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(57) Abstract: Disclosed are methods and compositions for treating or preventing a Staphylococcus bacterial infection using a non-toxigenic Protein A (SpA) variant.

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# DESCRIPTION

## [0001] COMPOSITIONS AND METHODS RELATED TO PROTEIN A (SpA) VARIANTS

## [0002] BACKGROUND OF THE INVENTION

### I. FIELD OF THE INVENTION

[0003] The present invention relates generally to the fields of immunology, microbiology, and pathology. More particularly, it concerns methods and compositions involving bacterial Protein A variants, which can be used to invoke an immune response against the bacteria.

### II. BACKGROUND

[0004] The number of both community acquired and hospital acquired infections have increased over recent years with the increased use of intravascular devices. Hospital acquired (nosocomial) infections are a major cause of morbidity and mortality, more particularly in the United States, where it affects more than 2 million patients annually. The most frequent infections are urinary tract infections (33% of the infections), followed by pneumonia (15.5%), surgical site infections (14.8%) and primary bloodstream infections (13%) (Emorl and Gaynes, 1993).

[0005] The major nosocomial pathogens include *Staphylococcus aureus*, coagulase-negative Staphylococci (mostly *Staphylococcus epidermidis*), *enterococcus* spp., *Escherichia coli* and *Pseudomonas aeruginosa*. Although these pathogens cause approximately the same number of infections, the severity of the disorders they can

produce combined with the frequency of antibiotic resistant isolates balance this ranking towards *S. aureus* and *S. epidermidis* as being the most significant nosocomial pathogens.

[0006] Staphylococci can cause a wide variety of diseases in humans and other animals through either toxin production or invasion. Staphylococcal toxins are also a common cause of food poisoning, as the bacteria can grow in improperly-stored food.

[0007] *Staphylococcus epidermidis* is a normal skin commensal which is also an important opportunistic pathogen responsible for infections of impaired medical devices and infections at sites of surgery. Medical devices infected by *S. epidermidis* include cardiac pacemakers, cerebrospinal fluid shunts, continuous ambulatory peritoneal dialysis catheters, orthopedic devices and prosthetic heart valves.

[0008] *Staphylococcus aureus* is the most common cause of nosocomial infections with a significant morbidity and mortality. It is the cause of some cases of osteomyelitis, endocarditis, septic arthritis, pneumonia, abscesses, and toxic shock syndrome. *S. aureus* can survive on dry surfaces, increasing the chance of transmission. Any *S. aureus* infection can cause the staphylococcal scalded skin syndrome, a cutaneous reaction to exotoxin absorbed into the bloodstream. It can also cause a type of septicemia called pyaemia that can be life-threatening. Problematically, Methicillin-resistant Staphylococcus aureus (MRSA) has become a major cause of hospital-acquired infections.

[0009] *S. aureus* and *S. epidermidis* infections are typically treated with antibiotics, with penicillin being the drug of choice, whereas vancomycin is used for methicillin resistant isolates. The percentage of staphylococcal strains exhibiting wide-spectrum resistance to antibiotics has become increasingly prevalent, posing a threat for effective antimicrobial therapy. In addition, the recent emergence of vancomycin resistant *S. aureus* strain has aroused fear that MRSA strains are emerging and spreading for which no effective therapy is available.

[0010] An alternative to antibiotic treatment for staphylococcal infections is under investigation that uses antibodies directed against staphylococcal antigens. This therapy involves administration of polyclonal antisera (WO00/15238, WO00/12132) or treatment with monoclonal antibodies against lipoteichoic acid (WO98/57994).

[0011] An alternative approach would be the use of active vaccination to generate an immune response against staphylococci. The *S. aureus* genome has been sequenced and many of the coding sequences have been identified (WO02/094868, EP0786519), which can lead to the identification of potential antigens. The same is true for *S. epidermidis* (WO01/34809). As a refinement of this approach, others have identified proteins that are recognized by hyperimmune sera from patients who have suffered staphylococcal infection (WO01/98499, WO02/059148).

[0012] *S. aureus* secretes a plethora of virulence factors into the extracellular milieu (Archer, 1998; Dinges *et al.*, 2000; Foster, 2005; Shaw *et al.*, 2004; Sibbald *et al.*, 2006). Like most secreted proteins, these virulence factors are translocated by the Sec machinery across the plasma membrane. Proteins secreted by the Sec machinery bear an N-terminal leader peptide that is removed by leader peptidase once the pre-protein is engaged in the Sec translocon (Dalbey and Wickner, 1985; van Wely *et al.*, 2001). Recent genome analysis suggests that Actinobacteria and members of the Firmicutes encode an additional secretion system that recognizes a subset of proteins in a Sec-independent manner (Pallen, 2002). ESAT-6 (early secreted antigen target 6 kDa) and CFP-10 (culture filtrate antigen 10 kDa) of *Mycobacterium tuberculosis* represent the first substrates of this novel secretion system termed ESX-1 or Snm in *M. tuberculosis* (Andersen *et al.*, 1995; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003). In *S. aureus,* two ESAT-6 like factors designated EsxA and EsxB are secreted by the Ess pathway (ESAT-6 secretion system) (Burts *et al.*, 2005).

[0013] The first generation of vaccines targeted against *S. aureus* or against the exoproteins it produces have met with limited success (Lee, 1996). There remains a need to develop effective vaccines against staphylococcal infections. Additional compositions for treating staphylococcal infections are also needed.

## SUMMARY OF THE INVENTION

[0014] Protein A (SpA)(SEQ ID NO:33), a cell wall anchored surface protein of *Staphylococcus aureus*, provides for bacterial evasion from innate and adaptive immune responses. Protein A binds immunoglobulins at their Fc portion, interacts with the VH3 domain of B cell receptors inappropriately stimulating B cell proliferation and apotosis, binds to von Willebrand factor A1 domains to activate intracellular clotting, and also binds to the TNF Receptor-1 to contribute to the

pathogenesis of staphylococcal pneumonia. Due to the fact that Protein A captures immunoglobulin and displays toxic attributes, the possibility that this surface molecule may function as a vaccine in humans has not been rigorously pursued. Here the inventors demonstrate that Protein A variants no longer able to bind to immunoglobulins, which are thereby removed of their toxigenic potential, *i.e.*, are non-toxigenic, stimulate humoral immune responses that protect against staphylococcal disease.

[0015] In certain embodiments the SpA variant is a full length SpA variant comprising a variant A, B, C, D, and E domain. In certain aspects, the SpA variant comprises or consists of the amino acid sequence that is 80, 90, 95, 98, 99, or 100% identical to the amino acid sequence of SEQ ID NO:34 In other embodiments the SpA variant comprises a segment of SpA. The SpA segment can comprise at least or at most 1, 2, 3, 4, 5 or more IgG binding domains. The IgG domains can be at least or at most 1, 2, 3, 4, 5 or more variant A, B, C, D, or E domains. In certain aspects the SpA variant comprises at least or at most 1, 2, 3, 4, 5, or more variant A domains. In a further aspect the SpA variant comprises at least or at most 1, 2, 3, 4, 5, or more variant B domains. In still a further aspect the SpA variant comprises at least or at most 1, 2, 3, 4, 5, or more variant C domains. In yet a further aspect the SpA variant comprises at least or at most 1, 2, 3, 4, 5, or more variant D domains. In certain aspects the SpA variant comprises at least or at most 1, 2, 3, 4, 5, or more variant E domains. In a further aspect the SpA variant comprises a combination of A, B, C, D, and E domains in various combinations and permutations. The combinations can include all or part of a SpA signal peptide segment, a SpA region X segment, and/or a SpA sorting signal segment. In other aspects the SpA variant does not include a SpA signal peptide segment, a SpA region X segment, and/or a SpA sorting signal segment. In certain aspects a variant A domain comprises a substitution at position(s) 7, 8, 34, and/or 35 of SEQ ID NO:4. In another aspect a variant B domain comprises a substitution at position(s) 7, 8, 34, and/or 35 of SEQ ID NO:6. In still anther aspect a variant C domain comprises a substitution at position(s) 7, 8, 34, and/or 35 of SEQ ID NO:5. In certain aspects a variant D domain comprises a substitution at position(s) 9, 10, 37, and/or 38 of SEQ ID NO:2. In a further aspect a variant E domain comprises a substitution at position(s) 6, 7, 33, and/or 34 of SEQ ID NO:3.

[0016] In certain aspects the SpA variant includes a substitution of (a) one or more amino acid substitution in an IgG Fc binding sub-domain of SpA domain A, B, C, D, and/or E that disrupts or decreases binding to IgG Fc, and (b) one or more amino acid substitution in a $V_H3$ binding sub-domain of SpA domain A, B, C, D, and/or E that disrupts or decreases binding to $V_H3$. In still further aspects the amino acid sequence of a SpA variant comprises an amino acid sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, or 100% identical, including all values and ranges there between, to the amino acid sequence of SEQ ID NOs:2-6.

[0017] In a further aspect the SpA variant includes (a) one or more amino acid substitution in an IgG Fc binding sub-domain of SpA domain D, or at a corresponding amino acid position in other IgG domains, that disrupts or decreases binding to IgG Fc, and (b) one or more amino acid substitution in a $V_H3$ binding sub-domain of SpA domain D, or at a corresponding amino acid position in other IgG domains, that disrupts or decreases binding to $V_H3$. In certain aspects amino acid residue F5, Q9, Q10, S11, F13, Y14, L17, N28, I31, and/or K35 (SEQ ID NO:2, QQNNFNKDQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNES) of the IgG Fc binding sub-domain of domain D are modified or substituted. In certain aspects amino acid residue Q26, G29, F30, S33, D36, D37, Q40, N43, and/or E47 (SEQ ID NO:2) of the $V_H3$ binding sub-domain of domain D are modified or substituted such that binding to Fc or $V_H3$ is attenuated. In further aspects corresponding modifications or substitutions can be engineered in corresponding positions of the domain A, B, C, and/or E. Corresponding positions are defined by alignment of the domain D amino acid sequence with one or more of the amino acid sequences from other IgG binding domains of SpA, for example see FIG. 1. In certain aspects the amino acid substitution can be any of the other 20 amino acids. In a further aspect conservative amino acid substitutions can be specifically excluded from possible amino acid substitutions. In other aspects only non-conservative substitutions are included. In any event, any substitution or combination of substitutions that reduces the binding of the domain such that SpA toxicity is significantly reduced is contemplated. The significance of the reduction in binding refers to a variant that produces minimal to no toxicity when introduced into a subject and can be assessed using in vitro methods described herein.

[0018] In certain embodiments, a variant SpA comprises at least or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more variant SpA domain D peptides. In certain aspects 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 or more amino acid residues of the variant SpA are substituted or modified - including but not limited to amino acids F5, Q9, Q10, S11, F13, Y14, L17, N28, I31, and/or K35 (SEQ ID NO:2) of the IgG Fc binding sub-domain of domain D and amino acid residue Q26, G29, F30, S33, D36, D37, Q40, N43, and/or E47 (SEQ ID NO:2) of the $V_H3$ binding sub-domain of domain D. In one aspect of the invention glutamine residues at position 9 and/or 10 of SEQ ID NO:2 (or corresponding positions in other domains) are mutated. In another aspect, aspartic acid residues 36 and/or 37 of SEQ ID NO:2 (or corresponding positions in other domains) are mutated. In a further aspect, glutamine 9 and 10, and aspartic acid residues 36 and 37 are mutated. Purified non-toxigenic SpA or SpA-D mutants/variants described herein are no longer able to significantly bind (i.e., demonstrate attenuated or disrupted binding affinity) Fcγ or $F(ab)_2$ $V_H3$ and also do not stimulate B cell apoptosis. These non-toxigenic Protein A variants can be used as subunit vaccines and raise humoral immune responses and confer protective immunity against *S. aureus* challenge. Compared to wild-type full-length Protein A or the wild-type SpA-domain D, immunization with SpA-D variants resulted in an increase in Protein A specific antibody. Using a mouse model of staphylococcal challenge and abscess formation, it was observed that immunization with the non-toxigenic Protein A variants generated significant protection from staphylococcal infection and abscess formation. As virtually all *S. aureus* strains express Protein A, immunization of humans with the non-toxigenic Protein A variants can neutralize this virulence factor and thereby establish protective immunity. In certain aspects the protective immunity protects or ameliorates infection by drug resistant strains of Staphylococcus, such as USA300 and other MRSA strains.

[0019] Embodiments include the use of Protein A variants in methods and compositions for the treatment of bacterial and/or staphylococcal infection. This application also provides an immunogenic composition comprising a Protein A variant or immunogenic fragment thereof. In certain aspects, the immunogenic fragment is a Protein A domain D segment. Furthermore, the present invention provides methods and compositions that can be used to treat (*e.g.*, limiting staphylococcal abscess formation and/or persistence in a subject) or prevent bacterial

infection. In some cases, methods for stimulating an immune response involve administering to the subject an effective amount of a composition including or encoding all or part of a Protein A variant polypeptide or antigen, and in certain aspects other bacterial proteins. Other bacterial proteins include, but are not limited to (i) a secreted virulence factor, and/or a cell surface protein or peptide, or (ii) a recombinant nucleic acid molecule encoding a secreted virulence factor, and/or a cell surface protein or peptide.

[0020] In other aspects, the subject can be administered all or part of a Protein A variant, such as a variant Protein A domain D segment. The polypeptide of the invention can be formulated in a pharmaceutically acceptable composition. The composition can further comprise one or more of at least or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 additional staphylococcal antigen or immunogenic fragment thereof (*e.g.*, Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla (*e.g.*, H35 mutants), IsdC, SasF, vWbp, or vWh). Additional staphylococcal antigens that can be used in combination with a Protein A variant include, but are not limited to 52kDa vitronectin binding protein (WO 01/60852), Aaa (GenBank CAC80837), Aap (GenBank accession AJ249487), Ant (GenBank accession NP_372518), autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein (see PCT publications WO2007/113222, WO2007/113223, WO2006/032472, WO2006/032475, WO2006/032500. The staphylococcal antigen or immunogenic fragment can be administered concurrently with the Protein A variant. The staphylococcal antigen or immunogenic fragment and the Protein A variant can be administered in the same composition. The Protein A variant can also be a recombinant nucleic acid

molecule encoding a Protein A variant. A recombinant nucleic acid molecule can encode the Protein A variant and at least one staphylococcal antigen or immunogenic fragment thereof. As used herein, the term "modulate" or "modulation" encompasses the meanings of the words "enhance," or "inhibit." "Modulation" of activity may be either an increase or a decrease in activity. As used herein, the term "modulator" refers to compounds that effect the function of a moiety, including up-regulation, induction, stimulation, potentiation, inhibition, down-regulation, or suppression of a protein, nucleic acid, gene, organism or the like.

[0021] In certain embodiments the methods and compositions use or include or encode all or part of the Protein A variant or antigen. In other aspects, the Protein A variant may be used in combination with secreted factors or surface antigens including, but not limited to one or more of an isolated Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, or vWh polypeptide or immunogenic segment thereof. Additional staphylococcal antigens that can be used in combination with a Protein A variant include, but are not limited to 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein. In certain embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more of Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, vWh, 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP,

Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein. can be specifically excluded from a formulation of the invention.

[0022] In still further aspects, the isolated Protein A variant is multimerized, *e.g.*, dimerized or a linear fusion of two or more polypeptides or peptide segments. In certain aspects of the invention, a composition comprises multimers or concatamers of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more isolated cell surface proteins or segments thereof. Concatamers are linear polypeptides having one or more repeating peptide units. SpA polypeptides or fragments can be consecutive or separated by a spacer or other peptide sequences, *e.g.*, one or more additional bacterial peptide. In a further aspect, the other polypeptides or peptides contained in the multimer or concatamer can include, but are not limited to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 of Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, vWh or immunogenic fragments thereof. Additional staphylococcal antigens that can be used in combination with a Protein A variant include, but are not limited to 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein.

[0023] The term “Protein A variant” or “SpA variant” refers to polypeptides that include a SpA IgG domain having two or more amino acid substitutions that disrupt

binding to Fc and $V_H3$. In certain aspect, a SpA variant includes a variant domain D peptide, as well as variants of SpA polypeptides and segments thereof that are non-toxigenic and stimulate an immune response against staphylococcus bacteria Protein A and/or bacteria expressing such.

[0024] Embodiments of the present invention include methods for eliciting an immune response against a staphylococcus bacterium or staphylococci in a subject comprising providing to the subject an effective amount of a Protein A variant or a segment thereof. In certain aspects, the methods for eliciting an immune response against a staphylococcus bacterium or staphylococci in a subject comprising providing to the subject an effective amount of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more secreted proteins and/or cell surface proteins or segments/fragments thereof. A secreted protein or cell surface protein includes, but is not limited to Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, and/or vWh proteins and immunogenic fragments thereof. Additional staphylococcal antigens that can be used in combination with a Protein A variant include, but are not limited to 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein.

[0025] Embodiments of the invention include compositions that include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to Protein A, or a second protein or peptide that is a secreted bacterial protein or a bacterial cell surface protein. In a further embodiment of the invention a composition may include a polypeptide,

peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a Protein A domain D polypeptide (SEQ ID NO:2), domain E (SEQ ID NO:3), domain A (SEQ ID NO:4), domain C (SEQ ID NO:5), domain B (SEQ ID NO:6), or a nucleic acid sequence encoding a Protein A domain D, domain E, domain A, domain C, or domain B polypeptide. In certain aspects a Protein A polypeptide segment will have an amino acid sequence of SEQ ID NO:8. Similarity or identity, with identity being preferred, is known in the art and a number of different programs can be used to identify whether a protein (or nucleic acid) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman (1981), by the sequence identity alignment algorithm of Needleman & Wunsch (1970), by the search for similarity method of Pearson & Lipman (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux *et al.* (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by using alignment tools known to and readily ascertainable to those of skill in the art. Percent identity is essentially the number of identical amino acids divided by the total number of amino acids compared times one hundred.

[0026] Still further embodiments include methods for stimulating in a subject a protective or therapeutic immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition including (i) a SpA variant, *e.g.*, a variant SpA domain D polypeptide or peptide thereof; or, (ii) a nucleic acid molecule encoding such a SpA variant polypeptide or peptide thereof, or (iii) administering a SpA variant domain D polypeptide with any combination or permutation of bacterial proteins described herein. In a preferred embodiment the composition is not a staphylococcus bacterium. In certain aspects the subject is a human or a cow. In a further aspect the composition is formulated in a pharmaceutically acceptable formulation. The staphylococci may be *Staphylococcus aureus*.

[0027] Yet still further embodiments include vaccines comprising a pharmaceutically acceptable composition having an isolated SpA variant polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein, wherein the composition is capable of stimulating an immune response against a staphylococcus bacterium. The vaccine may comprise an isolated SpA variant polypeptide, or any other combination or permutation of protein(s) or peptide(s) described. In certain aspects of the invention the isolated SpA variant polypeptide, or any other combination or permutation of protein(s) or peptide(s) described are multimerized, *e.g.*, dimerized or concatamerized. In a further aspect, the vaccine composition is contaminated by less than about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, 0.25, 0.05% (or any range derivable therein) of other Staphylococcal proteins. A composition may further comprise an isolated non-SpA polypeptide. Typically the vaccine comprises an adjuvant. In certain aspects a protein or peptide of the invention is linked (covalently or non-covalently) to the adjuvant, preferably the adjuvant is chemically conjugated to the protein.

[0028] In still yet further embodiments, a vaccine composition is a pharmaceutically acceptable composition having a recombinant nucleic acid encoding all or part of a SpA variant polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein, wherein the composition is capable of stimulating an immune response against a staphylococcus bacteria. The vaccine composition may comprise a recombinant nucleic acid encoding all or part of a SpA variant polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein. In certain embodiments the recombinant nucleic acid contains a heterologous promoter. Preferably the recombinant nucleic acid is a vector. More preferably the vector is a plasmid or a viral vector. In some aspects the vaccine includes a recombinant, non-staphylococcus bacterium containing the nucleic acid. The recombinant non-staphylococci may be Salmonella or another gram-positive bacteria. The vaccine may comprise a pharmaceutically acceptable excipient, more preferably an adjuvant.

[0029] Still further embodiments include methods for stimulating in a subject a protective or therapeutic immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition of a

SpA variant polypeptide or segment/fragment thereof and further comprising one or more of a Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, or vWh protein or peptide thereof. In a preferred embodiment the composition comprises a non-staphylococcus bacterium. In a further aspect the composition is formulated in a pharmaceutically acceptable formulation. The staphylococci for which a subject is being treated may be *Staphylococcus aureus*. Methods of the invention also include SpA variant compositions that contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more secreted virulence factors and/or cell surface proteins, such as Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, or vWh in various combinations. In certain aspects a vaccine formulation includes Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, and vWh. In certain aspects an antigen combination can include (1) a SpA variant and IsdA; (2) SpA variant and ClfB; (3) SpA variant and SdrD; (4) SpA variant and Hla or Hla variant; (5) SpA variant and ClfB, SdrD, and Hla or Hla variant; (6) SpA variant, IsdA, SdrD, and Hla or Hla variant; (7) SpA variant, IsdA, ClfB, and Hla or Hla variant; (8) SpA variant, IsdA, ClfB, and SdrD; (9) SpA variant, IsdA, ClfB, SdrD and Hla or Hla variant; (10) SpA variant, IsdA, ClfB, and SdrD; (11) SpA variant, IsdA, SdrD, and Hla or Hla variant; (12) SpA variant, IsdA, and Hla or Hla variant; (13) SpA variant, IsdA, ClfB, and Hla or Hla variant; (14) SpA variant, ClfB, and SdrD; (15) SpA variant, ClfB, and Hla or Hla variant; or (16) SpA variant, SdrD, and Hla or Hla variant.

[0030] In certain aspects, a bacterium delivering a composition of the invention will be limited or attenuated with respect to prolonged or persistent growth or abscess formation. In yet a further aspect, SpA variant(s) can be overexpressed in an attenuated bacterium to further enhance or supplement an immune response or vaccine formulation.

[0031] Certain embodiments are directed to methods for eliciting an immune response against a staphylococcus bacterium in a subject comprising providing to the subject an effective amount of a peptide comprising a coagulase polypeptide or an immunogenic segment thereof having an amino acid sequence that is at least 80, 85, 90, 95, 98, to 100% identical to SEQ ID NO:27 or a segment thereof or at least 80, 85,

90, 95, 98, to 100% identical to amino acids 27-508 of SEQ ID NO:32 or a segment thereof.

[0032] In certain aspects, the subject is provided with an effective amount of an coagulase polypeptide by administering to the subject a composition comprising: (i) an isolated coagulase polypeptide or segment thereof having an amino acid sequence that is at least 90% identical to SEQ ID NO:27 or a segment thereof or is at least 90% identical to amino acids 27-508 of SEQ ID NO:32 or a segment thereof; or (ii) at least one isolated recombinant nucleic acid molecule encoding a coagulase polypeptide or a segment thereof having an amino acid sequence that is at least 90% identical to SEQ ID NO:27 or a segment thereof or is at least 90% identical to amino acids 27-508 of SEQ ID NO:32 or a segment thereof. In a further aspect, the composition comprises an isolated coagulase polypeptide having the amino acid sequence of SEQ ID NO:27 or the amino acid sequence of amino acids 27-508 of SEQ ID NO:32.

[0033] Certain embodiments are directed to methods for treating a staphylococcal infection in a subject comprising providing to a subject having or suspected of having or at risk of developing a staphylococcal infection an effective amount of an isolated peptide comprising a coagulase polypeptide having an amino acid sequence that is at least 80, 85, 90, 95, 98, to 100% identical to SEQ ID NO:27 or is at least 80, 85, 90, 95, 98, to 100% identical to amino acids 27-508 of SEQ ID NO:32. In a particular aspect, the coagulase polypeptide has an amino acid sequence of SEQ ID NO:27 or has an amino acid identical to amino acids 27-508 of SEQ ID NO:32. In certain aspects, the subject is diagnosed with a persistent staphylococcal infection. In a frther aspect, the coagulase polypeptide elicits production of an antibody that binds Coa or vWbpvWh in the subject.

[0034] Embodiments include methods of preventing or treating staphylococcal infection comprising the step of administering an immunogenic composition comprising a Staphylococcal coagulase or an immunogenic segment thereof.

[0035] Certain embodiments are directed to methods of preparing an immunoglobulin for use in prevention or treatment of staphylococcal infection comprising the steps of immunizing a recipient with a coagulase polypeptide and isolating immunoglobulin from the recipient.

[0036] A further embodiment is directed to an immunoglobulin prepared by the method described herein.

[0037] A further embodiment is directed to methods for treatment or prevention of staphylococcal infection comprising a step of administering to a patient an effective amount of pharmaceutical preparation of immunoglobulin that binds a coagulase.

[0038] Other embodiments are directed to a use of the pharmaceutical preparation of coagulase immunoglobulins in the manufacture of a medicament for the treatment or prevention of staphylococcal infection.

[0039] Yet still further embodiments include vaccines comprising a pharmaceutically acceptable composition having an isolated coagulase polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein, wherein the composition is capable of stimulating an immune response against a staphylococcus bacterium. The vaccine may comprise an isolated coagulase polypeptide, or any other combination or permutation of protein(s) or peptide(s) described. In certain aspects of the invention the isolated coagulase polypeptide, or any other combination or permutation of protein(s) or peptide(s) described are multimerized, *e.g.*, dimerized or concatamerized. In a further aspect, the vaccine composition is contaminated by less than about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, 0.25, 0.05% (or any range derivable therein) of other Staphylococcal proteins. A composition may further comprise an isolated non-coagulase polypeptide. Typically the vaccine comprises an adjuvant. In certain aspects a protein or peptide of the invention is linked (covalently or non-covalently) to the adjuvant, preferably the adjuvant is chemically conjugated to the protein.

[0040] In still yet further embodiments, a vaccine composition is a pharmaceutically acceptable composition having a recombinant nucleic acid encoding all or part of a coagulase polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein, wherein the composition is capable of stimulating an immune response against a staphylococcus bacterium. The vaccine composition may comprise a recombinant nucleic acid encoding all or part of a coagulase polypeptide, or any other combination or permutation of protein(s) or peptide(s) described herein. In certain embodiments the recombinant nucleic acid

contains a heterologous promoter. Preferably the recombinant nucleic acid is a vector. More preferably the vector is a plasmid or a viral vector. In some aspects the vaccine includes a recombinant, non-staphylococcus bacterium containing the nucleic acid. The recombinant non-staphylococci may be Salmonella or another gram-positive bacteria. The vaccine may comprise a pharmaceutically acceptable excipient, more preferably an adjuvant.

[0041] Still further embodiments include methods for stimulating in a subject a protective or therapeutic immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition of a coagulase polypeptide or segment/fragment thereof and further comprising one or more of a Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, or vWh protein or peptide thereof. In a preferred embodiment the composition comprises a non-staphylococcus bacterium. In a further aspect the composition is formulated in a pharmaceutically acceptable formulation. The staphylococci for which a subject is being treated may be *Staphylococcus aureus*. Methods of the invention also include coagulase compositions that contain one or more of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more secreted virulence factors and/or cell surface proteins, such as Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, SpA and variants thereof, vWbp, or vWh in various combinations. In certain aspects a vaccine formulation includes Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, and vWh. In certain aspects an antigen combination can include (1) a Coa and/or vWbp, and IsdA; (2) a Coa and/or vWbp, and ClfB; (3) a Coa and/or vWbp, and SdrD; (4) a Coa and/or vWbp, and Hla or Hla variant; (5) a Coa and/or vWbp, and ClfB, SdrD, and Hla or Hla variant; (6) a Coa and/or vWbp, and IsdA, SdrD, and Hla or Hla variant; (7) a Coa and/or vWbp, and IsdA, ClfB, and Hla or Hla variant; (8) a Coa and/or vWbp, and IsdA, ClfB, and SdrD; (9) a Coa and/or vWbp, and IsdA, ClfB, SdrD and Hla or Hla variant; (10) a Coa and/or vWbp, and IsdA, ClfB, and SdrD; (11) a Coa and/or vWbp, and IsdA, SdrD, and Hla or Hla variant; (12) a Coa and/or vWbp, and IsdA, and Hla or Hla variant; (13) a Coa and/or vWbp, and IsdA, ClfB, and Hla or Hla variant; (14) a Coa and/or vWbp, and ClfB, and SdrD; (15) a Coa and/or vWbp, and

ClfB, and Hla or Hla variant; or (16) a Coa and/or vWbp, and SdrD, and Hla or Hla variant.

[0042] The term "EsxA protein" refers to a protein that includes isolated wild-type EsxA polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria EsxA proteins.

[0043] The term "EsxB protein" refers to a protein that includes isolated wild-type EsxB polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria EsxB proteins.

[0044] The term "SdrD protein" refers to a protein that includes isolated wild-type SdrD polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria SdrD proteins.

[0045] The term "SdrE protein" refers to a protein that includes isolated wild-type SdrE polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria SdrE proteins.

[0046] The term "IsdA protein" refers to a protein that includes isolated wild-type IsdA polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria IsdA proteins.

[0047] The term "IsdB protein" refers to a protein that includes isolated wild-type IsdB polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria IsdB proteins.

[0048] The term "Eap protein" refers to a protein that includes isolated wild-type Eap polypeptides from staphylococcus bacteria and segments thereof, as well as

variants that stimulate an immune response against staphylococcus bacteria Eap proteins.

[0049] The term "Ebh protein" refers to a protein that includes isolated wild-type Ebh polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria Ebh proteins.

[0050] The term "Emp protein" refers to a protein that includes isolated wild-type Emp polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria Emp proteins.

[0051] The term "EsaB protein" refers to a protein that includes isolated wild-type EsaB polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria EsaB proteins.

[0052] The term "EsaC protein" refers to a protein that includes isolated wild-type EsaC polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria EsaC proteins.

[0053] The term "SdrC protein" refers to a protein that includes isolated wild-type SdrC polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria SdrC proteins.

[0054] The term "ClfA protein" refers to a protein that includes isolated wild-type ClfA polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria ClfA proteins.

[0055] The term "ClfB protein" refers to a protein that includes isolated wild-type ClfB polypeptides from staphylococcus bacteria and segments thereof, as well as

variants that stimulate an immune response against staphylococcus bacteria ClfB proteins.

[0056] The term "Coa protein" refers to a protein that includes isolated wild-type Coa polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria Coa proteins.

[0057] The term "Hla protein" refers to a protein that includes isolated wild-type Hla polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria Hla proteins.

[0058] The term "IsdC protein" refers to a protein that includes isolated wild-type IsdC polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria IsdC proteins.

[0059] The term "SasF protein" refers to a protein that includes isolated wild-type SasF polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria SasF proteins.

[0060] The term "vWbp protein" refers to a protein that includes isolated wild-type vWbp (von Willebrand factor binding protein) polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria vWbp proteins.

[0061] The term "vWh protein" refers to a protein that includes isolated wild-type vWh (von Willebrand factor binding protein homolog) polypeptides from staphylococcus bacteria and segments thereof, as well as variants that stimulate an immune response against staphylococcus bacteria vWh proteins.

[0062] An immune response refers to a humoral response, a cellular response, or both a humoral and cellular response in an organism. An immune response can be measured by assays that include, but are not limited to, assays measuring the presence

or amount of antibodies that specifically recognize a protein or cell surface protein, assays measuring T-cell activation or proliferation, and/or assays that measure modulation in terms of activity or expression of one or more cytokines.

[0063] In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an EsxA protein. In certain aspects the EsxA protein will have all or part of the amino acid sequence of SEQ ID NO:11.

[0064] In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an EsxB protein. In certain aspects the EsxB protein will have all or part of the amino acid sequence of SEQ ID NO:12.

[0065] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an SdrD protein. In certain aspects the SdrD protein will have all or part of the amino acid sequence of SEQ ID NO:13.

[0066] In further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an SdrE protein. In certain aspects the SdrE protein will have all or part of the amino acid sequence of SEQ ID NO:14.

[0067] In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an IsdA protein. In certain aspects the IsdA protein will have all or part of the amino acid sequence of SEQ ID NO:15.

[0068] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an IsdB protein. In certain aspects the IsdB protein will have all or part of the amino acid sequence of SEQ ID NO:16.

[0069] Embodiments of the invention include compositions that include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a EsaB protein. In certain aspects the EsaB protein will have all or part of the amino acid sequence of SEQ ID NO:17.

[0070] In a further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a ClfB protein. In certain aspects the ClfB protein will have all or part of the amino acid sequence of SEQ ID NO:18.

[0071] In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an IsdC protein. In certain aspects the IsdC protein will have all or part of the amino acid sequence of SEQ ID NO:19.

[0072] In yet further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a SasF protein. In certain aspects the SasF protein will have all or part of the amino acid sequence of SEQ ID NO:20.

[0073] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a SdrC protein. In certain aspects the SdrC protein will have all or part of the amino acid sequence of SEQ ID NO:21.

[0074] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a ClfA protein. In certain aspects the ClfA protein will have all or part of the amino acid sequence of SEQ ID NO:22.

[0075] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an Eap protein. In certain

aspects the Eap protein will have all or part of the amino acid sequence of SEQ ID NO:23.

[0076] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an Ebh protein. In certain aspects the Ebh protein will have all or part of the amino acid sequence of SEQ ID NO:24.

[0077] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an Emp protein. In certain aspects the Emp protein will have all or part of the amino acid sequence of SEQ ID NO:25.

[0078] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to an EsaC protein. In certain aspects the EsaC protein will have all or part of the amino acid sequence of SEQ ID NO:26. Sequence of EsaC polypeptides can be found in the protein databases and include, but are not limited to accession numbers ZP_02760162 (GI:168727885), NP_645081.1 (GI:21281993), and NP_370813.1 (GI:15923279), each of which is incorporated herein by reference as of the priority date of this application.

[0079] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a Coa protein. In certain aspects the Coa protein will have all or part of the amino acid sequence of SEQ ID NO:27.

[0080] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a Hla protein. In certain aspects the Hla protein will have all or part of the amino acid sequence of SEQ ID NO:28.

[0081] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a vWa protein. In certain aspects the vWa protein will have all or part of the amino acid sequence of SEQ ID NO:29.

[0082] In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical or similar to a vWbp protein. In certain aspects the vWbp protein will have all or part of the amino acid sequence of SEQ ID NO:32.

[0083] In certain aspects, a polypeptide or segment/fragment can have a sequence that is at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% or more identical to the amino acid sequence of the reference polypeptide. The term "similarity" refers to a polypeptide that has a sequence that has a certain percentage of amino acids that are either identical with the reference polypeptide or constitute conservative substitutions with the reference polypeptides.

[0084] The polypeptides described herein may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more variant amino acids within at least, or at most 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 300, 400,

500, 550, 1000 or more contiguous amino acids, or any range derivable therein, of SEQ ID NO:2-30, or SEQ ID NO:32-34.

[0085] A polypeptide segment as described herein may include 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 300, 400, 500, 550, 1000 or more contiguous amino acids, or any range derivable therein, of SEQ ID NO:2-30, or SEQ ID NO:33-34.

[0086] The compositions may be formulated in a pharmaceutically acceptable composition. In certain aspects of the invention the staphylococcus bacterium is an *S. aureus* bacterium.

[0087] In further aspects, a composition may be administered more than one time to the subject, and may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or more times. The administration of the compositions include, but is not limited to oral, parenteral, subcutaneous, intramuscular, intravenous, or various combinations thereof, including inhalation or aspiration.

[0088] In still further embodiments, a composition comprises a recombinant nucleic acid molecule encoding a polypeptide described herein or segments/fragments thereof. Typically a recombinant nucleic acid molecule encoding a polypeptide described herein contains a heterologous promoter. In certain aspects, a recombinant nucleic acid molecule of the invention is a vector, in still other aspects the vector is a

plasmid. In certain embodiments the vector is a viral vector. In certain aspects a composition includes a recombinant, non-staphylococcus bacterium containing or expressing a polypeptide described herein. In particular aspects the recombinant non-staphylococcus bacteria is *Salmonella* or another gram-positive bacteria. A composition is typically administered to mammals, such as human subjects, but administration to other animals that are capable of eliciting an immune response is contemplated. In further aspects the staphylococcus bacterium containing or expressing the polypeptide is *Staphylococcus aureus*. In further embodiments the immune response is a protective immune response.

[0089] In further embodiments a composition comprises a recombinant nucleic acid molecule encoding all or part of one or more of a Eap, Ebh, Emp, EsaB, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, SpA, vWbp, or vWh protein or peptide or variant thereof. Additional staphylococcal antigens that can be used in combination with the polypeptides described herein include, but are not limited to 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein. In particular aspects, a bacteria is a recombinant non-staphylococcus bacteria, such as a *Salmonella* or other gram-positive bacteria.

[0090] Compositions of the invention are typically administered to human subjects, but administration to other animals that are capable of eliciting an immune response to a staphylococcus bacterium is contemplated, particularly cattle, horses, goats, sheep and other domestic animals, *i.e.*, mammals.

[0091] In certain aspects the staphylococcus bacterium is a *Staphylococcus aureus*. In further embodiments the immune response is a protective immune response. In still further aspects, the methods and compositions of the invention can be used to prevent, ameliorate, reduce, or treat infection of tissues or glands, *e.g.*, mammary glands, particularly mastitis and other infections. Other methods include, but are not limited to prophylactically reducing bacterial burden in a subject not exhibiting signs of infection, particularly those subjects suspected of or at risk of being colonized by a target bacteria, *e.g.*, patients that are or will be at risk or susceptible to infection during a hospital stay, treatment, and/or recovery.

