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(54) **IMMUNOGENIC COMPOSITION COMPRISING STAPHYLOCOCCAL ANTIGENS**

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(57) **ABSTRACT**

The invention provides an immunogenic composition comprising staphylococcal antigens, containing protein antigens and conjugates of capsular polysaccharides. Adjuvanted formulations are also provided. The invention may find use in the prevention and treatment of staphylococcal infections.

Specification includes a Sequence Listing.

FIG. 1A

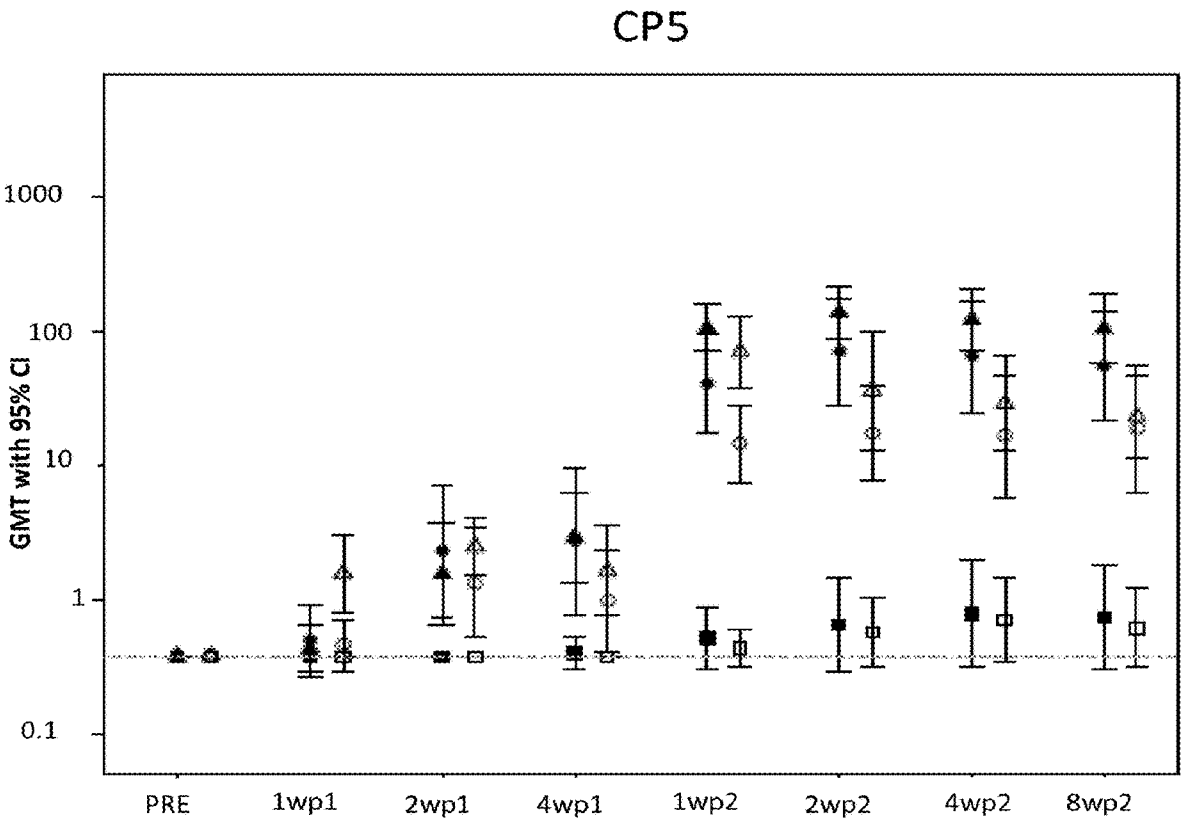

CP5
GMT with 95% CI
1000
100
10
1
0.1
PRE
1wp1
2wp1
4wp1
1wp2
2wp2
4wp2
8wp2

FIG. 1B

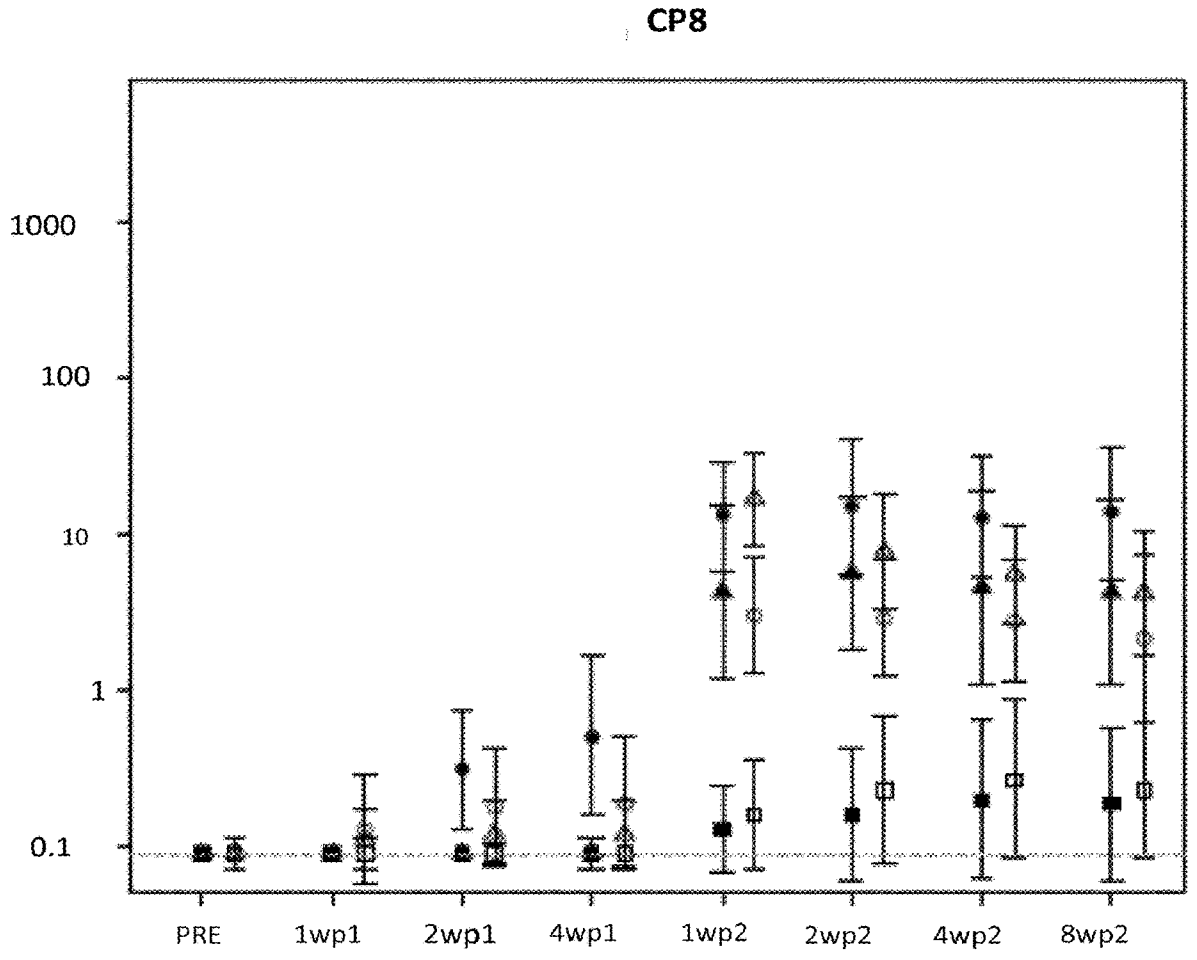


