops due to V(D)J (somatic) recombination during T cell development. However, there are a relatively limited number of possible TCR Vβ regions (~50 are functionally expressed in humans), and thus SAgs can activate T cells in orders of magnitude above conventional processes. SAgs also do this in an extremely potent manner, and in general, most SAgs can induce measurable activation of T cells in the picogram (10^{-12} g)

concentration range (18). T cell secretion of cytokines is APC-dependent and studies show that SAg stimulation results in the release of cytokines including interleukin (IL)-1, IL-2, IL-6, TNF-α, and interferon (IFN)-γ (76-78).

Experiments conducted with human peripheral blood mononuclear cells (PBMCs) exposed to SAgs resulted in a dose-dependent, Vβ specific increase in $CD25^+$ $FoxP3^+$ cells, indicative of a regulatory T cell (Treg) phenotype. The immunosuppressive qualities of these SAg-induced Tregs have been attributed to the expression of IL-10 and may have a role in prolonging commensalism (79).

1.5.1.1 T cell signaling

It is often assumed that SAg-mediated T cell activation follows the normal signaling rules for conventional pMHC-mediated T cell activation and indeed this is the case with at least one major distinction. As predicted, TCR ligation by SAg will induce signals through Lck (80), although Lck signaling is not actually required (81, 82). However, signaling can proceed in the absence of Lck through a Gα11/PLCβ-dependent pathway that converges with the canonical Lck-dependent pathway at the level of ERK1/2 (82). Since one function of the CD4/CD8 co-receptors is the recruitment of Lck, the ability of SAgs to bypass Lck is also likely related to the capability of SAgs to activate both $CD4^+$ and $CD8^+$ T cells, despite cross-linking with MHC class II molecules (83, 84). These signaling pathways ultimately activate transcription factors NF-κB, NFAT and AP-1 leading to cytokine production (85). Studies using rapamycin, which specifically inhibits mammalian target of rapamycin (mTOR) complex 1, to target SEB-induced shock has led to the phosphoinositide 3 kinase (PI3K)-mTOR pathway being recognized as an alternate signaling cascade (85).

1.5.1.2 T cell anergy

T cell anergy, a phenomenon where T cells become unresponsive to stimulation, has long been proposed to be an immune subversion tactic of *S. aureus*. Several studies have shown this occurs *ex vivo* following *in vivo* stimulation (86-89). However, SAg-induced anergy produced *ex vivo* does not necessarily translate into *in vivo* anergy (90). In

addition, high levels of purified toxin are often used in experimental mouse models that may not reflect physiological conditions. Also, there is no evidence to suggest that T cells are exhausted in nasal carriers of toxigenic *S. aureus* strains. Recently, a case study of TSS (likely induced by TSST-1) showed deletion followed by an expansion in the $V\beta2^+$ subset that normalized 70 days post-convalescence. In this important study, re-stimulation of PBMCs taken during the acute phase of disease with exogenous SAg resulted in proliferation of $V\beta2^+$ cells suggesting that T cells were not rendered anergic (91). Recurrent cases of TSS occur, usually as a combined result of insufficient eradication of *S. aureus* and the inability to form anti-SAg antibodies. The proliferative response of T cells was assessed from a patient with recurrent TSS and there was no reduction in the ability of the patients T cells to respond *in vitro* (92). Thus, at least in some patients, true anergy of Vβ specific T cell subsets may not occur and suggests that this activity is not the purpose of SAg activity for *S. aureus*. Clinically, recurrent TSS also implies that T cells are not rendered anergic as they are able to react to SAg stimulation during multiple episodes.

1.5.2 Major histocompatibility complex molecules

The HLA locus, which encodes the MHC molecules, is highly polymorphic and plays a significant role in determining the susceptibility of an individual to SAgs. Although SAgs bypass MHC restriction to activate T cells, different SAgs vary in their binding abilities to MHC molecules. For example, SEA, SEB, and TSST-1, show a preference for binding HLA-DR molecules while SEC1 prefers HLA-DQ over -DR (93). Polymorphic alleles also dictate the degree to which a certain SAg is presented to T cells, as well as the T cell response (94, 95). *In vivo* data in mice supports the notion that MHC polymorphisms dictate sensitivity or resistance to SAgs. In particular, transgenic mice expressing human MHC class II molecules (‘humanized transgenic mice’) have enhanced the sensitivity of mice to SAgs and improved the model for studying the effects of SAg toxicity (19, 96-98).

Activation of APCs by SAgs also contributes to cytokine release due to the involvement of MyD88, which upregulates NF-κB, leading to production of pro-inflammatory

molecules (56). Monocytes stimulated by SAgs are potent producers of IL-1 and TNF-α (99). SAgs have also been shown to upregulate the expression of toll-like receptor (TLR) 4 on monocytes which may explain the synergistic effect of LPS and SAg (100).

1.5.3 Costimulatory molecules

Recently, it was demonstrated that SEB can bind to the costimulatory molecule CD28, which is constitutively expressed on naïve T cells and binds B7 ligands on APCs. The CD28 binding site is divergent from both the TCR and MHC II binding domains of SEB, and is relatively conserved amongst the SAg family. Disruption of CD28 binding by peptide antagonists reduced mortality rates in mice administered with D-galactosamine and SEB by downregulating Th1, but not Th2 cytokines (101). These lines of evidence support the proposal that CD28 binding by SAgs is important to the function of SAgs. Further research elucidating downstream mechanisms will clarify the exact role of CD28 during T cell activation by SAgs.

Additionally, this same binding region on TSST-1 mediates interactions with CD40 on human vaginal epithelial cells in the absence of MHC II (102, 103). This novel binding site for epithelial cells may be important in the initiation process of disease in overcoming skin and mucosal barriers, especially in TSS.

1.6 Staphylococcal superantigen diseases

1.6.1 Food poisoning

The first disease linked to the staphylococcal SAgs was SFP, and evidence that a staphylococcal toxin caused the illness dates back to 1930, where filterable supernatants from a “yellow staphylococcus” was able to induce SFP in human volunteers (104, 105). The symptoms of SFP include nausea, emesis, and abdominal cramps with or without diarrhea. This common food-borne illness has rapid onset but is generally self-limiting and resolves within 24-48 hours. As SAgs are highly stable and resistant to heat, acid and proteolytic enzymes that would kill *S. aureus*, SFP is caused by pre-formed toxins in contaminated food that retain biological activity after ingestion (50, 106). Unlike the mechanisms of T cell activation, SAg emesis is poorly understood and divergent from its

immune-stimulating activities. It is thought that the cysteine-loop structure is responsible for emesis, although it is likely to be involved in stabilizing conformations required for activity rather than an absolute requirement (50).

1.6.2 Toxic shock syndrome

The other human disease clearly caused by the staphylococcal SAgs is TSS. This disease was described in 1927 by Franklin Stevens as staphylococcal scarlet fever (107), and was named "toxic shock syndrome" by Todd and colleagues in 1978 to describe a systemic illness in seven children caused by non-invasive *S. aureus* infections (108). The pathogenesis of TSS is due to a SAg-induced cytokine storm owing to the massive activation of T cells in individuals lacking neutralizing antibodies to the particular SAg. The disease is a capillary leak syndrome where patients develop fever, rash, hypotension, multi-organ involvement and convalescent desquamation (18). *S. aureus* can cause the menstrual form of TSS, which historically occurred in young women in association with high absorbency tampons, and non-menstrual TSS, which can occur from virtually any *S. aureus* infection, although infrequently from bacteremia (18). While most staphylococcal SAgs are functionally capable of inducing TSS in experimental animals, only a few select SAgs have historically been associated with the disease. This is somewhat surprising given the large "collection" of these extremely potent toxins. The TSST-1 SAg was linked to the menstrual form of TSS in 1981 (36, 37), although it is also clear that other SAgs, primarily TSST-1, SEB and SEC, are capable of causing the non-menstrual form (17, 18).

During the early 1980s, there were a high number of menstrual TSS cases in young women associated with the use of high absorbency tampons (109) and the estimated incidence of all forms of TSS at this time was 13.7/100,000 (110). By the mid-1980s, following the removal of these products from the market, and public awareness campaigns as well as product labeling, the overall incidence dropped to 0.53/100,000 with a case-fatality rate of ~4% (111). A recent population based surveillance for TSS in Minnesota between 2000 and 2006 demonstrates that this rate has been relatively stable and that TSST-1 was still the major cause in most cases. Of note, although the

community acquired methicillin-resistant *S. aureus* (CA-MRSA) clonal strain USA300 has dramatically increased in prevalence in the U.S., this strain does not appear to cause many cases of TSS (112). Although the overall incidence of TSS appears low, it has been suggested that severe SAg-mediated disease remains under-reported, due to both the strict CDC case definition (113) as well as prompt and appropriate medical attention that would prevent the most severe forms of SAg intoxication (112). Indeed, TSS is still a major problem, and cases of non-menstrual TSS pediatric burn patients can be extremely dangerous if not recognized early (114).

Apart from the more overt forms of SAg-mediated diseases, there is significant evidence that SAgs also can play a role in a number of other diseases and these will be discussed below.

1.6.3 Kawasaki disease

Kawasaki Disease (KD) was first described by Tomisaku Kawasaki in 1967 and is now the leading cause of acquired heart disease in children from developed nations (115). KD is an acute, self-limiting vasculitis, typically affecting the coronary arteries, and thought to be triggered by an infectious agent in genetically susceptible individuals (116). Although the etiology of KD is not known, there is compelling evidence that bacterial SAgs are involved, and could be causal in association with host genetic factors (117). First, the clinical presentation of KD has features reminiscent of TSS, including fever, a desquamating rash and erythema of the mucous membranes. SAg-producing *S. aureus* and *S. pyogenes* have been isolated from KD patients, and seroconversion with anti-SAg antibodies has also been demonstrated. Perhaps the strongest evidence of SAg involvement however, is the demonstration of Vβ skewing in KD patients (118). A number of studies have found primarily Vβ2 expansion (119) providing a link to either TSST-1 or SpeC which are both Vβ2-specific (59). Others, however, have found expansion of various Vβ families (120, 121), potentially implicating other SAgs with different Vβ profiles. Treatment of KD involves the use of intravenous immunoglobulin (IVIG) (122), and IVIG is well known to contain SAg neutralizing antibodies (123, 124). Although there is no direct evidence to suggest SAg involvement, there also exists the

Kawasaki-like syndrome, which in contrast to KD occurs primarily in adults with severe immunosuppression including HIV/AIDS (125).

1.6.4 Atopic dermatitis

Atopic dermatitis (AD) represents a chronic and relapsing T cell-mediated inflammatory skin disorder with immunoglobulin (Ig) E-mediated sensitization to allergens. AD most often affects infants and young children, but may persist into adulthood, or may first develop in adults as late-onset AD. AD has both genetic and environmental contributions but nearly all AD patients are colonized by *S. aureus*. This is likely due to both the damaged skin barrier and impaired host immune responses. The ability of staphylococcal δ-toxin to degranulate mast cells has recently been suggested as a link between staphylococcal colonization and the development of AD lesions (126). A variety of immune mechanisms have been proposed for how staphylococcal SAgs may be exacerbating the disease (103, 127). SAgs have long been known to induce the skin homing receptor cutaneous lymphocyte-associated antigen (CLA) on T cells to recruit these cells to the skin (128). Very recent evidence indicates that skin homing, phenotypically Treg ($CD4^+FoxP3^+$) cells from AD patients may actually display a Th2 phenotype in response to SEB stimulation (129). AD patients may also develop anti-SAg IgE antibodies that can further worsen the condition (130-132). AD is often treated with glucocorticoids and SAgs have been shown to induce glucocorticoid resistance in PBMCs (133). A recent study that examined essentially the entire staphylococcal SAg family found that isolates from steroid resistant AD patients contained significantly more SAgs genes than isolates from non-steroid resistant patients or menstrual isolates provoking the idea that steroid treatment may actually select for SAgs in these strains (134).

1.6.5 Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a group of disorders characterized by inflammation of the nose and paranasal sinuses for at least 3 months duration (135). CRS can occur with or without nasal polyps, and accumulated evidence is now convincing that *S. aureus* SAgs can contribute to, in some cases, CRS with nasal polyposis (136). In this disease, SAgs

are thought to skew the cytokine response towards a Th2 phenotype inducing both eosinophilia and the production of polyclonal IgE, which in turn could be further linked to asthma (137). There is no single SAg associated specifically to this disease (136, 138), and as noted (135), a causal relationship with *S. aureus* has not been established.

1.6.6 Guttate Psoriasis

Guttate psoriasis is an acute form of psoriasis mediated by autoreactive T cells that typically develops in young adults and children. This inflammatory skin disease is typically preceded by streptococcal pharyngitis, and the streptococcal SAgs, in particular SpeC, and $V\beta2^+$ T cells have been implicated (139). Some associations have also been made with *S. aureus* and chronic plaque psoriasis (140, 141).

1.7 *Staphylococcus aureus* overview

S. aureus is a Gram positive bacterium that is a frequent human commensal organism but also a highly versatile pathogen capable of causing a variety of infections in their hosts, resulting in high levels of morbidity and mortality. *S. aureus* is the most common cause of skin and soft tissue infections (SSTIs) worldwide (142), but can also cause life-threatening severe invasive infections such as endocarditis, osteomyelitis, bacteremia (which can progress to sepsis) and TSS (143). *S. aureus* infections are further complicated by an alarmingly high rate of antibiotic resistance acquisition and MRSA strains are of great concern, especially in hospital settings where nosocomial staphylococcal infections are common. *S. aureus* has now become a global epidemic, particularly MRSA clones (144, 145). Although MRSA was originally restricted to healthcare settings, outbreaks of CA-MRSA strains which target healthy young people are now becoming more common (146-150).

The ability to cause infections in virtually every tissue in the body is due to the vast arsenal of virulence factors possessed by *S. aureus*, many of which have evolved to specifically target the human immune system, including SAgs (151, 152). It is remarkable how an asymptomatic colonizing bacteria is capable of so many opportunistic infections and the different lifestyles of *S. aureus in vivo* – colonization versus invasive infections – will be discussed below.

1.8 *Staphylococcus aureus* nasal colonization

Staphylococcal colonization can be defined by the presence and multiplication of *S. aureus* in the absence of infection or disease. In humans, the most common area colonized by *S. aureus* is the anterior nares (143, 153). Prevalence of nasal colonization is high within the general population, and people have typically been classified into 3 groups based on their nasal carriage status: persistent, intermediate and non-carriers. While rates vary among studies, approximately 20% of the general population are persistent carriers of *S. aureus*, ~30% are intermittent carriers, and ~50% are non-carriers (154). In the event of an infection, carriers have a better prognosis than non-carriers (155, 156); however, nasal colonization increases the risk of infection by four-fold (157). Furthermore, it is believed that ~80% of *S. aureus* bloodstream infections come from an endogenous source (155), and this can be particularly dangerous in a hospital setting if a nasal carrier is immunocompromised and the colonizing strain is resistant to antibiotics.

1.8.1 Establishing nasal colonization

Successful establishment of nasal colonization is a result of bacterial, host, and environmental interactions. Hand-to-hand and hand-to-nose transmissions allow *S. aureus* to disseminate between people and within the individual host, eventually reaching the nose. Epidemiological studies have shown that household units, pets, and hospital workers positively influence carriage rates (154). In addition, transmission of nasal carriage has also been shown in mice (158). Behaviors, such as nose picking, have also been positively correlated with nasal carriage (159).

1.8.1.1 Bacterial factors that influence colonization

Once the bacteria reach the nose, *S. aureus* preferentially binds to keratinocytes and desquamated nasal epithelial cells in the anterior nares (160, 161). Histological sections of human cadavers have revealed *S. aureus* colonizing the cornified squamous epithelium, keratinized and mucous debris surfaces, as well as the hair follicles of the nose (162). Nasal colonization is a multifactorial process and depends on a number of adherence and immune-evasion factors. Interactions between microbial surface

components recognizing adhesive matrix molecules (MSCRAMMs) have been shown to promote binding to epithelial cells and colonization. Clumping factor B (ClfB) binds to cytokeratin 10 (K10), expressed on differentiated epithelial cells and has been shown to be important for nasal colonization in both rats and humans (163-165). K8 has been shown to be another possible target for ClfB (166), although it is not expressed on the stratified squamous epithelia. K10 is also a ligand for the adhesion iron-regulated surface determinant A (IsdA) (167). Loricrin, the major protein on keratinized epithelial cells, is a target for both IsdA and ClfB (167, 168). Although their host receptors have not been as well characterized, the MSCRAMMs serine-aspartic acid repeat protein (Sdr) C, SdrD, and surface protein SasG have been shown to mediate adherence to human nasal epithelial cells (161, 169). Wall teichoic acids (WTA) have also been identified as playing an important role in binding (170) to the host receptor SREC-1 on nasal epithelial cells (171). Both WTA and sortase A were found to be important for nasal colonization in a rodent model, although sortase A is necessary for persistence and not the initial stages of nasal colonization (172). A capsule-deficient strain of *S. aureus* also showed decreased colonization at later time points in a mouse colonization model (158). There are clear host differences involved in binding as *S. aureus* exhibits greater adherence to squamous cells from persistently colonized individuals than non-carriers (173, 174), which is likely influenced by both variances in MSCRAMMs between *S. aureus* strains, as well as host polymorphisms (175).

The two-component system WalKR has been shown to be upregulated in a cotton rat nasal model and is also important in human nasal carriage (176, 177). This global regulator is involved in cell wall metabolism and the positively regulated autolysins *sceD* and *atlA* have both been shown to be expressed in human carriers (177); moreover, SceD is essential in a rodent nasal colonization model (178).

Other than adhesion, bacterial factors that confer resistance to oxidative stress are instrumental for *S. aureus* nasal colonization in mice (179). In healthy human carriers, transcription of a number of immune evasion genes could be detected, including *spa* which encodes protein A and is involved in a number of pathogenic processes. Expression of staphylokinase (SAK) and chemotaxis inhibitory protein of *Staphylococcus*

(CHIPS) was high compared to *in vitro* growth and both are involved in immune evasion of the innate immune system (177). Expression of cytolytic toxins such as α-hemolysin and phenol-soluble modulins (PSMs) was generally repressed in healthy persistent carriers (177), while SAg toxin expression has been detected (180). It is likely that these factors are important during colonization to combat the host immune system (discussed below), but there has been no direct experimental evidence thus far.

1.8.1.2 Host immunity of the nasal cavity

Other than providing a physical barrier, the epidermis contains an abundance of antimicrobial defenses produced by epithelial cells and immune cells. Resident cells in the epidermis include Langerhans cells, melanocytes, Merkel cells and T cells (175). The anterior nares are primarily a keratinized environment protected by nasal secretions which contain antimicrobial peptides (AMPs) such as defensins and cathelicidins, lysozyme, lactoferrin, IgG and IgA amongst other antimicrobial components (154, 181). AMPs are produced by many resident skin cells, and play an integral role maintaining skin immunity by directly killing microbes and modulating both innate and adaptive immune responses (182). It is thought that differences in host immunity may be a determinant of nasal colonization but the mechanisms are not well understood. *S. aureus* nasal colonization can induce a subclinical immune response with elevated levels of the cathelicidin LL37, α defensins [human neutrophil peptides (HNP)]1-3 and human β defensin (HBD) 2 in some carriers, but not HBD-3 (183-186). It has also been suggested that colonization is due to carriers producing nasal secretions with poor antimicrobial activity as opposed to bacterial resistance (187), despite mechanisms of resistance against AMPs (188-190). HBD-3 has potent bactericidal activity (191), and keratinocytes are capable of killing *S. aureus* very quickly by directly depositing HBD-3 on the bacteria (192). Since the bactericidal effect relies on direct bacterial contact and was not observed with secretions into media, this may reconcile the fact that levels of HBD-3 are not different between carriers and non-carriers. *In vitro* studies have shown that HNP 1-3 and HBD-2 have poor bactericidal activity against *S. aureus* (184); however, planktonic *S. aureus* became susceptible to HBD-2 once biofilm formation was disrupted through interference by *S. epidermidis* (193). This suggests that *in vivo* conditions may have

different outcomes regarding susceptibility to AMPs and may be responsible for conflicting reports regarding the bactericidal activity of AMPs.

Although levels and presence of antibodies against *S. aureus* exhibit high inter-individual variability (194), it is clear that an anti-staphylococcal humoral response alone is not protective against colonization. Artificial nasal colonization in humans does not influence the anti-staphylococcal humoral response (195), and maternal IgG does not prevent infants from staphylococcal colonization – *S. aureus* colonization is actually higher in children than adults (154) with rates of approximately 50% *S. aureus* nasal carriage in neonates (196, 197). Thus, it is likely that the high levels of anti-staphylococcal antibodies in the population is due to transient breaches of the mucosa rather than colonization (195, 198). Carriers that do develop anti-staphylococcal antibodies are not protected from further colonization (199), although it is thought that these antibodies may protect against severe infection (200).

Clearance of nasally-colonized bacteria has been shown to be mediated by IL-17 and recruited neutrophils in mice (201). However, *S. aureus* colonizing strains have been shown to be able to dysregulate neutrophil-related IL-1 immunity (202), which is essential for host protection during staphylococcal cutaneous infections (203). Additionally, *S. aureus* has evolved many mechanisms to evade neutrophils which are essential for bacterial clearance (204).

1.8.1.3 Nasal microbiota and bacterial interference

In the 1960's, it was observed that colonization by one strain of *S. aureus* prevented colonization by a second strain. Thus, an avirulent strain was used to colonize newborns in hopes that it would protect against colonization by more virulent strains (205-208) but this practice was discontinued when the previously avirulent strain demonstrated pathogenic manifestations (209). The environmental presence of other bacterial species such as *Streptococcus pneumoniae, Staphylococcus epidermidis,* and *Corynebacterium* spp. has experimentally been found to interfere with *S. aureus* nasal colonization (193, 210-212). Interestingly, there is a negative correlation between *S. aureus* and *S. pneumoniae* rates in infants where *S. aureus* carriage is initially high but decreases while

S. penumoniae increases. Since the introduction of a pneumococcal vaccine, the pattern has shifted towards *S. aureus* carriage (213). Bacterial interference using non-pathogenic normal nasal microflora or avirulent strains of *S. aureus* as a method of treating staphylococcal colonization is still under consideration, largely due to antibiotic resistant strains of *S. aureus* (214, 215).

1.8.1.4 Persistence

Once *S. aureus* overcomes the barriers in the nares and colonization is established, the bacteria either persist, are cleared by the immune system, or invade the mucosal layer and cause infections. It has been experimentally shown that after artificial nasal inoculation by *S. aureus*, subjects had a tendency to revert to their original carrier state and carriers were re-colonized by their original strain (216). Further studies also showed that persistent carriers are preferentially colonized by the same strain of *S. aureus* whereas intermittent carriers tend to be colonized by different strains at different time points (217). This may be a result of the host adapting to long-term commensalism, perhaps in a way similar to commensal gut microbiota (218). Regardless, it appears that persistent carriers have attributes that allow a long-term commensal relationship to develop with the colonizing strain, and this remains an active area of research.

1.8.2 From colonization to infection

The particular molecular switch of how colonized bacteria become pathogenic has yet to be determined. It is likely a mixture of host-pathogen and environmental factors that causes a breach in the mucosal layer and subsequent infection or clearance. Although it has been observed that it is the elements of the virulon that determines the occurrence and extent of invasive infection (219), epidemiological studies have failed to assign a particular strain with either nasal or blood isolates (8). Furthermore, it has been shown that under the right circumstances, all strains of *S. aureus* have the ability to become invasive (10, 220). Controlling nasal colonization is a priority for health-care facilities since it predisposes the carrier to more severe staphylococcal infections. The increase in MRSA strains and emerging antibiotic resistance to mupirocin, commonly used to eradiate nasal *S. aureus*, is particularly alarming (221). Numerous studies which have

evaluated the efficacy of preventing staphylococcal infections by eradicating nasal carriage have thus far yielded mixed results, and does not always appear to be effective in preventing infections (222). The commercial vaccine StaphVax (capsular polysaccharide conjugates 5 and 8) failed to reduce levels of nasal colonization in humans (223) and many new targets and strategies are continuously being studied (194, 224).

The role of the two-component regulatory system, the accessory gene regulator (*agr*), has been classically associated with dissemination, release of secretory proteins, and down-regulation of surface associated proteins (225). Burian et al. showed that in persistent nasal carriers, both *agr* and *agr*-regulated toxins are generally not expressed (177). The presence of hemoglobin has been found to inhibit *agr* expression and promote nasal colonization in rats, while constitutive expression of RNAIII, the effector molecule of the *agr* system, reduced colonization (226). This may also partially explain why people who suffer from epitaxis (nosebleeds) are more prone to colonization by *S. aureus* (227). It is probable that *agr* is downregulated during nasal colonization to promote adhesion to the nasal epithelium. Environmental cues, after colonization has been established, may then result in the induction of RNAIII and secreted cytolysins, leading to breaches in the mucosal barrier and subsequent infection.

1.8.3 Nasal colonization and superantigens

Although a myriad of bacterial factors play a role in determining nasal colonization, it has not yet been established whether or not SAgs are involved. Epidemiological studies evaluating *S. aureus* SAg gene distribution in nasal swabs compared with blood isolates concluded that there were no differences between blood and nasal isolates in the number of toxins and there was no correlation to a particular toxin; toxin gene distribution was widespread and highly varied (8). Also, *S. aureus* strains encoding the same SAg genes can produce different amounts of toxin (228) and this can make correlations difficult in epidemiological studies, which often rely on genomic typing instead of protein quantification.

Many SAgs such as TSST-1 are regulated by *agr* (225), which appears to be dampened during colonization (177), suggesting that *agr*-controlled SAgs may not be involved in

colonization. Since it is likely that *agr* is downregulated during colonization as discussed previously, it has been suggested that certain SAgs such as SEA, which is not regulated by *agr*, may play a role early on in colonization (229). Despite the fact that many persistent carriers contain the bacteriophage that carries SEA, this genetic element does not appear to play a role early on during colonization (230). Furthermore, the *sea* gene has been correlated with sepsis, although the presence of SEA has yet to be confirmed in blood during sepsis (9). This work also demonstrated a correlation between the *egc* operon of SAgs and colonization. A follow-up study using recombinant SAgs found that both types of SAgs (*egc* and non-*egc*) induced similar proliferative activity on PBMCs (14). However, the proliferative potential of supernatants taken from patients with strains containing *egc* genes demonstrated that strains encoding *egc* SAgs do not have as high proliferative activity as strains encoding non-*egc* SAgs, suggesting that *egc* toxins are not made in quantities as high as non-*egc* ones. A lack of neutralizing antibodies against *egc*-encoded SAgs was also found in serum from healthy humans (13), although both *egc* and non-*egc* SAgs have been shown to be expressed in nasal carriers suggesting an inability to form antibodies against *egc* toxins (180). It is interesting that only non-*egc* encoded SAgs have been implicated in toxin-mediated diseases. Thus, the role of *egc*-encoded SAgs in colonization requires further investigation.

It is difficult to directly ascertain whether or not SAgs are produced *in vivo* during colonization mainly due to the presence of *S. aureus* protein A, which binds the Fc portion of antibodies, thereby causing background levels of antibody-mediated detection assays to be quite high. However, analysis of the immunological response can provide important information. In particular, both Vβ-specific T cell activation and SAg-neutralizing antibodies are indirect ways of determining if the immune system has encountered SAgs. While Vβ-skewing has been studied in the context of severe disease (231), it has long been known that the general population develops anti-SAg antibodies capable of neutralizing these toxins (232). Also, persistent nasal carriers of *S. aureus* have been found to have neutralizing antibodies against the SAgs produced by the colonizing strain (198, 200). Levels of neutralizing antibodies against TSST-1 and SEA were significantly higher in persistent nasal carriers than non-carriers (233), again suggesting that these SAgs may be actively produced during nasal colonization.