[0092] Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well. In particular, any embodiment discussed in the context of a SpA variant polypeptide or peptide or nucleic acid may be implemented with respect to other antigens, such as Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, vWh, 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein (or nucleic acids), and vice versa. It is also understood that any one or more of Eap, Ebh, Emp, EsaC, EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, Coa, Hla, IsdC, SasF, vWbp, vWh, 52kDa vitronectin binding protein (WO 01/60852), Aaa, Aap, Ant, autolysin glucosaminidase, autolysin amidase, Cna, collagen binding protein (US6288214), EFB (FIB), Elastin binding protein (EbpS), EPB, FbpA, fibrinogen binding protein (US6008341), Fibronectin binding protein (US5840846), FnbA, FnbB, GehD (US 2002/0169288), HarA, HBP, Immunodominant ABC transporter, IsaA/PisA, laminin receptor, Lipase GehD, MAP, Mg2+ transporter, MHC II analogue (US5648240), MRPII, Npase, RNA III activating

protein (RAP), SasA, SasB, SasC, SasD, SasK,SBI, SdrF(WO 00/12689), SdrG / Fig (WO 00/12689), SdrH (WO 00/12689), SEA exotoxins (WO 00/02523), SEB exotoxins (WO 00/02523), SitC and Ni ABC transporter, SitC/MntC/saliva binding protein (US5,801,234), SsaA, SSP-1, SSP-2, and/or Vitronectin binding protein can be specifically excluded from a claimed composition.

[0093] Embodiments of the invention include compositions that contain or do not contain a bacterium. A composition may or may not include an attenuated or viable or intact staphylococcal bacterium. In certain aspects, the composition comprises a bacterium that is not a staphylococcal bacterium or does not contain staphylococcal bacteria. In certain embodiments a bacterial composition comprises an isolated or recombinantly expressed staphylococcal Protein A variant or a nucleotide encoding the same. The composition may be or include a recombinantly engineered staphylococcus bacterium that has been altered in a way that comprises specifically altering the bacterium with respect to a secreted virulence factor or cell surface protein. For example, the bacteria may be recombinantly modified to express more of the virulence factor or cell surface protein than it would express if unmodified.

[0094] The term "isolated" can refer to a nucleic acid or polypeptide that is substantially free of cellular material, bacterial material, viral material, or culture medium (when produced by recombinant DNA techniques) of their source of origin, or chemical precursors or other chemicals (when chemically synthesized). Moreover, an isolated compound refers to one that can be administered to a subject as an isolated compound; in other words, the compound may not simply be considered "isolated" if it is adhered to a column or embedded in an agarose gel. Moreover, an "isolated nucleic acid fragment" or "isolated peptide" is a nucleic acid or protein fragment that is not naturally occurring as a fragment and/or is not typically in the functional state.

[0095] Moieties of the invention, such as polypeptides, peptides, antigens, or immunogens, may be conjugated or linked covalently or noncovalently to other moieties such as adjuvants, proteins, peptides, supports, fluorescence moieties, or labels. The term "conjugate" or "immunoconjugate" is broadly used to define the operative association of one moiety with another agent and is not intended to refer solely to any type of operative association, and is particularly not limited to chemical "conjugation." Recombinant fusion proteins are particularly contemplated.

Compositions of the invention may further comprise an adjuvant or a pharmaceutically acceptable excipient. An adjuvant may be covalently or non-covalently coupled to a polypeptide or peptide of the invention. In certain aspects, the adjuvant is chemically conjugated to a protein, polypeptide, or peptide.

[0096] The term "providing" is used according to its ordinary meaning to indicate "to supply or furnish for use." In some embodiments, the protein is provided directly by administering the protein, while in other embodiments, the protein is effectively provided by administering a nucleic acid that encodes the protein. In certain aspects the invention contemplates compositions comprising various combinations of nucleic acid, antigens, peptides, and/or epitopes.

[0097] The subject will have (*e.g.*, are diagnosed with a staphylococcal infection), will be suspected of having, or will be at risk of developing a staphylococcal infection. Compositions of the present invention include immunogenic compositions wherein the antigen(s) or epitope(s) are contained in an amount effective to achieve the intended purpose. More specifically, an effective amount means an amount of active ingredients necessary to stimulate or elicit an immune response, or provide resistance to, amelioration of, or mitigation of infection. In more specific aspects, an effective amount prevents, alleviates or ameliorates symptoms of disease or infection, or prolongs the survival of the subject being treated. Determination of the effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. For any preparation used in the methods of the invention, an effective amount or dose can be estimated initially from *in vitro* studies, cell culture, and/or animal model assays. For example, a dose can be formulated in animal models to achieve a desired immune response or circulating antibody concentration or titer. Such information can be used to more accurately determine useful doses in humans.

[0098] The embodiments in the Example section are understood to be embodiments of the invention that are applicable to all aspects of the invention.

[0099] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives

and "and/or." It is also contemplated that anything listed using the term "or" may also be specifically excluded.

[00100] Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[00101] Following long-standing patent law, the words "a" and "an," when used in conjunction with the word "comprising" in the claims or specification, denotes one or more, unless specifically noted.

[00102] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments are given by way of illustration only, since various changes and modifications will become apparent to those skilled in the art from this detailed description.

## DESCRIPTION OF THE DRAWINGS

[00103] So that the matter in which the above-recited features, advantages and objects of the invention as well as others which will become clear are attained and can be understood in detail, more particular descriptions and certain embodiments of the invention briefly summarized above are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate certain embodiments of the invention and therefore are not to be considered limiting in their scope.

[00104] **FIGs. 1A – 1E Generation of a non-toxigenic protein A vaccine.** FIG. 1A Translational protein A (SpA) product of *S. aureus* Newman and USA300 LAC with an N-terminal signal peptide (white box), five immunoglobulin binding domains (IgBDs designated E, D, A, B and C), variable region X and C-terminal sorting signal (black box). FIG. 1B, Amino acid sequence of the five IgBDs as well as nontoxigenic SpA-$D_{KKAA}$, with the positions of triple α-helical bundles (H1, H2 and H3) as well as glutamine (Q) 9, 10 and aspartate (D) 36, 37 indicated. FIG. 1C, Coomassie Blue-stained SDS-PAGE of SpA, SpA-D, SpA-$D_{KKAA}$ or SrtA purified on Ni-NTA

sepharose in the presence or absence of human immunoglobulin (hIgG). FIG. 1D, ELISA examining the association of immobilized SpA, SpA-D or SpA-$D_{KKAA}$ with human IgG as well as its Fc or $F(ab)_2$ fragments and von Willebrand factor (vWF). FIG. 1E, CD19+ B lymphocytes in splenic tissue of BALB/c mice that had been mock immunized or treated with SpA-D or SpA-$D_{KKAA}$ were quantified by FACS.

[00105] **FIG. 2** Non-toxigenic protein A vaccine prevents abscess formation. Histopathology of renal tissue isolated during necropsy of BALB/c mice that had been mock immunized (PBS) or vaccinated with SpA, SpA-D as well as SpA-$D_{KKAA}$ and challenged with *S. aureus* Newman. Thin sectioned tissues were stained with hematoxylin-eosin. White arrows identify polymorphonuclear leukocyte (PMN) infiltrates. Dark arrows identify staphylococcal abscess communities.

[00106] **FIGs. 3A-C** Antibodies raised by the non-toxigenic protein A vaccine block the B cell superantigen function of SpA. FIG 3A, Rabbit antibodies raised against SpA-$D_{KKAA}$ were purified on a matrix with immobilized antigen and analyzed by Coomassie Blue-stained SDS-PAGE. Antibodies were cleaved with pepsin and F(ab)2 fragments were purified by a second round of affinity chromatography on SpA-$D_{KKAA}$ matrix. FIG 3B, SpA-$D_{KKAA}$ specific F(ab)2 interfere with the binding of SpA or SpA-D to human immunoglobulin (hIgG) or, FIG 3C, to von Willebrand Factor (vWF).

[00107] **FIGs. 4A-D** Full-length non-toxigenic protein A generates improved immune responses. FIG 4A, Full-length $SpA_{KKAA}$ was purified on Ni-NTA sepharose and analyzed by Coomassie-Blue stained SDS-PAGE. FIG 4B, CD19+ B lymphocytes in splenic tissue of BALB/c mice that had been mock immunized or treated with SpA or $SpA_{KKAA}$ were quantified by FACS. FIG 4C, ELISA examining the association of immobilized SpA or $SpA_{KKAA}$ with human IgG as well as its Fc or F(ab)2 fragments or von Willebrand factor (vWF). FIG 4D, Human or mouse serum antibody titers to diphtheria toxoid (CRM197) and non-toxigenic $SpA_{KKAA}$ or SpA-$D_{KKAA}$. Human volunteers with a history of DTaP immunization and staphylococcal infection (n=16) as well as mice (n=20) that had been infected with *S. aureus* Newman or USA 300 LAC or immunized with $SpA_{KKAA}$ or SpA-$D_{KKAA}$ were examined by quantitative dot blot.

[00108] **FIG. 5** Protein A is required for the pathogenesis of lethal *S. aureus* infections in mice. Cohorts of BALB/c mice (n=8) were injected with suspensions of $2 \times 10^8$ CFU S. aureus Newman or its isogenic protein A deletion variant (Δspa) in PBS. Infected animals were monitored for survival over a period of 15 days.

[00109] **FIGs. 6A-B** Antibodies against protein A protect mice against lethal *S. aureus* infections. FIG 6A Cohorts of BALB/c mice (n=10) were injected with 5 mg $kg^{-1}$ affinity purified rabbit IgG specific for $SpA_{KKAA}$ (α-$Sp_{AKKAA}$) or the plague vaccine antigen rV10 (DeBord *et al.*, 2006) (mock). Four hours later, each animal was infected by intraperitoneal injection with a suspension of $3 \times 10^8$ CFU *S. aureus* Newman and monitored for survival over a period of 10 days. Data are representative of three independent experiments FIG 6B Cohorts of BALB/c mice (n=10) were prime-booster immunized with $SpA_{KKAA}$ or PBS/adjuvant control (mock). Each animal was subsequently infected by intraperitoneal injection with a suspension of $6 \times 10^8$ CFU *S. aureus* Newman and monitored for survival over a period of 10 days. Statistical significance (P) was analyzed with the unpaired two-tailed log-rank test. Data are representative of all three independent experiments.

[00110] **FIG. 7** $SpA_{KKAA}$ immunization protects mice against challenge with the vancomycin-resistant MRSA isolated Mu50. Cohorts of BALB/c mice (n=15) were prime-booster immunized with $SpA_{KKAA}$ or PBS/adjuvant control (mock). Each animal was subsequently infected by intravenous injection with a suspension of $3 \times 10^7$ CFU *S. aureus* Mu50. Staphylococcal load, calculated as log10 CFU $g^{-1}$, was determined in homogenized renal tissues 4 days following infection. Statistical significance was calculated with the unpaired two-tailed Students t-test and P-value recorded.

[00111] **FIGs. 8A-B** Lack of protective immune responses to staphylococcal infections. FIG 8A Staphylococcal infection does not generate protective immunity. BALB/c mice (n=10) were infected with *S. aureus* Newman or mock challenged (PBS) for thirty days and infection cleared with chloramphenicol treatment. Both cohorts of animals were then challenged with *S. aureus* Newman and bacterial load (CFU) in kidney tissue homogenate analyzed following necropsy on day 4. Data are representative of three independent analyses. FIG 8B IsdB immunization does not protect mice against *S. aureus* USA300 (LAC) challenge. BALB/c mice (n=10) were

immunized with IsdB (100 μg IsdB emulsified in CFA followed by IFA/IsdB booster on day 11) and challenged by retro-orbital injection with $5\times10^6$ CFU *S. aureus* USA300 (LAC) on day 21. Four days following challenge, kidneys were removed during necropsy and staphylococcal load per gram of homogenized tissue enumerated by colony formation on agar plates. Compared to mock immunized (PBS/adjuvant) animals with 6.93 (±0.24) log10 CFU $g^{-1}$, IsdB vaccination was associated with 6.25 (±0.46) log10 CFU $g^{-1}$ and did not generate statistically significant protection (P=0.2138, two-tailed Student's t-test) from USA300 (LAC) challenge. Data are representative of three independent analyses.

[00112] **FIG. 9** Comparison of abscess formation in mice treated with PBS, SpA, SpA-D and SpA-$D_{KKAA}$.

[00113] **FIGs. 10A-10H** Localization of prothrombin, fibrinogen, coagulase (Coa), and von Willebrand factor binding protein (vWbp) in staphylococcal abscesses. BALB/c mice infected by intravenous inoculation with $1 \times 10^7$ CFU *S. aureus* Newman were killed 5 days post infection. Kidneys were removed, embedded in paraffin, thin-sectioned and stained by immunochemistry using rabbit antibodies (α) specific for mouse prothrombin (FIG. 10A, 10C), mouse fibrinogen / fibrin (FIG. 10B, 10D), *S. aureus* Coa (FIG. 10E, 10G) or *S. aureus* vWbp (FIG. 10F, 10H). Displayed images are representative of three sampled kidneys. Panels FIG. 10C, 10D, 10G, and 10H illustrate antibody staining within a single abscess analyzed as four sequential sections, enlarged from an area in panels FIG. 10A, 10B, 10E, and 10F that is defined by box with white margins.

[00114] **FIGs. 11A-11C** *Staphylococcus aureus coa* and *vWbp* mutants display defects in blood clotting. (FIG. 11A) Diagram illustrating the primary translational product of coa and vWbp including signal peptide (S), the D1 and D2 domain from prothrombin binding, a domain of unknown function, von Willebrand factor (vWF) binding site on vWbp, and the fibrinogen binding repeats (R) of Coa. Numbers indicate amino acid residues. (FIG. 11B) Culture supernatants from *S. aureus* Newman (wild-type) or isogenic variants lacking *coa* (Δ*coa*), vWbp (Δ*vWbp*) or both genes (Δ*coa*, Δ*vWbp*) were examined by immunoblotting with antibodies specific for Coa (αCoa) or vWbp (αvWbp). For complementation studies, plasmids expressing the wild-type alleles of *coa* (p*coa*) or *vWbp* (p*vWbp*) were electroporated into

staphylococcal strains and subsequently analyzed by immunoblotting. (FIG. 11C) Lepirudin-treated mouse blood was mock treated or infected with *S. aureus* Newman or its isogenic coagulase variants and incubated for up to 48 hours at 25°C. Tubes were tilted to assess for coagulation. Data are representative of four independent determinations.

[00115] **FIGs. 12A-12R** Contributions of *coa* and *vWbp* to bacterial survival in blood and *S. aureus* induced lethal bacteremia of mice. (FIG. 12A) Staphylococcal strains Newman, Δcoa, ΔvWbp or Δcoa, ΔvWbp and the complemented variants were incubated with lepirudin anticoagulated mouse blood for 30 minutes and bacterial survival assessed by colony formation on agar plates. Data were generated from three separate trials. (FIG. 12B) Cohorts of 10 mice were injected into the retro-orbital plexus with $1\times10^8$ CFU of *S. aureus* Newman (wild-type) as well as Δcoa, ΔvWbp or Δcoa, ΔvWbp. Animal survival over time was recorded over 10 days. Similar to B, mice were given $1x10^7$ CFU of staphylococcal strains Newman (FIG. 12C, E and K, M), ΔvWbp (FIG. 14D, F and M, L), Δcoa (FIG. 14G, I and O, Q) or Δcoa, ΔvWbp (FIG. 12H, J and P, R), harvested on days 5 (FIG. 12C-J) or 15 (FIG. 12K-R) and assessed for bacterial load in the renal tissue (Table 7) and histopathological abscess formation. All animal data are representative of two independent experiments.

[00116] **FIGs. 13A-13D** Antibodies against Coa and vWbp block the clotting of blood by staphylococcal coagulases. (FIG. 13A) $His_6$-Coa and $His_6$-vWbp were purified by affinity chromatography from *E. coli* and analyzed on Coomassie-stained SDS-PAGE. (FIG. 13B) Rabbit antibodies raised against $His_6$-Coa or $His_6$-vWbp were affinity purified and analyzed by ELISA for immune reactivity with purified coagulases. Data are averaged from three independent experimental determinations. (FIG. 13C) Lepirudin-treated mouse blood was treated with PBS (mock), irrelevant antibodies (αV10) or antibodies directed against Coa (αCoa), vWbp (αvWbp) or both coagulases (αCoa/ αvWbp) prior to infection with *S. aureus* Newman and incubation for 48 hours at 25°C. (FIG. 13D) Lepirudin-treated mouse blood was treated with antibodies as above. Blood samples were then incubated with functionally active Coa or vWbp and coagulation time recorded.

[00117] **FIGs. 14A-14F** Biological effects of antibodies directed against staphylococcal coagulases. Surface plasmon resonance measurement of antibody

perturbing the association between Coa or vWbp and prothrombin or fibrinogen. Response differences upon addition of coagulase (Coa) to either prothrombin (FIG. 14A) or fibrinogen (FIG. 14B) were compared to response differences in the presence of increasing amounts of antibodies (αCoa - 1:1, 1:2, 1:4, 1:8). Response differences upon addition of vWbp to either prothrombin (FIG. 16A) or fibrinogen (FIG. 14B) were compared to response differences in the presence of increasing amounts of antibodies (αvWbp - 1:1, 1:2, 1:4, 1:8). (FIG. 14E, F) Purified active Coa or vWbp was incubated in a 1:1 molar ratio with human prothrombin. The enzymatic ability of the complex was assessed by monitoring the rate of S-2238 cleavage (fibrinogen substitute chromogenic substrate, given in excess). The assay was repeated in presence of specific or cross antibodies added in 3M excess and the data was normalized to the % average activity without inhibition. Data are an average of three independent trials.

[00118] **FIG. 15** Contribution of coagulase specific antibodies to the survival of mice with staphylococcal bacteremia. Twenty-four hours prior to infection, BALB/c mice (n=15) were injected into the peritoneum with purified rabbit antibodies (5 mg antibody/kg body weight). Animals were then challenged with $1 \times 10^8$ CFU *S. aureus* Newman injected into the retro-orbital plexus and monitored for survival. Data are representative of two independent experiments.

[00119] **FIGs. 16A-16H** Passive transfer of coagulase antibodies confers protection against *S. aureus* abscess formation. An experimental mock (PBS, FIG. 18A and 18C) or purified rabbit antibodies directed against vWbp (αvWbp, FIG. 18B and 18D), Coa (αCoa, FIG. 18E and 18G) or both coagulases (αCoa / αvWbp, FIG. 18F and 18H) were injected into the peritoneal cavity of BALB/c mice (n=10) and antibody titers analyzed by ELISA (Table 8). Passively immunized animals were infected by injecting 1 x $10^7$CFU *S. aureus* Newman into the retro-orbital plexus. Bacterial load and abscess formation were determined following necropsy in the kidneys of animals that had been killed five days following infection. Renal tissues were fixed with paraformaldehyde, embedded in paraffin, thin sectioned, stained with hematoxylin-eosin and histopathology images acquired by light microscopy. Data are representative of two separate experiments.

[00120] **FIG. 17s A-H** Immunization with coagulases protects mice against *S. aureus* abscess formation. BALB/c mice (n=15) were immunized with 50 μg $His_6$-Coa, $His_6$-vWbp, $His_6$-Coa and $His_6$-vWbp or mock (PBS) emulsified with adjuvant on day 0 and 11 and antibody titers analyzed by ELISA on day 21 (Table 8). On day 21, animals were challenged by injecting 1 x $10^7$CFU *S. aureus* Newman into the retro-orbital plexus. Bacterial load and abscess formation were determined following necropsy in the kidneys of animals that had been killed five days following infection. Renal tissues were fixed with paraformaldehyde, embedded in paraffin, thin sectioned, stained with hematoxylin-eosin and histopathology images acquired by light microscopy. Data are representative of two separate experiments.

## DETAILED DESCRIPTION

[00121] *Staphylococcus aureus* is a commensal of the human skin and nares, and the leading cause of bloodstream, skin and soft tissue infections (Klevens *et al.*, 2007). Recent dramatic increases in the mortality of staphylococcal diseases are attributed to the spread of methicillin-resistant *S. aureus* (MRSA) strains often not susceptible to antibiotics (Kennedy *et al.*, 2008). In a large retrospective study, the incidence of MRSA infections was 4.6% of all hospital admissions in the United States (Klevens *et al.*, 2007). The annual health care costs for 94,300 MRSA infected individuals in the United States exceed $2.4 billion (Klevens *et al.*, 2007). The current MRSA epidemic has precipitated a public health crisis that needs to be addressed by development of a preventive vaccine (Boucher and Corey, 2008). To date, an FDA licensed vaccine that prevents *S. aureus* diseases is not available.

[00122] The inventors describe here the use of Protein A, a cell wall anchored surface protein of staphylococci, for the generation of variants that can serve as subunit vaccines. The pathogenesis of staphylococcal infections is initiated as bacteria invade the skin or blood stream via trauma, surgical wounds, or medical devices (Lowy, 1998). Although the invading pathogen may be phagocytosed and killed, staphylococci can also escape innate immune defenses and seed infections in organ tissues, inducing inflammatory responses that attract macrophages, neutrophils, and other phagocytes (Lowy, 1998). The responsive invasion of immune cells to the site of infection is accompanied by liquefaction necrosis as the host seeks to prevent staphylococcal spread and allow for removal of necrotic tissue debris (Lam *et al.*,

1963). Such lesions can be observed by microscopy as hypercellular areas containing necrotic tissue, leukocytes, and a central nidus of bacteria (Lam *et al.*, 1963). Unless staphylococcal abscesses are surgically drained and treated with antibiotics, disseminated infection and septicemia produce a lethal outcome (Sheagren, 1984).

## III. Staphylococcal antigens

### A. Staphylcoccal Protein A (SpA)

[00123] All *Staphylococcus aureus* strains express the structural gene for Protein A (spa) (Jensen, 1958 ; Said-Salim *et al.*, 2003), a well characterized virulence factor whose cell wall anchored surface protein product (SpA) encompasses five highly homologous immunoglobulin binding domains designated E, D, A, B, and C (Sjodahl, 1977). These domains display ~ 80% identity at the amino acid level, are 56 to 61 residues in length, and are organized as tandem repeats (Uhlen *et al.*, 1984). SpA is synthesized as a precursor protein with an N-terminal YSIRK/GS signal peptide and a C-terminal LPXTG motif sorting signal (DeDent *et al.*, 2008; Schneewind *et al.*, 1992). Cell wall anchored Protein A is displayed in great abundance on the staphylococcal surface (DeDent *et al.*, 2007; Sjoquist *et al.*, 1972). Each of its immunoglobulin binding domains is composed of anti-parallel α-helices that assemble into a three helix bundle and bind the Fc domain of immunoglobulin G (IgG) (Deisenhofer, 1981; Deisenhofer *et al.*, 1978), the VH3 heavy chain (Fab) of IgM (*i.e.*, the B cell receptor) (Graille *et al.*, 2000), the von Willebrand factor at its A1 domain [vWF AI is a ligand for platelets] (O'Seaghdha *et al.*, 2006) and the tumor necrosis factor α (TNF-α) receptor I (TNFRI) (Gomez *et al.*, 2006), which is displayed on surfaces of airway epithelia (Gomez *et al.*, 2004; Gomez *et al.*, 2007).

[00124] SpA impedes neutrophil phagocytosis of staphylococci through its attribute of binding the Fc component of IgG (Jensen, 1958; Uhlen *et al.*, 1984). Moreover, SpA is able to activate intravascular clotting via its binding to von Willebrand factor AI domains (Hartleib *et al.*, 2000). Plasma proteins such as fibrinogen and fibronectin act as bridges between staphylococci (CIfA and CIfB) and the platelet integrin GPIIb/IIIa (O'Brien *et al.*, 2002), an activity that is supplemented through Protein A association with vWF AI, which allows staphylococci to capture platelets via the GPIb-α platelet receptor (Foster, 2005; O'Seaghdha *et al.*, 2006). SpA also binds TNFRI and this interaction contributes to the pathogenesis of

staphylococcal pneumonia (Gomez *et al.*, 2004). SpA activates proinflammatory signaling through TNFR1 mediated activation of TRAF2, the p38/c-Jun kinase, mitogen activate protein kinase (MAPK) and the Rel-transcription factor NF-KB. SpA binding further induces TNFR1 shedding, an activity that appears to require the TNF-converting enzyme (TACE)(Gomez *et al.*, 2007). All of the aforementioned SpA activities are mediated through its five IgG binding domains and can be perturbed by the same amino acid substitutions, initially defined by their requirement for the interaction between Protein A and human IgG1 (Cedergren *et al.*, 1993.

[00125] SpA also functions as a B cell superantigen by capturing the Fab region of VH3 bearing IgM, the B cell receptor (Gomez *et al.*, 2007; Goodyear *et al.*, 2003; Goodyear and Silverman, 2004; Roben *et al.*, 1995). Following intravenous challenge, staphylococcal Protein A (SpA) mutations show a reduction in staphylococcal load in organ tissues and dramatically diminished ability to form abscesses (described herein). During infection with wildtype *S. aureus*, abscesses are formed within forty-eight hours and are detectable by light microscopy of hematoxylin-eosin stained, thin-sectioned kidney tissue, initially marked by an influx of polymorphonuclear leukocytes (PMNs). On day 5 of infection, abscesses increase in size and enclosed a central population of staphylococci, surrounded by a layer of eosinophilic, amorphous material and a large cuff of PMNs. Histopathology revealed massive necrosis of PMNs in proximity to the staphylococcal nidus at the center of abscess lesions as well as a mantle of healthy phagocytes. The inventors also observed a rim of necrotic PMNs at the periphery of abscess lesions, bordering the eosinophilic pseudocapsule that separated healthy renal tissue from the infectious lesion. Staphylococcal variants lacking Protein A are unable to establish the histopathology features of abscesses and are cleared during infection.

[00126] In previous studies, Cedergren *et al.* (1993) engineered five individual substitutions in the Fc frgament binding sub-domain of the B domain of SpA, L17D, N28A, I31A and K35A. These authors created these proteins to test data gathered from a three dimensional structure of a complex between one domain of SpA and $Fc_1$. Cedergren *et al.* determined the effects of these mutations on stability and binding, but did not contemplate use of such substitutions for the production of a vaccine antigen.

[00127] Brown *et al.* (1998) describe studies designed to engineer new proteins based on SpA that allow the use of more favorable elution conditions when used as affinity ligands. The mutations studied included single mutations of Q13A, Q14H, N15A, N15H, F17H, Y18F, L21H, N32H, or K39H. Brown *et al.* report that Q13A, N15A, N15H, and N32H substitutions made little difference to the dissociation constant values and that the Y18F substitution resulted in a 2 fold decrease in binding affinity as compared to wild type SpA. Brown *et al.* also report that L21H and F17H substitutions decrease the binding affinity by five-fold and a hundred-fold respectively. The authors also studied analogous substitutions in two tandem domains. Thus, the Brown *et al.* studies were directed to generating a SpA with a more favorable elution profile, hence the use of His substitutions to provide a pH sensitive alteration in the binding affinity. Brown *et al.* ia silent on the use of SpA as a vaccine antigen.

[00128] Graille *et al.* (2000) describe a crystal structure of domain D of SpA and the Fab fragment of a human IgM antibody. Graille *et al.* define by analysis of a crystal structure the D domain amino acid residues that interact with the Fab fragment as residues Q26, G29, F30, Q32, S33, D36, D37, Q40, N43, E47, or L51, as well as the amino acid residues that form the interface between the domain D sub-domains. Graille *et al.* define the molecular interactions of these two proteins, but is silent in regard to any use of substitutions in the interacting residues in producing a vaccine antigen.

[00129] O'Seaghdha *et al.* (2006) describe studies directed at elucidating which sub-domain of domain D binds vWF. The authors generated single mutations in either the Fc or VH3 binding sub-domains, i.e., amino acid residues F5A, Q9A, Q10A, F13A, Y14A, L17A, N28A, I31A, K35A, G29A, F30A, S33A, D36A, D37A, Q40A, E47A, or Q32A.. The authors discovered that vWF binds the same sub-domain that binds Fc. O'Seaghda *et al.* define the sub-domain of domain D responsible for binding vWF, but is silent in regard to any use of substitutions in the interacting residues in producing a vaccine antigen.

[00130] Gomez *et al.* (2006) describe the identifcation of residues responsible for activation of the TNFR1 by using single mutations of F5A, F13A, Y14A, L17A,

N21A, I31A, Q32A, and K35A. Gomez *et al.* is silent in regard to any use of substitutions in the interacting residues in producing a vaccine antigen.

[00131] Recombinant affinity tagged Protein A, a polypeptide encompassing the five IgG domains (EDCAB) (Sjodahl, 1977) but lacking the C-terminal Region X (Guss *et al.*, 1984), was purified from recombinant *E. coli* and used as a vaccine antigen (Stranger-Jones *et al.*, 2006). Because of the attributes of SpA in binding the Fc portion of IgG, a specific humoral immune response to Protein A could not be measured (Stranger-Jones *et al.*, 2006). The inventors have overcome this obstacle through the generation of SpA-DQ9,10K;D36,37A. BALB/c mice immunized with recombinant Protein A (SpA) displayed significant protection against intravenous challenge with *S. aureus* strains: a 2.951 log reduction in staphylococcal load as compared to the wild-type ($P > 0.005$; Student's t-test) (Stranger-Jones *et al.*, 2006). SpA specific antibodies may cause phagocytic clearance prior to abscess formation and/or impact the formation of the aforementioned eosinophilic barrier in abscesses that separate staphylococcal communities from immune cells since these do not form during infection with Protein A mutant strains. Each of the five SpA domains (*i.e.*, domains formed from three helix bundles designated E, D, A, B, and C) exerts similar binding properties (Jansson *et al.*, 1998). The solution and crystal structure of the domain D has been solved both with and without the Fc and VH3 (Fab) ligands, which bind Protein A in a non-competitive manner at distinct sites (Graille *et al.*, 2000). Mutations in residues known to be involved in IgG binding (FS, Q9, Q10, S11, F13, Y14, L17, N28, I31 and K35) are also required for vWF AI and TNFR1 binding (Cedergren *et al.*, 1993; Gomez *et al.*, 2006; O'Seaghdha *et al.*, 2006), whereas residues important for the VH3 interaction (Q26, G29, F30, S33, D36, D37, Q40, N43, E47) appear to have no impact on the other binding activities (Graille *et al.*, 2000; Jansson *et al.*, 1998). SpA specifically targets a subset of B cells that express VH3 family related IgM on their surface, *i.e.,* VH3 type B cell receptors (Roben *et al.*, 1995). Upon interaction with SpA, these B cells proliferate and commit to apoptosis, leading to preferential and prolonged deletion of innate-like B lymphocytes (*i.e.,* marginal zone B cells and follicular B2 cells)(Goodyear *et al.*, 2003; Goodyear *et al.*, 2004).

[00132] ***Molecular basis of Protein A surface display and function.*** Protein A is synthesized as a precursor in the bacterial cytoplasm and secreted via its YSIRK signal peptide at the cross wall, *i.e.* the cell division septum of staphylococci (FIG. 1) (DeDent *et al.*, 2007; DeDent *et al.*, 2008). Following cleavage of the C-terminal LPXTG sorting signal, Protein A is anchored to bacterial peptidoglycan crossbridges by sortase A (Mazmanian *et al.*, 1999; Schneewind *et al.*, 1995; Mazmanian *et al.*, 2000). Protein A is the most abundant surface protein of staphylococci; the molecule is expressed by virtually all *S. aureus* strains (Cespedes *et al.*, 2005; Kennedy *et al.*, 2008; Said-Salim *et al.*, 2003). Staphylococci turn over 15-20% of their cell wall per division cycle (Navarre and Schneewind, 1999). Murine hydrolases cleave the glycan strands and wall peptides of peptidoglycan, thereby releasing Protein A with its attached C-terminal cell wall disaccharide tetrapeptide into the extracellular medium (Ton-That *et al.*, 1999). Thus, by physiological design, Protein A is both anchored to the cell wall and displayed on the bacterial surface but also released into surrounding tissues during host infection (Marraffini *et al.*, 2006).

[00133] Protein A captures immunoglobulins on the bacterial surface and this biochemical activity enables staphylococcal escape from host innate and acquired immune responses (Jensen, 1958; Goodyear *et al.*, 2004). Interestingly, region X of Protein A (Guss *et al.*, 1984), a repeat domain that tethers the IgG binding domains to the LPXTG sorting signal / cell wall anchor, is perhaps the most variable portion of the staphylococcal genome (Said-Salim, 2003; Schneewind *et al.*, 1992). Each of the five immunoglobulin binding domains of Protein A (SpA), formed from three helix bundles and designated E, D, A, B, and C, exerts similar structural and functional properties (Sjodahl, 1977; Jansson *et al.*, 1998). The solution and crystal structure of the domain D has been solved both with and without the Fc and $V_H3$ (Fab) ligands, which bind Protein A in a non-competitive manner at distinct sites (Graille 2000).

[00134] In the crystal structure complex, the Fab interacts with helix II and helix III of domain D via a surface composed of four VH region β-strands (Graille 2000). The major axis of helix II of domain D is approximately 50° to the orientation of the strands, and the interhelical portion of domain D is most proximal to the C0 strand. The site of interaction on Fab is remote from the Ig light chain and the heavy chain constant region. The interaction involves the following domain D residues: Asp-36 of

helix II, Asp-37 and Gln-40 in the loop between helix II and helix III and several other residues (Graille 2000). Both interacting surfaces are composed predominantly of polar side chains, with three negatively charged residues on domain D and two positively charged residues on the 2A2 Fab buried by the interaction, providing an overall electrostatic attraction between the two molecules. Of the five polar interactions identified between Fab and domain D, three are between side chains. A salt bridge is formed between Arg-H19 and Asp-36 and two hydrogen bonds are made between Tyr-H59 and Asp-37 and between Asn-H82a and Ser-33. Because of the conservation of Asp-36 and Asp-37 in all five IgG binding domains of Protein A, the inventors mutated these residues.

[00135] The SpA-D sites responsible for Fab binding are structurally separate from the domain surface that mediates Fcγ binding. The interaction of Fcγ with domain D primarily involves residues in helix I with lesser involvement of helix II (Gouda *et al.*, 1992; Deisenhofer, 1981). With the exception of the Gln-32, a minor contact in both complexes, none of the residues that mediate the Fcγ interaction are involved in Fab binding. To examine the spatial relationship between these different Ig-binding sites, the SpA domains in these complexes have been superimposed to construct a model of a complex between Fab, the SpA-domain D, and the Fcγ molecule. In this ternary model, Fab and Fcγ form a sandwich about opposite faces of the helix II without evidence of steric hindrance of either interaction. These findings illustrate how, despite its small size (*i.e.*, 56–61 aa), an SpA domain can simultaneously display both activities, explaining experimental evidence that the interactions of Fab with an individual domain are noncompetitive. Residues for the interaction between SpA-D and Fcγ are Gln-9 and Gln-10.

[00136] In contrast, occupancy of the Fc portion of IgG on the domain D blocks its interaction with vWF A1 and probably also TNFR1 (O'Seaghdha *et al.*, 2006). Mutations in residues essential for IgG Fc binding (F5, Q9, Q10, S11, F13, Y14, L17, N28, I31 and K35) are also required for vWF A1 and TNFR1 binding (O'Seaghdha *et al.*, 2006; Cedergren *et al.*, 1993; Gomez *et al.*, 2006), whereas residues critical for the VH3 interaction (Q26, G29, F30, S33, D36, D37, Q40, N43, E47) have no impact on the binding activities of IgG Fc, vWF A1 or TNFR1 (Jansson *et al.*, 1998; Graille *et al.*, 2000). The Protein A immunoglobulin Fab binding activity targets a subset of

B cells that express $V_H3$ family related IgM on their surface, *i.e.*, these molecules function as VH3type B cell receptors (Roben *et al.*, 1995). Upon interaction with SpA, these B cells rapidly proliferate and then commit to apoptosis, leading to preferential and prolonged deletion of innate-like B lymphocytes (*i.e.*, marginal zone B cells and follicular B2 cells) (Goodyear and Silverman, 2004; Goodyear and Silverman, 2003). More than 40% of circulating B cells are targeted by the Protein A interaction and the $V_H3$ family represents the largest family of human B cell receptors to impart protective humoral responses against pathogens (Goodyear and Silverman, 2004; Goodyear and Silverman, 2003). Thus, Protein A functions analogously to staphylococcal superantigens (Roben *et al.*, 1995), albeit that the latter class of molecules, for example SEB, TSST-1, TSST-2, form complexes with the T cell receptor to inappropriately stimulate host immune responses and thereby precipitating characteristic disease features of staphylococcal infections (Roben *et al.*, 1995; Tiedemann *et al.*, 1995). Together these findings document the contributions of Protein A in establishing staphylococcal infections and in modulating host immune responses.

[00137] In sum, Protein A domains can viewed as displaying two different interfaces for binding with host molecules and any development of Protein A based vaccines must consider the generation of variants that do not perturb host cell signaling, platelet aggregation, sequestration of immunoglobulins or the induction of B cell proliferation and apoptosis. Such Protein A variants should also be useful in analyzing vaccines for the ability of raising antibodies that block the aforementioned SpA activities and occupy the five repeat domains at their dual binding interfaces. This goal is articulated and pursued here for the first time and methods are described in detail for the generation of Protein A variants that can be used as a safe vaccine for humans. To perturb IgG Fcγ, vWF AI and TNFR1 binding, glutamine (Q) 9 and 10 [numbering derived from the SpA domain D as described in Uhlen *et al.*, 1984] were mutated, and generated lysine substitutions for both glutamines with the expectation that these abolish the ligand attributes at the first binding interface. To perturb IgM Fab VH3 binding, aspartate (D) 36 and 37 were mutated, each of which is required for the association with the B cell receptor. D36 and D37 were both substituted with alanine. Q9,10K and D36,37A mutations are here combined in the recombinant molecule SpA-DQ9,10K;D36,37A and tested for the binding attributes of Protein A.

Further, SpA-D and SpA-DQ9,10K;D36,37A are subjected to immunization studies in mice and rabbits and analyzed for [1] the production of specific antibodies (SpA-D Ab); [2] the ability of SpA-D Ab to block the association between Protein A and its four different ligands; and, [3] the attributes of SpA-D Ab to generate protective immunity against staphylococcal infections. (*See* Examples section below).