FIG. 1C

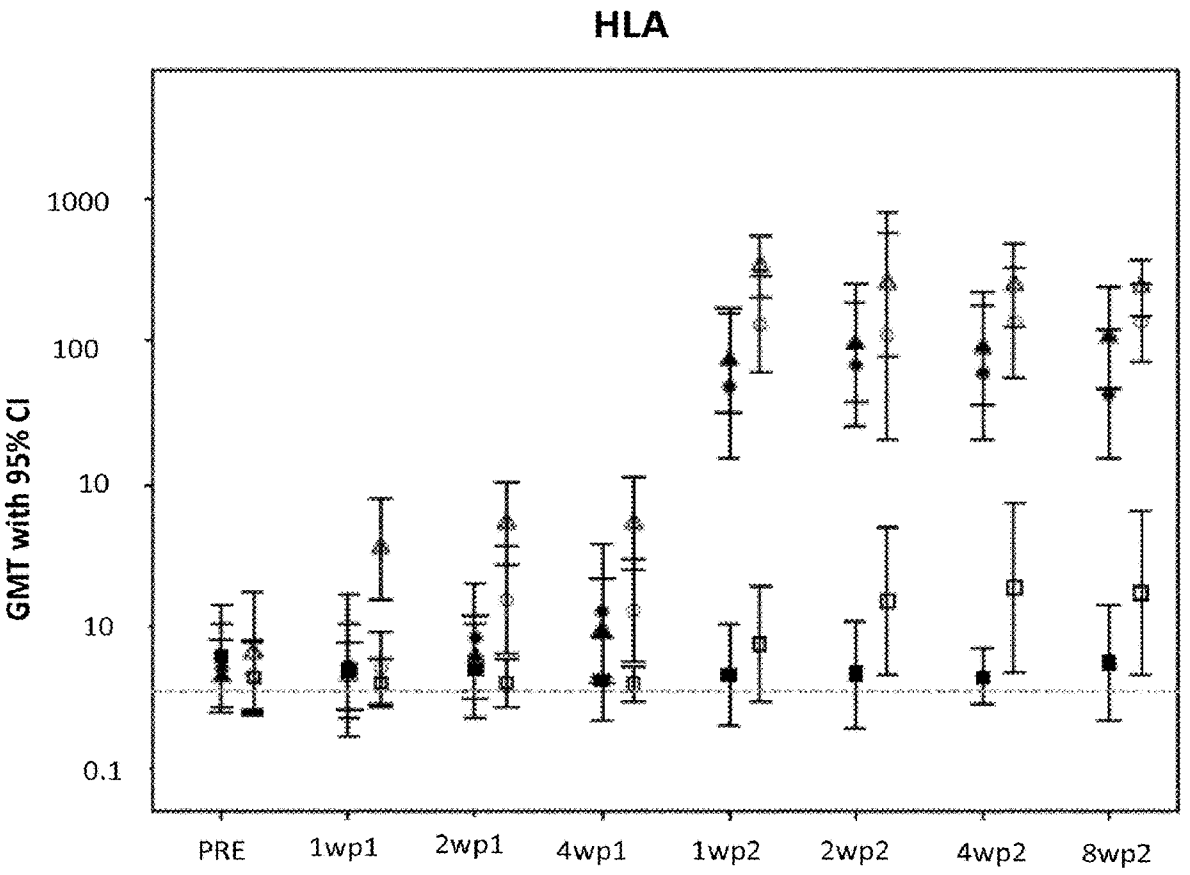

HLA
GMT with 95% CI
1000
100
10
10
0.1
PRE
1wp1
2wp1
4wp1
1wp2
2wp2
4wp2
8wp2

FIG. 1D

