THE ROLE OF CD44 AND ITS ISOFORMS IN *S. AUREUS*-INDUCED INFLAMMATION

by

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ABSTRACT

BRITTANY KAYE HILL
THE ROLE OF CD44 AND ITS ISOFORMS IN *S. AUREUS*-INDUCED INFLAMMATION
Under the direction of ROBERT J. MCKALLIP, Ph. D.

Hospital-acquired *S. aureus* infections have long been an issue in public health in the United States, but community-acquired infections are now on the rise as well. While *S. aureus* are sensitive to numerous antibiotics, there is no known effective treatment for the superantigen activity produced by the bacteria. Superantigens are virulence factors secreted by *S. aureus* that cause complications during infection due to cross-linking of MCH class II antigens on antigen presenting cells and T-cell receptors. This process stimulates a large number of T cells causing increased pro-inflammatory cytokine production, vascular leak, and severe inflammation [5]. This study examines the role of CD44 during *S. aureus*-induced inflammation and identifies CD44 isoforms CD44v6v7 and CD44v8v10 as potential targets for treatment of superantigen-induced inflammation. The results show that CD44 isoforms CD44v6v7 and CD44v8-v10 are elevated in PBMC's isolated from patients bacteremic with *S. aureus* and in mice exposed to *S. aureus*. Consequently, these isoforms may be potential targets to reduce the effects of superantigen activity. The role of CD44 is shown through the use of CD44KO mice, which have reduced pro-inflammatory cytokine production and reduced

immune cell migration into the lungs after *S. aureus* exposure when compared to WT C57BL/6 mice. The CD44KO mice do, however, show increased bacterial loads in the lungs. This suggests that while not all CD44 molecules can be targeted due to reduced clearance of bacteria, perhaps the isoforms CD44v6v7 and CD44v8-v10 can. Future studies to target CD44v6v7 and/or CD44v8-v10 to examine the effects of the immune response following *S. aureus* infection are warranted.

CHAPTER 1

REVIEW OF THE LITERATURE

Staphylococcus aureus

Staphylococcus aureus is a gram-positive cocci bacterium that grows in clusters [1]. As a gram positive bacteria, it contains a thick cell wall of peptidoglycan with teichoic and lipoteichoic acids. Most staphylococci have capsules to evade host responses. The most typical infections caused by *S. aureus* are skin and soft tissue infections. However, severe infections can occur and result in endocarditis, pneumonia, sepsis, and even death. *S. aureus* is a commensal organism that naturally colonizes 30-50% of healthy adults at any given time, while 10-20% of those are persistently colonized [1]. It can colonize many areas such as the nares, axillae, vagina, pharynx, and skin surfaces. However,, infection does not always occur. *S. aureus* is opportunistic, and infections are initiated when there is a breach in the skin or mucosa that allows the bacteria to invade the tissues or bloodstream. Although the bacteria is usually harmless to the host it can be passed on to others through skin-to-skin contact, fomites, and wound contact [1].

S. aureus Virulence Factors and Superantigens

S. aureus produces several virulence factors that can cause host injury and promote evasion of the host immune system. These include cell surface components,

which are a collection of protein adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). MSCRAMMs promote bacterial adherence to components of the extracellular matrix of the host to initiate colonization [2]. These include fibronectin-binding proteins, collagen-binding proteins, and fibrinogen-binding proteins. It also includes protein A, a surface protein which can mediate attachment of the bacteria to von Willebrand factor and bind to the Fc domain of IgG to inhibit phagocytosis by the host immune system [3]. The toxins that *S. aureus* produce are perhaps what make the bacteria extremely virulent. There are two main types toxins produced: cytotoxins and superantigens. Cytotoxins (alpha, beta, delta, gamma, and PV-leukocidin) act locally to kill immune cells by forming pores in their membranes. Alpha, Beta, Delta, and Gamma-toxins are hemolysins attacking erythrocytes, while PV-leukocidin attacks neutrophils and macrophages. Pores in the membrane result in an efflux of K^+ out of the cell and an influx of Na^+ and Ca^{2+} into the cell, which interrupts cell metabolism to cause cell death [4].

S. aureus strains can produce up to 24 different superantigens. The different superantigens include Toxic Shock Syndrome Toxin-1 (TSST-1), staphylococcal enterotoxins A, B, C, D, E, and G, and staphylococcal-like enterotoxins H, I and J-X [5]. Superantigens act by cross-bridging the V-beta region of T cell receptors with major histocompatiblility complex class II molecules on antigen-presenting cells without an antigen being present (Figure 1). Typically, antigens stimulate about 1 in 10,000 T cells through the interaction with a specific peptide associated with the MHC complex. However, when superantigens are present, they can stimulate up to 50% of T cells and

cause increased activation of antigen presenting cells (APCs) [5]. This results in a "cytokine storm" involving massive release of pro-inflammatory cytokines IL-1, IL-2, IL-6, and TNF-α. A cytokine release of this magnitude can lead to endothelial cell injury, severe inflammation, vascular collapse, and ultimately even death. Even a small amount of superantigen in the bloodstream can raise these cytokines to toxic levels [5].

S. aureus and the immune response

Many cell types are involved in superantigen/*S. aureus*-induced inflammation. Neutrophils are the most prominent cell component of the innate immune system and provide the primary defense against *S. aureus*. They are recruited quickly to sites of infection and produce reactive oxygen species (ROS), antimicrobial proteins and other enzymes to kill the bacteria [6]. Macrophages are also an important cell type in the clearance of *S. aureus*. They capture and kill bacteria like neutrophils, but they also release proinflammatory cytokines IL-1, IL-6, and TNF-α and other cytokines such as IL-12 to help activate T cells. [7]. The role of T cells is perhaps the most important part of superantigen/*S. aureus* clearance. Conventional T cells are important for the generation of opsonizing antibodies. They also promote phagocytosis by recruiting neutrophils and macrophages from the bone marrow to the infection site and produce many cytokines, such as IFN-γ, that help innate cells kill persisting bacteria [8]. However, when superantigens are present, these cells are overactivated and cause additional problems in the body. Regulatory T cells are important for reestablishing homeostasis to the body after infection [9].

Clinical Relevance

Hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) has long been an issue in public health with approximately 20% mortality rate, but unfortunately, community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is now on the rise as well [10]. In the United States, MRSA is now the most common cause of soft tissue infections in the community setting [11]. Although strains of CA-MRSA usually cause skin or soft tissue infection, they can also cause bacteremia/sepsis, necrotizing pneumonia, and infective endocarditis under certain conditions. One CA-MRSA strain, USA300, has reached predominance in the United States recently. It expresses high amounts of the exotoxin PV-leukocidin which causes tissue damage and destruction of leukocytes [12]. The MRSA bacteria is resistant to penicillin because it expresses an enzyme called β-lactamase, an enzyme that breaks open the β -lactam ring of the antibiotic. Resistance to methicillin results from the bacteria containing the *mecA* gene, which encodes for penicillin-binding protein 2a [13].

Current Treatments

Many *S. aureus* infections that are limited to the skin and soft tissue resolve of their own. Deeper infections usually respond well to surgical incision and drainage with antimicrobial therapy. At the present time, in the United States, trimethoprim-sulfamethoxazole and clindamycin are the most common prescribed drugs for the outpatient treatment of CA-MRSA infections [14]. Other drugs used are doxycycline, minocycline, linezolid, and rifampin. For more serious infections that require hospitalizations, vancomycin, daptomycin, linezolid, and tigecycline are commonly given intravenously [14]. These drugs kill the bacteria causing infections but do not neutralize

the superantigen toxins already wreaking havoc in the body. The current treatment for this consists of using intravenous human antibodies to specific suspected superantigens to neutralize the effects [15]. However, this has severe limitations since neutralization is only effective at the early stages of exposure. The fact that many strains of *S. aureus* can produce multiple superantigens also presents a problem. It is improbable to treat for every superantigen possible within a particular *S. aureus* strain. The cost of intravenous antibodies themselves present a problem along with the numerous side effects such as fever, headache, chills, nausea/vomiting, tachycardia, and even serious renal or neurological issues [15].

CD44 and Inflammation

The Extracellular Matrix

The extracellular matrix is made up of several different components including collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins [16]. While initially it was just considered to provide mechanical support to tissues, recent years have found many other functions for the extracellular matrix. Beyond providing scaffolding between cells, the ECM is also important for cell migration, differentiation, proliferation, apoptosis and adhesion during embryonic development [17]. It serves as a storage site for a variety of soluble molecules such as cytokines and growth factors. Cells within the extracellular matrix secret the ECM molecules building their own support network and degrade/replace the matrix depending on different signals. This subject is of great interest since the process of

extracellular matrix repair and degradation is altered in many different disease processes and injuries.