The extent to which, if any, SAgs play during colonization has not yet been experimentally addressed. Intranasal vaccination in rodents with deactivated TSST-1 was able to decrease mortality rates from TSST-1 producing *S. aureus* septic challenge and significantly decreased the bacterial load in organs (234). This was a TSST-1-specific response, as challenge with non-TSST-1 producing *S. aureus* did not result in a significant reduction in bacterial load when compared to non-vaccinated mice. The same vaccination strategy protected against nasal challenge only during the initial colonization phase (days 1 and 3). Since the model only evaluated colonization up to day 7, it is difficult to assess whether or not this is able to have a lasting effect against *S. aureus* nasal persistence, since there were not significant effects at day 5 (234).

Staphylococcal peptidoglycan-embedded molecules have been found to downregulate the immune response stimulated by SAgs (98). This effect was most notable at high cell densities suggesting that it is important in biofilms or established colonizers, as opposed to free-living planktonic cells. Thus, if a colonized population of *S. aureus* is producing SAgs, any invading "rogue" cells that are not a part of the main colony may be killed by an activated immune system, while the dense colony is able to downregulate this response in the local area to prevent clearance. This suggests a role for SAgs as checkpoints of dissemination. Evidence suggests that when SAgs are systemic as in the case of TSS (231), *S. aureus* is able to prevent dissemination, which may be partly why bacteremia is rarely associated with staphylococcal TSS. This is also supported by the observation that sepsis patients lack SAg-specific Vβ-skewing unlike TSS patients (235), suggesting that bacterial dissemination could prevent toxin production.

1.9 *Staphylococcus aureus* severe infections

1.9.1 Bacteremia

Bacteremia, or bloodstream infection, is defined by the presence of viable bacteria in blood (236). Bacteremia can be transient and induced by activities such as tooth-brushing or biopsy, where bacteria are normally cleared without signs of inflammation (237, 238). However in a clinical setting, even a single positive blood culture should be treated as significant since associated complications of *S. aureus* bacteremia (SAB) are damaging

and severe (239, 240). Up to one third of SAB cases are complicated by endocarditis, metastatic infections, or progress to sepsis (143, 241, 242). Bacteremia incidence is on the rise and *S. aureus* is among one of the highest etiological agents of Gram positive blood infection among inpatients in hospitals (243, 244), and the second highest among outpatients (245). In Western countries SAB is associated with mortality rates of 20-30% (246-248), increased hospital stays and economic burdens (244, 249).

The most common causes of SAB are unknown primary sources, intravascular catheters, and infections from secondary sources such as genitourinary, gastrointestinal, respiratory, and biliary tracts, abdominal or cutaneous infections (237). Hematogenous seeding from abscesses or another distal site can cause intermittent and recurring episodes of SAB (237). Expert opinion on successful treatment of SAB includes not only antimicrobial therapy but removal or drainage of the foci, thus making it important to distinguish between primary and secondary infections (240, 250).

1.9.2 Infectious endocarditis

Endocarditis is specifically an infection of the heart endothelium and can develop as vegetations or intracardiac abscesses on heart valves (251, 252). Vegetative lesions are most commonly found on the aortic and mitral valves (253), and are aggregates of fibrin, platelets, bacteria and recruited immune cells which can embolize and establish infections elsewhere (252, 254).

S. aureus is the most common cause worldwide of infectious endocarditis (IE) and a major risk factor for the development of IE is SAB. IE is typically associated with intravenous (IV) drug use and intravascular medical devices such as catheters, pacemakers and prosthetic heart valves (143, 251, 253). IE in IV drug users typically are right-sided while non-drug related cases of IE tend to be left-sided and occur in older patients (143, 255). Patients with *S. aureus* IE also have worse outcomes than non-*S. aureus* IE with a mortality rate of up to 30% (253).

1.9.3 Sepsis

Sepsis is a severe clinical condition that results from systemic inflammation in response to infection. Since shock can occur in the absence of infection, the term systemic inflammatory response syndrome (SIRS) has been created to describe the syndrome of systemic host response in general, which can arise as a result of infection, among other reasons. If SIRS is a result of an infectious insult either by the pathogen itself, toxins or microbial mediators, then the term sepsis is applied (238, 256). During sepsis, microbial components interact with endothelial and immune cells which lead to dysregulation of the coagulation cascade, formation of fibrin clots and activation of various inflammatory mediators. This leads to vascular instability, impaired tissue perfusion, capillary leak, vasodilation and can ultimately result in organ failure (257).

Historically, sepsis was typically associated with Gram negative infections due to the key role that the cell wall factor lipopolysaccharide (LPS) plays in inducing a potent inflammatory response (258). LPS is recognized by the acute phase protein LPS-binding protein, which then binds to the CD14 receptor on the surface of immune cells such as monocytes and macrophages. Cell signaling is induced by TLR4, an integral part of the innate immune system, which is able to mount an immediate response to the presence of LPS (257). However, Gram positive sepsis has risen in prominence over the past 20 years (258, 259), with the most common culprits being *S. aureus*, CoNS, pneumococci and streptococci (258, 260). While Gram positive organisms lack LPS, other cell wall factors such as lipoteichoic acid (LTA) and peptidoglycan have been found to elicit inflammation which can synergize and contribute to the induction of sepsis (261-263). Lipoproteins also induce inflammation via TLR2 (264). Additionally, Gram positive pathogens secrete inflammatory exotoxins, such as SAgs which can cause shock in animal models as well as TSS in humans; and it is thought that SAgs can also contribute to the development of septic shock (260, 265), although the extent of superantigenic activity to the development of sepsis is not clear (257). Staphylococcal sepsis is associated with worse outcomes than SAB with mortality rates as high as 86% being reported, although the severity of SAB is a good predictor of 30-day mortality rates of sepsis (242).

1.10 Mechanisms of *S. aureus* pathogenicity

1.10.1 Adhesion and coagulation

Adhesion to cells is the first step not only in colonization, but pathogenesis as well. *S. aureus* have cell surface factors that facilitate binding to host ligands and colonization. MSCRAMMs bind extracellular matrix molecules (ECM) such as fibrinogen, fibronectin and collagen (266), as well as other cell host receptors such as cytokeratin and loricrin. Adhesion to damaged endothelial cells or heart valves begins the infection process of IE (254); coagulase (Coa) and ClfA are important mediators of adhesion to fibrin and fibrinogen, leading to the development of IE in a rat model (267).

Coagulation is an important host process that limits the spread of infection and also helps to trap bacteria within clots in the bloodstream. However, pathogen interference with the coagulation cascade further contributes to the development of sepsis. Coa and von Willebrand factor binding protein (vWbp) cause fibrinogen cleavage while ClfA mediates binding to fibrin, creating thrombotic lesions (268). In contrast, SAK interacts with plasminogen and acts as a thrombolytic agent that digests fibrin clots, allowing dissemination of bacteria (269). It is clear that *S. aureus* has evolved many mechanisms to interfere with host processes that are designed to protect against pathogens.

1.10.2 Invasion factors

In order to invade, *S. aureus* has many factors that allow it to penetrate epithelial and endothelial layers. Fibronectin-binding proteins (FnBP) not only mediate adhesion but bacterial uptake by endothelial cells, and has been shown enhance virulence and lethality during sepsis (270). A variety of enzymes such as proteases, nucleases, lipases, collagenases and hyaluronidase are thought to be involved in disruption of the epithelial layer, but these mechanisms are not well-defined (143, 271). Exfoliative toxins target desmosomes, creating loss of cell-to-cell adherence (272). α-hemolysin binds not only erythrocytes but epithelial cells via a disintegrin and metalloprotease 10 (ADAM-10), and causes cytolytic pores to form in a variety of cell types (273). ADAM-17 has been shown to be upregulated in vaginal epithelial cells in response to TSST-1 and it is thought that the inflammatory response is essential in the initial stages of disease (274).

1.10.3 Immune evasion factors

Survival in blood and tissues involves evasion of the host's immune system. Our immune system has evolved powerful ways of eliminating pathogens such as *S. aureus*, but these bacteria have in turn, evolved ways to counteract our defenses. *S. aureus* uses multiple factors to neutralize both the innate and adaptive components of our immune system.

Phagocytes are crucial for bacterial clearance and neutrophils and macrophages are the main antagonists of *S. aureus*. Not surprisingly, there are many anti-phagocytic mechanisms that target these immune cells. SSL5 and extracellular adherence protein (Eap) prevent neutrophil recruitment and extravasation to the site of inflammation via receptor blockage. Interference with chemokine signaling via receptor blocking is mediated by SSL3 and SSL5, as well as formyl peptide receptor (FPR) antagonists: CHIPS and FPR-like inhibitory proteins (FLIPr and FLIPr-like). Proteases such as Staphopain A and aureolysin are also involved in degrading components of the immune system and inhibit neutrophil recruitment (204). Protein A binds the Fc portion of antibodies, preventing opsonization and phagocytosis killing (275). Additionally, protein A can also interact with the B cell receptor (BCR) and induce apoptosis of B cells (276). Staphylococcal complement inhibitor (SCIN) prevents deposition of complement proteins on bacterial surfaces and appears to work cooperatively with CHIPS to evade early immune responses (277). Extracellular fibrinogen binding protein (Efb) has been shown to bind both fibrinogen and proteins from the complement cascade that protects the bacterium from being phagocytosed, similar to the function of bacterial capsule (278).

S. aureus is also highly resistant to neutrophil killing as it also has the ability to survive within neutrophils. Staphyloxanthin, the golden pigment that gives *S. aureus* its name, permits resistance to reactive oxygen species among other such factors including catalase (151, 204). Cells of the leukocyte lineage are directly targeted and lysed by pore-forming toxins which include α-hemolysin, Panton-Valentine leukocidin (PVL), γ-hemolysin, leukotoxin ED and leukotoxin AB/GH, as well as small cytolytic peptides known as PSMs (279, 280). PSMs mediate lysis of neutrophils during intracellular growth and facilitate bacterial escape (204). Many of these virulence factors play multiple roles

during infections, and highlight the dynamic and versatile nature of *S. aureus* interactions with the immune system.

1.10.4 Superantigen contribution to infections

Other than SFP and TSS, there is no definitive disease that is caused by SAgs. However, there are substantial experimental data supporting a contribution of SAgs towards overall staphylococcal pathogenesis. In animal models, SAgs have been shown to be critical for the development of infectious endocarditis, dermatitis and arthritis (252, 281, 282). SAg-deletion strains also lower mortality rates in models of necrotizing pneumonia and sepsis/IE (6, 252). Vaccination with SAg or SAg toxoids, or monoclonal antibodies (mAbs) against SAgs have shown efficacy in reducing lethality and virulence in various models of invasive staphylococcal infections (234, 283, 284). The mechanisms of pathogenesis are likely due to a positive feedback loop from tissue and endothelial damage as a result of inflammatory mediators released by SAg activation. Due to the synergistic effects of LPS and SAg-induced shock in experimental models, it has been suggested that SAgs play a biological role during polymicrobial infections, enhancing Gram negative shock (260); whether this is the case during human staphylococcal infections has yet to be determined.

Although the patient sample size was small, a clinical study comparing the Vβ profiles of patients with either *S. aureus* sepsis or TSS revealed that most of the sepsis patients' Vβ profiles showed no skewing which is normally indicative of SAg activation. Of the sepsis samples that showed Vβ-skewing, they did not match the corresponding SAg-induced Vβ profiles of the isolated strain. In contrast, all the TSS patients' Vβ profiles did show skewing which corresponded with specific SAg Vβ signatures of the isolated strain. This is suggestive that SAgs may not be produced in significant quantities during 'typical' sepsis, or that their effects are masked by lymphopenia (in this study, found in all sepsis patients) as a result of sepsis (231). This report suggests that SAgs may not be actively involved during sepsis as previously thought, despite the similarities between TSS and septic shock.

1.11 Rationale and hypothesis

The human immune system has evolved to be able to recognize and eliminate pathogens and their antigens. However, SAgs represent the only known microbial virulence factor whose primary role is to deliberately force the activation of the adaptive immune system. This is counter-intuitive given the numerous staphylococcal virulence factors apparently designed for immune subversion and evasion (151). Since SAgs are primarily encoded on mobile genetic elements, it is likely that these genes would be lost if they did not contribute to the overall fitness of *S. aureus*. It is clear that SAgs enhance the lethality and virulence of staphylococcal infections; however, this still does not explain why *S. aureus* produce SAgs. Enhancing mortality is not evolutionarily prudent for survival and transmission to new organisms, and obviously death of the host would deprive *S. aureus* of a viable niche. With such widespread distribution and variability of SAg genes, the question arises as to what purpose SAgs serve for *S. aureus* – one that has remained largely unanswered. Given the adaptive nature of *S. aureus* to asymptomatically colonize as a commensal as well as causing a variety of pathogenic infections, it is likely that SAgs may have different contributions to these opposing lifestyles, or may play a role in the molecular switch from one lifestyle to another. Although traditionally viewed as disease-causing virulence factors that encourages the pathogenicity of *S. aureus*, I propose a different function of SAgs in the context of colonization versus dissemination and hypothesize that SAgs maintain *S. aureus* colonization by acting as 'checkpoints' to prevent bacterial dissemination within the host. Furthermore, with the numerous array of virulence factors designed to evade phagocytosis, I hypothesize that during invasive *S. aureus* infections, SAgs function to increase bacterial fitness by preventing bacterial clearance.

1.12 Specific aims

The specific aims of this thesis were to evaluate isogenic SAg-negative strains of *S. aureus* against their wild-type counterparts in i) a SAg-sensitive murine model of nasal colonization and ii) a bacteremia model, in order to elucidate the role that SAgs play during these opposing lifestyles.

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Chapter 2 Expression of superantigen influences *Staphylococcus aureus* nasal colonization[2]

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2.1 Introduction

Staphylococcus aureus is a commensal bacterium that is also one of the most common sources of nosocomial infections. These infections can range in severity from superficial skin infections to invasive deep tissue infections, infective endocarditis and sepsis (1) and the increasing prevalence of methicillin-resistant *S. aureus* strains (MRSA) has resulted in an increase of morbidity and mortality (1-4). *S. aureus* carriage is ubiquitous in the population, being found most typically on the skin and nasal cavities. Nasal carriers have been defined as persistent, intermittent, or non-carriers, although the definitions for each group can vary between studies. In general, persistent and intermittent carriers account for least 50% of the population, with some studies showing even higher levels of colonization (4, 5). *S. aureus* typically resides in the vestibulum nasi of the anterior nares and has been found colonizing the cornified layer of stratified squamous epithelium, keratinized surfaces and mucous debris, as well as hair follicles of human noses (6). Given these anatomical findings, it is not surprising that *S. aureus* are able to bind to keratinized cells and desquamated nasal epithelial cells as key host cells upon which to initiate colonization (7, 8). Bacterial components contributing to staphylococcal colonization are multifactorial and include host genetic factors that influence carrier status (9), as well as a variety of bacterial adhesins and cell-wall associated factors such as clumping factor B (ClfB) (10), wall teichoic acids (11), surface protein SasG (12), and iron-regulated surface determinant A (IsdA) (13).

Nasal carriers of *S. aureus* are generally asymptomatic and healthy, forming a commensal relationship with the bacteria. However, colonization status increases the risk of a severe infection from the carrier strain, although nasal carriers tend to have a better prognosis in the event of a staphylococcal infection (14). This is thought to be due to specific immunity built up against the colonizing strain which is usually dominant in a persistent carrier (15). The humoral response of persistent carriers show a robust response against the adhesins ClfA and ClfB, as well as the superantigens (SAgs) toxic shock syndrome toxin-1 (TSST-1), and staphylococcal enterotoxin A (SEA) (16).

SAgs are a group of toxins produced by bacteria including *S. aureus* that mediate interactions between peptide-MHC class II and the CDR2 loop of the variable chain of

the T cell receptor that bypasses antigen-specificity (17). This can result in activation of up to ~20% of the exposed T cell population and the subsequent release of excessive amounts of cytokines, known as a 'cytokine storm'. These toxins are the causative agents of toxic shock syndrome (TSS), and have been implicated in many other diseases including infectious endocarditis, Kawasaki disease, atopic dermatitis, and various autoimmune diseases (17, 18). To date, more than twenty *S. aureus* SAgs have been identified including an operon of SAgs, the enterotoxin gene cluster (*egc*), encoding staphylococcal enterotoxins (SE) G, I and SE-like (SEl) M, N, O and U (17, 19). Epidemiological studies of clinical isolates reveal the high prevalence of *egc* SAgs (20), as well as a negative correlation of these toxins with severe septic shock (21). Assessment of the humoral response from persistently colonized individuals have shown that these carriers produce high titres of neutralizing antibodies with high specificity for the SAgs produced by the carrier strain (15, 22). Nasal swabs from persistent carriers revealed that *sea*, *sec* and *sel-o* were actively transcribed; however, neutralizing antibodies against SEA and SEC but not SEl-O were detected in this cohort (23). It was concluded that the robust antibody response against the non-*egc* SAgs was due to minor infections rather than colonization, although this was not tested. Vaccination of mice with SAg toxoids seems to protect only against the early phase of colonization (days 1 and 3) (24). This study suggests that SAgs may be involved in initial colonization, but further implications are difficult to extrapolate. Collectively, these studies have shed light on the highly complex nature of nasal colonization and hinted at a role for SAgs in humans and mouse infection models. However, the role of SAgs during nasal colonization, either for establishing initial colonization, or involvement in dissemination, has not been experimentally addressed.

Human studies reveal low levels of bacteria in the nose, with 10^1-10^4 colony forming units (CFU) of *S. aureus* being isolated from nasal swabs (25). We hypothesize that secreted SAgs act as 'checkpoints' of colonization in order to maintain this state of commensalism and to prevent high bacterial densities through activation of the immune system and subsequent elimination of invasive organisms. In order to test our hypothesis, we created isogenic SAg deletions of two well-characterized strains of *S. aureus*, and tested these strains against their wild-type counterparts in a SAg-sensitized murine model

of staphylococcal nasal colonization. We discovered that the deletion of SEA from *S. aureus* Newman transiently increased nasal colonization compared to wild-type Newman colonization, while expression of SEB lowered the ability of *S. aureus* COL to nasally colonize mice compared to its *seb*-negative counterpart. These experiments demonstrate that SAgs play a role in modulating bacterial numbers in the nasal cavity during colonization.

2.2 Materials and Methods

2.2.1 Mice

Six-to-twelve week old male and female HLA-DR4-IE (DRB1*0401) humanized transgenic mice lacking endogenous mouse MHC-II on a C57BL/6 (B6) background (herein referred to as DR4-B6 mice) were used for all *in vivo* infection experiments (26). B6 mice were purchased from Charles River. All animal experiments were performed according to protocols approved by the Animal Use Subcommittee at Western University and in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

2.2.2 Bacterial strains, media and growth conditions

Escherichia coli DH5α was used as a cloning host, grown in Luria Bertani (LB) broth (Difco; Mississauga, ON, Canada) supplemented with 150 μg/mL ampicillin where necessary, at 37°C with shaking at 250rpm. Strains of *S. aureus* are listed in Table 2.1 and were grown in tryptic soy broth or agar (TSB/TSA) (Difco) at either 30°C or 37°C with shaking, and supplemented with appropriate antibiotics (Sigma Aldrich; Oakville, ON, Canada). Endogenous microbiota strains were isolated from mice on either TSA or mannitol salt agar (MSA) plates grown at 37^{o}C. Growth curves were performed using a Bioscreen C MBR system (Thermo Labsystems; Milford, MA, USA).

2.2.3 Selection of a streptomycin-resistant *S. aureus* strain

Staphylococcus aureus strain Newman is an early methicillin-sensitive clinical isolate from the 1950's that is commonly used in experimental studies of staphylococcal pathogenesis (27). Initial attempts to colonize mice resulted in competition with endogenous bacterial species and poor *S. aureus* colonization. This phenomenon has been documented previously in the literature (28) and represents an additional challenge for *S. aureus* to colonize in nature. However, for the purposes of testing our hypothesis, an antibiotic dosing regime was instated with streptomycin sulfate (Sm) in order to reduce the endogenous murine microbiota, as previously described (28). Since *S. aureus* Newman is not naturally resistant to Sm, a mutated strain was engineered by plating

Table 2.1 Strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
S. aureus Newman	Early methicillin sensitive isolate from secondary infection in a patient with tubercular osteomyelitis (Sm sensitive)	(27)
S. aureus Newman (SmR)	*S. aureus* Newman resistant to Sm	This study
S. aureus Newman Δ*sea* (SmR)	*sea*-null *S. aureus* Newman (with resistance to Sm)	This study
S. aureus RN4220	Restriction-deficient derivation of NCTC8325-4	(51)
S. aureus COL	Early methicillin-resistant strain of *S. aureus* isolated in the 1960s	(33)
S. aureus COL Δ*seb*	*seb* deletion strain of *S. aureus* COL	This study
E. coli DH5α	Cloning strain	Invitrogen
E. coli BL21 (DE3)	Protein expression strain	New England Biolabs
Plasmids		
pET28	Protein expression vector	Novagen
pET28::*sea*	Recombinant SEA expression vector	This study
pDG1513	Source of tet^R gene	(32)
pMAD	Integration plasmid	(31)

S. aureus Newman on Sm gradient TSA plates and selecting for bacteria that gained increased resistance until an isolated strain, *S. aureus* Newman SmR, was able to be grown in TSB + 500 μg/mL Sm. No loss of resistance was observed after daily 1% subcultures in TSB without Sm for up to 6 days. Since the growth rate was reduced with the inclusion of Sm (data not shown), preparations of bacteria for inoculation into mice were cultured without Sm. *spa* genotyping (29) showed that *S. aureus* Newman SmR had the same genetic background as Sm-sensitive Newman and qRT-PCR showed normal levels of *sea* expression (data not shown). Isogenicity was retained during construction of the *sea* deletion strain Newman Δ*sea* as described below, by using the Sm resistant strain of *S. aureus* Newman as wild-type host so that the Sm resistance marker was identical in both strains. Thus for the remainder of the experiments, Newman SmR will be referred to as Newman and the isogenic *sea* deletion strain as Newman Δ*sea*.

2.2.4 Construction of *S. aureus* Newman Δ*sea*

Restriction enzymes were purchased from New England Biolabs (Whitby, ON, Canada) and primers were designed using Primer3 software (30) and supplied by Sigma Aldrich. Standard techniques were used for the following molecular cloning procedures. The gene encoding for staphylococcal enterotoxin A (SEA) in *S. aureus* strain Newman was insertionally inactivated with a tetracycline-resistant cassette using an established protocol and as depicted in Figure 2.1 (31). Wild-type *sea* along with its corresponding upstream (Up) and downstream fragments (Down) were PCR amplified from the genome of Newman using seaFP 5'-AACGGGATCCCATGTGCTTGAACTTAGAGAGGAA-3' and seaRP 5'-TTCGGTCGACCCCAATAGCTTTTGCGATGT-3' and directionally cloned into pMAD via *Bam*HI and *Sal*I sites. A 261 bp fragment was excised from the middle of *sea* using *Cla*I and *Eco*RI, and replaced with a tetracycline resistance marker (*tetR*) excised from pDG1513 (32). This construct was then transformed into *S. aureus* Newman after undergoing methylation in *S. aureus* RN4220. Allelic replacement of the wild-type *sea* with *tetR* via homologous recombination was conducted as described (31). The resulting *sea*-null *S. aureus* strain Newman Δ*sea* was confirmed to be tetracycline resistant and erythromycin sensitive, with the *tetR* insertion verified by PCR with primers flanking the genomic region outside of the original amplicon and DNA sequencing.

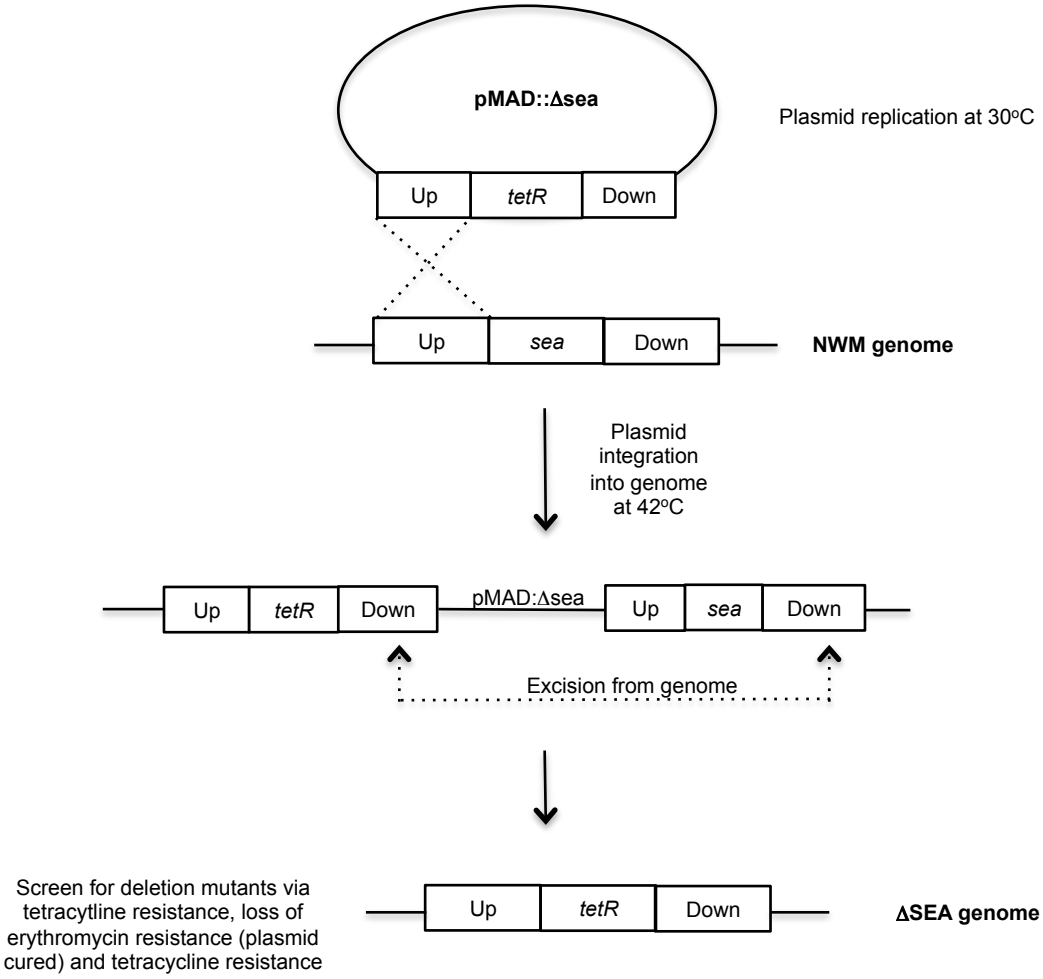


Figure 2.1 Deletion of *sea* from *S. aureus* Newman. Schematic and protocol of *sea* deletion from *S. aureus* Newman using double homologous recombination. Up and Down designates the region upstream and downstream respectively of *sea* from the genome of *S. aureus* Newman and *tetR* denotes the tetracycline resistance cassette. This schematic represents one of two possible scenarios for homologous recombination (upstream vs. downstream) and excision

2.2.5 Construction of *S. aureus* COL Δ*seb*

S. aureus COL is one of the earliest MRSA strains to be isolated in the 1960's and data mining of the sequenced COL genome (33) revealed three SAgs: SEB, SEl-K and SEl-I (formerly SEQ (34)). COL was found to be inherently resistant to Sm and thus did not require a new Sm-resistant strain to be generated. A markerless deletion was created in *seb* based on previously described methods as depicted in Figure 2.2 (31). Briefly, a 524 bp fragment upstream of *seb* was amplified using the primers 5'-TAGGGATCCAGCTCGTGATATGTTGGGTAAA-3' and 5'-GGGCGGGTCGACTGAAATAAATAATCTCTTATACA-3' along with a 505 bp region downstream of *seb* amplified by the primers 5'-CGATGTCGACTATCTTACGACAAAGAAAAAGTGAAA-3' and 5'-TCAGGAATTCGAGATGCTTTGAAAGAAGCAAA-3'. These products were directionally cloned into pMAD, creating pMAD::*seb* which only includes 54 bp of the original 801 bp encoding *seb*. This knockout construct was methylated by *S. aureus* RN4220 and electroporated into *S. aureus* COL. To create the *seb* knockout, a single-integration event was first isolated, followed by subcultures in TSB without antibiotics grown at 30^{o}C. Since pMAD contains β-galactosidase, patching of white colonies detected colonies that had lost resistance to erythromycin, evident of plasmid curing and screened by PCR to verify successful deletion of *seb*.