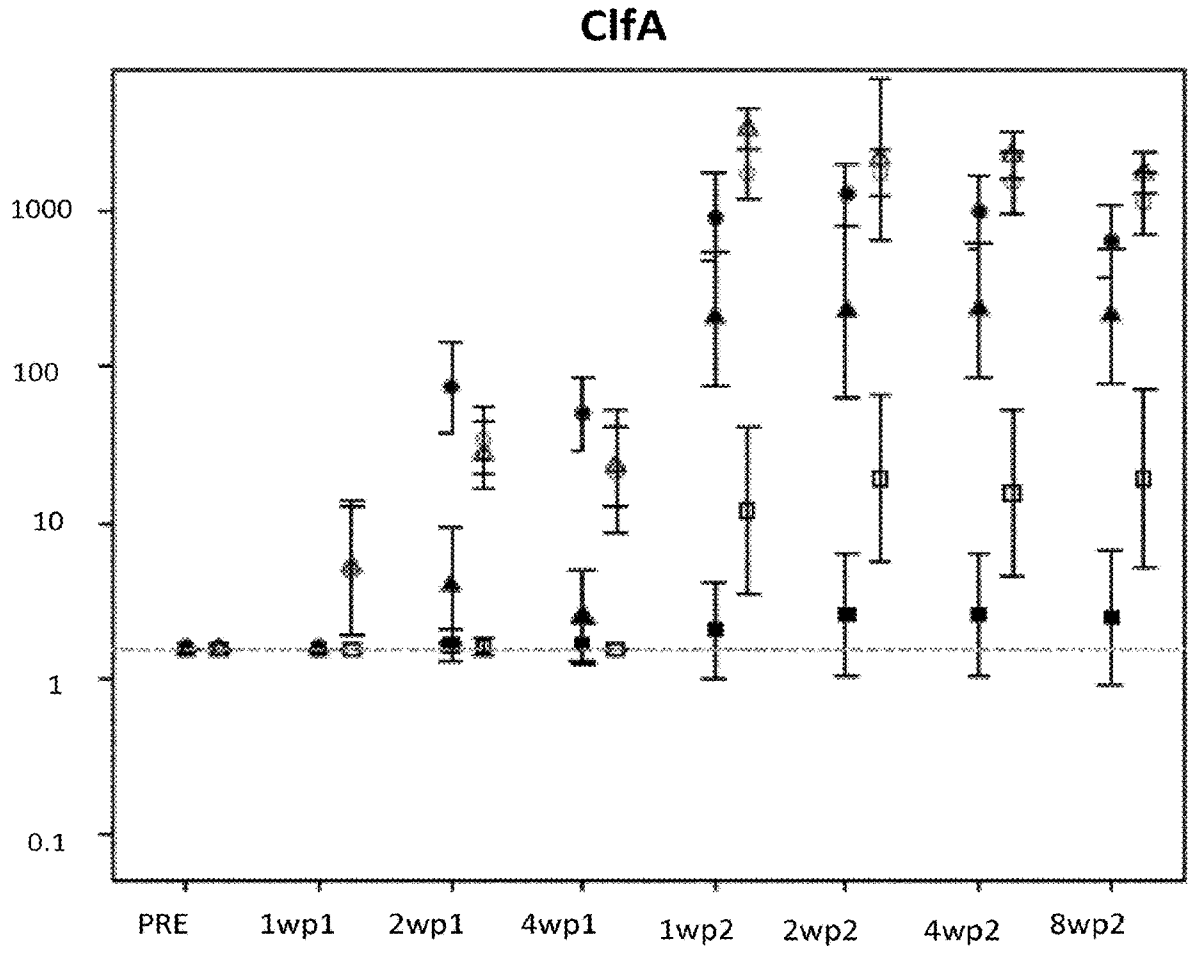


FIG. 1E

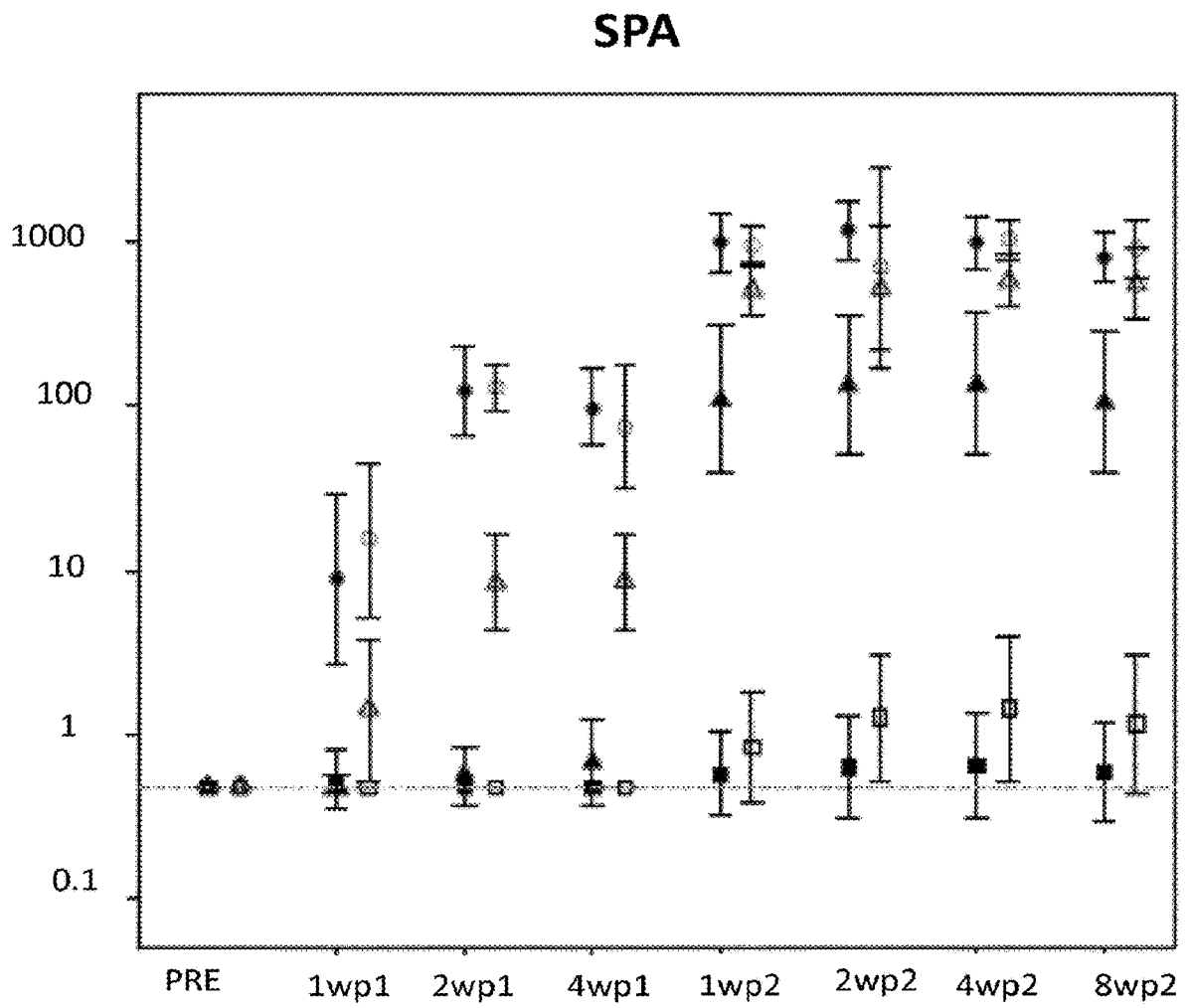


FIG. 2A

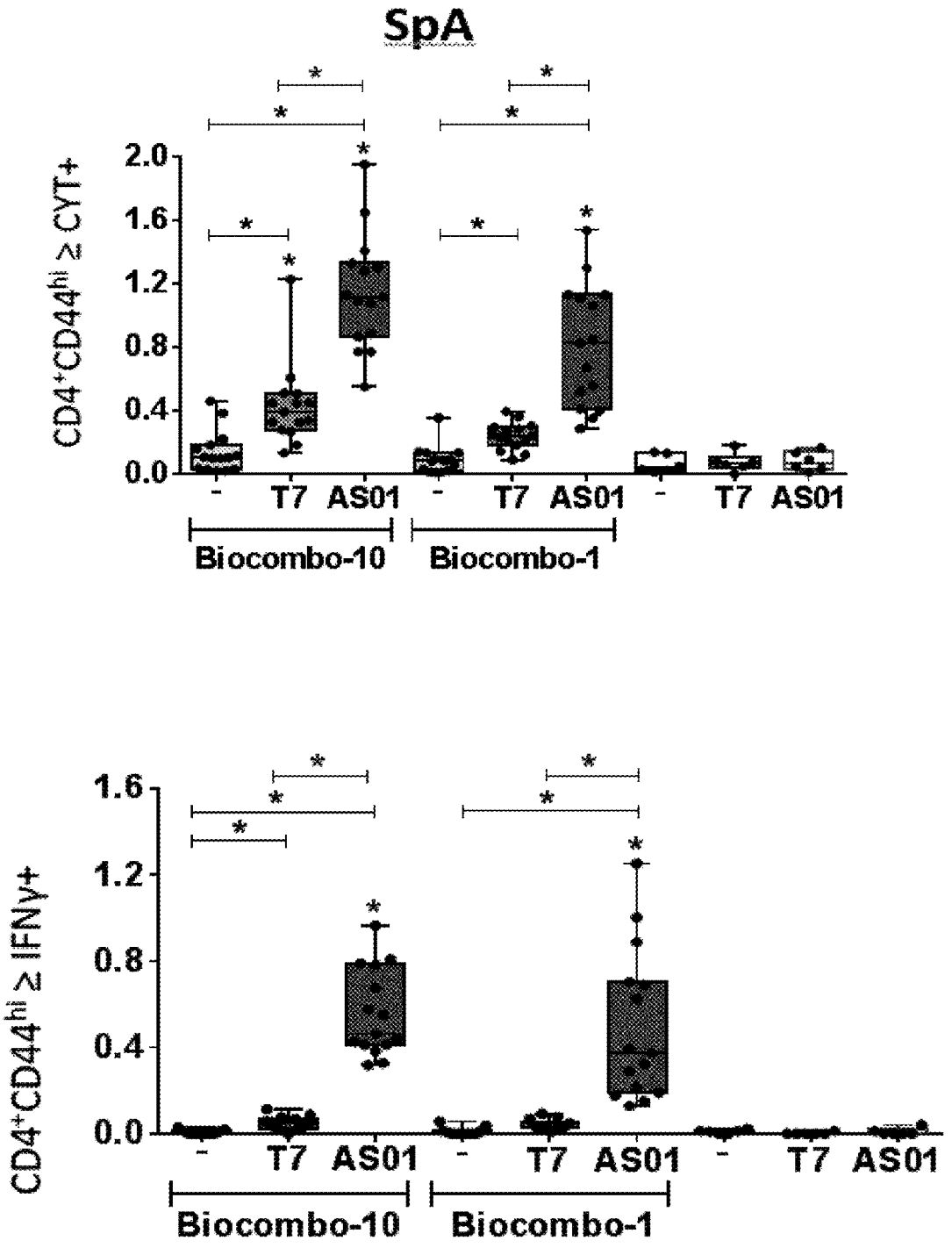
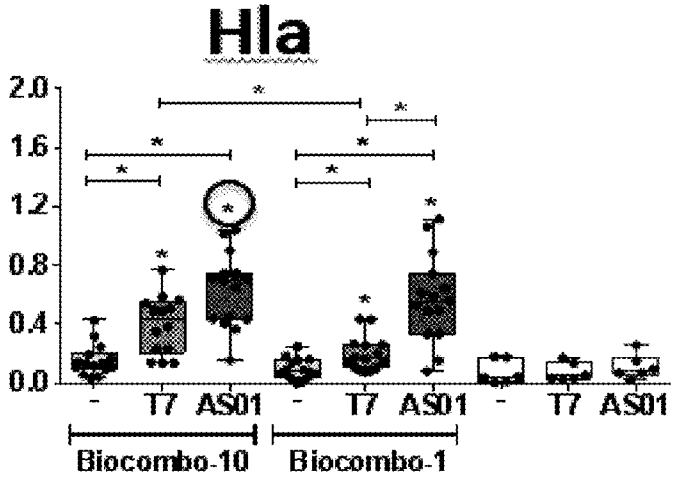

