Role of Different Adjuvants on Outcome of *Staphylococcus aureus* Bacteremia in Mice Following Immunization with a Whole Cell or Toxoid Vaccine.

By

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B.S. Biochemistry and Molecular Biology (Gettysburg College) 2016

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

In

BIOMEDICAL SCIENCE

In the

GRADUATE SCHOOL

Of

HOOD COLLEGE

November 2021

Accepted:

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ABSTRACT

Despite the public health threat that *Staphylococcus aureus* (SA) poses, there is no available vaccine. Clinical trials testing vaccine candidates targeting SA surface antigens have had to be ended prematurely due to higher rates of detrimental effects in vaccinated groups when compared to placebo groups. Post-study analyses of both preclinical and clinical data show that high levels of IFNγ and low levels of IL-17a may be the main drivers of detrimental effects following surface antigen immunization. Here we show that in a mouse bacteremia model, detrimental effects following whole cell vaccination can be lessened depending on the adjuvant used with immunization. We also show that when immunizing with inert mutants of SA superantigens and pore-forming toxins, using certain adjuvants results in a decrease of disease severity by means of producing a stronger antibody response against superantigens as well as greater production of regulatory cytokines (IL-10, TNFα) known to be effective in combating SA.

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
BHI	Brain-Heart Infused
BSA	Bovine Serum Albumin
BSI	Blood Stream Infection
C3	Complement Component 3
CD	Cluster of Differentiation
CFU	Colony Forming Unit
ELISA	Enzyme-Linked Immunosorbent Assay
F_c	Fragment Crystallizable Region
FoxP3	Forkhead Box P3
Hla	Alpha-Toxin
HRP	Horseradish-Peroxidase
IFNγ	Interferon-Gamma
IgG	Immunoglobulin G
IL-2	Interleukin-2
IL-8	Interleukin-8
IL-17a	Interleukin-17a
IsdB	Iron-Regulated Surface Determinant Protein B
kGy	Kilo-Gray
LukAB	Leukocidin AB
LukED	Leukocidin ED
MHC II	Major Histocompatibility Complex Class II
NHP	Non-Human Primate

PE	Phycoerythrin
PE-Cy7	Phycoerythrin/Cyanine7
PFT	Pore-Forming Toxin
PVL	Panton Valentine Leukocidin
SA	*Staphylococcus aureus*
SAg	Superantigen
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEK	Staphylococcal enterotoxin K
SEQ	Staphylococcal enterotoxin Q
TH1	T-Cell Helper-1
TH2	T-Cell Helper-2
TH17	T-Cell Helper-17
TMB	3,3´,5,5´-tetramentylbenzidine
TNFα	Tumor Necrosis Factor-Alpha
TSST-1	Toxic Shock Syndrome Toxin-1

INTRODUCTION

Staphylococcus aureus (SA) is a leading cause of infectious morbidity and mortality around the world. SA is a Gram-positive bacterium belonging to the Firmicutes family and is a part of the natural skin flora in humans. Despite this relationship, as well as the fact that between 20% and 30% of the human population being long-term carriers, SA presents a global health threat (1,2). SA bloodstream infections (BSI) are among the most consequential SA infections with 10-20% treatment failure and mortality, with no vaccines or therapies currently available (1). SA's mortality is the result of its production of both superantigens (SAgs) and pore-forming toxins (PFTs) (2). SAgs like staphylococcal enterotoxins A and B (SEA, SEB) and Toxic Shock syndrome toxin (TSST-1) crosslink the Vβ chain of T cells receptors, resulting in antigen-independent stimulation of T cells, ultimately leading to a cytokine storm (2, 17). The main purpose of PFTs like alpha-toxin (Hla), Panton Valentine Leukocidin (PVL), Leukocidin AB (LukAB), and LukED is to incapacitate the host defenses by compromising the cell membrane of immune cells by both forming trans-membrane pores as well as opening already existing Ca^{2+} channels (2, 16).

SA has been observed to be able to evade the innate immune system by secreting proteases that cleave innate immune molecules, such as CD31, a member of the immunoglobulin family necessary for the signaling pathway that discourages predatory macrophages, resulting in dampening neutrophil activity, and C3, a complement component leading to compromised bacterial opsonization (3). In addition, SA also manages to disrupt the extracellular and intracellular matrices of tissues by secreting superantigen-like proteins that prevent the cleaveage of IL-8, which is required for neutrophil chemotaxis (3). Other methods of immune invasion include non-opsonic binding of immunoglobulins by means of Protein A, which binds non-specifically to the F_c region of IgG antibodies (4). SA also evades the immune system via the impairment of phagocyte recruitment by means of secreting Chemotaxis Inhibitory Proteins (CHIPs), and the lysis of neutrophils using PFTs such as Hla, PVL, LukAB and LukED (4). All vaccines and immunotherapies developed to target SA surface antigens have failed to protect against invasive SA

infections in human trials. Recently, a clinical trial of an active vaccine targeting the surface-associated iron-scavenger protein IsdB had to be terminated prematurely due to a significantly higher incidence in multiorgan failure and death in the vaccinated group who developed a SA infection following cardiothoracic surgery (5,6). A post-hoc analysis indicated that serum levels of certain CD4 T cell-derived cytokines (low levels of IL-2 and IL-17A) at the time of vaccination might have predisposed the patients to this catastrophic outcome (7).

A further study immunized C57BL/6J mice with lethally irradiated MRSA, followed by an intravenous challenge with SA USA300 four weeks later. The results from this study corroborated what was seen in the aforementioned clinical trial using the IsdB vaccine: priming the immune system with inactivated MRSA, used as a multiepitope vaccine resulted in a worse clinical outcome than the control (7). In addition, low levels of T cell derived IL-17a were seen in mice immunized with whole cell MRSA, indicating that the whole cell vaccine (WCV) did not induce a strong T_H17 response. At the same time, high levels of T cell derived Interferon γ (IFNγ) were seen in mice that were immunized with WCV, indicating that this poor outcome was the result of a T_H1-dependent immune response (8). It has been theorized that by generating a T_H17 rather than aT_H1 response, a more desirable clinical outcome could be achieved (9). Previous preclinical studies indicate that immunizing with non-toxic mutants of SAgs secreted by SA may result in a favorable outcome (10). These toxins play an important role in the pathogenesis of SA and are primarily responsible for clinical signs of disease progression in SA infection. Toxins of particular interest are PFTs such as alpha hemolysin (Hla) and Panton-Valentine leucocidin (PVL), and SAgs (comprising of staphylococcal enterotoxins including SEA, SEB, SEC, SED, SEK, SEQ and many others). Cytolytic toxin functions appear to be mainly related to immune subversion and tissue destruction, while the superantigenic toxins cause non-specific polyclonal activation of T cells which leads to the dysregulation of the immune system, which can ultimately prove fatal (2, 11 ,12). Since using surface antigens as vaccine targets, such as in the case of the previously mentioned IsdB vaccine as well as the failed StaphVax vaccine

(which produced no differences between the vaccinated and placebo groups), has not been successful, as seen in the clinical trial mentioned previously, it is possible that vaccines that target toxins produced by SA would have a more favorable clinical outcome (5,6, 18). Recently, a multicomponent toxoid vaccine targeting three PFTs and three SAgs was shown to be highly effective in protecting against primary and secondary skin infections caused by SA (13). However, it is not yet clear as to whether a similar toxoid-based vaccine would provide the same protection during a systemic infection.

If the detrimental response seen following whole cell or surface antigen immunization is the result of priming the TH1 response, it is possible to diminish any adverse outcomes by shifting the immune response in a different direction. Adjuvants are typically used in vaccines to increase and/or modulate the resulting immune response (14). As mentioned previously, poor outcomes in previous experiments in mice were found to be related to high levels of IFNγ, suggesting that driving the response towards a TH17 response might prevent a detrimental outcome (8). Otherwise, it could show that the harmful effects of SA whole cell immunization are inherent to surface antigens used. IFNγ is a cytokine associated with the TH1 response, the result of which is the activation of the cellular immune response and the activation of macrophages and monocytes, while IL-17a is associated with the TH17 response, the point of which is to recruit neutrophils to the site of infection (15).

We will evaluate the differences in the immune response when vaccinating with a toxoid vaccine versus a WCV. We will investigate if and how harmful effects following whole cell vaccination seen in previous experiments can be mitigated when polarizing the immune response away from a TH1 response and towards a TH17 response by using of different adjuvants.

