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UNIVERSITY OF CALIFORNIA SAN DIEGO

The group B *Streptococcus* virulence factor C5a peptidase role in complement cleavage and vaccine protection

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

Sinead McCabe

Committee in charge:

Professor Victor Nizet, Chair
Professor Fabian Rivera Chavez, Co-Chair
Professor Cressida Madigan

2022

The thesis of Sinead McCabe is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

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The results section in part is currently being prepared for submission for publication for the material. The results section was co-authored by Uchiyama, Satoshi; Zurich, Raymond; Nizet, Victor. The thesis author was the primary author of this material.

ABSTRACT OF THE THESIS

The group B *Streptococcus* virulence factor C5a peptidase role in complement cleavage and vaccine protection

by

Sinead McCabe

Master of Science in Biology

University of California San Diego, 2022

Professor Victor Nizet, Chair
Professor Fabian Rivera Chavez, Co-chair

Infections with the bacterial pathogen group B *Streptococcus* (GBS, *S. agalactiae*) cause significant morbidity and mortality in pregnant women and neonates, especially in resource-poor countries where implementation of universal screening programs and intrapartum antibiotic prophylaxis remain challenging. Furthermore, potential negative impacts of antibiotic treatment used for GBS prophylaxis on the developing neonatal microbiome is gaining increased scrutiny. For these reasons, a vaccine to prevent GBS infection is an important public health priority. Our laboratory has been collaborating with Vaxcyte, Inc. (Foster City, CA) in advanced preclinical

development of the multivalent vaccine, VAX-A1, for prevention of infections by another common childhood pathogen, group A *Streptococcus* (GAS, *S. pyogenes*). Due to high sequence homology (98%) between one key VAX-A1 component, the surface anchored GAS C5a peptidase (ScpA) and its GBS homolog (ScpB), we predicted that VAX-A1 could provide a measure of cross-protection against GBS infection. In addition, the functional role of ScpB in GBS virulence remains unclear. Here we generate a GBS ScpB deletion mutant (ΔScpB) paired with heterologous expression of the ScpB gene in nonvirulent *Lactococcus lactis* for loss and gain of function analyses of the role of C5a peptidase in both GBS virulence and VAX-A1 efficacy. Rabbits immunized with VAX-A1 were protected against lethal infection with three GBS strains of different serotypes prevalent in human newborn infection. Serum from rabbits immunized with VAX-A1 cross-react with WT GBS, but not the ΔScpB mutant, suggesting the protein is the target antigen responsible for cross protection. These were corroborated by cross reactivity of the antisera specific to *L. lactis* expressing GBS ScpB. WT GBS opsonized with serum from rabbits immunized with VAX-A1 were more readily killed by human neutrophils. C5a peptidase in both GAS and GBS cleaves human C5a, a major chemoattractant and component of the complement cascade, to impair host immunity. We thus applied our loss- and gain-of-function GBS bacterial reagents to study the contribution of ScpB to GBS neutrophil resistance and virulence in a murine challenge model. Our research highlights potential added efficacy of VAX-A1 in preventing not only GAS but also GBS infection, a clear added benefit of its future adoption into the routine immunization schedule. Likewise, our studies shed new light on the significance of C5a peptidase as a GBS virulence factor.

INTRODUCTION:

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae,* is a beta-hemolytic, Gram-positive, bacterial pathogen that can cause severe morbidity and mortality in pregnant women, neonates, and immunocompromised adults. GBS was initially isolated from cow's milk and thought to be a non-pathogenic colonizing species until it was first recognized as a human pathogen in 1938 after causing a puerperal sepsis infection (1,2). GBS is frequently identified in the maternal vaginal tract but also the lower gastrointestinal tract and can undergo ascending infection of the placental membranes during pregnancy or be acquired by the infant during vaginal passage to cause disease (3,4). These infections include but are not limited to sepsis of the mother and miscarriages as well as meningitis and pneumonia in the fetus or neonate.

Due to the major health issues GBS can cause, preventive measures aimed at reducing the risk of invasive early-onset GBS disease in newborns have focused on intrapartum antibiotic prophylaxis given to the mother (8). Antibiotics, often penicillin or ampicillin, are given to women during labor have been effective at preventing severe early onset GBS infection, but there are increasing concerns around the use of intrapartum antibiotics due to the selective pressure for antibiotic resistance and potential negative impact of antibiotic administration on maternal-to-neonatal microbiome transfer (5,6). GBS is also increasingly antibiotic-resistant itself, particularly to non-beta-lactam antibiotics, with the CDC reporting 42% of GBS isolates with clindamycin resistance and 58% of GBS isolate with erythromycin resistance, lessening potential treatment options for penicillin allergic patients each year (7). Development of preventative measures against GBS such as a vaccine necessitates gaining a better understanding of its virulence mechanisms.The Nizet laboratory is collaborating with Vaxcyte, Inc. (Foster City, CA) in advanced preclinical development of the multivalent vaccine VAX-A1, for prevention of

infections by the related pathogen group A *Streptococcus* (GAS). VAX-A1 has been effective at preventing GAS infection in mice. Due to one of the conserved bacterial protein components that VAX-A1 targets, C5a peptidase, it is possible that this vaccine developed to prevent GAS disease may provide some cross-protections against GBS. Another VAX-A1 component is the polyrhamnose backbone of the GAS-species conserved Lancefield group A carbohydrate conjugated to the GAS SpyAD protein and administered together with two other recombinant GAS proteins SLO and C5a peptidase (8). GBS cell wall carbohydrate shares stretches of polyrhamnose backbone resembling that of the GAS cell wall carbohydrate, and there is 98% sequence homology between the surface anchored C5a peptidase (ScpA) and its GBS homolog (ScpB) (12). Thus we predicted that VAX-A1 could also be effective at protecting against GBS infection.