Hyaluronic Acid and Inflammation

Hyaluronic acid (HA) is a main component of the ECM. It is a linear glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine [16]. HA is important for the maintenance of tissue homeostasis, and production and degradation occurs continuously [18]. Under normal conditions, the large molecular mass form of hyaluronan predominates in healthy tissues. However, during inflammation such as in the case of *S. aureus* infection, the ECM and hyaluronan are broken down into smaller fragments and accumulate [19]. Low molecular weight HA is known to be associated with the inflammatory response, allowing inflammatory cytokines to be produced, inducing CXC chemokines for cell migration, and causing proliferation of immune cells. High molecular weight HA is associated with anti-inflammatory responses [18-19]. Signal transduction pathways to achieve these inflammatory responses are initiated by HA binding to its receptors, CD44 and RHAMM.

CD44 and Its Isoforms

CD44 is a cell surface glycoprotein expressed by many different types of cells. CD44 binding to its ligand HA plays an important role in recruiting immune cells to the sites of inflammation. It also plays a direct role in the production of pro-inflammatory cytokines. Naïve lymphocytes express primarily the standard form of CD44, CD44s . Alternative splicing and differential N- and O-glycosylation produces different variants of

CD44 (v1-v10) (Figure 2). Under various situations, different isoforms of CD44 are more prevalent than the standard form [20]. This suggests that the different isoforms may play distinct roles in the immune response and may give us a potential target for preventing the effects of superantigens. Previous studies have shown that different cytokines modulate CD44 isoform expression. In work shown by Mackay *et al*, CD44v9 and CD44v6 were the main variants that were upregulated on T cells after stimulation by TNF-α and IFN-γ respectively [20]. Interestingly, this upregulation was much more apparent than that of CD44s. Not all variants are upregulated however. No modulation of CD44v4 was observed when stimulated by cytokines. In another study, CD44v6 and CD44v7 were found to be distinctly expressed on subpopulations of activated lymphocytes with CD44v6 found on T cells only and CD44v7 found on $CD4^+$ T cells, B cells, and monocytes. Furthermore, antigenic stimulation of lymphocytes was impaired *in vitro* when anti-CD44v6 and anti-CD44v7 was present. Specifically, anti-CD44v6 treatment down-regulated IL-2 and IFN-γ production by $CD8^+$ T cells while anti-CD44v7 treatments led to a decrease in IL-12 production [21]

CD44 and HA Binding Effects

There are many functions of CD44 binding to its ligand HA. Most notably, interaction between CD44 and HA is highly involved in leukocyte trafficking to areas of inflammation. The movement of leukocytes into tissues involves a series of steps mediated by adhesion molecules on the cells themselves and their ligands on the endothelium. The cells first establish loose interactions with the endothelium and start to 'roll' along the surface. This rolling slows the cells so that they can then firmly attach

to the endothelium and crawl along the vessel wall until they arrive at a place for migration into the vasculature [22]. Within the tissue, leukocytes are guided by chemokines toward the area where they are needed. At signs of infection or damage within a tissue, immune cells secrete pro-inflammatory cytokines such as IL-1 and TNF-α [22]. These act on the endothelium in the vasculature to upregulate HA. It has been shown *in vitro* and *in vivo* that under flow conditions, upregulation of HA on activated endothelium leads to T cell recruitment into the tissue [22]. This implies that the binding of CD44 and HA is needed for the initial rolling step of migration. For neutrophils, the rolling step of recruitment is not dependent upon CD44-HA binding. When antibodies are used to block CD44, neutrophil adhesion but not rolling is attenuated [22]. Because there are already well-established roles of selectin and integrin molecules for leukocyte trafficking, CD44-HA binding most likely provides an additional method of recruitment of cells into tissue. The importance of CD44-HA interactions for leukocyte trafficking have been established in many inflammatory disease cases. Antibodies used against CD44, CD44 genetic deficiencies, or HA depletion by hyaluronidase has shown to decrease neutrophil, monocyte, and lymphocyte recruitment to tissues and lessen disease activity in arthritis, dermatitis, peritonitis, myositis, allergic asthma, liver disease, and graft-vs-host disease. In one study, the blockade of CD44-HA interactions led to a 50-70% reduction in the number of adherent neutrophils [22].

CHAPTER 2

INTRODUCTION

Staphylococcus aureus is a commonly isolated human pathogen whose infection can result in problems from skin and soft-tissue infection to pneumonia, endocarditis, sepsis, and even death [11]. Hospital-acquired methicillin-resistant *S. aureus* has long been an issue in public health with approximately 20% mortality rate, but unfortunately, community-acquired methicillin-resistant *S. aureus* is now on the rise as well [10, 11]. Many *S. aureus* related illnesses are due to the presence of superantigens, such as staphylococcal enterotoxin B (SEB), produced by the bacteria. Superantigens are produced by many different bacteria and cause complications during infection by cross-linking of MCH class II antigens on antigen presenting cells and T-cell receptors to stimulate large numbers of T cells [5]. Even a small amount of superantigen can greatly raise the levels of pro-inflammatory cytokines IL-1β, IL-2, IL-6, TNF-α, and IFN-γ that can lead to endothelial cell injury, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and vascular collapse [5]. There are no known effective treatments to reduced or eliminate the downstream effects of superantigens. The current treatment consists of using intravenous human antibodies (IVIG) to specific suspected superantigens to neutralize their effects. However, this has severe limitations since neutralization is only effective at the early stages of exposure. The fact that many strains

of *S. aureus* can produce multiple superantigens also presents a problem. It is improbable to treat for every superantigen possible produced by a particular *S. aureus* strain. The cost of IVIG's themselves present a problem along with the numerous side effects such as fever, headache, chills, nausea/vomiting, tachycardia, and even serious renal or neurological issues [15].

CD44 is a cell surface glycoprotein expressed by many different types of cells. CD44 binding to its ligand HA plays an important role in recruiting immune cells to the sites of inflammation. It also plays a direct role in the production of pro-inflammatory cytokines. Naïve lymphocytes express primarily the standard form of CD44, CD44s. Alternative splicing and differential N- and O-glycosylation produces different variants of CD44 (v1-v10). Across various situations, different isoforms of CD44 are more prevalent than the standard form [21]. This suggests that the different isoforms may play distinct roles in the immune response and may give us a potential target for preventing the effects of superantigens/S. aureus infection. Previous experiments in the McKallip lab have identified CD44 isoforms CD44v6v7 and CD44v8v10 as possible targets for superantigen/*S. aureus*-induced inflammation. This was the focus of the current study.

We hypothesize that superantigens/*S. aureus* strains of bacteria cause an increase in the expression of specific CD44 isoforms CD44v6-v7 and/or CD44v8-v10 and that CD44 plays an important role in *S. aureus*- and superantigen-induced inflammation (Figure 3). Furthermore we hypothesize that targeting the CD44 isoforms CD44v6-v7 and/or CD44v8-v10 may lead to novel treatments for the harmful effects associated with superantigen exposure.

CHAPTER 3

METHODOLOGY

PBMC Exposure to Superantigens

Human blood was drawn from 6 different donors, approximately 10 mL per donor. PBMCs were separated from the whole blood by a density gradient centrifugation method using Ficoll Histopaque. We then cultured the cells (1 x 10^6 cells/mL) in 100 ul plates with unstimulated media and stimulated with SEB, SEA, and TSST-1 superantigen toxins (2 ug/ml each). The cells were harvested after 24 and 48 hours.

Quantification of gene expression for PBMC's

RNA isolation was performed with GeneJet RNA Purification Kit (Thermo Scientific, Waltham, MA), and cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transciptase Kit (Applied Biosystems, Carlsbad, CA). qPCR amplification was performed with the isoform specific primers and SYBR green (Applied Biolsystems, Carlsbad, CA) using the Applied Biosystems Model 7300 qPCR system. The forward and reverse primers used were as follows: 18s 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3', CD44s 5'- GACACATATTGCTTCAATGCTTCAGC-3'and 5'-GTGGAATGTGCTTGGTCTC-3', CD44v3 5'-GTACGTCTTCAAATACCATCTCAGC-3'and 5'-GGTGCTGGAGATAAATCTTCATC-3', CD44v6v7 5'-TCCAGGCAACTCCTAGTAGTAC-3' and 5'-

CCATCCTTCTTCCTGCTTG-3', CD44v8v10 5'-TGGACTCCAGTCATAGTATAACGC-3'and 5'-CGATTGACATTAGAGTTGGAATCTCC-3'. Expression was examined using comparative CT values compared to unstimulated cell groups.