2.2.6 Construction and purification of recombinant SAgs.

Wild-type *sea* lacking the signal peptide was PCR-amplified from the genome of *S. aureus* Newman using the primers 5'- GGGCCATGGGCAGCCATCATCATCATCATCACAGCAGCGGCGAAAACTTGTATTTCCAAAGCGAGAAAAGCGAAGAAAT-3' and 5'-GGGGGATCCTTAACTTGTATATAAATATATATC-3', introducing nucleotide sequences encoding a His_6-tag and tobacco etch virus (TEV) protease cleavage site (ENLYFQ↓G) onto the N-terminus of *sea*. This PCR product was inserted into pET28a (Novagen) via *Bam*HI and *Nco*I sites to create pET28a::*sea* and transformed into *E. coli* BL21 (DE3) for protein purification. Cells were induced with 200 μM isopropyl β-D-1-thiogalactopyranoside (Sigma Aldrich) to express His_6-tagged SEA and purified using nickel column chromatography as previously described (35). The His_6-tag was removed with TEV protease and dialyzed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

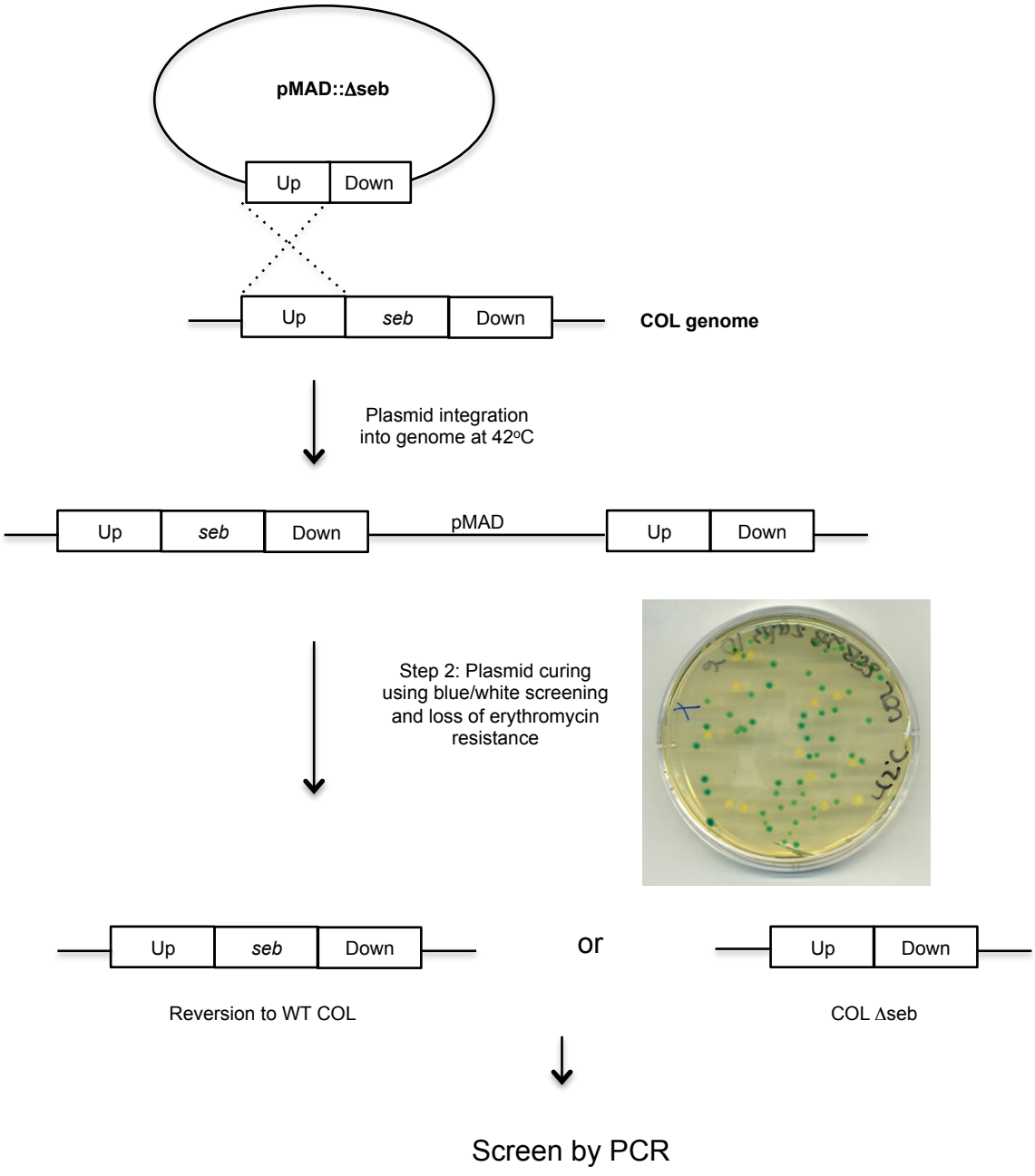


Figure 2.2 Deletion of *seb* from *S. aureus* COL. Schematic and protocol of markerless *seb* deletion from *S. aureus* COL using a two-step double homologous recombination method. Up and Down designates the region upstream and downstream respectively of *seb* from the genome of *S. aureus* COL.

(HEPES) or phosphate buffered saline (PBS) before use. Proteins were quantified using a bicinchoninic acid (BCA) commercial kit (Pierce Biotechnology; Rockford, IL, USA) according to manufacturer's instructions. Recombinant SEB was generated as previously described (36).

2.2.7 Detection of SAgs in cultural supernatants *in vitro*

Bacterial cultures were grown overnight in TSB, cells were pelleted, and cell-free supernatants equivalent to 5.0 OD_{600} units of culture were collected. Proteins were precipitated with 10% trichloroacetic acid (TCA) overnight on ice, washed twice with ice-cold 70% ethanol and resuspended in Laemmli buffer as previously described (37). Samples were analyzed on 12% polyacrylamide gels stained with Coomassie Brilliant Blue R-250. For Western blot analysis of SEB expression, samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore; Etobicoke, ON, Canada) at 100V for 1 hour. The membrane was blocked at roomed temperature for 1 hour with PBS supplemented with 10% skim milk and 5% horse serum (Gibco; Burlington, ON, Canada). Following removal of the blocking buffer, the membrane was incubated with rabbit polyclonal anti-SEB antibodies (kindly provided by Dr. Patrick Schlievert) diluted 1:100 in PBS supplemented with 5% skim milk and 2.5% horse serum. The membrane was washed three times with PBS supplemented with 0.02% Tween-20 (Fischer Scientific; Ottawa, ON, Canada) (PBST), followed by incubation with IRDye-conjugated goat anti-rabbit secondary antibody (LI-COR Biosciences; Lincoln, NB, USA) diluted 1:10 000 in PBST supplemented with 5% skim milk and 2.5% horse serum for 1 hour in the dark. The membrane was imaged using an Odyssey imager (LI-COR Biosciences).

2.2.8 Anti-SEA antibody production in rabbits

Twenty-five μg of recombinant SEA suspended in PBS was emulsified in incomplete Freund's adjuvant in a total volume of 1mL and injected subcutaneously into a New Zealand rabbit by Animal Care and Veterinary Service at the University of Western Ontario. Two and four weeks after the initial vaccination, booster injections at the same SEA concentration were administered. A sample of baseline blood was taken prior to vaccination and further blood samples taken two and five weeks after the initial

vaccination. Sera from the bleeds were collected and frozen at -80°C and anti-SEA antibodies were detected by enzyme-linked immunosorbent assay (ELISA), as previously described (38). 96-well certified high-bind plates (Costar; Oakville, ON, Canada) were coated with 10 μg/mL of recombinant SEA in coating buffer (eBioscience) overnight. Plates were washed twice with distilled water and blocked with PBST supplemented with 1% bovine serum albumin (BSA) (Sigma Aldrich) for 2 hours. Plates were washed three times with PBST, followed by three washes with distilled water and serially-diluted serum samples were added in the plate and incubated for 2 hours. Plates were washed as before with PBST followed by addition of 1:10 000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Rockland; Gilbertsville, PA, USA) for 2 hours. Plates were washed five times as before and developed for 15 minutes with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BD). The reaction was stopped by addition of 1 N H_2SO_4 and the absorbance was determined at 450 nm with subtraction of 570 nm wavelength background. All steps were carried out at room temperature.

2.2.9 Cellular proliferation quantification.

The ability of B6 and DR4-B6 mice to respond to SEA was assessed using the incorporation of [^{3}H]thymidine as described (39). Mouse spleens were collected and broken into a single cell suspension, followed by erythrocyte lysis in ammonium-chloride-potassium (ACK) buffer. The remaining cells were suspended in RPMI (Invitrogen Life Technologies) supplemented with 10% FBS (Sigma Aldrich), 100 μg/mL streptomycin and 100 U/mL penicillin (Gibco), 2 mM L-glutamine (Gibco), 1 mM MEM sodium pyruvate (Gibco), 100 μM nonessential amino acid (Gibco), and 25 mM HEPES (pH 7.2) (Gibco), and seeded into 96-well plates at a density of 1×10^6 cells/mL. Various concentrations of recombinant SEA were added to cells and incubated for 72 hours at 37°C. Cells were then pulsed with 1 μCi/well of [^{3}H]thymidine for an additional 18 hours prior to harvesting on fiberglass filters. Counts were measured using a 1450 Microbeta liquid scintillation counter (Wallac; Woodbridge, ON, Canada).

2.2.10 Assessment of superantigenic activity of *S. aureus* SAg deletion strains *in vitro*

Supernatants from *S. aureus* strains were tested for SAg activity using DR4-B6 splenocytes seeded into 96-well plates as described above. Titrations of recombinant SEA or SEB, and supernatants from overnight cultures of *S. aureus* Newman, Newman Δ*sea*, COL and COL Δ*seb* diluted 1:10 were added to splenocytes for 18 hours at 37°C, and supernatants were assayed for IL-2 by ELISA according to manufacturer's instructions (eBioscience; San Diego, CA, USA).

2.2.11 *Staphylococcus aureus* nasal colonization model

Twenty-four hours prior to inoculation, mice were administered drinking water supplemented with 2.0 mg/mL of Sm *ad libitum*, which was changed every 3-4 days for the duration of the experiment. Bacteria picked from a TSA plate were grown in 5 mL TSB overnight (16-18 hours), OD_{600} was adjusted to 1.0, subcultured 2% into 50 mL TSB and grown to exponential phase (OD_{600} ~ 3.0-3.5). The bacterial pellet was washed 3 times with Hank's Buffered Salt Solution (HBSS) (Hyclone; Logan, UT, USA) and suspended at a concentration of 1×10^{10} CFU/mL in HBSS. Isofluorane-anesthetized mice were nasally inoculated by slowly pipetting 5 μL into each nare and allowing the animal to breathe in the suspension naturally, resulting in a total inoculum of 1×10^8 CFU *S. aureus* per mouse. Mice were weighed and monitored daily according to animal ethics use protocol and sacrificed at days 3, 7, 10, and 14. To enumerate the amount of bacteria in the nose, euthanized mice were decapitated and the lower jaws removed. The entire snout was excised using the back of the mouth opening as an anatomical marker in order to include any bacteria in the nasal passage. The whiskers and surrounding skin were removed without touching the nose and the remaining tissue was collected in HBSS. The kidneys, hearts, lungs, livers and spleens were also collected and all organs were homogenized, serially diluted and plated on MSA (Difco) to differentiate between *S. aureus* and endogenous bacteria. Counts were not different between plates containing Sm and without Sm (data not shown), thus Sm was not included in plates. Plates were enumerated after being incubated at 37°C for 24 hours. Counts less than 3 CFU/10 μL were considered below the detectable limit.

2.2.12 Determination of SAg function *in vivo*

Lymph nodes (cervical, axillary, brachial, inguinal, and popliteal) were isolated *in toto* from mice and pushed through a cell strainer to create a single cell suspension in PBS. Cells were stained with APC-conjugated anti-CD3 (clone 145-2C11) (eBioscience) and FITC-conjugated anti-Vβ3 (clone KJ25) (BD Pharmingen; Missisauga, ON, Canada) or FITC-conjugated anti-Vβ8 (clone KJ16) (eBioscience) and assayed using a FACSCanto II (BD). Data were analyzed using FlowJo v.8.7. (Treestar; Ashland, OR, USA).

2.2.13 Detection of serum anti-SAg antibodies

Sera from mice were collected at time of sacrifice and stored at -20^{o}C. Anti-SAg serum immunoglobulin G (IgG) were determined by ELISA as described above for rabbit anti-SEA IgG. Detection of IgG positive antibody titres were determined as greater than four times the average of control serum. HRP-conjugated goat anti-mouse IgG (Sigma Aldrich) was used as the secondary antibody.

2.2.14 Statistical analyses

Data were analyzed using unpaired student's t-tests. All statistical analyses were performed using Prism v5.0 (GraphPad; La Jolla, CA, USA) with $p < 0.05$ being considered significant.

2.3 Results

2.3.1 DR4-B6 mice are sensitized to superantigens

A general feature of most bacterial SAgs is that these toxins do not efficiently bind mouse MHC-II molecules (40, 41). *S. aureus* Newman encodes the SEA SAg (42) so we first tested the ability of recombinant SEA protein to activate splenocytes isolated from both B6 and DR4-B6 transgenic mice. SEA resulted in a dose-dependent proliferative response as low as 1 pg for splenocytes from DR4-B6 mice, while proliferation of B6 splenocytes was not detected above background levels (Fig. 2.3). Thus, remaining experiments were conducted in DR4-B6 mice.

2.3.2 SAg deletion strains have reduced superantigen production and activity *in vitro*.

A *sea* deletion mutant was generated in *S. aureus* Newman as described in the materials and methods. The exoprotein profile of *S. aureus* Newman Δ*sea* lacked detectable SEA by Western blot (Fig. 2.4A), confirming the deletion. Additionally, the supernatants from *S. aureus* Newman and Newman Δ*sea* strains were tested for SAg activity on DR4-B6 splenocytes using IL-2 production as a measure of T cell activation. Ten-fold diluted supernatants from wild-type *S. aureus* Newman induced ~50 pg/mL IL-2 from DR4-B6 splenocytes which extrapolated to secreted SEA concentrations of ~100 ng/mL. In contrast, we did not detect IL-2 production from Newman Δ*sea* supernatants confirming both the genetic deletion and that other functional DR4-B6 reactive SAgs, such as the genome-encoded SEl-X (which is the only other known SAg encoded by Newman) (43), do not display superantigenic activity for DR4-B6 splenocytes in these growth conditions (Fig. 2.4B). Similarly, a *seb* deletion mutant created in *S. aureus* COL, described in the materials and methods, did not produce SEB as detected by Western blot in the exoprotein profile of *S. aureus* COL Δ*seb* (Fig. 2.5A). IL-2 production was barely detected above background when DR4-B6 splenocytes were treated with cultural supernatants from COL Δ*seb* compared to wild-type COL (Fig. 2.5B). The minute levels of IL-2 induced by COL Δ*seb* can be attributed to the remaining two SAgs, SEI and SEK, although it appears that SEB is the main SAg produced by *S. aureus* COL *in vitro*. Growth curve analysis of SAg deletion strains compared to their wild-type counterparts

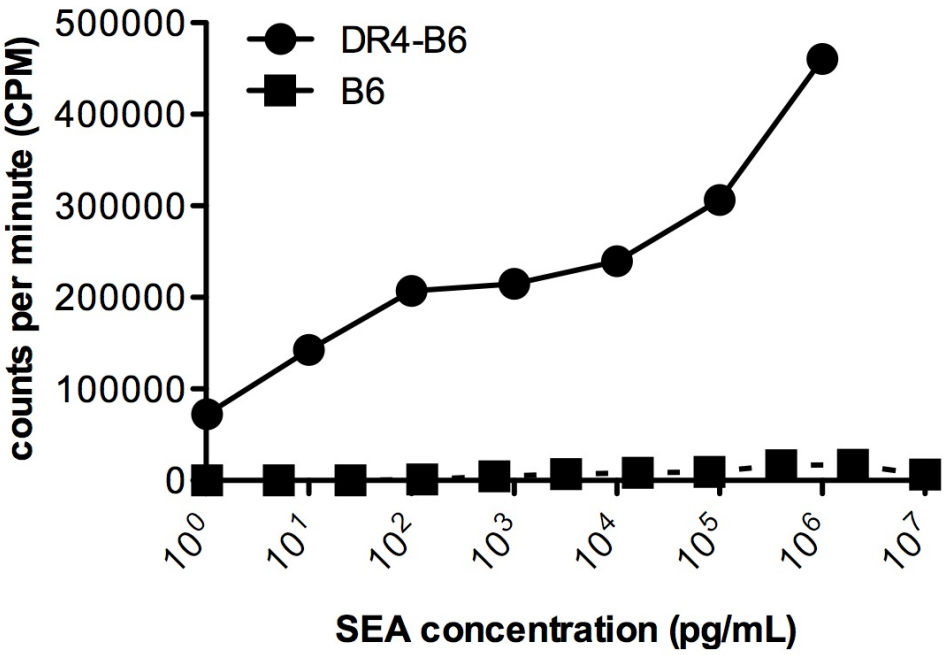
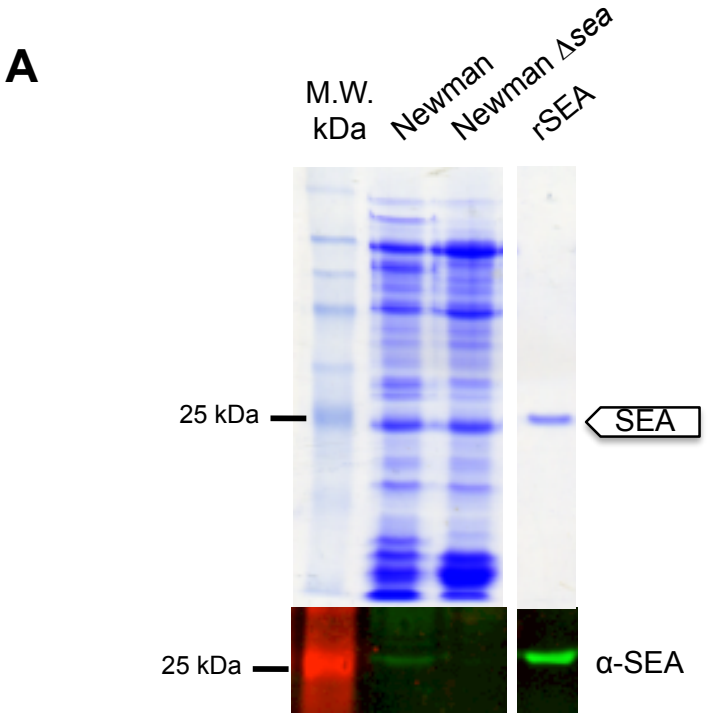


Figure 2.3 DR4-B6 transgenic mouse splenocytes proliferate more in response to SEA than B6 mouse splenocytes. Splenocytes from DR4-B6 (black circles) and B6 (black squares) mice were treated with increasing concentrations of recombinant SEA for 72 hours, followed by the addition of tritiated thymidine. Proliferation was recorded by a scintillation counter as radioactive counts per minute. Results show a representative data set.

Figure 2.4 *S. aureus* Newman Δ*sea* does not produce SEA and has greatly reduced superantigenic activity. A) TCA-precipitated supernatants (5 OD units) showing the exoprotein and SAg profiles of *S. aureus* Newman and Newman Δ*sea* and detection of SEA production by anti-SEA antibodies using Western blot. B) IL-2 production from DR4-B6 splenocytes activated with increasing concentrations of recombinant SEA (white bars) and bacterial supernatants diluted 1:10 from *S. aureus* Newman and Newman Δ*sea* (black bars). Results shown as the mean ± SEM from a representative data set.


A
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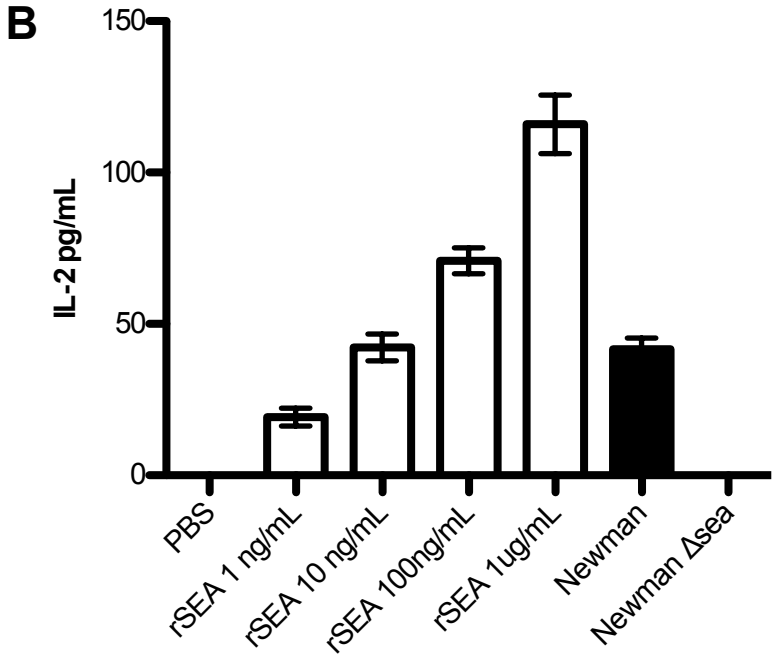
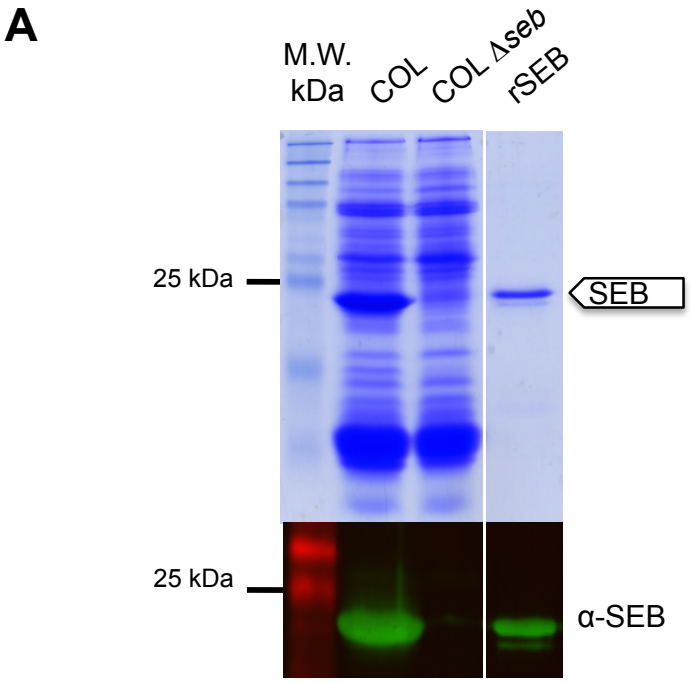

B
IL-2 pg/mL
150
100
50
0
PBS
rSEA 1 ng/mL
rSEA 10 ng/mL
rSEA 100ng/mL
rSEA 1ug/mL
Newman
Newman Δsea

Figure 2.5 *S. aureus* COL Δ*seb* does not produce SEB and has greatly reduced superantigenic activity. A) TCA-precipitated supernatants (5 OD units) showing the exoprotein and SAg profiles of *S. aureus* COL and COL Δ*seb* and detection of SEB production by anti-SEB antibodies using Western blot. B) IL-2 production from DR4-B6 splenocytes activated with increasing concentrations of recombinant SEB (white bars) and bacterial supernatants diluted 1:10 from *S. aureus* COL and COL Δ*seb* (black bars). Results shown as the mean ± SEM from a representative data set.


A
M.W.
kDa
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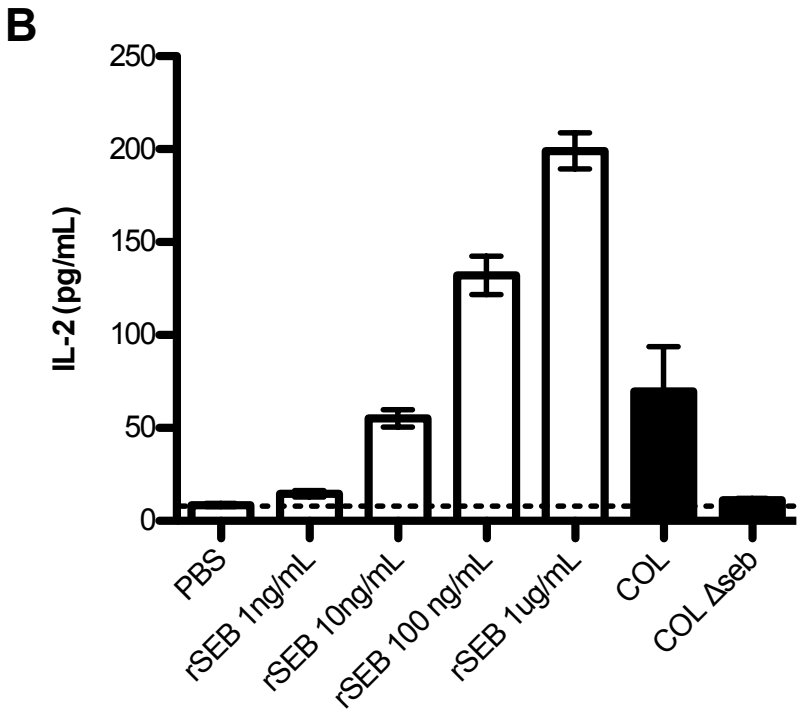
B
IL-2 (pg/mL)
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200
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0
PBS
rSEB 1ng/mL
rSEB 10ng/mL
rSEB 100 ng/mL
rSEB 1ug/mL
COL
COL Δseb

showed no obvious growth defects *in vitro* (Fig. 2.6); thus, it is not likely that lack of SAg production is due to an inability to reach the same growth phases as wild-type strains. Furthermore, comparison of the exoproteins between wild-type and their counterpart SAg-deletion strains reveal no major differences in secreted proteins other than the presence or absence of SAg (Fig. 2.4A and 2.5A), confirming the isogenicity of the deletion strains.