MATERIALS AND METHODS

Model and Infectious Disease in Study

C57BL/6 mice were ordered from Jackson Labs in Bar Harbor, Maine. CD1 mice were ordered from Envigo in Frederick, Maryland. Six-week old female mice were used for active vaccination studies. Fourteen-week old mice were used in experiments where mice receive infection only. *Staphylococcus aureus* strain USA300 (SF8300) was used for both infections, while lethally irradiated SA USA300 (SF8300) referred to as WCV was used for immunizations.

Wild type SF8300 was grown in a liquid TSB culture overnight with shaking (240 rpm, 37° C), washed the following day, and then frozen in freezing medium (10% glycerol in 1X PBS). Aliquots were frozen at -80° C and back titers were done to establish the CFU/mL of the SF8300 stocks. Additional aliquots of SA USA300 were lethally irradiated (30 KGy) by the Integrated Research Facility at Fort Detrick, Maryland. Inactivation of SA USA300 was done by gamma-irradiation rather than other means to better conserve the structure of SA surface antigens.

Once the concentration of the SA USA300 stock was determined, groups of female C57BL/6 mice were challenged with varying infective doses of bacteria. Naïve mice were used as controls. The mice were monitored for a period of two weeks for weight loss and survival. After the two weeks ended, the survival rates of the different groups were analyzed to determine which doses would serve best for lethality and sub-lethality for future experiments.

Evaluating Differences Between Whole Cell Vaccination versus Toxoid Vaccination

To establish the differences between a whole cell vaccine and toxoid vaccine in a bacteremia model, one group of female C57BL/6 mice (n=10) were vaccinated with a toxoid cocktail containing mutants of seven purified toxins (10 µg each) secreted by SA (Hla toxoid, LukS toxoid, LukF toxoid, SEA toxoid, SEB toxoid,

TSST-1 toxoid and LukAB toxoid). Two groups (n=10) of female C57BL/6 mice were vaccinated with different doses of lethally-irradiated SA USA300, and a final group (n=10) was vaccinated with BSA. Vaccinations occured three times, two weeks apart each. Two weeks following the final immunization, serum was collected from the mice. Four weeks following the final vaccination, each group was split in half, with one half being challenged with a lower dose of SA USA300, and the remaining half challenged with a higher, but still sublethal, dose. The mice receiving the lower dose of SA USA300 were monitored and terminated on day 7 following challenge and harvested for spleens, lymph nodes, kidneys, serum and whole blood. The mice receiving the higher dose were monitored for weight loss and overall health and terminated on day 14 after challenge. Spleens and pooled lymph nodes were analyzed using flow cytometry, while the serum was used for IgG detection assays and toxin neutralization assays. Whole Blood and kidneys from terminated mice were used to count CFU.

Evaluating Role of Humoral Immunity on Outcome of Infection

To determine the role of the humoral immune response in USA300 infection, one group of female C57BL/6 mice (n=15) was vaccinated with a toxoid cocktail, a second (n=15) was immunized with wild type SA USA300, a third (n=15) with SA USA300 mutant lacking Protein A, and a final group (n=15) was immunized with BSA. All vaccine components were adjuvated in $Al(OH_3)$. A total of three immunizations occured, each two weeks apart.

Fourteen days after the final immunization, mice were be bled and serum separated and pooled per group. Sera was transferred into naïve female C57BL/6 mice and infected with SA USA300 four hours later. The mice were monitored for weight loss and overall health for 14 days.

Evaluating Effects of Different Adjuvants on CD4 T-Cell Response

To observe whether polarizing the immune response towards TH17 alters poor outcomes following whole cell immunization, mice were vaccinated with a toxoid cocktail adjuvated in either Alhydrogel ($Al(OH)_3$)

(Group 1), QS21 (Group 2) or Freund's Adjuvant (Group 3) or immunized with lethally irradiated SA USA300 formulated in $Al(OH)_3$ (Group4), QS21 (Group 5), or Freund's Adjuvant (Group 6). A fourth group was immunized with lethally irradiated SA USA300 without adjuvant and a control group for each adjuvant received an immunization with adjuvant only. All mice were immunized three times, two weeks apart each, with serum collected from each mouse two weeks after the final immunization. Four weeks after the final immunization, the mice were challenged with live SA USA300.

Following infection, the mice were monitored for 7 days for weight loss, then terminated. The spleens and pooled lymph nodes of these mice were processed, stained, and analyzed on a BD FACSymphony. Whole blood from the mice was streaked out on BHI plates for CFU counts. The serum from a test bleed occurring after the third vaccination as well as from termination was be analyzed for total and neutralizing IgG titers against each wild type toxin that made up the toxoid cocktail previously mentioned.

Phenotypic and functional characterization of CD4 T cells by Flow Cytometry

All flow cytometry was done using an optimized panel to characterize CD4 T cell responses in mice. The following gating strategy was utilized: exclusion of dead cells -> exclusion of doublets -> gating on CD45+ events -> gating on lymphocytes -> gating on CD4+ events -> gating on FoxP3- events -> gating on $CD44^{medium/high}$ and Ki-67+ events. The resulting events were evaluated for intracellular cytokine expression. At termination, spleen and pooled lymph nodes were processed into single cell suspensions. The cells were incubated with CD4-BUV805 (BD Biosciences, 612900), CD8-BV480 (BD Biosciences, 566096), CD44-BB515 (BD Biosciences, 564587), CD45-AF700 (eBioscience, 56-0451-82), and CD62L-BUV396 (BD Biosciences, 640218) at 4° C for 30 minutes, following by staining with LIVE/DEAD Near-IR (ThermoFisher, L10119) at 4° C for 15 minutes. The cells were fixed overnight using Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, 00-5523-00), followed by intracellular staining with FoxP3-eFluor450 (Invitrogen, 48-5773-82), IL-4-BV605 (BD Biosciences, 564004), IL-10-BV711 (BD Biosciences,

564081), IL-13-PE (Invitrogen, 12-7133-82), IL-17a-PE/Dazzle594 (BioLegend, 506937), IL-22-APC (Invitrogen, 17-7222-82), IFN_{γ} -BUV737 (BD Biosciences, 612769), Ki-67-PerCP-Cy5.5 (BD Biosciences, 561284), and TNF_{α}-PE-Cy7 (Invitrogen, 25-7321-81) at 4° C for 30 minutes. The stained cells were acquired on a BD FACSymphony A3 (800,000 events/sample), and the resulting data analyzed using FlowJo 10.

Luminex

IgG titers were determined by a multiplex assay using the Luminex (LX200) platform. Wildtype target antigens (Hla, LukS-PV, LukF-PV, LukAB, SEA, SEB, and TSST-1) were coupled to carboxylated MagPlex microsphere beads with distinct spectral regions via a carbodiimide reaction. Antigen-coupled beads were incubated with serum samples at a starting dilution of 1:40 in a two-fold 8-point dilution series at room temperature (RT) for 2 hours. Samples were washed and incubated with a PE-conjugated goat anti-mouse IgG antibody (Biolegend, San Diego, CA.) for 1 hour at RT. The samples were washed and acquired using a Luminex200. Data were analyzed using a 4-parameter (4PL) curve fit in XLFit (Microsoft). IgG titers were expressed as the effective dilution at the point of the 4PL curve where 50% (ED50) of antigen was detected by toxin-specific antibodies present in the serum sample.

Whole Cell ELISA

Whole cell ELISAs were used to evaluate anti-SA IgG levels in mouse serum. In brief, plates were coated with $1x10^7$ *S. aureus* (NE296) lacking Protein A (Spa) and incubated at 37° C for one hour. The plates were washed, blocked for an hour at room temperature with StartingBlock (PBS) Blocking Buffer (ThermoFisher), washed and diluted serum samples were added and incubated at room temperature for one hour, followed by a wash and incubation with an anti-mouse IgG antibody conjugated to Horseradish Peroxidase at room temperature for one hour in the dark. After another wash, the plates were developed with TMB Ultra (Thermofisher), and then read at OD_{650}. IgG titers were expressed as the effective dilution

at the point where 50% of antigen is detected by the mouse-IgG specific secondary antibody, calculated by Graphpad/Prism version 9.

SEK and SEQ ELISAs

ELISAs were used to evaluate anti-SEK and anti-SEQ IgG levels in mouse serum. Plates were coated with either a 3 μg/mL solution of either SEK of SEQ and incubated at 37° C for one hour, then washed and blocked for an hour at room temperature with StartingBlock (PBS) Blocking Buffer (ThermoFisher). The plates were washed and diluted samples were added and incubator at room temperature for one hour, followed by a wash and incubation with an anti-mouse IgG antibody conjugated to Horseradish Peroxidase at room temperature for one hour in the dark. After another wash, the plates were developed with TMB Ultra (ThermoFisher) and read at OD_{650}. IgG titers were expressed as the effective dilution at the point where 50% of antigen is detected by the mouse-IgG specific secondary antibody, calculate by Graphpad/Prism version 9.