Pro-inflammatory Cytokine Gene Expression *in vivo*

We intranasally injected WT mice (C57BL/6) and CD44KO mice with *S. aureus* USA 300 strain (50 ul of 6 x 10^8 CFU/ml) or PBS (50 ul) as control. To determine the amount of inflammation and cytokines produced, we extracted lung tissue 24 hrs after exposure and used a laboratory homogenizer and AKC lysing buffer to extract leukocytes. RNA isolation, reverse transcription and RT-PCR were performed as described above. Various inflammatory cytokines (IFN-γ, IL-1, IL-6, and TNF-α) with specific primers were tested to determine the increase in inflammation in mice exposed to *S. aureus* compared to PBS control mice. The forward and reverse primers used were as follows: B-actin 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and 5'-ATGGAGCCACCGATCCACA-3', IL-1 5'-GAAATGCCACCTTTTGACAGTG-3' and 5'-CTGGATGCTCTCATCAGGACA-3', IL-6 5'- ATGGATGCTACCAAACTGGAT-3' and 5'-TGAAGGACTCTGGCTTTGTCT-3', IFN-γ 5'-ATGAACGCTACACACTGCATC-3' and 5'-CTAGGCTTTCAATGACTGTGC-3', TNF-α 5'-CCAGTGTGGGAAGCTGTCTT-3' and 5'-AAGCAAAAGAGGAGGCAACA-3'.

CD44 Isoform Gene Expression *in vivo*

We intranasally injected WT mice (C57BL/6) and CD44KO mice with *S. aureus* USA 300 strain (50 ul of 8.7 x 10^8 CFU/ml) or PBS (50 ul) as control. Lung tissue was harvested 24 hrs after exposure and a laboratory homogenizer and AKC lysing buffer

was used to extract leukocytes. RNA isolation, reverse transcription and RT-PCR were performed as described above.CD44 isoforms CD44s, CD44v3, CD44v6v7, and CD44v8-v10 were examined. The forward and reverse primers used were as follows: CD44s 5'-GCACCCCAGAAGGCTACATTTT-3' and 5'-TTCTGCCCACCCTTCTCCTACTA-3', CD44v3 5'-ACGGAGTCAAATACCAACCC-3' and 5'-GGGTATTGTCTGTTTCATCTTC-3', CD44v6v7 5'-GTACAGCAGAAGCAGCAGCTAC-3' and 5'-CTGTCCAGGAAACATCCTCTTG-3', CD44v8v10 5'-CCAGTCATAGTACAACCC-3' and 5'-CAAAGACCTCAGTTTTAGCAG-3'. The B-actin primers used were same as described above.

Lung Histology

We intranasally injected WT mice and CD44KO mice with *S. aureus* (50 ul of 9 x 10^8 CFU/ml) or PBS (50 ul) as control. We then sacrificed the mice after 24 hours and collected the lung tissue. The samples were put in 10% formalin solution after harvesting. The lungs were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Pictures of particular places in the slides were taken.

Flow Cytometric Analysis

WT and CD44KO mice were exposed to USA 300 *S. aureus* (50 ul of 9 x 10^8 CFU/mL) or PBS (50 ul) intranasally. The mice were sacrificed 24 hours later, and the lungs were harvested. The mononuclear cells were separated using Histopaque and density gradient centrifugation. The phenotype of the cells was examined using flow cytometric analysis with a BD FACSAria II cell sorter (BD Bioscience, San Jose, CA, USA). For flow, antibodies conjugated to fluorescent probes were used. T cells were characterized by anti-CD3 antibody. Macrophages were characterized using anti-Mac-3

antibody. Neutrophils were identified using anti-Gr-1 antibodies. The total cell number for the phenotypes was calculated by multiplying the total number of mononuclear cells from the lungs x the percentage of the particular phenotype population.

Degree of Infection

WT and KO mice were injected intranasally with PBS (50 ul) or *S. aureus* USA 300 strain (50 ul of 3.67 x 10^8 CFU/mL in PBS). They were sacrificed 24 hours later and the lungs were harvested for culturing. The lungs were homogenized with the laboratory homogenizer. For the control samples, only 1 mL of each was plated. The samples were centrifuged and resuspended in 50-100 ul of saline. They were then plated on TSA agar plates. For the test samples, each sample was plated 3 times each at 100 ul and 10 ul concentrations. They were also plated on TSA agar plates. The number of colonies recorded was adjusted to the 1 mL concentration of control samples.

CHAPTER 4

RESULTS

PBMC's Stimulated with Superantigens SEB, SEA, and TSST-1 Result in Varied CD44v Expression

An important first step to targeting certain CD44 isoforms for treatment in *S. aureus* and/or superantigen-induced inflammation, we must first determine whether the isoforms of interest are elevated after exposure to the bacteria. Initially, we observed the effect of three different superantigens, SEB, SEA, and TSST-1, on CD44 isoforms of PBMC's. We used donor blood and separated PBMC's by a density gradient centrifugation method using Ficoll. Cells were stimulated with 2µg/ml of each superantigen toxin, SEB, SEA, and TSST-1. After 24 and 48 hours, the cells were harvested and the RNA isolation, RT, and real-time RT-PCR was performed with isoform-specific primers to monitor CD44s, CD44v3, CD44v6v7, and CD44v8-10. CD44v3 was not expected to be elevated after superantigen exposure, and therefore used as a control. The effect of superantigens on gene expression of these isoforms is shown in figures 4, 5 and 6. Figure 4a-d shows the isoforms after exposure to SEB. Noteworthy observations consist of CD44s elevated 3.38 fold on average at 48 hours, CD44v6v7 13.87 fold elevated on average at 48 hours, and CD44v8-v10 elevated 5.44 fold on average at 24

hours and elevated 3.13 fold on average at 48 hours. Figure 5 a-d shows the isoforms after exposure to SEA. CD44v6v7 is the only isoform elevated on average after SEA exposure at 2.95 fold at 48 hours. In the case of TSST-1, none of the isoforms were particularly elevated as shown in figure 6 a-d. This suggests that different superantigens effect the immune system in slightly different ways. Each strain of *S. aureus* however can produce more than one superantigen at a time. Therefore, it is still reasonable to believe that targeting particular CD44 isoforms CD44v6v7 and CD44v8-v10 could result in treatment of *S. aureus*/superantigen-induced inflammation.

Exposure to *S.aureus* Results in Elevation in CD44v6v7 and CD44v8-v10 in Patients and Mice

The next set of experiments were designed to determine whether exposure to *S. aureus* results in elevation of any CD44 isoforms. We analyzed CD44 expression in mice following exposure to *S. aureus* and in patient blood samples bacteremic with *S .aureus*. In the mouse model, WT mice were exposed to 50 µl of 8.7 x 10^8 CFU/mL of *S.aureus* bacteria in PBS or vehicle control. After 24 hours, the lungs were harvested and immune cells were isolated. The gene expression of CD44v6v7 and CD44v8-v10 was examined using RT-PCR with isoform-specific primers and compared to control mice. The effect of *S .aureus* on CD44v6v7 and CD44v8-v10 is shown in figure 7. Both isoforms were more elevated than the standard form of CD44. CD44v6v7 was increased 2.00 fold from control mice. CD44v8-v10 was 4.59 fold increased from control mice. We observed even more of an increase on average in human samples. Patient blood bacteremic with presumptive *S .aureus* along with healthy donor blood were supplied by a physician. The

immune cells were isolated and RT-PCR with isoform-specific primers was performed compared with the healthy donors. The effect *S. aureus* had on CD44v6v7 and CD44v8-v10 expression in the bacteremic patients is shown in figure 8. On average, CD44v6v7 gene expression was 3.00 fold higher than the controls and CD44v8-v10 was 18.8 fold higher than the controls. These results tell us that these isoforms are in fact elevated, by a great amount in the human patients, and could potentially be appropriate targets in the case of *S .aureus*/superantigen infections.

Exposure to *S. aureus* Results in Reduced Inflammation in CD44KO Mice

To determine the role CD44 plays in *S.aureus*/superantigen infections, we monitored the immune response in CD44KO mice. Pro-inflammatory cytokine production can be analyzed as an indication of inflammation in tissues. In this experiment, we examined pro-inflammatory cytokine production in the lung tissue of WT and CD44KO mice after exposure to 50 µl of 6 x 10^8 CFU/mL *S .aureus* bacteria in PBS compared to PBS control mice. After 24 hours, the lungs of these mice were harvested, and mononuclear immune cells were isolated. Cytokine mRNA levels were determined by real-time RT-PCR. As shown in figure 9, the pro-inflammatory cytokines of IL-1, IL-6, IFN-γ, and TNF-α were all decreased in the CD44KO mice. Specifically, whereas WT *S. aureus* exposed mice increased IL-1 19.9 fold, IL-6 19.2 fold, IFN-γ 7.4 fold, and TNF-α 7.0 fold, CD44KO *S. aureus* exposed mice only increased IL-1 about 1.7 fold and IL-6 about 2.8 fold, and actually downregulated IFN-γ and TNF-α. Therefore, inflammation in the lungs of the CD44KO mice was greatly reduced compared to the WT mice. This suggests that CD44 plays a role in the production of pro-inflammatory cytokines.