2.3.3 Lack of SEA transiently increases *S. aureus* Newman Δ*sea* nasal colonization.

To investigate if SEA plays a role during murine nasal colonization, DR4-B6 mice pre-treated with Sm were inoculated with 1×10^8 CFUs of *S. aureus* Newman or *S. aureus* Newman Δ*sea*. *S. aureus* was detected in the nasal passages of both *S. aureus* Newman and *S. aureus* Newman Δ*sea*-infected mice up to day 14 post-inoculation. Generally, CFU counts were higher during the first week of colonization compared to the second week (Fig. 2.7). Infected mice did not show overt signs of infection (lack of piloerection, conjunctivitis, skin rashes, and dehydration, with normal activity levels), had no weight loss, and were generally healthy for the duration of the experiment (data not shown). Despite the apparent lack of infection, the lungs and livers of both infection groups revealed spread of bacteria beyond the nose, although the bacterial burdens in these organs were lower than in the nasal passage and generally very low by day 14 (Fig. 2.7B and C). Bacteria were not detected in the kidneys, heart or spleen (data not shown). No significant differences in bacterial loads were observed between bacterial strains on days 3 or 7 in the nose. However, by day 10, *S. aureus* Newman Δ*sea*-colonized mice had increased counts of nasal bacteria compared to wild-type-colonized mice (Fig. 2.7A); although, this phenotype reverted to no differences between treatment groups by day 14. These data suggest that SEA does not play a major role during the initial stages of colonization, but may prevent higher bacterial densities from forming in the nose. While the lack of SEA production did allow higher bacterial densities to form, this transient growth did not result in better colonization at later time points, suggesting that it does not enhance the overall colonization capabilities of *S. aureus* Newman. No significant

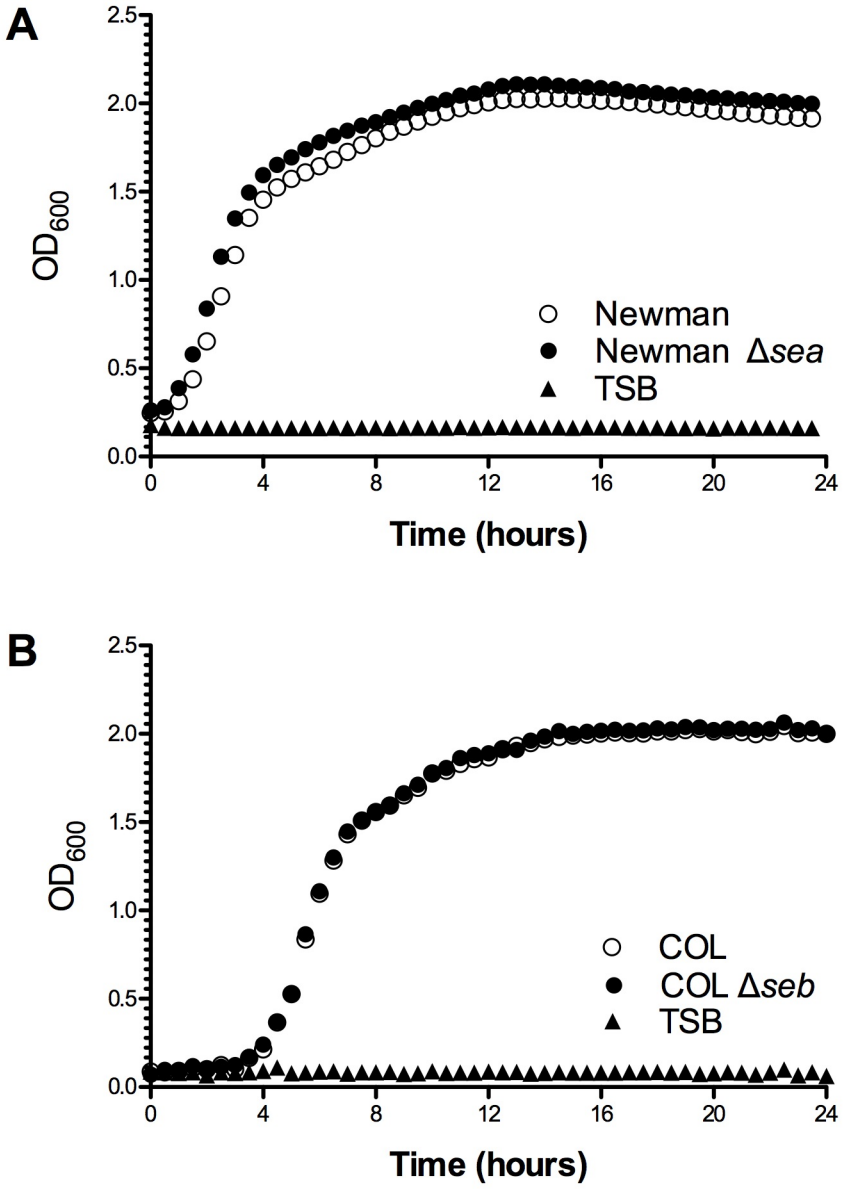
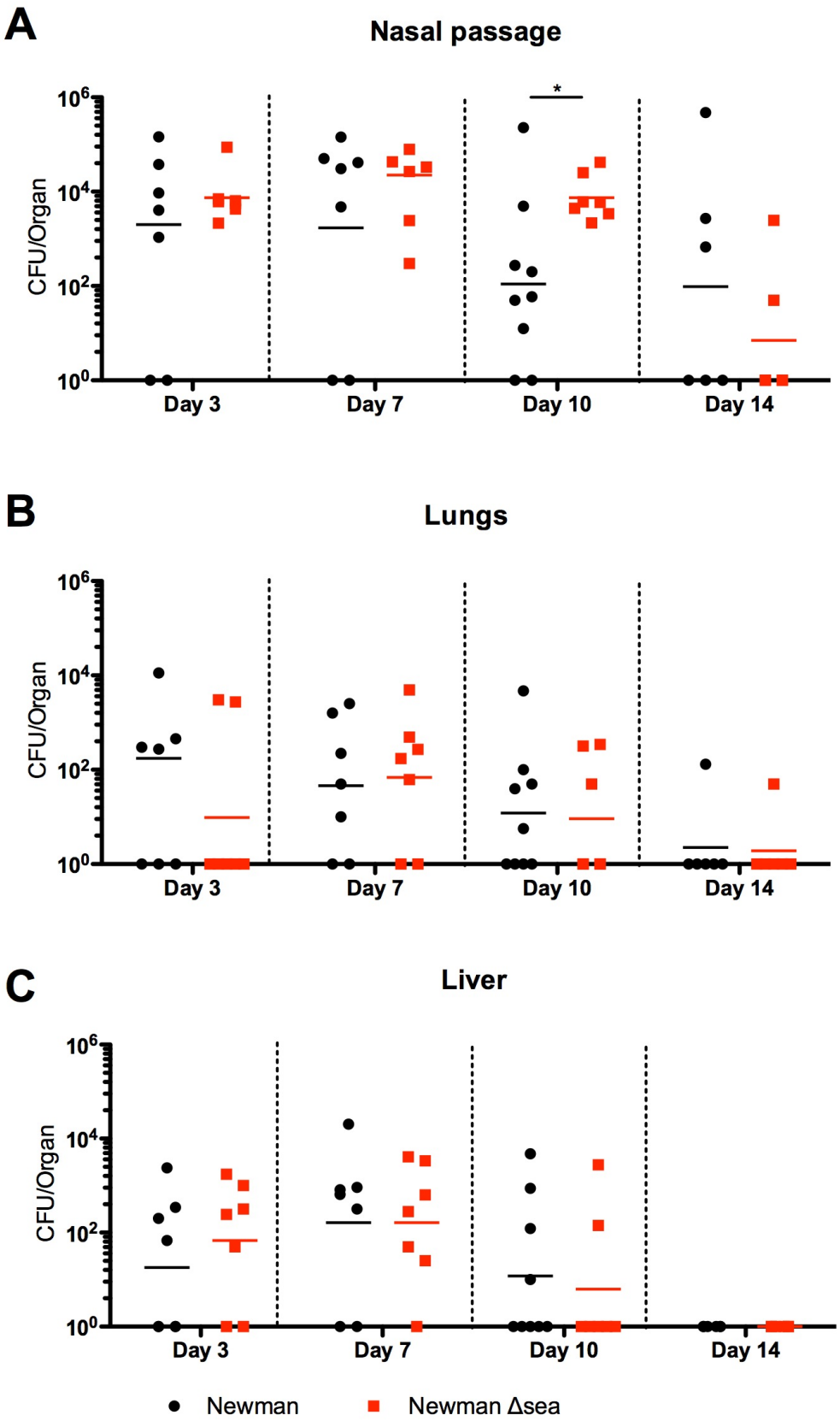


Figure 2.6 SAg deletion does not affect *S. aureus* growth *in vitro*. Growth curve analysis of A) *S. aureus* Newman (open circles) and Newman Δ*sea* (black circle) in TSB, and TSB only (black triangle) and B) *S. aureus* COL (open circles) and COL Δ*seb* (black circle) in TSB, and TSB only (black triangle) grown in triplicate.

Figure 2.7 Nasal colonization of DR4-B6 mice with *S. aureus* Newman Δ*sea* results in a transient increase in bacterial load compared to wild-type Newman. DR4-B6 mice were infected nasally with 1×10^8 CFUs of *S. aureus* Newman (n = 6-9) or Newman Δ*sea* (n = 6-7). Mice were sacrificed on days 3, 7, 10 and 14 and the A) nasal passage, B) lungs and C) livers were assessed for overall *S. aureus* burdens. Each point represents an individual mouse and the line in each treatment group represents the mean. Counts below the limit of detection are interpreted as having no counts. Data are representative of at least three independent experiments. Significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *.


A
Nasal passage
CFU/Organ
10^6
10^4
10^2
10^0
*
Day 3
Day 7
Day 10
Day 14
B
Lungs
CFU/Organ
10^6
10^4
10^2
10^0
Day 3
Day 7
Day 10
Day 14
C
Liver
CFU/Organ
10^6
10^4
10^2
10^0
Day 3
Day 7
Day 10
Day 14
Newman
Newman Δsea

differences were observed in the spread of infection to other organs between wild-type and *sea*-null infection indicating that SEA likely does not influence dissemination.

2.3.4 SEA does not skew Vβ3 subsets *in vivo*

We aimed to determine if SEA was produced during *S. aureus* colonization by examining the Vβ profiles of infected mice. As SEA is known to target murine $V\beta3^+$ T cells (44), we analyzed the Vβ3 subset as well as levels of serum IgG against SEA in order to assess if SEA had *in vivo* activity. Analysis of the $V\beta3^+CD3^+$ lymphocytes from lymph nodes revealed no significant changes in this subset between *S. aureus* Newman or Newman Δ*sea*-inoculated mice on any of the days analyzed (Fig. 2.8), although there is a trend of decreased $V\beta3^+$ T cells in wild-type Newman-colonized mice. These data suggest that SEA may not be produced in large amounts or is weakly active during the length of the experiment. Additionally, no IgG against SEA could be detected in Newman and Newman Δ*sea*-inoculated mice sera. Collectively, these data suggest that SEA was not produced in functionally detectable quantities *in vivo* during colonization. This may explain that lack of differences seen in bacterial burdens at earlier time points (Fig. 2.7A), since the lack of SEA production by *S. aureus* Newman is functionally equivalent to infection with Newman Δ*sea*.

2.3.5 SEB influences nasal colonization

Unlike SEA, SEB is transcriptionally activated by the accessory gene regulator (*agr*) quorum-sensing system during exponential and late stages of growth (45) and may result in differential expression in response to environmental cues. Similar to colonization with *S. aureus* Newman, bacteria were found in the nasal passages of infected mice in both treatment groups; however, colonization with wild-type *S. aureus* COL persisted with higher bacterial numbers (10^3 –10^4) (Fig. 2.9A) compared to wild-type Newman (10^2–10^3) (Fig. 2.7A) especially at later time points, suggesting that COL may be a better nasal colonizer than Newman. When the mice were colonized with *S. aureus* COL Δ*seb*, bacteria recovered from the nasal passages was ~100-fold higher CFUs at all time points compared with wild-type COL colonization alone (Fig. 2.9A). As with nasal colonization by *S. aureus* Newman, all mice were apparently healthy for the duration of the

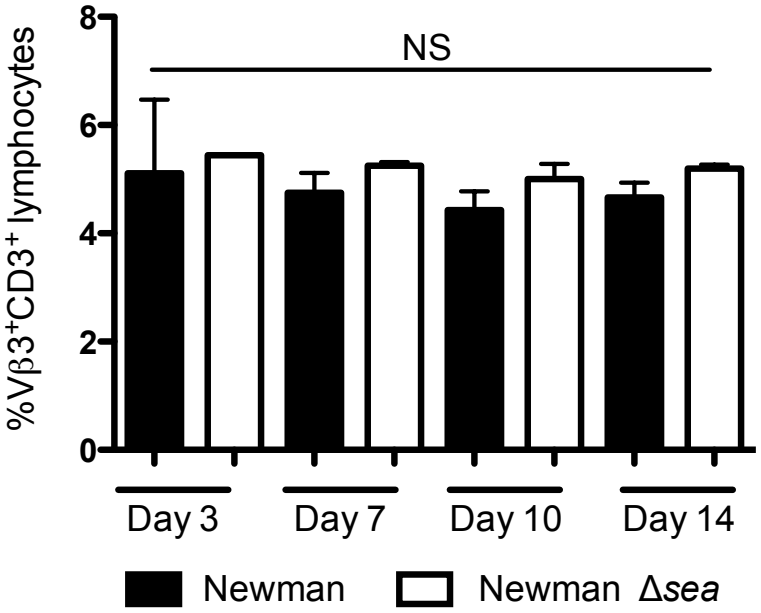
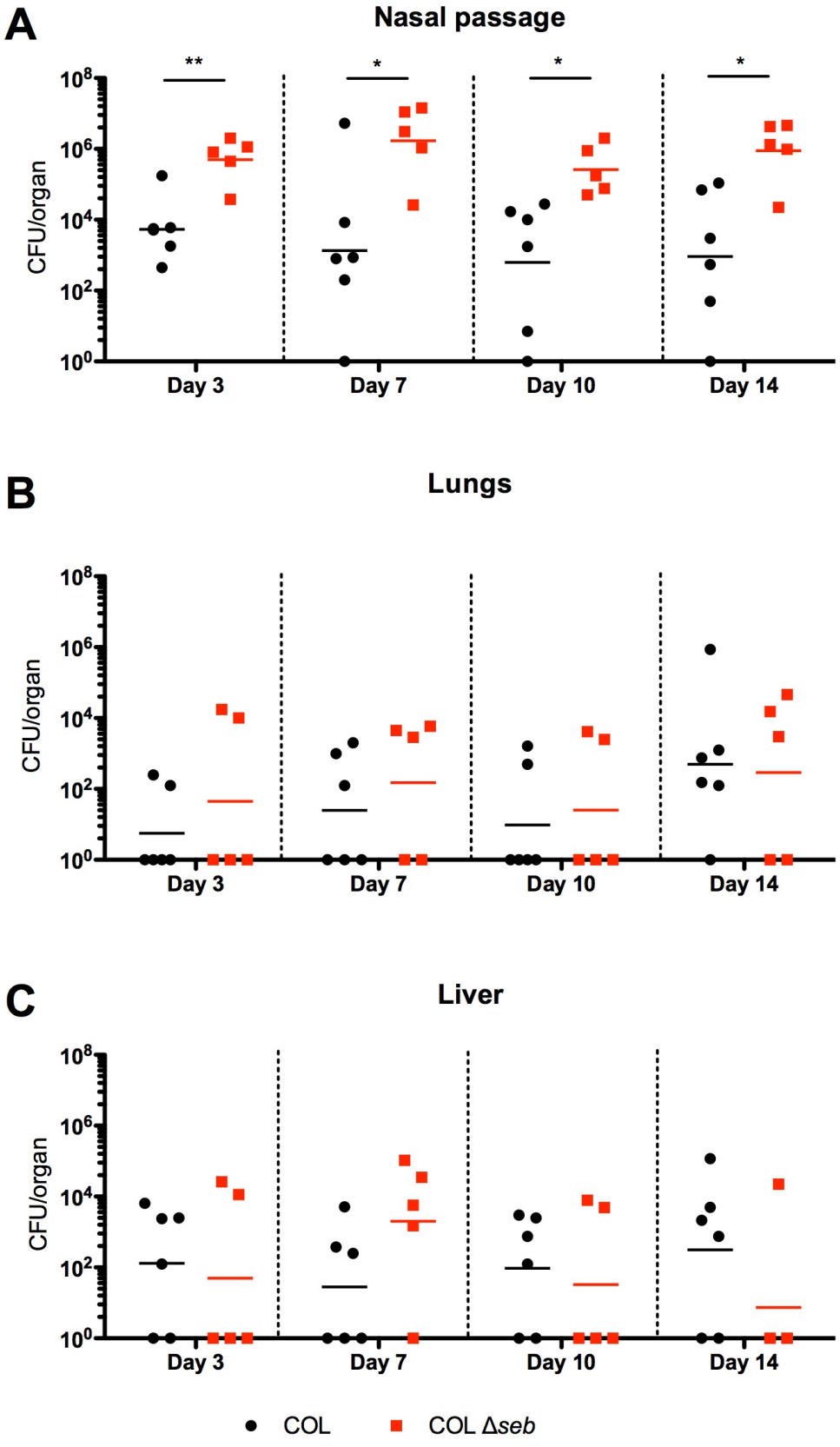


Figure 2.8 *S. aureus* Newman nasal colonization does not result in significant changes in the percentage of Vβ3$^+$CD3$^+$ T cells. Analysis of lymphocytes from lymph nodes isolated from DR4-B6 mice nasally inoculated with 1×10^8 CFU *S. aureus* Newman or Newman Δ*sea* (n = 2-4). Cells were stained with antibodies against CD3 and Vβ3 and gated on CD3$^+$ lymphocytes, followed by gating on the Vβ3$^+$CD3$^+$ population. Data are shown as the mean ± SEM and significant differences ($p < 0.05$) were determined by unpaired student's t-test (NS = no significance).

Figure 2.9 Murine nasal colonization with *S. aureus* COL Δ*seb* results in enhanced bacterial counts compared to wild-type COL. DR4-B6 mice infected nasally with 1×10^8 CFUs of *S. aureus* COL (black circles, n = 6) or COL Δ*seb* (red squares, n = 5) were sacrificed on days 3, 7, 10 and 14. The A) nasal passage B) lungs and C) livers were assessed for overall *S. aureus* loads. Each point represents an individual mouse and the line in each treatment group represents the mean. Counts below the limit of detection are interpreted as having no counts. Data are representative of at least three independent experiments. Significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *, $p < 0.01$ is denoted by **.


A
Nasal passage
**
*
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CFU/organ
10^8
10^6
10^4
10^2
10^0
Day 3
Day 7
Day 10
Day 14
B
Lungs
CFU/organ
Day 3
Day 7
Day 10
Day 14
C
Liver
CFU/organ
Day 3
Day 7
Day 10
Day 14
COL
COL Δseb

experiment with no obvious signs of infection. Spread of the infection to the lungs and livers were also observed during *S. aureus* COL and COL Δ*seb* colonization, although no significant differences were observed between the two strains (Fig. 2.9B and C). No bacteria were detected in the kidneys, hearts or spleens (data not shown). While a complete SAg-negative strain was not assessed, this data suggests that the presence of SEB inhibits high-density colonization of the nasal passage.

2.3.6 SEB induces late Vβ8 skewing but not anti-SEB IgG during nasal colonization

To evaluate if the phenotype observed during *S. aureus* COL colonization was SEB-dependent, we assessed Vβ-skewing in mice colonized with *S. aureus* COL and COL Δ*seb* to test for functional SEB activity. SEB targets Vβ8.1/8.2 (henceforth Vβ8) T cells in mice (44) and Vβ3 was used as an internal control as it is not targeted by SEB. The murine Vβ subsets targeted by SEl-K and SEl-I are unknown to date and thus could not be assessed for *in vivo* activity although these SAgs showed little superantigenic activity *in vitro* (Fig. 2.5B). While no differences could be detected at early time points (days 3 and 7), by day 10 there was a trend of decreased $V\beta8^+$ T cells which was significantly decreased by day 14 (Fig. 2.10). Interestingly, anti-SEB IgG antibodies were not detected from either COL or COL Δ*seb*-colonized mice, except for one mouse at day 3 (data not shown). The demonstrated Vβ-skewing by day 14 indicates that SEB was produced and functional during *S. aureus* COL nasal colonization. Furthermore, the difference in bacterial loads between COL and COL Δ*seb* (Fig.2.9A) at early time points suggests that SEB is functioning early on during colonization although we were not able to detect functional activity until the later time points.

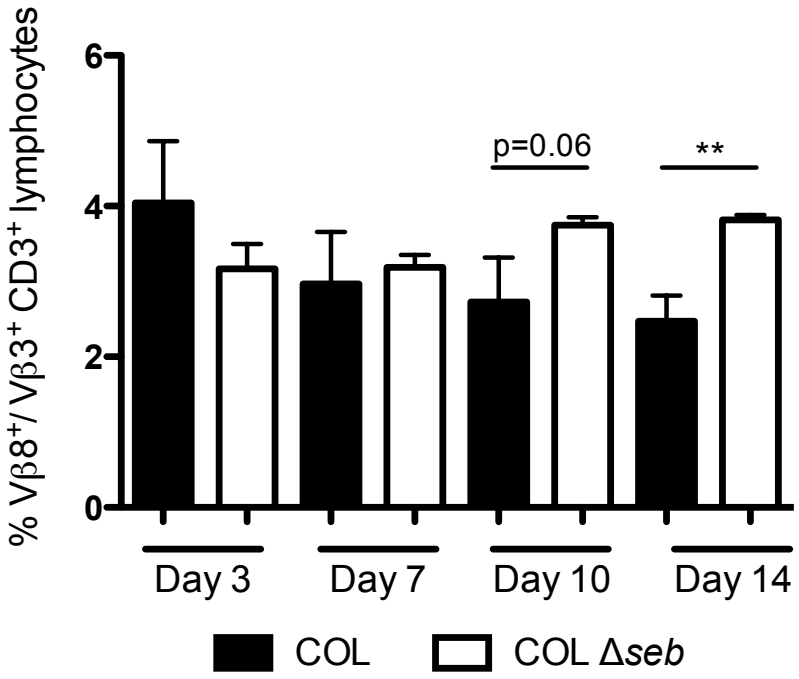

Figure 2.10 SEB is produced during *S. aureus* COL nasal colonization and specifically interacts with Vβ8$^+$CD3$^+$ lymphocytes. Lymphocytes from lymph nodes isolated from DR4-B6 mice nasally inoculated with 1×10^8 CFU *S. aureus* COL or COL Δ*seb* were analyzed using flow cytometry (COL n = 3, COL Δ*seb* n = 2-5). Samples were stained with antibodies against either CD3 and Vβ3 or CD3 and Vβ8. Each mouse sample was stained with both Vβ3 and Vβ8, using Vβ3 as the internal control. Samples were gated on CD3$^+$ lymphocytes, followed by gating on the Vβ3$^+$CD3$^+$ or Vβ8$^+$CD3$^+$ population and expressed as a ratio of Vβ8$^+$ CD3$^+$ to Vβ3$^+$CD3$^+$ cells per mouse. Data are shown as the mean ± SEM and ** denotes $p < 0.01$, as determined by student's-test.

2.4 Discussion

This is the first study where the role of SAgs has been directly and experimentally assessed during a controlled model of nasal colonization using SAg-sensitive, humanized transgenic mice. Our experiments revealed that different SAgs may play distinctive roles during colonization as SEA did not alter CFUs for *S. aureus* Newman nasal colonization, while SEB reduced *S. aureus* COL colonization. Although *S. aureus* Newman also encodes *sel-x* and COL encodes *sei* and *sel-k*, the *in vitro* stimulation data suggests that in our growth conditions, these SAgs are not made in high quantities by these strains and thus may not play a major role in our model. However, future studies should assess a complete SAg deletion strain in comparison to wild-type colonization.

Data from previous human studies suggest that SAgs may be involved during *S. aureus* colonization from two lines of evidence: real-time PCR analysis of nasal swabs from persistent carriers show transcription of *sea* (23) and the finding that persistently-colonized individuals have high levels of neutralizing antibodies against SEA and TSST-1 (16). Although it has been suggested that non-*agr* regulated SAgs such as SEA may be involved during the early phases of colonization (46), this was not supported by our model when we inoculated DR4-B6 mice with *S. aureus* Newman. SEA expression during Newman colonization is supported by the increase in bacterial colonization at day 10 by *S. aureus* Newman Δ*sea* despite the lack of significant Vβ-skewing. These data suggest that SEA was expressed in small amounts and inhibited the formation of high bacterial densities in the nasal cavities. Conversely, the decrease in Vβ8 T cells during colonization with *S. aureus* COL compared to COL Δ*seb* mice is indicative of SEB expression by COL, which is responsible for the difference in nasal bacterial burdens. Direct comparison of the role of SEA versus SEB is difficult because they are encoded by two distinct strains. However, a notable difference between SEA and SEB lies in their regulation and expression: SEA is generally not produced in large amounts, whereas SEB production can reach high concentrations *in vitro*, likely due to the activation of the *agr* two-component system (Fig. 2.4A and 2.5A). Thus, the high expression of SEB by *S. aureus* COL may have resulted in colonization with lower bacterial counts due to its

inflammatory properties at all time points while lower expression of SEA by *S. aureus* Newman did not have as dramatic differences.

The absence of anti-SAg antibodies by day 14 is suggestive that either the SAgs were not processed as conventional antigens and presented to B cells, or that anti-SAg antibodies were not IgG isotypes and thus could not be detected by the assay employed. Human studies have concluded that colonization by *S. aureus* does not appear to induce a strong humoral response (23, 47). Thus, the high levels of anti-SEA antibodies in healthy subjects (16) may not be a result of persistent colonization, but rather breaches of the nasal mucosa from colonizing *S. aureus*, or mild skin infections. It has also been noted that anti-SAg antibodies are not always produced when the immune system is subjected to wild-type SAg, whereas SAg toxoids are much more immunogenic and are capable of forming robust anti-SAg antibodies (24, 48), suggesting that SAgs can dysregulate the antibody response. Furthermore, it has been shown that naïve T cells exposed to SAgs will restrict antibody production, but will not affect 'primed' T cells (49) which may likewise explain the lack of anti-SAg IgG in our colonized mice (who have not been previously exposed to SAgs). TSS patients that fail to seroconvert after an episode may lead to recurrence, which has been attributed to the mechanisms of TSST-1 that prevent the development of Th2 responses, and thus T-cell dependent B cell activation (50).

Our study was extended to 14 days to observe differences in dissemination to other organs. *agr*-regulated SAgs such as SEB and TSST-1 may be involved in dissemination from the main bacterial colony, during which many exoproteins and virulence factors are produced, as opposed to cell-surface factors such as MSCRAMMs required for the initial colonization phase (46). Surprisingly, we found bacteria in the lungs and livers of colonized mice as early as 3 days even though the mice did not show any overt signs of infection. There were no significant differences in the bacterial loads in these extra-nasal locations between the wild-type strains and their SAg deletion counterparts, suggesting that neither SEA nor SEB were involved in dissemination from the nasal cavity.

While SAgs are generally thought to enhance virulence, the deletion of SAgs actually increased bacterial CFUs in the nasal cavity indicative of greater bacterial fitness.

Interestingly, although colonization with *S. aureus* Newman Δ*sea* resulted in higher bacterial counts at day 10, this did not translate into long-term fitness and actually decreased back to wild-type levels by day 14. This suggests that higher bacterial densities in the nose may not be beneficial for asymptomatic colonization. Extending the length of the study may further clarify this theory since COL Δ*seb* maintained a higher bacterial density throughout the duration of the experiment. Although we did not observe differences in dissemination in our model during *S. aureus* COL and COL Δ*seb* colonization, the highest bacterial counts in the lungs were mostly COL Δ*seb*–inoculated mice, suggesting increased seeding from the higher bacterial counts in the nasal cavity. Given that bacteria colonizing the anterior nares are poised for both transmission between people and dissemination within the host, the vestibulum nasi is a desirable environment for *S. aureus* to reside in. Thus, *S. aureus* may utilize SAgs to prevent nasal bacteria from overwhelming this niche and breaching the mucosa, potentially leading to elimination by the immune system, thus acting as 'checkpoints' of dissemination. Since higher densities of bacteria may result in a greater inflammatory response, maintaining a low presence in the nose may be an evolutionarily prudent tactic to maintain long-term asymptomatic colonization. This is supported by the low bacterial burdens isolated from human nasal carriers during asymptomatic colonization (25). Thus, this work supports the clinical finding that SAgs are expressed during nasal colonization (23), and that these toxins may play an important role for influencing bacterial densities during this commensal lifestyle. This provides evidence for a novel role for SAgs, contrary to the traditional role of having been associated with enhancing virulence in severe invasive diseases.