Toxin Neutralization Assay

Serum samples were used to determine the extent to which neutralizing antibodies against alpha-hemolysin and SAgs (SEA, SEB and TSST-1) are produced. In brief, samples were diluted and incubated with diluted rabbit red blood cells (RBCs) in a 96-well plate for 30 minutes at 37° C. The plates were centrifuged at 3500 rpm at 20° C for three minutes, then 100 μL of the supernatant was transferred to a fresh plate and read on a SpectraMax at OD_{416}. Resulting data were analyzed using a 4-parameter (4PL) curve fit in XLFit (Microsoft). Toxin neutralizing activity was defined as the effective dilution of sera at the point of the 4PL curve at which 50% of toxin activity is neutralized.

CFU Counts

To determine bacterial load in various organs, following termination, whole blood and homogenized kidney, liver, lung, and spleen were plated at various dilutions on Brain Heart Infused Agar Plates (Teknova) to determine CFU counts.

Organs were placed in 2 mL tissue homogenizing tubes (FisherScientific) upon harvest and stored at -20°C until they are enumerated. After thawing, 500 µL 1X PBS were added to each tube. Kidneys, lungs, and spleens were homogenized using a Precellys Evolution (Bertin Technologies) at 5800 rpm for 30 seconds twice, with a five second pause in between. Liver was homogenized using the same instrument, but at 6500 rpm for 30 seconds twice, with a five second pause in between.

Statistics

Survival curves were generated for each experiment in which mice are infected with SA and monitored, as well as graphs displaying changes in weight following challenge. CFU counts were calculated as the total CFU per organ.

When analyzing differences in cytokine expression, CFU counts, total IgG titers and neutralizing IgG titers, two-way ANOVA and t-test were performed using GraphPad PRISM. Statistical differences in survival were determined by using Log-Rank Mantel-Cox tests in GraphPad PRISM.

Results

Natural Course of USA300 Infection

Infection with a sublethal dose of USA300 resulted in weight loss and noticeable amounts of bacteria in organs as well as observable IgG titers against several toxoids. Most groups experienced significant weight loss (5-10%) following infection. One group (D14) at some points over a two-week interval managed to gain weight, albeit much less than the uninfected control (Figure 1). CFU counts from homogenized organs show discernible amounts of bacteria in each organ, however by Day 14 only the lungs showed high levels of USA300 (Figure 2).

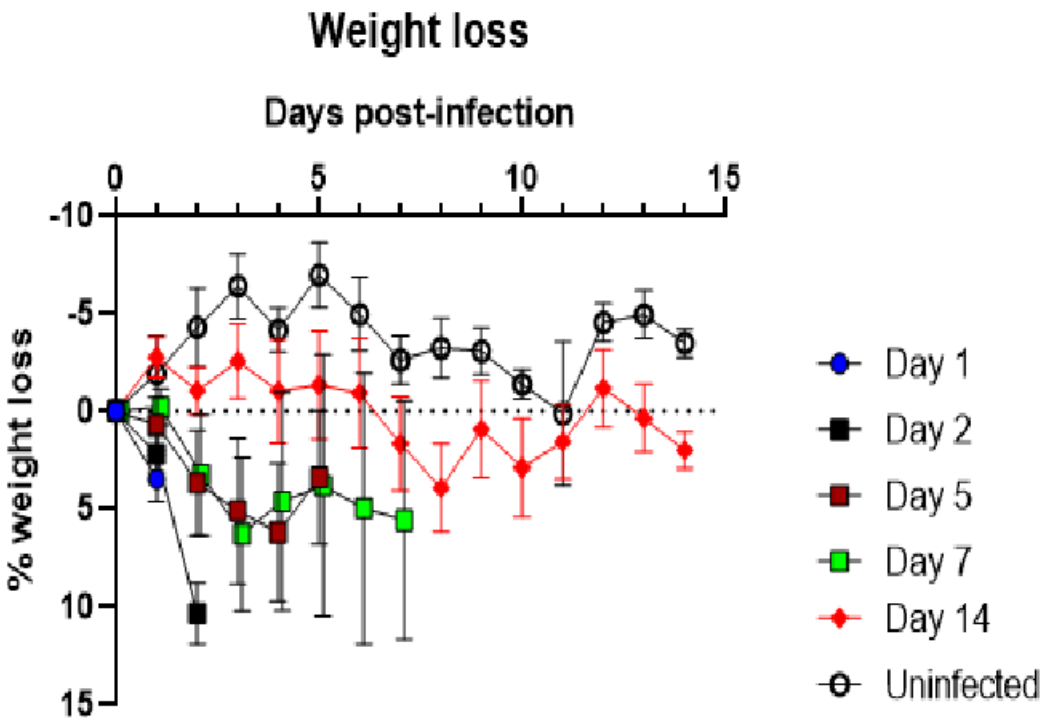


Figure 1. A. Weight loss in C57BL/6 mice following challenge with a sublethal dose of USA300.

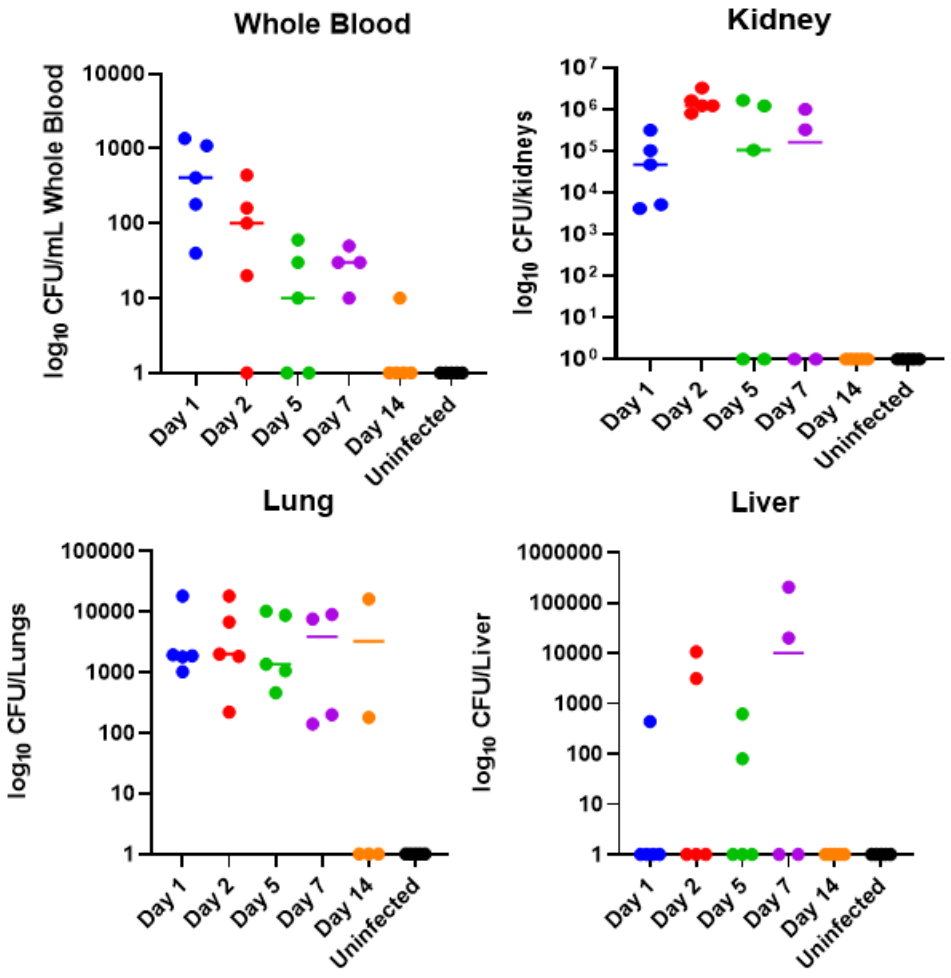


Figure 2. CFU counts in C57BL/6 mouse tissues at various time points following USA300 challenge.

Whole Cell ELISAs demonstrate that IgG titers against SA increase with time post-infection (Figure 3A). IgG titers for almost all tested USA300 toxoids increased with time, but no neutralizing titers were observed at any timepoint (Figures 3B, 3C).