Exposure to *S. aureus* Leads to Decreased Immune Cell Infiltration to the Lungs in CD44KO Mice

To examine further roles of CD44 in response to *S. aureus*, we next looked at immune cell trafficking into the lungs after exposure to the bacteria with flow analysis and histology slides. Using flow analysis, we separated the particular immune cells with cell surface-specific antibody staining. After exposure to *S .aureus* (50 µl of 9 x 10^8 CFU/ml bacteria in PBS) or PBS control for 24 hours, mice were euthanized. The lungs were harvested and mononuclear immune cells were isolated. Staining for macrophages with anti-Mac1, staining for neutrophils with anti-Gr1, and staining for T cells with anti-CD3 was performed. The results are shown in figure 10. Macrophage and neutrophil recruitment was shown to be most effected in the CD44KO mice. Specifically, CD44KO macrophage numbers increased by 8,017 cells and neutrophil numbers increased by only 1,612 cells when exposed to *S .aureus*. This is compared to the WT *S. aureus* group which had macrophage number increase by 32,890 cells and neutrophil number increase by 35,757 cells when compared to control. These results are supported by the H&E histology slides. The WT *S. aureus* exposed slides show a much greater amount of cells and nuclei in the lung tissue compared to CD44KO mice as shown in figure 11.

Exposure to *S. aureus* Results in Increased Colony Counts in the Lungs of CD44KO Mice

After observing that CD44KO mice have decreased immune cell trafficking to the lungs, we questioned whether this affected bacterial load in the lungs. WT and CD44KO mice were exposed to *S. aureus* bacteria (50 µl of 3.67 x 10^8 CFU/mL in PBS) or PBS control. Lungs were harvested after 24 hours, and the tissue was plated on TSA plates

for *S. aureus* colony growth. CD44KO *S. aureus* exposed mice seemed unable to clear the infection. WT *S. aureus* exposed groups showed 2089 CFU/mL of bacteria after 24 hours, while the CD44KO *S. aureus* exposed groups showed approximately 12.4 fold more bacteria (~25,911 CFU/mL). The results are shown in figure 12. This suggests that the influx of immune cells into the lungs of the CD44KO mice is insufficient to clear the *S. aureus* infection compared to the WT mice.

CHAPTER 5

DISCUSSION

Hospital-acquired methicillin-resistant *Staphylococcus aureus* has long been an issue in public health with approximately 20% mortality rate, but unfortunately, community-acquired methicillin-resistant *S. aureus* is now on the rise as well [10, 11]. Because of this, new treatments for the effect of superantigens within the *S. aureus* bacteria need to be discovered. Superantigens cause complications during infection with *S. aureus* due to cross-linking of MCH class II antigens on antigen presenting cells and T-cell receptors to stimulate large numbers of T cells [5]. Even a small amount of superantigen can greatly raise the levels of pro-inflammatory cytokines IL-1β, IL-2, IL-6, TNF-α, and IFN-γ and can lead to endothelial cell injury, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and vascular collapse [5]. There are no known effective treatments for these effects to date, which presents a problem in today's health care.

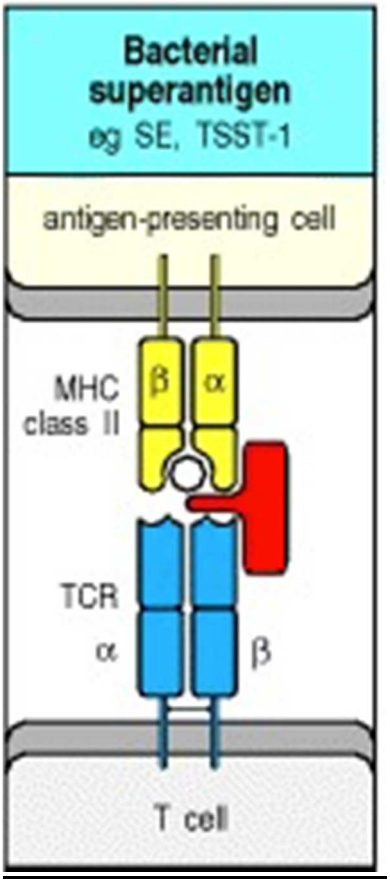
In this study, we examined the role of CD44 and its isoforms in *S. aureus*-induced inflammation. We first examined the expression of CD44 isoforms CD44v6v7 and CD44v8-v10 after *S. aureus* infection and by stimulation with three different superantigens. In the human patients with presumptive *S. aureus* contaminated blood, CD44v6v7 and CD44v8-v10 expression were both elevated. In the mice, CD44v6v7 and CD44v8-v10 expression were also both elevated after exposure to *S. aureus* although

not quite to the extent as the human patients. After exposure to superantigens SEB, SEA, and TSST-1, we saw varied expressions of the CD44 isoforms in human PBMCs. SEB greatly elevated CD44v6v7 and CD44v8-v10, while SEA and TSST-1 did not. However, most strains of *S. aureus* produce more than one superantigen. Therefore, it is reasonable to think that we can target CD44v6v7 and CD44v8-v10 for treatment of *S. aureus*/superantigen-induced inflammation. Next, we examined the role of CD44 after *S. aureus* infection by comparing results from WT and CD44KO mice . The CD44KO mice had decreased pro-inflammatory cytokine production and decreased immune cell migration to the lungs. This suggests that without any CD44 molecules, the mice have a lessened immune response to the *S. aureus* bacteria. This is demonstrated in figure 12. When CD44KO and WT mice are exposed to *S. aureus*, CD44KO mice have a much greater bacterial load present. This suggests that the bacteria are not being cleared efficiently in the absence of CD44, implying that the immune response is diminished.

In conclusion, this study has shown that CD44 plays a significant role in *S. aureus*/superantigen-induced inflammation. While elimination of all CD44 isoforms would greatly diminish the immune response and reduce clearance of the bacteria, targeting particular isoforms of CD44 may provide a potential treatment for patients suffering from a superantigen-induced response. Further studies could also examine the particular types of immune cells that differentially expressing the CD44 isoforms. As there are no effective treatments for superantigen-induced immune responses, this would be a great discovery. While the *S. aureus* bacteria can be cleared by numerous antibiotics, the downstream effects of superantigens are not easily corrected .

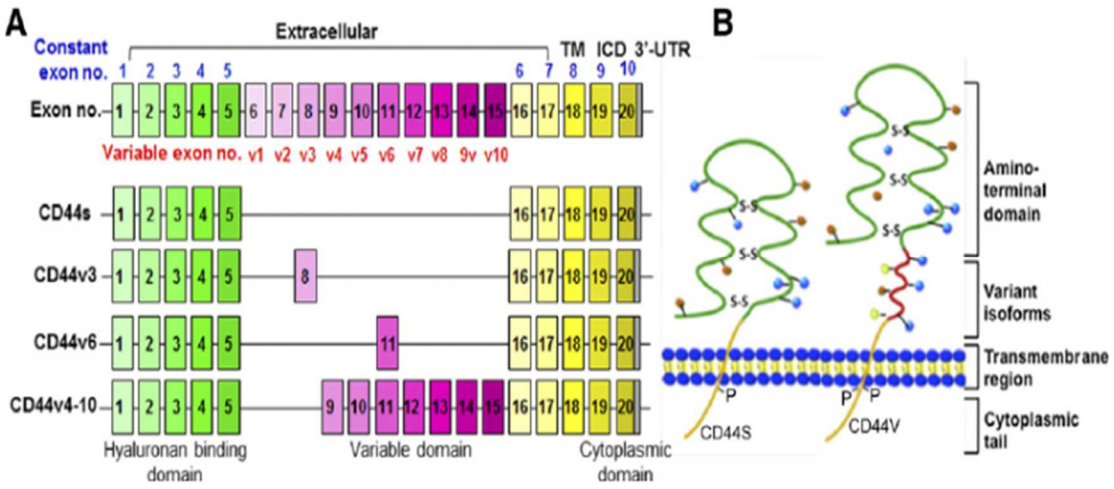
Therefore, identification of treatments that mitigate the effect of superantigens is urgent. This study suggests that CD44v6v7 and/or CD44v8-v10 may provide suitable targets for therapeutic intervention. Further studies are required to determine whether targeting these isoforms produces the desired effect on the immune system.

Figure 1: Superantigen pictorial showing non-specific activation of a T cell through an antigen-presenting cell's TCR.