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Chapter 3 Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival *in vivo*[3]

[3] Parts of this chapter have been previously published and are adapted from:

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3.1 Introduction

Staphylococcus aureus is a common human commensal equipped with numerous virulence factors that allow this organism to successfully colonize and infect host tissues. Staphylococcal diseases most frequently manifest as skin and soft tissue infections with a high propensity for abscess formation (1-3); however, *S. aureus* is also readily capable of disseminating into deeper tissues to cause invasive and life-threatening infections including endocarditis, osteomyelitis and sepsis (1-3). Moreover, *S. aureus* can also induce toxin-driven diseases such as food poisoning, staphylococcal scalded skin syndrome, and the toxic shock syndrome (TSS) (4). The versatility of this bacterium as a successful commensal and pathogen, coupled with the development of resistance to a wide array of antibiotics, has led to the establishment of *S. aureus* as a leading cause of both hospital- and community-associated infections (5, 6).

Many of the specialized *S. aureus* virulence factors have evolved to target innate immune mechanisms, primarily neutrophils and macrophages, which are key cells involved in the clearance of *S. aureus* (7-9). In contrast, *S. aureus* also secretes superantigens (SAgs) that directly target and activate cells of the adaptive immune system (10, 11). The family of SAgs in *S. aureus* now includes over 20 genetically distinct SAg variants that comprise the staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like (SEls) toxins, and toxic shock syndrome toxin-1 (TSST-1) (12). These functionally unique exotoxins circumvent antigen presentation by engaging lateral surfaces of MHC class II (MHC-II) molecules (13-16), and complementarity determining region (CDR) 2 of the T cell receptor (TCR) β-chain variable region (Vβ) (17-20). Thus, SAgs alter the conventional TCR-peptide-MHC-II activation complex to prevent antigen recognition by the CDR loops (21), leading to the activation and expansion of numerous T cells in a Vβ-restricted manner (22). In cases of severe SAg intoxication, excessive T cell activation can result in a cytokine storm leading to the development of TSS (11, 23).

In vivo mouse experiments using the injection of purified SAgs have demonstrated many important features of SAg biology, yet these experiments cannot recapitulate the complex interactions between *S. aureus* and the host. Although *S. aureus* has been intensively studied using live *in vivo* infection models, relatively few reports have examined the role

of SAgs using genetically controlled SAg-knockout strains. Early work by Tarkowski and colleagues has demonstrated a pathogenic role of TSST-1 for the onset of dermatitis, arthritis and septic mortality in mice (24, 25). In addition, vaccination with SAg toxoids, or neutralization of SAgs with monoclonal antibodies, have prevented or reduced mortality from experimental *S. aureus* sepsis (26-28). Rabbits are particularly sensitive to the effects of SAgs and using this animal species, deletion of the gene encoding SEl-X from *S. aureus* USA300 demonstrated reduced mortality from necrotizing pneumonia (29) and deletion of the gene encoding staphylococcal enterotoxin C (*sec*) from *S. aureus* MW2 prevented mortality in a rabbit model of sepsis/infective endocarditis (30). Furthermore, engineered high-affinity SAg inhibitors, or vaccination with SAg toxoids, can protect rabbits from *S. aureus* pneumonia, infective endocarditis and sepsis (31-33). Collectively, these studies show unequivocally that SAgs enhance the severity and lethality of staphylococcal infection.

The majority of the human population has circulating antibodies against SAgs that are protective against TSS – which rarely develops (34, 35) – indicating that SAg exposure does not usually result in overt disease. Furthermore, at least 80% of clinical strains of *S. aureus* are genetically positive for at least one SAg gene (36), although this preceded the discovery of *selx* which been found in ~95% of *S. aureus* strains (29) suggesting that the prevalence of SAg genes is even higher than previously thought. Thus, the high prevalence and widespread distribution of SAgs in *S. aureus* suggests these toxins provide an evolutionary advantage to *S. aureus*. Although SAg-induced virulence has been attributed to the cytokine storm that results in immune cell infiltration, pyrexia, hypotension, endothelial damage (29, 30) and ultimately death, enhanced host mortality may not provide an evolutionarily prudent tactic for bacterial survival and propagation. We reasoned that there are other biologically relevant SAg functions that contribute to *S. aureus* fitness and given that *S. aureus* is one of the most common sources of bacteremia (37), we set out to study the role of SAgs in this context. Using an isogenic *sea* knockout strain of *S. aureus*, we found that SEA manipulates the immune system and recruits neutrophils to promote formation of hepatic abscesses, forming a protective niche for staphylococcal survival *in vivo*.

3.2 Materials and Methods

3.2.1 Mice

Six-to-twelve week old male and female HLA-DR4-IE (DRB1*0401) humanized transgenic mice (DR4-B6) (38) were bred and housed in specific pathogen-free facilities at Western University. All animal experiments were in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, and the animal protocol was approved by the Animal Use Subcommittee at Western University.

3.2.2 Bacterial strains, media and growth conditions

S. aureus strains listed in Table 3.1 were grown aerobically at 37°C in tryptic soy broth (TSB) (Difco) with shaking (250 rpm), or on tryptic soy agar (TSA), supplemented with the appropriate antibiotics (Sigma Aldrich). *Escherichia coli* DH5α was used as a cloning host and was grown in Luria Bertani broth (LB) (Difco) or LB agar supplemented with appropriate antibiotics at 37°C with shaking (250 rpm).

3.2.3 *in trans* complementation of Newman Δ*sea*

The *sea*-null strain Newman Δ*sea* (as described in Chapter 2) was complemented by amplifying the native *sea* promoter and complete *sea* gene from Newman using the primers seaFP and seaRP and cloned into the *Bam*HI and *Sal*I sites of the plasmid pALC2073 (39). This construct (pALC2073::*sea*) was electroporated into Newman Δ*sea*, generating the complementation strain *S. aureus* Newman Δ*sea* (pSEA).

3.2.4 Staphylococcal bacteremia model

Single bacterial colonies were picked from a TSA plate and grown in a 5 mL TSB overnight (16-18 hours) and the OD_{600} was adjusted to 1.0. Cells were subsequently subcultured (2%) into TSB and grown to exponential phase (OD_{600} ~3.0-3.5). The bacterial pellet was washed 3× with HBSS (Hyclone) and resuspended in HBSS to an OD_{600} = 0.15, corresponding to ~5×10^7 CFU/mL. Mice were injected via tail vein with 5×10^6 CFU of *S. aureus* in a total volume of 100 µL. Mice were weighed and monitored daily. At 8 or 96 hours post-infection, mice were sacrificed and the heart, lungs, kidneys, and liver were aseptically harvested. All organs were homogenized and plated on mannitol salt agar (Difco) and incubated at 37°C overnight. *S. aureus* colonies were

Table 3.1. Strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
S. aureus Newman	Early methicillin sensitive isolate from secondary infection in a patient with tubercular osteomyelitis	(83)
S. aureus Newman Δ*sea*	*sea*-null *S. aureus* Newman	Chapter 2
S. aureus Newman Δ*sea* (pSEA)	*sea*-null S. aureus Newman complemented with wild-type *sea*	This study
Plasmids		
pALC2073	Complementation vector	(39)
pSEA	*sea* complementation plasmid	This study

enumerated the following day with a limit of detection determined to be 3 CFU per 10 μL.

3.2.5 Determination of Vβ populations targeted by SAgs using flow cytometry

Lymph nodes (cervical, axillary, brachial, inguinal, and popliteal) were isolated *in toto* from mice and pushed through a cell strainer to create a single cell suspension. Cells were stained with APC-conjugated anti-CD3 (clone 145-2C11) (eBioscience) and FITC-conjugated anti-Vβ3 (clone KJ25) (BD Pharmingen) or FITC-conjugated anti-Vβ8 (clone KJ16) (eBioscience). Events were acquired using a FACSCanto II (BD Biosciences) and data were analyzed using FlowJo v.8.7 (Treestar).

3.2.6 Detection of cytokines and chemokines *in vivo*

Eight hours post-infection, serum supernatants and livers were collected. Supernatants were obtained from whole livers by homogenization in HBSS supplemented with the complete protease inhibitor cocktail (Roche). Samples were analyzed using a 32-multiplex array against mouse cytokines and chemokines (Eve Technologies; Calgary, AB, Canada).

3.2.7 Liver leukocyte isolation, staining and cytofluorimetric analysis

Livers were extracted from mice and pushed through a fine mesh. Leukocytes were isolated from livers as previously described using a 33.75% percoll gradient (GE Healthcare) (40). Cells were stained with FITC-conjugated anti-F4/80 (clone BM8), FITC-conjugated anti-Ly6G (clone RB6-8C5), PE-conjugated anti-CD11b (clone M1/70) or APC-conjugated anti-CD3 (eBioscience). Events were acquired and data analyzed as outlined above.

3.2.8 Histological analysis

Standard histology techniques were used. Briefly, tissues were fixed in 10% formalin, embedded in paraffin and thin-sectioned. Sections were stained with a combination Hematoxalin and Eosin/Gram stain and images were captured using a BX-61 upright microscope (Olympus).

3.2.9 Assessment of β-hemolysin activity

Livers and liver abscesses from mice infected with 5×10^6 CFUs of either *S. aureus* Newman or Newman Δ*sea* were homogenized 96 hours post-infection and plated on blood agar (sheep) and incubated at 37°C for 24 hours, followed by incubation for 24 hours at 4°C. Dark zones of hemolysis surrounding individual colonies were measured after the cold-shock incubation. To compare β-hemolysis expression between *in vivo* and *in vitro* conditions, *S. aureus* Newman, Newman Δ*sea* and COL were grown overnight at 37°C in TSB before plating on sheep blood agar with the hot-cold incubation as described for *in vivo* samples.

3.2.10 Statistical analyses

Data were analyzed using unpaired student's *t*-test or one-way ANOVA with Tukey's post-test analysis. All statistical analyses were performed using Prism v5.0 (GraphPad) with $p < 0.05$ being considered significant.

3.3 Results

3.3.1 SEA is produced *in vivo* during staphylococcal bacteremia

S. aureus Newman is known to produce SEA during the exponential phase of growth *in vitro* (41); however, the exact environmental triggers *in vivo* are not well-defined. We aimed to determine if SEA was produced during *S. aureus* Newman infection by examining the Vβ profiles of infected mice, as SEA is known to target $V\beta3^+$ T cells but not $V\beta8^+$ T cells (42, 43). Vβ-specific T cell subpopulations from lymph nodes were measured using flow cytometry from mice inoculated with *S. aureus* strains Newman, Newman Δ*sea*, Newman Δ*sea* (pSEA), or vehicle-treated mice. Ninety-six hours post-inoculation, mice infected with *S. aureus* Newman Δ*sea* did not show a difference in the $V\beta3^+CD3^+$ lymphocyte population compared to vehicle-treated mice. Conversely, wild-type *S. aureus* Newman and Newman Δ*sea* (pSEA) infection demonstrated a significant decrease in $V\beta3^+CD3^+$ cells compared to vehicle-treated mice, indicating Vβ-specific targeting by SEA (Figure 3.1A). Concurrent analysis of $V\beta8^+CD3^+$ cells was used as an internal control since it is an irrelevant T cell subpopulation that is not targeted by SEA. Thus, the significant decrease in the ratio of $V\beta3^+CD3^+$ to $V\beta8^+CD3^+$ cells from 0.33 (vehicle) to 0.16 (Newman) during infection with *S. aureus* Newman showed indirectly that SEA was specifically targeting the $V\beta3^+CD3^+$ population (Fig. 3.1B), thereby confirming the production of SEA *in vivo* during infection in our model.

3.3.2 Bacterial survival is enhanced in the livers of mice infected with SEA-producing *S. aureus*

To evaluate a role for SEA in *S. aureus* bacteremia, we injected 5×10^6 CFUs of *S. aureus* Newman, or *S. aureus* Newman Δ*sea*, into the tail vein of DR4-B6 mice and assessed bacterial burden in multiple organs at 96 hours post-infection. Bacterial loads were highest in the kidneys and livers, but were also found in the heart and lungs (Fig. 3.2A-D). Although bacterial load was not statistically different in the kidneys or lungs, we observed a ~100-fold decrease in bacterial burden in the livers of mice infected with *S. aureus* Newman Δ*sea* compared with wild-type *S. aureus* Newman-infected mice. There was also a significant difference between the bacterial loads in the heart

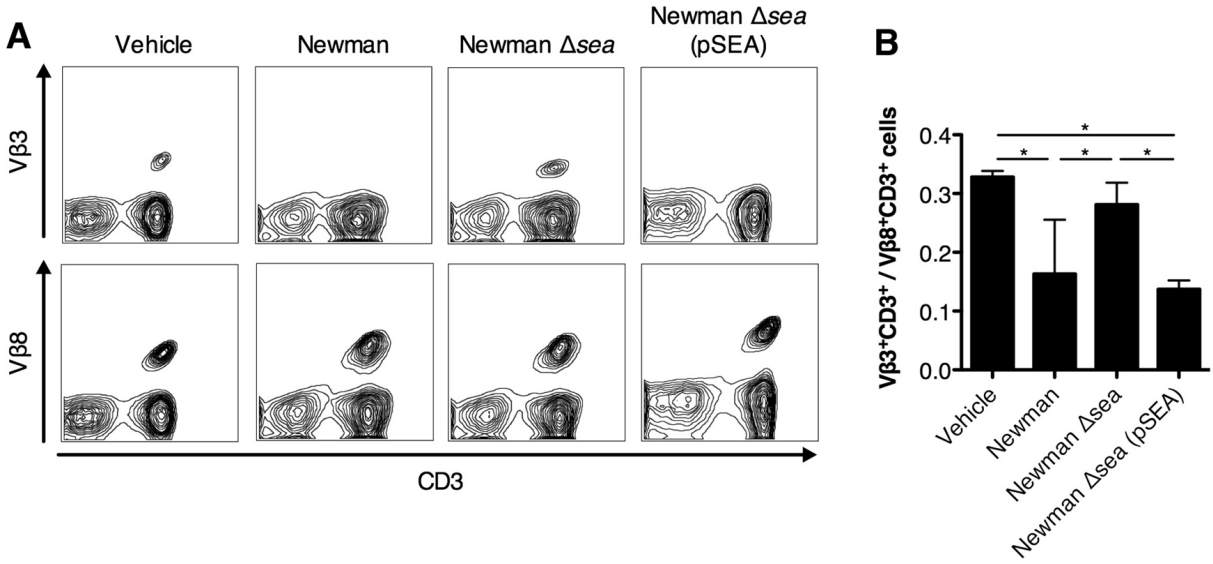
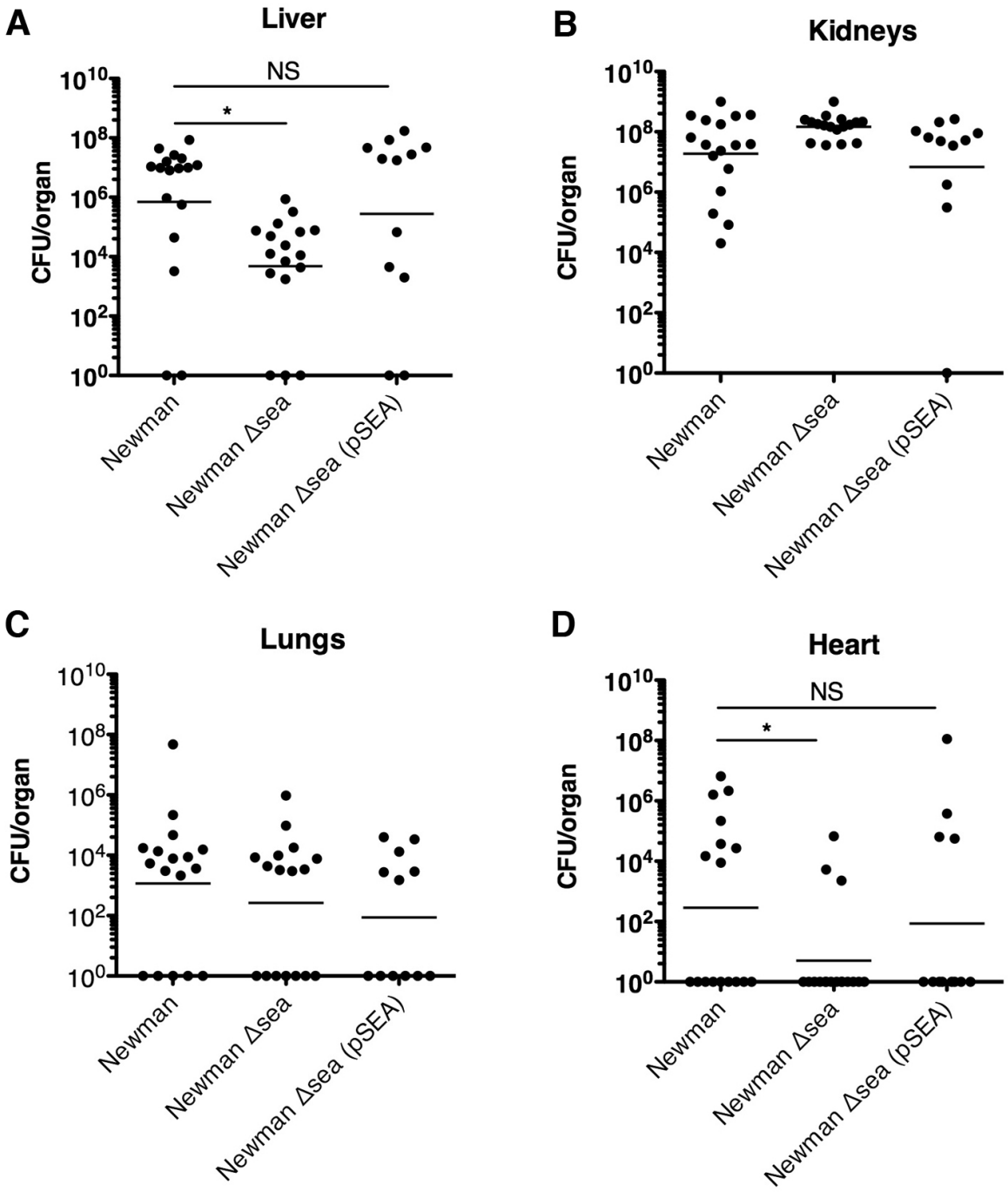


Figure 3.1. SEA is produced during *S. aureus* bacteremia and interacts specifically with the Vβ3$^+$ subset of T cells. Flow cytometry analysis of lymph node populations 96 hours post-infection [vehicle n = 3, Newman n = 4, Newman Δ*sea* n = 4, and Newman Δ*sea* (pSEA) n = 5] A) Representative FACS plots from each infection group stained with antibodies against either CD3 and Vβ3 or CD3 and Vβ8. Vβ3 and Vβ8 staining were from the same mouse with Vβ8 acting as the internal control for each mouse. Each sample was gated for the Vβ$^+$CD3$^+$ population. B) Ratio of Vβ3$^+$CD3$^+$ to Vβ8$^+$CD3$^+$ cells per mouse for each infection group. Data shown as the mean ± SEM, significant differences ($p < 0.05$) as determined by one-way ANOVA with Tukey's post-test are denoted with *.

Figure 3.2. Septic infection with SEA-producing *S. aureus* results in higher bacterial loads in the liver than SEA-deficient *S. aureus.* Bacterial counts of mice infected with *S. aureus* Newman (n = 17), Newman Δ*sea* (n = 17) or Newman Δ*sea* (pSEA) (n = 12) from A) Liver B) Kidneys C) Lungs and D) Heart 96 hours post-infection. Each point represents data from one mouse. Results reflect 3 independent experiments. The line in each treatment group represents the mean and counts below the limit of detection are interpreted as having no counts. Significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *. NS = no significance.


A
Liver
NS
*
CFU/organ
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
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Newman
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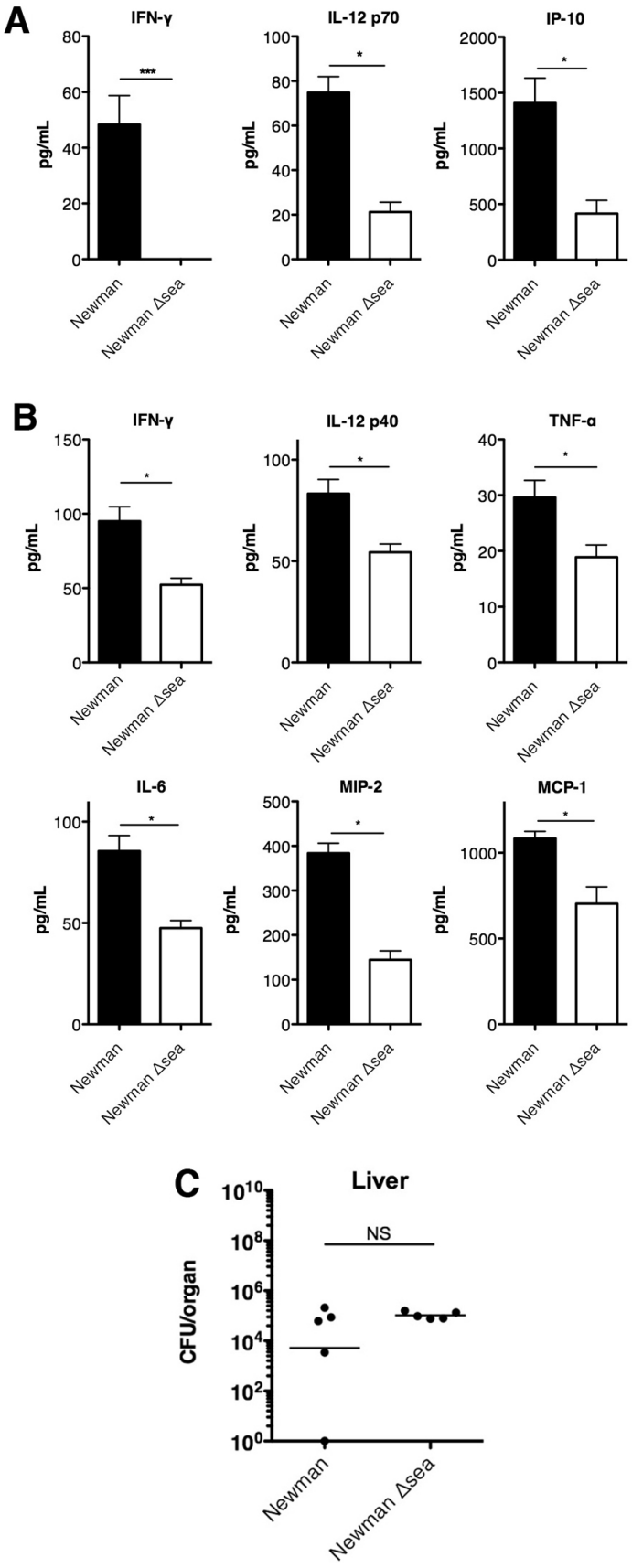
Newman Δsea (pSEA)
B
Kidneys
CFU/organ
Newman
Newman Δsea
Newman Δsea (pSEA)
C
Lungs
CFU/organ
Newman
Newman Δsea
Newman Δsea (pSEA)
D
Heart
NS
*
CFU/organ
Newman
Newman Δsea
Newman Δsea (pSEA)

(Figure 3.2D) between *S. aureus* Newman- and Newman Δ*sea*-infected mice. In order to confirm this pronounced phenotype was SEA-dependent, and not due to an inadvertent secondary site mutation in *S. aureus* Newman Δ*sea*, we restored SEA expression *in trans* using the pSEA plasmid. The complemented strain Newman Δ*sea* (pSEA) restored the virulence phenotype in both the liver and heart as seen with wild-type *S. aureus* Newman (Fig. 3.2). These data indicate that expression of SEA by *S. aureus* Newman promotes infection within the liver and heart, but does not apparently alter bacterial burden in other organs tested.

3.3.3 SEA induces production of IFN-γ and other inflammatory cytokines and chemokines both locally and systemically during *S. aureus* infection

Since it is well known that SAgs function to induce cytokine production, we reasoned that the survival advantage seen during infection with *S. aureus* Newman was a downstream result of SAg-mediated immune activation. We investigated early cytokine production to assess both local and systemic inflammation of infected mice 8 hours post-infection. Liver homogenate supernatants and sera from Newman- and Newman Δ*sea*-infected mice were analyzed for 32 cytokines and chemokines (Appendices 3 and 4). Systemically, IFN-γ and IL-12p70 were upregulated in wild-type-infected mice sera compared to Newman Δ*sea* infection, as well as the chemokine interferon-induced protein 10 (IP-10) (Fig. 3.3A). Elevated levels of IFN-γ, TNF-α, IL-6 and IL-12p40 were detected from mouse livers infected with *S. aureus* Newman compared to Newman Δ*sea*-infected mice (Fig. 3.3B), which are known to be induced by SAgs (23, 44, 45). Additionally, the chemokines MIP-2 and MCP-1 were upregulated in Newman-infected livers (Fig. 3.3B). Bacterial burdens in the liver at 8 hours post-infection were not significantly different between wild-type and Newman Δ*sea*-infected mice (Figure 3.3C), and no bacteria were detected in blood from any mice (data not shown), indicating that the differences in chemokine and cytokine production are not likely due to differences in bacterial load. Overall, these data demonstrate that SEA is an important driver of SAg-induced inflammation during our model of *S. aureus* bacteremia in DR4-B6 mice.

Figure 3.3. Cytokines and chemokines induced by *S. aureus* Newman and Newman Δ*sea* infection. Serum and liver supernatants were collected from mice 8 hours post infection from *S. aureus* Newman and Newman Δ*sea*-infected mice. Blinded samples were sent for multiplex cytokine array analysis (n = 3-4 per experimental group). A) Serum levels of cytokines and chemokines produced significantly different in Newman- than Newman Δ*sea*-infection. B) Local production of liver chemokines and cytokines significantly different during infection with Newman compared to Newman Δ*sea*. C) Bacterial burdens in the liver at 8 hours post-infection (n = 5 per group). Data shown as the mean ± SEM, significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *; *** $p < 0.001$.


A
IFN-γ
IL-12 p70
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*
Newman
Newman Δsea
B
IFN-γ
IL-12 p40
TNF-α
IL-6
MIP-2
MCP-1
C
Liver
CFU/organ
NS

3.3.4 CD11b$^+$Ly6G$^+$ neutrophils are recruited to the liver during *S. aureus* infection in an SEA-dependent manner

Given the production of the MIP-2 and MCP-1 chemokines in the liver induced by SEA from *S. aureus* Newman-infected mice, we predicted that there would also be a difference in the number of immune cells trafficking to the liver. Since macrophages and neutrophils are the primary cells responsible for the clearance of *S. aureus*, we examined these populations to evaluate if there was a defect in phagocyte recruitment during staphylococcal infection with SAgs. Additionally, the liver is known to contain high numbers of resident macrophages (Kupffer cells) so we hypothesized that SEA would have an effect on the macrophage population. Leukocytes were isolated from mouse livers 96 hours post-infection and stained for various surface markers. Analysis of F4/80$^+$ macrophages showed no significant difference between mice infected with *S. aureus* Newman and Newman Δ*sea* (Fig. 3.4A). Similarly, CD3$^+$ T cells were also not significantly different (Fig. 3.4B), despite the decreased number of Vβ3$^+$ T cells detected in lymph nodes (Fig. 3.1). However, mice infected with *S. aureus* Newman showed an increased frequency of CD11b$^+$Ly6G$^+$ neutrophils (Fig. 3.5C), suggesting that SEA-induced chemokines (Fig. 3.3) resulted in the recruitment of neutrophils to the liver.