C5a peptidase has not been well studied in the context of GBS therefore a better understanding of this virulence factor can greatly benefit in the search for alternative treatment and prevention. In GAS, C5a peptidase is a highly specific protease and adhesin/invasin that cleaves complement factor human C5a and contributes to GAS evasion of the immune response during infection (10,11). Human C5a is a neutrophil chemoattractant and the loss of active human C5a is detrimental to the function of innate immunity. In addition to determining the role of C5a peptidase in vaccine protection against GBS, this study will also elucidate the function of C5a peptidase in the context of GBS virulence.

We developed a genetic knockout of the ScpB gene in GBS (ΔScpB) to both better understand the role of C5a peptidase in GBS pathogenicity and to test the potential for VAX-A1 to be effective against GBS infection. We also developed a gain-of-function bacterial reagent through the introduction of the ScpB gene into non-virulent *Lactococcus lactis.* We used these

tools to study the contribution of ScpB to GBS immune evasion and virulence. Here we show, using the genetically manipulated bacterial strains, that VAX-A1 has some efficacy against GBS and that C5a peptidase ScpB is a significant virulence factor in GBS.

RESULTS:

Development and confirmation of mutant strains

The main shared component of GAS and GBS is the 98% homology in the ScpA and ScpB gene. This similarity in the genes that encode for C5a peptidase is hypothesized to be the main reason behind VAX-A1 protection against GBS as well as GAS. To study the specific role of C5a peptidase as a virulence factor and to study its contribution to VAX-A1 protection against GBS, we made a gain of function (GOF) mutant in the nonvirulent bacteria *Lactococcus lactis* and a loss of function (LOF) genetic knockout of the ScpB gene in WT A909 GBS. *L. lactis* was transformed via electroporation with a plasmid carrying the full length ScpB gene, including its promoter region to get continuous expression. The GBS ΔScpB was generated by inserting a plasmid into the active region of the ScpB gene to disrupt the enzymatic activity of the gene. To confirm that the cloning was successful, cross reactivity between the mutated bacteria was analyzed via flow cytometry. The *L. lactis* expressing ScpB, GBS ΔScpB, WT *L. lactis* control, and WT A909 GBS control was incubated with both heat inactivated pre-immune and heat inactivated immune serum taken from rabbits immunized with recombinant GAS C5a peptidase and measured binding of IgG via flow cytometry. The WT L. lactis (non-ScpB expressing) displayed low IgG binding to the pre-immunization and immunized serum. The *L. lactis* expressing ScpB displayed low IgG binding to the pre-immunized serum, but high binding to the immunized serum. This data shows that antibodies raised against the GAS C5a peptidase also recognize the GBS C5a peptidase as well as confirm that the *L. lactis* with the insertion of the ScpB gene enzymatically expresses the ScpB gene (Figure 1A). The GBS ΔScpB #1 and GBS ΔScpB #2 both lost binding to the pre-immune and immune serum indicating that the ScpB gene's enzymatic activity was lost and that the cross-reactivity of GBS to IgG antibodies raised against GAS C5a peptidase is attributable to the ScpB gene (Figure 1B).

VAX-A1 IgG antibody cross reactivity to GBS strains

To evaluate if VAX-A1 would provide protection against GBS infection, immune serum produced by rabbits immunized with VAX-A1 was provided by collaborator Vaxcyte Inc. to determine if IgG antibodies in the serum would cross react to the WT GBS and *L. lactis* expressing ScpB strains. Cross reactivity of antibodies produced by VAX-A1 provides insight into understanding the vaccine's interaction against GBS. To test the IgG binding to the various strains of bacteria (WT *L. lactis*, *L. lactis* expressing ScpB, WT GBS, GBS ΔScpB, and Revertant) were incubated with heat inactivated pre-immune serum and heat inactivated VAX-A1 immunized serum for 30 minutes at room temperature. Analysis of the IgG binding was done on the FacsCanto Flow Cytometer. The WT GBS had an average of 43.7% increased binding affinity and the *L. lactis* expressing ScpB had an average of 77.1% increased binding affinity to the VAX-A1 immune serum in comparison to the binding to pre-immune serum (Figure 2B). We believe the increase in binding in the *L.lactis* expressing ScpB in comparison to the WT GBS is due to *L.lactis* lacking the bacterial capsule that GBS has, making antibody binding easier. The binding in the GBS ΔScpB was lower at an average of 9.8% increased binding affinity and the WT *L. lactis* control had the lowest affinity with an average of 5.7% increased binding affinity (Figure 2B). Figure 2A is the representative analysis of the study repeated in triplicate. We saw a significant amount of binding of IgG antibodies produced by the VAX-A1 immunization to the WT GBS and *L. lactis* expressing ScpB and a loss of binding in the GBS ΔScpB strain. ScpB expression is needed for VAX-A1 and bacterial cross reactivity.

VAX-A1 has many components to the vaccine. To understand which specific components of the VAX-A1 vaccine the WT GBS was interacting with, we looked at IgG binding of the bacteria to serum from rabbits immunized with singular components of the vaccine. These

components were SpyAD conjugated to the GAS polyrhamnose backbone, SpyAD alone, and recombinant GAS C5a peptidase. The different immune sera was incubated with the WT GBS and GBS ΔScpB under the same conditions stated above. The WT GBS only interacted with the IgG antibodies produced in response to the recombinant GAS C5a peptidase. GBS ΔScpB interacted with none of the individual components of the vaccine (Figure 2C). The cross reactivity of the WT GBS to VAX-A1 is due to the presence of the recombinant GAS C5a peptidase.