### B. Staphylococcal Coagulases

[00138] Coagulases are enzymes produced by *Staphylococcus* bacteria that convert fibrinogen to fibrin. Coa and $vW_h$ activate prothrombin without proteolysis (Friedrich *et al.*, 2003). The coagulase·prothrombin complex recognizes fibrinogen as a specific substrate, converting it directly into fibrin. The crystal structure of the active complex revealed binding of the D1 and D2 domains to prothrombin and insertion of its Ile1-$Val^2$ N-terminus into the $Ile^{16}$ pocket, inducing a functional active site in the zymogen through conformational change (Friedrich *et al.*, 2003). Exosite I of $\alpha$-thrombin, the fibrinogen recognition site, and proexosite I on prothrombin are blocked by the D2 of Coa (Friedrich *et al.*, 2003). Nevertheless, association of the tetrameric $(Coa{\cdot}prothrombin)_2$ complex binds fibrinogen at a new site with high affinity (Panizzi *et al.*, 2006). This model explains the coagulant properties and efficient fibrinogen conversion by coagulase (Panizzi *et al.*, 2006).

[00139] Fibrinogen is a large glycoprotein (*M*r ~340,000), formed by three pairs of A$\alpha$-, B$\beta$-, and $\gamma$-chains covalently linked to form a "dimer of trimers," where A and B designate the fibrinopeptides released by thrombin cleavage (Panizzi *et al.*, 2006). The elongated molecule folds into three separate domains, a central fragment E that contains the N-termini of all six chains and two flanking fragments D formed mainly by the C-termini of the B$\beta$- and $\gamma$-chains. These globular domains are connected by long triple-helical structures. Coagulase-prothrombin complexes, which convert human fibrinogen to the self-polymerizing fibrin, are not targeted by circulating thrombin inhibitors (Panizzi *et al.*, 2006). Thus, staphylococcal coagulases bypass the physiological blood coagulation pathway.

[00140] All *S. aureus* strains secrete coagulase and vWbp (Bjerketorp *et al.*, 2004; Field and Smith, 1945). Although early work reported important contributions of coagulase to the pathogenesis of staphylococcal infections (Ekstedt and Yotis, 1960; Smith et al., 1947), more recent investigations with molecular genetics tools

challenged this view by observing no virulence phenotypes with endocarditis, skin abscess and mastitis models in mice (Moreillon *et al.*, 1995; Phonimdaeng *et al.*, 1990). Generating isogenic variants of *S. aureus* Newman, a fully virulent clinical isolate (Duthie *et al.*, 1952), it is described herein that *coa* mutants indeed display virulence defects in a lethal bacteremia and renal abscess model in mice. In the inventors experience, *S. aureus* 8325-4 is not fully virulent and it is presumed that mutational lesions in this strain may not be able to reveal virulence defects *in vivo*. Moreover, antibodies raised against Coa or vWbp perturb the pathogenesis of *S. aureus* Newman infections to a degree mirroring the impact of gene deletions. Coa and vWbp contribute to staphylococcal abscess formation and lethal bacteremia and may also function as protective antigens in subunit vaccines.

[00141] Biochemical studies document the biological value of antibodies against Coa and vWbp. By binding to antigen and blocking its association with clotting factors, the antibodies prevent the formation of Coa·prothrombin and vWbp·prothrombin complexes. Passive transfer studies revealed protection of experimental animals against staphylococcal abscess formation and lethal challenge by Coa and vWbp antibodies. Thus, Coa and vWbp neutralizing antibodies generate immune protection against staphylococcal disease.

[00142] Earlier studies revealed a requirement of coagulase for resisting phagocytosis in blood (Smith *et al.*, 1947) and the inventors observed a similar phenotype for Δ*coa* mutants in lepirudin-treated mouse blood (see Example 3 below). As vWbp displays higher affinity for human prothrombin than the mouse counterpart, it is suspected the same may be true for Δ*vWbp* variants in human blood. Further, expression of Coa and vWbp in abscess lesions as well as their striking distribution in the eosinophilic pseudocapsule surrounding (staphylococcal abscess communities (SACs) or the peripheral fibrin wall, suggest that secreted coagulases contribute to the establishment of these lesions. This hypothesis was tested and, indeed, Δ*coa* mutants were defective in the establishment of abscesses. A corresponding test, blocking Coa function with specific antibodies, produced the same effect. Consequently, it is proposed that the clotting of fibrin is a critical event in the establishment of staphylococcal abscesses that can be targeted for the development of protective

vaccines. Due to their overlapping function on human prothrombin, both Coa and vWbp are considered excellent candidates for vaccine development.

### C. Other Staphylococcal Antigens

[00143] Research over the past several decades identified *S. aureus* exotoxins, surface proteins and regulatory molecules as important virulence factors (Foster, 2005; Mazmanian *et al.*, 2001; Novick, 2003). Much progress has been achieved regarding the regulation of these genes. For example, staphylococci perform a bacterial census via the secretion of auto-inducing peptides that bind to a cognate receptor at threshold concentration, thereby activating phospho-relay reactions and transcriptional activation of many of the exotoxin genes (Novick, 2003). The pathogenesis of staphylococcal infections relies on these virulence factors (secreted exotoxins, exopolysaccharides, and surface adhesins). The development of staphylococcal vaccines is hindered by the multifaceted nature of staphylococcal invasion mechanisms. It is well established that live attenuated micro-organisms are highly effective vaccines; immune responses elicited by such vaccines are often of greater magnitude and of longer duration than those produced by non-replicating immunogens. One explanation for this may be that live attenuated strains establish limited infections in the host and mimic the early stages of natural infection. Embodiments of the invention are directed to compositions and methods including variant SpA polypeptides and peptides, as well as other immunogenic extracellular proteins, polypeptides, and peptides (including both secreted and cell surface proteins or peptides) of gram positive bacteria for the use in mitigating or immunizing against infection. In particular embodiments the bacteria is a staphylococcus bacteria. Extracellular proteins, polypeptides, or peptides include, but are not limited to secreted and cell surface proteins of the targeted bacteria.

[00144] The human pathogen *S. aureus* secretes EsxA and EsxB, two ESAT-6 like proteins, across the bacterial envelope (Burts *et al.*, 2005). Staphylococcal *esxA* and *esxB* are clustered with six other genes in the order of transcription: *esxA esaA essA esaB essB essC esaC esxB*. The acronyms esa, ess, and esx stand for ESAT-6 secretion accessory, system, and extracellular, respectively, depending whether the encoded proteins play an accessory (esa) or direct (ess) role for secretion, or are secreted (esx) in the extracellular milieu.

The entire cluster of eight genes is herein referred to as the Ess cluster. EsxA, esxB, essA, essB, and essC are all required for synthesis or secretion of EsxA and EsxB. Mutants that fail to produce EsxA, EsxB, and EssC display defects in the pathogenesis of *S. aureus* murine abscesses, suggesting that this specialized secretion system may be a general strategy of human bacterial pathogenesis. Secretion of non-WXG100 substrates by the ESX-1 pathway has been reported for several antigens including EspA, EspB, Rv3483c, and Rv3615c (Fortune *et al.*, 2005; MacGurn *et al.*, 2005; McLaughlin *et al.*, 2007; Xu *et al.*, 2007). The alternate ESX-5 pathway has also been shown to secrete both WXG100 and non-WXG100 proteins in pathogenic mycobacteria (Abdallah *et al.*, 2007; Abdallah *et al.*, 2006).

[00145] The *Staphylococcus aureus* Ess pathway can be viewed as a secretion module equipped with specialized transport components (Ess), accessory factors (Esa) and cognate secretion substrates (Esx). EssA, EssB and EssC are required for EsxA and EsxB secretion. Because EssA, EssB and EssC are predicted to be transmembrane proteins, it is contemplated that these proteins form a secretion apparatus. Some of the proteins in the *ess* gene cluster may actively transport secreted substrates (acting as motor) while others may regulate transport (regulator). Regulation may be achieved, but need not be limited to, transcriptional or post-translational mechanisms for secreted polypeptides, sorting of specific substrates to defined locations (*e.g.,* extracellular medium or host cells), or timing of secretion events during infection. At this point, it is unclear whether all secreted Esx proteins function as toxins or contribute indirectly to pathogenesis.

[00146] Staphylococci rely on surface protein mediated-adhesion to host cells or invasion of tissues as a strategy for escape from immune defenses. Furthermore, *S. aureus* utilize surface proteins to sequester iron from the host during infection. The majority of surface proteins involved in staphylococcal pathogenesis carry C-terminal sorting signals, *i.e.*, they are covalently linked to the cell wall envelope by sortase. Further, staphylococcal strains lacking the genes required for surface protein anchoring, *i.e.,* sortase A and B, display a dramatic defect in the virulence in several different mouse models of disease. Thus, surface protein antigens represent a validated vaccine target as the corresponding genes are essential for the development of staphylococcal disease and can be exploited in various embodiments of the

invention. The sortase enzyme superfamily are Gram-positive transpeptidases responsible for anchoring surface protein virulence factors to the peptidoglycan cell wall layer. Two sortase isoforms have been identified in *Staphylococcus aureus*, SrtA and SrtB. These enzymes have been shown to recognize a LPXTG motif in substrate proteins. The SrtB isoform appears to be important in heme iron acquisition and iron homeostasis, whereas the SrtA isoform plays a critical role in the pathogenesis of Gram-positive bacteria by modulating the ability of the bacterium to adhere to host tissue via the covalent anchoring of adhesins and other proteins to the cell wall peptidoglycan. In certain embodiments the SpA variants described herein can be used in combination with other staphylococcal proteins such as Coa, Eap, Ebh, Emp, EsaC, EsaB, EsxA, EsxB, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, IsdC, SasF, vWbp, and/or vWh proteins.

[00147] Certain aspects of the invention include methods and compositions concerning proteinaceous compositions including polypeptides, peptides, or nucleic acid encoding SpA variant(s) and other staphylococcal antigens such as other proteins transported by the Ess pathway, or sortase substrates. These proteins may be modified by deletion, insertion, and/or substitution.

[00148] The Esx polypeptides include the amino acid sequence of Esx proteins from bacteria in the Staphylococcus genus. The Esx sequence may be from a particular staphylococcus species, such as *Staphylococcus aureus*, and may be from a particular strain, such as Newman. In certain embodiments, the EsxA sequence is SAV0282 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number Q99WU4 (gi|68565539). In other embodiments, the EsxB sequence is SAV0290 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number Q99WT7 (gi|68565532). In further embodiments, other polypeptides transported by the Ess pathway may be used, the sequences of which may be identified by one of skill in the art using databases and internet accessible resources.

[00149] The sortase substrate polypeptides include, but are not limited to the amino acid sequence of SdrC, SdrD, SdrE, IsdA, IsdB, ClfA, ClfB, IsdC or SasF proteins

from bacteria in the Staphylococcus genus. The sortase substrate polypeptide sequence may be from a particular staphylococcus species, such as *Staphylococcus aureus*, and may be from a particular strain, such as Newman. In certain embodiments, the SdrD sequence is from strain N315 and can be accessed using Genbank Accession Number NP_373773.1 (gi|15926240). In other embodiments, the SdrE sequence is from strain N315 and can be accessed using Genbank Accession Number NP_373774.1 (gi|15926241). In other embodiments, the IsdA sequence is SAV1130 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number NP_371654.1 (gi|15924120). In other embodiments, the IsdB sequence is SAV1129 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number NP_371653.1 (gi|15924119). In further embodiments, other polypeptides transported by the Ess pathway or processed by sortase may be used, the sequences of which may be identified by one of skill in the art using databases and internet accessible resources.

[00149] Examples of various proteins that can be used in the context of the present invention can be identified by analysis of database submissions of bacterial genomes, including but not limited to accession numbers NC_002951 (GI:57650036 and GenBank CP000046), NC_002758 (GI:57634611 and GenBank BA000017), NC_002745 (GI:29165615 and GenBank BA000018), NC_003923 (GI:21281729 and GenBank BA000033), NC_002952 (GI:49482253 and GenBank BX571856), NC_002953 (GI:49484912 and GenBank BX571857), NC_007793 (GI:87125858 and GenBank CP000255), NC_007795 (GI:87201381 and GenBank CP000253).

[00150] As used herein, a "protein" or "polypeptide" refers to a molecule comprising at least ten amino acid residues. In some embodiments, a wild-type version of a protein or polypeptide are employed, however, in many embodiments of the invention, a modified protein or polypeptide is employed to generate an immune response. The terms described above may be used interchangeably. A "modified protein" or "modified polypeptide" or a "variant" refers to a protein or polypeptide whose chemical structure, particularly its amino acid sequence, is altered with respect

to the wild-type protein or polypeptide. In some embodiments, a modified/variant protein or polypeptide has at least one modified activity or function (recognizing that proteins or polypeptides may have multiple activities or functions). It is specifically contemplated that a modified/variant protein or polypeptide may be altered with respect to one activity or function yet retain a wild-type activity or function in other respects, such as immunogenicity.

[00152] In certain embodiments the size of a protein or polypeptide (wild-type or modified) may comprise, but is not limited to, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2250, 2500 amino molecules or greater, and any range derivable therein, or derivative of a corresponding amino sequence described or referenced herein. It is contemplated that polypeptides may be mutated by truncation, rendering them shorter than their corresponding wild-type form, but also they might be altered by fusing or conjugating a heterologous protein sequence with a particular function (*e.g.*, for targeting or localization, for enhanced immunogenicity, for purification purposes, *etc.*).

[00153] As used herein, an "amino molecule" refers to any amino acid, amino acid derivative, or amino acid mimic known in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

[00154] Accordingly, the term "proteinaceous composition" encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid.

[00155] Proteinaceous compositions may be made by any technique known to those of skill in the art, including (i) the expression of proteins, polypeptides, or peptides through standard molecular biological techniques, (ii) the isolation of proteinaceous compounds from natural sources, or (iii) the chemical synthesis of proteinaceous materials. The nucleotide as well as the protein, polypeptide, and peptide sequences for various genes have been previously disclosed, and may be found in the recognized computerized databases. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (on the World Wide Web). The coding regions for these genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art.

[00156] Amino acid sequence variants of SpA, coagulases and other polypeptides of the invention can be substitutional, insertional, or deletion variants. A variation in a polypeptide of the invention may affect 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more non-contiguous or contiguous amino acids of the polypeptide, as compared to wild-type. A variant can comprise an amino acid sequence that is at least 50%, 60%, 70%, 80%, or 90%, including all values and ranges there between, identical to any sequence provided or referenced herein, e.g., SEQ ID NO:2-8 or SEQ ID NO:11-30, A variant can include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more substitute amino acids. A polypeptide processed or secreted by the Ess pathway or other surface proteins (see Table 1) or sortase substrates from any staphylococcus species and strain are contemplated for use in compositions and methods described herein.

[00157] Deletion variants typically lack one or more residues of the native or wild-type protein. Individual residues can be deleted or a number of contiguous amino acids can be deleted. A stop codon may be introduced (by substitution or insertion) into an encoding nucleic acid sequence to generate a truncated protein. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of one or more residues. Terminal additions, called fusion proteins, may also be generated. These fusion proteins

include multimers or concatamers of one or more peptide or polypeptide described or referenced herein.

[00158] Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, with or without the loss of other functions or properties. Substitutions may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Alternatively, substitutions may be non-conservative such that a function or activity of the polypeptide is affected. Non-conservative changes typically involve substituting a residue with one that is chemically dissimilar, such as a polar or charged amino acid for a nonpolar or uncharged amino acid, and vice versa.

Table 1. Exemplary surface proteins of *S. aureus* strains.

| SAV # | SA# | Surface | MW2 | Mu50 | N315 | Newman | MRSA252* | MSSA476* |
|---|---|---|---|---|---|---|---|---|
| SAV0111 | SA0107 | Spa | 492 | 450 | 450 | 520 | 516 | 492 |
| SAV2503 | SA2291 | FnBPA | 1015 | 1038 | 1038 | 741 | - | 1015 |
| SAV2502 | SA2290 | FnBPB | 943 | 961 | 961 | 677 | 965 | 957 |
| SAV0811 | SA0742 | ClfA | 946 | 935 | 989 | 933 | 1029 | 928 |
| SAV2630 | SA2423 | ClfB | 907 | 877 | 877 | 913 | 873 | 905 |
| Np | Np | Cna | 1183 | - | - | - | 1183 | 1183 |
| SAV0561 | SA0519 | SdrC | 955 | 953 | 953 | 947 | 906 | 957 |
| SAV0562 | SA0520 | SdrD | 1347 | 1385 | 1385 | 1315 | - | 1365 |
| SAV0563 | SA0521 | SdrE | 1141 | 1141 | 1141 | 1166 | 1137 | 1141 |
| Np | Np | Pls | - | - | - | - | - | - |
| SAV2654 | SA2447 | SasA | 2275 | 2271 | 2271 | 2271 | 1351 | 2275 |
| SAV2160 | SA1964 | SasB | 686 | 2481 | 2481 | 2481 | 2222 | 685 |
| | SA1577 | SasC | 2186 | 213 | 2186 | 2186 | 2189 | 2186 |
| SAV0134 | SA0129 | SasD | 241 | 241 | 241 | 241 | 221 | 241 |
| SAV1130 | SA0977 | SasE/IsdA | 350 | 350 | 350 | 350 | 354 | 350 |
| SAV2646 | SA2439 | SasF | 635 | 635 | 635 | 635 | 627 | 635 |
| SAV2496 | | SasG | 1371 | 525 | 927 | - | - | 1371 |
| SAV0023 | SA0022 | SasH | 772 | - | 772 | 772 | 786 | 786 |
| SAV1731 | SA1552 | SasI | 895 | 891 | 891 | 891 | 534 | 895 |
| SAV1129 | SA0976 | SasJ/IsdB | 645 | 645 | 645 | 645 | 652 | 645 |
| | SA2381 | SasK | 198 | 211 | 211 | - | - | 197 |
| | Np | SasL | - | 232 | - | - | - | - |
| SAV1131 | SA0978 | IsdC | 227 | 227 | 227 | 227 | 227 | 227 |

[00159] Proteins of the invention may be recombinant, or synthesized *in vitro*. Alternatively, a non-recombinant or recombinant protein may be isolated from bacteria. It is also contemplated that a bacteria containing such a variant may be implemented in compositions and methods of the invention. Consequently, a protein need not be isolated.

[00160] The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 2, below).

Table 2 Codon Table

| Amino Acids | | | Codons |
|---|---|---|---|
| Alanine | Ala | A | GCA GCC GCG GCU |
| Cysteine | Cys | C | UGC UGU |
| Aspartic acid | Asp | D | GAC GAU |
| Glutamic acid | Glu | E | GAA GAG |
| Phenylalanine | Phe | F | UUC UUU |
| Glycine | Gly | G | GGA GGC GGG GGU |
| Histidine | His | H | CAC CAU |
| Isoleucine | Ile | I | AUA AUC AUU |
| Lysine | Lys | K | AAA AAG |
| Leucine | Leu | L | UUA UUG CUA CUC CUG CUU |
| Methionine | Met | M | AUG |
| Asparagine | Asn | N | AAC AAU |
| Proline | Pro | P | CCA CCC CCG CCU |
| Glutamine | Gln | Q | CAA CAG |
| Arginine | Arg | R | AGA AGG CGA CGC CGG CGU |
| Serine | Ser | S | AGC AGU UCA UCC UCG UCU |
| Threonine | Thr | T | ACA ACC ACG ACU |
| Valine | Val | V | GUA GUC GUG GUU |
| Tryptophan | Trp | W | UGG |
| Tyrosine | Tyr | Y | UAC UAU |

[00161] It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids, or 5' or 3' sequences, respectively, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity (*e.g.*, immunogenicity) where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region.

[00162] The following is a discussion based upon changing of the amino acids of a protein to create a variant polypeptide or peptide. For example, certain amino acids may be substituted for other amino acids in a protein structure with or without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with a desirable

property. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes.

[00163] It is contemplated that in compositions of the invention, there is between about 0.001 mg and about 10 mg of total polypeptide, peptide, and/or protein per ml. The concentration of protein in a composition can be about, at least about or at most about 0.001, 0.010, 0.050, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 mg/ml or more (or any range derivable therein). Of this, about, at least about, or at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% may be an SpA variant or a coagulase, and may be used in combination with other peptides or polypeptides, such as other bacterial peptides and/or antigens.

[00164] The present invention contemplates the administration of variant SpA polypeptides or peptides to effect a preventative therapy or therapeutic effect against the development of a disease or condition associated with infection by a staphylococcus pathogen.

[00165] In certain aspects, combinations of staphylococcal antigens are used in the production of an immunogenic composition that is effective at treating or preventing staphylococcal infection. Staphylococcal infections progress through several different stages. For example, the staphylococcal life cycle involves commensal colonization, initiation of infection by accessing adjoining tissues or the bloodstream, and/or anaerobic multiplication in the blood. The interplay between *S. aureus* virulence determinants and the host defense mechanisms can induce complications such as endocarditis, metastatic abscess formation, and sepsis syndrome. Different molecules on the surface of the bacterium are involved in different steps of the infection cycle. Combinations of certain antigens can elicit an immune response which protects against multiple stages of staphylococcal infection. The effectiveness of the immune response can be measured either in animal model assays and/or using an opsonophagocytic assay.

### D. Polypeptides and Polypeptide Production

[00166] The present invention describes polypeptides, peptides, and proteins and immunogenic fragments thereof for use in various embodiments of the present invention. For example, specific polypeptides are assayed for or used to elicit an immune response. In specific embodiments, all or part of the proteins of the invention can also be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979).

[00167] Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

[00168] One embodiment of the invention includes the use of gene transfer to cells, including microorganisms, for the production and/or presentation of polypeptides or peptides. The gene for the polypeptide or peptide of interest may be transferred into appropriate host cells followed by culture of cells under the appropriate conditions. The generation of recombinant expression vectors, and the elements included therein, are well known in the art and briefly discussed herein. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell that is isolated and purified.

[00169] Another embodiment of the present invention uses autologous B lymphocyte cell lines, which are transfected with a viral vector that expresses an immunogen product, and more specifically, a protein having immunogenic activity. Other examples of mammalian host cell lines include, but are not limited to Vero and HeLa cells, other B- and T- cell lines, such as CEM, 721.221, H9, Jurkat, Raji, as well as cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, 3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or that modifies and processes the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein.

Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

[00170] A number of selection systems may be used including, but not limited to HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes, in tk-, hgprt- or aprt- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection: for dhfr, which confers resistance to trimethoprim and methotrexate; gpt, which confers resistance to mycophenolic acid; neo, which confers resistance to the aminoglycoside G418; and hygro, which confers resistance to hygromycin.

[00171] Animal cells can be propagated *in vitro* in two modes: as non-anchorage-dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

[00172] Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

[00173] Where a protein is specifically mentioned herein, it is preferably a reference to a native or recombinant protein or optionally a protein in which any signal sequence has been removed. The protein may be isolated directly from the staphylococcal strain or produced by recombinant DNA techniques. Immunogenic fragments of the protein may be incorporated into the immunogenic composition of the invention. These are fragments comprising at least 10 amino acids, 20 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, or 100 amino acids, including all values and ranges there between, taken contiguously from the amino acid sequence of the protein. In addition, such immunogenic fragments are immunologically reactive with antibodies generated against the Staphylococcal proteins or with antibodies generated by infection of a mammalian host with Staphylococci. Immunogenic fragments also include fragments that when administered at an effective

dose, (either alone or as a hapten bound to a carrier), elicit a protective or therapeutic immune response against Staphylococcal infection, in certain aspects it is protective against *S. aureus* and/or *S. epidermidis* infection. Such an immunogenic fragment may include, for example, the protein lacking an N-terminal leader sequence, and/or a transmembrane domain and/or a C-terminal anchor domain. In a preferred aspect the immunogenic fragment according to the invention comprises substantially all of the extracellular domain of a protein which has at least 80% identity, at least 85% identity, at least 90% identity, at least 95% identity, or at least 97-99% identity, including all values and ranges there between, to a sequence selected segment of a polypeptide described or referenced herein.

[00174] Also included in immunogenic compositions of the invention are fusion proteins composed of one or more Staphylococcal proteins, or immunogenic fragments of staphylococcal proteins. Such fusion proteins may be made recombinantly and may comprise one portion of at least 1, 2, 3, 4, 5, or 6 staphylococcal proteins or segments. Alternatively, a fusion protein may comprise multiple portions of at least 1, 2, 3, 4 or 5 staphylococcal proteins. These may combine different Staphylococcal proteins and/or multiples of the same protein or proten fragment, or immunogenic fragments in the same protein (forming a multimer or a concatamer). Alternatively, the invention also includes individual fusion proteins of Staphylococcal proteins or immunogenic fragments thereof, as a fusion protein with heterologous sequences such as a provider of T-cell epitopes or purification tags, for example: β-galactosidase, glutathione-S-transferase, green fluorescent proteins (GFP), epitope tags such as FLAG, myc tag, poly histidine, or viral surface proteins such as influenza virus haemagglutinin, or bacterial proteins such as tetanus toxoid, diphtheria toxoid, or CRM197.

## IV. Nucleic Acids

[00175] In certain embodiments, the present invention concerns recombinant polynucleotides encoding the proteins, polypeptides, peptides of the invention. The nucleic acid sequences for SpA, coagulases and other bacterial proteins are included and can be used to prepare peptides or polypeptides.

[00176] As used in this application, the term "polynucleotide" refers to a nucleic acid molecule that either is recombinant or has been isolated free of total genomic nucleic acid. Included within the term "polynucleotide" are oligonucleotides (nucleic acids of 100 residues or less in length), recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like. Polynucleotides include, in certain aspects, regulatory sequences, isolated substantially away from their naturally occurring genes or protein encoding sequences. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be RNA, DNA (genomic, cDNA or synthetic), analogs thereof, or a combination thereof. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide.

[00177] In this respect, the term "gene," "polynucleotide," or "nucleic acid" is used to refer to a nucleic acid that encodes a protein, polypeptide, or peptide (including any sequences required for proper transcription, post-translational modification, or localization). As will be understood by those in the art, this term encompasses genomic sequences, expression cassettes, cDNA sequences, and smaller engineered nucleic acid segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a polypeptide may contain a contiguous nucleic acid sequence of: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1095, 1100, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 9000, 10000, or more nucleotides, nucleosides, or base pairs, including all values and ranges therebetween, of a polynucleotide encoding one or more amino acid sequence described or referenced herein. It also is contemplated that a particular polypeptide may be encoded by nucleic acids containing variations having slightly different nucleic acid sequences but, nonetheless, encode the same or substantially similar protein (see Table 2 above).

[00178] In particular embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode a variant SpA or coagulase. The term "recombinant" may be used in conjunction with a polynucleotide or polypeptide and generally refers to a polypeptide or polynucleotide produced and/or manipulated *in vitro* or that is a replication product of such a molecule.

[00179] In other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode a variant SpA or coagulase polypeptide or peptide to generate an immune response in a subject. In various embodiments the nucleic acids of the invention may be used in genetic vaccines.

[00180] The nucleic acid segments used in the present invention can be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant nucleic acid protocol. In some cases, a nucleic acid sequence may encode a polypeptide sequence with additional heterologous coding sequences, for example to allow for purification of the polypeptide, transport, secretion, post-translational modification, or for therapeutic benefits such as targeting or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a polypeptide that is not the same as the modified polypeptide.

[00181] In certain other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence from SEQ ID NO:1 (SpA domain D) or SEQ ID NO:3 (SpA) or any other nucleic acid sequences encoding coagulases or other secreted virulence factors and/or surface proteins including proteins transported by the Ess pathway, processed by sortase, or proteins.

[00182] In certain embodiments, the present invention provides polynucleotide variants having substantial identity to the sequences disclosed herein; those comprising at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher sequence identity, including all values and ranges there between, compared to a polynucleotide sequence of this invention using the methods described herein (e.g., BLAST analysis using standard parameters).

[00183] The invention also contemplates the use of polynucleotides which are complementary to all the above described polynucleotides.

### E. Vectors

[00184] Polypeptides of the invention may be encoded by a nucleic acid molecule comprised in a vector. The term "vector" is used to refer to a carrier nucleic acid molecule into which a heterologous nucleic acid sequence can be inserted for introduction into a cell where it can be replicated and expressed. A nucleic acid sequence can be "heterologous," which means that it is in a context foreign to the cell in which the vector is being introduced or to the nucleic acid in which is incorporated, which includes a sequence homologous to a sequence in the cell or nucleic acid but in a position within the host cell or nucleic acid where it is ordinarily not found. Vectors include DNAs, RNAs, plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (for example Sambrook *et al.*, 2001; Ausubel *et al.*, 1996). In addition to encoding a variant SpA polypeptide the vector can encode other polypeptide sequences such as a one or more other bacterial peptide, a tag, or an immunogenicity enhancing peptide. Useful vectors encoding such fusion proteins include pIN vectors (Inouye *et al.*, 1985), vectors encoding a stretch of histidines, and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage.

[00185] The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an

operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described herein.

### 1. Promoters and Enhancers

[00186] A "promoter" is a control sequence. The promoter is typically a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[00187] Naturally, it may be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression (see Sambrook *et al.*, 2001). The promoters employed may be constitutive, tissue-specific, or inducible and in certain embodiments may direct high level expression of the introduced DNA segment under specified conditions, such as large-scale production of recombinant proteins or peptides.

[00188] Various elements/promoters may be employed in the context of the present invention to regulate the expression of a gene. Examples of such inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus, include but are not limited to Immunoglobulin Heavy Chain (Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Atchinson *et al.*, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990), Immunoglobulin Light Chain (Queen *et al.*, 1983; Picard *et al.*, 1984), T Cell Receptor (Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990), HLA

DQ α and/or DQ β (Sullivan *et al.*, 1987), β Interferon (Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988), Interleukin-2 (Greene *et al.*, 1989), Interleukin-2 Receptor (Greene *et al.*, 1989; Lin *et al.*, 1990), MHC Class II 5 (Koch *et al.*, 1989), MHC Class II HLA-DRα (Sherman *et al.*, 1989), β-Actin (Kawamoto *et al.*, 1988; Ng *et al.*; 1989), Muscle Creatine Kinase (MCK) (Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989), Prealbumin (Transthyretin) (Costa *et al.*, 1988), Elastase I (Ornitz *et al.*, 1987), Metallothionein (MTII) (Karin *et al.*, 1987; Culotta *et al.*, 1989), Collagenase (Pinkert *et al.*, 1987; Angel *et al.*, 1987), Albumin (Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990), α-Fetoprotein (Godbout *et al.*, 1988; Campere *et al.*, 1989), γ-Globin (Bodine *et al.*, 1987; Perez-Stable *et al.*, 1990), β-Globin (Trudel *et al.*, 1987), c-fos (Cohen *et al.*, 1987), c-Ha-Ras (Triesman, 1986; Deschamps *et al.*, 1985), Insulin (Edlund *et al.*, 1985), Neural Cell Adhesion Molecule (NCAM) (Hirsh *et al.*, 1990), α1-Antitrypain (Latimer *et al.*, 1990), H2B (TH2B) Histone (Hwang *et al.*, 1990), Mouse and/or Type I Collagen (Ripe *et al.*, 1989), Glucose-Regulated Proteins (GRP94 and GRP78) (Chang *et al.*, 1989), Rat Growth Hormone (Larsen *et al.*, 1986), Human Serum Amyloid A (SAA) (Edbrooke *et al.*, 1989), Troponin I (TN I) (Yutzey *et al.*, 1989), Platelet-Derived Growth Factor (PDGF) (Pech *et al.*, 1989), Duchenne Muscular Dystrophy (Klamut *et al.*, 1990), SV40 (Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988), Polyoma (Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell *et al.*, 1988), Retroviruses (Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman *et al.*, 1989), Papilloma Virus (Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987), Hepatitis B Virus (Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988), Human Immunodeficiency Virus (Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et*

*al.*, 1989), Cytomegalovirus (CMV) IE (Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986), Gibbon Ape Leukemia Virus (Holbrook *et al.*, 1987; Quinn *et al.*, 1989).

[00189] Inducible elements include, but are not limited to MT II - Phorbol Ester (TFA)/Heavy metals (Palmiter *et al.*, 1982; Haslinger *et al.*, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989); MMTV (mouse mammary tumor virus) – Glucocorticoids (Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988); β-Interferon - poly(rI)x/poly(rc) (Tavernier *et al.*, 1983); Adenovirus 5 E2 – ElA (Imperiale *et al.*, 1984); Collagenase - Phorbol Ester (TPA) (Angel *et al.*, 1987a); Stromelysin - Phorbol Ester (TPA) (Angel *et al.*, 1987b); SV40 - Phorbol Ester (TPA) (Angel *et al.*, 1987b); Murine MX Gene - Interferon, Newcastle Disease Virus (Hug *et al.*, 1988); GRP78 Gene - A23187 (Resendez *et al.*, 1988); α-2-Macroglobulin - IL-6 (Kunz *et al.*, 1989); Vimentin – Serum (Rittling *et al.*, 1989); MHC Class I Gene H-2κb – Interferon (Blanar *et al.*, 1989); HSP70 – ElA/SV40 Large T Antigen (Taylor *et al.*, 1989, 1990a, 1990b); Proliferin - Phorbol Ester/TPA (Mordacq *et al.*, 1989); Tumor Necrosis Factor – PMA (Hensel *et al.*, 1989); and Thyroid Stimulating Hormone α Gene - Thyroid Hormone (Chatterjee *et al.*, 1989).

[00190] The particular promoter that is employed to control the expression of peptide or protein encoding polynucleotide of the invention is not believed to be critical, so long as it is capable of expressing the polynucleotide in a targeted cell, preferably a bacterial cell. Where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a bacterial, human or viral promoter.

[00191] In embodiments in which a vector is administered to a subject for expression of the protein, it is contemplated that a desirable promoter for use with the vector is one that is not down-regulated by cytokines or one that is strong enough that even if down-regulated, it produces an effective amount of a variant SpA for eliciting an immune response. Non-limiting examples of these are CMV IE and RSV LTR. Tissue specific promoters can be used, particularly if expression is in cells in which

expression of an antigen is desirable, such as dendritic cells or macrophages. The mammalian MHC I and MHC II promoters are examples of such tissue-specific promoters.

### 2. Initiation Signals and Internal Ribosome Binding Sites (IRES)

[00192] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals.

[00193] In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of $5^{\prime}$ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988; Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patents 5,925,565 and 5,935,819).

### 3. Selectable and Screenable Markers

[00194] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by encoding a screenable or selectable marker in the expression vector. When transcribed and translated, a marker confers an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

### F. Host Cells

[00195] As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors or viruses. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid, such as a recombinant protein-encoding sequence, is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

[00196] Host cells may be derived from prokaryotes or eukaryotes, including bacteria, yeast cells, insect cells, and mammalian cells for replication of the vector or expression of part or all of the nucleic acid sequence(s). Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials.

### G. Expression Systems

[00197] Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

[00198] The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents 5,871,986, 4,879,236 and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

[00199] In addition to the disclosed expression systems of the invention, other examples of expression systems include STRATAGENE®'s COMPLETE

CONTROL™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast Pichia methanolica. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

## V. POLYSACCHARIDES

[00200] The immunogenic compositions of the invention may further comprise capsular polysaccharides including one or more of PIA (also known as PNAG) and/or *S. aureus* Type V and/or type VIII capsular polysaccharide and/or *S. epidermidis* Type I, and/or Type II and/or Type III capsular polysaccharide.

### H. PIA (PNAG)

[00201] It is now clear that the various forms of staphylococcal surface polysaccharides identified as PS/A, PIA and SAA are the same chemical entity - PNAG (Maira-Litran *et al.*, 2004). Therefore the term PIA or PNAG encompasses all these polysaccharides or oligosaccharides derived from them.

[00202] PIA is a polysaccharide intercellular adhesin and is composed of a polymer of β-(1→6)-linked glucosamine substituted with N-acetyl and O-succinyl constituents. This polysaccharide is present in both *S. aureus* and *S. epidermidis* and can be isolated from either source (Joyce *et al.*, 2003; Maira-Litran *et al.*, 2002). For example, PNAG may be isolated from *S. aureus* strain MN8m (WO04/43407). PIA isolated from *S. epidermidis* is a integral constituent of biofilm. It is responsible for mediating cell-cell adhesion and probably also functions to shield the growing colony from the host's immune response. The polysaccharide previously known as poly-N-succinyl-β-(1→6)-glucosamine (PNSG) was recently shown not to have the expected structure since the identification of N- succinylation was incorrect (Maira-Litran *et*

*al.*, 2002). Therefore the polysaccharide formally known as PNSG and now found to be PNAG is also encompassed by the term PIA.

[00203] PIA (or PNAG) may be of different sizes varying from over 400kDa to between 75 and 400kDa to between 10 and 75kDa to oligosaccharides composed of up to 30 repeat units (of β-(1→6)-linked glucosamine substituted with N-acetyl and O-succinyl constituents). Any size of PIA polysaccharide or oligosaccharide may be use in an immunogenic composition of the invention, in one aspect the polysaccharide is over 40kDa. Sizing may be achieved by any method known in the art, for instance by microfluidization, ultrasonic irradiation or by chemical cleavage (WO 03/53462, EP497524, EP497525). In certain aspects PIA (PNAG) is at least or at most 40-400kDa, 40-300kDa, 50-350kDa, 60-300kDa, 50-250kDa and 60-200kDa.

[00204] PIA (PNAG) can have different degree of acetylation due to substitution on the amino groups by acetate. PIA produced *in vitro* is almost fully substituted on amino groups (95- 100%). Alternatively, a deacetylated PIA (PNAG) can be used having less than 60%, 50%, 40%, 30%, 20%, 10% acetylation. Use of a deacetylated PIA (PNAG) is preferred since non-acetylated epitopes of PNAG are efficient at mediating opsonic killing of Gram positive bacteria, preferably *S. aureus* and/or *S. epidermidis*. In certain aspects, the PIA (PNAG) has a size between 40kDa and 300kDa and is deacetylated so that less than 60%, 50%, 40%, 30% or 20% of amino groups are acetylated.

[00205] The term deacetylated PNAG (dPNAG) refers to a PNAG polysaccharide or oligosaccharide in which less than 60%, 50%, 40%, 30%, 20% or 10% of the amino agroups are acetylated. In certain aspects, PNAG is deaceylated to form dPNAG by chemically treating the native polysaccharide. For example, the native PNAG is treated with a basic solution such that the pH rises to above 10. For instance the PNAG is treated with 0.1-5 M, 0.2-4 M, 0.3-3 M, 0.5-2 M, 0.75-1.5 M or 1 M NaOH , KOH or $NH_4OH$. Treatment is for at least 10 to 30 minutes, or 1, 2, 3, 4, 5, 10, 15 or 20 hours at a temperature of 20-100, 25-80, 30-60 or 30-50 or 35-45 °C. dPNAG may be prepared as described in WO 04/43405.