3.3.5 SEA promotes the formation of hepatic abscesses that contain viable bacteria in high densities

During organ retrieval following the bacteremia model, we observed numerous white hepatic lesions that commonly formed on the surface of livers of *S. aureus* Newman-infected mice (Fig. 3.5A). An abscess score was established whereby livers were examined on a lobe-by-lobe basis for visible surface lesions and enumerated. We observed a significant increase in the number of abscesses formed in the livers of *S. aureus* Newman-infected mice compared with mice infected with *S. aureus* Newman Δ*sea*. The number of abscesses from *S. aureus* Newman Δ*sea* complemented with pSEA was similar to wild-type *S. aureus* Newman infected mice, demonstrating that this phenotype was SEA-dependent (Fig. 3.5B). H&E/Gram staining of thin sections from both groups showed high neutrophilic infiltration into the abscess with polymorphonuclear cells and associated tissue damage. Abscesses contained high numbers of Gram positive cocci in the centre (Fig. 3.5C insets i and ii). However, the

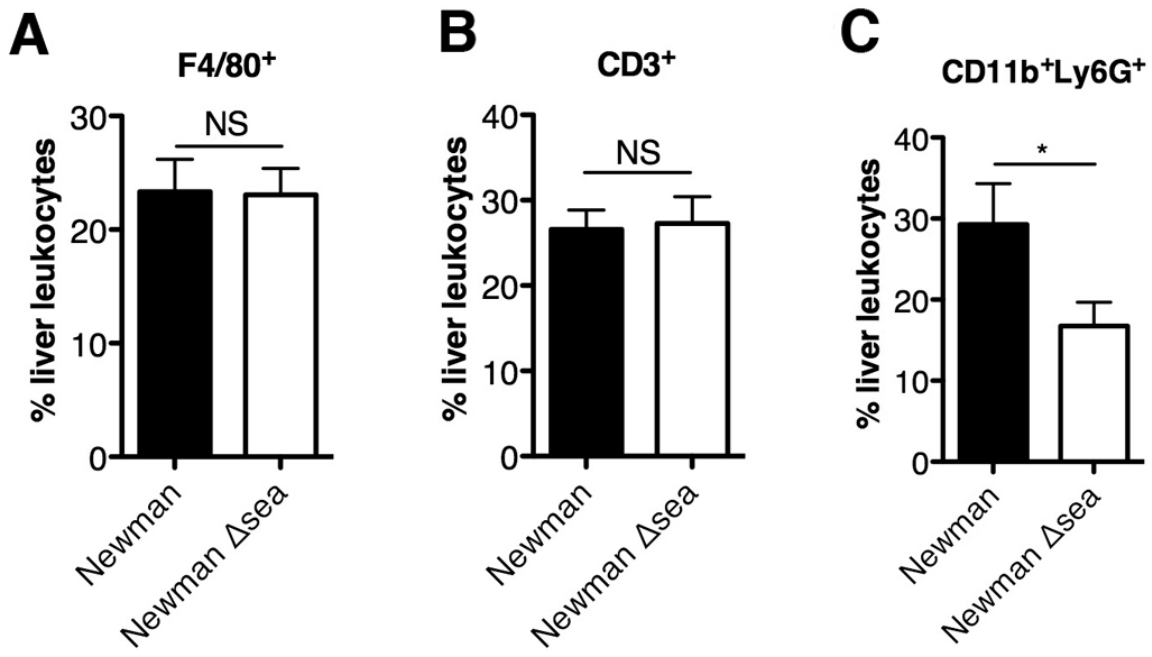
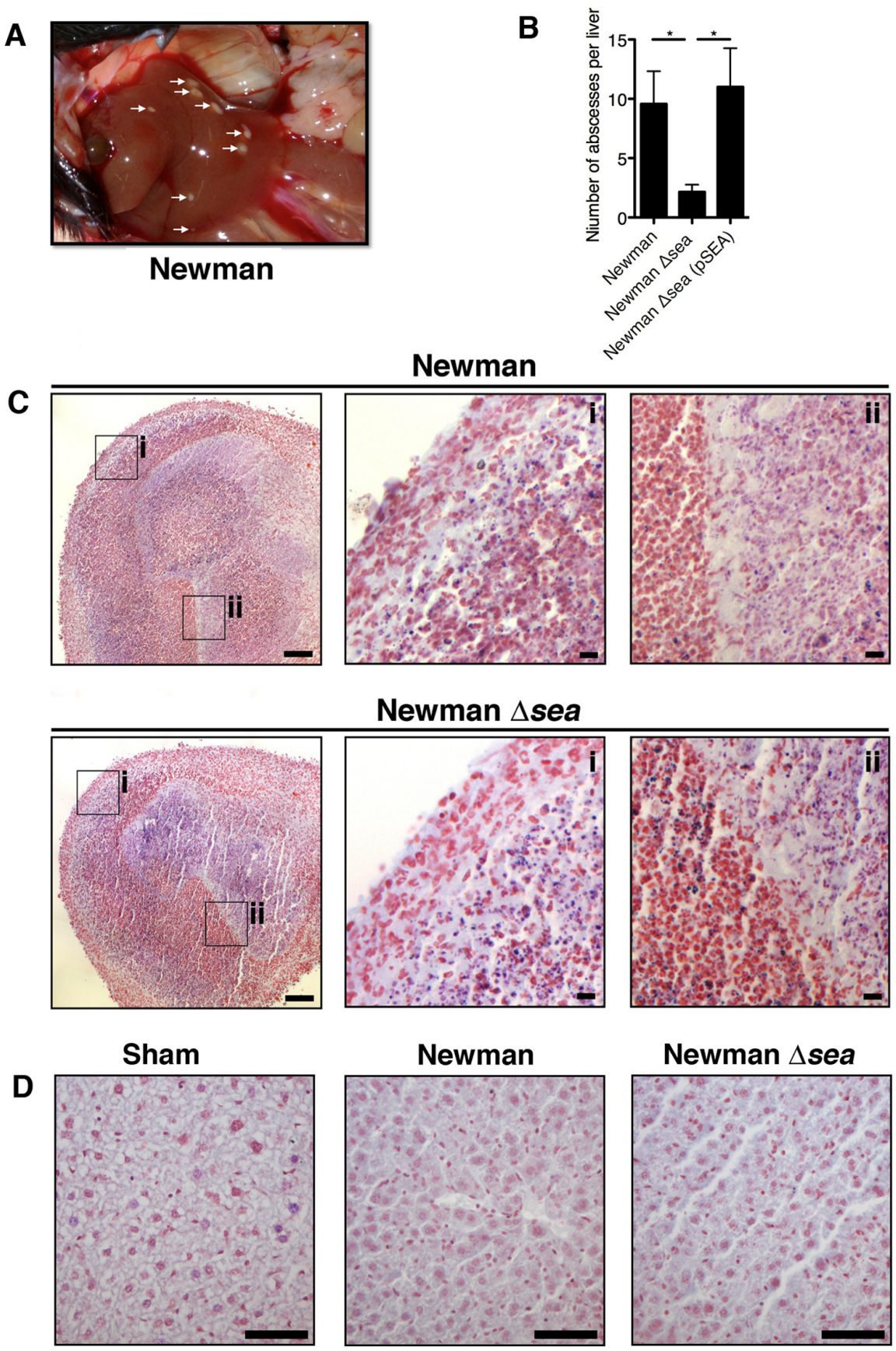


Figure 3.4. Livers of mice infected with *S. aureus* Newman show an increase in CD11b$^+$ Ly6G$^+$ neutrophils but not F4/80$^+$ macrophages or CD3$^+$ T cells. Livers of infected mice 96 hours post-infection were broken into single cell suspension and leukocytes isolated by percoll gradient. Samples were stained with antibodies against F4/80, CD3 or CD11b and Ly6G and analyzed by flow cytometry. Samples underwent doublet discrimination with debris gated out. Subsequently, cells were gated on A) F4/80$^+$ macrophages, B) CD3$^+$ T cells, or C) CD11b$^+$ Ly6G$^+$ neutrophils. Data shown as the mean ± SEM from three independent experiments, with n = 12 for each group. Significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *, NS = no significance.

Figure 3.5. Infection with *S. aureus* Newman results in greater abscess formation than infection with *S. aureus* Newman Δ*sea*. A) Visible white lesions (abscesses) on a representative liver of a Newman-infected mouse, indicated by white arrows. B) Liver abscess score from Newman (n = 14), Newman Δ*sea* (n = 13), and Newman Δ*sea* (pSEA)-infected mice (n = 5). Data shown as mean ± SEM from at least three independent experiments. Significant differences ($p < 0.05$) as determined by unpaired student's t-test are denoted with *. C) Representative H&E/Gram-stained histological sections of abscesses from Newman and Newman Δ*sea*-infected mice. Black bar indicates 100μm and 10μm on the insets of i and ii. D) Representative sections of Gram-stained liver parenchyma surrounding abscesses from sham, Newman and Newman Δ*sea*-infected mice. The black bar indicates 50 μm.


A
Newman
B
Niumber of abscesses per liver
15
10
5
0
*
*
Newman
Newman Δsea
Newman Δsea (pSEA)
C
Newman
i
ii
Newman Δsea
D
Sham
Newman
Newman Δsea

overall abscess structure appeared very similar between wild-type *S. aureus* Newman and Newman Δ*sea* infections (Fig. 3.5C). Abscesses were also excised from the liver, homogenized, and compared to hepatic immune cells isolated from the surrounding liver tissue. Compared to non-abscessed liver tissue, abscesses contained few live host cells as assessed by trypan blue staining, and loss of forward and side scatter when analyzed with flow cytometry (data not shown). Additionally, individually excised abscesses yielded high counts of viable *S. aureus* (10^6 - 10^7 CFU/abscess) (data not shown). We did not detect staphylococci distant from the abscesses within the surrounding liver parenchyma or in sham-infected mice (Fig. 3.5D). These data indicated that the enhanced fitness phenotype of *S. aureus* Newman we observed (Fig. 3.2A) is attributed to an increase in abscess formation that confers greater bacterial survival and growth in the liver.

3.3.6 β-hemolysin is not activated during *S. aureus* Newman bacteremia.

The gene *sea* is encoded on a β-hemolysin (*hlb*)-converting phage (ϕNM3), which disrupts β-hemolysin function upon integration into the Newman genome. However, it has been shown that *S. aureus* strains carrying these phage can be excised and become *hlb*+, especially during *in vivo* infection (46, 47). In *S. aureus* Newman, the lack of excisionase (*xis*), prevents ϕNM3 from excising from the genome (44, 48, 49). To ensure that a secondary mutation did not occur during genetic manipulation of *S. aureus* Newman Δ*sea*, we tested the strains both *in vitro* and following *in vivo* infection, to ensure that the *sea*-encoding ϕNM3 was not excised during infection. Compared to COL which is hlb^+ (9, 50, 51), the areas of hemolysis were significantly lower in all Newman and Newman Δ*sea* samples. There were no differences between Newman and Newman Δ*sea* samples, in either *in vivo*, *in vitro* or abscess conditions (Fig. 3.6). Thus, we confirmed that β-hemolysin was not activated, and that ϕNM3 (and therefore *sea*) was not excised during infection.

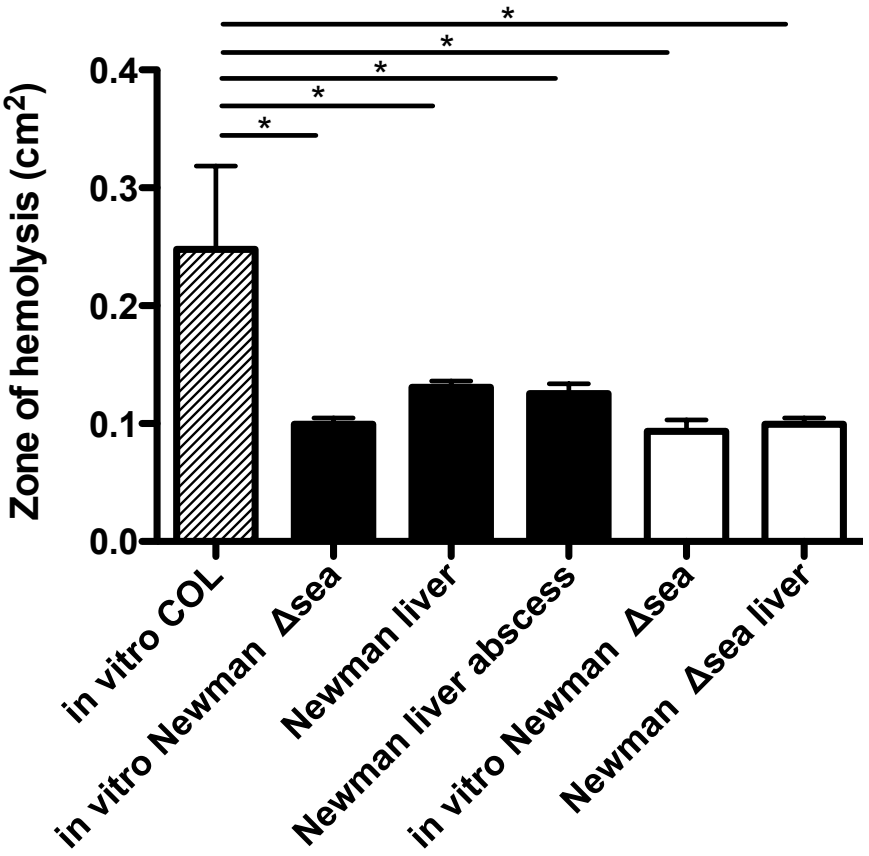


Fig. 3.6. β-hemolysin is not activated in *S. aureus* Newman or Newman Δ*sea.* The thatched bar represents the *hlb*$^{+}$ COL colonies grown *in vitro*, the black bars represent wild-type Newman colonies, and the white bars represent Newman Δ*sea*. At least 3 colonies and their zones of hemolysis were measured per plate. *denotes $p < 0.05$ as determined by one-way ANOVA with Tukey's post-test.

3.4 Discussion

In this work, we combined SAg-sensitive humanized transgenic DR4-B6 mice with an isogenic *sea* knockout strain of *S. aureus* to study the role of SAgs during staphylococcal bacteremia. By using a live infection model, we were able to not only study the detrimental effects of SAg intoxication on the host, but also the advantageous effects of SAg expression for *S. aureus*. We demonstrated an SEA-specific downstream effect that enhanced the number of abscesses formed in the liver, although individual abscesses appeared similar in both morphology and bacterial counts from both strains. This in turn increased bacterial persistence in the liver overall since staphylococcal abscess communities are sustained within a fibrin pseudocapsule that is protective against immune cells and permits bacterial survival *in vivo* (44, 51). Abscess formation is an important host immune response during infection for limiting the spread of infection to other tissues. Host immunity against *S. aureus* infection is dependent on abscess formation by neutrophils (46) and suppurative abscesses have long been recognized as a hallmark of *S. aureus* infection (44, 49). However, successful eradication of *S. aureus* by neutrophils exists in a balance, with staphylococci actively subverting neutrophil responses in order to persist *in vivo* (9, 51). The presence of abscesses during staphylococcal bacteremia is clinically significant since hematogenous spread from the abscess is well documented (44, 52, 53).

A basal level of abscess formation could still be observed during *S. aureus* Newman Δ*sea* infection, albeit with lower frequency than wild-type infection, since the former still retains essential cell-surface proteins required for abscess formation (51). The lower bacterial counts seen in Newman Δ*sea* is not likely due to an inherent growth defect (Fig. 2.7A), or an inability to survive within neutrophils since viable bacteria were observed within both Newman and Newman Δ*sea* abscesses. To our knowledge, this model is the first to describe a liver tropism for *S. aureus* related to SAg expression. Although we also observed renal abscesses in the infected mice, no differences were detected in bacterial counts between SEA-expressing and *sea*-null infections. We speculate that given the paucity of residence T cells in the kidney (54), the initial infection within the kidney remained independent of SEA function. Additionally, high densities of staphylococcal

cell wall (such as the loads observed in the kidneys) have been shown to downregulate SAg-mediated T cell activation (55), which may nullify SAg activity locally.

Consistent with our findings, SEC has been shown to increase renal damage during experimental infective endocarditis/sepsis in rabbits, including the formation of kidney abscesses, although this was attributed to the embolization of valve vegetations (30). Similarly, blocking SEC function using a high-affinity SEC binding inhibitor resulted in a drastic reduction of vegetation size (32). Although Newman Δ*sea* demonstrated decreased counts within the hearts, we did not observe any obvious aortic valve vegetations from wild-type Newman, although our protocol is not an endocarditis model as valve damage is not actively induced. Neutralization of SEB also decreased abscess size using in a murine thigh infection model (28). Although it is difficult to aggregate these collective findings, an overall picture is now emerging that SAg-induced inflammation contributes to the formation and severity of *S. aureus* abscesses in multiple experimental settings.

Compared to Newman Δ*sea* infection, wild-type Newman infection produced significantly higher quantities of cytokines and chemokines that correspond to those induced by SAgs reported in the literature (23, 45, 56, 57). Although IL-2 is a cytokine typically used to measure T cell-dependent superantigenic activity *in vitro*, we did not detect differences in IL-2 production from the *in vivo* liver samples. This finding may be explained by IL-2 levels peaking at 2-4 hour *in vivo* in response to SAg (58), and by the very short half-life of IL-2 *in vivo* (59, 60). The SEA-driven inflammatory milieu likely mediates the promotion of abscesses and seems to be driven by the early production of both IL-12 and IFN-γ, detected in both serum and liver supernatants 8 hours post-infection. IL-12 enhances production of IFN-γ after SAg challenge (58), productively boosting the cytokine and chemokine response. McLoughlin et al. have shown that IFN-γ is a master regulator during *S. aureus* infections, mediating chemokine responses that allow for neutrophil recruitment and trafficking (61, 62). This is consistent with our observations in Newman-infected mice where we observed an increase in IFN-γ and chemokines that are chemotactic for neutrophils and monocytes. Presumably, activated monocytes trafficking to the liver undergo differentiation into macrophages, although we

did not observe any significant differences in the macrophage population in the liver. Purified SAgs have been shown to recruit neutrophils (but not T cells) mediated by TNF-α and chemokines (63). Notably, our study is the first report showing SEA increases trafficking of $CD11b^{+}Ly6G^{+}$ neutrophils during a live infection. Given that abscess formation is largely driven by neutrophils, the infiltration into the liver correlates well with the increased incidence of hepatic abscesses.

It appears paradoxical that an increased influx of $CD11b^{+}Ly6G^{+}$ neutrophils had an inverse correlation to bacterial survival, considering the important role of neutrophils in staphylococcal clearance. As a successful human pathogen, *S. aureus* has evolved many mechanisms to counteract neutrophils (9). While neutrophils are absolutely necessary for the eradication of staphylococcal infections (64, 65), their presence during infection has also been described as pathogenic (61, 62). IP-10, which we showed to be upregulated systemically by SEA, can promote phagocytosis (66); however, MIP-2, also upregulated, is capable of enhancing intracellular bacterial survival within neutrophils (61, 62). The avoidance of neutrophil bactericidal activity likely contributed to *S. aureus* survival during early abscess formation and subsequently, the staphylococcal community in the mature abscess. This supports the paradigm that neutrophils can be pathogenic during systemic infection due to SAgs usurping the immune system to form abscesses, thereby conferring staphylococcal fitness and survival *in vivo*. The *sea* gene is encoded on the same immune evasion cluster (IEC) of β-hemolysin converting phage which includes staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein of *S. aureus* (CHIPS) and staphylokinase (SAK) (67, 68). It has been proposed that CHIPS and SCIN 'stall' early neutrophil recruitment to successfully establish an infection. This is due to their inhibitory action against complement proteins, an early innate response (68, 69). While this seems counterintuitive to the neutrophil recruiting activities of SEA, it has been also proposed that this early blockade of neutrophils allows later modulation of the immune system by SEA and SAK. This theory fits with our model where we see an accumulation of neutrophils later on at 96 hours, in response to SEA. SCIN may work in tandem with SEA by inhibiting phagocytosis and bactericidal activity of recruited neutrophils (68), which may help form neutrophilic abscesses that *S. aureus* can survive in. SAK may be involved in dissemination from abscesses due to its ability to cleave

fibrin (70), which are characteristic of abscesses. However, it should be noted that the IEC factors, SEA included, are highly human-specific (67), and thus may not be active in our murine model which is sensitized to SEA only.

SAg function has typically been attributed towards crippling the adaptive arm of the immune system by inducing T cell anergy and deletion of T cell-dependent B cell responses (71, 72). Indeed, an inability to form neutralizing antibodies has been linked to many cases of TSS (11, 73); however, SAgs are also highly immunogenic and the majority of the population is able to form both anti-SAg and anti-staphylococcal antibodies (74). Although purified SAgs have long been shown to induce T cell anergy (75-78), to our knowledge, the role of SAg-mediated T cell anergy has not been demonstrated during a live infection. In our model, SEA-expressing *S. aureus* caused a decrease in the detectable $V\beta3^+CD3^+$ cells, although SAg-activated T cells usually undergo early expansion (79). This decrease may be a result of Vβ-specific TCR internalization (80, 81), T cell deletion (82), or a combination thereof. Injection of mice with purified SEA similarly resulted in Vβ3-specific $CD4^+$ T cell suppression mediated by IFN-γ and myeloid-derived suppressor cells (79) and this may represent an additional role for SAgs to subvert the immune response. The effect of T cell anergy during staphylococcal disease may inhibit numerous T cells in the context of chronic infection; however, SAgs do not target T cells in an antigen-specific manner so it is unclear how Vβ-specific anergy would contribute to staphylococcal infections. Thus, it will be important to dissect the role of SAg-mediated T cell suppression during live infections in future studies. Given that SAgs have an inherent ability to impact numerous immune cells, it is highly likely that these toxins are multi-functional virulence factors and thus are able to influence both the adaptive and the innate immune systems. Overall, this work shows that SAgs are used by *S. aureus* during infection to not only target T cells directly, but also neutrophils as a result of the SAg-elicited cytokines. While the recruitment of neutrophils appears to be counterintuitive to survival, our work demonstrates that SAg expression by *S. aureus* enables a sophisticated method of *in vivo* survival by subverting the neutrophil response into a protective niche, demonstrating a biologically relevant and highly novel role for SAgs during infection.

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Chapter 4 Discussion and Conclusions[4]

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S. aureus has evolved with humans as a commensal organism, consistently colonizing ~20% of the population, as well as a prominent and successful pathogen. This dualistic role is due to the abundance of virulence factors encoded by *S. aureus*, many of which play multiple roles in both colonization and disease. SAgs have fascinated scientists since Marrack and Kappler presented their discoveries almost 30 years ago. Since then, the structure and function of these toxins have been studied in great detail, as well as their causal role in provoking TSS and food poisoning. Arguably the most interesting question that remains in this field is why do *S. aureus* possess such a large, genetically and antigenically distinct, extremely potent, and seemingly redundant group of these toxins?

SAgs skew immune responses toward Th1 during severe disease, but toward Th2 during atopic disease in genetically predisposed individuals. Th1 skewing can result in delayed development of neutralizing antibody (1) and perhaps this is an important *in vivo* strategy that promotes persistence. Many patients following menstrual TSS fail to develop anti-TSST-1 antibodies (1, 2). Conversely, humans clearly develop anti-TSST-1 antibodies such that by age 1, ~50% have antibody titers considered to be protective (3). An interesting hypothesis has been proposed where excessive T cell expansion may act as a 'sponge' to titrate IL-2 necessary for further T cell expansion, essentially causing immunosuppression (4). Similarly, massive expansion of Vβ-specific T cells may induce a loss of overall receptor diversity filling up the “space,” providing an alternative method of immune escape. Continued efforts into understanding the complex biology of SAgs will undoubtedly answer many of these questions. It is clear that these remarkable toxins represent a highly unique and well adapted virulence factor, although the evolutionary function of these toxins in the life cycle of *S. aureus* still remains unclear. The body of work presented in this thesis aimed to answer the biological purpose of staphylococcal SAgs by utilizing SAg-deletion strains in SAg-sensitive humanized transgenic mice, studying both commensal and pathogenic lifestyles.

4.1 Chapter 2 summary and conclusions

In Chapter 2, I discovered that *S. aureus* strains encoding SAgs had reduced bacterial burdens in the nasal passages of mice compared to isogenic counterparts that did not

express SEA or SEB. Colonization with *S. aureus* Newman Δ*sea* caused a transient increase compared to wild-type Newman at day 10 (Fig. 2.7A). Furthermore, *S. aureus* COL Δ*seb* resulted in higher bacterial densities throughout the entire duration of the experiment, compared to wild-type COL (Fig. 2.9A). The phenotypic differences between Newman and COL strains may be due, in part, to *agr*-mediated regulation of SEB which is massively overexpressed (5) compared to SEA, which is not controlled by *agr* (6). Interestingly, *agr* appears to be repressed in a cotton rat model of nasal colonization (7); however, *agr* and exotoxin expression can become uncoupled *in vivo* where repression of *agr* did not result in a corresponding inhibition of SAg expression (8). In our model, the presence of Vβ-skewing and the difference in bacterial loads is indicative of SEB production during murine colonization. In humans, *sea* appears to be expressed even in persistently colonized individuals (9) while *agr* does not appear to be expressed (10). Real-time PCR analysis of bacteria isolated from colonized DR4-B6 mice may shed light on the kinetics of *agr* and SEB expression to reconcile our findings with previous studies. It is possible that SAg expression is important for the initial stages of establishing colonization, before *agr* expression is repressed. Overall, the bacterial count data suggest that the deletion of SAgs enhanced nasal colonization *in vivo*. In particular, colonization with *S. aureus* COL suggests that SAgs attenuate the ability to colonize the nose, which can be explained by the inflammatory properties of SAgs, leading to subsequent bacterial clearance due to activation of the immune system. Notably however, deletion of *sea* from Newman did not result in enhanced colonization long-term (compared to their wild-type counterparts), as bacterial loads decreased to match wild-type levels after the transient increase (Fig. 2.7A). This data suggests that increased pathogenicity may not be beneficial for the bacteria long-term. Indeed, a higher number of bacteria in the nose may result in activation of *agr* via quorum sensing and production of cytotoxins, proteases and other exoproteins typically associated with dissemination and invasion, in opposition to colonization tactics (11). I propose that in the context of nasal colonization, maintenance of a lower bacterial burden may be beneficial for the organism's long-term survival and continued colonization. The idea that high numbers of bacteria forming biofilms in the nose during colonization has been challenged by Krismer et al. who propose a dispersed mode of growth during nasal colonization (12). Their

model is in agreement with our studies and supported by clinical observations that nasal swabs and histological human nose sections contain relatively low amounts of *S. aureus* (10^1-10^4 CFU) (13, 14). Moreover, formation of biofilms may not persist in the nose due to physical forces (e.g. nose blowing). There is also growing evidence of differing immune states in persistent carriers vs. non-carriers, with intermediate carriers resembling non-carriers (15-18), which may explain why persistently-colonized individuals have higher densities of bacteria in their noses (14). During colonization, expression of SAgs may preclude a higher level of inflammation by regulating bacterial density and thus, peptidoglycan and other bacterial moieties that can induce further inflammation. Since SAgs are inflammatory molecules, SAg-mediated immune responses could maintain the density of bacteria in check. Thus, SAgs may act as 'checkpoints' of dissemination – by preventing the bacterial colony from growing too large and dispersing before a niche can be established. Future experiments that extend the length of colonization in our model may elaborate the overall fitness advantage of SAg-expressing vs. SAg-negative strains.