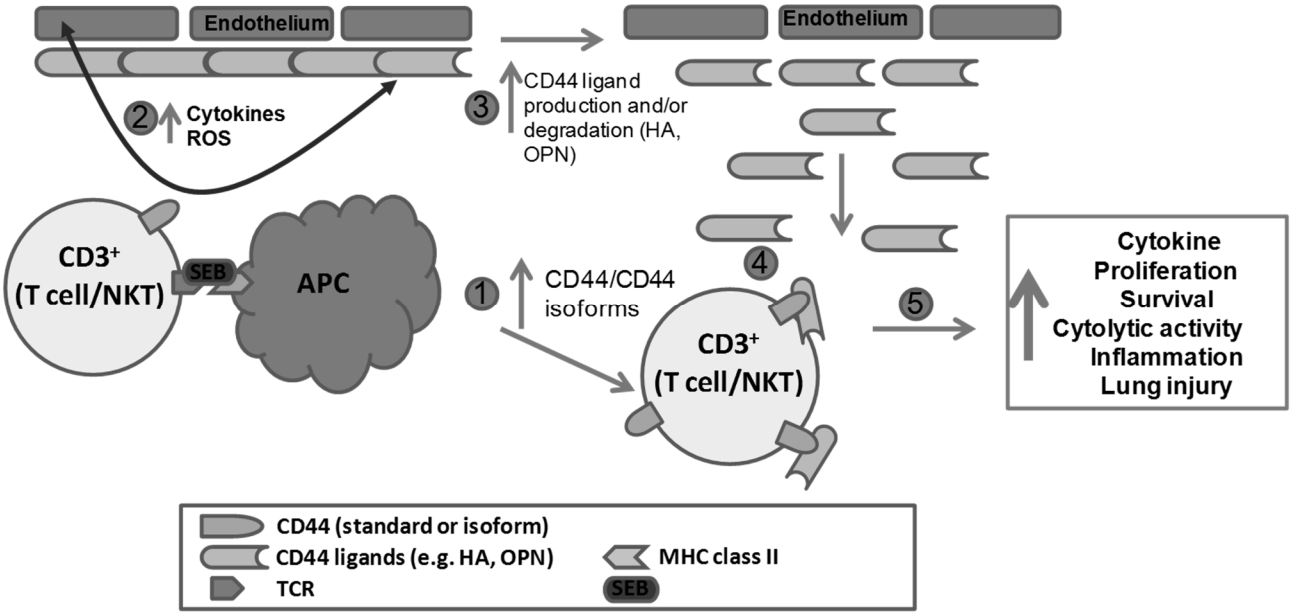
Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. The major histocompatibility complex and its functions. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27156/

Figure 2: The structure of the CD44 gene showing the difference between CD44s and CD44 isoforms.



Yan, Y., Zuo, X., & Wei, D. (2015). Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med*, *4*(9), 1033–1043. http://doi.org/10.5966/sctm.2015-0048

Figure 3: Current Hypothesis Figure
When a superantigen activates numerous T cells through MHCII/TCR interactions, CD44 and CD44 isoforms are upregulated. The superantigen causes an increase in pro-inflammatory cytokines and reactive oxygen species, which in turn act on the endothelium to increase CD44 ligand production and/or degradation. These CD44 ligands can then bind to CD44 and CD44 isoforms to result in further cytokine production, cell proliferation, cell survival, and inflammation.



McKallip, Robert. "The role of hyaluronic acid in SEB-induced acute lung inflammation." *Clinical Immunology*. 146.1 (2013): 56. Web. 16 Mar. 2014.

Figure 4 a-d: CD44v6v7 and CD44v8-v10 gene expression after exposure to SEB
Figure 5 a-d: CD44v6v7 and CD44v8-v10 gene expression after exposure to SEA
Figure 6 a-d: CD44v6v7 and CD44v8-v10 gene expression after exposure to TSST-1
Donor blood was used and PBMC's were separated using Ficoll and density gradient centrifugation. Cells were stimulated with 2 ug/mL of each superantigen toxin. After 24 and 48 hours, the cells were harvested. RNA isolation, RT, and real-time RT-PCR was performed with primers specific for CD44s, CD44v3, CD44v6v7, and CD44v8-v10. Relative fold expression compared to control is shown in figure 4 a-d, figure 5 a-d, and figure 6 a-d.