[00206] The polysaccharide(s) can be conjugated or unconjugated to a carrier protein.

## I. Type 5 and Type 8 polysaccharides from *S. aureus*

[00207] Most strains of *S. aureus* that cause infection in man contain either Type 5 or Type 8 polysaccharides. Approximately 60% of human strains are Type 8 and approximately 30% are Type 5. The structures of Type 5 and Type 8 capsular polysaccharide antigens are described in Moreau *et al.*, (1990) and Fournier *et al.*, (1984). Both have FucNAcp in their repeat unit as well as ManNAcA which can be used to introduce a sulfhydryl group. The structures are:

[00208] Type 5

[00209] →4)-β-D-ManNAcA(3OAc)-(1→4)-α-L-FucNAc(1→3)-β-D-FucNAc-(1→

[00210] Type 8

[00211] →3)-β-D-ManNAcA(4OAc)-(1→3)-α-L-FucNAc(1→3)-β-D-FucNAc-(1→

[00212] Recently (Jones, 2005) NMR spectroscopy revised the structures to:

[00213] Type 5

[00214] →4)-β-D-ManNAcA-(1→4)-α-L-FucNAc(3OAc)-(1→3)-β-D-FucNAc-(1→

[00215] Type 8

[00216] →3)-β-D-ManNAcA(4OAc)-(1→3)-α-L-FucNAc(1→3)-α-D-FucNAc(1→

[00217] Polysaccharides may be extracted from the appropriate strain of *S. aureus* using method well known to of skill in the art, *See* U.S. Patent 6,294,177. For example, ATCC 12902 is a Type 5 *S. aureus* strain and ATCC 12605 is a Type 8 *S. aureus* strain.

[00218] Polysaccharides are of native size or alternatively may be sized, for instance by microfluidisation, ultrasonic irradiation, or by chemical treatment. The invention also covers oligosaccharides derived from the type 5 and 8 polysaccharides

from *S. aureus*. The type 5 and 8 polysaccharides included in the immunogenic composition of the invention are preferably conjugated to a carrier protein as described below or are alternatively unconjugated. The immunogenic compositions of the invention alternatively contains either type 5 or type 8 polysaccharide.

### J. *S. aureus* 336 antigen

[00219] In an embodiment, the immunogenic composition of the invention comprises the *S. aureus* 336 antigen described in U.S. Patent 6,294,177. The 336 antigen comprises β-linked hexosamine, contains no O-acetyl groups, and specifically binds to antibodies to *S. aureus* Type 336 deposited under ATCC 55804. In an embodiment, the 336 antigen is a polysaccharide which is of native size or alternatively may be sized, for instance by microfluidisation, ultrasonic irradiation, or by chemical treatment. The invention also covers oligosaccharides derived from the 336 antigen. The 336 antigen can be unconjugated or conjugated to a carrier protein.

### K. Type I, II and III polysaccharides from *S. epidermidis*

[00220] Amongst the problems associated with the use of polysaccharides in vaccination, is the fact that polysaccharides *per se* are poor immunogens. It is preferred that the polysaccharides utilized in the invention are linked to a protein carrier which provide bystander T-cell help to improve immunogenicity. Examples of such carriers which may be conjugated to polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, DT CRM197 and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD), *Pseudomonas aeruginosa* exoprotein A (rEPA), protein D from *Haemophilus influenzae*, pneumolysin or fragments of any of the above. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular the protein D fragment from *H. influenza* will preferably contain the N-terminal 1/3 of the protein. Protein D is an IgD-binding protein from *Haemophilus influenzae* (EP 0 594 610 B1) and is a potential immunogen. In addition, staphylococcal proteins may be used as a carrier protein in the polysaccharide conjugates of the invention.

[00221] A carrier protein that would be particularly advantageous to use in the context of a staphylococcal vaccine is staphylococcal alpha toxoid. The native form may be conjugated to a polysaccharide since the process of conjugation reduces

toxicity. Preferably genetically detoxified alpha toxins such as the His35Leu or His35Arg variants are used as carriers since residual toxicity is lower. Alternatively the alpha toxin is chemically detoxified by treatment with a cross-linking reagent, formaldehyde or glutaraldehyde. A genetically detoxified alpha toxin is optionally chemically detoxified, preferably by treatment with a cross-linking reagent, formaldehyde or glutaraldehyde to further reduce toxicity.

[00222] The polysaccharides may be linked to the carrier protein(s) by any known method (for example those methods described in U.S. Patents 4,372,945, 4,474,757, and 4,356,170). Preferably, CDAP conjugation chemistry is carried out (see WO95/08348). In CDAP, the cyanylating reagent 1-cyano-dimethylaminopyridinium tetrafluoroborate (CDAP) is preferably used for the synthesis of polysaccharide-protein conjugates. The cyanilation reaction can be performed under relatively mild conditions, which avoids hydrolysis of the alkaline sensitive polysaccharides. This synthesis allows direct coupling to a carrier protein.

[00223] Conjugation preferably involves producing a direct linkage between the carrier protein and polysaccharide. Optionally a spacer (such as adipic dihydride (ADH)) may be introduced between the carrier protein and the polysaccharide.

## IV. Immune Response and Assays

[00224] As discussed above, the invention concerns evoking or inducing an immune response in a subject against a variant SpA or coagulase peptide. In one embodiment, the immune response can protect against or treat a subject having, suspected of having, or at risk of developing an infection or related disease, particularly those related to staphylococci. One use of the immunogenic compositions of the invention is to prevent nosocomial infections by inoculating a subject prior to undergoing procedures in a hospital or other environment having an increased risk of infection.

### A. Immunoassays

[00225] The present invention includes the implementation of serological assays to evaluate whether and to what extent an immune response is induced or evoked by compositions of the invention. There are many types of immunoassays that can be implemented. Immunoassays encompassed by the present invention include, but are

not limited to, those described in U.S. Patent 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent 4,452,901 (western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

[00226] Immunoassays generally are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. In one example, antibodies or antigens are immobilized on a selected surface, such as a well in a polystyrene microtiter plate, dipstick, or column support. Then, a test composition suspected of containing the desired antigen or antibody, such as a clinical sample, is added to the wells. After binding and washing to remove non specifically bound immune complexes, the bound antigen or antibody may be detected. Detection is generally achieved by the addition of another antibody, specific for the desired antigen or antibody, that is linked to a detectable label. This type of ELISA is known as a "sandwich ELISA." Detection also may be achieved by the addition of a second antibody specific for the desired antigen, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

[00227] Competition ELISAs are also possible implementations in which test samples compete for binding with known amounts of labeled antigens or antibodies. The amount of reactive species in the unknown sample is determined by mixing the sample with the known labeled species before or during incubation with coated wells. The presence of reactive species in the sample acts to reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal. Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non specifically bound species, and detecting the bound immune complexes.

[00228] Antigen or antibodies may also be linked to a solid support, such as in the form of plate, beads, dipstick, membrane, or column matrix, and the sample to be analyzed is applied to the immobilized antigen or antibody. In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a

solution of the antigen or antibody, either overnight or for a specified period. The wells of the plate will then be washed to remove incompletely-adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein, and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

### B. Diagnosis of Bacterial Infection

[00229] In addition to the use of proteins, polypeptides, and/or peptides, as well as antibodies binding these polypeptides, proteins, and/or peptides, to treat or prevent infection as described above, the present invention contemplates the use of these polypeptides, proteins, peptides, and/or antibodies in a variety of ways, including the detection of the presence of Staphylococci to diagnose an infection, whether in a patient or on medical equipment which may also become infected. In accordance with the invention, a preferred method of detecting the presence of infections involves the steps of obtaining a sample suspected of being infected by one or more staphylococcal bacteria species or strains, such as a sample taken from an individual, for example, from one's blood, saliva, tissues, bone, muscle, cartilage, or skin. Following isolation of the sample, diagnostic assays utilizing the polypeptides, proteins, peptides, and/or antibodies of the present invention may be carried out to detect the presence of staphylococci, and such assay techniques for determining such presence in a sample are well known to those skilled in the art and include methods such as radioimmunoassay, western blot analysis and ELISA assays. In general, in accordance with the invention, a method of diagnosing an infection is contemplated wherein a sample suspected of being infected with staphylococci has added to it the polypeptide, protein, peptide, antibody, or monoclonal antibody in accordance with the present invention, and staphylococci are indicated by antibody binding to the polypeptides, proteins, and/or peptides, or polypeptides, proteins, and/or peptides binding to the antibodies in the sample.

[00230] Accordingly, antibodies in accordance with the invention may be used for the prevention of infection from staphylococcal bacteria (*i.e.*, passive immunization),

for the treatment of an ongoing infection, or for use as research tools. The term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, such as those fragments which maintain the binding specificity of the antibodies, including the products of an Fab immunoglobulin expression library. Accordingly, the invention contemplates the use of single chains such as the variable heavy and light chains of the antibodies. Generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art. Specific examples of the generation of an antibody to a bacterial protein can be found in U.S. Patent Application Pub. No. 20030153022.

[00231] Any of the above described polypeptides, proteins, peptides, and/or antibodies may be labeled directly with a detectable label for identification and quantification of staphylococcal bacteria. Labels for use in immunoassays are generally known to those skilled in the art and include enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances, including colored particles such as colloidal gold or latex beads. Suitable immunoassays include enzyme-linked immunosorbent assays (ELISA).

### C. Protective Immunity

[00232] In some embodiments of the invention, proteinaceous compositions confer protective immunity to a subject. Protective immunity refers to a body's ability to mount a specific immune response that protects the subject from developing a particular disease or condition that involves the agent against which there is an immune response. An immunogenically effective amount is capable of conferring protective immunity to the subject.

[00233] As used herein in the specification and in the claims section that follows, the term polypeptide or peptide refer to a stretch of amino acids covalently linked there amongst via peptide bonds. Different polypeptides have different functionalities according to the present invention. While according to one aspect, a polypeptide is derived from an immunogen designed to induce an active immune response in a recipient, according to another aspect of the invention, a polypeptide is derived from an antibody which results following the elicitation of an active immune response in,

for example, an animal, and which can serve to induce a passive immune response in the recipient. In both cases, however, the polypeptide is encoded by a polynucleotide according to any possible codon usage.

[00234] As used herein the phrase "immune response" or its equivalent "immunological response" refers to the development of a humoral (antibody mediated), cellular (mediated by antigen-specific T cells or their secretion products) or both humoral and cellular response directed against a protein, peptide, carbohydrate, or polypeptide of the invention in a recipient patient. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibody, antibody containing material, or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules, to activate antigen-specific CD4 (+) T helper cells and/or CD8 (+) cytotoxic T cells. The response may also involve activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. As used herein "active immunity" refers to any immunity conferred upon a subject by administration of an antigen.

[00235] As used herein "passive immunity" refers to any immunity conferred upon a subject without administration of an antigen to the subject. "Passive immunity" therefore includes, but is not limited to, administration of activated immune effectors including cellular mediators or protein mediators (*e.g.*, monoclonal and/or polyclonal antibodies) of an immune response. A monoclonal or polyclonal antibody composition may be used in passive immunization for the prevention or treatment of infection by organisms that carry the antigen recognized by the antibody. An antibody composition may include antibodies that bind to a variety of antigens that may in turn be associated with various organisms. The antibody component can be a polyclonal antiserum. In certain aspects the antibody or antibodies are affinity purified from an animal or second subject that has been challenged with an antigen(s). Alternatively, an antibody mixture may be used, which is a mixture of monoclonal and/or polyclonal antibodies to antigens present in the same, related, or different microbes or organisms, such as gram-positive bacteria, gram-negative bacteria, including but not limited to staphylococcus bacteria.

[00236] Passive immunity may be imparted to a patient or subject by administering to the patient immunoglobulins (Ig) and/or other immune factors obtained from a donor or other non-patient source having a known immunoreactivity. In other aspects, an antigenic composition of the present invention can be administered to a subject who then acts as a source or donor for globulin, produced in response to challenge with the antigenic composition ("hyperimmune globulin"), that contains antibodies directed against Staphylococcus or other organism. A subject thus treated would donate plasma from which hyperimmune globulin would then be obtained, *via* conventional plasma-fractionation methodology, and administered to another subject in order to impart resistance against or to treat staphylococcus infection. Hyperimmune globulins according to the invention are particularly useful for immune-compromised individuals, for individuals undergoing invasive procedures or where time does not permit the individual to produce their own antibodies in response to vaccination. See U.S. Patents 6,936,258, 6,770,278, 6,756,361, 5,548,066, 5,512,282, 4,338,298, and 4,748,018 for exemplary methods and compositions related to passive immunity.

[00237] For purposes of this specification and the accompanying claims the terms "epitope" and "antigenic determinant" are used interchangeably to refer to a site on an antigen to which B and/or T cells respond or recognize. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, Epitope Mapping Protocols (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. T-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by $^{3}$H-

thymidine incorporation by primed T cells in response to an epitope (Burke *et al.*, 1994), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges *et al.*, 1996) or by cytokine secretion.

[00238] The presence of a cell-mediated immunological response can be determined by proliferation assays (CD4 (+) T cells) or CTL (cytotoxic T lymphocyte) assays. The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating IgG and T-cells from an immunized syngeneic animal and measuring protective or therapeutic effect in a second subject.

[00239] As used herein and in the claims, the terms "antibody" or "immunoglobulin" are used interchangeably and refer to any of several classes of structurally related proteins that function as part of the immune response of an animal or recipient, which proteins include IgG, IgD, IgE, IgA, IgM and related proteins.

[00240] Under normal physiological conditions antibodies are found in plasma and other body fluids and in the membrane of certain cells and are produced by lymphocytes of the type denoted B cells or their functional equivalent. Antibodies of the IgG class are made up of four polypeptide chains linked together by disulfide bonds. The four chains of intact IgG molecules are two identical heavy chains referred to as H-chains and two identical light chains referred to as L-chains.

[00241] In order to produce polyclonal antibodies, a host, such as a rabbit or goat, is immunized with the antigen or antigen fragment, generally with an adjuvant and, if necessary, coupled to a carrier. Antibodies to the antigen are subsequently collected from the sera of the host. The polyclonal antibody can be affinity purified against the antigen rendering it monospecific.

[00242] Monoclonal antibodies can be produced by hyperimmunization of an appropriate donor with the antigen or *ex-vivo* by use of primary cultures of splenic cells or cell lines derived from spleen (Anavi, 1998; Huston *et al.*, 1991; Johnson *et al.*, 1991; Mernaugh *et al.*, 1995).

[00243] As used herein and in the claims, the phrase "an immunological portion of an antibody" includes a Fab fragment of an antibody, a Fv fragment of an antibody, a

heavy chain of an antibody, a light chain of an antibody, a heterodimer consisting of a heavy chain and a light chain of an antibody, a variable fragment of a light chain of an antibody, a variable fragment of a heavy chain of an antibody, and a single chain variant of an antibody, which is also known as scFv. In addition, the term includes chimeric immunoglobulins which are the expression products of fused genes derived from different species, one of the species can be a human, in which case a chimeric immunoglobulin is said to be humanized. Typically, an immunological portion of an antibody competes with the intact antibody from which it was derived for specific binding to an antigen.

[00244] Optionally, an antibody or preferably an immunological portion of an antibody, can be chemically conjugated to, or expressed as, a fusion protein with other proteins. For purposes of this specification and the accompanying claims, all such fused proteins are included in the definition of antibodies or an immunological portion of an antibody.

[00245] As used herein the terms "immunogenic agent" or "immunogen" or "antigen" are used interchangeably to describe a molecule capable of inducing an immunological response against itself on administration to a recipient, either alone, in conjunction with an adjuvant, or presented on a display vehicle.

### D. Treatment Methods

[00246] A method of the present invention includes treatment for a disease or condition caused by a staphylococcus pathogen. An immunogenic polypeptide of the invention can be given to induce an immune response in a person infected with staphylococcus or suspected of having been exposed to staphylococcus. Methods may be employed with respect to individuals who have tested positive for exposure to staphylococcus or who are deemed to be at risk for infection based on possible exposure.

[00247] In particular, the invention encompasses a method of treatment for staphylococcal infection, particularly hospital acquired nosocomial infections. The immunogenic compositions and vaccines of the invention are particularly advantageous to use in cases of elective surgery. Such patients will know the date of surgery in advance and could be inoculated in advance. The immunogenic

compositions and vaccines of the invention are also advantageous to use to inoculate health care workers.

[00248] In some embodiments, the treatment is administered in the presence of adjuvants or carriers or other staphylococcal antigens. Furthermore, in some examples, treatment comprises administration of other agents commonly used against bacterial infection, such as one or more antibiotics.

[00249] The use of peptides for vaccination can require, but not necessarily, conjugation of the peptide to an immunogenic carrier protein, such as hepatitis B surface antigen, keyhole limpet hemocyanin, or bovine serum albumin. Methods for performing this conjugation are well known in the art.

## VI. Vaccine and other Pharmaceutical Compositions and Administration

### E. Vaccines

[00250] The present invention includes methods for preventing or ameliorating staphylococcal infections, particularly hospital acquired nosocomial infections. As such, the invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared from immunogenic SpA polypeptide(s), such as a SpA domain D variant, or immunogenic coagulases. In other embodiments SpA or coagulases can be used in combination with other secreted virulence proteins, surface proteins or immunogenic fragments thereof. In certain aspects, antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

[00251] Other options for a protein/peptide-based vaccine involve introducing nucleic acids encoding the antigen(s) as DNA vaccines. In this regard, recent reports described construction of recombinant vaccinia viruses expressing either 10 contiguous minimal CTL epitopes (Thomson, 1996) or a combination of B cell, cytotoxic T-lymphocyte (CTL), and T-helper (Th) epitopes from several microbes (An, 1997), and successful use of such constructs to immunize mice for priming protective immune responses. Thus, there is ample evidence in the literature for successful utilization of peptides, peptide-pulsed antigen presenting cells (APCs), and peptide-encoding constructs for efficient *in vivo* priming of protective immune

responses. The use of nucleic acid sequences as vaccines is exemplified in U.S. Patents 5,958,895 and 5,620,896.

[00252] The preparation of vaccines that contain polypeptide or peptide sequence(s) as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions: solid forms suitable for solution in or suspension in liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants that enhance the effectiveness of the vaccines. In specific embodiments, vaccines are formulated with a combination of substances, as described in U.S. Patents 6,793,923 and 6,733,754.

[00253] Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1% to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

[00254] The polypeptides and polypeptide-encoding DNA constructs may be formulated into a vaccine as neutral or salt forms. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the peptide) and

those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like.

[00255] Typically, vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including the capacity of the individual's immune system to synthesize antibodies and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms of active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

[00256] The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application within a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection and the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size and health of the subject.

[00257] In certain instances, it will be desirable to have multiple administrations of the vaccine, *e.g.*, 2, 3, 4, 5, 6 or more administrations. The vaccinations can be at 1, 2, 3, 4, 5, 6, 7, 8, to 5, 6, 7, 8, 9 ,10, 11, 12 twelve week intervals, including all ranges there between. Periodic boosters at intervals of 1-5 years will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies against the antigens, as described in U.S. Patents 3,791,932; 4,174,384 and 3,949,064.

### 1. Carriers

[00258] A given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as

ovalbumin, mouse serum albumin, or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimyde, and bis-biazotized benzidine.

### 2. Adjuvants

[00259] The immunogenicity of polypeptide or peptide compositions can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins, or synthetic compositions. A number of adjuvants can be used to enhance an antibody response against a variant SpA polypeptide or coagulase, or any other bacterial protein or combination contemplated herein. Adjuvants can (1) trap the antigen in the body to cause a slow release; (2) attract cells involved in the immune response to the site of administration; (3) induce proliferation or activation of immune system cells; or (4) improve the spread of the antigen throughout the subject's body.

[00260] Adjuvants include, but are not limited to, oil-in-water emulsions, water-in-oil emulsions, mineral salts, polynucleotides, and natural substances. Specific adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ-interferon, GMCSP, BCG, aluminum salts, such as aluminum hydroxide or other aluminum compound, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM), and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion. MHC antigens may even be used. Others adjuvants or methods are exemplified in U.S. Patents 6,814,971, 5,084,269, 6,656,462).

[00261] Various methods of achieving adjuvant affect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by

reactivating with pepsin-treated (Fab) antibodies to albumin; mixture with bacterial cells (e.g., *C. parvum*), endotoxins or lipopolysaccharide components of Gram-negative bacteria; emulsion in physiologically acceptable oil vehicles (*e.g.*, mannide mono-oleate (Aracel A)); or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed to produce an adjuvant effect.

[00262] Examples of and often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants, and aluminum hydroxide.

[00263] In some aspects, it is preferred that the adjuvant be selected to be a preferential inducer of either a Th1 or a Th2 type of response. High levels of Th1-type cytokines tend to favor the induction of cell mediated immune responses to a given antigen, while high levels of Th2-type cytokines tend to favor the induction of humoral immune responses to the antigen.

[00264] The distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4+ T cell clones by Mosmann and Coffman (Mosmann, and Coffman, 1989). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of IL- 4, IL-5, IL-6, IL-10.

[00265] In addition to adjuvants, it may be desirable to co-administer biologic response modifiers (BRM) to enhance immune responses. BRMs have been shown to upregulate T cell immunity or downregulate suppresser cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); or low-dose Cyclophosphamide (CYP; 300 $mg/m^2$) (Johnson/ Mead, NJ) and cytokines

such as γ-interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B-7.

### F. Lipid Components and Moieties

[00266] In certain embodiments, the present invention concerns compositions comprising one or more lipids associated with a nucleic acid or a polypeptide/peptide. A lipid is a substance that is insoluble in water and extractable with an organic solvent. Compounds other than those specifically described herein are understood by one of skill in the art as lipids, and are encompassed by the compositions and methods of the present invention. A lipid component and a non-lipid may be attached to one another, either covalently or non-covalently.

[00267] A lipid may be a naturally occurring lipid or a synthetic lipid. However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glucolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof.

[00268] A nucleic acid molecule or a polypeptide/peptide, associated with a lipid may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid or otherwise associated with a lipid. A lipid or lipid-poxvirus-associated composition of the present invention is not limited to any particular structure. For example, they may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape. In another example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. In another non-limiting example, a lipofectamine(Gibco BRL)-poxvirus or Superfect (Qiagen)-poxvirus complex is also contemplated.

[00269] In certain embodiments, a composition may comprise about 1%, about 2%, about 3%, about 4% about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%,

about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or any range therebetween, of a particular lipid, lipid type, or non-lipid component such as an adjuvant, antigen, peptide, polypeptide, sugar, nucleic acid or other material disclosed herein or as would be known to one of skill in the art. In a non-limiting example, a composition may comprise about 10% to about 20% neutral lipids, and about 33% to about 34% of a cerebroside, and about 1% cholesterol. In another non-limiting example, a liposome may comprise about 4% to about 12% terpenes, wherein about 1% of the micelle is specifically lycopene, leaving about 3% to about 11% of the liposome as comprising other terpenes; and about 10% to about 35% phosphatidyl choline, and about 1% of a non-lipid component. Thus, it is contemplated that compositions of the present invention may comprise any of the lipids, lipid types or other components in any combination or percentage range.

### G. Combination Therapy

[00270] The compositions and related methods of the present invention, particularly administration of a secreted virulence factor or surface protein, including a variant SpA polypeptide or peptide, and/or other bacterial peptides or proteins to a patient/subject, may also be used in combination with the administration of traditional therapies. These include, but are not limited to, the administration of antibiotics such as streptomycin, ciprofloxacin, doxycycline, gentamycin, chloramphenicol, trimethoprim, sulfamethoxazole, ampicillin, tetracycline or various combinations of antibiotics.

[00271] In one aspect, it is contemplated that a polypeptide vaccine and/or therapy is used in conjunction with antibacterial treatment. Alternatively, the therapy may precede or follow the other agent treatment by intervals ranging from minutes to

weeks. In embodiments where the other agents and/or a proteins or polynucleotides are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and antigenic composition would still be able to exert an advantageously combined effect on the subject. In such instances, it is contemplated that one may administer both modalities within about 12-24 h of each other or within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for administration significantly, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[00272] Various combinations may be employed, for example antibiotic therapy is "A" and the immunogenic molecule given as part of an immune therapy regime, such as an antigen, is "B":

[00273] A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

[00274] B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

[00275] B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[00276] Administration of the immunogenic compositions of the present invention to a patient/subject will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the SpA composition, or other compositions described herein. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, such as hydration, may be applied in combination with the described therapy.

### H. General Pharmaceutical Compositions

[00277] In some embodiments, pharmaceutical compositions are administered to a subject. Different aspects of the present invention involve administering an effective amount of a composition to a subject. In some embodiments of the present invention, staphylococcal antigens, members of the Ess pathway, including polypeptides or peptides of the Esa or Esx class, and/or members of sortase substrates may be

administered to the patient to protect against infection by one or more staphylococcus pathogens. Alternatively, an expression vector encoding one or more such polypeptides or peptides may be given to a patient as a preventative treatment. Additionally, such compounds can be administered in combination with an antibiotic or an antibacterial. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

[00278] In addition to the compounds formulated for parenteral administration, such as those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including creams, lotions, mouthwashes, inhalants and the like.

[00279] The active compounds of the present invention can be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains a compound or compounds that increase the expression of an MHC class I molecule will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for use to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and, the preparations can also be emulsified.

[00280] Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[00281] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil, or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that it may be easily injected. It also should be stable

under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[00282] The proteinaceous compositions may be formulated into a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[00283] The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[00284] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00285] Administration of the compositions according to the present invention will typically be via any common route. This includes, but is not limited to oral, nasal, or buccal administration. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal, intranasal, or intravenous injection. In certain embodiments, a vaccine composition may be inhaled (*e.g.*, U.S. Patent 6,651,655). Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. As used herein, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem complications commensurate with a reasonable benefit/risk ratio. The term "pharmaceutically acceptable carrier," means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent.

[00286] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in isotonic NaCl solution and either added to hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, Remington's Pharmaceutical Sciences, 1990). Some variation in dosage will necessarily occur depending on the condition of the subject. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

[00287] An effective amount of therapeutic or prophylactic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined quantity of the composition calculated to produce the desired responses

discussed above in association with its administration, *i.e.*, the appropriate route and regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

[00288] Precise amounts of the composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the subject, route of administration, intended goal of treatment (alleviation of symptoms versus cure), and potency, stability, and toxicity of the particular composition.

[00289] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically or prophylactically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above.

### I. *In Vitro*, *Ex Vivo*, or *In Vivo* Administration

[00290] As used herein, the term *in vitro* administration refers to manipulations performed on cells removed from or outside of a subject, including, but not limited to cells in culture. The term *ex vivo* administration refers to cells which have been manipulated *in vitro*, and are subsequently administered to a subject. The term *in vivo* administration includes all manipulations performed within a subject.

[00291] In certain aspects of the present invention, the compositions may be administered either *in vitro*, *ex vivo*, or *in vivo*. In certain *in vitro* embodiments, autologous B-lymphocyte cell lines are incubated with a virus vector of the instant invention for 24 to 48 hours or with a variant SpA and/or cogaulase and/or any other composition described herein for two hours. The transduced cells can then be used for *in vitro* analysis, or alternatively for *ex vivo* administration. U.S. Patents 4,690,915 and 5,199,942 disclose methods for *ex vivo* manipulation of blood mononuclear cells and bone marrow cells for use in therapeutic applications.

### J. Antibodies And Passive Immunization

[00292] Another aspect of the invention is a method of preparing an immunoglobulin for use in prevention or treatment of staphylococcal infection

comprising the steps of immunizing a recipient or donor with the vaccine of the invention and isolating immunoglobulin from the recipient or donor. An immunoglobulin prepared by this method is a further aspect of the invention. A pharmaceutical composition comprising the immunoglobulin of the invention and a pharmaceutically acceptable carrier is a further aspect of the invention which could be used in the manufacture of a medicament for the treatment or prevention of staphylococcal disease. A method for treatment or prevention of staphylococcal infection comprising a step of administering to a patient an effective amount of the pharmaceutical preparation of the invention is a further aspect of the invention.

[00293] Inocula for polyclonal antibody production are typically prepared by dispersing the antigenic composition in a physiologically tolerable diluent such as saline or other adjuvants suitable for human use to form an aqueous composition. An immunostimulatory amount of inoculum is administered to a mammal and the inoculated mammal is then maintained for a time sufficient for the antigenic composition to induce protective antibodies.

[00294] The antibodies can be isolated to the extent desired by well known techniques such as affinity chromatography (Harlow and Lane, 1988). Antibodies can include antiserum preparations from a variety of commonly used animals, *e.g.* goats, primates, donkeys, swine, horses, guinea pigs, rats or man.

[00295] An immunoglobulin produced in accordance with the present invention can include whole antibodies, antibody fragments or subfragments. Antibodies can be whole immunoglobulins of any class (*e.g.,* IgG, IgM, IgA, IgD or IgE), chimeric antibodies or hybrid antibodies with dual specificity to two or more antigens of the invention. They may also be fragments (*e.g.*, F(ab')2, Fab', Fab, Fv and the like) including hybrid fragments. An immunoglobulin also includes natural, synthetic, or genetically engineered proteins that act like an antibody by binding to specific antigens to form a complex.

[00296] A vaccine of the present invention can be administered to a recipient who then acts as a source of immunoglobulin, produced in response to challenge from the specific vaccine. A subject thus treated would donate plasma from which hyperimmune globulin would be obtained via conventional plasma fractionation

methodology. The hyperimmune globulin would be administered to another subject in order to impart resistance against or treat staphylococcal infection. Hyperimmune globulins of the invention are particularly useful for treatment or prevention of staphylococcal disease in infants, immune compromised individuals, or where treatment is required and there is no time for the individual to produce antibodies in response to vaccination.

[00297] An additional aspect of the invention is a pharmaceutical composition comprising two of more monoclonal antibodies (or fragments thereof; preferably human or humanised) reactive against at least two constituents of the immunogenic composition of the invention, which could be used to treat or prevent infection by Gram positive bacteria, preferably staphylococci, more preferably *S. aureus* or *S. epidermidis*. Such pharmaceutical compositions comprise monoclonal antibodies that can be whole immunoglobulins of any class, chimeric antibodies, or hybrid antibodies with specificity to two or more antigens of the invention. They may also be fragments (*e.g.*, F(ab')2, Fab', Fab, Fv and the like) including hybrid fragments.

[00298] Methods of making monoclonal antibodies are well known in the art and can include the fusion of splenocytes with myeloma cells (Kohler and Milstein, 1975; Harlow and Lane, 1988). Alternatively, monoclonal Fv fragments can be obtained by screening a suitable phage display library (Vaughan *et al.*, 1998). Monoclonal antibodies may be humanized or part humanized by known methods.

## VII. EXAMPLES

[00299] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

## EXAMPLE 1

## NON-TOXIGENIC PROTEIN A VARIANTS AS SUBUNIT VACCINES TO PREVENT *STAPHYLOCOCCUS AUREUS* INFECTIONS

[00300] ***An animal model for S. aureus infection*** BALB/c mice were infected by intravenous injection with $1\times10^7$ CFU of the human clinical isolate *S. aureus* Newman (Baba *et al.*, 2007). Within 6 hours following infection, 99.999% of staphylococci disappeared from the blood stream and were distributed via the vasculature. Staphylococcal dissemination to peripheral tissues occurred rapidly, as the bacterial load in kidney and other peripheral organ tissues reached $1\times10^5$ CFU $g^{-1}$ within the first three hours. The staphylococcal load in kidney tissues increased by 1.5 log CFU within twenty-four hours. Forty-eight hours following infection, mice developed disseminated abscesses in multiple organs, detectable by light microscopy of hematoxylin-eosin stained, thin-sectioned kidney tissue. The initial abscess diameter was 524 μM (± 65 μM); lesions were initially marked by an influx of polymorphonuclear leukocytes (PMNs) and harbored no discernable organization of staphylococci, most of which appeared to reside within PMNs. On day 5 of infection, abscesses increased in size and enclosed a central population of staphylococci, surrounded by a layer of eosinophilic, amorphous material and a large cuff of PMNs. Histopathology revealed massive necrosis of PMNs in proximity to the staphylococcal nidus at the center of abscess lesions as well as a mantle of healthy phagocytes. A rim of necrotic PMNs were observed at the periphery of abscess lesions, bordering eosinophilic, amorphous material that separates healthy renal tissue from lesions. Abscesses eventually reached a diameter of ≥ 1,524 μM on day 15 or 36. At later time intervals, the staphylococcal load was increased to $10^4$-$10^6$ CFU $g^{-1}$ and growing abscess lesions migrated towards the organ capsule. Peripheral lesions were prone to rupture, thereby releasing necrotic material and staphylococci into the peritoneal cavity or the retroperitoneal space. These events resulted in bacteremia as well as a secondary wave of abscesses, eventually precipitating a lethal outcome.

[00301] To enumerate staphylococcal load in renal tissue, animals were killed, their kidneys excised and tissue homogenate spread on agar media for colony formation. On day 5 of infection, a mean of $1\times10^6$ CFU $g^{-1}$ renal tissue for *S. aureus* Newman was observed. To quantify abscess formation, kidneys were visually inspected, and each individual organ was given a score of one or zero. The final sum

was divided by the total number of kidneys to calculate percent surface abscesses (Table 3). In addition, randomly chosen kidneys were fixed in formalin, embedded, thin sectioned, and stained with hematoxylin-eosin. For each kidney, four sagittal sections at 200 μM intervals were viewed by microscopy. The numbers of lesions were counted for each section and averaged to quantify the number of abscesses within the kidneys. *S. aureus* Newman caused 4.364 ± 0.889 abscesses per kidney, and surface abscesses were observed on 14 out of 20 kidneys (70%) (Table 3).

[00302] When examined by scanning electron microscopy, *S. aureus* Newman was located in tightly associated lawns at the center of abscesses. Staphylococci were contained by an amorphous pseudocapsule that separated bacteria from the cuff of abscesses leukocytes. No immune cells were observed in these central nests of staphylococci, however occasional red blood cells were located among the bacteria. Bacterial populations at the abscess center, designated *staphylococcal abscess communities* (SAC), appeared homogenous and coated by an electron-dense, granular material. The kinetics of the appearance of infectious lesions and the morphological attributes of abscesses formed by *S. aureus* Newman were similar to those observed following mouse infection with *S. aureus* USA300 (LAC), the current epidemic community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clone in the United States (Diep *et al.*, 2006).

**Table 3.** Genetic requirements for *S. aureus* Newman abscess formation in mice

| Genotype | Staphylococcal load in kidney tissue | | | Abscess formation in kidney tissue | | |
|---|---|---|---|---|---|---|
| | [a]$log_{10}$ CFU $g^{-1}$ tissue | [b]Significance (P-value) | [c]Reduction ($log_{10}$ CFU $g^{-1}$) | [d]Surface abscesses (%) | [e]Number of abscesses per kidney | [f] Significance (P-value) |
| wild-type | 6.141 ± 0.192 | – | – | 70 | 4.364 ± 0.889 | – |
| Δ*srtA* | 4.095 ± 0.347 | $6.7\times10^{-6}$ | 2.046 | 0 | 0.000 ± 0.000 | 0.0216 |
| *spa* | 5.137 ± 0.374 | 0.0144 | 1.004 | 13 | 0.375 ± 0.374 | 0.0356 |

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 5 days following infection in cohorts of fifteen BALB/c mice per challenge strain. Standard error of the means (±SEM) is indicated.
[b]Statistical significance was calculated with the Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.
[c]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.
[d]Abscess formation in kidney tissues five days following infection was measured by macroscopic inspection (% positive)
[e]Histopathology of hematoxylin-eosin stained, thin sectioned kidneys from eight to ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).
[f]Statistical significance was calculated with the Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[00303] ***S. aureus* Protein A (*spa*) mutants are avirulent and cannot form abscesses** Sortase A is a transpeptidase that immobilizes nineteen surface proteins in the envelope of *S. aureus* strain Newman (Mazmanian *et al.*, 1999; Mazmanian *et al.*, 2000). Earlier work identified sortase A as a virulence factor in multiple animal model systems, however the contributions of this enzyme and its anchored surface proteins to abscess formation or persistence have not yet been revealed (Jonsson *et al.*, 2002; Weiss *et al.*, 2004). Compared to the wild-type parent (Baba *et al.*, 2007), an isogenic *srtA* variant (Δ*srtA*) failed to form abscess lesions on either macroscopic or histopathology examination on days 2, 5, or 15. In mice infected with the *strA* mutant, only $1\times10^4$ CFU $g^{-1}$ was recovered from kidney tissue on day 5 of infection, which is a 2.046 $\log_{10}$ CFU $g^{-1}$ reduction compared to the wild-type parent strain ($P=6.73\times10^{-6}$). A similar defect was observed for the *srtA* mutant of MRSA strain USA300 (data not shown). Scanning electron microscopy showed that *srtA* mutants were highly dispersed and often associated with leukocytes in otherwise healthy renal tissue. On day fifteen following infection, *srtA* mutants were cleared from renal tissues, a $\geq$ 3.5 $\log_{10}$ CFU $g^{-1}$ reduction compared to the wild-type (Table 3). Thus, sortase A anchored surface proteins enable the formation of abscess lesions and the persistence of bacteria in host tissues, wherein staphylococci replicate as communities embedded in an extracellular matrix and shielded from surrounding leukocytes by an amorphous pseudocapsule.

[00304] Sortase A anchors a large spectrum of proteins with LPXTG motif sorting signals to the cell wall envelope, thereby providing for the surface display of many virulence factors (Mazmanian *et al.*, 2002). To identify surface proteins required for staphylococcal abscess formation, *bursa aurealis* insertions were introduced in 5' coding sequences of genes that encode polypeptides with LPXTG motif proteins (Bae *et al.*, 2004) and these mutations were transduced into *S. aureus* Newman. Mutations in the structural gene for Protein A (*spa)* reduced the staphylococcal load in infected mouse kidney tissues by 1.004 $\log_{10}$ (P=0.0144). When analyzed for their ability to form abscesses in kidney tissues by histopathology, we observed that the *spa* mutants were unable to form abscesses as compared with the wild-type parent strain *S. aureus* Newman (wild-type *S. aureus* Newman 4.364 $\pm$ 0.889 abscesses per kidney vs. the isogenic *spa* mutant with 0.375 $\pm$ 0.374 lesions; P = 0.0356).