VAX-A1 serum interaction with Neutrophils

Neutrophils are an essential component to the innate immune system, therefore, neutrophil interaction to the VAX-A1 serum was important to understand. This assay was performed with 30 minute incubation of the WT GBS and GBS ΔScpB strains with the heat-inactivated VAX-A1 immune serum and pre-immune serum. Freshly isolated human neutrophils from human donors were incubated with 4% BRC and 4% heat-inactivated FBS to replenish the complement components lost in the heat inactivation of the serum. Bacteria were exposed to the neutrophils at multiplicity of infection (MOI) = 0.1 bacteria per neutrophil for 1 hour at 37ºC, 5% CO_2. The VAX-A1 antisera induced statically significant increases of neutrophil killing of the WT GBS by unpaired t-test versus pre-immune serum (Figure 3). There was no significant difference between the VAX-A1 immune and pre-immune serum response in the GBS ΔScpB showing that the interaction between the VAX-A1 immune serum is dependent on the ScpB gene and the presence of the GBS C5a peptidase. WT GBS opsonized with VAX-A1 immune serum more readily killed my human neutrophils.

GBS C5a peptidase virulence in vivo

C5a peptidase's role in GBS virulence is understudied. C5a peptidase cleaving C5a alters the chemotaxis effects of the complement system potentially leading to a more severe infection. To gain a better understanding of the C5a peptidase as a virulence factor, we investigated through the use in vivo mouse models infected by the GBS ΔScpB to gain a better understanding of the role of GBS C5a peptidase virulence. For all in vivo work, CD-1 female mice (Charles River) between the ages of 8-10 weeks old were used throughout each study. The intratracheal (IT) infection was done to mimic a pneumonia infection. GBS often infects neonates through the lungs and causes severe pneumonia in the new born baby. Mice were infected with $3x10^8$ CFU/mL WT GBS and GBS ΔScpB in groups of 10 mice. Mice were euthanized and lungs were excised after 20 hours and taken for CFU enumeration. There was significantly more WT GBS in the lungs of the mice in comparison to the mice infected with GBS ΔScpB. Mice infected with GBS ΔScpB cleared the bacteria significantly better than the mice infected with WT GBS (Figure 4A).

GBS also causes systemic infections, impacting major organs such as the spleen. Mice were given an intravenous (IV) infection by retro-orbital injection with $1x10^9$ CFU/mL ($1x10^8$ CFU/mouse). After 24 hours, the mice were euthanized and spleens were excised and taken for CFU enumeration. There was no significant difference here but there was an increase in the CFU burden in the spleens of the WT GBS infected mice in comparison to that of the GBS ΔScpB (Figure 4B). We expect, if the experiment is repeated, that there will be a significant difference.

A survival study was conducted to gain a better understanding of the virulence of GBS C5a peptidase. Mice in groups of 10 were injected intraperitoneally (IP) with $1x10^9$ CFU/mL ($1x10^8$ CFU/mouse) of the WT GBS, GBS ΔScpB, and revertant strain. Mice were monitored for survival every 8 hours for 8 days. 70% of mice infected with GBS ΔScpB survived in comparison to 20% survival of the WT GBS and revertant infected mice. The P value of the study was P=0.06 for the WT GBS to GBS ΔScpB comparison and P=0.07 for the revertant to

GBS ΔScpB comparison. The statistics of Kaplan-Meier survival curves calculated using a log-rank Mantel-Cox test indicated that there is association between increased survival probability in mice infected with GBS ΔScpB in comparison to mice infected with WT GBS due to the loss of C5a peptidase cleavage of C5a(Figure 4C).

These three mice studies give us insight into the significance of GBS C5a peptidase as a virulence factor.

Complement Cleavage of Human C5a

Complement cleavage of human C5a of GBS C5a peptidase was used to confirm enzymatic activity of the mutated strains but also to confirm whether the ScpB gene is both sufficient and necessary for human C5a cleavage. Bacteria strains (WT GBS, GBS ΔScpB, *L. lactis* expressing ScpB, WT *L. lactis*, and revertant) were incubated for 16 hours at 37 ºC with recombinant human C5a. After the 16 hour incubation, samples were run on a 4-12% Bis-Tris gel and visualized using coomassie staining. The WT GBS, revertant and *L. lactis* expressing ScpB cleaved the human C5a with the band being lower indicating cleavage (Figure 5). The WT *L. lactis* and GBS ΔScpB did not cleave the human C5a. Of note, *L. lactis* expressing ScpB was able to cleave the human C5a with the presence of the ScpB gene alone and the GBS ΔScpB was not able to cleave the human C5a due to the loss of the enzymatic activity of the GBS C5a peptidase. This indicates that the presence of the ScpB gene and active GBS C5a peptidase is both sufficient and necessary for GBS to cleave human C5a and act as a significant virulence factor.

The results section in part is currently being prepared for submission for publication for the material. The results section was co-authored by Uchiyama, Satoshi; Zurich, Raymond; Nizet, Victor. The thesis author was the primary author of this material.

MATERIALS AND METHODS:

Isolation of the genome DNA (gDNA) from GBS

To isolate the genome DNA (gDNA) from GBS, the overnight culture of GBS was centrifuged, resuspended in RLT buffer (Qiagen), added to Lying Matrix B (Qbiogene), and mechanically lysed with Mini-Beadbeater-96 (Biospec Products). Bacterial gDNA was purified from the lysate by using NucleoSpin gDNA Clean-up kit (Macherey-Nagel), according to the manufacturer's guidelines.