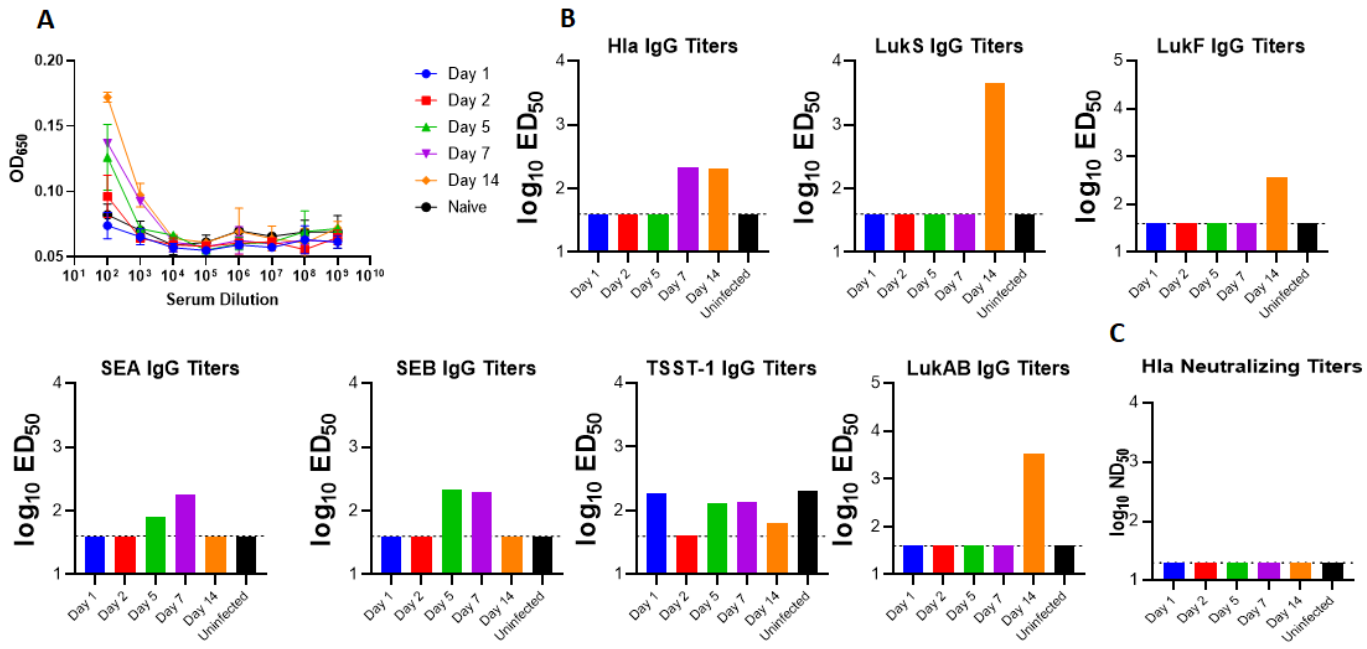


Figure 3. IgG Titers Against Toxoids and Whole Cell USA300 Following Sublethal Infection A. OD_{650} data in pooled C57BL/6 mouse serum at various time points following USA300 challenge. **B.** Total IgG titers in pooled C57BL/6 mouse serum against various wild type SAgs and PFTs at various time points following USA300 challenge. **C.** Neutralizing antibody titers against various wild type SAgs and PFTs at various time points following USA300 challenge in C57BL/6 mice.

Effects of Toxoid Vaccine versus Whole Cell Vaccine on Survival

Type of vaccination has little effect on outcome of USA300 infection. Naïve mice were immunized with either a toxoid cocktail, three different concentrations of lethally irradiated USA300 (1E7, 1E6, 1E5 CFU/mL), or BSA. One month post-immunization, the mice were split into two groups, one receiving a lethal dose (8E5 CFU/mL) of USA300 and the other receiving a sublethal dose (1E5 CFU/mL) of USA300. No statistically significant difference was observed in survival rates or weight loss in either the lethal or sublethal dose (Figures 4A, 4B).

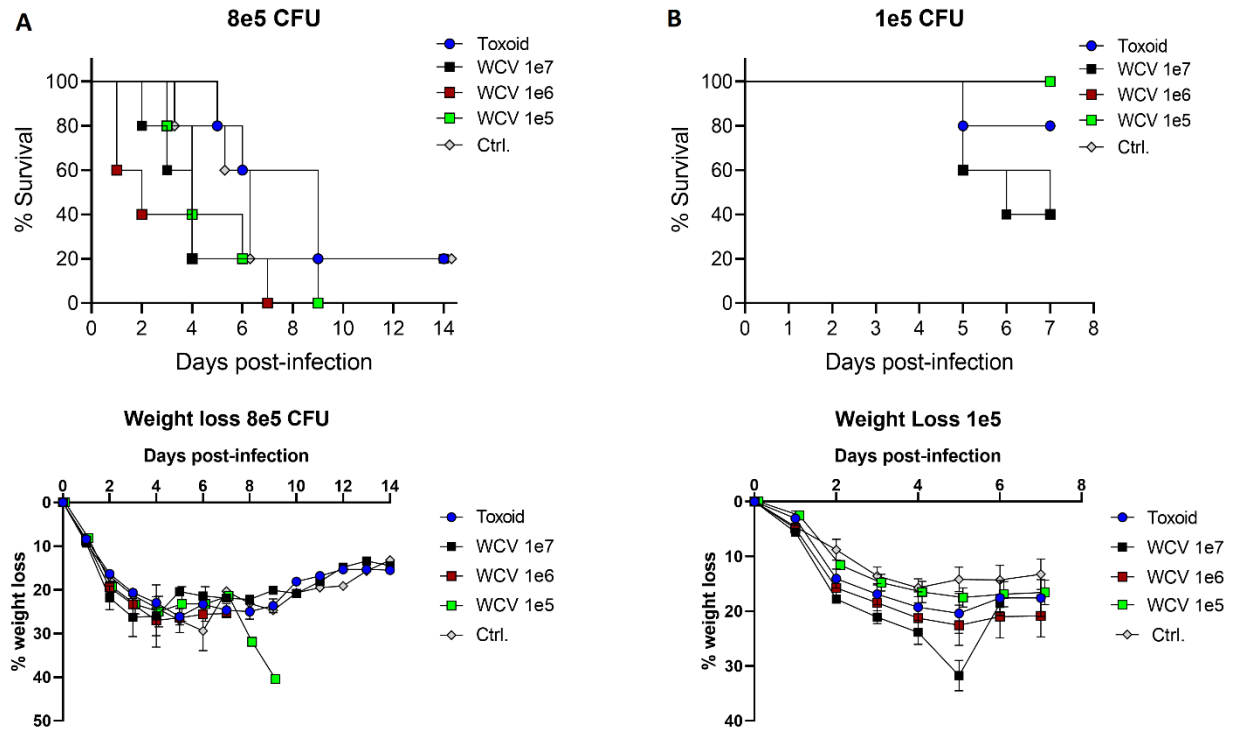
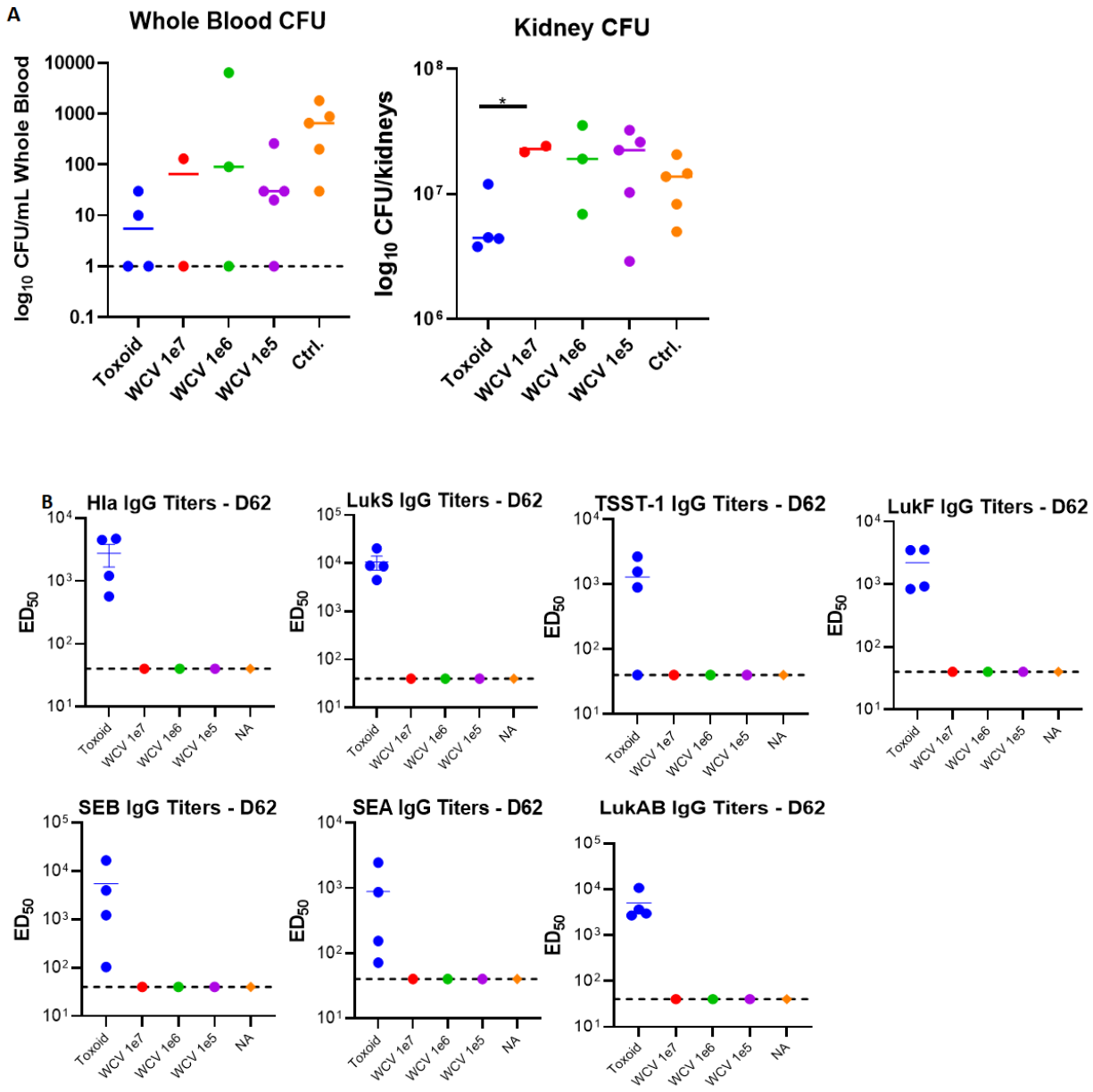