[00305] ***Protein A blocks innate and adaptive immune responses.*** Studies identified Protein A as a critical virulence factor during the pathogenesis of *S. aureus* infections. Earlier work demonstrated that Protein A impedes phagocytosis of staphylococci by binding the Fc component of immunoglobulin (Jensen 1958; Uhlén *et al.*, 1984), activates platelet aggregation via the von Willebrand factor (Hartleib *et al.*, 2000), functions as a B cell superantigen by capturing the $F(ab)_2$ region of VH3 bearing IgM (Roben *et al.*, 1995), and, through its activation of TNFR1, can initiate staphylococcal pneumonia (Gomez *et al.*, 2004). Due to the fact that Protein A captures immunoglobulin and displays toxic attributes, the possibility that this surface molecule may function as a vaccine in humans has not been rigorously pursued. The inventors demonstrate for the first time that Protein A variants no longer able to bind to immunoglobulins, vWF and TNFR-1 are removed of their toxigenic potential and are able to stimulate humoral immune responses that protect against staphylococcal disease.

[00306] ***Molecular basis of Protein A surface display and function.*** Protein A is synthesized as a precursor in the bacterial cytoplasm and secreted via its YSIRK signal peptide at the cross wall, *i.e.*, the cell division septum of staphylococci (FIG. 1A). (DeDent *et al.*, 2007; DeDent *et al.*, 2008). Following cleavage of the C-terminal LPXTG sorting signal, Protein A is anchored to bacterial peptidoglycan crossbridges by sortase A (Schneewind *et al.*, 1995; Mazmanian *et al.*, 1999; Mazmanian *et al.*, 2000). Protein A is the most abundant surface protein of staphylococci; the molecule is expressed by virtually all *S. aureus* strains (Saïd-Salim *et al.*, 2003; Cespedes *et al.*, 2005; Kennedy *et al.*, 2008). Staphylococci turn over 15-20% of their cell wall per division cycle (Navarre and Schneewind 1999). Murine hydrolases cleave the glycan strands and wall peptides of peptidoglycan, thereby releasing Protein A with its attached C-terminal cell wall disaccharide tetrapeptide into the extracellular medium (Ton-That *et al.*, 1999). Thus, by physiological design, Protein A is both anchored to the cell wall and displayed on the bacterial surface but also released into surrounding tissues during host infection (Marraffini *et al.*, 2006).

[00307] Protein A captures immunoglobulins on the bacterial surface and this biochemical activity enables staphylococcal escape from host innate and acquired immune responses (Jensen 1958; Goodyear and Silverman 2004). Interestingly,

region X of Protein A (Guss *et al.*, 1984), a repeat domain that tethers the IgG binding domains to the LPXTG sorting signal/cell wall anchor, is perhaps the most variable portion of the staphylococcal genome (Schneewind *et al.*, 1992; Saïd-Salim *et al.*, 2003). Each of the five immunoglobulin binding domains of Protein A (SpA), formed from three helix bundles and designated E, D, A, B, and C, exerts similar structural and functional properties (Sjödahl 1977; Jansson *et al.*, 1998). The solution and crystal structure of domain D has been solved both with and without the Fc and $V_H3$ (Fab) ligands, which bind Protein A in a non-competitive manner at distinct sites (Graille *et al.*, 2000).

[00308] In the crystal structure complex, the Fab interacts with helix II and helix III of domain D via a surface composed of four VH region β-strands (Graille *et al.*, 2000). The major axis of helix II of domain D is approximately 50° to the orientation of the strands, and the interhelical portion of domain D is most proximal to the C0 strand. The site of interaction on Fab is remote from the Ig light chain and the heavy chain constant region. The interaction involves the following domain D residues: Asp-36 of helix II as well as Asp-37 and Gln-40 in the loop between helix II and helix III, in addition to several other residues with SpA-D (Graille *et al.*, 2000). Both interacting surfaces are composed predominantly of polar side chains, with three negatively charged residues on domain D and two positively charged residues on the 2A2 Fab buried by the interaction, providing an overall electrostatic attraction between the two molecules. Of the five polar interactions identified between Fab and domain D, three are between side chains. A salt bridge is formed between Arg-H19 and Asp-36 and two hydrogen bonds are made between Tyr-H59 and Asp-37 and between Asn-H82a and Ser-33. Because of the conservation of Asp-36 and Asp-37 in all five IgG binding domains of Protein A, these residues were selected for mutagenesis.

[00309] The SpA-D sites responsible for Fab binding are structurally separate from the domain surface that mediates Fcγ binding. The interaction of Fcγ with domain B primarily involves residues in helix I with lesser involvement of helix II (Deisenhofer 1981; Gouda *et al.*, 1992). With the exception of the Gln-32, a minor contact in both complexes, none of the residues that mediate the Fcγ interaction are involved in Fab binding. To examine the spatial relationship between these different Ig-binding sites,

the SpA domains in these complexes have been superimposed to construct a model of a complex between Fab, the SpA-domain D, and the Fcγ molecule. In this ternary model, Fab and Fcγ form a sandwich about opposite faces of the helix II without evidence of steric hindrance of either interaction. These findings illustrate how, despite its small size (*i.e.*, 56–61 aa), a SpA domain can simultaneously display both activities, explaining experimental evidence that the interactions of Fab with an individual domain are noncompetitive. Residues for the interaction between SpA-D and Fcγ are Gln-9 and Gln-10.

[00310] In contrast, occupancy of the Fc portion of IgG on the domain D blocks its interaction with vWF A1 and probably also TNFR1 (O'Seaghdha *et al.*, 2006). Mutations in residues essential for IgG Fc binding (F5, Q9, Q10, S11, F13, Y14, L17, N28, I31 and K35) are also required for vWF A1 and TNFR1 binding (Cedergren *et al.*, 1993; Gómez *et al.*, 2006; O'Seaghdha *et al.* 2006), whereas residues critical for the $V_H3$ interaction (Q26, G29, F30, S33, D36, D37, Q40, N43, E47) have no impact on the binding activities of IgG Fc, vWF A1 or TNFR1 (Jansson *et al.*, 1998; Graille *et al.*, 2000). The Protein A immunoglobulin Fab binding activity targets a subset of B cells that express VH3 family related IgM on their surface, *i.e.* these molecules function as VH3 type B cell receptors (Roben *et al.*, 1995). Upon interaction with SpA, these B cells rapidly proliferate and then commit to apoptosis, leading to preferential and prolonged deletion of innate-like B lymphocytes (*i.e.* marginal zone B cells and follicular B2 cells) (Goodyear and Silverman 2003; Goodyear and Silverman 2004). It is important to note that more than 40% of circulating B cells are targeted by the Protein A interaction and the VH3 family represents the largest family of human B cell receptors to impart protective humoral responses against pathogens (Goodyear and Silverman 2003; Goodyear and Silverman 2004). Thus, Protein A functions analogously to staphylococcal superantigens (Roben *et al.*, 1995), albeit that the latter class of molecules, for example SEB, TSST-1, TSST-2, form complexes with the T cell receptor to inappropriately stimulate host immune responses and thereby precipitating characteristic disease features of staphylococcal infections (Roben *et al.*, 1995; Tiedemann *et al.*, 1995). Together these findings document the contributions of Protein A in establishing staphylococcal infections and in modulating host immune responses.

[00311] ***Non-toxigenic variant of Protein A.*** The inventors have developed a non-toxigenic variant of staphylococcal Protein A and, with this reagent in hand, aimed for the first time to measure the immune response of animals to Protein A immunization. Further, the inventors address whether immunization of animals with a non-toxigenic variant of Protein A could generate immune responses that raise protective immunity against staphylococcal infection.

[00312] To perturb the IgG Fc, vWF A1 and TNFR1 binding activities of Protein A, glutamine (Q) residues 9 and 10 [the numbering here is derived from that established for the SpA domain D] were modified generating lysine or glycine substitutions for both glutamines with the expectation that these substitutions abolish the ion bonds formed between wild-type Protein A and its ligands. The added effect of the dual lysine substitutions may be that these positively charged residues institute a repellent charge for immunoglobulins. To perturb IgM Fab VH3 binding, the inventors selected the aspartate (D) residues 36 and 37 of SpA-D, each of which is required for the association of Protein A with the B cell receptor. D36 and D37 were both substituted with alanine. The Q9,10K and D36,37A mutations were combined in the recombinant molecule SpA-$D_{Q9,10K;D36,37A}$ and examined for the binding attributes of Protein A.

[00313] In brief, the Protein A (*spa*) genomic sequence of *Staphylococcus aureus* N315 was PCR amplified with the primers (GCTGCACATATGGCGCAACACGATGAAGCTCAAC [5'primer](SEQ ID NO:35) and AGTGGATCCTTATGCTTTGTTAGCATCTGC [3' primer] (SEQ ID NO:36)), cloned into the pET15b vector (pYSJ1, codons 48-486) (Stranger-Jones, *et al.*, 2006) and recombinant plasmid transformed into *E. coli* BL21(DE3) (Studier *et al.*, 1990). The Protein A product derived from pYSJ1 harbors SpA residues 36-265 fused to the N-terminal His tag (MGSSHHHHHHSSGLVPRGS (SEQ ID NO:37)). Following IPTG inducible expression, recombinant N-terminal $His_6$-tagged SpA was purified by affinity chromatography on Ni-NTA resin (Stranger-Jones *et al.*, 2006). The domain D of SpA (SpA-D) was PCR amplified with a pair of specific primers (AACATATGTTCAACAAAGATCAACAAAGC [5' primer](SEQ ID NO:38) and AAGGATCCAGATTCGTTTAATTTTTTAGC [3' primer] (SEQ ID NO:39)), sub-cloned into the pET15b vector (pHAN1, *spa* codons 212-261) and recombinant

plasmid transformed into *E. coli* BL21(DE3) to express and purify recombinant N-terminal $His_6$-tagged protein. To generate mutations in the SpA-D coding sequence, sets of two pairs of primers were synthesized (for D to A substitutions: CTTCATTCAAAGTCTTAAAGCCGCCCCAAGCCAAAGCACTAAC [5' primer] (SEQ ID NO:40) and GTTAGTGCTTTGGCTTGGGGCGGCTTTAAGACTTTGAATGAAG [3' primer] (SEQ ID NO:41); for Q to K substitutions CATATGTTCAACAAAGATAAAAAAAGCGCCTTCTATGAAATC [5' primer] (SEQ ID NO:42) and GATTTCATAGAAGGCGCTTTTTTTATCTTTGTTGAACATATG [3' primer] (SEQ ID NO:43); for Q to G substitutions CATATGTTCAACAAAGATGGAGGAAGCGCCTTCTATGAAATC [5' primer] (SEQ ID NO:44) and GATTTCATAGAAGGCGCTTCCTCCATCTTTGTTGAACATATG' [3' primer] (SEQ ID NO:45). Primers were used for quick-change mutagenesis protocols. Following mutagenesis, DNA sequences were confirmed for each of the recombinant proteins: SpA, SpA-D and SpA-$D_{Q9,10G;D36,37A}$ and SpA-$D_{Q9,10K;D36,37A}$. All proteins were purified from lysates of recombinant *E. coli* using Ni-NTA chromatography and subsequently dialyzed against PBS and stored at 4°C.

[00314] To measure binding of immunoglobulin to Protein A and its variants, 200 μg of purified protein was diluted into a 1 ml volume using column buffer (50 mM Tris-HCl, 150 mM NaCl, pH7.5) and then loaded onto a pre-equilibrated Ni-NTA column (1 ml bed volume). Columns were washed with 10 ml of column buffer. 200 μg of purified human IgG was diluted in a total volume of 1 ml column buffer and then applied to each of the columns charged with Protein A and its variants. The columns were subsequently washed with 5 ml wash buffer (10 mM imidazole in column buffer) and 5 ml column buffer. Protein samples were eluted with 2 ml elution buffer (500 mM imidazole in column buffer), fractions collected and aliquots subjected to SDS-PAGE gel electrophoresis, followed by Coomassie-Blue staining. As shown in FIG. 1C, wild-type Protein A (SpA) and its SpA-domain D both retained immunogobulin during chromatography. In contrast, the SpA-$D_{Q9,10K;D36,37A}$ variant did not bind to immunoglobulin.

[00315] To quantify the binding of Protein A and its variants to the Fc portion of immunoglobulin and the VH3 domain of Fab, HRP conjugated human immunoglobulin G [hIgG], the Fc portion of human IgG [hFc] and the $F(ab)_2$ portion of human IgG [$hF(ab)_2$] as well as ELISA assays were used to quantify the relative amount binding to Protein A and its variants. The data in FIG. 1D demonstrate the binding of SpA and SpA-D to hIgG and hFc, whereas SpA-$D_{Q9,10G;D36,37A}$ and SpA-$D_{Q9,10K;D36,37A}$ displayed only background binding activities. SpA bound similar amounts of hFc and $hF(ab)_2$, however the binding of SpA-D to $hF(ab)_2$ was reduced compared to full length SpA. This result suggests that the presence of multiple IgG binding domains may cooperatively increase the ability of Protein A to bind to the B cell receptor. When compared with the reduced binding power of SpA-D for $hF(ab)_2$, of the two variants only SpA-$D_{Q9,10K;D36,37A}$ displayed a significant reduction in the ability to bind the VH3 domain of immunoglobulin. To examine the toxigenic attributes of SpA-D and its variants, purified proteins were injected into mice, which were sacrificed after 4 hours to remove their spleens. Organ tissue was homogenized, capsular material removed and B cells stained with fluorescent CD19 antibodies. Following FACS analysis to quantify the abundance of B cells in splenic tissues, it was observed that SpA-D caused a 5% drop in the B cell count compared to a mock (PBS) control (FIG. 1E). In contrast, SpA-$D_{Q9,10K;D36,37A}$ did not cause a reduction in B-cell counts, indicating that the mutant molecule had lost its toxigenic attributes of stimulating B cell proliferation and death (FIG. 1E). In summary, amino acid substitutions in the SpA-D residues Q9, Q10, D36, and D37 abolished the ability of Protein A domains to bind immunoglobulins or exert toxigenic functions in human and animal tissues.

[00316] ***Non-toxigenic Protein A variants elicit vaccine protection.*** To test whether or not Protein A and its variants can function as vaccine antigens, SpA, SpA-D, SpA-$D_{Q9,10K;D36,37A}$, and SpA-$D_{Q9,10K;D36,37A}$ were emulsified with complete or incomplete Freund's adjuvant and immunized 4 week old BALB/c mice on day 1 and day 11 with 50 μg of purified protein. Cohort of animals (n=5) were analyzed for humoral immune responses to immunization by bleeding the animals before (day 0) and after the immunization schedule (day 21). Table 4 indicates that immunized mice generated only a modest humoral immune response directed at wild-type Protein A or its SpA-D module, whereas the amount of antibody raised following immunization

with SpA-$D_{Q9,10K;D36,37A}$ or SpA-$D_{Q9,10K;D36,37A}$ was increased four to five fold. Following intravenous challenge with $1 \times 10^7$ CFU *S. aureus* Newman, animals were killed on day 4, their kidneys removed and either analyzed for staphylococcal load (by plating tissue homogenate on agar plates and enumerating colony forming units, CFU) or histopathology. As expected, mock (PBS) immunized mice (n=19) harbored 6.46 $\log_{10}$ (± 0.25) CFU in kidney tissue and infectious lesions were organized into 3.7 (± 1.2) abscesses per organ (n=10)(Table 4). Immunization of animals with SpA led to a 2.51 $\log_{10}$ CFU reduction on day 5 (P=0.0003) with 2.1 (± 1.2) abscesses per organ. The latter data indicate that there was no significant reduction in abscess formation (P=0.35). Immunization with SpA-D generated similar results: a 2.03 $\log_{10}$ CFU reduction on day 5 (P=0.0001) with 1.5 (± 0.8) abscesses per organ (P=0.15). In contrast, immunization with SpA-$D_{Q9,10K;D36,37A}$ or SpA-$D_{Q9,10G;D36,37A}$ created increased protection, with 3.07 $\log_{10}$ and 3.03 $\log_{10}$ CFU reduction on day 4, respectively (statistical significance P<0.0001 for both observations). Further, immunization with both SpA-$D_{Q9,10K;D36,37A}$ and SpA-$D_{Q9,10G;D36,37A}$ generated significant protection from staphylococcal abscess formation, as only 0.5 (± 0.4) and 0.8 (± 0.5) infectious lesions per organ (P=0.02 and P=0.04) were identified. Thus, immunization with non-toxigenic Protein A variants generates increased humoral immune responses for Protein A and provides protective immunity against staphylococcal challenge. These data indicate that Protein A is an ideal candidate for a human vaccine that prevents *S. aureus* disease.

[00317] These exciting results have several implications for the design of a human vaccine. First, the generation of substitution mutations that affect the ability of the immunoglobulin binding domains of Protein A, either alone or in combination of two or more domains, can generate non-toxigenic variants suitable for vaccine development. It seems likely that a combination of mutant IgG binding domains closely resembling the structure of Protein A can generate even better humoral immune responses as is reported here for the SpA-domain D alone. Further, a likely attribute of Protein A specific antibodies may be that the interaction of antigen binding sites with the microbial surface can neutralize the ability of staphylococci to capture immunoglobulins via their Fc portion or to stimulate the B cell receptor via the VH3 binding activities.

**Table 4. Non-toxigenic Protein A variants as vaccine antigens that prevent *S. aureus* disease**

| | Bacterial load in kidney (n=number of mice) | | | IgG titer | Abscess formation in mice (n=number of mice) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Antigen | [a]$log_{10}$ CFU $g^{-1}$ | [b]Reduction | [c]P value | | [d]Surface abscess | Reduction | [e]Histopathology | Reduction | [f]P value |
| Mock | 6.46 ± 0.25 (n=19) | ____ | ____ | <100 | 14/19 (70%) | ____ | 3.7 ± 1.2 (n=10) | ____ | ____ |
| SpA | 3.95 ± 0.56 (n=20) | 2.51 | 0.0003 | 1706 ± 370 | 10/20 (50%) | 32% | 2.1 ± 1.2 (n=10) | 2.2 | 0.35 |
| SpA-D | 4.43 ± 0.41 (n=18) | 2.03 | 0.0001 | 381 ± 27 | 10/18 (55%) | 25% | 1.5 ± 0.8 (n=10) | 2.2 | 0.15 |
| SpA-D1 | 3.39 ± 0.50 (n=19) | 3.07 | <0.0001 | 5600 ± 801 | 6/20 (30%) | 59% | 0.5 ± 0.4 (n=10) | 3.2 | 0.02 |
| SpA-D2 | 3.43 ± 0.46 (n=19) | 3.03 | <0.0001 | 3980 ± 676 | 6/19 (32%) | 57% | 0.8 ± 0.5 (n=10) | 2.9 | 0.04 |

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of 18 to 20 BALB/c mice. Standard error of the means (±SEM) is indicated.
[c]Statistical significance was calculated with the Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.
[b]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.
[d]Abscess formation in kidney tissues four days following infection was measured by macroscopic inspection (% positive)
[e]Histopathology of hematoxylin-eosin stained, thin sectioned kidneys from ten animals; the number of abscesses per kidney was recorded and averaged for the final mean (±SEM).
[f]Statistical significance was calculated with the Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.
SpA-D1 and SpA-D2 represent SpA-$D_{Q9,10K;D36,37A}$ and SpA-$D_{Q9,10G;D36,37A}$, respectively.

[00318] **Vaccine protection in murine abscess, murine lethal infection, and murine pneumonia models.** Three animal models have been established for the study of *S. aureus* infectious disease. These models are used here to examine the level of protective immunity provided via the generation of Protein A specific antibodies.

[00319] **MATERIALS AND METHODS**

[00320] **Murine abscess** – BALB/c mice (24-day-old female, 8-10 mice per group, Charles River Laboratories, Wilmington, MA) are immunized by intramuscular injection into the hind leg with purified protein (Chang *et al.*, 2003; Schneewind *et al.*, 1992). Purified SpA, SpA-D or SpA-DQ9,10K;D36,37A (50 μg protein) is administered on days 0 (emulsified 1:1 with complete Freund's adjuvant) and 11 (emulsified 1:1 with incomplete Freund's adjuvant). Blood samples are drawn by retroorbital bleeding on days 0, 11, and 20. Sera are examined by ELISA for IgG titers for specific SpA-D and SpA-DQ9,10K;D36,37A binding activity. Immunized animals are challenged on day 21 by retroorbital injection of 100 μl of *S. aureus* Newman or *S. aureus* USA300 suspension ($1 \times 10^7$ cfu). For this, overnight cultures of *S. aureus* Newman are diluted 1:100 into fresh tryptic soy broth and grown for 3 h at 37°C. Staphylococci are centrifuged, washed twice, and diluted in PBS to yield an $A_{600}$ of 0.4 ($1 \times 10^8$ cfu per ml). Dilutions are verified experimentally by agar plating and colony formation. Mice are anesthetized by intraperitoneal injection of 80-120 mg of ketamine and 3-6 mg of xylazine per kilogram of body weight and infected by retroorbital injection. On day 5 or 15 following challenge, mice are euthanized by compressed $CO_2$ inhalation. Kidneys are removed and homogenized in 1% Triton X-100™. Aliquots are diluted and plated on agar medium for triplicate determination of cfu. For histology, kidney tissue is incubated at room temperature in 10% formalin for 24 h. Tissues are embedded in paraffin, thin-sectioned, stained with hematoxylinleosin, and examined by microscopy.

[00321] **Murine lethal infection** - BALB/c mice (24-day-old female, 8-10 mice per group, Charles River Laboratories, Wilmington, MA) are immunized by intramuscular injection into the hind leg with purified SpA, SpA-D or SpA-$D_{Q9,10K;D36,37A}$ (50 μg protein). Vaccine is administered on days 0 (emulsified 1:1 with complete Freund's adjuvant) and 11 (emulsified 1:1 with incomplete Freund's

adjuvant). Blood samples are drawn by retroorbital bleeding on days 0, 11, and 20. Sera are examined by ELISA for IgG titers with specific SpA-D and SpA-$D_{Q9,10K;D36,37A}$ binding activity. Immunized animals are challenged on day 21 by retroorbital injection of 100 µl of *S. aureus* Newman or *S. aureus* USA300 suspension (15 x $10^7$ cfu) (34). For this, overnight cultures of *S. aureus* Newman are diluted 1:100 into fresh tryptic soy broth and grown for 3 h at 37°C. Staphylococci are centrifuged, washed twice, diluted in PBS to yield an $A_{600}$ of 0.4 (1 x $10^8$ cfu per ml) and concentrated. Dilutions are verified experimentally by agar plating and colony formation. Mice are anesthetized by intraperitoneal injection of 80-120 mg of ketamine and 3-6 mg of xylazine per kilogram of body weight. Immunized animals are challenged on day 21 by intraperitoneal inject with 2 x $10^{10}$ cfu of *S. aureus* Newman or 3-10 x $10^9$ cfu of clinical *S. aureus* isolates. Animals are monitored for 14 days, and lethal disease is recorded.

[00322] **Murine pneumonia model** - *S. aureus* strains Newman or USA300 (LAC) are grown at 37°C in tryptic soy broth/agar to $OD_{660}$ 0.5. 50-ml culture aliquots are centrifuged, washed in PBS, and suspended in 750 µl PBS for mortality studies (3-4 x $10^8$ CFU per 30-µl volume), or 1,250 µl PBS (2 x $10^8$ CFU per 30-µl volume) for bacterial load and histopathology experiments (2, 3). For lung infection, 7-wk-old C57BL/6J mice (The Jackson Laboratory) are anesthetized before inoculation of 30 µl of *S. aureus* suspension into the left nare. Animals are placed into the cage in a supine position for recovery and observed for 14 days. For active immunization, 4-wk-old mice receive 20 µg SpA-D or SpA-$D_{Q9,10K;D36,37A}$ in CFA on day 0 via the i.m. route, followed by a boost with 20 µg SpA-D or SpA-$D_{Q9,10K;D36,37A}$ in incomplete Freund's adjuvant (IFA) on day 10. Animals are challenged with *S. aureus* on day 21. Sera are collected before immunization and on day 20 to assess specific antibody production. For passive immunization studies, 7-wk-old mice receive 100 µl of either NRS (normal rabbit serum) or SpA-D-specific rabbit antisera via i.p. injection 24 h before challenge. To assess the pathological correlates of pneumonia, infected animals are killed via forced $CO_2$ inhalation before removal of both lungs. The right lung is homogenized for enumeration of lung bacterial load. The left lung is placed in 1% formalin and paraffin embedded, thin sectioned, stained with hematoxylin-eosin, and analyzed by microscopy.

[00323] **Rabbit antibodies** - Purified 200 μg SpA-D or SpA-$D_{Q9,10K;D36,37A}$ is used as an immunogen for the production of rabbit antisera. 200 μg protein is emulsified with CFA for injection at day 0, followed by booster injections with 200 μg protein emulsified with IFA on days 21 and 42. Rabbit antibody titers are determined by ELISA. Purified antibodies are obtained by affinity chromatography of rabbit serum on SpA-D or SpA-$D_{Q9,10K;D36,37A}$ sepharose. The concentration of eluted antibodies is measured by absorbance at $A_{280}$ and specific antibody titers are determined by ELISA.

[00324] **Active immunization with SpA-domain D variants.** - To determine vaccine efficacy, animals are actively immunized with purified SpA-D or $SpAD_{Q9,10K;D36,37A}$. As a control, animals are immunized with adjuvant alone. Antibody titers against Protein A preparations are determined using SpA-D or SpA-$D_{Q9,10K;D36,37A}$ as antigens; note that the SpA-$D_{Q9,10K;D36,37A}$ variant cannot bind the Fc or Fab portion of IgG. Using infectious disease models described above, any reduction in bacterial load (murine abscess and pneumonia), histopathology evidence of staphylococcal disease (murine abscess and pneumonia) and protection from lethal disease (murine lethal challenge and pneumonia) is measured.

[00325] **Passive immunization with affinity purified rabbit polyclonal antibodies generated against SpA-domain D variants.** To determine protective immunity of Protein A specific rabbit antibodies, mice are passively immunized with 5 mg/kg of purified SpA-D or SpA-$D_{Q9,10K;D36,37A}$ derived rabbit antibodies. Both of these antibody preparations are purified by affinity chromatography using immobilized SpA-D or SpA-$D_{Q9,10K;D36,37A}$. As a control, animals are passively immunized with rV10 antibodies (a plague protective antigen that has no impact on the outcome of staphylococcal infections). Antibody titers against all Protein A preparations are determined using SpA-$D_{Q9,10K;\ D36,37A}$ as an antigen, as this variant cannot bind the Fc or Fab portion of IgG. Using the infectious disease models described above, the reduction in bacterial load (murine abscess and pneumonia), histopathology evidence of staphylococcal disease (murine abscess and pneumonia), and the protection from lethal disease (murine lethal challenge and pneumonia) is measured.

## EXAMPLE 2

## NON-TOXIGENIC PROTEIN A VACCINE FOR METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* INFECTION

[00326] Clinical isolates of *S. aureus* express protein A (Shopsin *et al.*, 1999, whose primary translational product is comprised of an N-terminal signal peptide (DeDent *et al.*, 2008), five Ig-BDs (designated E, D, A, B and C)(Sjodahl, 1977), region X with variable repeats of an eight residue peptide (Guss *et al.*, 1984), and C-terminal sorting signal for the cell wall anchoring of SpA (Schneewind *et al.*, 1992; Schneewind *et al.*, 1995) (FIG. 1A-1B). Guided by amino acid homology (Uhlen *et al.*, 1984), the triple α-helical bundle structure of IgBDs (Deisenhofer *et al.*, 1978; Deisenhofer *et al.*, 1981) and their atomic interactions with Fab $V_H3$ (Graille *et al.*, 2000) or Fcγ (Gouda *et al.*, 1998), glutamine 9 and 10 were selected as well as aspartate 36 and 37 as critical for the association of SpA with antibodies or B cell receptor, respectively. Substitutions Gln9Lys, Gln10Lys, Asp36Ala and Asp37Ala were introduced into the D domain to generate SpA-$D_{KKAA}$ (FIG. 1B). The ability of isolated SpA-D or SpA-$D_{KKAA}$ to bind human IgG was analyzed by affinity chromatography (FIG. 1D). Polyhistidine tagged SpA-D as well as full-length SpA retained human IgG on Ni-NTA, whereas SpA-$D_{KKAA}$ and a negative control (SrtA) did not (FIG. 1C). A similar result was observed with von Willebrand factor (Hartleib *et al.*, 2000), which, along with tumor necrosis factor receptor 1 (TNFR1)(Gomez *et al.*, 2004), can also bind protein A via glutamine 9 and 10 (FIG. 1D). Human immunoglobulin encompasses 60-70% $V_H3$-type IgG. The inventors distinguish between Fc domain and B cell receptor activation of Igs and measured association of human Fcγ and $F(ab)_2$ fragments, both of which bound to full-length SpA or SpA-D, but not to SpA-$D_{KKAA}$ (FIG. 1D). Injection of SpA-D into the peritoneal cavity of mice resulted in B cell expansion followed by apoptotic collapse of CD19+ lymphocytes in spleen tissue of BALB/c mice (Goodyear and Silverman, 2003)(FIG. 1E). B cell superantigen activity was not observed following injection with SpA-$D_{KKAA}$, and TUNEL-staining of splenic tissue failed to detect the increase in apoptotic cells that follows injection of SpA or SpA-D (FIG. 1E).

[00327] **Antibodies against SpA-$D_{KKAA}$ protect against MSSA and MRSA infections.** Naive six week old BALB/c mice were injected with 50μg each of purified SpA, SpA-D or SpA-$D_{KKAA}$ emulsified in CFA and boosted with the same

antigen emulsified in IFA. In agreement with the hypothesis that SpA-D promotes the apoptotic collapse of activated clonal B cell populations, the inventors observed a ten-fold higher titer of SpA-$D_{KKAA}$ specific antibodies following immunization of mice with the non-toxigenic variant as compared to the B cell superantigen (Spa-D vs. SpA-$D_{KKAA}$ P <0.0001, Table 5). Antibody titers raised by immunization with full-length SpA were higher than those elicited by SpA-D (P=0.0022), which is likely due to the larger size and reiterative domain structure of this antigen (Table 5). Nevertheless, even SpA elicited lower antibody titers than SpA-$D_{KKAA}$ (P=0.0003), which encompasses only 50 amino acids of protein A (520 residues, SEQ ID NO:33). Immunized mice were challenged by intravenous inoculation with *S. aureus* Newman and the ability of staphylococci to seed abscesses in renal tissues was examined by necropsy four days after challenge. In homogenized renal tissue of mock (PBS/adjuvant) immunized mice, an average staphylococcal load of 6.46 $\log_{10}$ CFU $g^{-1}$ was enumerated (Table 5). Immunization of mice with SpA or SpA-D led to a reduction in staphylococcal load, however SpA-$D_{KKAA}$ vaccinated animals displayed an even greater, 3.07 $\log_{10}$ CFU $g^{-1}$ reduction of *S. aureus* Newman in renal tissues (P <0.0001, Table 5). Abscess formation in kidneys was analyzed by histopathology (FIG. 2). Mock immunized animals harbored an average of 3.7 (±1.2) abscesses per kidney (Table 5). Vaccination with SpA-$D_{KKAA}$ reduced the average number of abscesses to 0.5 (±0.4)(P= 0.0204), whereas immunization with SpA or SpA-D did not cause a significant reduction in the number of abscess lesions (Table 5). Lesions from SpA-$D_{KKAA}$ vaccinated animals were smaller in size, with fewer infiltrating PMNs and characteristically lacked staphylococcal abscess communities (Cheng *et al.*, 2009)(FIG. 2). Abscesses in animals that had been immunized with SpA or SpA-D displayed the same overall structure of lesions in mock immunized animals (FIG. 2).

[00328] The inventors examined whether SpA-$D_{KKAA}$ immunization can protect mice against MRSA strains and selected the USA300 LAC isolate for animal challenge (Diep *et al.*, 2006). This highly virulent CA-MRSA strain spread rapidly throughout the United States, causing significant human morbidity and mortality (Kennedy *et al.*, 2008). Compared to adjuvant control mice, SpA-$D_{KKAA}$ immunized animals harbored a 1.07 $\log_{10}$ CFU $g^{-1}$ reduction in bacterial load of infected kidney tissues. Histopathology examination of renal tissue following *S. aureus* USA300

challenge revealed that the average number of abscesses was reduced from 4.04 (±0.8) to 1.6 (±0.6)(P=0.02774). In contrast, SpA or SpA-D immunization did not cause a significant reduction in bacterial load or abscess formation (Table 5).

[00329] **SpA-$D_{KKAA}$ antibodies prevent immunoglobulin-protein A interaction.** Rabbits were immunized with SpA-$D_{KKAA}$ and specific antibodies were purified on SpA-$D_{KKAA}$ affinity column followed by SDS-PAGE (FIG. 3). SpA-$D_{KKAA}$ specific IgG was cleaved with pepsin to generate Fcγ and $F(ab)_2$ fragments, the latter of which were purified by chromatography on SpA-$D_{KKAA}$ column (FIG. 3). Binding of human IgG or vWF to SpA or SpA-D was perturbed by SpA-$D_{KKAA}$ specific $F(ab)_2$, indicating that SpA-$D_{KKAA}$ derived antibodies neutralize the B cell superantigen function of protein A as well as its interactions with Ig (FIG. 3).

[00330] **$SpA_{KKAA}$ generates improved protective immune responses.** To further improve the vaccine properties for non-toxigenic protein A, the inventors generated $SpA_{KKAA}$, which includes all five IgBDs with four amino acid substitutions – substitutions corresponding to Gln9Lys, Gln10Lys, Asp36Ala and Asp37Ala of domain D - in each of its five domains (E, D, A, B and C). Polyhistidine tagged $SpA_{KKAA}$ was purified by affinity chromatography and analyzed by Coomassie Blue-stained SDS-PAGE (FIG. 4). Unlike full-length SpA, $SpA_{KKAA}$ did not bind human IgG, Fc and $F(ab)_2$ or vWF (FIG. 4). $SpA_{KKAA}$ failed to display B cell superantigen activity, as injection of the variant into BALB/c mice did not cause a depletion of CD19+ B cells in splenic tissue (FIG. 4). $SpA_{KKAA}$ vaccination generated higher specific antibody titers than SpA-$D_{KKAA}$ immunization and provided mice with elevated protection against *S. aureus* USA300 challenge (Table 5). Four days following challenge, $SpA_{KKAA}$ vaccinated animals harbored 3.54 $\log_{10}$ CFU $g^{-1}$ fewer staphylococci in renal tissues (P =0.0001) and also caused a greater reduction in the number of abscess lesions (P=0.0109) (Table 5). As a test whether protein A vaccines impact other MRSA strains, mice were challenged with the Japanese vancomycin-resistant MRSA isolate Mu50 (Hiramatsu *et al.*, 1997). Similar to the data observed with the MRSA isolate USA300, $SpA_{KKAA}$ vaccinated animals harbored fewer Mu50 staphylococci in renal tissues than mock immunized animals (P=0.0248, FIG. 7).

[00331] **Passive transfer of SpA-specific antibodies prevents staphylococcal disease.** $SpA_{KKAA}$ was used to immunize rabbits. Rabbit antibodies specific for SpA-

$D_{KKAA}$ or $SpA_{KKAA}$ were affinity purified on matrices with immobilized cognate antigen and injected at a concentration of 5 mg $kg^{-1}$ body weight into the peritoneal cavity of BALB/c mice (Table 6). Twenty-four hours later, specific antibody titers were determined in serum and animals challenged by intravenous inoculation with *S. aureus* Newman. Passive transfer reduced the staphylococcal load in kidney tissues for SpA-$D_{KKAA}$ (P=0.0016) or $SpA_{KKAA}$ (P=0.0005) specific antibodies. On histopathology examination, both antibodies reduced the abundance of abscess lesions in the kidneys of mice challenged with *S. aureus* Newman (Table 6). Together these data reveal that vaccine protection following immunization with SpA-$D_{KKAA}$ or $SpA_{KKAA}$ is conferred by antibodies that neutralize protein A.

[00332] The inventors also sought to ascertain whether protein A-specific antibodies can protect animals against lethal challenge. BALB/c mice were actively or passively immunized to raise antibodies against $SpA_{KKAA}$ and then challenged by intraperitoneal injection with lethal doses of *S. aureus* Newman (FIG. 6). Antibodies against $SpA_{KKAA}$, whether raised by active (P=0.0475, $SpA_{KKAA}$ vs. mock) or passive immunization (P=0.0493, $SpA_{KKAA}$ vs. mock), conferred protection against lethal challenge with *S. aureus* Newman (FIG. 6)

Table 5. Active immunization of mice with protein A vaccines.

| Antigen | Staphylococcal load and abscess formation in renal tissue | | | | | |
|---|---|---|---|---|---|---|
| | [a]$log_{10}$ CFU $g^{-1}$ | [b]P-value | [c]Reduction ($log_{10}$ CFU $g^{-1}$) | [d]IgG Titer | [e]Number of abscesses | [b]P-value |
| *S. aureus* Newman challenge | | | | | | |
| Mock | 6.46 ± 0.25 | – | – | <100 | 3.7 ± 1.2 | – |
| SpA | 3.95 ± 0.56 | 0.0003 | 2.51 | 1706 ± 370 | 2.1 ± 1.2 | 0.3581 |
| SpA-D | 4.43 ± 0.41 | 0.0001 | 2.03 | 381 ± 27 | 1.5 ± 0.8 | 0.1480 |
| SpA-$D_{KKAA}$ | 3.39 ± 0.50 | <0.0001 | 3.07 | 5600 ± 801 | 0.5 ± 0.4 | 0.0204 |
| *S. aureus* USA300 (LAC) challenge | | | | | | |
| Mock | 7.20 ± 0.24 | – | – | <100 | 4.0 ± 0.8 | – |
| SpA | 6.81 ± 0.26 | 0.2819 | 0.39 | 476 ± 60 | 3.3 ± 1.0 | 0.5969 |
| SpA-D | 6.34 ± 0.52 | 0.1249 | 0.86 | 358 ± 19 | 2.2 ± 0.6 | 0.0912 |
| SpA-$D_{KKAA}$ | 6.00 ± 0.42 | 0.0189 | 1.20 | 3710 ± 1147 | 1.6 ± 0.6 | 0.0277 |
| $SpA_{KKAA}$ | 3.66 ± 0.76 | 0.0001 | 3.54 | 10200 ± 2476 | 1.2 ± 0.5 | 0.0109 |

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of fifteen to twenty BALB/c mice per immunization. A representative of three independent and reproducible animal experiments is shown. Standard error of the means (±SEM) is indicated.