In contrast to *S. aureus*, establishment of nasal colonization by *Streptococcus pyogenes* (group A *Streptococcus*; GAS) in a similar murine model is heavily dependent on SAg presence (19). During asymptomatic colonization with GAS, the location differs between the two SAg-expressing organisms: *S. pyogenes* primarily colonize murine nasal-associated lymphoid tissue (NALT) or the upper nasal turbinates, while *S. aureus* does not (19-21). This may in part explain the different usage of SAgs by these two organisms, as T cell activation by SAgs appears to be a requirement for the establishment of acute colonization by GAS (19). Thus, the evolution of SAg function appears to be divergent between these organisms, likely dependent on their different survival tactics, and the establishment of a niche environment that is favourable for long-term survival and transmission. This may also contribute to the understanding of the differences seen between staphylococcal and streptococcal TSS where the latter is often associated with bacteremia (and thus higher mortality rates) compared to TSS caused by *S. aureus* (22).

The idea of 'virulence attenuation' is an emerging concept, where pathogens produce factors that protect host cells instead of inducing cell death (23). By regulating their own

virulence, pathogenic organisms 'play the long game' so that the infection can be prolonged as long as the host is not killed, thereby depriving them of a niche. Often these host-protective factors attenuate cytotoxicity induced by another virulence factor and enhances pathogenicity; moreover, removal of the host-protective factor reduces pathogenicity (23). For example, *Bartonellae* inject BepE into host cells which prevents cytotoxicity induced by BepC; however, a *bepE* deletion mutant was unable to disseminate *in vivo* and thus reducing pathogenicity (24). *S. aureus* produce a cell-wall associated factor that downregulates SAg-induced T cell activation once a high density is reached, which has been proposed as a reason for the low prevalence of staphylococcal TSS and may be a host-protective factor that allows *S. aureus* to regulate SAg toxicity (25). Although SAgs are not host-protective factors, they appear to regulate virulence indirectly by controlling bacterial density. The WalKR regulatory system, which regulates cell wall dynamics, is expressed during nasal colonization (7, 10), further supporting the notion that controlling bacterial density is an important determinant of colonization. Thus, *S. aureus* may have evolved mechanisms and virulence factors that target and fine-tune the inflammatory response for long-term survival in humans.

4.1.1 Limitations and future work

In humans, the nasal cavities are sources of endogenous infections, likely due to hematogenous seeding (26). While our mouse model was able to show some dissemination to other organs, namely the liver and lungs, establishment of an extra-nasal infection did not occur. Thus conclusions regarding the role of SAgs in the regulation of dissemination from nasal colonization could not be made. Also, the inoculum of bacteria administered to the mice (1×10^8) is not clinically relevant, especially considering the comparative sizes of the mouse vs. human nose. Moreover, it is unlikely that a highly concentrated colony of bacteria is transmitted to the noses of humans during hand-to-nose passage. Thus, the initial colonization steps to establish a successful colony may be mechanistically different and have different roles for SAgs.

Since bacteria are prepared and introduced to mice during their exponential phase of growth, the expression of virulence factors will be affected and may impact survival *in vivo*. Deposition of C3 on bacterial cells is decreased during stationary phase even in the

absence of capsule, reducing bacterial opsonization (27) and it has been shown that bacteria in early exponential growth phase were more susceptible to neutrophil bactericidal activity than cells in stationary phase (28). Future work on this model should expand the duration of the experiment, in order to evaluate if SAg-expressing strains are able to colonize longer than their SAg-deletion counterpart. Further analysis of immune cell infiltrates to the NALT and nasal passage may also help elucidate the mechanism of how SAg expression reduces bacterial densities. Lastly, histological analyses may reveal if bacteria have been internalized by keratinocytes or if they remain extracellular. There is an increasing amount of evidence emerging regarding the intracellular lifestyle of *S. aureus*, particularly during chronic infections, as reservoirs of infection (29).

4.2 Chapter 3 summary and conclusions

In contrast to a commensal lifestyle, I demonstrated in Chapter 3 that the expression of SEA during staphylococcal bacteremia increased bacterial counts and thus, survival *in vivo* (Fig. 3.2). Although there were lower bacterial counts in Newman-infected livers compared to Newman Δ*sea* ones, there were a higher percentage of $CD11b^{+}Ly6G^{+}$ neutrophils in the former compared to the latter. This initially appeared counterintuive as neutrophils have been shown to be crucial for the clearance of staphylococcal infections (30, 31); however, *S. aureus* in turn has evolved multiple mechanisms to counteract neutrophil activity (32). Additionally, chemokines induced by SEA and *S. aureus* Newman infection have been shown to enhance survival of *S. aureus* within neutrophils (33). Thus it is likely that the liver-specific phenotype we observed was caused by SEA-induced inflammation that resulted in increased migration of neutrophils (34).

I also showed that the higher number of neutrophils in Newman-infected livers corresponded to the number of neutrophilic hepatic abscesses compared to Newman Δ*sea* mice (Fig. 3.5B). Since these abscesses contained viable bacteria (Fig. 3.5C), *S. aureus* was able to utilize abscesses as specialized niches to survive *in vivo* and be protected from immune clearance. Thus, even though Newman Δ*sea* was capable of forming abscesses, the presence of SEA was able to enhance this process via its inflammatory properties. Abscesses are a natural host response to infection, limiting the spread of pathogens and preventing further dissemination, and is a hallmark of *S. aureus* infection

(35). Many staphylococcal cell surface components have been identified as crucial for the formation and persistence of abscesses (36), supporting the notion that *S. aureus* modifies this host process in order to persist *in vivo* (37). Thus, by enhancing neutrophil recruitment and abscess formation, SAgs represent another virulence factor that *S. aureus* has evolved in order to subvert the immune system.

Interestingly, Vojtov et al. showed that a TSST-1 knockout strain resulted in increased inflammation and larger abscesses in a subcutaneous model (38), in conflict with our observations. However, the same strains were also used in a previous cutaneous infection model that demonstrated decreased inflammation in the absence of TSST-1 (39), which is in agreement with our findings. Other reports show that the abrogation of SEC using isogenic knockout strains and an anti-SEC inhibitor decreased inflammation, lethality, bacterial counts, the number of vegetations formed, and vegetation size in a rabbit model of IE (40, 41). Furthermore, treatment with an anti-SEB monoclonal antibody (mAb) reduced inflammation, abscess size and bacterial counts in various mouse models of infection (42). Although these studies were conducted in different animal models, utilized different strains of *S. aureus*, and studied different SAgs, an overall trend that can be observed in many of these studies is the change in abscess or vegetation formation in the absence of SAg, lending support to our findings. Since we used a sublethal dose of *S. aureus*, we were able to further discern the effects of SEA which may have been masked in previous studies that used mortality as a parameter of bacterial fitness.

Although high bacterial counts and abscesses were also detected in the kidneys, we did not see a difference between *S. aureus* Newman and Newman Δ*sea* infection in bacterial loads. This may be due to the local downregulation of SAg-mediated T cell activation by cell-wall embedded molecules (25) or the low numbers of T cells in the kidneys (43) for a SAg-specific response in this organ. While we were able to detect an SEA-specific T cell response in the form of $V\beta3^+CD3^+$ lymphocytes from the lymph nodes (Fig. 3.1), no changes were observed in the percentage of $CD3^+$ cells in the livers (Fig. 3.4B), nor did we detect changes in IL-2 *in vivo*. This is likely due to the short half-life of IL-2 *in vivo*, as well as sampling time (44, 45). Typically, an expansion of the SAg-targeted Vβ subset is seen in humans (46), whereas in our mice we observed a SEA-specific decrease.

Whether this is due to deletion of activated T cells, anergy or internalization of the TCR has yet to be determined. Thus, within the context of infection I showed that the purpose of SAgs is to enhance fitness by creating *in vivo* niches to promote survival. By subverting the neutrophil response to infection, and evolving to survive within neutrophilic abscesses, *S. aureus* has coordinated the role of SAgs well with its other virulence factors.

4.2.1 Limitations and future work

In humans, staphylococcal bacteremia and sepsis typically presents with lung complications such as pneumonia (47), which our model was unable to mimic. Since we did not use clinical parameters to assess if the mice were truly septic, the disease severity is difficult to correlate to humans. Thus far, there is no concrete clinical data linking SAgs and abscess formation. However, there are hints that SAgs may play a clinically relevant role regarding abscesses. A recent case study presented a patient with lung and pharyngeal abscesses was attributed to a strain of *S. aureus* that produced copious amounts of SEG and SEI (48). Furthermore, *S. aureus* has been found to be the most common etiological agent of liver abscesses in children (49). As *S. aureus* is the most common cause of SSTIs, the role of SAgs and abscess formation and persistence should be expanded from earlier studies. Since abscess formation is influenced by so many staphylococcal factors, epidemiological studies may have trouble discerning a relationship. However, it is important to determine if the phenotype and mechanisms of pathogenicity in mice translate to humans who are much more sensitive to the effects of SAgs.

4.3 Overall conclusions

Comparison of the two lifestyles of *S. aureus* reveals seemingly conflicting roles for SAgs. On one hand, the expression of SAgs during nasal colonization decreases bacterial loads; however, SAgs enhanced bacterial survival during bacteremic infection. The answer to this paradox may be answered by analyzing the different survival tactics taken by *S. aureus* in these two different lifestyles. It has been shown that there are different expression patterns in the virulon between invasive infection and colonization; thus, it

was surprising that our model showed expression of SAg during both lifestyles. However, by coordinating SAg function with other staphylococcal virulence factors, *S. aureus* appeared to be successful at manipulating the inflammatory response so that it could persist in specialized *in vivo* niches: the nose during colonization, and abscesses during invasive infection. The anterior nares are a highly desirable niche for colonization and transmission between people; therefore, by attenuating virulence, propagation can continue. A low-level of colonization may be what allows for an asymptomatic state of colonization, which SAgs may be a contributing factor towards as suggested in Chapter 2. Persistent nasal colonizers may have dysregulated immune responses that permit higher bacterial densities (15-18), and may explain why SAg-activation of the immune system does not cause bacterial clearance. In the body, neutrophils and macrophages are highly effective at bacterial clearance. Thus, the tactic of maintaining a low bacterial density does not increase overall bacterial fitness once the mucosa is breached since bacteria are introduced to a myriad of immune components that function to clear the infection. Thus, the formation of an immune-protected niche *in vivo* is important for continued survival. *S. aureus* has evolved many mechanisms to persist as staphylococcal abscess communities and SAgs enhance this process as shown in Chapter 3. Overall, it appears that *S. aureus* utilize SAgs in order to adapt to *in vivo* niches by manipulating the immune system to promote overall survival and persistence. The work presented in this thesis shows a novel purpose for SAgs and reconciles the seemingly counterintuitive inflammatory properties of these toxins, with a role for enhancing biological fitness.

4.4 Future directions for SAg research

The collective SAg research community has contributed enormously to an advanced understanding of SAg biology. Yet, there remain a number of important avenues for further study and consideration. Although SAgs are defined by Vβ-specificity, different human MHC II molecules are also clearly important for the response to SAgs (50-55). Mouse models (such as B6 and BALB/c) have been hampered by the fact that mouse MHC II do not respond in the same way, and are not as sensitive to SAgs, as human MHC (50). Alternative models include rabbits that respond more appropriately (56-58), as well as transgenic mouse strains that express human MHC class II molecules (50, 59).

Models of TSS also often utilize a liver-damaging reagent such as D-galactosamine in conjunction with high levels of SAg protein. Liver and gut pathology has recently been implicated in the course of TSS in a humanized transgenic HLA-DR3 mouse model without the use of D-galactosamine (60, 61) and thus, D-galactosamine may mask pathologies normally induced via TSS. Lastly, although many studies using purified recombinant SAgs have yielded many insights, SAg function is still rarely studied in the context of live infections using genetically defined bacterial knockout strains. More work using live infections with appropriate SAg-responsive models is needed to be able to coordinate SAg production with other virulence factors.

Although a number of studies have evaluated the presence of *S. aureus*, and correlations of particular SAg genes with particular clinical syndromes, the presence of the gene does not equate to expression and function of the actual toxin. Indeed, the original discovery of TSST-1 as the causal agent of menstrual TSS was made due to the high level production of this toxin from menstrual TSS strains (62, 63). For many human diseases where SAgs may contribute to, or drive the pathology, there is likely not a single toxin responsible given that they can all activate numerous T cells. As we now know the Vβ skewing patterns of virtually all the known staphylococcal SAgs in humans (64-66), further systematic evaluations focused on SAg expression coupled with function in relation to particular clinical syndromes (46), are warranted.

The large family of SAgs continue to grow, and the YPM and MAM SAgs seem to have developed their SAg-activity through convergent evolution as these toxins are not orthologous to the pyrogenic toxin SAgs, or to each other. Also, the animal model of KD utilizes an uncharacterized SAg from the cell wall preparation of *Lactobacillus casei* to induce the disease in mice (67). *L. casei* is found commonly in the intestinal tract, is widely used by the dairy industry, and is clearly not a pathogen. It is easy to speculate that uncharacterized SAgs could be produced by other microorganisms.

Given the findings presented in this thesis, it is worth exploring the ways that SAgs benefit other organisms other than contributing to experimental or clinical shock, as this is likely not the biological purpose for these toxins. The redundancy of these toxins

should also be explored as there is evidence for preferential SAg expression. Determining the environmental cues that activate SAgs or 'alternative' toxins may help prevent enhanced morbidity associated with staphylococcal infections. Given the high prevalence of colonization in the population, complete eradication of *S. aureus* at this time is not feasible. However, vaccine efforts can concentrate on reducing morbidity and mortality of staphylococcal infections. Since there is no single virulence factor responsible for staphylococcal pathogenicity, vaccine candidates need to include multiple targets and may require activation of immune components other than the humoral response (68). As SAgs have been shown to help establish *in vivo* niches regardless of colonization or infection, they may be good candidates to include in cocktail vaccines to reduce bacterial fitness and ameliorate morbidity and mortality levels. As such, there is promising pre-clinical data using SAgs in polyvalent vaccines against staphylococcal infections (69, 70). As the only known virulence factor to purposefully activate the adaptive immune system, SAgs are evolutionarily unique. Since engagement of the immune system is so crucial for the success of *S. aureus* as both a commensal and pathogen, there is untapped potential in these toxins yet to be explored.

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Appendices

Appendix 1. Animal ethics approval

AUP Number: 2011-074
AUP Title: Staphylococcal Infections
Yearly Renewal Date: 04/01/2013

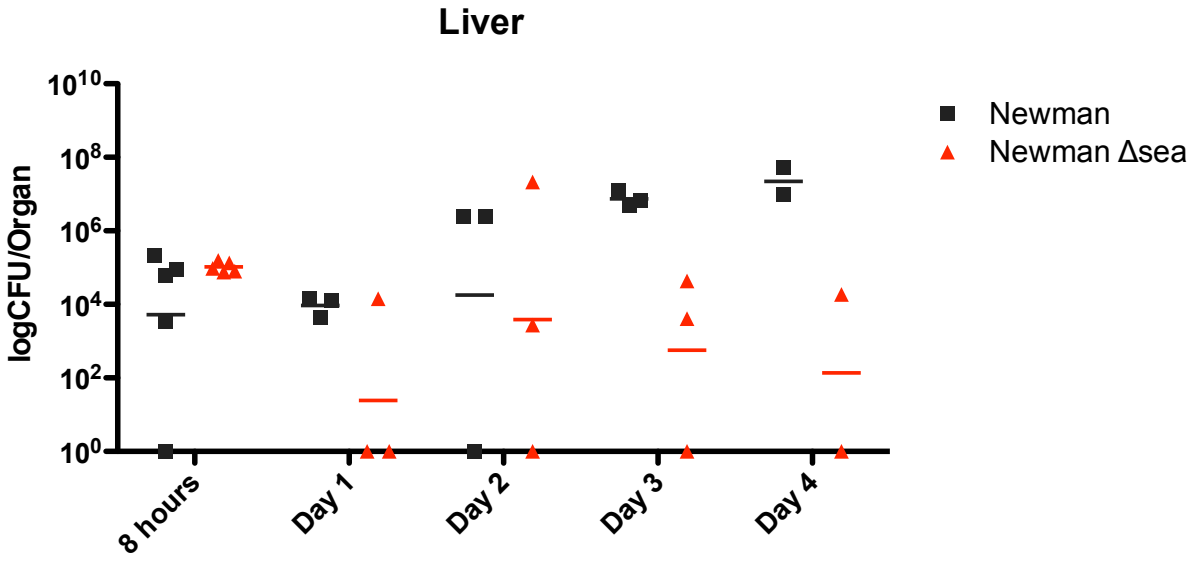
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2011-074 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Mollard, Maureen
on behalf of the Animal Use Subcommittee



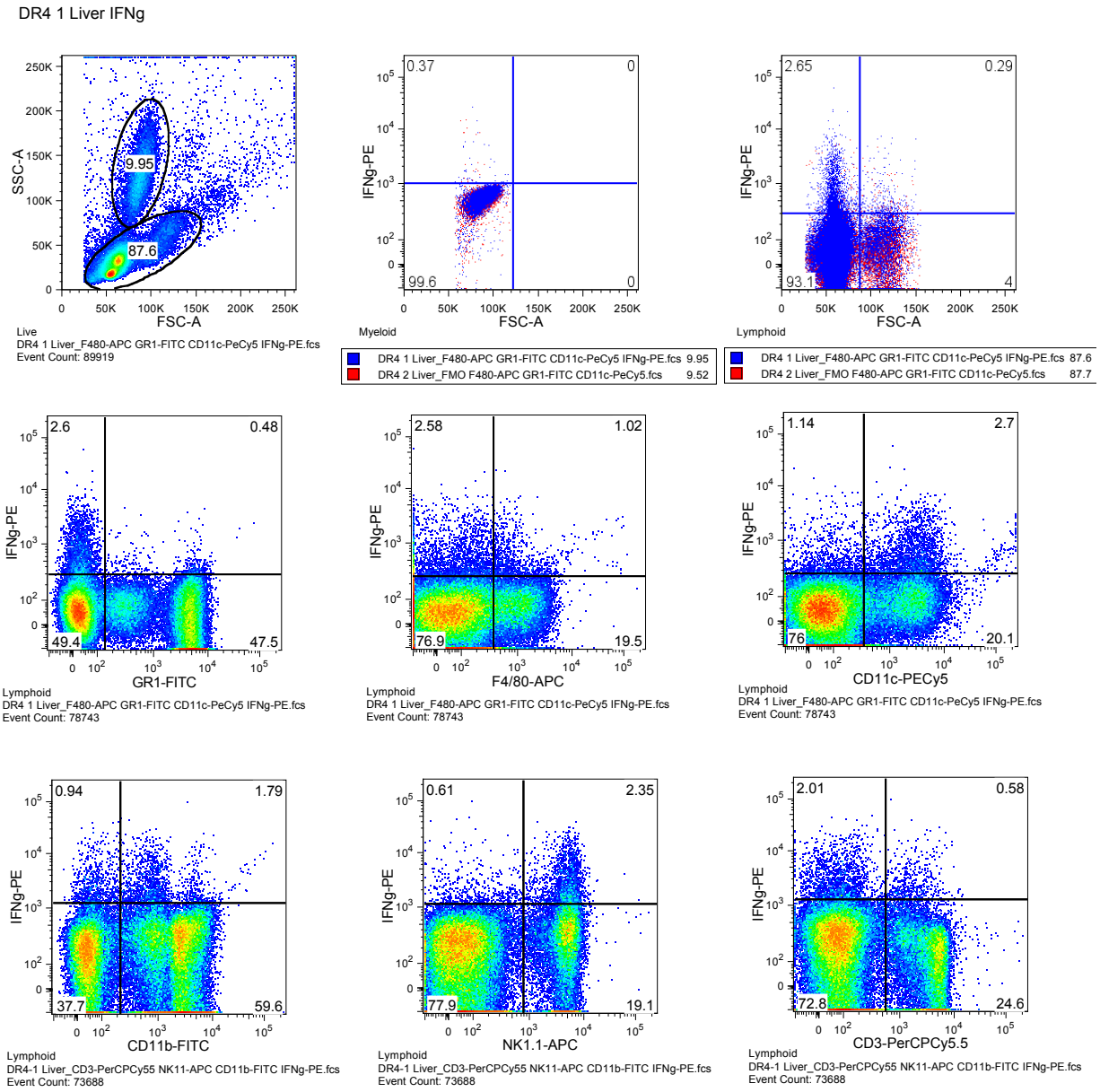
Appendix 2. Kinetics of bacterial counts in the liver during *S. aureus* bacteremia. Bacterial burdens in the livers of DR4-B6 mice infected with 5×10^6 CFU *S. aureus* Newman (black squares) or Newman Δ*sea* (red triangles) over time. Each point represents one mouse and the line represents the mean.

Appendix 3. Cytokines and chemokines detected from liver supernatants of Newman and Newman Δ*sea*-infected mice. DR4-B6 mice were infected with 5×10^6 CFU *S. aureus* Newman and samples taken 8 hours post-infection (pg/mL). Each column represents an individual mouse.

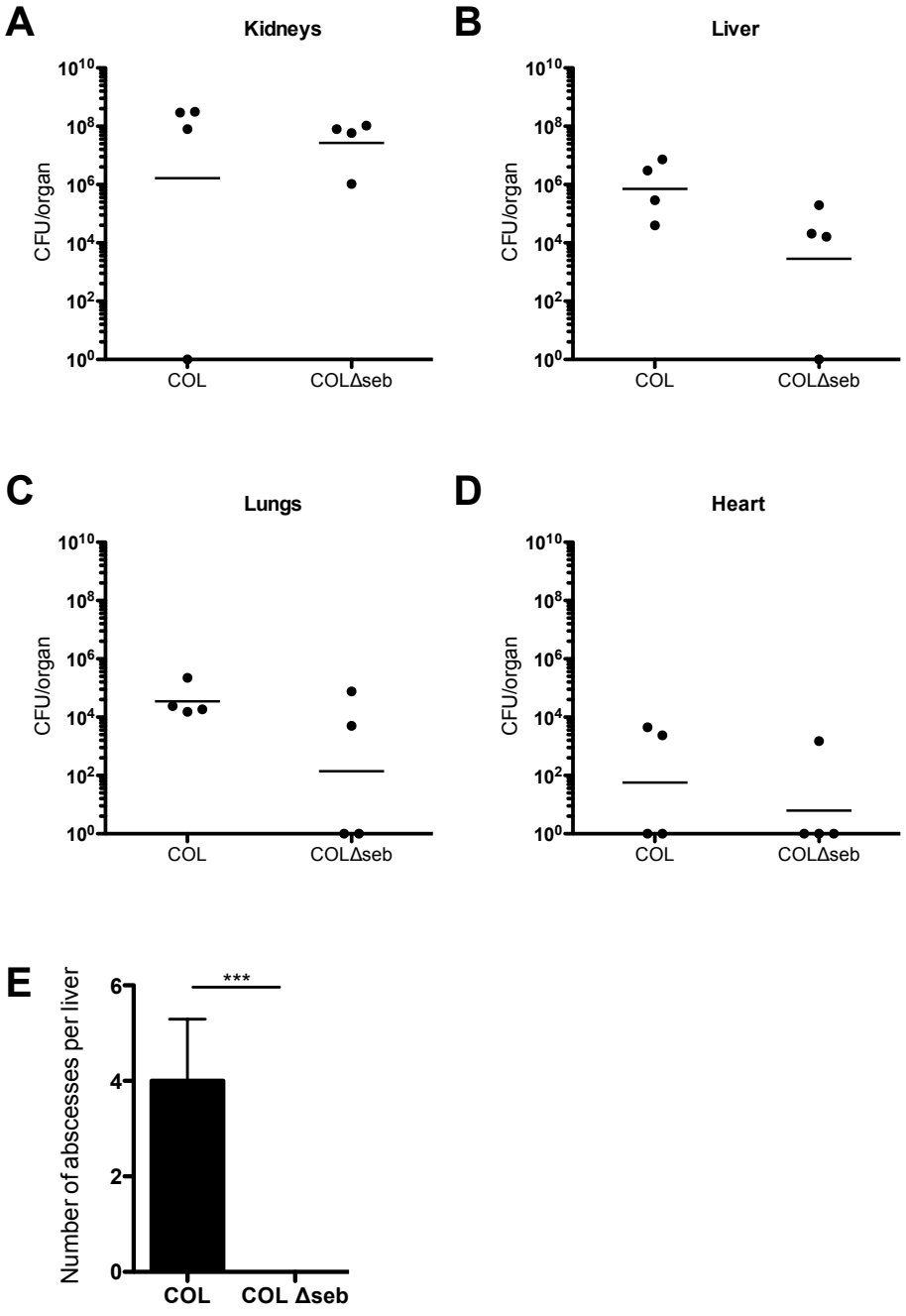
LIVER	NWM-1	NWM-2	NWM-3	NWM Δsea-1	NWM Δsea-2	NWM Δsea-3
Eotaxin	195.42	175.94	149.99	165.2	148.26	118.68
GCSF	330.77	93.12	137.37	58.75	146.13	34.94
GMCSF	233.29	144.01	85.02	150.39	68.78	58.9
IFNy	113.86	81.05	90.24	51.97	59.98	44.89
IL-1a	626.22	314.74	479.11	437.73	474.64	234.05
IL-1b	95.64	73.99	103.6	75.3	76.6	52.3
IL-2	68.7	59.37	48.38	54.91	64.45	51.29
IL-3	43.78	15.03	15.92	25.43	23.96	17.05
IL-4	9.9	6.06	5.99	5.99	3.48	2.91
IL-5	5.93	3.4	3.61	3.23	3.44	2.48
IL-6	100.66	75.58	80.13	46.75	54.3	41.47
IL-7	85.82	33.34	18.2	53.72	35.69	19.08
IL-9	5.03	6.12	25.67	30.33	39.7	36.35
IL-10	140.71	80.55	59.7	75.71	70.92	54.02
p40	90.46	90.18	69.22	62.43	49.43	51.33
p70	170.03	110.01	86.72	121.88	98.72	84.64
IL-13	209.34	45.08	10.62	87.96	2.04	0
IL-15	301.09	142.13	128.15	119.2	186.21	108.86
IL-17	29.11	15.98	14.03	19.94	10.27	9.46
IP-10	3267.3	2399.6	2761.4	2079	2496.26	1563.8
KC	403.13	677.11	660.32	201.26	469.35	401.46
LIF	1.21	0.27	1.06	2.26	0	0
LIX	0	0	0	0	0	0
MCP-1	1157.86	1015.85	1077.51	818.48	783.79	509.5
MCSF	73.25	34.68	39.14	40.94	59.52	40.74
MIG	1594.78	1236.46	1489.09	1565.37	1701.95	826.3
MIP-1a	130.99	87.44	81.12	82.49	82.95	51.18
MIP-1b	265.04	151.8	101.87	167.5	35.94	45.12
MIP-2	356.66	366.65	427.87	132.54	184.15	116.86
RANTES	84.76	52.71	49.35	60.08	52.66	35.2
TNF-a	35.63	25.68	27.5	23.21	16.55	16.89
VEGF	7.06	3.5	2.84	5.26	5.31	1.94

Appendix 4. Cytokines and chemokines detected from sera of Newman and Newman Δ*sea*-infected mice. DR4-B6 mice were infected with 5×10^6 CFU *S. aureus* Newman and samples taken 8 hours post-infection (pg/mL). Each column represents an individual mouse.