Figure 4. Survival and Weight Loss in Mice Following Immunization and Infection with USA300 A. Survival in mice immunized with either a toxoid vaccine, lethally irradiated USA300 or BSA following infection with a lethal or sublethal dose of USA300. **B.** weight loss in mice immunized with either a toxoid vaccine, lethally irradiated USA300 or BSA following infection with a lethal or sublethal dose of USA300.

Effects of Toxoid Vaccine versus Whole Cell Vaccine on Immunogenicity

Following three immunizations with either a toxoid vaccine, lethally irradiated USA300 or a mock vaccine and challenge with a sublethal dose of live USA300, whole blood and kidneys were collected from each mouse and enumerated to determine bacterial burden. While mice that received the toxoid vaccine had observably less CFU counts in the whole blood, it was not significant. However, mice that received the toxoid vaccine had significantly less bacteria in their kidneys than mice that were vaccinated with the highest dose of lethally irradiated bacteria ($p<0.05$). The differences between the other groups were not significant (Figure 5A.) When analyzed for IgG titers, it was seen that the group immunized with a toxoid vaccine developed higher titers against the wild type of each toxin, while the groups immunized with lethally irradiated USA300 did not (Figure 5B).

Spleens from each surviving mouse were processed into a single cell suspension and stained for flow cytometry. The collected events were gated to specifically look at CD4+ effector cells ($CD44^{med/hi}$, $Ki\text{-}67^{+}$) (Figure 5C). No significant differences were seen in IL-17a of TNFα production upon restimulation with lethally irradiated USA300. It was determined that mice immunized with 1E5 and 1E6 CFU/mL lethally irradiated USA300 expressed significantly more ($p<0.05$, $p<0.01$ respectively) IFNγ than mice immunized with the toxoid vaccine (Figure 5D).



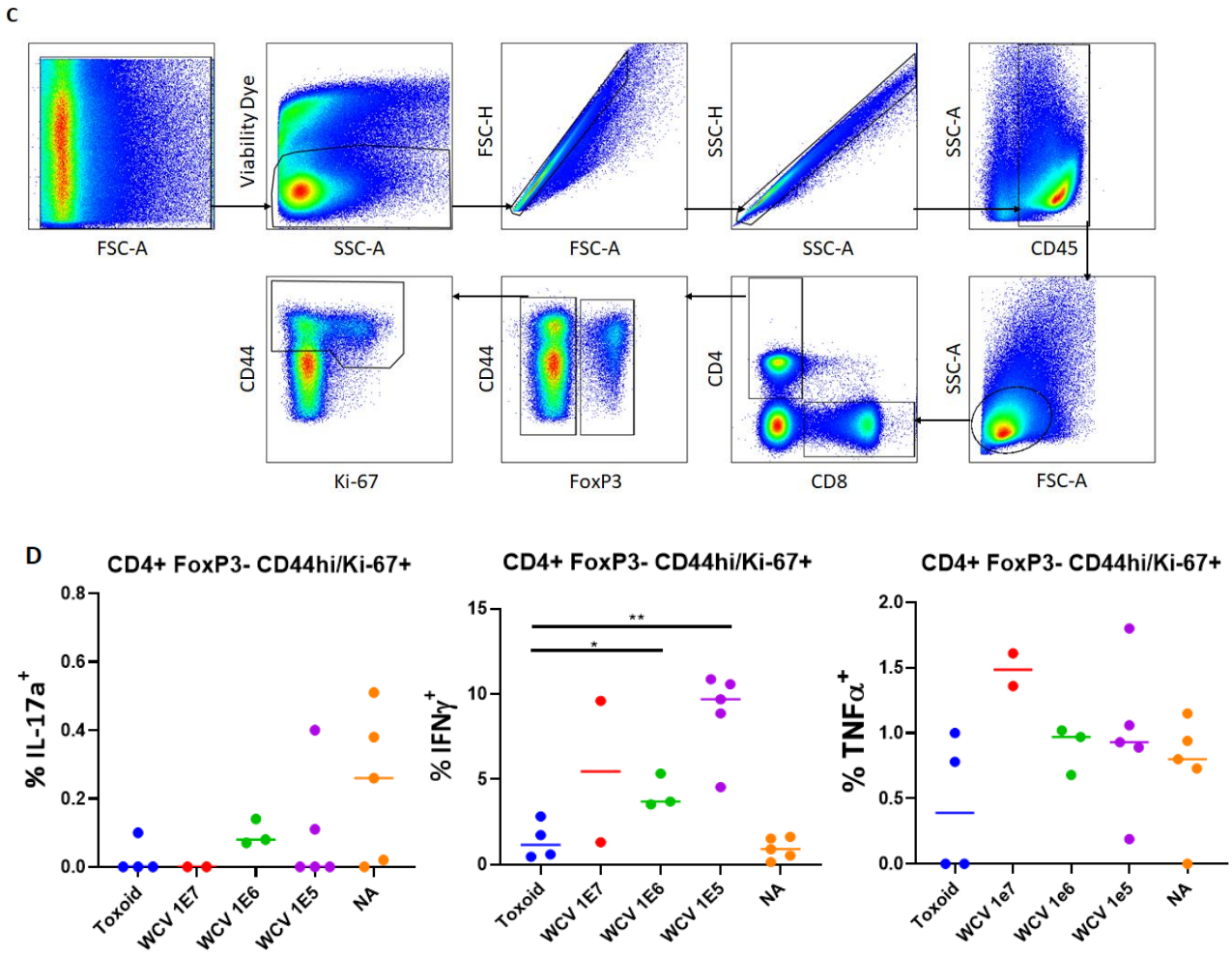


Figure 5. Immunogenicity of Both Whole Cell and Toxoid Vaccinations. A. CFU counts in whole blood and kidneys in mice immunized with either a toxoid vaccine, lethally irradiated USA300 or a mock vaccine two weeks following USA300 challenge. **B.** Total IgG titers against various SAgs and PFTs in C57BL/6 mouse serum following immunization and challenge with USA300. **C.** Gating strategy used to focus on effector cells (CD4+ CD44hi Ki-67+). **D.** Expression of IL-17a, IFNγ and TNFα in mouse splenocytes following restimulation with lethally irradiated USA300.

Role of Humoral Immunity on Outcome of Infection

Humoral immunity plays a negligible role in protection from USA300 infection. Serum was transferred from mice immunized with either a toxoid cocktail, lethally irradiated USA300, or BSA into naïve mice, who were subsequently infected with a lethal dose of USA300. Passive transfer of serum did not have a positive effect on outcome or on clinical signs of USA300 infection (Figures 6A, 6B). Serum from

immunized mice were tested for both overall IgG titers of several PFTs and SAgs, demonstrating that mice immunized with the toxoid cocktail showed high overall IgG titers against proteins of interest (Figure 6C) as well as high neutralizing antibody titers against Hla (Figure 6D). IgG titers against whole cell USA300 (both WT and ΔSpa) were examined in the serum from the immunized mice. Mice immunized with lethally irradiated USA300 showed higher titers against whole cell USA300 than mice immunized with the toxoid cocktail or BSA (Figure 6E). These data indicate that humoral immunity alone is not enough to control USA300 infection.