[b]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[c]Reduction in bacterial load calculated as $\log_{10}$ CFU $g^{-1}$.

[d]Means of five randomly chosen serum IgG titers were measured prior to staphylococcal infection by ELISA.

[e]Histopathology of hematoxylin-eosin stained, thin sectioned kidneys from ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).

Table 6. Passive immunization of mice with antibodies against protein A.

| [a]Antibody | Staphylococcal load and abscess formation in renal tissue | | | | | |
|---|---|---|---|---|---|---|
| | [b]$log_{10}$ CFU $g^{-1}$ | [c]P-value | [d]Reduction ($log_{10}$ CFU $g^{-1}$) | [e]IgG Titer | [f]Number of abscesses | [c]P-value |
| Mock | 7.10 ± 0.14 | – | – | <100 | 4.5 ± 0.8 | – |
| α-SpA-$D_{KKAA}$ | 5.53 ± 0.43 | 0.0016 | 1.57 | 466 ± 114 | 1.9 ± 0.7 | 0.0235 |
| α-$SpA_{KKAA}$ | 5.69 ± 0.34 | 0.0005 | 1.41 | 1575 ± 152 | 1.6 ± 0.5 | 0.0062 |

[a]Affinity purified antibodies were injected into the peritoneal cavity of BALB/c mice at a concentration of 5 mg · $kg^{-1}$ twenty-four hours prior to intravenous challenge with 1 × $10^7$ CFU *S. aureus* Newman.

[b]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of fifteen BALB/c mice per immunization. A representative of two independent and reproducible animal experiments is shown. Standard error of the means (±SEM) is indicated.

[c]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[d]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.

[e]Means of five randomly chosen serum IgG titers were measured prior to staphylococcal infection by ELISA.

[f]Histopathology of hematoxylin-eosin stained, thin sectioned kidneys from ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).

[00333] **Immune response to protein A following staphyloccal infection or $SpA_{KKAA}$ immunization.** Following infection with virulent *S. aureus*, mice do not develop protective immunity against subsequent infection with the same strain (Burts *et al.*, 2008)(FIG. 8). The average abundance of SpA-$D_{KKAA}$ specific IgG in these animals was determined by dot blot as 0.20 μg $ml^{-1}$ (±0.04) and 0.14 μg $ml^{-1}$ (±0.01) for strains Newman and USA300 LAC, respectively (FIG. 4). The minimal concentration of protein A-specific IgG required for disease protection in $SpA_{KKAA}$ or SpA-$D_{KKAA}$ vaccinated animals (P .0.05 $log_{10}$ reduction in staphylococcal CFU $g^{-1}$ renal tissue) was calculated as 4.05 μg $ml^{-1}$ (± 0.88). Average serum concentration of SpA-specific IgG in adult healthy human volunteers (n=16) was 0.21 μg $ml^{-1}$ (± 0.02). Thus, *S. aureus* infections in mice or humans are not associated with immune responses that raise significant levels of neutralizing antibodies directed against protein A, which is likely due to the B cell superantigen attributes of this molecule. In contrast, the average serum concentration of IgG specific for diphtheria toxin in human volunteers, 0.068 μg $ml^{-1}$ (± 0.20), was within range for protective immunity against diphtheria (Behring, 1890; Lagergard *et al.*, 1992).

[00334] Clinical *S. aureus* isolates express protein A, an essential virulence factor whose B cell surperantigen activity and evasive attributes towards opsono-phagocytic clearance are absolutely required for staphylococcal abscess formation (Palmqvist *et al.*, 2005; Cheng *et al.*, 2009; Silverman and Goodyear, 2006). Protein A can thus be thought of as a toxin, essential for pathogenesis, whose molecular attributes must be neutralized in order to achieve protective immunity. By generating non-toxigenic variants unable to bind Igs via Fcγ or $VH_3$-Fab domains, the inventors measure here for the first time protein A neutralizing immune responses as a correlate for protective immunity against *S. aureus* infection. In contrast to many methicillin-sensitive strains, CA-MRSA isolate USA300 LAC is significantly more virulent (Cheng *et al.*, 2009). For example, immunization of experimental animals with the surface protein IsdB (Kuklin *et al.*, 2006; Stranger-Jones *et al.,* 2006) raises antibodies that confer protection against *S. aureus* Newman (Stranger-Jones *et al.,* 2009) but not against USA300 challenge.

[00335] **MATERIAL AND METHODS**

[00336] **Bacterial strains and growth.** *Staphylococcus aureus* strains Newman and USA300 were grown in tryptic soy broth (TSB) at 37°C. *Escherichia coli* strains DH5α and BL21 (DE3) were grown in Luria-Bertani (LB) broth with 100 μg $ml^{-1}$ ampicillin at 37°C.

[00337] **Rabbit Antibodies.** The coding sequence for SpA was PCR-amplified with two primers, gctgcacatatggcgcaacacgatgaagctcaac (SEQ ID NO:35) and agtggatccttatgcttgagctttgttagcatctgc (SEQ ID NO:36) using *S. aureus* Newman template DNA. SpA-D was PCR-amplified with two primers, aacatatgttcaacaaagatcaacaaagc (SEQ ID NO:38) and aaggatccagattcgtttaatttttttagc (SEQ ID NO:39). The sequence for $SpA\text{-}D_{KKAA}$ was mutagenized with two sets of primers catatgttcaacaaagataaaaaaagcgccttctatgaaatc (SEQ ID NO:42) and gatttcatagaaggcgctttttttatctttgttgaacatatg (SEQ ID NO:43) for for Q9K, Q10K as well as cttcattcaaagtcttaaagccgccccaagccaaagcactaac (SEQ ID NO:40) and gttagtgctttggcttggggcggctttaagactttgaatgaag (SEQ ID NO:41) for D36A,D37A. The sequence of $SpA_{KKAA}$ was synthesized by Integrated DNA Technologies, Inc. PCR products were cloned into pET-15b generating N-terminal $His_6$ tagged recombinant protein. Plasmids were transformed into BL21(DE3). Overnight cultures of transformants were diluted 1:100 into fresh media and grown at 37°C to an $OD_{600}$ 0.5, at which point cultures were induced with 1 mM isopropyl β-D-1-thiogalatopyranoside (IPTG) and grown for an additional three hours. Bacterial cells were sedimented by centrifugation, suspended in column buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and disrupted with a French pressure cell at 14,000 psi. Lysates were cleared of membrane and insoluble components by ultracentrifugation at 40,000 ×*g*. Proteins in the soluble lysate were subjected to nickel-nitrilotriacetic acid (Ni-NTA, Qiagen) affinity chromatography. Proteins were eluted in column buffer containing successively higher concentrations of imidazole (100-500 mM). Protein concentrations were determined by bicinchonic acid (BCA) assay (Thermo Scientific). For antibody generation, rabbits (6 month old New-Zealand white, female, Charles River Laboratories) were immunized with 500 μg protein emulsified in Complete Freund's Adjuvant (Difco) by subscapular injection. For booster immunizations, proteins emulsified in Incomplete Freund's Adjuvant and injected 24

or 48 days following the initial immunization. On day 60, rabbits were bled and serum recovered.

[00338] **Antibody Isolation.** Purified antigen (5 mg protein) was covalently linked to HiTrap™ NHS-activated HP columns (GE Healthcare). Antigen-matrix was used for affinity chromatography of 10-20 ml of rabbit serum at 4°C. Charged matrix was washed with 50 column volumes of PBS, antibodies eluted with elution buffer (1 M glycine, pH 2.5, 0.5 M NaCl) and immediately neutralized with 1M Tris-HCl, pH 8.5. Purified antibodies were dialyzed overnight against PBS at 4°C.

[00339] **F(ab)$_2$ fragments.** Affinity purified antibodies were mixed with 3 mg of pepsin at 37°C for 30 minutes. The reaction was quenched with 1 M Tris-HCl, pH 8.5 and F(ab)$_2$ fragments were affinity purified with specific antigen-conjugated HiTrap™ NHS-activated HP columns. Purified antibodies were dialyzed overnight against PBS at 4°C, loaded onto SDS-PAGE gel and visualized with Coomassie Blue staining.

[00340] **Active and passive immunization.** BALB/c mice (3 week old, female, Charles River Laboratories) were immunized with 50 µg protein emulsified in Complete Freund's Adjuvant (Difco) by intramuscular injection. For booster immunizations, proteins were emulsified in Incomplete Freund's Adjuvant and injected 11 days following the initial immunization. On day 20 following immunization, 5 mice were bled to obtain sera for specific antibody titers by enzyme-linked immunosorbent assay (ELISA).

[00341] BALB/c mice were immunized by intramuscular injection and boosted with the same antigen in Alum after 11 and 25 days. On day 34, mice were bled to obtain serum for specific antibody titers. Affinity purified antibodies were injected into the peritoneal cavity of BALB/c mice either 24 hours or 4 hours prior to sub-lethal or lethal challenge, respectively. Animal blood was collected via periorbital vein puncture and antigen specific serum antibody titers measured by ELISA.

[00342] **Mouse renal abscess.** Overnight cultures of *S. aureus* Newman or USA300 (LAC) were diluted 1:100 into fresh TSB and grown for 2 hours at 37°C. Staphylococci were sedimented, washed and suspended PBS at $OD_{600}$ of 0.4 (~$1 \times 10^8$ CFU $ml^{-1}$). Inocula were quantified by spreading sample aliquots on TSA and enumerating colonies formed. BALB/c mice (6 week old, female, Charles River

Laboratories) were anesthetized via intraperitoneal injection with 100 mg $ml^{-1}$ ketamine and 20 mg $ml^{-1}$ xylazine per kilogram of body weight. Mice were infected by retro-obital injection with $1 \times 10^7$ CFU of *S. aureus* Newman or $5 \times 10^6$ CFU of *S. aureus* USA300. On day 4 following challenge, mice were killed by $CO_2$ inhalation. Both kidneys were removed, and the staphylococcal load in one organ was analyzed by homogenizing renal tissue with PBS, 1% Triton X-100. Serial dilutions of homogenate were spread on TSA and incubated for colony formation. The remaining organ was examined by histopathology. Briefly, kidneys were fixed in 10% formalin for 24 hours at room temperature. Tissues were embedded in paraffin, thin-sectioned, stained with hematoxylin-eosin, and inspected by light microscopy to enumerate abscess lesions. All mouse experiments were performed in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

[00343] **Mouse infection.** Staphylococci were used to infect anesthetized mice by retro-orbital injection ($1 \times 10^7$ CFU of *S. aureus* Newman, $5 \times 10^6$ CFU of *S. aureus* USA300 or $3 \times 10^7$ CFU of *S. aureus* Mu50). On day 4, 15 or 30, mice were killed, kidneys removed, and homogenized tissue spread on agar for colony formation. Organ tissue was also thin-sectioned, stained with hematoxylin-eosin, and viewed by microscopy. Animal experiments were performed in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

[00344] For lethal challenge experiments, BALB/c mice (cohorts of 8-10 animals per experiment) were injected with a suspension of $2\text{-}6 \times 10^8$ CFU of *S. aureus* Newman or its isogenic Δspa variant into the peritoneal cavity. Animal survival was monitored over a period of 15 days and statistical significance of survival data analyzed with the log-rank test.

[00345] **Protein A binding.** For human IgG binding, Ni-NTA affinity columns were pre-charged with 200 μg of purified proteins (SpA, SpA-D, SpA-$D_{KKAA}$, and SrtA) in column buffer. After washing, 200 μg of human IgG (Sigma) was loaded onto the column. Protein samples were collected from washes and elutions and

subjected to SDS-PAGE gel electrophoresis, followed by Coomassie Blue staining. Purified proteins (SpA, $SpA_{KKAA}$, SpA-D and $SpA\text{-}D_{KKAA}$) were coated onto MaxiSorp ELISA plates (NUNC) in 0.1M carbonate buffer (pH 9.5) at 1 μg $ml^{-1}$ concentration overnight at 4°C. Plates were next blocked with 5% whole milk followed by incubation with serial dilutions of peroxidase-conjugated human IgG, Fc or $F(ab)_2$ fragments for one hour. Plates were washed and developed using OptEIA ELISA reagents (BD). Reactions were quenched with 1 M phosphoric acid and $A_{450}$ readings were used to calculate half maximal titer and percent binding.

[00346] **von Willebrand Factor (vWF) binding assays.** Purified proteins (SpA, $SpA_{KKAA}$, SpA D and $SpA\text{-}D_{KKAA}$) were coated and blocked as described above. Plates were incubated with human vWF at 1 μg $ml^{-1}$ concentration for two hours, then washed and blocked with human IgG for another hour. After washing, plates were incubated with serial dilution of peroxidase-conjugated antibody directed against human vWF for one hour. Plates were washed and developed using OptEIA ELISA reagents (BD). Reactions were quenched with 1 M phosphoric acid and $A_{450}$ readings were used to calculate half maximal titer and percent binding. For inhibition assays, plates were incubated with affinity purified $F(ab)_2$ fragments specific for $SpA\text{-}D_{KKAA}$ at 10 μg $ml^{-1}$ concentration for one hour prior to ligand binding assays.

[00347] **Splenocyte apoptosis.** Affinity purified proteins (150 μg of SpA, SpA-D, $SpA_{KKAA}$, and $SpA\text{-}D_{KKAA}$) were injected into the peritoneal cavity of BALB/c mice (6 week old, female, Charles River Laboratories). Four hours following injection, animals were killed by $CO_2$ inhalation. Their spleens were removed and homogenized. Cell debris were removed using cell strainer and suspended cells were transferred to ACK lysis buffer (0.15 M $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA) to lyse red blood cells. White blood cells were sedimented by centrifugation, suspended in PBS and stained with 1:250 diluted R-PE conjugated anti-CD19 monoclonal antibody (Invitrogen) on ice and in the dark for one hour. Cells were washed with 1% FBS and fixed with 4% formalin overnight at 4°C. The following day, cells were diluted in PBS and analyzed by flow cytometry. The remaining organ was examined for histopathology. Briefly, spleens were fixed in 10% formalin for 24 hours at room temperature. Tissues were embedded in paraffin, thin-sectioned, stained with the Apoptosis detection kit (Millipore™), and inspected by light microscopy.

[00348] **Antibody quantification.** Sera were collected from healthy human volunteers or BALB/c mice that had been either infected with *S. aureus* Newman or USA300 for 30 days or that had been immunized with SpA-$D_{KKAA}$/ $SpA_{KKAA}$ as described above. Human/mouse IgG (Jackson Immunology Laboratory), $SpA_{KKAA}$, and $CRM_{197}$ were blotted onto nitrocellulose membrane. Membranes were blocked with 5% whole milk, followed by incubation with either human or mouse sera. IRDye 700DX conjugated affinity purified anti-human/mouse IgG (Rockland) was used to quantify signal intensities using the Odyssey™ infrared imaging system (Li-cor). Experiments with blood from human volunteers involved protocols that were reviewed, approved and performed under regulatory supervision of The University of Chicago's Institutional Review Board (IRB).

[00349] **Statistical Analysis.** Two tailed Student's *t* tests were performed to analyze the statistical significance of renal abscess, ELISA, and B cell superantigen data. Animal survival data were analyzed with the log-rank test (Prism).

## EXAMPLE 3

## COAGULASES OF *STAPHYLOCOCCUS AUREUS* CONTRIBUTE TO ABSCESSES FORMATION AND FUNCTION AS PROTECTIVE ANTIGENS

[00350] All clinical *S. aureus* isolates display coagulase activity - the clotting of blood or plasma through non-proteolytic activation of prothrombin to cleave fibrinogen. The inventors identified prothrombin, fibrinogen, coagulase (Coa) and von Willebrand-factor binding protein (vWbp) in staphylococcal abscess lesions of infected mice. Secreted Coa and vWbp both contributed to *S. aureus* Newman coagulase activity, thereby enabling abscess formation as well as lethal disease in mice. Antibodies raised against purified Coa or vWbp specifically block association of the corresponding polypeptide with prothrombin and fibrinogen. Coa- and vWbp-specific antibodies, whether raised by active or passive immunization, prevented abscess formation and mortality of mice infected with staphylococci.

### VIII. Results

[00351] **Localization of coagulase and coagulation factors in staphylococcal abscesses.** Previous work established the mouse renal abscess model, whereby $1\times10^7$ CFU of the human clinical isolate *S. aureus* Newman (Baba *et al.,* 2007) are injected

into the blood stream of BALB/c mice (Albus *et al.*, 1991). Forty-eight hours following infection, mice develop disseminated abscesses in multiple organs, detectable by light microscopy of hematoxylin-eosin stained, thin-sectioned kidney tissue initially as an accumulation of polymorphonuclear leukocytes (PMNs) with few bacteria (Cheng *et al.,* 2009). By day 5 of infection, abscesses increase in size and enclose a central population of staphylococci (staphylococcal abscess community - SAC), surrounded by a layer of eosinophilic, amorphous material (the pseudocapsule) and a large cuff of PMNs (Cheng *et al.*, 2009). Histopathology reveals massive necrosis of PMNs in proximity to the staphylococcal nidus at the center of abscess lesions as well as a mantle of healthy phagocytes. At later time intervals, SACs increase and abscesses rupture, releasing necrotic material and staphylococci into the bloodstream. A new round of abscess formation is initiated, eventually precipitating a lethal outcome of infections (Cheng *et al.*, 2009).

[00352] To localize coagulases in abscess lesions, kidneys of mice that had been infected for 5 days with *S. aureus* Newman were thin-sectioned and stained by immuno-histochemistry with affinity purified Coa- or vWbp-specific rabbit antibodies (FIG. 10). The inventors observed intense Coa staining in the pseudocapsule surrounding SACs and in the periphery of abscess lesions, *i.e.,* the fibrin capsule bordering uninfected tissue. vWbp staining occurred throughout abscess lesions, but also with accumulation at the periphery. Prothrombin specific antibodies revealed staining of the zymogen in the pseudocapsule and in the periphery, whereas fibrinogen/fibrin specific staining occurred throughout abscess lesions. Together these data indicate that the eosinophilic pseudocapsule of staphylococcal abscesses harbors prothrombin and fibrinogen, which co-localize with Coa. At the periphery of abscess lesions, Coa, vWbp, prothrombin and fibrinogen/fibrin are co-localized. These observations prompted further investigation in to whether Coa and vWbp are crucial contributors to the establishment of abscesses by triggering prothombin-mediated conversion of fibrinogen to fibrin.

[00353] **Staphylococcus aureus coa and vWbp contribute to the clotting of mouse blood.** The *coa* and/or *vWbp* genes on the chromosome of *S. aureus* Newman were deleted by allelic replacement using pKOR1 technology (Bae and Schneewind, 2005). Two complementing plasmids, p*coa* and p*vWbp*, were generated by cloning

*coa* or *vWbp* structural genes as well as their upstream promoter sequences into pOS1 (Schneewind *et al.*, 1993). Plasmids were electroporated into staphylococci and their continued replication selected on media supplemented with chloramphenicol (Schneewind *et al.,* 1992). When probed for coagulases with specific antibodies, the inventors observed Coa secretion by the wild-type as well as the Δ*vWbp* strain, but not by Δ*coa* or Δ*coa*/Δ*vWbp* variants (FIG. 11). The phenotypic defect of Δ*coa* and Δ*coa*/Δ*vWbp* mutants was restored by electroporation with p*coa* but not by p*vWbp* (FIG. 11). Similarly, secretion of vWbp was observed in *S. aureus* Newman (wild-type) as well as Δ*coa* mutant cultures, but not in Δ*vWbp* or Δ*coa*/Δ*vWbp* variants (FIG. 11). This defect was restored by electroporation with p*vWbp*, but not by p*coa*.

[00354] Clotting of blood is effectively inhibited by hirudin (lepirudin) (Harvey *et al.*, 1986), a 65 residue peptide from leech that forms a 1:1 complex with thrombin, thereby blocking proteolytic conversion of fibrinogen to fibrin (Markwardt, 1955). Inoculation of fresh lepirudin-treated mouse blood with *S. aureus* Newman triggered clotting in less than 12 hours, whereas mock infected blood remained without clots for more than 48 hours (FIG. 11C). Using this assay, it was observed that staphylococcal variants lacking coagulase activity displayed delays in clotting time, Δ*coa* 36 hours and Δ*vWbp* 24 hours (FIG. 11C). The double mutant, Δ*coa*/Δ*vWbp*, was unable to clot mouse blood. These defects were complemented by electroporation with plasmids p*vWbp* as well as p*coa*. Taken together, these data indicate that the two coagulases, Coa and vWbp, contribute to the ability of *S. aureus* Newman to clot mouse blood (FIG. 11C).

[00355] **Coa and vWbp are required for staphylococcal survival in blood, abscess formation and lethal bacteremia in mice.** To analyze the virulence contributions of coagulases, the inventors first examined staphylococcal survival in lepirudin-treated blood. Wild-type strain *S. aureus* Newman was not killed in mouse blood, however isogenic variants lacking Coa, *i.e.* Δ*coa* and Δ*coa*/Δ*vWbp*, each displayed a significant reduction in CFU after 30 min incubation. This defect in survival was restored by p*coa*, but not by p*vWbp*, suggesting that only Coa is required for staphylococcal survival in mouse blood.

[00356] Staphylococcal bacteremia is a frequent cause of human mortality in hospital settings (Klevens *et al.*, 2007). The inventors sought to ascertain whether

coagulases are required for lethal challenge of BALB/c mice, following intravenous injection of $1 \times 10^8$ CFU *S. aureus* Newman. All animals infected with the wild-type parent strain Newman succumbed to infection within 24 hours (FIG. 12B). Animals infected with single mutants, Δ*coa* or Δ*vWbp*, each displayed a short but statistically significant delay in time-to-death (FIG. 12B). The double mutant strain was significantly more impaired than mutants with single deletions and animals infected with the Δ*coa*/Δ*vWbp* strain displayed the largest reduction in virulence as compared to the wild-type (FIG. 12B).

[00357] The inventors next analyzed abscess formation in renal tissues of infected mice and observed that Δcoa variants were impaired in their ability to form abscesses by day 5 and 15 of infection (Table 7, FIG. 12G, 12I)). The ΔvWbp mutant continued to form abscesses, although the bacterial load, the overall size of staphylococcal abscess communities and the amount of immune cell infiltrates were reduced in these variants (Table 7, FIG. 12D, 12F)). Mutants in coagulase are slightly more attenuated in virulence than those in vWbp, as Δcoa has lower abscess formation and bacterial load by day 15. However, the Δcoa / ΔvWbp double mutants markedly incapacitated in their ability to form abscesses and persist in infected tissues (Table 7, FIG. 12H, 12K)). Thus, both coagulase and von Willebrand factor binding protein are important for staphylococcal survival in the host, whether in the bloodstream or end organ tissues.

**Table 7.** Virulence *of S. aureus* Newman *coa, vWbp*, and *coa/vWbp mutants*

| Strain | Staphylococcal load in kidney tissue* | | | Abscess formation in kidney tissue* | | |
|---|---|---|---|---|---|---|
| | [a]$log_{10}$ CFU $g^{-1}$ of kidney tissue | [b]Significance (P-value) | [c]Reduction in [a]$log_{10}$ CFU $g^{-1}$ | [e]Surface abscesses (%) | [f]Number of abscesses per kidney | [g]Significance (P-value) |
| Day 5 analysis of staphylococcal load and abscess formation | | | | | | |
| PBS | 6.034 ± 0.899 | – | – | 75 | 2.333 ± 0.623 | – |
| *Coa* | 5.538 ± 0.560 | 0.3750 | 0.492 | 38 | 1.111 ± 0.389 | 0.1635 |
| *vWbp* | 5.247 ± 0.311 | 0.0859 | 0.783 | 56 | 1.750 ± 0.650 | 0.6085 |
| *coa/vWbp* | 4.908 ± 0.251 | 0.0044 | 1.395 | 25 | 0.750 ± 0.342 | 0.0786 |
| Day 15 analysis of staphylococcal load and abscess formation | | | | | | |
| PBS | 5.380 ± 0.294 | – | – | 81 | 3.000 ± 1.234 | – |
| *Coa* | 4.023 ± 0.324 | 0.0077 | 1.357 | 44 | 1.400 ± 0.452 | 0.1862 |
| *vWbp* | 5.140 ± 0.689 | 0.0688 | 0.240 | 50 | 1.625 ± 0.298 | 0.2974 |
| *coa/vWbp* | 3.300 ± 0.552 | 0.0056 | 2.080 | 20 | 0.556 ± 0.154 | 0.0341 |

*BALB/c mice (n=18-20) were injected into the peritoneum with 100 µl each of affinity purified rabbit antibodies against vWbp (α-vWbp), Coa (α-Coa) or vWbp and Coa (α-vWbp/Coa) on day 0 . Twenty four hours later, animals were examined for IgG antibody titers in serum and were challenged by intravenous inoculation with $1\times10^7$ colony forming units (CFU) *S. aureus* Newman or mutants thereof. Five or fifteen days later, animals were killed and both kidneys removed. One kidney was fixed in formaldehyde, embedded in paraffin, thin sectioned, hemaotoxylin-eosin stained and four saggital sections per kidney were analyzed for abscess formation. The other kidney was homogenized in PBS buffer, homogenate spread on agar medium for colony formation, and staphylococcal load enumerated as CFU.

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of eighteen to twenty BALB/c mice per immunization. Standard error of the means (±SEM) is indicated.

[b]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[c]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.

[e]Abscess formation in kidney tissues four days following infection was measured by macroscopic inspection (% positive)

[f]Histopathology of hematoxylene-eosin stained, thin sectioned kidneys from ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).

[g]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.



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[00358] **Antibodies against coagulases and their effect on blood clotting induced during staphylococcal infection.** Recombinant $His_6$-Coa and $His_6$-vWbp were purified by affinity chromatography on Ni-NTA (FIG. 13A), emulsified in adjuvant and injected into rabbits to raise specific antibodies that were purified on affinity matrices harboring recombinant protein. Antibodies directed against Coa preferentially bound to Coa, not to vWbp (FIG. 13B). The reciprocal was true for antibodies directed against vWbp (FIG. 13B). When added to lepirudin-treated mouse blood infected with *S. aureus* Newman, the inventors observed that antibodies directed against Coa, vWbp or Coa and vWbp each blocked the coagulation of blood (FIG. 13C). As controls, mock treated samples or the irrelevant V10 antibody (which provides protection against *Yersinia pestis* type III injection (DeBord *et al.,* 2006)) had no effect (FIG. 13C).

[00359] To examine the role of antibodies on isolated Coa or vWbp, the inventors purified recombinant, functionally active proteins (Friedrich *et al.*, 2003) that were then added to lepirudin treated mouse blood. Coa or vWbp treated mouse blood coagulated in less than 30 minutes (FIG. 13D). As a control, mock (PBS) or treatment with irrelevant V10 antibody did not affect clotting. Antibodies directed against Coa or vWbp delayed clotting of mouse blood treated with recombinant proteins and this occurred even for the cross-reacting homologous factor (FIG. 13D). Minimal cross reactivity of the antibodies was observed by ELISA and western blot, yet there is cross inhibition of function.

[00360] **Antibodies that block association between coagulases and prothrombin or fibrinogen.** Surface plasmon resonance (SPR) was used to investigate how αCoa and αvWbp antibodies interfere with the physiological functions of coagulases. Prothrombin was immobilized on a CM5 chip. Flowing purified Coa over the sample, a dissociation constant $K_D$ 28 nM was caluclated, a measurement that is commensurate with other reports in the literature (Friedrich *et al.,* 2003). The addition of αCoa led to a concentration-dependent decrease in response signal for the formation of prothrombin·Coa, indicating that these antibodies block association of Coa with prothrombin (FIG. 14A). SPR further confirmed association between coagulase and fibrinogen ($K_D$ 93.1 nM, FIG. 14B). Upon pre-incubation with αCoa, the inventors observed a dramatic decrease in the binding of Coa to

fibrinogen (FIG. 14B). Taken together, these results indicate that antibodies directed against Coa block the association of this molecule with blood coagulation factors.

[00361] Purified vWbp displayed strong affinity for prothrombin ($K_D$ 38.4 nM, FIG. 13C) and fibrinogen (484 nM, FIG. 13D), the latter of which had hitherto not been appreciated (Kroh *et al.*, 2009). Further, pre-incubation with antibodies raised against vWbp blocked the association between vWbp and prothrombin or fibrinogen in a dose-dependent manner (FIG. 13C, 13D). These findings support results from the blood coagulation assays, demonstrating that specific polyclonal antibodies can block the interaction between Coa or vWbp and specific components of the coagulation cascade (FIG. 12).

[00362] To test whether antibodies specific for coagulases block the conversion of fibrinogen to fibrin, the ability of prothrombin•coagulase complexes to cleave S-2238 was measured, a surrogate for the cleavage of fibrinogen to fibrin (FIG. 14E, 14F). Addition of specific antibodies to prothrombin•Coa or prothrombin•vWbp reduced the ability of these complexes to convert substrate to product. Further, cross-inhibition of coagulase-specific antibodies was observed, where the addition of cross-reacting antibodies caused a reduction in activity of the prothrombin•vWbp complex. These data suggest that specific antibodies directed against Coa or vWbp neutralize the pathophysiological effect of the secreted product to which they bind.

[00363] **Antibodies against coagulases provide protection against staphylococcal disease.** IgG type antibodies specific for Coa or vWbp were isolated from rabbit serum by chromatography over an affinity column, generated by covalent crosslinking of the antigen to CNBr sepharose™. The inventors attempted to perturb staphylococcal pathogenesis by administration of neutralizing antibodies, directed against Coa and/or vWbp. Mice were administered rabbit antibodies and challenged with a lethal dose of *S. aureus* strain Newman. Injection of Coa or vWbp specific antibodies significantly prolonged murine survival (FIG. 15).

[00364] To test antibody reagents for possible vaccine protection against lethal bacteremia, affinity purified IgG (5 mg $kg^{-1}$ body weight) were injected into the peritoneal cavity of mice. Twenty-four hours later, animals were injected with a suspension of $1 \times 10^8$ CFU *S. aureus* Newman in PBS into the retro-orbital plexus.

Monitoring animals over time, the inventors observed that antibodies directed against vWbp (αvWbp) led to increased time-to-death and to 10% survival, as compared to animals that had received irrelevant αV10 antibodies and died within 12-48 hours (FIG. 15). Antibodies against Coa (αCoa) further increased the time-to-death of passively immunized mice (FIG. 15). A mixture of both antibodies (αCoa/αvWbp) did not generate a statistically significant improvement in survival or time-to-death over αCoa antibodies.

[00365] To examine the passive immunization for protection against staphylococcal abscess formation, purified antibodies (5 mg kg-1 body weight) were injected into the peritoneal cavity of mice and abscess formation was monitored for five days after intravenous challenge with $1 \times 10^7$ CFU S. aureus Newman. Antibodies against vWbp did not lead to a significant reduction in staphylococcal load or in the number of inflammatory lesions (Table 8), although the observed lesions harbored smaller abscess communities and reduced PMN infiltrates as compared to mock immunized mice (FIG. 16). Antibodies against coagulase reduced the staphylococcal load (P=0.042) as well as the number of inflammatory lesions (P=0.039); abscess lesions with staphylococcal communities at the nidus of large PMN infiltrates were not detected (FIG. 16 and Table 8). Animals that received both antibodies, αWbp and αCoa, displayed an even greater reduction in staphylococcal load (P=0.013) and a reduction in the abundance of inflammatory lesions (P=0.0078) (Table 8). Together, these data indicate that antibodies against coagulases, administered by passive immunization, protect mice against abscess formation and enable clearance of the invading pathogen from host tissues. Antibodies against vWbp contribute relatively little to vaccine protection, in agreement with the finding that vWbp does not play the same critical role as Coa during the pathogenesis of S. aureus infections in mice (Table 8).

**Table 8.** Passive immunization of mice with rabbit antibodies against Coa and/or vWbp

| | Staphylococcal load in kidney tissue* | | | | Abscess formation in kidney tissue* | | |
|---|---|---|---|---|---|---|---|
| **Purified Rabbit Antibody** | **[a]$log_{10}$ CFU $g^{-1}$ of kidney tissue** | **[b]Significance (P-value)** | **[c]Reduction in [a]$log_{10}$ CFU $g^{-1}$** | **[d]IgG Titer** | **[e]Surface abscesses (%)** | **[f]Number of abscesses per kidney** | **[g]Significance (P-value)** |
| Mock | 5.86 ± 0.29 | – | – | <100 | 75 | 4.6 ± 1.4 | – |
| α-vWbp | 5.25 ± 0.36 | 0.3554 | 0.60 | 1,100 ± 200 | 39 | 1.4 ± 0.5 | 0.0592 |
| α-Coa | 4.68 ± 0.47 | 0.0420 | 1.18 | 1,300 ± 250 | 20 | 1.2 ± 0.7 | 0.0396 |
| α-vWbp/Coa | 4.29 ± 0.52 | 0.0130 | 1.53 | 1,000 ± 300 | 25 | 0.3 ± 0.2 | 0.0078 |

*BALB/c mice (n=18-20) were injected into the peritoneum with 100 µl each of affinity purified rabbit antibodies against vWbp (α-vWbp), Coa (α-Coa) or vWbp and Coa (α-vWbp/Coa) on day 0 . Twenty four hours later, animals were examined for IgG antibody titers in serum and were challenged by intravenous inoculation with $1\times10^7$ colony forming units (CFU) *S. aureus* Newman. Five days later, animals were killed and both kidneys removed. One kidney was fixed in formaldehyde, embedded in paraffin, thin sectioned, hemaotoxylin-eosin stained and four saggital sections per kidney were analyzed for abscess formation. The other kidney was homogenized in PBS buffer, homogenate spread on agar medium for colony formation, and staphylococcal load enumerated as CFU.

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of eighteen to twenty BALB/c mice per immunization. Standard error of the means (±SEM) is indicated.

[b]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[c]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.

[e]Abscess formation in kidney tissues four days following infection was measured by macroscopic inspection (% positive)

[f]Histopathology of hematoxylene-eosin stained, thin sectioned kidneys from ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).

[g]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[00366] **Coagulases function as protective antigens for staphylococcal infections.** Poly-histidine tagged CoA and vWbp were purified from *E. coli* and used as subunit vaccine antigens. Proteins (100 μg emulisified in CFA or IFA) were injected into naïve BALB/c mice on day 0 (CFA) or 11 (IFA). Animals were challenged on day 21 by intravenous inoculation of *S. aureus* Newman. Five control animals were bled at the time of challenge and serum antibody titers against vaccine antigens were determined by ELISA (Table 9). Animals were killed five or fifteen days following challenge staphylococcal load and histopathology of abscess lesions were analyzed. Immunization with Coa reduced the bacterial load by day 5 (P=0.03, PBS mock vs. Coa) and day 15 ($P=4.286 \times 10^{-5}$, PBS mock vs. Coa, see Table 9). Coa vaccination also diminished the number of infectious lesions that formed in kidney tissues, mock vs. Coa, P=0.03 (day 5) and P=0.0522 (day 15) (Table 9). Of note, none of the Coa-immunized mice developed typical abscess lesions (FIG. 17). On occasion small accumulations of PMNs that were not associated with staphylococcal abscess communities were observed (FIG. 17). Immunization with vWbp did not significantly reduce staphylococcal load on day 5 (P=0.39, PBS mock vs. vWbp) or on day 15 (P=0.09, PBS mock vs. vWbp). The total number of inflammatory lesions was not reduced. Nevertheless, the architecture of abscesses had changed following immunization with vWbp. Staphylococcal communities were not detected at the center of abscesses and instead PMN infiltrations were observed (FIG. 17). The combination vaccine, vWbp-Coa, further reduced the number of inflammatory cells in kidney tissues and infected animals did not display abscess lesions on day 5 or 15 (Table 9).

**Table 9.** Active immunization of mice with Coa and/or vWbp

| | Purified Vaccine Antigen | Staphylococcal load in kidney tissue* | | | | Abscess formation in kidney tissue* | | |
|---|---|---|---|---|---|---|---|---|
| | | [a]$log_{10}$ CFU $g^{-1}$ of kidney tissue | [b]Significance (P-value) | [c]Reduction in [a]$log_{10}$ CFU $g^{-1}$ | [d]IgG Titer | [e]Surface abscesses (%) | [f]Number of abscesses per kidney | [g]Significance (P-value) |
| Day 5[h] | Mock | 5.75 ± 0.42 | – | – | <100 | 56 | 1.3 ± 0.3 | – |
| | vWbp | 4.94 ± 0.46 | 0.1413 | 0.81 | 14,000 ± 5,000 | 45 | 1.8 ± 0.5 | 0.39 |
| | Coa | 4.86 ± 0.50 | 0.1417 | 0.88 | 19,000 ± 4,000 | 25 | 0.3 ± 0.3 | 0.03 |
| | vWbp/Coa | 4.84 ± 0.38 | 0.1195 | 0.90 | 7,000 ± 1,500 | 25 | 0.3 ± 0.3 | 0.03 |
| Day 15[i] | Mock | 6.68 ± 0.22 | – | – | <100 | 75 | 6.0 ± 1.9 | – |
| | vWbp | 3.41 ± 0.47 | 0.4503 | 3.27 | 14,000 ± 5,000 | 20 | 1.8 ± 1.1 | 0.09 |
| | Coa | 3.43 ± 0.54 | 0.1681 | 3.25 | 19,000 ± 4,000 | 20 | 1.2 ± 0.8 | 0.05 |
| | vWbp/Coa | 3.79 ± 0.37 | 0.0263 | 2.89 | 7,000 ± 1,500 | 30 | 0.7 ± 0.5 | 0.01 |

*BALB/c mice (n=18-20) were injected with 100 µg each of purified vWbp, Coa or vWbp and Coa emulsified in CFA on day 0 and boosted with the same antigen emulsified in IFA on day 11. On day 20, animals were examined for IgG antibody titers and on day 21 animals were challenged by intravenous inoculation with either $1\times10^7$ colony forming units (CFU) *S. aureus* Newman. On day 25, animals were killed and both kidneys removed. One kidney was fixed in formaldehyde, embedded in paraffin, thin sectioned, hemaotoxylin-eosin stained and four saggital sections per kidney were analyzed for abscess formation. The other kidney was homogenized in PBS buffer, homogenate spread on agar medium for colony formation, and staphylococcal load enumerated as CFU.