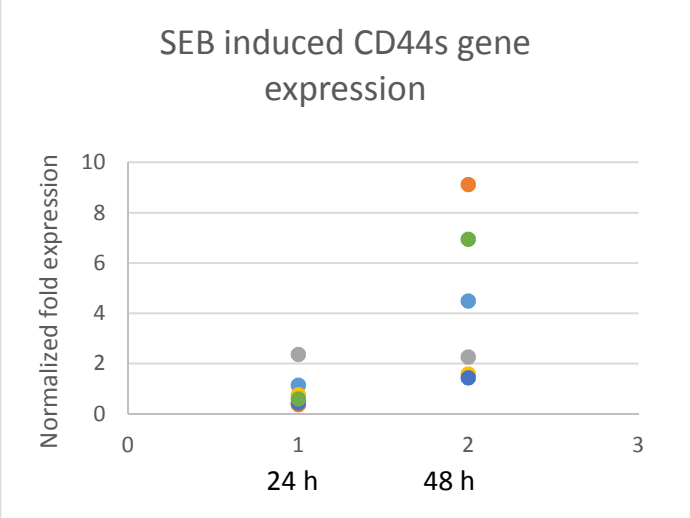


Figure 4a: SEB induced CD44s gene expression

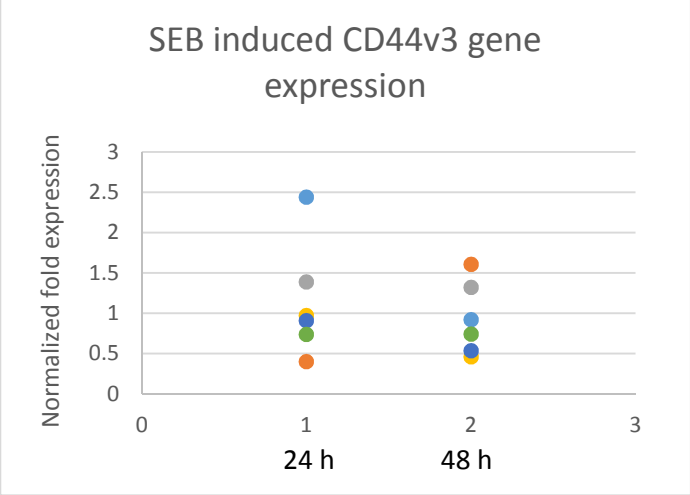


Figure 4b: SEB induced CD44v3 gene expression

Figure 4c: SEB induced CD44v6v7 gene expression

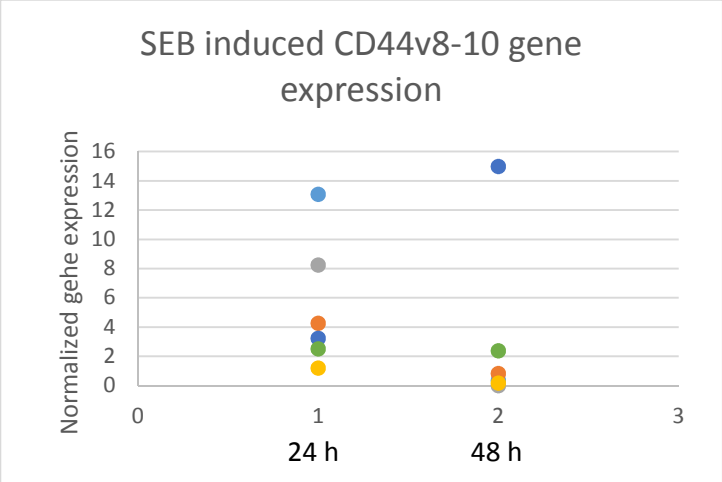


Figure 4d: SEB induced CD44v8-v10 gene expression

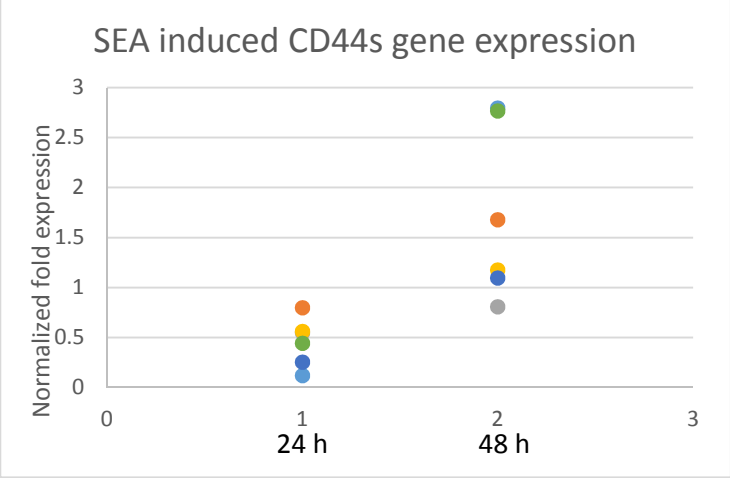


Figure 5a: SEA induced CD44s gene expression

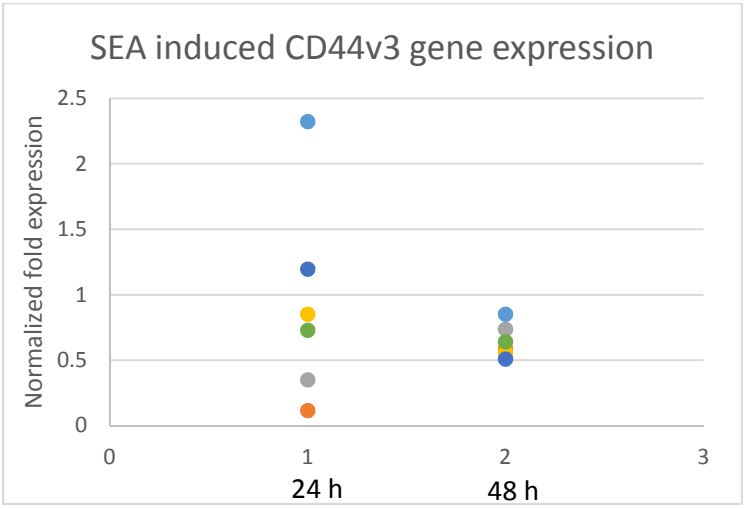


Figure 5b: SEA induced CD44v3 gene expression

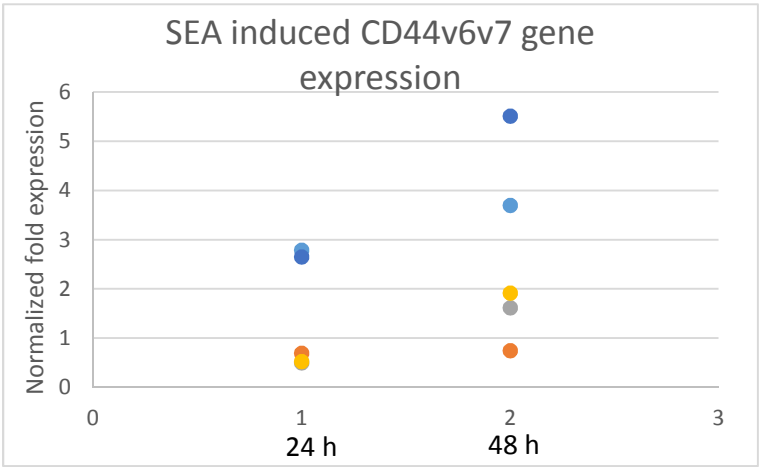


Figure 5c: SEA induced CD44v6v7 gene expression

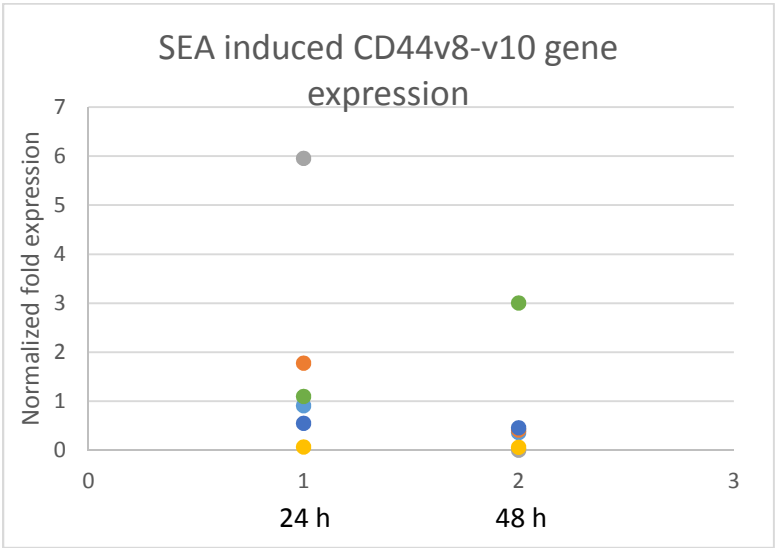


Figure 5d: SEA induced CD44v8-v10 gene expression

Figure 6a: TSST-1 induced CD44s gene expression

Figure 6b: TSST-1 induced CD44v3 gene expression

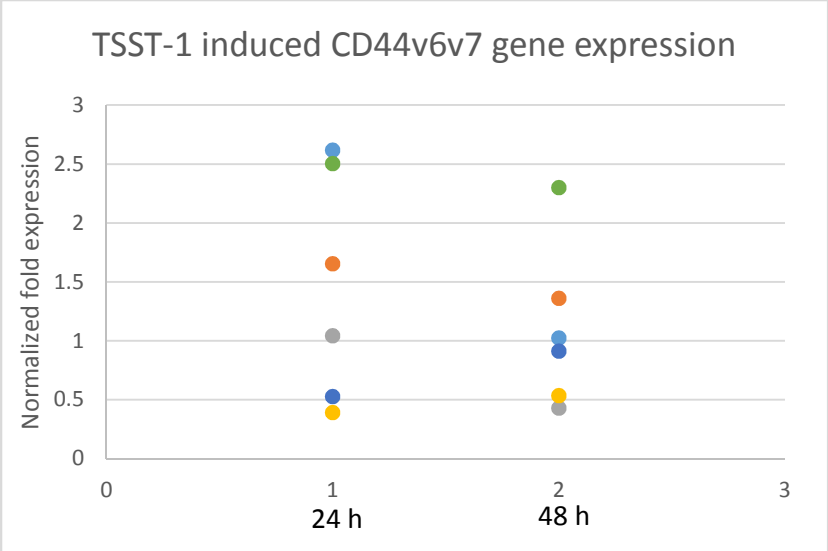


Figure 6c: TSST-1 induced CD44v6v7 gene expression

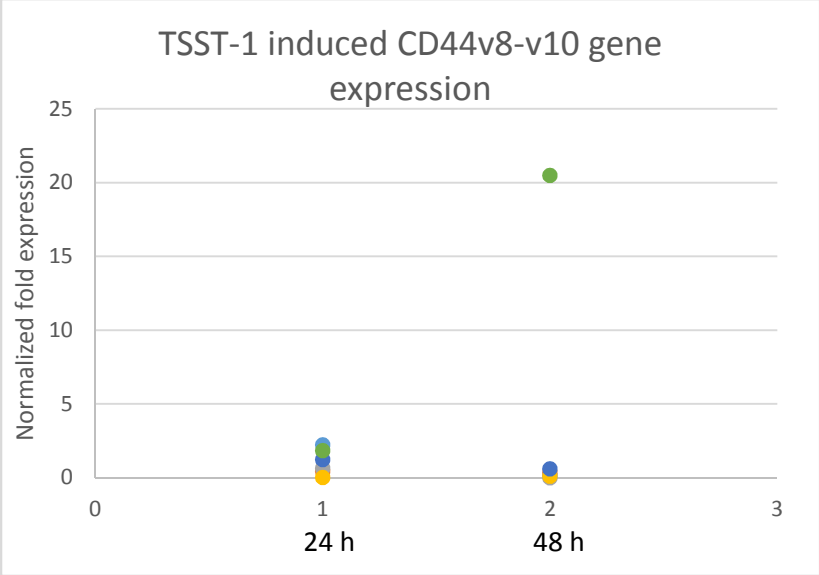


Figure 6d: TSST-1 induced CD44v8-v10 gene expression

Figure 7: CD44v6v7 and CD44v8-v10 gene expression is elevated after exposure to *S. aureus* in mice.
WT mice were exposed to 8.7 x 10^8 CFU/mL of *S. aureus*. 24 hours later, the lungs were harvested and homogenized. CD44s, CD44v3, CD44v6v7, and CD44v8-v10 isoform expression was determined using real-time RT-PCR. Shown is the relative fold expression of CD44 isoform mRNA after *S. aureus* exposure compared to PBS control. Asterisks indicate statistically significant difference when compared with vehicle controls, $p \leq 0.05$.