***Lactococcus Lactis* (*L. lactis*) expressing ScpB**

L. lactis expressing ScpB was generated through transformation of the expression vector pdcERM expressing the ScpB gene. Genomic DNA was extracted from GBS A909 and the ScpB gene was amplified from GBS strain A909 with the addition of restriction enzyme sites EcoR1 and Bam1. These restriction enzyme sites correspond with the pdcERM multiple cloning site. Amplified ScpB was subcloned into the TOPO PCR Cloning Vector (Thermo-Fisher) and transformed into TOP10 *E. coli* Competent Cells (Thermo-Fisher). *E. coli* carrying the vector with the inserted ScpB DNA was confirmed through antibiotic selection on Luria agar plates with 100 ug/mL ampicillin. The purified TOPO PCR Cloning Vector carrying the ScpB gene was restriction digested with EcoR1and BamH1(NEB) according to manufacturer's protocol. Digested product was run on a agarose gel and the visualized band corresponding to the size of the ScpB gene was cut out to purify DNA by DNA gel purification kit (Qiagen).according to the manufacturer's instructions. This ScpB insert DNA was ligated with pdcERM plasmid using T4 DNA Ligase and T4 DNA Ligase Buffer (NEB). After ligation, the product was cloned into the TOP10 *E. coli* Competent Cell (Thermo-Fisher). To check for successful ligation and that the

insert ScpB DNA had ligated into the pdcERM plasmid, we used pdcERM specific primers to amplify the ScpB insertion in the plasmid. Once confirmed the pdcERM plasmid was transformed into a *L. lactis* MG1363 Electrocompetent Cells (Intact Genomics Inc.) and selected for Erythromycin (Erm) resistance introduced by the pdCERM plasmid on Todd Hewitt Agar plates containing 3 ug/mL Erm.

GBS ΔScpB Mutant

Genomic DNA was extracted from GBS strain A909. Forward primer (InserMutFor+ Xho1: TGCTCGAGTATCAGAGATGCTATCAACTTGGGAGCTAAGGTG) and Reverse primer (InserMutRev+Xbal: GATCTAGAGGAAGCCCTTGTCCTGATTGTCATAGATCA) were made to amplify about 500bp within the active region of the ScpB gene with the addition of restriction enzymes sites Xho1 and Xbal. The PCR product was recovered and cloned into a TOPO PCR Cloning Vector (Thermo-Fisher) and transformed into TOP10 *E. coli* Competent Cells (Thermo-Fisher). *E. coli* carrying the vector with the inserted DNA was confirmed through antibiotic selection on Luria agar plates with 100 ug/mL ampicillin and X-gal for color selection with colonies with the insert DNA remaining white and colonies lacking the insert DNA turning blue. The purified TOPO PCR Cloning Vector carrying the insert DNA was then digested with restriction enzymes Xho1 and Xbal. Insert DNA was visualized on a DNA agarose gel and cut out followed by purification using a DNA gel purification kit (Qiagen). pHY304 plasmid with temperature sensitivity and selective Erm resistance was digested with Xbal, Xho1, and rCutSmart Buffer (NEB) according to the manufacturer's protocol. Digested pHY304 was cleaned and purified using the DNA clean and purification kit (Qiagen) according to the manufacturer’s instructions. Fragments from the digested insert DNA and pHY304 plasmid were

then ligated with T4 DNA Ligase and T4 DNA Ligase Buffer (NEB). After ligation, the product was cloned into the TOP10 *E. coli* Competent Cell (Thermo-Fisher) and plated on LB agar plates with Erm. To check for successful ligation and that the insert DNA had ligated into the pHY304 plasmid, pHY304 specific primers to amplify the insertion DNA in the plasmid were used. Once confirmed the pHY304 plasmid was transformed into a wild type A909 GBS electrocompetent cell. Colonies with the pHY304 plasmid were selected for on Erm 3ug/mL THB agar plates. Single crossover chromosomal insertions were achieved by shifting to the non-permissive temperature (37°C) while maintaining 3 ug/ mL Erm selection. The loss of ScpB surface expression and enzymatic activity was confirmed in two ways, loss of binding to IgG antibodies produced by rabbits immunized with recombinant GAS C5a peptidase (surface expression) and human C5a cleavage visualized on through a coomassie stain (enzymatic activity).

Revertant strain

Revertant strain was produced by relaxation of the GBS ΔScpB strain to cause the release of the pHY304 plasmid from the bacteria. GBS ΔScpB was grown in non-antibiotic conditions in THB alone at 37 ºC and passaged daily. With each passage, 10 ul of diluted culture was plated onto THB plates and colonies were individually selected for loss of Cm resistance. Once a colony with sensitivity to Cm was found, loss of the pHY304 plasmid was confirmed by confirming that the revertant strain could cleave human C5a through coomassie stain visualization.

IgG binding assay

Pre- immunized and immunized serum from rabbits immunized with recombinant GAS C5a peptidase and rabbits immunized with VAX-A1 was obtained from Vaxcyte inc. Samples (*L.*

lactis expressing ScpB, GBS ΔScpB, WT GBS, revertant or WT *L. lactis* control) were first blocked with 10% heat inactivated Donkey Serum for 1 hour at room temperature. After blocking, bacteria were incubated with either pre-immunization serum or immunized serum for 1 hour at room temperature. After serum incubation, Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody (Thermo-Fisher) was added and samples were incubated for 30 minutes in the dark. IgG binding to bacteria was determined via flow cytometry analysis on a FACs Canto. Results were analyzed on FlowJo Version 10.