SERUM	NWM-1	NWM-2	NWM-3	NWM-4	NWM Δsea-1	NWM Δsea-2	NWM Δsea-3
Eotaxin	1413.98	1067.95	1300.86	1169.51	1316.71	1855.2	1306.32
GCSF	3270.89	2627.33	2510.03	1365.86	1653.55	6473.47	5019.4
GMCSF	60.73	0	78.25	98.57	0	34.46	49.49
IFNy	59.66	43.69	68.64	21.32	0	0	0
IL-1a	96.99	64.89	34.29	0	44.14	75.65	44.14
IL-1b	0.39	39.36	48.38	3.39	18.3	6.4	21.17
IL-2	22.94	9.52	7.16	0	3.44	0.56	4.17
IL-3	5.73	0	0	0	0	0	0
IL-4	0.31	0	0	0.8	0.16	0	0
IL-5	57.27	29.19	16.56	29.48	16.14	55.79	49.27
IL-6	495.84	270.9	193.13	244.44	315.99	488.26	490.49
IL-7	0	0	0	0	0.84	0	0
IL-9		35.04	33.52	423.14	0	ND	113.4
IL-10	5.33	25.95	0	1.28	38.41	17.56	43.59
p40	0	0	44.71	38.58	6.4	0	34.25
p70	53.88	79.12	83.27	83.27	28.9	13.65	21.08
IL-13	155.34	83.04	215.99	102.08	83.04	215.99	136.24
IL-15	0	0	0	0	0	0	6.4
IL-17	0	0	0	0	0	0	1.79
IP-10	979.71	1346.43	2032.9	1277.95	408.14	212.34	626.06
KC	5460.82	4864.12	3346.85	4486.14	3188.64	7825.15	3553.02
LIF	0	0	0	0	0	0	0
LIX	9051.91	2767.26	4635.6	673.01	3979.11	2518.47	7071.74
MCP-1	1816.63	3515.82	4216.75	2883.08	1336.44	2853.34	3527.23
MCSF	2.72	4.22	4.74	1.76	5	4.74	3.46
MIG	103.68	152.84	170.77	109.46	52.5	293.42	77.16
MIP-1a	148.89	71.53	124.46	159.59	127.58	114.73	188.86
MIP-1b	171.05	188.02	237.79	211.43	66.05	163.77	272.49
MIP-2	0	50.19	56.23	0	32	80.25	74.28
RANTES	48.17	45.13	86.01	10.28	32.02	51.13	39.88
TNF-a	19.51	23.1	17.43	13.93	13.93	12.07	22.32
VEGF	0.04	0.46	0.48	0.25	0.25	0.29	0.34

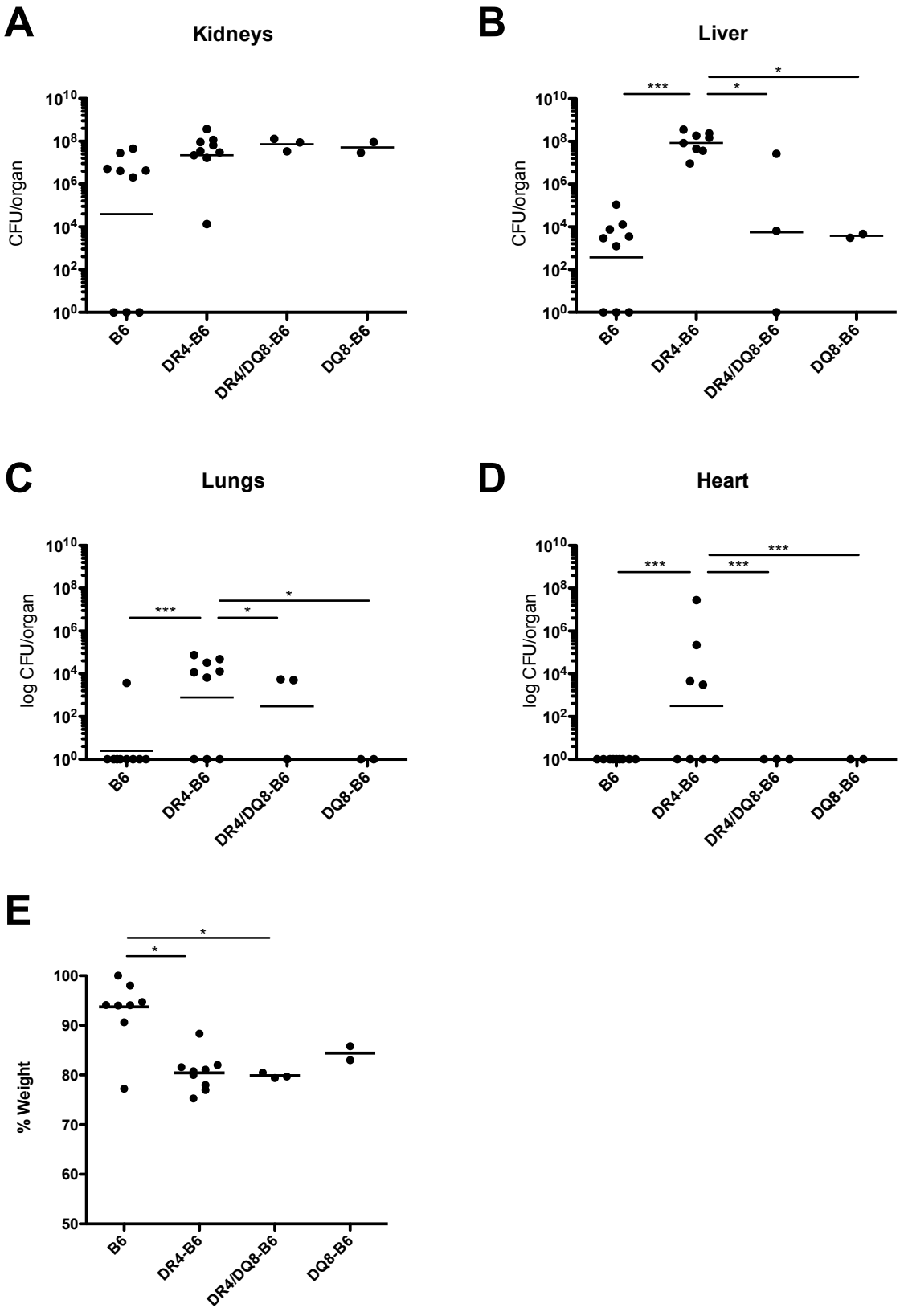


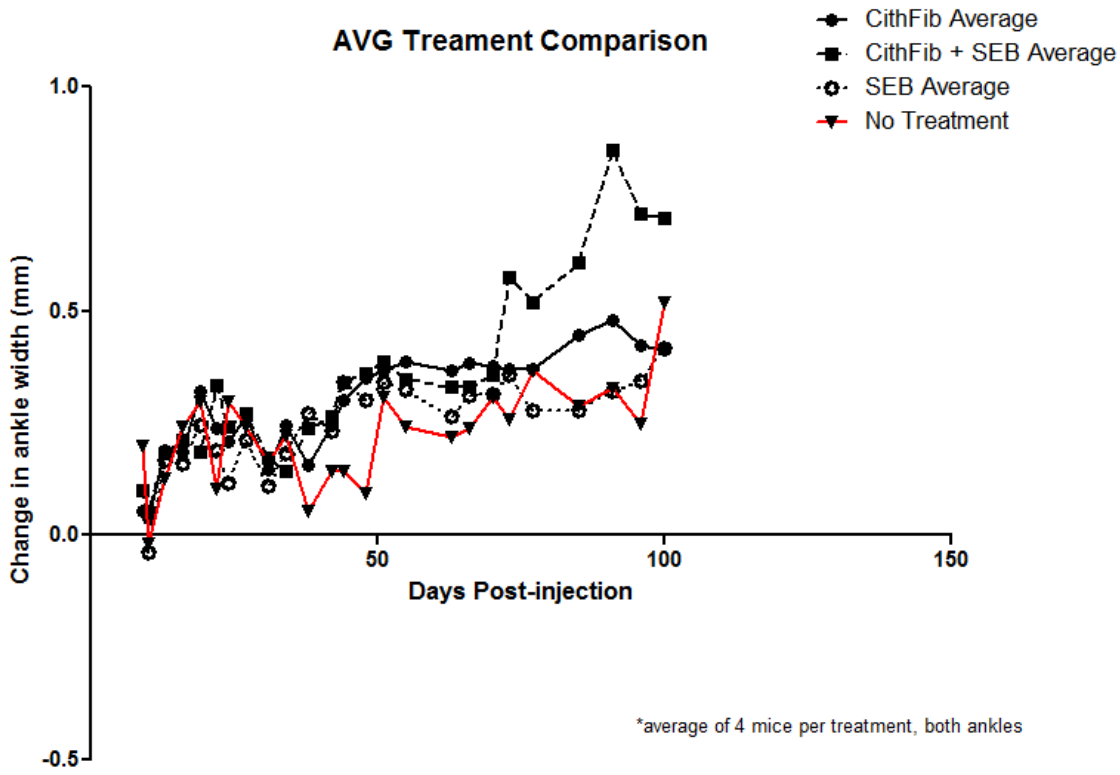
Appendix 5. Intracellular detection of IFN-γ in Newman-infected mice. Representative mouse of liver leukocyte populations examined for intracellular IFN-γ, 8 hours post-infection with 5×10^6 CFU *S. aureus* Newman via tail-vein. Dot plots represent analysis of lymphocyte populations as this was shown to be IFN-γ positive, and not the myeloid population. Gating was based on FMO controls. Panels consisted of either anti-GR-1, anti-F4/80 and anti-CD11c or anti-CD11b, anti-NK1.1 and anti-CD3.



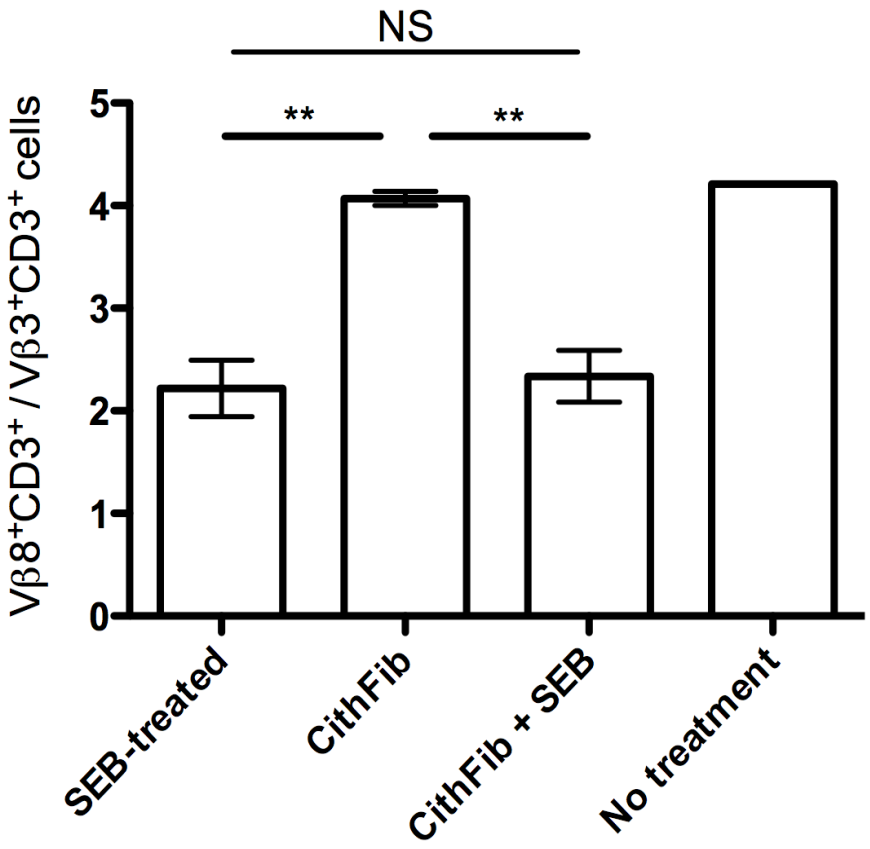
Appendix 6. SEB contributes to abscess formation during *S. aureus* COL bacteremia. Bacterial burdens 96 hours post-infection of DR4-B6 mice infected with 5×10^6 CFU *S. aureus* COL or COL Δ*seb* in A) livers, B) kidneys, C) lungs and D) hearts. E) Liver abscess scores from infected mice. (n=4 per group); data are representative of one experiment. ***denotes $p < 0.001$ as determined by unpaired student's t-test

Appendix 7. MHC class II influences susceptilibity to *S. aureus* COL bacteremia. B6, DR4-B6, DR4-B6/DQ8 or B6-DQ8 mice were injected with 5×10^6 CFU *S. aureus* COL via tail-vein and bacterial loads were enumerated 96 hours post-infection in the A) liver, B) kidneys, C) lungs and D) heart. E) Weight loss was calculated as change in percentage from pre-infection weights and measured at 96 hours post-infection. Statistical significance are denoted by * $p < 0.05$ and *** $p < 0.001$ as determined by one-way ANOVA with Tukey's post-test


A
Kidneys
CFU/organ
B
Liver
CFU/organ
C
Lungs
log CFU/organ
D
Heart
log CFU/organ
E
% Weight
B6
DR4-B6
DR4/DQ8-B6
DQ8-B6
***
*



Appendix 8. SEB enhances the arthritogenicity of citrullinated fibrinogen in DR4-B6 mice. DR4-B6 mice were immunized (subdermal) with 100µg of citrullinated human fibrinogen (CithFib), 10µg of SEB or both with complete Freund's adjuvant (CFA), and boosted at day 27 with incomplete Freund's adjuvant (IFA). (SEB-treated n = 3, CithFib n=3, CithFib + SEB n = 4, No treatment n = 1). Ankle measurements were taken for both feet up to 100 days post-vaccination. Data are represented as average ankle measurements per group



Appendix 9. SEB-treatment results in chronic decrease in Vβ8$^+$CD3$^+$ T cells. Flow cytometry analysis of splenocytes 100 days after the initial vaccination (SEB-treated n = 3, CithFib n = 3, CithFib + SEB n = 4, No treatment n = 1). Data are expressed as a ratio of Vβ8$^+$CD3$^+$ to Vβ3$^+$CD3$^+$ cells per mouse for each treatment group. Data shown as the mean ± SEM, significant differences ($p < 0.005$) as determined by one-way ANOVA with Tukey's post-test are denoted with **. No treatment was not included in statistical analyses and is present for comparison only.

Curriculum Vitae

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Education

Doctorate Candidate (PhD) in Microbiology and Immunology
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Honors Bachelor of Medical Sciences BMSc (Hons) Specialization in Microbiology and Immunology, Scholar's Electives
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September 2005 – April 2009

Awards and Scholarships

2009-2014 **Western Graduate Research Scholarship** ($35 500)
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2013 **Cedarlane/ATCC 1st Place Oral Presentation ($500)**
Infection and Immunity Research Forum, London, Canada

2013 **Microbiology and Immunology Travel Award** ($500)
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2013-2014 **Ontario Graduate Scholarship** ($15 000)
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2013 **Poster Award – Infection and Immunity** ($500)
London Health Research Day, London, Canada

2012 **Nomination for Graduate Teaching Award**
Western University

2012-2013 **Ontario Graduate Scholarship** ($15 000)
Province of Ontario, Western University

2012-2013 **Queen Elizabeth II Graduate Scholarship in Science and Technology** ($15 000)
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2011 **Microbiology and Immunology Graduate Entrance Award** ($2000)
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Schulich School of Medicine and Dentistry, University of Western Ontario

2010-2011 **Schulich Scholarship for Medical Research** ($4500)
Schulich School of Medicine and Dentistry, University of Western Ontario

2010 **Diamed Poster Award** ($100)
Infection and Immunity Research Forum, London, Canada

2008-2009 **Dean's Honor Roll**
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Publications

1. S. Sayedyahossein, **S.X. Xu**, A. Rudkousakaya, M.J. McGavin, J.K. McCormick, and L. Dagnino. (2014) *Staphylococcus aureus* keratinocyte invasion is mediated by Integrin-Linked-Kinase and Rac 1. Accepted to *FASEB*.

2. R.V. Anantha, D.M. Mazzuca, **S.X. Xu**, S.A. Porcelli, D.D. Fraser, C.M. Martin, I. Welch, T. Mele, S.M.M. Haeryfar, and J.K. McCormick. (2014) Th2-polarized invariant natural killer T cells reduce disease severity in acute intra-abdominal sepsis. *Clin Exp Immunol.* Epub ahead of print.

3. **S.X. Xu**, K.J. Gilmore, P. Szabo, M.L. Baroja, S.M.M. Haeryfar, and J.K. McCormick. (2014) Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival

in vivo. *Infect Immun.* **82**(9):3588-98.

4. K.J. Kasper, J.J. Zeppa, A.T. Wakabayashi, **S.X. Xu**, D.M. Mazzuca, I. Welch, M. Kotb, E. Cairns, P.P. Cleary, S.M.M. Haeryfar and J.K. McCormick. (2014) Bacterial superantigens promote acute nasopharyngeal infection by Streptococcus pyogenes in a human MHC class II-dependent manner. *PLoS Pathog.* **10**(5): e1004155

5. B. Shrum, R.V. Anantha, **S.X. Xu**, M. Donnelly, S.M.M. Haeryfar, J.K. McCormick, and T. Mele. (2014) A Robust Scoring System to Evaluate Sepsis Severity in an Animal Model. *BMC Res Notes.* **7:**233

6. **S.X. Xu** and J.K. McCormick (2012) Staphylococcal superantigens in colonization and disease. *Front Cell Inf Microbio.* **2:**52

7. J. Li, Wang W., **S.X. Xu**, N.A. Magarvey, and J.K. McCormick (2011) *Lactobacillus reuteri*-produced cyclic dipeptides quench *agr*-mediated expression of toxic shock syndrome toxin-1 in staphylococci. *Proc Natl Acad Sci*. **108**(8):3360-5.

Presentations/Abstracts

*denotes presenter

S.X. Xu, K.J. Gilmore, P. Szabo, M.L. Baroja, S.M.M. Haeryfar, and J.K. McCormick 'Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival *in vivo*' London Health Research Day (Poster presentation) London, Canada. March 20, 2014.

Samar Sayedyahossein, **S.X. Xu**, A. Rudkouskaya, J.K. McCormick, and L. Dagnino 'Integrin Linked Kinase Modulates *Staphylococcus aureus* Invasion of Epidermal Keratinocytes' Physiology and Pharmacology Research Day (Poster presentation) London, Canada. November 6, 2013.

Alena Rudkouskaya, S. Sayedyahossein**, S.X. Xu**, John K. McCormick, and L. Dagnino 'The integrity and barrier function of the epidermis are modulated by Integrin-Linked Kinase' (Poster presentation) London, Canada. November 6, 2013.

Stacey X. Xu*, K.J. Gilmore, M.L. Baroja, K. Summers, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated immune suppression in the liver during *Staphylococcus aureus* sepsis' 8th Annual Infection and Immunity Research Forum (Oral presentation) London, ON, Canada. November 1, 2013.

Stacey X. Xu*, K.J. Gilmore, M.L. Baroja, K. Summers, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated immune suppression in the liver during

Staphylococcus aureus sepsis' Staphylococcal Diseases Gordon Research Conference (Poster presentation) Waterville Valley, New Hampshire, USA. July 28-August 2, 2013

Stacey X. Xu*, K.J. Gilmore, M.L. Baroja, K. Summers, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated immune suppression in the liver during *Staphylococcus aureus* sepsis' Staphylococcal Diseases Gordon Research Seminar (Poster presentation) Waterville Valley, New Hampshire, USA. July 27-28, 2013

Stacey X. Xu*, M.L. Baroja, K. Summers, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated immune suppression in the liver during *Staphylococcus aureus* sepsis' 26th Annual Canadian Student Health Research Forum (Poster presentation) Winnipeg, Canada. June 4-6, 2013

Stacey X. Xu*, M.L. Baroja, K. Summers, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated immune suppression in the liver during *Staphylococcus aureus* sepsis' London Health Research Day (Poster presentation) London, Ontario, Canada. March 2013

Samar Sayedyahossein**, S.X. Xu**, J.K. McCormick, L. Dagnino 'Integrin Linked Kinase Modulates Staphylococcus aureus Invasion of Epidermal Keratinocytes' London Health Research Day (Oral presentation) London, Ontario, Canada. March 2013

Samar Sayedyahossein**, S.X. Xu**, J.K. McCormick, L. Dagnino 'Integrin Linked Kinase Modulates *Staphylococcus aureus* Invasion of Epidermal Keratinocytes' The Fibronectin, Integrins & Related Molecules Gordon Research Seminar (Oral presentation) Ventura, USA. February 2013

Samar Sayedyahossein**, S.X. Xu**, J.K. McCormick, L. Dagnino 'Integrin Linked Kinase Modulates *Staphylococcus aureus* Invasion of Epidermal Keratinocytes' 7th Annual Infection and Immunity Research Forum (Poster presentation) London, Ontario, Canada. November 2012

Samar Sayedyahossein**, S.X. Xu**, J.K. McCormick, L. Dagnino 'Integrin Linked Kinase Modulates *Staphylococcus aureus* Invasion of Epidermal Keratinocytes' Physiology and Pharmacology Research Day (Poster presentation) London, Ontario, Canada. November 2012

Stacey X. Xu*, M.L. Baroja, S.M.M. Haeryfar and J.K. McCormick 'Superantigen-mediated alteration of macrophages in the liver during staphylococcal bacteremia' 15th International Symposium on Staphylococci and Staphylococcal Infections (Poster presentation) Lyon, France. August 2012

Jingru Li, M.L. Baroja, W. Wang, **S. Xu**, N.A. Magarvey, and J.K. McCormick. 'Interspecies communication between *Lactobacillus reuteri* and *Staphylococcus aureus* quenches *agr*-mediated expression of toxic shock syndrome toxin-1' 15th

International Symposium on Staphylococci and Staphylococcal Infections (Poster presentation) Lyon, France. August 2012

Katherine J. Kasper, **S.X Xu**, D.M. Mazzuca, L. Baroja and J.K. McCormick 'Development of a model to assess the role of streptococcal virulence factors in nasal-associated lymphoid tissue colonization in mice' London Health Research Day (Poster presentation) London, Ontario, Canada. March 2012

Stacey Xu*, and J.K. McCormick 'The role of superantigens in *Staphylococcus aureus* nasal colonization and disease' 6th Annual Infection and Immunity Research Forum (Poster presentation) London, Ontario, Canada. November 2011

Katherine J Kasper, **S.X Xu**, D.M. Mazzuca, and J.K. McCormick, 'Development of a model to assess the role of streptococcal virulence factors in nasal-associated lymphoid tissue colonization in mice' Canadian Society of Microbiologists 61st Annual Conference (Poster presentation) St. John's, Newfoundland, Canada. June 2011

Stacey Xu* and J.K. McCormick 'The role of superantigens in *Staphylococcus aureus* nasal colonization and disease' 5th Annual Infection and Immunity Research Forum (Poster presentation) London, Ontario, Canada. November 2010

Jingru Li, **S. Xu**, and J.K. McCormick 'Probiotic Interspecies cell-to-cell communication between *Lactobacillus* and *Staphylococcus aureus* quenches *agr*-mediated quorum sensing and represses expression of toxic shock syndrome toxin-1' Society of Industrial Microbiology 60th Annual Meeting (Poster presentation) San Francisco, USA. August 2010

Jingru Li, **S. Xu**, and J.K. McCormick 'Interspecies cell-to-cell communication between probiotic *Lactobacillus* and *Staphylococcus aureus* inhibits production of the staphylococcal superantigen toxic shock syndrome toxin-1' 2nd TNO Beneficial Microbes Conference (Poster presentation) Noordwijkerhout, the Netherlands. March 2010

Stacey Xu* and J.K. McCormick 'The role of superantigens in *Staphylococcus aureus* nasal colonization' 4th Annual Infection and Immunity Research Forum (Poster presentation) London, Ontario, Canada. November 2009

Research Experience

2009 -2014 **PhD Candidate** (Supervisor: Dr. John K. McCormick)
University of Western Ontario (London, Canada)
Thesis: 'The role of superantigens in *Staphylococcus aureus* nasal colonization and disease'
- PhD qualifying candidacy exam - Passed with Distinction (2011)

2006 – 2011 **Animal Lab Assistant** (Supervisor: Dr. Arthur Brown)
Robarts Research Institute (London, Canada)
- Responsible for care of research animals (mice) and assisted in animal behavioural testing

2009 **Summer student** (Supervisor: Dr. John K. McCormick)
University of Western Ontario (London, Canada)
Research Project: *Lactobacillus reuteri* RC-14 suppression of *Staphylococcus aureus* two-component systems and virulence factors

2008-2009 **4th Year Honors Thesis Student** (Supervisor: Dr. John K. McCormick)
University of Western Ontario (London, Canada
Thesis: 'Interspecies cell-to-cell communication: *Lactobacillus reuteri* RC-14 represses expression of *Staphylococcus aureus* two-component systems and virulence factors'

2008 **Summer Student** (Supervisor: Dr. Arthur Brown)
Robarts Research Institute – UWO (London, Canada)
Research topic: Spinal cord injuries and the inflammatory sequelae

2007 **Lab Volunteer** (Supervisor: Dr. Jun Wang)
Dalhousie University (Halifax, Canada)
Research topic: construction of a vaccine vector for *Chlamydia trachomatis*

2006-2007 **Scholar's Electives Student** (Supervisor: Dr. Kathleen Hill)
University of Western Ontario (London, Canada)
Thesis: Spontaneous mutations measured in the cerebrum of *Harlequin* mice using the *cII* assay

2005-2006 **Scholar's Electives Student** (Supervisor: Dr. Laura Hertel)
University of Western Ontario (London, Canada)
Thesis: The Evolution of Viruses

2005 **Lab Volunteer** (Supervisor: Dr. Gonzalo Hortelano)
McMaster University (Hamilton, Canada)

Teaching and Mentoring Experience

2011-2014 **Science Fair Judge**
London District Science and Technology Fair (London, Canada)

2011-2013 **Undergraduate supervisor/mentor**
Western University (London, Canada)

2011-2013 **Guest Lecturer**
4th Year Current Concepts in Biotechnology (Dr. Todd Hyrciw)
Fanshawe College (London, Canada)

2009-2013 **Teaching Assistant** (Dr. Susan Koval)
Microbiology and immunology 2100a
University of Western Ontario (London, Canada)
- Nominated for a Graduate Teaching Award (2012)

2009-2013 **Tutor** (Dr. Kelly Summers and Dr. David Colby)
Microbiology and Immunology 3810 for Nursing students
University of Western Ontario (London, Canada)

2011-2013 **Let's Talk Science Classroom Volunteer**
University Heights Public School (London, Canada)

2008-2009 **Scholar's Electives Connections Program Mentor**
Western University (London, Canada)

Volunteer Work

2012-2014 **Children's Hospital subcommittee member**
Strong Bones, Strong Minds, Strong Muscles

2011-2014 **Co-chair**
MNI Outreach Committee

2011-2014 **R.E.A.D. program volunteer**
London Public Libraries

2010-2014 **Community Volunteer**
Let's Talk Science

2009-2014 **MNI Outreach Volunteer**
Western University

2009-2014 **MNI Social Committee**
Western University

2012-2013 **Past-Chair/Advisor**
IIRF Organizing Committee

2011-2012 **Chair**
IIRF Organizing Committee

2010-2011 **Co-Chair, VP Promotions**

IIRF Organizing Committee

2009-2010 **VP Promotions**
IIRF Organizing Committee

2009 **Logistics Coordinator**
Scholar's Electives Big Ideas Conference, Western University
Western University