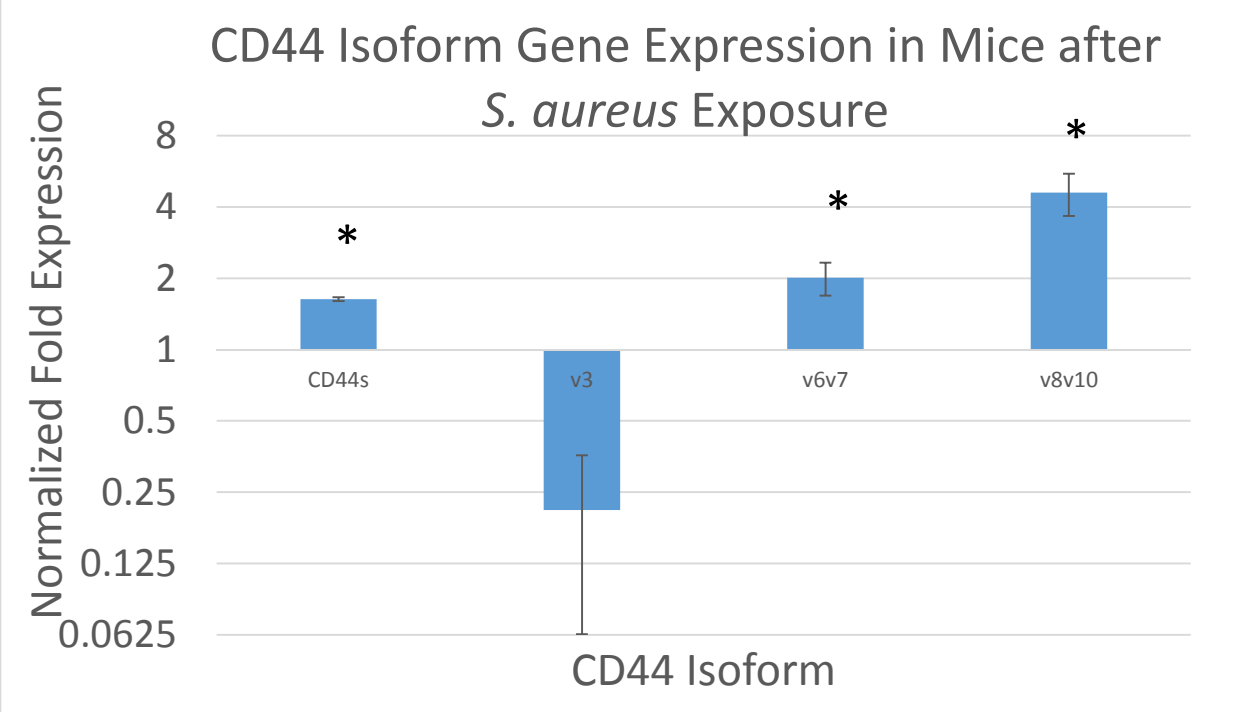


Figure 8: CD44v6v7 and CD44v8-v10 gene expression is elevated in PBMC's isolated from bacteremic patients

Blood was isolated from bacteremic patients with presumptive *S. aureus* infections. CD44v6v7 and CD44v8-v10 mRNA levels were determined by real-time RT-PCR. Shown is the relative fold expression of the isoform mRNA for each individual patient and on average when compared to healthy donors.

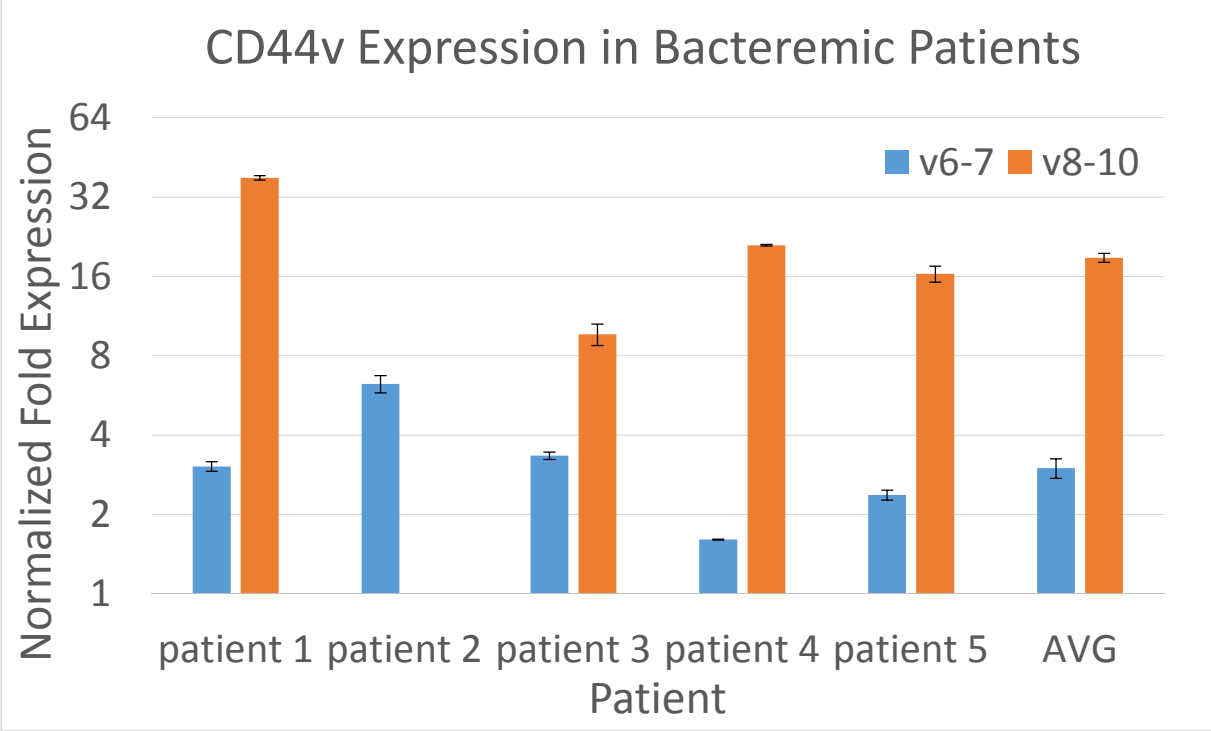


Figure 9: CD44KO mice have a reduced inflammatory response to *S. aureus*.
WT and CD44KO mice were exposed to 6 x 10^8 CFU/mL of *S. aureus* intranasally. 24 hours later, the lungs were harvested and homogenized. Cytokine mRNA expression was determined by real-time RT-PCR. Shown is the relative fold expression of pro-inflammatory cytokine mRNA for IL-1, IL-6, IFN-γ, and TNF-α of WT and CD44KO mice when compared to their respective control mice groups.

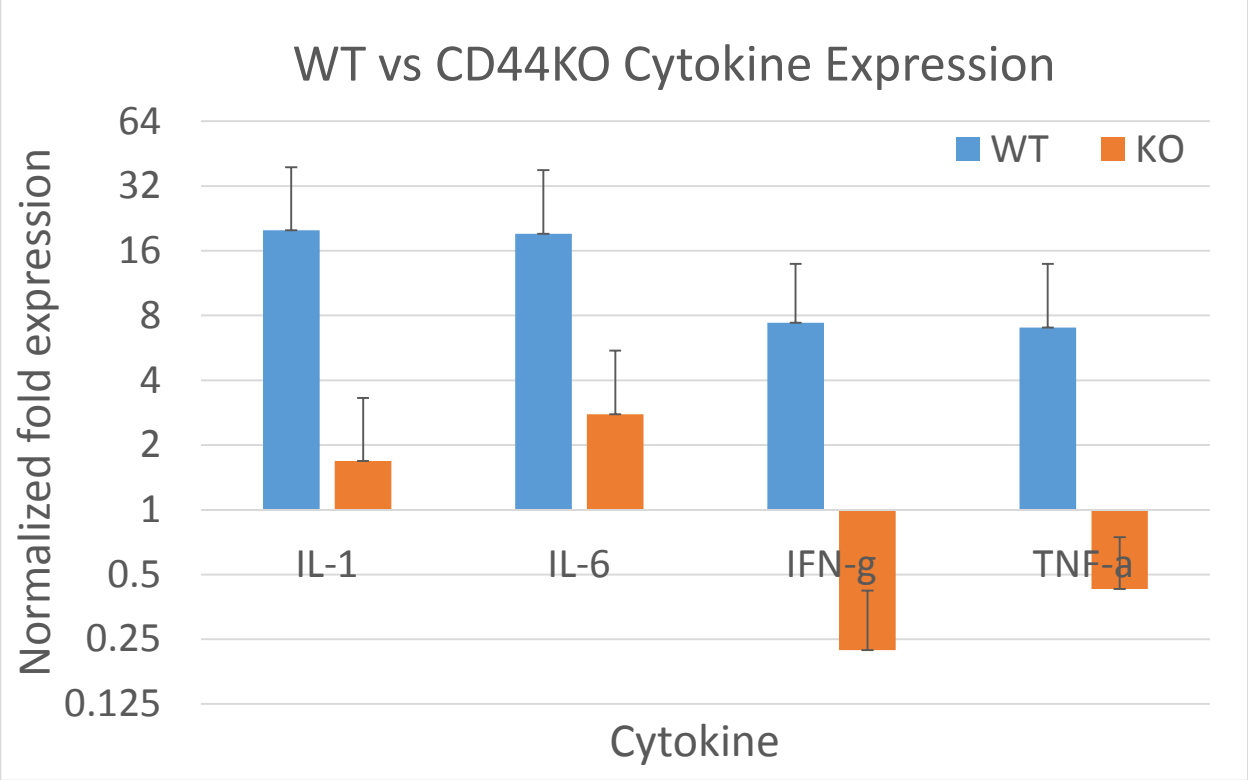


Figure 10: CD44KO mice have a reduced number of immune cells in the lungs after *S. aureus* infection
WT and CD44KO mice were exposed to 9 x 10^8 CFU/mL of *S. aureus* intranasally. The lungs were harvested 24 hours later, and the mononuclear cells were isolated with Ficoll density gradient centrifugation. The cells were counted then stained with fluorescently-labeled antibodies and analyzed using flow cytometry to get cell number of each individual population. The total cell number for each subset was determined using the following format: Subset cell number= total cell number X % positive for phenotypic markers.