Neutrophil Killing Assay

Neutrophils were isolated from blood drawn from healthy human donors with consent, as approved by UC San Diego institutional review board (Protocol #131002X). Neutrophils at $5x10^6$ cells/mL were pre-incubated with Baby rabbit complement (BRC) (PelFreez cat#31061) and heat inactivated fetal bovine serum (FBS). The various GBS strains were grown to mid-logarithmic growth phase of an OD600 nm=0.4 to get and washed with HBSS calcium, magnesium (Thermo-Fisher). The bacteria were diluted to working concentration of $5x10^6$ colony forming units (CFU)/ mL and incubated with heat-inactivated rabbit antisera for 30 min at room temperature. Neutrophils were added to bacteria at a MOI = 0.1 bacteria per neutrophil in a 96 well Tissue Culture plate and centrifuged for 5 minutes at 500 g to ensure contact. The plate was allowed to incubate for 1 hour at 37°C with 5% CO_2. Samples were serially diluted in PBS and plated onto THB agar plates for CFU enumeration.

In vivo mouse pneumonia model

All mouse experiments were approved by the UC San Diego Institutional Animal Care and Use Committee (Protocol #S00227M) and conducted per accepted veterinary standards. 30 ul of the WT A909 GBS, GBS ΔScpB,and Revertant bacteria at a concentration $3x10^8$ CFU/mL were delivered intratracheally to each anesthetized wild-type female CD-1 mice (Charles River), 8-10 weeks of age. After 20 hours, mice were euthanized and lungs were excised and homogenized using a Mini-BeadBeater 96 (BioSpec). Homogenized tissue was serially diluted and plated on THB agar plates for CFU enumeration. Statistics of CFUs recovered were calculated using unpaired t-test.

In vivo mouse systemic infection model

All mouse experiments were approved by the UC San Diego Institutional Animal Care and Use Committee (Protocol #S00227M) and conducted per accepted veterinary standards. 100 ul of the WT A909 GBS and GBS ΔScpB bacteria at a concentration $1x10^9$ CFU/mL ($1x10^8$ CFU/mouse) were delivered by retro-orbital injection to each anesthetized wild-type female CD-1 mice (Charles River), 8-10 weeks of age. After 24 hours, mice were euthanized and spleen were excised and homogenized using a Mini-BeadBeater 96 (BioSpec). Homogenized tissue were serially diluted and plated on THB agar plates for CFU enumeration. Statistics of CFUs recovered were calculated using unpaired t-test.

In vivo mouse survival study

All mouse experiments were approved by the UC San Diego Institutional Animal Care and Use Committee (Protocol #S00227M) and conducted per accepted veterinary standards. 100 ul of the WT A909 GBS and GBS ΔScpB bacteria at a concentration $1x10^9$ CFU/mL ($1x10^8$ CFU/mouse) were delivered by intraperitoneal injection to each wild-type female CD-1 mice (Charles River), 8-10 weeks of age. Mice were monitored for 8 days every 8 hours and tracked for survival. Statistics of Kaplan-Meier survival curves were calculated using a log-rank Mantel-Cox test.

Cleavage of human C5a and SDS-Page visualization

Bacteria strains (*L. lactis* expressing ScpB, GBS ΔScpB, WT GBS, revertant or WT *L. lactis*) were grown overnight. Overnight cultures were washed twice with PBS and brought to a mid-logarithmic growth phase of an OD600 nm=0.4 to get to a $2x10^8$ CFU/mL. Bacteria was concentrated to get to a final concentration of $2x10^9$ CFU/mL. 100ug/mL concentration of recombinant human C5a complement component (R&D Systems, cat#: 2037-C5-025/CF) was incubated with the bacteria for 16 hours at 37ºC. After 16 hours, bacteria was pelleted and the supernatant was separated. NuPAGE™ LDS Sample Buffer 4x (Thermo-Fisher, cat#:NP0007) was added to the sample supernatant and boiled for 5 minutes. Samples were run on a 4-12% Bis-Tris gel (Thermo-Fisher) at 100 volts and stained with SimplyBlue™ SafeStain (Thermo-Fisher, cat#:LC6065) for 1 hour and 30 minutes. After staining, gel was washed with DI water for optimal visualization (13).

DISCUSSION:

GBS is an important human pathogen to understand and study. GBS continues to cause severe infections in the fetus, pregnant women, and neonates (4). As treatment options become limited and potentially harmful for highly impacted groups, alternative treatment options such as VAX-A1 and gaining a deeper understanding of virulence factors like C5a peptidase is important to study. In this work, we present preliminary data to the protection against GBS infection VAX-A1 can provide. Through the use of mutated strains of GBS and *L. lactis*, we were able to gain a better understanding of if and how VAX-A1 provides protection. The high affinity of the IgG antibodies from recombinant GAS C5a peptidase- immunized rabbits to GBS ScpB-expressing *L. lactis* suggests that the GAS C5a peptidase component of VAX-A1 provides protection against GBS infection as well as gave us insight into confirming the cloning steps went as intended. This same binding was lost in the GBS ΔScpB strain allowing us to attribute this potential interaction of the antibodies to the presence of ScpB as well as allowing us to visualize the loss of ScpB enzymatic activity in the mutated strain. With this knowledge we moved into study how IgG antibodies produced by rabbits immunized with VAX-A1 would interact with our various strains. The IgG antibodies cross reacted to the WT GBS and L lactis expressing ScpB strains indicating that there will be protection provided by this vaccine. This data was further confirmed by then looking into the neutrophil interactions with the bacteria when incubated with the vaccine. Neutrophils were significantly increased in their ability to kill WT GBS when the bacteria was incubated with the VAX-A1 immune serum. This neutrophil killing was lost in the GBS ΔScpB as expected due to the loss of the key C5a peptidase.