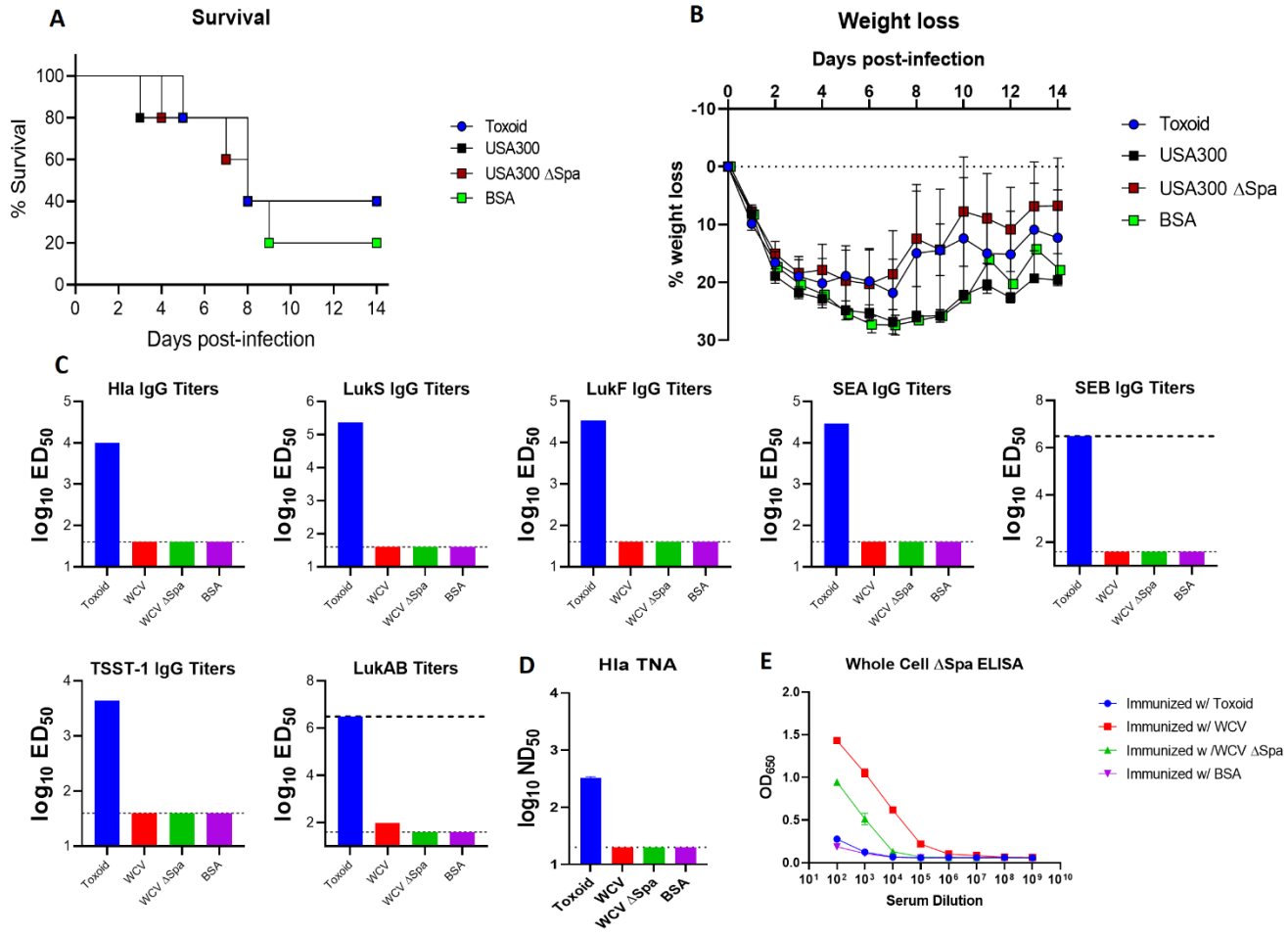


Figure 6. Effect of Passive Immunization on USA300 Disease Progression in C57BL/6 mice. A. Survival in passive immunized mice following challenge with a lethal dose of USA300. **B.** Weight loss in passively

immunized mice following challenge with a lethal dose of USA300. **C.** Overall IgG titers against several PFTs and SAgs in C57BL/6 mice immunized with a toxoid cocktail and with lethally irradiated USA300. **D.** Neutralizing IgG titers against Hemolysin-alpha in immunized C57BL/6 mice. **E.** IgG titers against whole cell USA300 (WT and ΔSpa) in immunized C57BL/6 mice.

Effects of Adjuvants on Disease Progression

The impact that adjuvants have on USA300 disease progression following immunization depends on the type of vaccine they accompany. Mice immunized with a toxoid vaccine adjuvanted in Alhydrogel lost significantly less weight than mice immunized with toxoid vaccines adjuvated in other substances ($p<0.05$) when compared to mice that received toxoid vaccines adjuvated in either QS21 or Freund's Adjuvant. Simultaneously, mice immunized with a whole cell vaccine adjuvated in Alhydrogel lost significantly more weight ($p<0.05$) than mice receiving a whole cell vaccine adjuvated in either QS21 or Freund's Adjuvant (Figure 7A). There was no difference in weight loss in mice receiving either vaccine adjuvated in either QS21 or Freund's Adjuvant.

Adjuvants had no observable effect on the bacterial burden observed in whole blood isolated from mice one week after USA300 infection. Mice immunized with lethally irradiated USA300 adjuvanted in Alum exhibited significantly higher levels ($p<0.05$) of USA300 than mice receiving the whole cell vaccine adjuvated in other substances (Figure 7B).

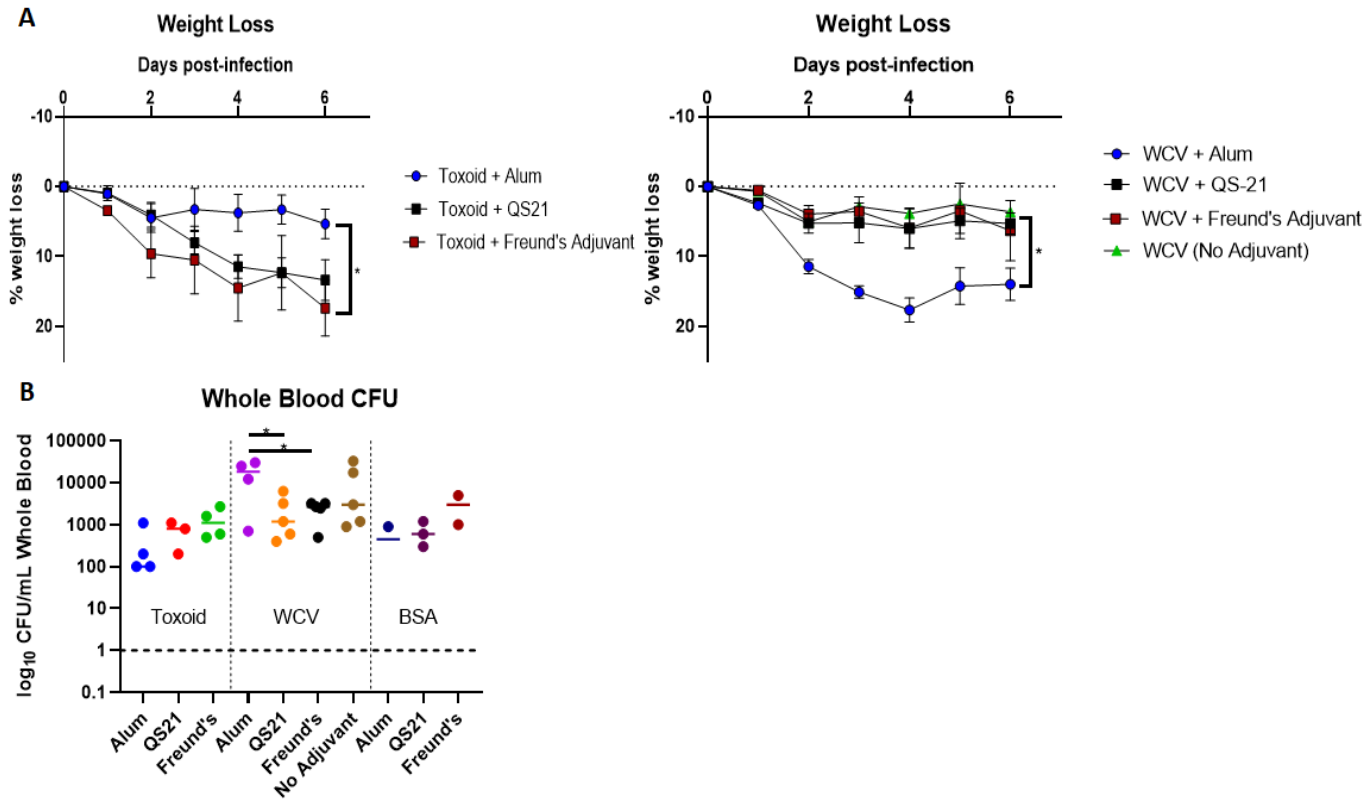


Figure 7. Disease Progression in Mice Immunized with a Toxoid Vaccine or Whole Cell Vaccine Adjuvated in Different Substances A. Weight loss in mice challenged with USA300 following immunization with either a toxoid vaccine or lethally irradiated USA300 adjuvated in different substances. **B.** CFU in immunized mouse whole blood following challenge with USA300.