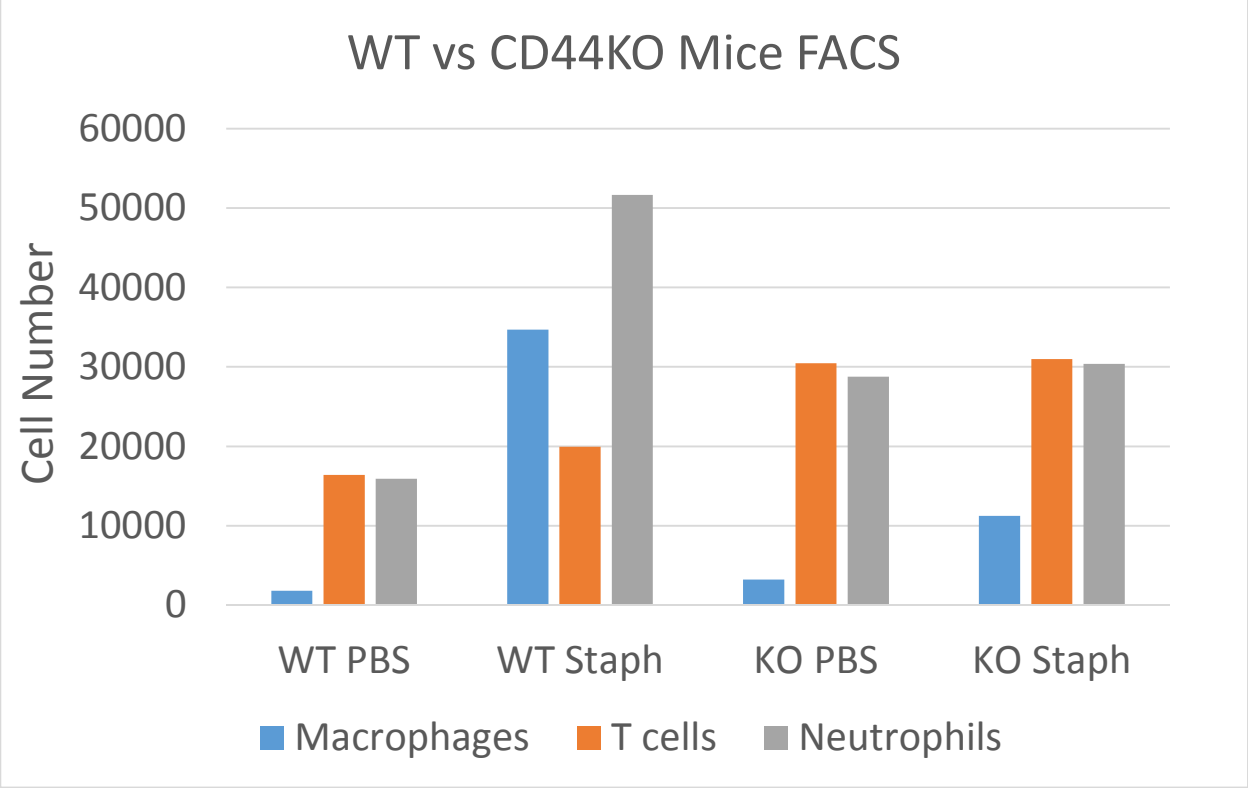
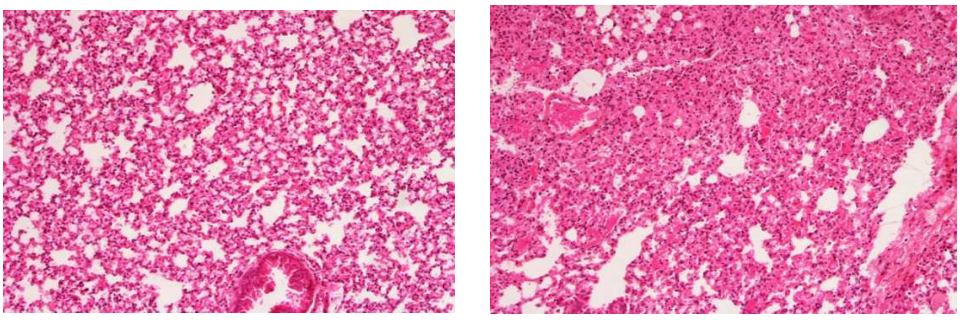
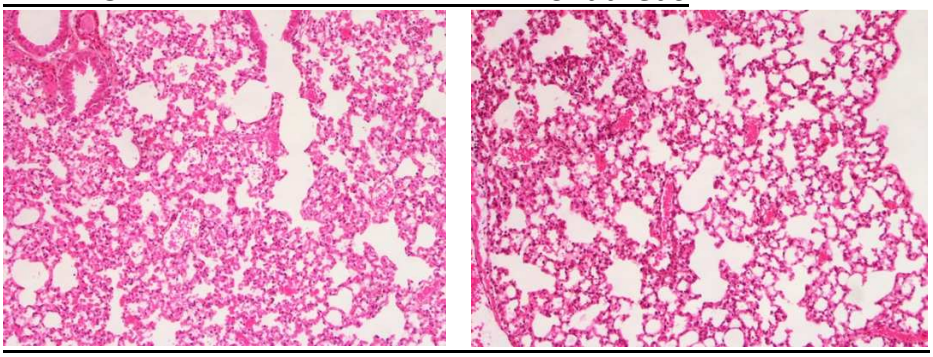


Figure 11: CD44KO mice have a reduced number of immune cells in the lungs after *S. aureus* exposure
WT and CD44KO mice were exposed intranasally to 9 x 10^8 CFU/mL of *S. aureus*. 24 hours later, the lungs were harvested and placed in formalin. H&E staining was performed.

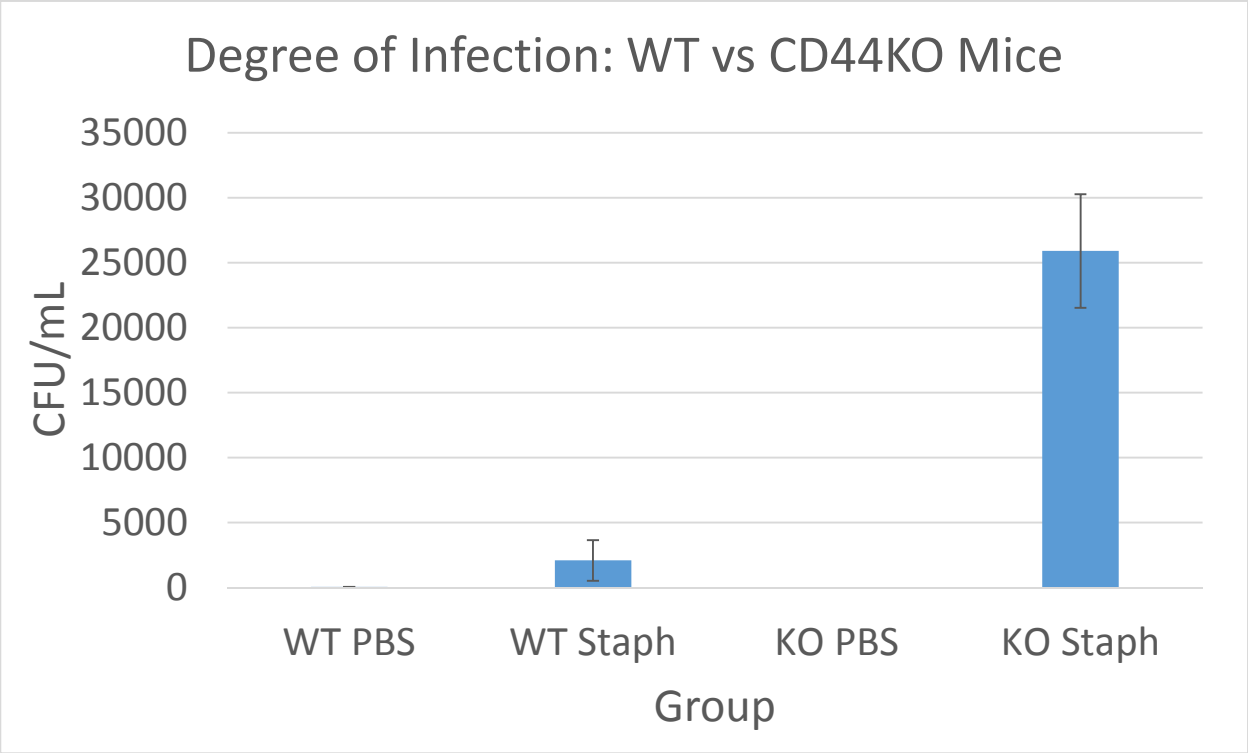


WT PBS WT *S. aureus*



CD44KO PBS CD44KO *S. aureus*

Figure 12: CD44KO mice have and increased bacterial load in the lungs after exposure to *S. aureus*
WT and CD44KO mice were exposed to 3.67 x 10^8 CFU/mL of *S. aureus* intranasally. After 24 hours, the lungs were harvested, homogenized, and plated on TSA plates. Colonies of *S. aureus* were counted.



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