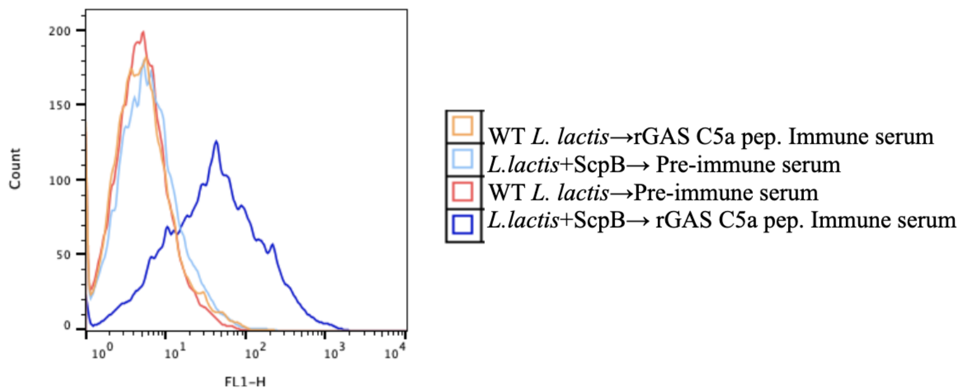
To gain a better understanding of C5a peptidase as a virulence factor in the context of GBS and not GAS we decided to perform three in vivo studies to highlight the main ways GBS

can cause severe infection. GBS often causes pneumonia in the lungs of neonates passing through the vaginal tract of the GBS colonized mother and by causing systemic infections as well. To model this, we performed IT infection to create a pneumonia model as well as IP and IV infections to identify bacterial burden in the spleen and measure survival. These three studies showed how the presence of C5a peptidase in GBS creates a more severe infection and makes the infection much harder to clear than infection from the GBS ΔScpB. This was particularly notable in the lungs with the bacterial burden in the lungs of mice infected with WT GBS being much worse than that of GBS ΔScpB infection. This data highlights the significant virulence factor that C5a peptidase is in GBS.

Understanding the significance of GBS C5a peptidase was furthered by visualizing through the use of a coomassie stain to understand if the ScpB gene was sufficient and necessary to cleave human C5a. Human C5a was cleaved by WT GBS and L. lactis expressing ScpB. No other strains were able to cleave C5a, resulting in the conclusion that ScpB is sufficient and necessary for human C5a cleavage.

Additional studies are needed to completely understand the protection VAX-A1 provides against GBS. As the vaccine continues through clinical trials more research into the protection against GBS alongside GAS will be further investigated. There is also additional research that needs to be done to completely understand the role of C5a peptidase in GBS virulence.

A



B

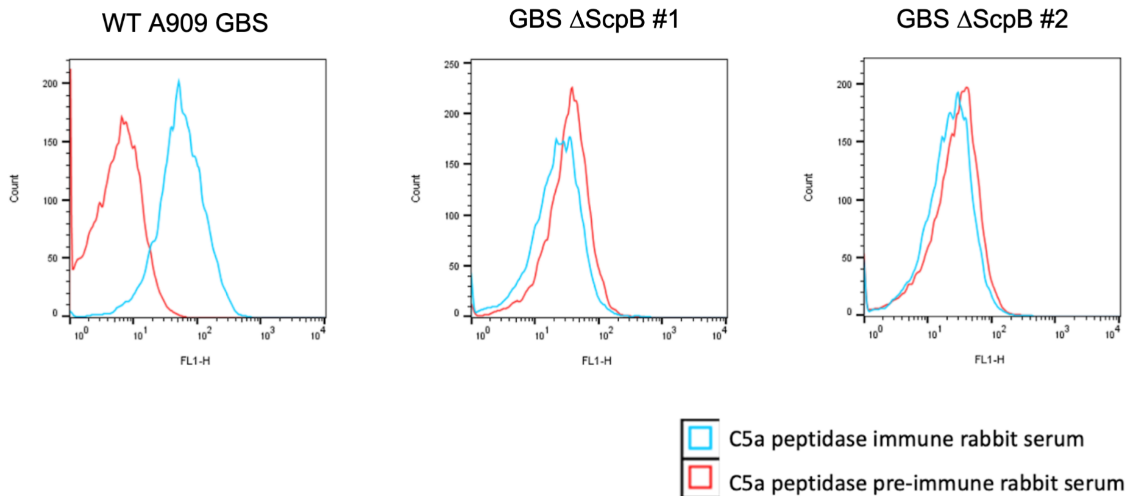


Figure 1. GOF and LOF Mutant cloning.

A: GOF *L. lactis* expressing ScpB binds to IgG antibodies in rGAS C5a peptidase showing that ScpB is being expressed and that there is cross reactivity between GAS C5a peptidase and GBS. B: LOF GBS ΔScpB does not bind to rGAS C5a peptidase showing that the expression of the ScpB gene was lost and that the cross reactivity of GAS C5a peptidase and GBS is due to ScpB expression.