Effects of Adjuvants on Immunogenicity

Mice immunized with the toxoid vaccine adjuvated in alum developed significantly higher titers ($p<0.05$) against SEA and SEB following challenge with USA300 (Figure 8). As USA300 does not produce SEA or SEB, the serum from mice that received the toxoid cocktail were tested for cross-reactivity against SEK and SEQ, and it was observed that mice receiving the toxoid cocktail adjuvant in Alhydrogel did not have higher SEK IgG titers, but had statistically higher IgG titers against SEQ when compared to mice receiving the toxoid vaccine adjuvanted in Freund's Adjuvant ($p<0.05$). While an observable difference was seen in anti-SEQ IgG titers between mice receiving the toxoid vaccine adjuvated in Alhydrogel or QS21, the difference was not determined to be statistically significant (Figure 9).

Mice immunized with lethally irradiated USA300 adjuvated in alum had noticeably lower IgG titers against SA surface antigens following three immunizations when compared to mice that received the same vaccine but with differing adjuvants. There were no differences in the same IgG titers in mice immunized with lethally irradiated USA300 in the time point following challenge and disease progression (Figure 10).

Higher levels of regulatory cytokines were observed in effector cells from mice that showed less severe signs of USA300 disease progression. T-regulatory cells (Tregs, CD4+ FoxP3+) had significantly higher rates of IL-10 production in mice that were immunized with lethally irradiated USA300 adjuvated in QS21 or Freund's Adjuvant than mice receiving the same vaccine adjuvanted in Alum upon restimulated with lethally irradiated USA300 ($p<0.05$). In addition, higher rates of IFNγ and TNFα were observed in groups of mice that exhibited less severe signs of USA300 disease progression ($p<0.05$). It was also noticed that mice immunized with BSA adjuvated in QS21 typically had high rates of intracellular cytokine production, regardless of the restimulation condition (Figure 11).

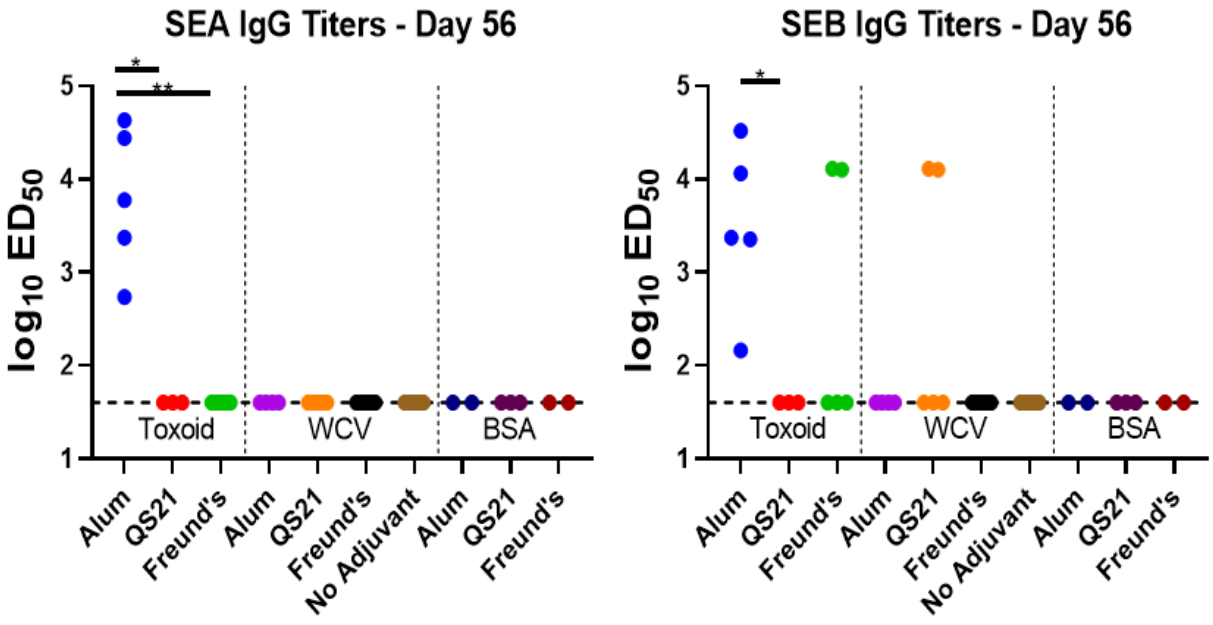


Figure 8. IgG titers against SEA and SEB in immunized C57BL/6 mice.

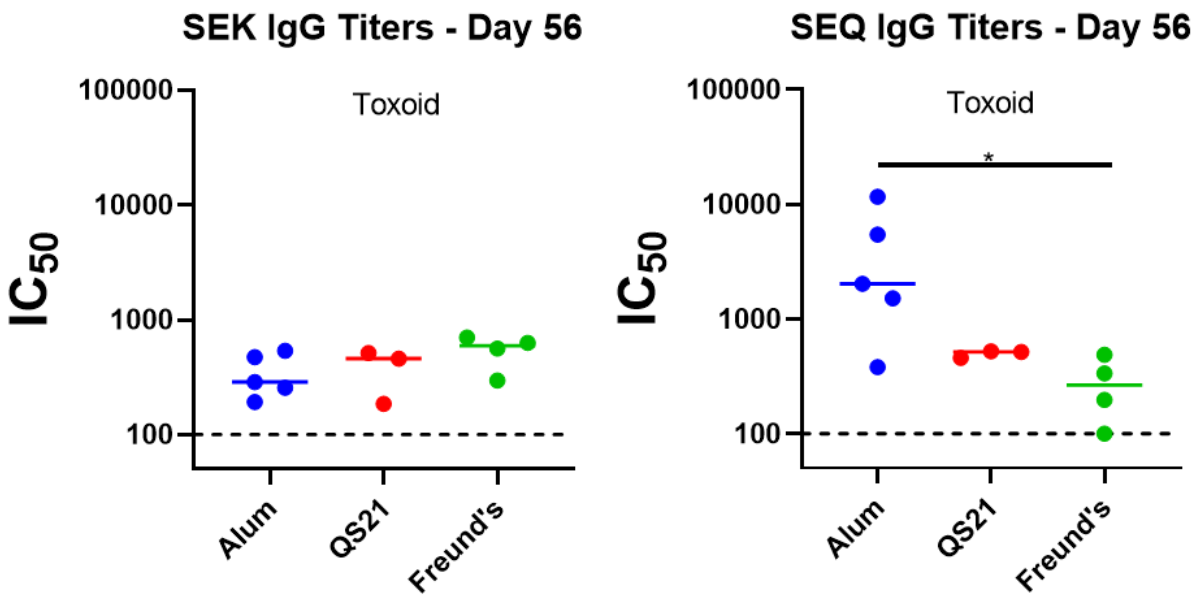


Figure 9. IgG titers against SEK and SEQ in immunized C57BL/6 mice.