[a]Means of staphylococcal load calculated as $log_{10}$ CFU $g^{-1}$ in homogenized renal tissues 4 days following infection in cohorts of eighteen to twenty BALB/c mice per immunization. Standard error of the means (±SEM) is indicated.

[b]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[c]Reduction in bacterial load calculated as $log_{10}$ CFU $g^{-1}$.

[d]Means of five randomly chosen serum IgG titers were measured prior to staphylococcal infection by ELISA with SpA-$D_{KKAA}$ antigen

[e]Abscess formation in kidney tissues four days following infection was measured by macroscopic inspection (% positive)

[f]Histopathology of hematoxylene-eosin stained, thin sectioned kidneys from ten animals; the average number of abscesses per kidney was recorded and averaged again for the final mean (±SEM).

[g]Statistical significance was calculated with the unpaired two-tailed Students *t*-test and P-values recorded; P-values <0.05 were deemed significant.

[h]Analysis of mice 5 days following infection with *S. aureus* Newman.

[i]Analysis of mice 15 days following infection with *S. aureus* Newman.

## IX. Materials and Methods

[00367] **Bacterial strains and growth of cultures.** Staphylococci were cultured on tryptic soy agar or broth at 37°C. *E. coli* strains DH5α and BL21(DE3) (Studier *et al.*, 1990) were cultured on Luria agar or broth at 37°C. Ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) were used for pET15b (Studier *et al.*, 1990) and pOS1 (Schneewind *et al.,* 1993) plasmid selection, respectively.

[00368] **Generation of mutants.** DNA sequences 1 kb upstream and downstream of *coa* and *vWbp* were PCR amplified using the primers attB1_Coa, Coa1_BamHI, Coa2_BamHI, attbB2_Coa and attB1_vWF, vWF1_BamHI, vWF2_BamHI, attbB2_vWF (Table 10). The fragments were exchanged onto pKOR1 using the BP clonase II kit (Invitrogen) (Bae and Schneewind, 2005). These vectors were electroporated into *S. aureus* Newman and subjected to temperature shift induced allelic exchange to generate the corresponding deletion (Bae and Schneewind, 2005). Mutants were verified by PCR amplification of the gene locus, DNA sequencing, and immunoblot analysis.

[00369] To generate complementing plasmids, the primers Coa_promoter_BamHI_F, Coa_out_PstI_R, vWbp_promoter_BamHI_F, vWbp_out_PstI_R (Table 10) were designed to include the upstream promoter region of *vWbp* or *coa* and the amplified regions were cloned into pOS1. These plasmids were verified by sequencing and then electroporated into staphylococcal strains. For immunoblot analysis, overnight cultures of staphylococci grown in tryptic soy broth (Difco) were refreshed 1:100 and grown with shaking at 37°C until they reached $OD_{600}$ of 0.4. One ml samples of each culture centrifuged at 13,000 ×g for 10 min in a table top centrifuge and the supernatant was recovered. Trichloroacetic acid, 75 μl of 100% w/v solution, was added and samples were incubated on ice for 10 min, followed by centrifugation and wash with 1 ml ice-cold 100% acetone. Samples were air dried overnight and solubilized in 50 μl sample buffer (4% SDS, 50 mM Tris-HCl, pH8, 10% glycerol, and bromophenol blue).

[00370] **Blood survival assay and blood coagulation.** Overnight cultures of staphylococcal strains were diluted 1:100 into fresh TSB and grown at 37°C until they reached an $OD_{600}$ 0.4. One ml of culture was centrifuged, and staphylococci washed and suspended in 10 ml of sterile PBS to generate a suspension of $1\times10^7$ CFU/ml.

Whole blood from naïve 6 week old Balb/c mice was collected and REFLUDAN™ (lepirudin, Bayer) was added to a final concentration 50 μg/ml. 450 μL blood was aliquoted into a 1 ml eppendorf tube and mixed with 50 μl bacterial sample ($1x10^5$ CFU/ml). Samples were incubated at 37°C with slow rotation. 100 μl aliquots were removed at times 0 min and 30 min, mixed 1:1 with 2% saponin/PBS and incubated on ice for 30 minutes. Five 1:10 serial dilutions were prepared and 10 μl aliquots spread on TSA agar for colony formation and enumeration.

[00371] To assess bacterial blood coagulating activity, 10 μl of the above stock bacterial culture was added to 100 μl of anti-coagulated mouse blood in a sterile plastic test tube (BD falcon) to achieve an end concentration of $1x10^5$ CFU/ml. For antibody perturbation, an additional 10 ul of PBS containing $3x10^{-5}$ Mol of antibody was added to the mixture. To assess recombinant proteins, 10 μl of protein in PBS buffer added to an end concentration of 50 μM. Test tubes were incubated at room temperature and blood liquidity or coagulation was verified by tipping the tubes to 45° angles in timed intervals.

[00372] **Protein purification.** For vaccination studies, full-length coding sequence of mature Coa or vWbp was cloned into pET15b vector using the primers Coa_foward_XhoI, Coa_reverse_BamHI, vWbp_forward_XhoI, vWbp_reverse_BamHI (Table 10) to obtain $His_6$-Coa and $His_6$-vWbp. *E. coli* BL21(DE3) harboring expression vectors were grown at 37°C and induced with 1 mM IPTG after two hours. Four hours after induction, cells were centrifuged at 6,000 ×g, supended in 1 × column buffer (0.1 M Tris-HCl pH 7.5, 0.5 M NaCl) and lysed in a French press at 14,0000 lb/in$^2$. Lysates were subjected to ultracentrifugation at 40,000 ×g for 30 min and the supernatant was subjected to Ni-NTA chromatography, washed with column buffer containing 25 μM imidazole, followed by elution with 500 μM imidazole. Eluate was dialyzed with 1 × PBS. To remove endotoxin, 1:1,000 Triton-X114 was added and the solution was chilled for 5 min, incubated at 37°C for 10 min, and centrifuged at 13,000 ×g. Supernatant was loaded onto a HiTrap desalting column to remove any remnant of Triton-X114.

**Table 10. Primers used in this study**

| Primer name | Sequence |
|---|---|
| attB1_Coa | GGGGACAAGTTTGTACAAAAAAGCAGGCTgatgactaagttgaaaaaagaag (SEQ ID NO:46) |
| Coa1_BamHI | aaGGATCCcctccaaaatgtaattgccc (SEQ ID NO:47) |
| Coa2_BamHI | aaGGATCCgtttgtaactctatccaaagac (SEQ ID NO:48) |
| attbB2_Coa | GGGGACCACTTTGTACAAGAAAGCTGGGTgacacctattgcacgattcg (SEQ ID NO:49) |
| attB1_vWF | GGGGACAAGTTTGTACAAAAAAGCAGGCTcagatagcgattcagattcag (SEQ ID NO:50) |
| vWF1_BamHI | aaGGATCCctgtattttctccttaattttcc (SEQ ID NO:51) |
| vWF2_BamHI | aaGGATCCcatggctgcaaagcaaataatg (SEQ ID NO:52) |
| attbB2_vWF | GGGGACCACTTTGTACAAGAAAGCTGGGTgccctggtgtaacaaatttatg (SEQ ID NO:53) |
| Coa_promoter_BamHI_F | gaaGGATCCgtttattctagttaatatatagttaatg (SEQ ID NO:54) |
| Coa_out_PstI_R | gaaCTGCAGctgtatgtctttggatagagttac (SEQ ID NO:55) |
| vWbp_promoter_BamHI_F | gaaGGATCCggtggctttttacttggattttc (SEQ ID NO:56) |
| vWbp_out_PstI_R | gaaCTGCAGcgacaaactcattatttgctttgc (SEQ ID NO:57) |
| Coa_foward_XhoI | GAACTCGAGTCTAGCTTATTTACATGG (SEQ ID NO:58) |
| Coa_Xho_factorXa_F | GAACTCGAGatagaaggcagaatagtaacaaaggattatagtggg (SEQ ID NO:59) |
| Coa_reverse_BamHI | GTAGGATCCTGGGATAGAGTTACAAAC (SEQ ID NO:60) |
| vWbp_forward_XhoI | GAACTCGAGgcattatgtgtatcacaaatttggg (SEQ ID NO:61) |
| vWbp_Xho_factorXa_F | GAACTCGAGatagaaggcagagtggtttctggggagaagaatc (SEQ ID NO:62) |
| vWbp_reverse_BamHI | GAACTCGAGgcagccatgcattaattatttgcc (SEQ ID NO:63) |

[00373] For enzymatic studies, ELISA, and SPR, full-length coding sequence of mature Coa or vWbp was cloned into pET15b with primers Coa_Xho_factorXa_F, Coa_reverse_BamHI, vWbp_Xho_factorXa_F, vWbp_reverse_BamHI (Table 10) which contain a Factor Xa site preceding the initial Ile-Val-Thr-Lys of coagulase and Val-Val-Ser-Gly of vWbp. These proteins were expressed and purified using the above protocol, then cleaved with 10 units Factor Xa/1ml for 1 hour at 25°C to remove the $His_6$ tag from the N-terminus. Proteins were then loaded onto a Superdex 75 (GE Healthcare) column for final purification. All eluted proteins were stored in 1× PBS.

[00374] **Rabbit antibodies.** Protein concentration was determined using a BCA kit (Thermo Scientific). Purity was verified by SDS page gel analysis and Coomassie Brilliant Blue staining. Six month old New-Zealand white female rabbits (Charles River Laboratories) were immunized with 500 μg protein emulsified in CFA (Difco) for initial immunization or IFA for booster immunizations on day 24 and 48. On day 60, rabbits were bled and serum recovered for immunoblotting or passive transfer experiments. For antibody purification, recombinant $His_6$-Coa or $His_6$-vWbp (5 mg) was covalently linked to HiTrap NHS-activated HP columns (GE Healthcare). This antigen-matrix was then used for affinity chromatography of 10-20 ml of rabbit serum

at 4°C. Charged matrix was washed with 50 column volumes of PBS, antibodies eluted with elution buffer (1 M glycine pH 2.5, 0.5 M NaCl) and immediately neutralized with 1M Tris-HCl, pH 8.5. Purified antibodies were dialyzed overnight against PBS at 4°C.

[00375] **Surface Plasmon Resonance.** Affinity and rates of association and dissociation were measured on a BIAcore 3000. Buffers were sterile filtered and degassed. A CM5 chip was prepared for amine linkage by injection of human prothrombin (500 nM, pH 4.0) (Innovative Research) and human fibrinogen (200 nM, pH 4.5) (Innovative Research) in presence of 0.2 M EDC and 0.05 M NHS. To measure the interaction of coagulase with prothrombin and fibrinogen, Coa was diluted into HBS-P buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.005% [vol/vol] surfactant P20) at concentrations 0 – 75 nM with successive injections of coagulase for 300 seconds followed by 300 seconds for dissociation followed by regeneration with NaOH (50 μL, 30 seconds). $K_D$ and $\chi^2$ were determined using the BiaEvaluation software and best fit was determined with a 1:1 binding model with drifting baseline and local $R_{max}$. The interaction of von Willebrand factor with prothrombin and fibrinogen was measured in the same way. All experiments were repeated in triplicate. Inhibition experiments with polyclonal antibodies were conducted by successive injections of coagulase (25 nM) incubated with αCoa at 0 nM – 200 nM under the same injection conditions described above. vWF (50 nM) was similarly incubated with αvWF at 0 nM – 400 nM. Response difference was measured as the change in response units from before the injection to the end of the injection.

[00376] **Measurements of coagulase activity.** $1x10^{-16}$ M prothrombin (Innovative Research) was pre-incubated for 20 min with an equimolar amount of functional coagulase or vWbp at room temperature, followed by addition of S-2238 (a chromogenic substrate) to an end concentration of 1 mM in a total reaction buffer of 100 μl 1 × PBS. The change in absorbance was measured at 450 nm for 10 minutes in a spectrophotometer, plotted as a function of time and fitted to a linear curve. The slope of the curve (dA/dt) was interpreted to be the rate of S-2238 hydrolysis, and thus reflective of enzymatic function (% coagulase-prothrombin or vWbp-prothrombin complex activity). The assay was repeated in presence of specific or

cross antibodies added in 3M excess ($3x10^{-16}$M) and the data was normalized to the % average activity without inhibition.

[00377] **Renal abscess model and lethal challenge.** Overnight cultures of staphylococcal strains were diluted 1:100 into fresh TSB and grown until they reached an $OD_{600}$ of 0.4. 10 ml of bacteria were centrifuged at 7,500 ×g, washed, and suspended in 10 ml of 1 ×PBS. Six week old female BALB/c mice (Charles River) were injected retro-orbitally with $1\times10^7$ CFU staphylococcal suspension in 100 µl of PBS. Cohorts of 10 mice were used. On the fifth day post infection, these mice were killed by $CO_2$ asphyxiation and their kidneys were excised. All organs were examined for surface lesions and 8-10 right kidneys were sent for histopathology sectioning and hematoxylin-eosin staining. These slides were examined by light microscopy for internal abscesses. For the lethal challenge model, all experimental conditions remain the same except that 1 x $10^8$ CFU staphylococci were administered and that the mice were monitored for 10 days post infection for survival.

[00378] **Immunohistochemistry staining of renal sections.** Sectioned kidneys were deparafinized and rehydrated through xylene and serial dilutions of EtOH to distilled water. They were incubated in antigen retrieval buffer (DAKO, pH 6.0) and heated in steamer at over 96°C for 20 minutes. After rinsing, the slides were incubated in 3% hydrogen peroxide for 5 minutes and then 10% normal serum in 0.025%Tritonx-100 -PBS for 30 minutes. 10% human IgG was used as blocking reagent for 30 minutes incubation (Sigma-Aldrich). Primary antibody was applied on the slides for over night incubation at 4°C degree in a humidity chamber. The primary antibodies used were 1:500 rat anti-mouse Prothrombin (Innovative Research), 1:500 rabbit anti-mouse fibrinogen (Innovative Research), 1:250 rabbit-anti staphylocoagulase, or 1:250 rabbit anti-staphylococcus vwbp. Following TBS wash, the slides were incubated with biotinylated secondary antibody (1:50 dilution of biotinylated anti-rat IgG, BA-4001 from Vector Laboratories; or 1:200 dilution of biotinylated anti-rabbit IgG, BA-1000 from Vector), and then ABC reagents (Vector Laboratories). The antigen-antibody binding was detected by DAB substrate chromogen system. The slide were briefly immersed in hematoxylin for counterstaining and evaluated under light microscope.

[00379] **Active immunization.** Three week old BALB/c mice were injected with 50 μg protein each, emulsified in 100 μl CFA. Cohorts of 15 mice were used, with 5 mice reserved for bleeding and antibody titers. Eleven days post vaccination, these mice were boosted with 50 μg protein each, emulsified in 100 μl IFA. On day 21, mice were injected with 1x107 CFU of staphylococci for the renal abscess model or 1x108 CFU for lethal challenge. At the time of infection 5 mice were bled to obtain antibody titers.

[00380] **Passive transfer of antibodies.** Twenty four hours prior to infection, six week old BALB/c mice were injected with purified antibodies against Coa and/or vWbp at a dose of 5 mg/kg body weight. Cohorts of 10 mice were used. These mice were challenged by retro-orbital injection with $1x10^7$ CFU (renal abscess model) or $1x10^8$ CFU staphylococci (lethal bacteremia).

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U.S. Patent 4,474,757
U.S. Patent 4,554,101
U.S. Patent 4,578,770
U.S. Patent 4,596,792
U.S. Patent 4,599,230
U.S. Patent 4,599,231
U.S. Patent 4,601,903
U.S. Patent 4,608,251
U.S. Patent 4,683,195
U.S. Patent 4,683,202
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U.S. Patent 5,591,616
U.S. Patent 5,610,042
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PCT Appln. WO 03/53462

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CLAIMS

1. An immunogenic isolated polypeptide comprising a Staphylococcal Protein A (SpA) variant domain D segment having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 and having (a) two amino acid substitutions that disrupt Fc binding comprising amino acid substitutions with a lysine at positions corresponding to amino acid positions 9 and 10 of SEQ ID NO:2 and (b) two amino acid substitutions that disrupt VH3 binding comprising amino acid substitutions with an alanine at positions corresponding to amino acid positions 36 and 37 of SEQ ID NO:2.

2. The immunogenic isolated polypeptide of claim 1, further comprising one or more SpA variant domain E, A, B, and C segments.

3. The immunogenic isolated polypeptide of claim 2, wherein the polypeptide comprises SpA variant domains D, E, A, B, and C and wherein each domain has substitutions with a lysine at amino acid positions corresponding to amino acids 9 and 10 of SEQ ID NO:2 and substitutions with an alanine at amino acid positions corresponding to amino acids 36 and 37 of SEQ ID NO:2.

4. The immunogenic isolated polypeptide of claim 1, comprising two or more variant domain D segments.

5. The immunogenic isolated polypeptide of claim 1, further comprising a non-Protein A segment.

6. The immunogenic isolated polypeptide of claim 5, wherein the non-Protein A segment is a second staphylococcal antigen segment comprising an Emp, EsxA, EsxB, EsaC, Eap, Ebh, EsaB, Coa, vWbp, vWh, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, ClfA, ClfB, or SasF segment.

7. A pharmaceutical composition comprising a carrier and an immunogenic polypeptide comprising a non-toxigenic Staphylococcal Protein A (SpA) variant domain D segment having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 and having amino acid substitutions that attenuate the binding of the Protein A domain D to IgG, Fcγ, VH3 $F(ab)_2$, von Willebrand factor (vWF), and tumor necrosis factor α receptor 1

(TNFR1), wherein the composition stimulates an immune response in a subject in need thereof; wherein the amino acid substitutions comprise amino acid substitutions with a lysine at amino acid positions corresponding to positions 9 and 10 of SEQ ID NO:2 and amino acid substitutions with an alanine at amino acid positions corresponding to positions 36 and 37 of SEQ ID NO:2.

8. The composition of claim 7, wherein the composition or polypeptide further comprises one or more SpA variant domain E, A, B, and C segments.

9. The composition of claim 8, wherein the composition or polypeptide comprises SpA variant domains D, E, A, B, and C and wherein each domain has substitutions with a lysine at amino acid positions corresponding to amino acids 9 and 10 of SEQ ID NO:2 and substitutions with an alanine at amino acid positions corresponding to amino acids 36 and 37 of SEQ ID NO:2.

10. The composition of claim 8, further comprising at least a second staphylococcal antigen segment, the second staphylococcal antigen segment comprising an EsaB, Emp, EsxA, EsxB, EsaC, Eap, Ebh, Coa, vWbp, vWh, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, ClfA, ClfB, or SasF peptide.

11. The composition of claim 7, wherein the composition contains less than 1% by weight of staphylococcal bacterial components other than the polypeptide comprising the SpA variant domain D segment.

12. The composition of claim 7, wherein the composition further comprises an adjuvant.

13. The composition of claim 12, wherein the adjuvant is coupled to the SpA variant domain D segment.

14. An immunogenic composition comprising a carrier and an immunogenic isolated polypeptide comprising a Staphylococcal Protein A (SpA) variant domain D segment having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 and having an amino acid substitution at amino acid positions corresponding to positions 9, 10, 36, and 37 of SEQ ID NO:2; wherein the substitutions at positions 9 and 10 are with a lysine and the substitutions at positions 36 and 37 are with an alanine.

15. The polypeptide of any one of claims 1-6 or the composition of any one of claims 8-16, further comprising a Staphylococcal PIA polysaccharide or oligosaccharide.

16. The polypeptide of any one of claims 1-6 or the composition of any one of claims 8-16 further comprising a type V or type VIII capsular polysaccharide or oligosaccharide from *S. aureus*.

17. The composition of any one of claims 7-14 further comprising a staphylococcal capsular polysaccharide conjugated to a protein carrier.

18. The composition of claim 17, wherein the protein carrier is selected from the group consisting of tetanus toxoid, diphtheria toxoid, CRM197, *Haemophilus influenzae* protein D, pneumolysin and alpha toxoid.

19. A vaccine comprising the polypeptide of any one of claims 1-6 or the composition of any one of claims 7-14 and a pharmaceutically acceptable excipient.

20. A method of making a vaccine comprising the composition of any one of claims 7-14, the method comprising the step of mixing the polypeptide defined in any one of claim 1 to 6 and a carrier.

21. A use of the polypeptide of any one of claims 1-6 or the composition of any one of claims 7-14 in the manufacture of a vaccine for treatment or prevention of staphylococcal infection.

22. Use of a composition comprising a carrier and a polypeptide comprising a Staphylococcal Protein A (SpA) variant domain D segment having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 and having amino acid substitutions at amino acid positions corresponding to amino acid positions 9, 10, 36, and 37 of SEQ ID NO:2 in the manufacture of a medicament for eliciting an immune response against a staphylococcus bacterium in a subject; wherein the amino acid substitutions at amino acid positions 9 and 10 are with a lysine and the amino acid substitutions at amino acid positions 36 and 37 are with an alanine.

23. The use of claim 22, for use in combination with an adjuvant and a carrier.

24. The use of claim 22, wherein the composition further comprises an adjuvant.

25. The use of claim 24, wherein the SpA variant domain D segment is coupled to the adjuvant.

26. The use of claim 22, wherein the composition further comprises one or more SpA variant domain E, A, B, and C segments.

27. The use of claim 22, wherein the composition comprises SpA variant domains D, E, A, B, and C and wherein each domain has substitutions with a lysine at amino acid positions corresponding to amino acids 9 and 10 of SEQ ID NO:2 and substitutions with an alanine at amino acid positions corresponding to amino acids 36 and 37 of SEQ ID NO:2.

28. The use of claim 22, wherein the staphylococcus bacterium is a *S. aureus* bacterium.

29. The use of claim 22, wherein the staphylococcus bacterium is resistant to one or more antibiotics.

30. The use of claim 29, wherein the bacterium is methicillin resistant.

31. The use of claim 22, wherein the composition is for oral, parenteral, subcutaneous, intramuscular, or intravenous administration.

32. The use of claim 22, wherein the composition is for use with a second staphylococcal antigen, the second staphylococcal antigen comprising one or more of Emp, EsxA, EsxB, EsaC, Eap, Ebh, EsaB, Coa, vWbp, vWh, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, ClfA, ClfB, and SasF.

33. The use of claim 22, wherein the composition comprises a recombinant, non-staphylococcus bacterium expressing the SpA variant domain segment.

34. The use of claim 33, wherein the recombinant non-staphylococcus bacterium has a recombinant nucleic molecule encoding the SpA variant domain segment.

35. The use of claim 33, wherein the recombinant non-staphylococcus bacterium is a Salmonella.

36. The use of claim 22, wherein the subject is a mammal.

37. The use of claim 22, wherein the subject is human.

38. The use of claim 22, wherein the immune response is a protective immune response.

39. Use of an immunogenic isolated polypeptide comprising a Staphylococcal Protein A (SpA) variant domain D segment having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 and having amino acid substitutions at amino acid positions corresponding to amino acid positions 9, 10, 36, and 37 of SEQ ID NO:2 in the manufacture of a medicament for treating a staphylococcal infection in a subject having, suspected of having, or at risk of developing a staphylococcal infection, wherein the amino acid substitutions at amino acid positions 9 and 10 are with a lysine and the amino acid substitutions at amino acid positions 36 and 37 are with an alanine.

40. The use of claim 39, wherein the subject has been previously diagnosed with a persistent staphylococcal infection.

41. The use of claim 39, wherein the SpA variant domain D segment elicits production of an antibody that binds Protein A in the subject.

42. The use of claim 39, wherein the polypeptide further comprises one or more SpA variant domain E, A, B, and C segments.

43. The use of claim 42, wherein the polypeptide comprises SpA variant domains D, E, A, B, and C and wherein each domain has substitutions with a lysine at amino acid positions corresponding to amino acid positions 9 and 10 of SEQ ID NO:2 and substitutions with an alanine at amino acid positions corresponding to amino acid positions 36 and 37 of SEQ ID NO:2.

44. The use of claim 39, wherein the SpA variant domain D segment is for use in combination with an adjuvant.

45. The use of claim 44, wherein the SpA variant domain D segment is coupled to the adjuvant.

46. The use of claim 39, wherein the SpA variant domain D segment is for use in combination with a second staphylococcal antigen comprising one or more of Emp, EsxA, EsxB, EsaC, Eap, Ebh, EsaB, Coa, vWbp, vWh, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, ClfA, ClfB, and SasF.

47. The use of claim 46, wherein the second staphylococcal antigen is for concurrent administration with the SpA variant domain segment.

48. The use of claim 39, wherein the immunogenic isolated polypeptide is formulated with a second staphylococcal antigen comprising one or more of Emp, EsxA, EsxB, EsaC, Eap, Ebh, EsaB, Coa, vWbp, vWh, Hla, SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, ClfA, ClfB, and SasF peptide.

49. The use of claim 48, wherein the second staphylococcal antigen is fused with the SpA variant domain D segment.

50. The use of claim 39, wherein the staphylococcal infection is a *Staphylococcus aureus* infection.

51. The use of claim 39, wherein the polypeptide is for oral, parenteral, transdermal, transmucosal, subcutaneous, or intramuscular administration, or administration by inhalation.

52. The use of claim 39, wherein the subject is a mammal.

53. The use of claim 39, wherein the subject is human.

54. A nucleic acid encoding the polypeptide of any one of claims 1-6.

55. The immunogenic isolated polypeptide of claim 2, wherein the polypeptide comprises SpA variant domains D, E, A, B, and C and wherein each domain is 100% identical to SEQ ID

NOS:2, 3, 4, 6, and 5, respectively, except for having substitutions with a lysine at amino acid positions corresponding to amino acids 9 and 10 of SEQ ID NO:2 and substitutions with an alanine at amino acid positions corresponding to amino acids 36 and 37 of SEQ ID NO:2.

56. The immunogenic isolated polypeptide of claim 1, wherein the SpA variant domain D segment has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2.

57. The use of claim 22 or 39, wherein the SpA variant domain D segment is at least 90% identical to SEQ ID NO:2.

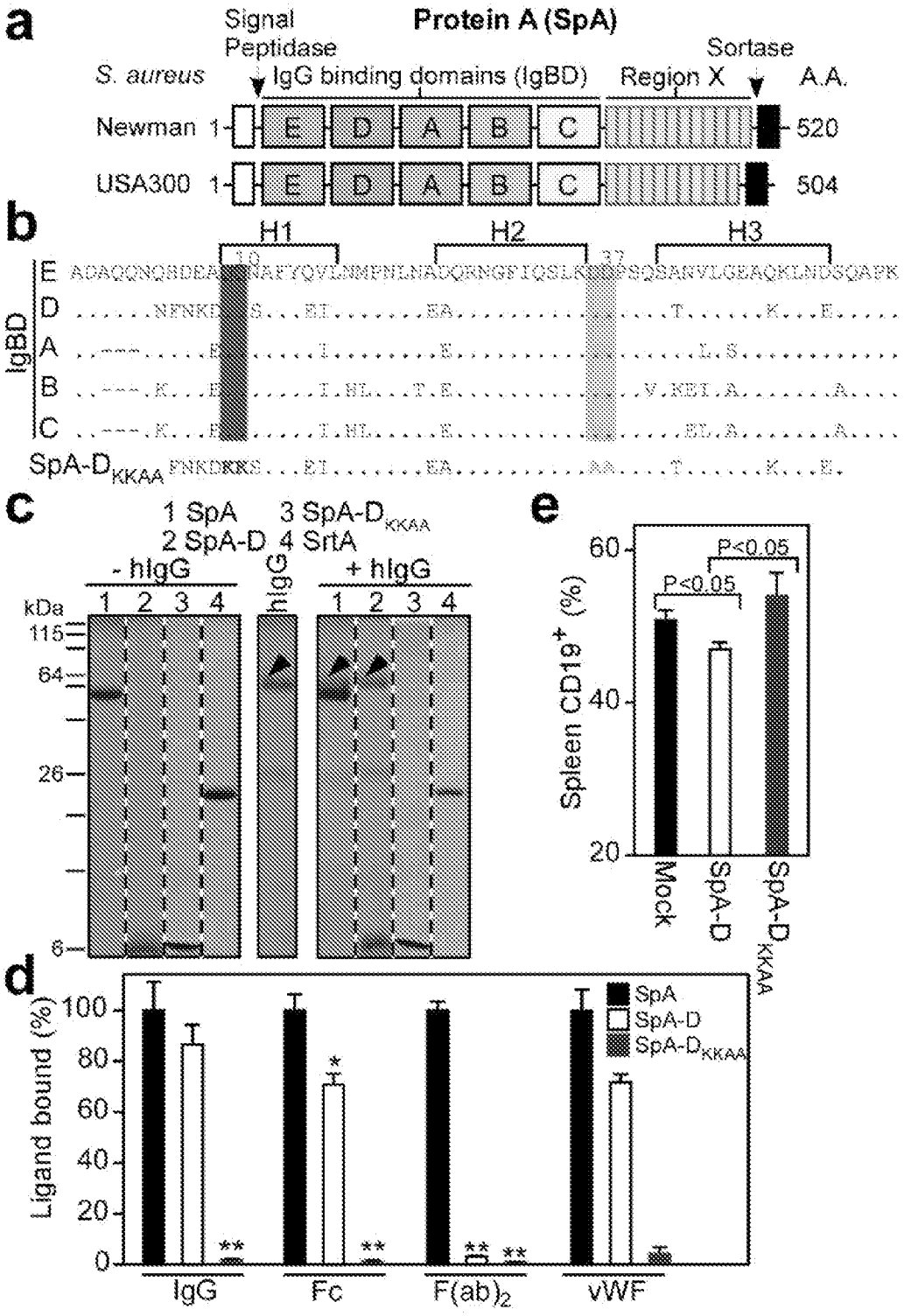

a
Signal Peptidase
Protein A (SpA)
Sortase
S. aureus
IgG binding domains (IgBD)
Region X
A.A.
Newman 1
E D A B C
520
USA300 1
E D A B C
504
b
H1
H2
H3
IgBD
E
D
A
B
C
SpA-D KKAA
c
1 SpA
2 SpA-D
3 SpA-D KKAA
4 SrtA
- hIgG
hIgG
+ hIgG
kDa
1 2 3 4
115
64
26
6
e
Spleen CD19+ (%)
60
40
20
P<0.05
P<0.05
Mock
SpA-D
SpA-D KKAA
d
Ligand bound (%)
100
80
60
40
20
0
SpA
SpA-D
SpA-D KKAA
IgG
Fc
F(ab)2
vWF

FIG. 1

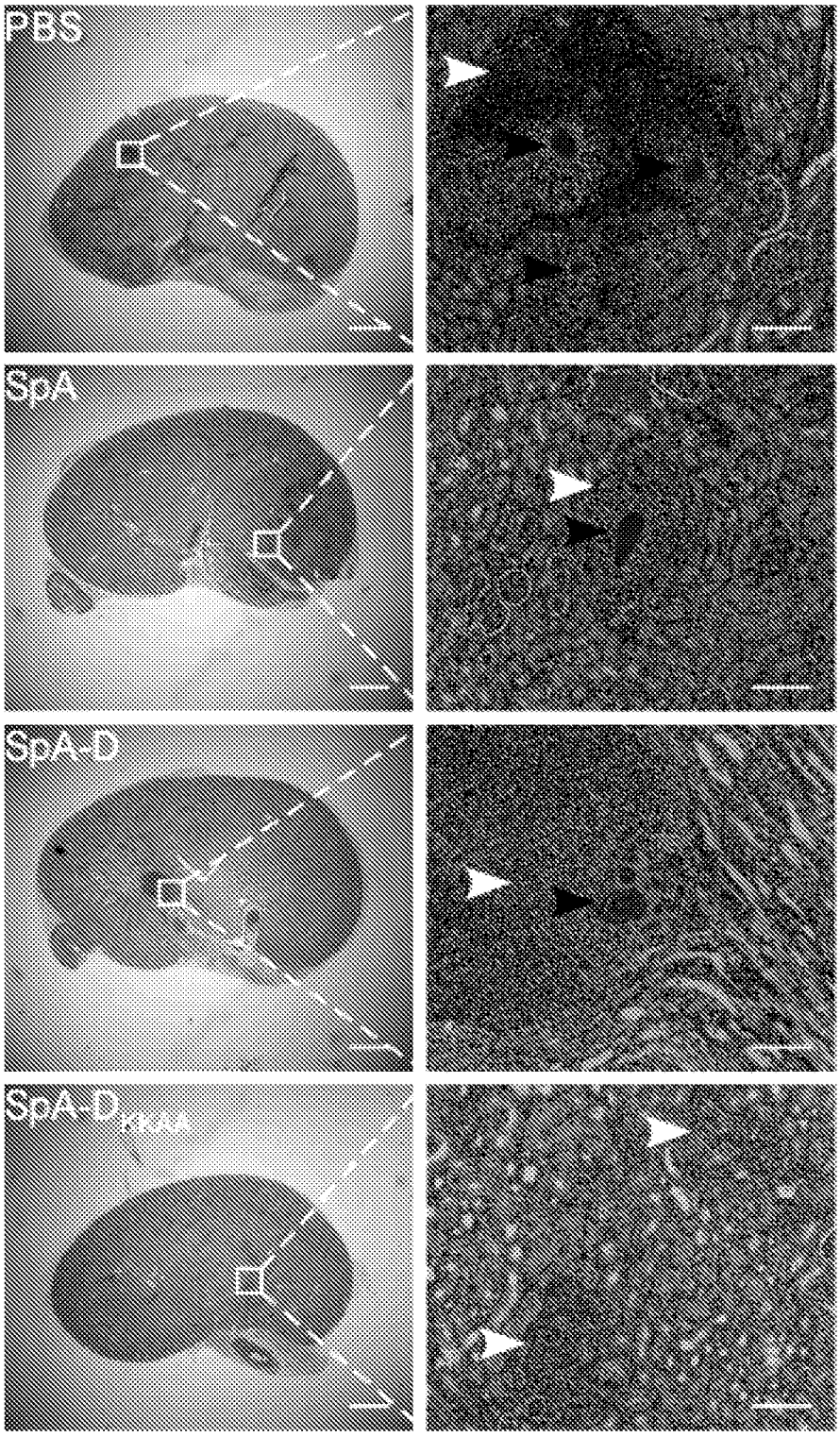



FIG. 2

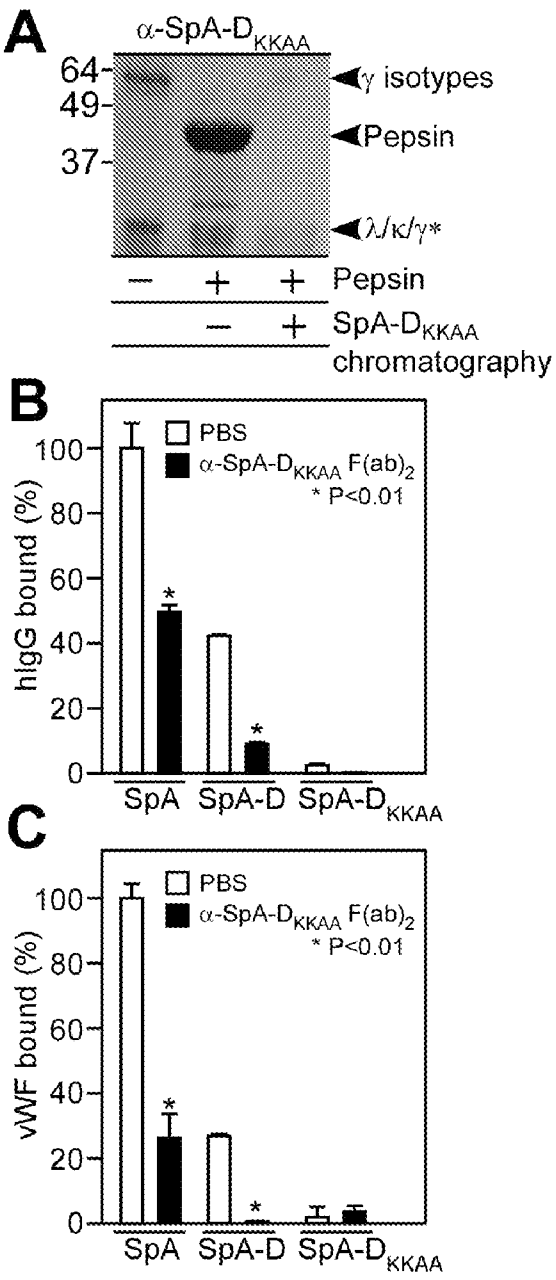

A
α-SpA-D$_{KKAA}$
64-
49-
37-
γ isotypes
Pepsin
λ/κ/γ*
— + + Pepsin
— + SpA-D$_{KKAA}$
chromatography
B
PBS
α-SpA-D$_{KKAA}$ F(ab)$_2$
* P<0.01
hIgG bound (%)
100
80
60
40
20
0
SpA
SpA-D
SpA-D$_{KKAA}$
C
PBS
α-SpA-D$_{KKAA}$ F(ab)$_2$
* P<0.01
vWF bound (%)
100
80
60
40
20
0
SpA
SpA-D
SpA-D$_{KKAA}$