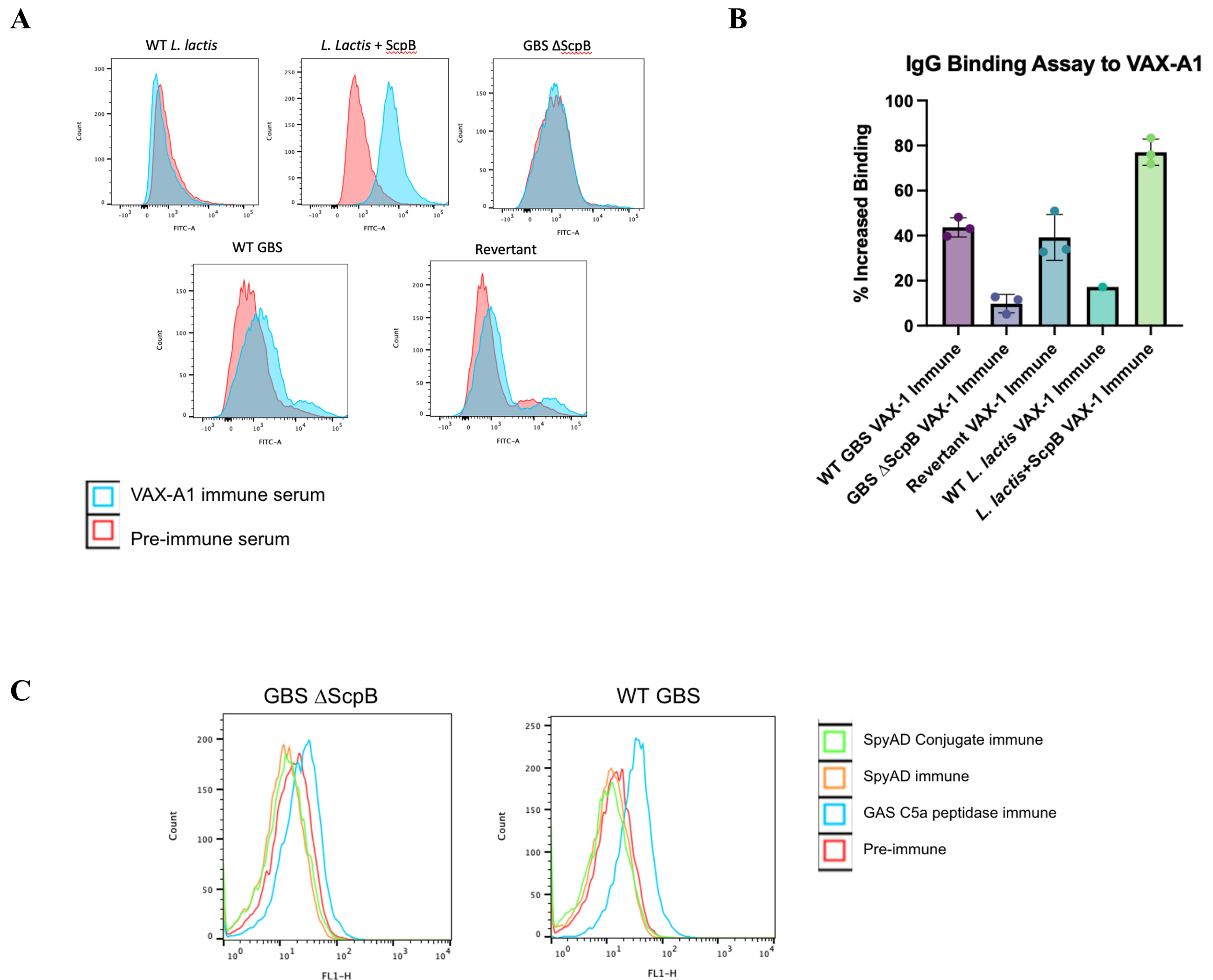


Figure 2. IgG binding of the antibodies produced against VAX-A1 to ScpB expressing strains.

A: GBS strains were used to determine VAX-A1 rabbit serum IgG binding to native antigens by flow cytometry. Histograms show representative fluorescent signals from IgG binding; red represents pre-immune sera and blue immune sera. B: Combined percent increase binding of the IgG antibodies to bacterial strains for 3 separate VAX-A1 IgG binding assays. C: Serum from specific components of VAX-A1 were tested for IgG binding the WT GBS and ΔScpB. Recombinant GAS C5a peptidase was the only component with significant binding.

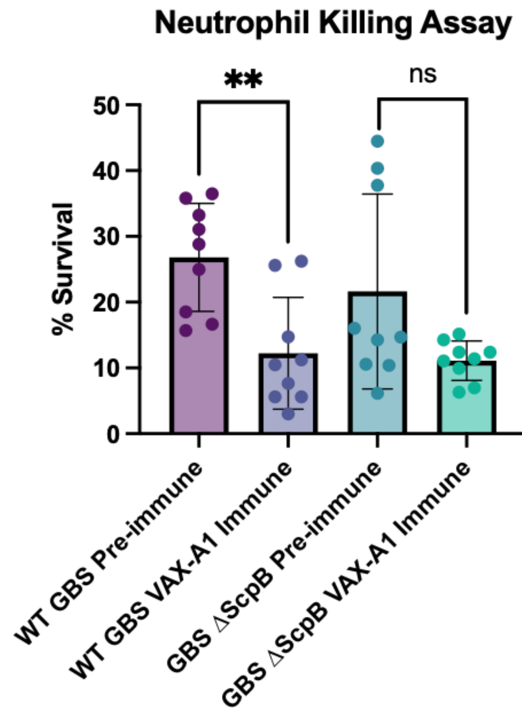


Figure 3: WT GBS opsonized with serum from rabbits immunized with VAX-A1 were more readily killed by human neutrophils.

WT GBS opsonized with VAX-A1 immune serum was killed by neutrophil isolated from human blood significantly better (**=P<0.01) than the WT GBS opsonized by pre-immune serum. GBS ΔScpB opsonized with pre-immune and VAX-A1 immune serum saw no difference between the two conditions. Statistical analysis was performed by non-parametric t-test.