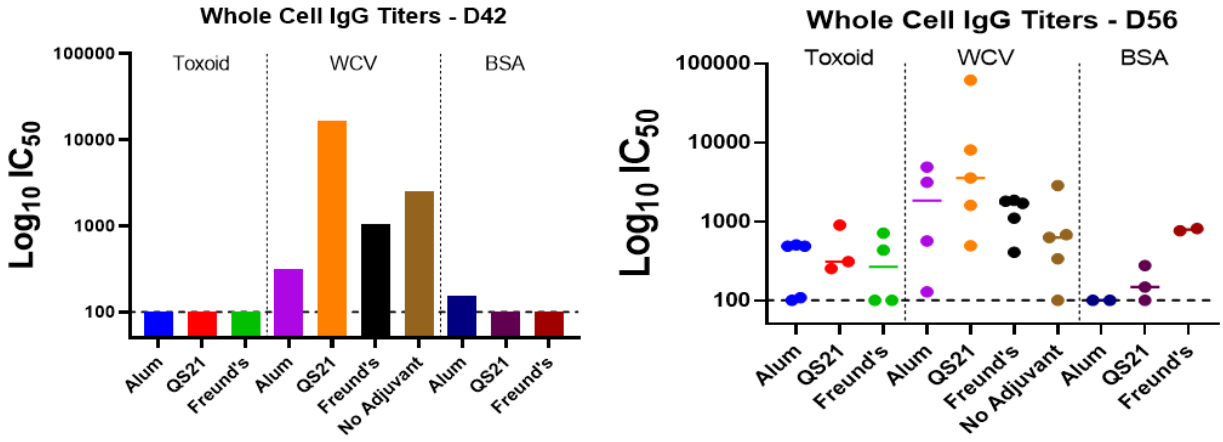


Figure 10. IgG titers against lethally irradiated USA300 in immunized C57BL/6 mice both prior to and after challenge with USA300.

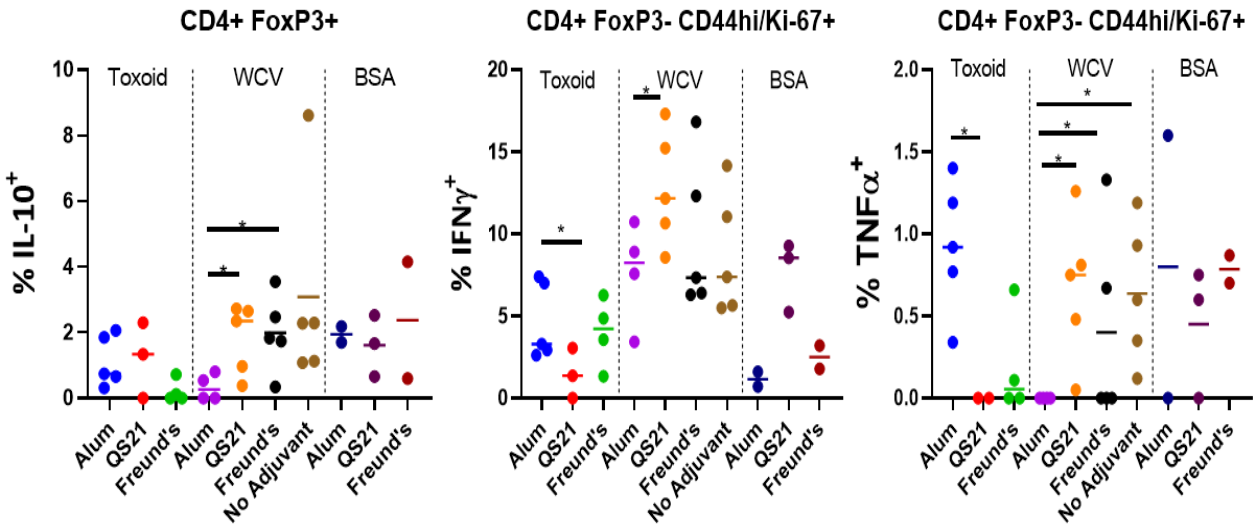


Figure 11. Expression of IL-10 in Tregs (CD4+ FoxP3+) and effector cells isolated from C57BL/6 (CD4+ CD44hi Ki-67+) mouse splenocytes.

Discussion

When comparing a whole cell vaccine to a toxoid vaccine in a mouse USA300 bacteremia model, very little effects can be seen in terms of overall survival or in signs of disease progression such as weight loss. However, this does not discount the potential benefits of multicomponent vaccines that specifically target SAgs and PFTs produced by SA, as other published literature support the benefits of such a vaccine in conferring some form of protection in both primary and secondary skin infections by SA (13). The data do support that a toxoid vaccine results in lower levels of IFNγ, which previous literature has implicated in being responsible for the detrimental effects observed when immunizing with a whole cell vaccine or with SA surface antigens (8). In addition, the data also showed that a toxoid vaccine could be more beneficial than immunization with surface antigens in terms of lessening the bacterial burden observed in the kidneys of infected mice. Consistent with previously published literature, the rates of IFNγ production was shown to be increased in mice immunized with lethally irradiated USA300, but survival rates and weight loss did not differ between groups, suggesting that simply lowering IFNγ levels may not be enough to produce the desired outcome.

The data demonstrate that adjuvants play a significant role in altering USA300 disease progression depending on the type of vaccine they accompany. Alum was observed to have the greatest effect, either significantly slowing USA300 disease progression when used in conjunction with a toxoid vaccine, or significantly contribute to the severity of disease. When assessing the immunogenicity of the adjuvants, immunizing with a toxoid vaccine adjuvated in Alum resulted in higher IgG titers against SA superantigens SEA and SEB. These antibodies were shown to have a high degree of cross-reactivity with superantigen SEQ, which unlike SEA and SEB, is expressed in USA300. The role that SEQ plays in USA300 infections is not as well described as are other superantigens, although it operates in the same method as other superantigens in cross-linking the Vβ chain of the TCR non-specifically to MHCs. Future studies should be

planned to investigate whether specifically targeting SEQ would provide any sort of protection against poor outcomes in USA300 infections.

The data suggest that adjuvating a whole cell vaccine with different substances results in differing levels of IgG specific to USA300 surface antigens, and that higher levels of anti-SA IgG may confer some protection. Pooled serum from WCV-immunized mice showed higher IgG titers when adjuvated in QS21 or Freund's Adjuvant following three immunizations, although following challenge with USA300 there appeared to be no differences in IgG titers against whole cell SA despite the substance the lethally irradiated USA300 was adjuvated in.

While high levels of antibodies that are cross-reactive to various SAgs may indeed play a role in more positive outcomes, the data resulting from passive transfer of serum from immunized mice show that alone it is not capable of controlling SA infection. A similar study immunized mice with Clumping Factor A (ClfA) adjuvated in c-di-GMP, an immunomodulator and immune enhancer which has been shown to stimulate a protective innate immune response. Data show that immunized mice had a significantly higher survival rate and higher levels of IgG2a, IgG2b and IgG3 compared to the controls (19). Further studies examining the role adjuvants play in vaccine-mediated immunogenicity should assess the differences in levels of IgG subtypes following immunization.

Higher levels of regulatory cytokines were observed in mice that received the toxoid vaccine adjuvated in Alum, as well as in mice immunized with lethally irradiated USA300 adjuvated in anything other than Alum. These data seem to correlate with the weight loss data in that the groups expressing higher levels of regulatory cytokines (IL-10, TNFα) showed much less weight loss than groups that expressed lower levels of the same cytokines. Other studies have implicated both these cytokines in a protective role in SA infection. One study found that patients with psoriasis and rheumatologic conditions that required them to go on immunosuppressive drugs that diminished TNFα were more likely to become colonized

with SA and thus more likely to develop complications as a result (20). Other studies show that IL-10, along PD-L1 antagonists may boost immunity against SA as well as other bacteria, and that dysregulated IL-10 and TNFα levels could be used as predictors for poor outcomes of SA bacteremia in patients (21, 22).

IL-17a levels were not observed to be significantly or observably different under any condition tested. Groups of mice still did better than others in terms of USA300 disease progression following challenge despite no apparent increase in a TH17 response. This does not invalidate our hypothesis or what has been seen in previous clinical and preclinical studies, rather it suggests that a TH17 response is not the only component necessary to be encouraged by immunization. Further studies should investigate the use of adjuvants other than Freund's Adjuvant which can stimulate a noticeable TH17 response, as well as the addition of adjuvants that are likely to be used in clinical studies. While Freund's Adjuvant has shown to elicit a strong TH17 response at times, it is highly immunoreactive and typically not advised for use in humans.

The data showing protection from a toxoid vaccine adjuvated in Alum corroborate what was seen in other preclinical studies. Another multicomponent SA vaccine that targeted four different antigens (Clumping Factor A, Capsular Polysaccharide Type 5 and Type 8, and Manganese Transporter Subunit C) that was adjuvated in $AlPO_4$ (similar to, but not the same as Alum) was shown to reduce bacterial burden in a mouse model of surgical site infection, as well as providing protection against USA300 challenge in a bacteremia model (23).

While some data did prove to be significant, further studies should use more mice per group to more clearly demonstrate variances between different immunization conditions and to discern differences that were not observed in these experiments. If possible, similar experiments should be conducted using non-human primates (NHPs) instead of mice. NHPs are colonized by SA just like humans, as opposed to murine models in which colonization by SA is highly irregular.

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