FIG. 3

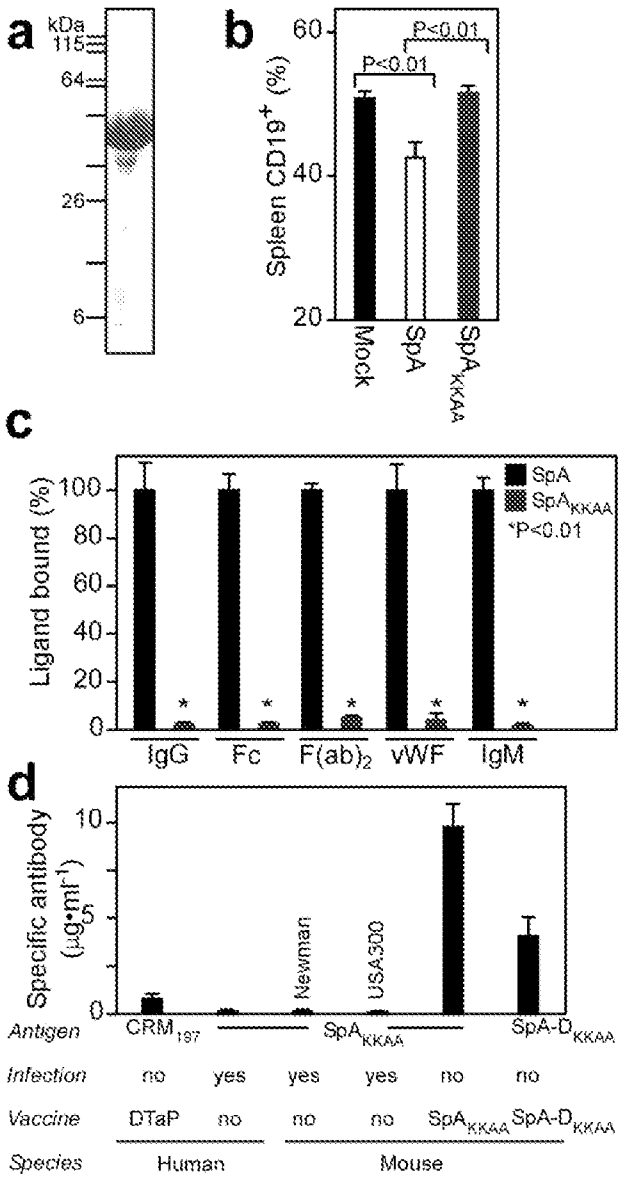

a
kDa
115
64
26
6
b
Spleen CD19+ (%)
60
40
20
P<0.01
P<0.01
Mock
SpA
SpAKKAA
c
Ligand bound (%)
100
80
60
40
20
0
SpA
SpAKKAA
*P<0.01
IgG
Fc
F(ab)2
vWF
IgM
d
Specific antibody (μg•ml-1)
10
5
0
Newman
USA300
Antigen
CRM197
SpAKKAA
SpA-DKKAA
Infection
no
yes
yes
yes
no
no
Vaccine
DTaP
no
no
no
SpAKKAA
SpA-DKKAA
Species
Human
Mouse

**FIG. 4**

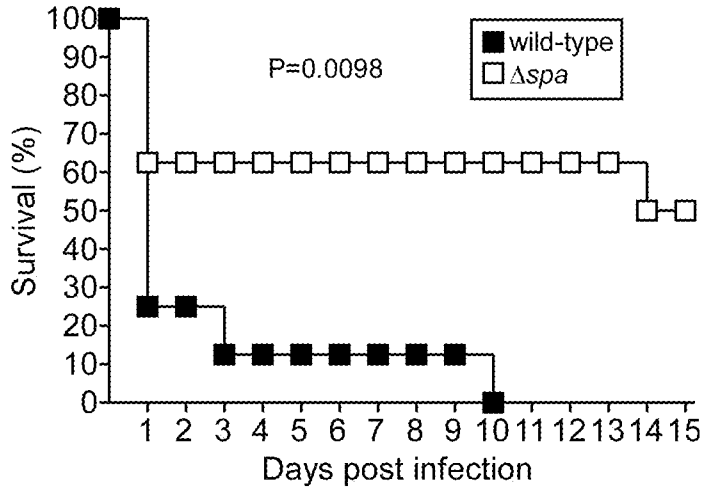

P=0.0098
wild-type
Δspa
Survival (%)
Days post infection

FIG. 5

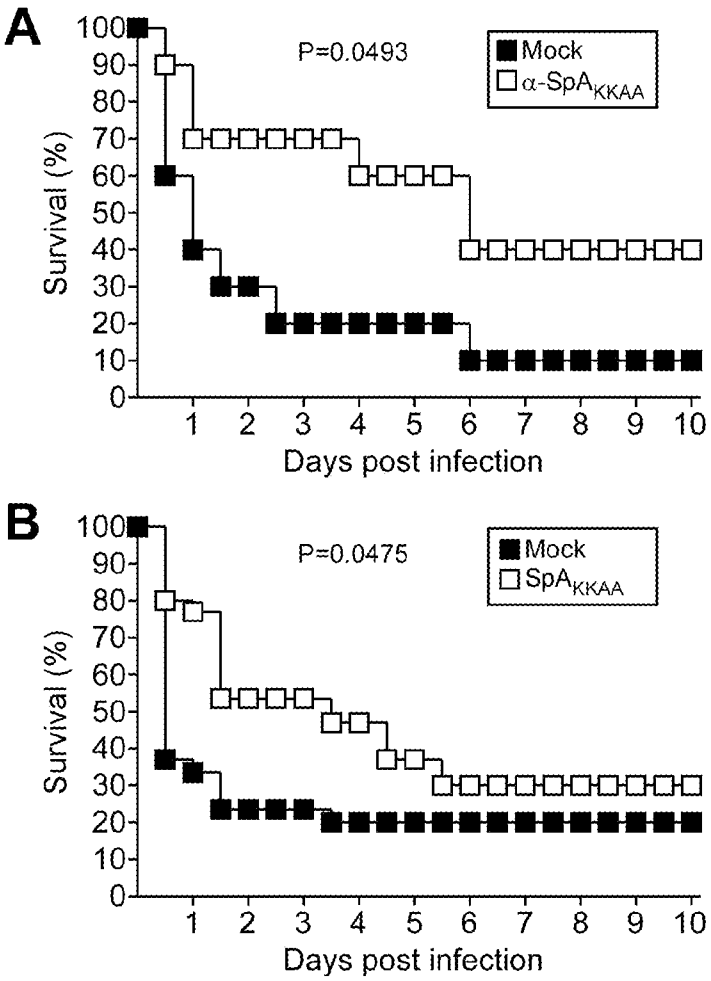

A
P=0.0493
Mock
α-SpA$_{KKAA}$
Survival (%)
Days post infection
B
P=0.0475
Mock
SpA$_{KKAA}$
Survival (%)
Days post infection

FIG. 6

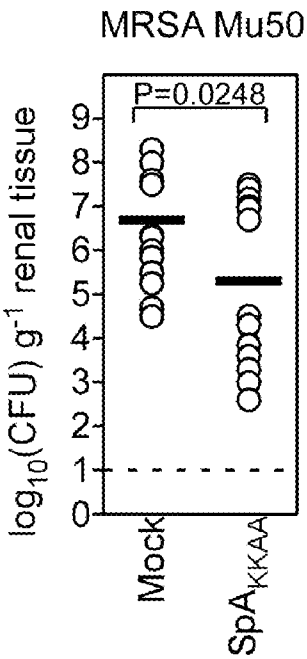

MRSA Mu50
P=0.0248
log10(CFU) g-1 renal tissue
0
1
2
3
4
5
6
7
8
9
Mock
SpAKKAA

FIG. 7

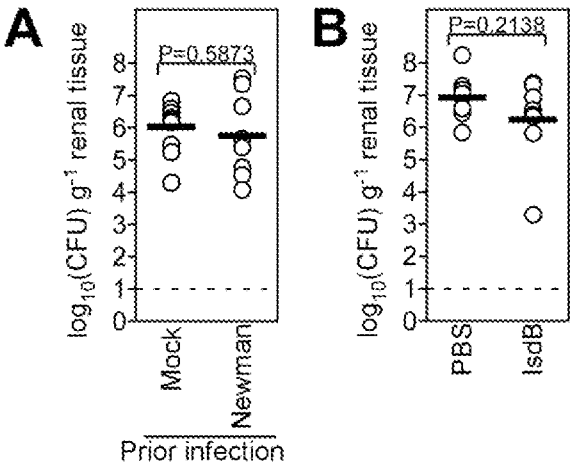

A
P=0.5873
log10(CFU) g-1 renal tissue
Mock
Newman
Prior infection
B
P=0.2138
log10(CFU) g-1 renal tissue
PBS
IsdB

FIG. 8

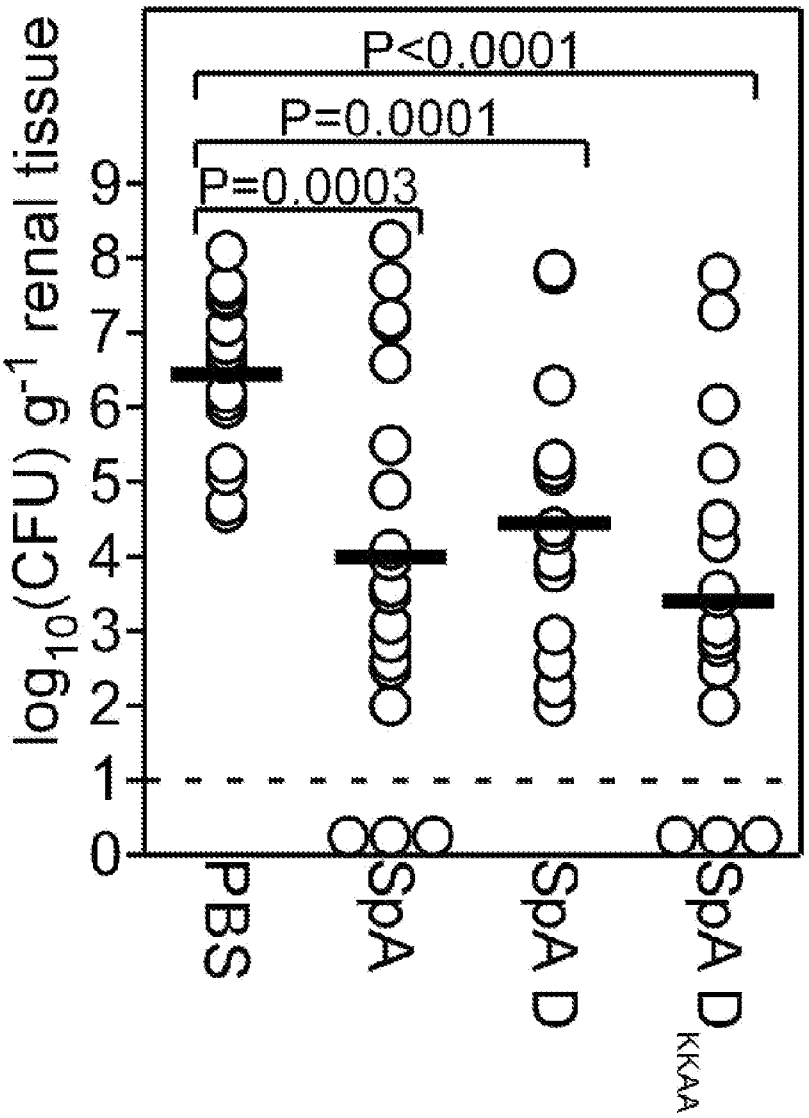

P<0.0001
P=0.0001
P=0.0003
log10(CFU) g-1 renal tissue
0
1
2
3
4
5
6
7
8
9
PBS
SpA
SpA D
SpA DKKAA

FIG. 9

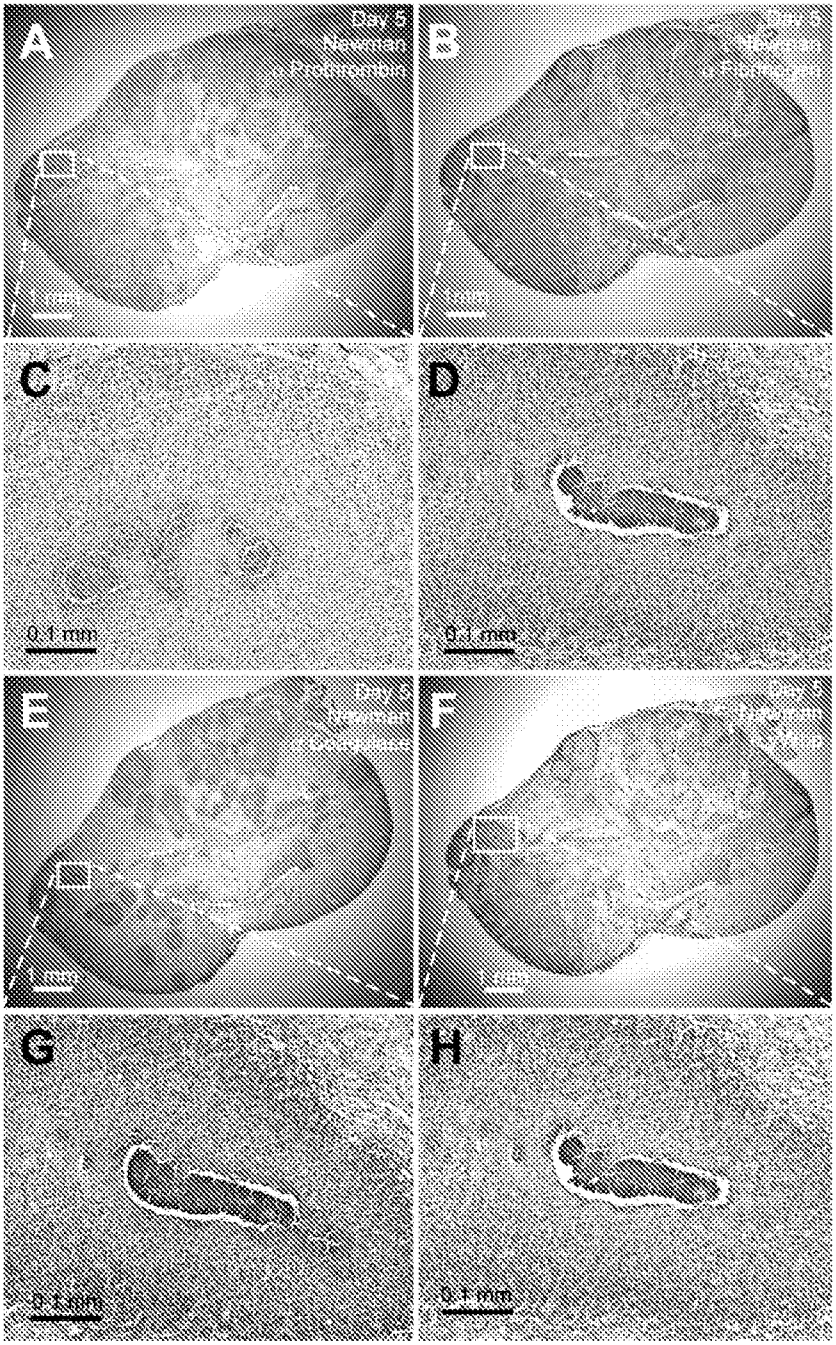



FIG. 10

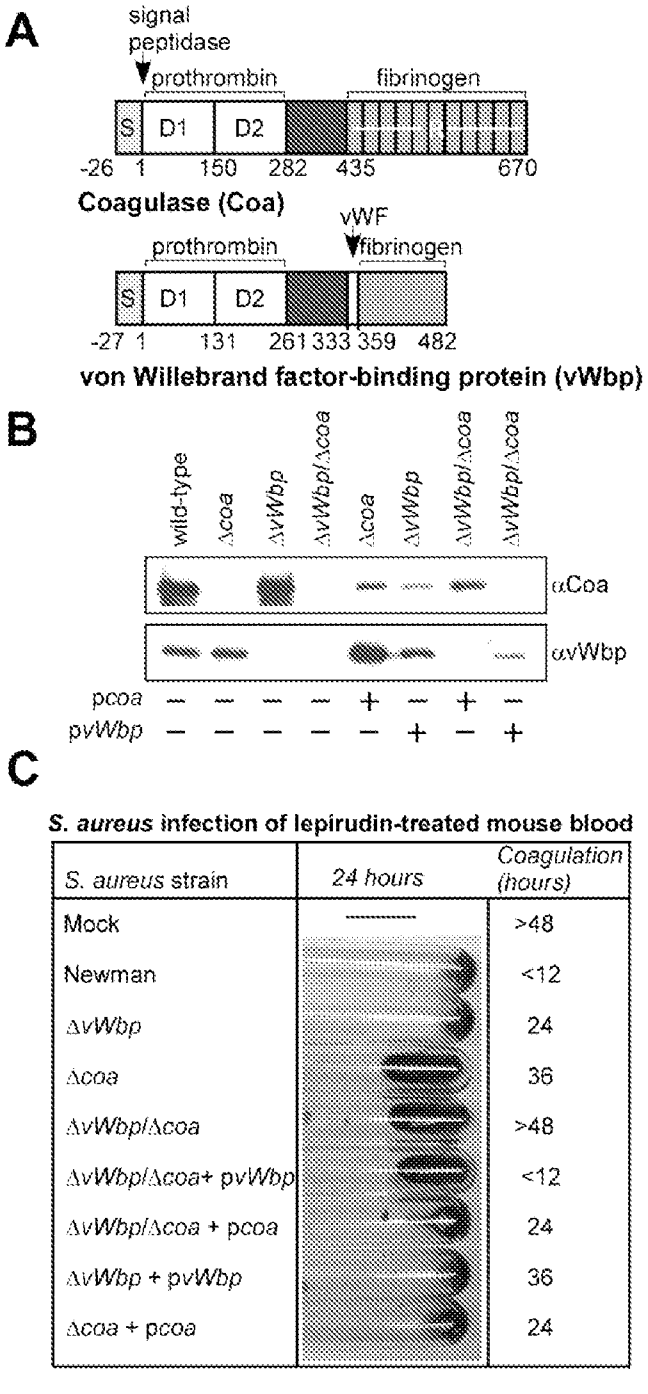

A
signal peptidase
prothrombin
fibrinogen
S
D1
D2
-26 1 150 282 435 670
Coagulase (Coa)
vWF
prothrombin
fibrinogen
S
D1
D2
-27 1 131 261 333 359 482
von Willebrand factor-binding protein (vWbp)
B
wild-type
Δcoa
ΔvWbp
ΔvWbp/Δcoa
Δcoa
ΔvWbp
ΔvWbp/Δcoa
ΔvWbp/Δcoa
αCoa
αvWbp
pcoa
pvWbp
C
S. aureus infection of lepirudin-treated mouse blood
S. aureus strain
24 hours
Coagulation (hours)
Mock >48
Newman <12
ΔvWbp 24
Δcoa 36
ΔvWbp/Δcoa >48
ΔvWbp/Δcoa+ pvWbp <12
ΔvWbp/Δcoa + pcoa 24
ΔvWbp + pvWbp 36
Δcoa + pcoa 24

**FIG. 11**

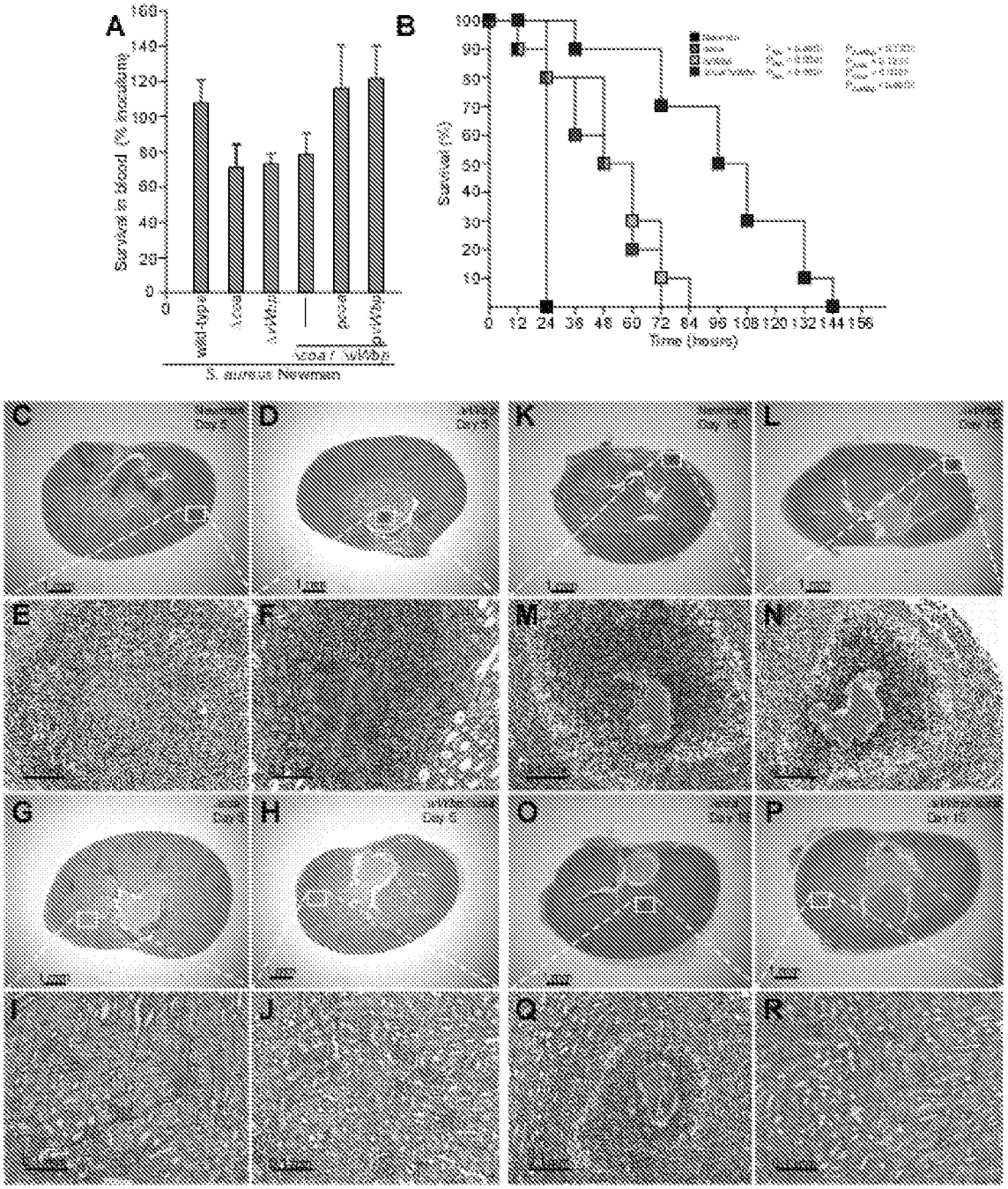
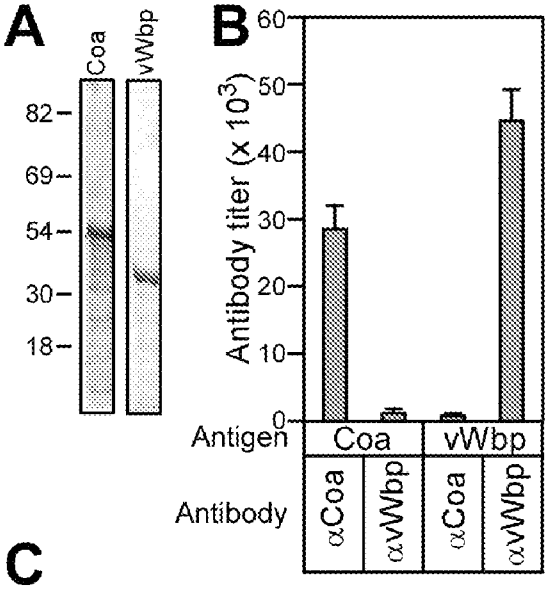

A
B
Survival in blood (% inoculum)
Survival (%)
Time (hours)
S. aureus Newman
C
D
K
L
E
F
M
N
G
H
O
P
I
J
Q
R

FIG. 12




C

***S. aureus* infection of mouse blood**

| Antibody | Coagulation 24 hours | Coagulation Completed |
|---|---|---|
| Mock | — | 24 |
| αV10 | | 24 |
| αCoa | | >48 |
| αvWbp | | >48 |
| αCoa/αvWbp | | >48 |

D

| Mouse blood with | Coagulation time |
|---|---|
| PBS | indefinite |
| Coagulase | < 30 min |
| Coagulase + αV10 | < 30 min |
| Coagulase + αCoa | 2 hrs |
| Coagulase + αvWbp | 1 hr |
| vWbp | < 30 min |
| vWbp + αV10 | < 30 min |
| vWbp + αCoa | 2 hrs |
| vWbp + αvWbp | 2 hrs |

**FIG. 13**

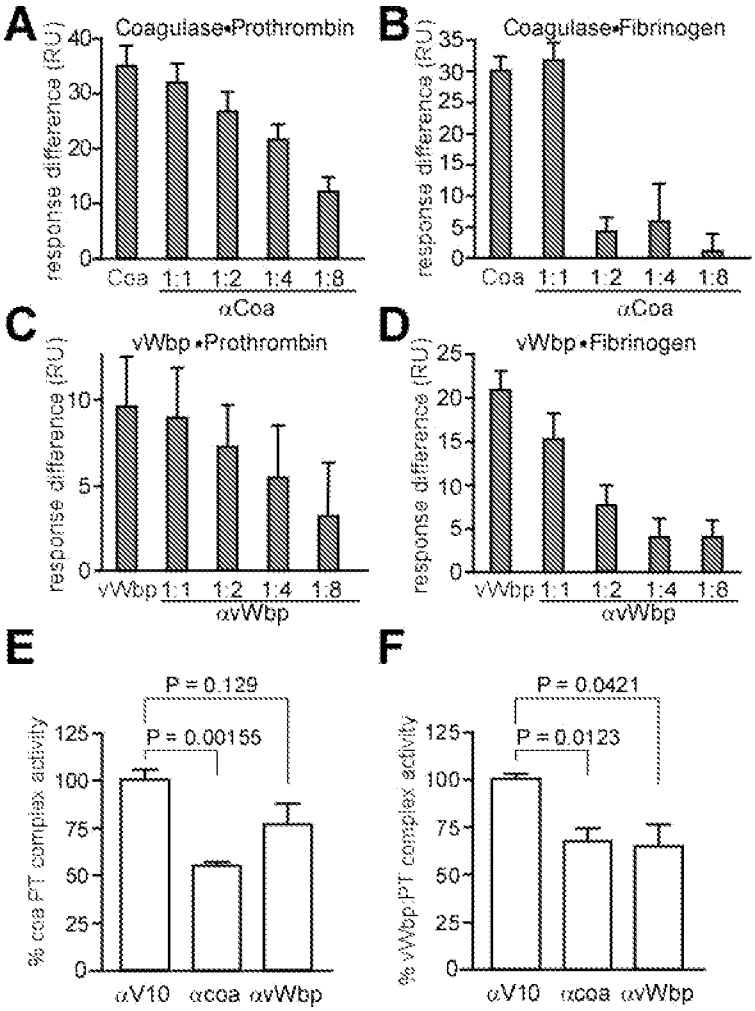

A
Coagulase•Prothrombin
response difference (RU)
Coa 1:1 1:2 1:4 1:8
αCoa
B
Coagulase•Fibrinogen
response difference (RU)
Coa 1:1 1:2 1:4 1:8
αCoa
C
vWbp•Prothrombin
response difference (RU)
vWbp 1:1 1:2 1:4 1:8
αvWbp
D
vWbp•Fibrinogen
response difference (RU)
vWbp 1:1 1:2 1:4 1:8
αvWbp
E
P = 0.129
P = 0.00155
% coa PT complex activity
αV10 αcoa αvWbp
F
P = 0.0421
P = 0.0123
% vWbp:PT complex activity
αV10 αcoa αvWbp

**FIG. 14**

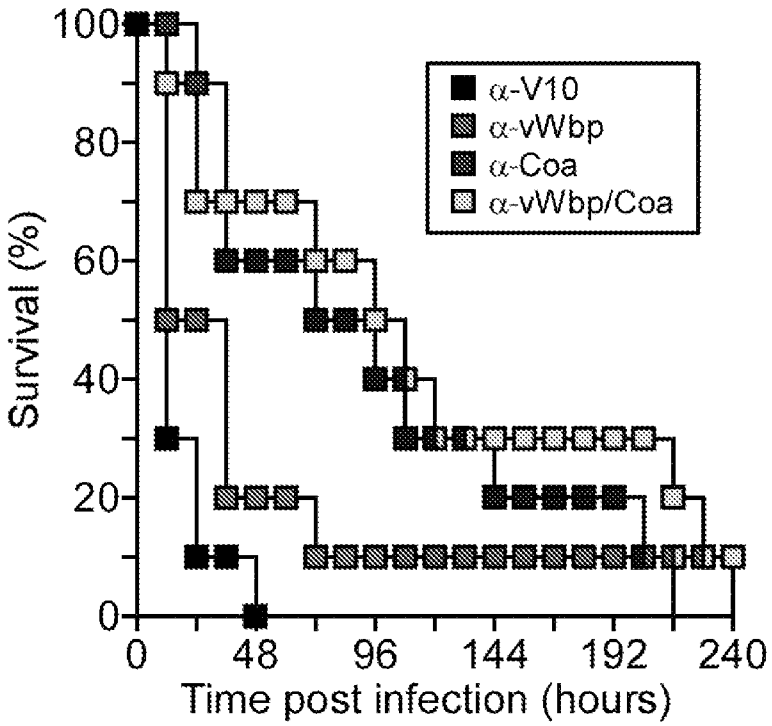

100
80
60
40
20
0
Survival (%)
0
48
96
144
192
240
Time post infection (hours)
α-V10
α-vWbp
α-Coa
α-vWbp/Coa

FIG. 15

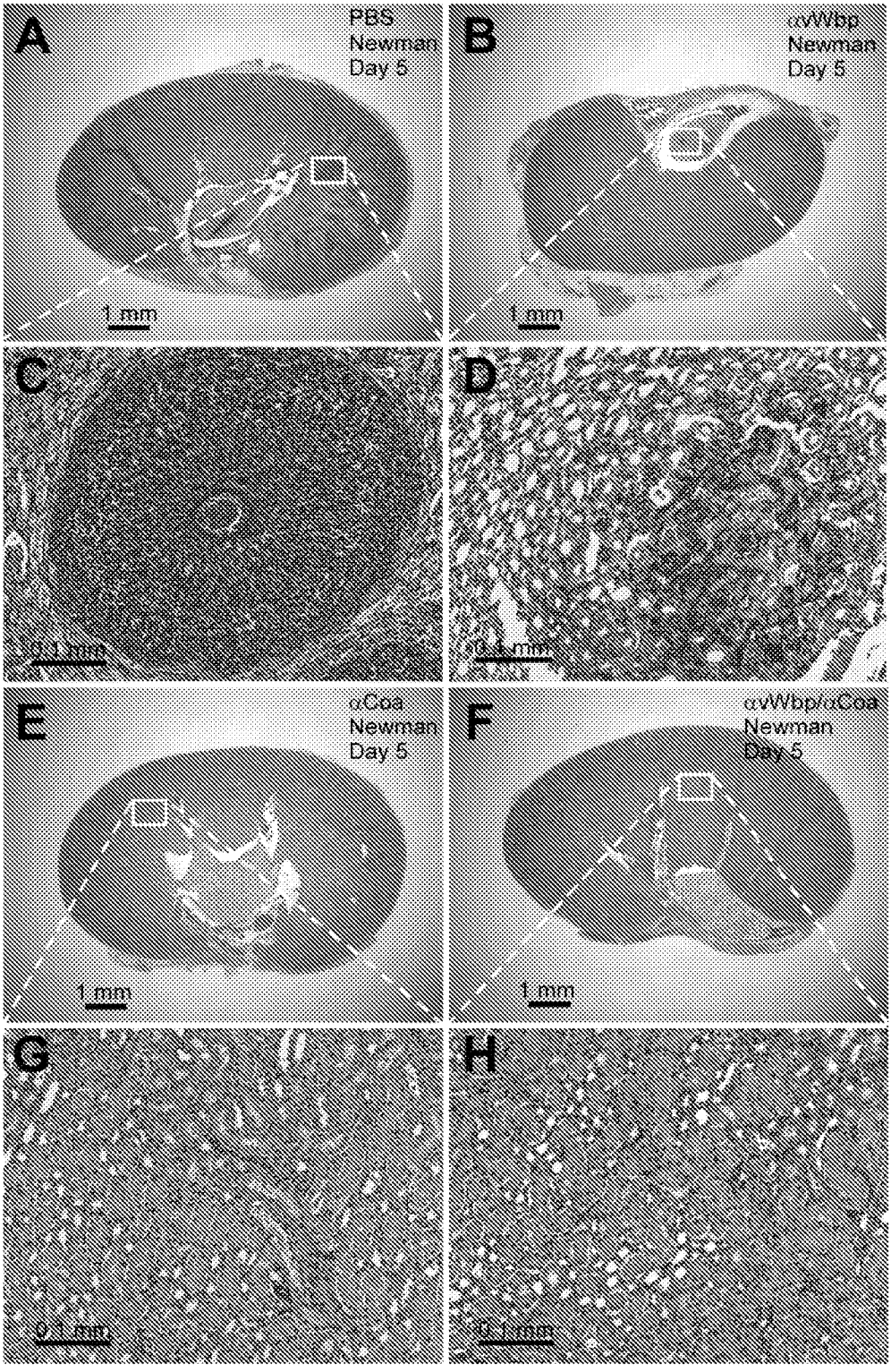

A
PBS
Newman
Day 5
1 mm
B
αvWbp
Newman
Day 5
1 mm
C
0.1 mm
D
0.1 mm
E
αCoa
Newman
Day 5
1 mm
F
αvWbp/αCoa
Newman
Day 5
1 mm
G
0.1 mm
H
0.1 mm

FIG. 16

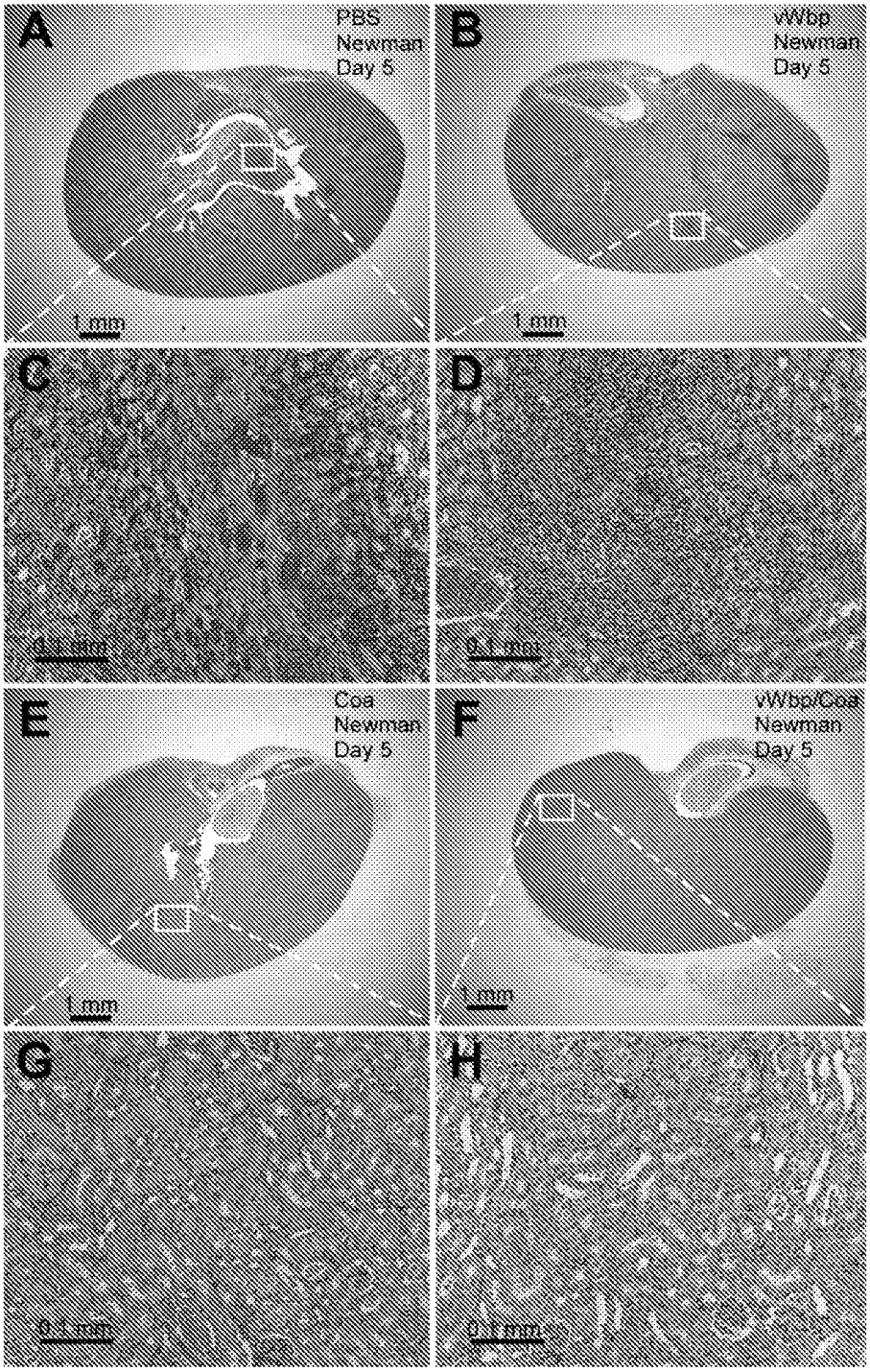

A
PBS
Newman
Day 5
1 mm
B
vWbp
Newman
Day 5
1 mm
C
0.1 mm
D
0.1 mm
E
Coa
Newman
Day 5
1 mm
F
vWbp/Coa
Newman
Day 5
1 mm
G
0.1 mm
H
0.1 mm

FIG. 17