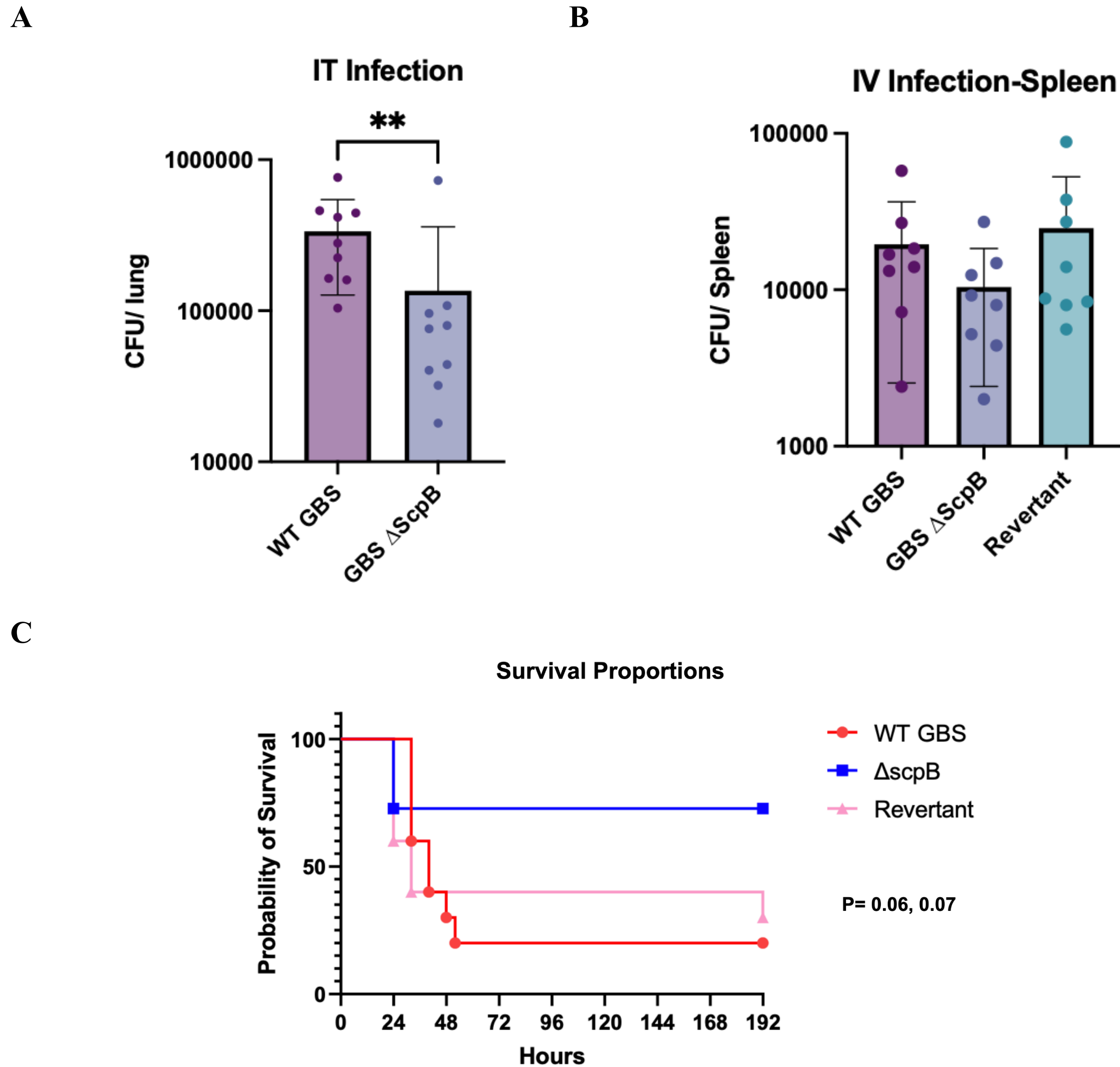


Figure 4: GBS C5a peptidase leads to more severe GBS infection in mice models.

A: Intratracheal infection of CD-1 mice were infected with $3x10^8$ CFU of WT GBS and ΔScpB. Lungs were excised and homogenized for CFU enumeration. WT GBS had a significantly higher bacterial burden in the lungs than that of ΔScpB. B: Systemic infection of CD-1 mice were infected with $3x10^8$ CFU of WT GBS and ΔScpB. Spleens were excised and homogenized for CFU enumeration. WT GBS had a higher bacterial burden in the spleen than that of ΔScpB. C: CD-1 female mice received an intravenous lethal dose challenge with strain WT GBS and ΔScpB. WT GBS and revertant strain percent survival was lower than ΔScpB. Statistics are calculated from the log rank Mantel Cox test for Kaplan-Meier plots.

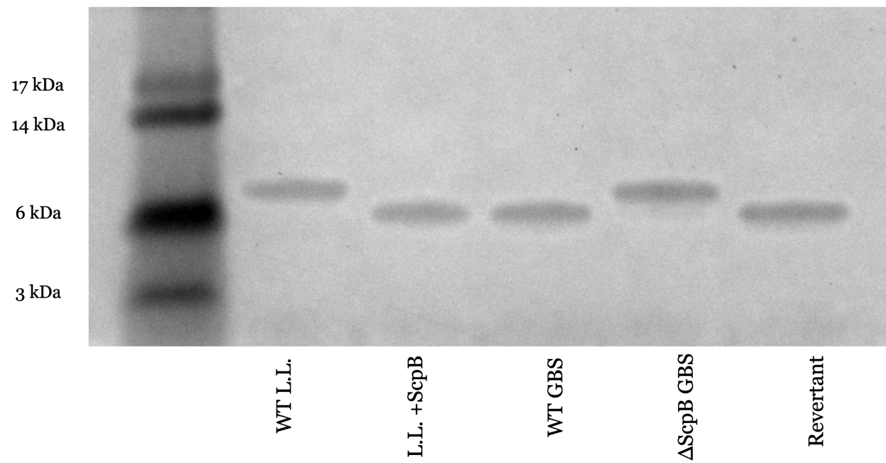


Figure 5: ScpB is sufficient and necessary for human C5a cleavage.

Cleavage of human C5a by whole cell pellets ($2x10^8$ CFU) of WT L. lactis, L. lactis expressing ScpB, WT GBS, GBS ΔScpB, and revertant strain. Only bacteria expressing ScpB (WT GBS, L. lactis+ScpB, revertant) were able to cleave C5a. Cleaved C5a is indicated by a lower positioning on the gel.

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