SpA
CD4+CD44hi ≥ CYT+
2.0
1.6
1.2
0.8
0.4
0.0
-
T7
AS01
Biocombo-10
Biocombo-1
CD4+CD44hi ≥ IFNγ+

FIG. 2B


Hla
2.0
1.6
1.2
0.8
0.4
0.0
-
T7
AS01
-
T7
AS01
-
T7
AS01
Biocombo-10
Biocombo-1

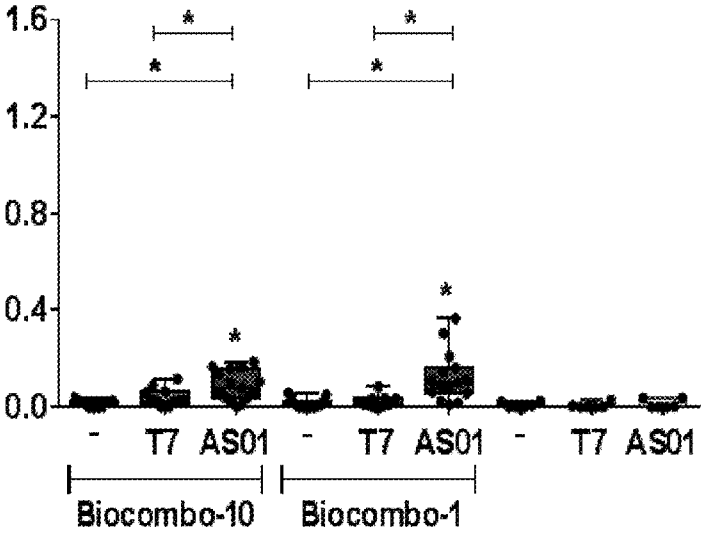

1.6
1.2
0.8
0.4
0.0
-
T7
AS01
-
T7
AS01
-
T7
AS01
Biocombo-10
Biocombo-1

FIG. 2C

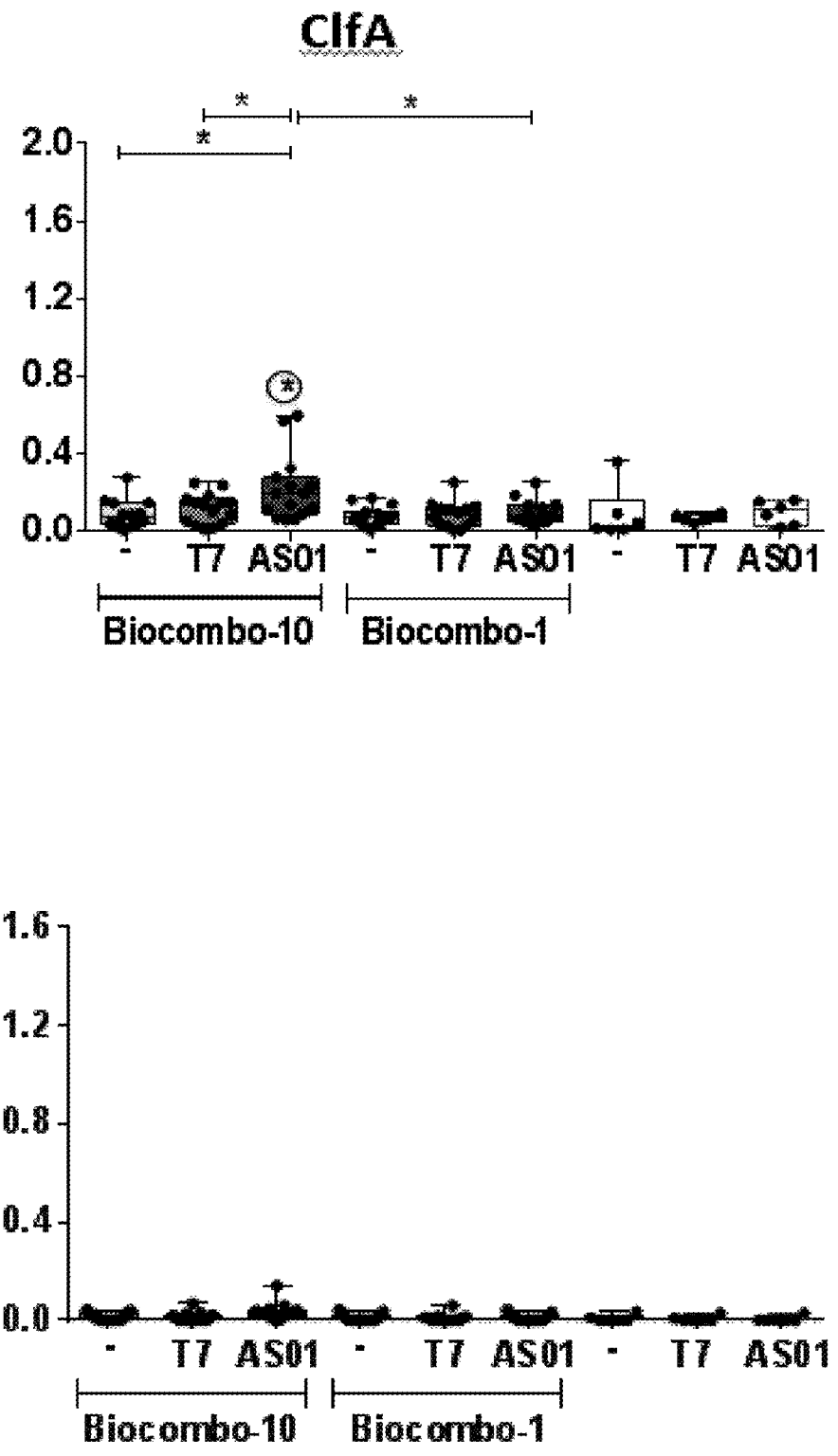

ClfA
2.0
1.6
1.2
0.8
0.4
0.0
*
*
*
-
T7
AS01
-
T7
AS01
-
T7
AS01
Biocombo-10
Biocombo-1
1.6
1.2
0.8
0.4
0.0
-
T7
AS01
-
T7
AS01
-
T7
AS01
Biocombo-10
Biocombo-1

FIG. 3A

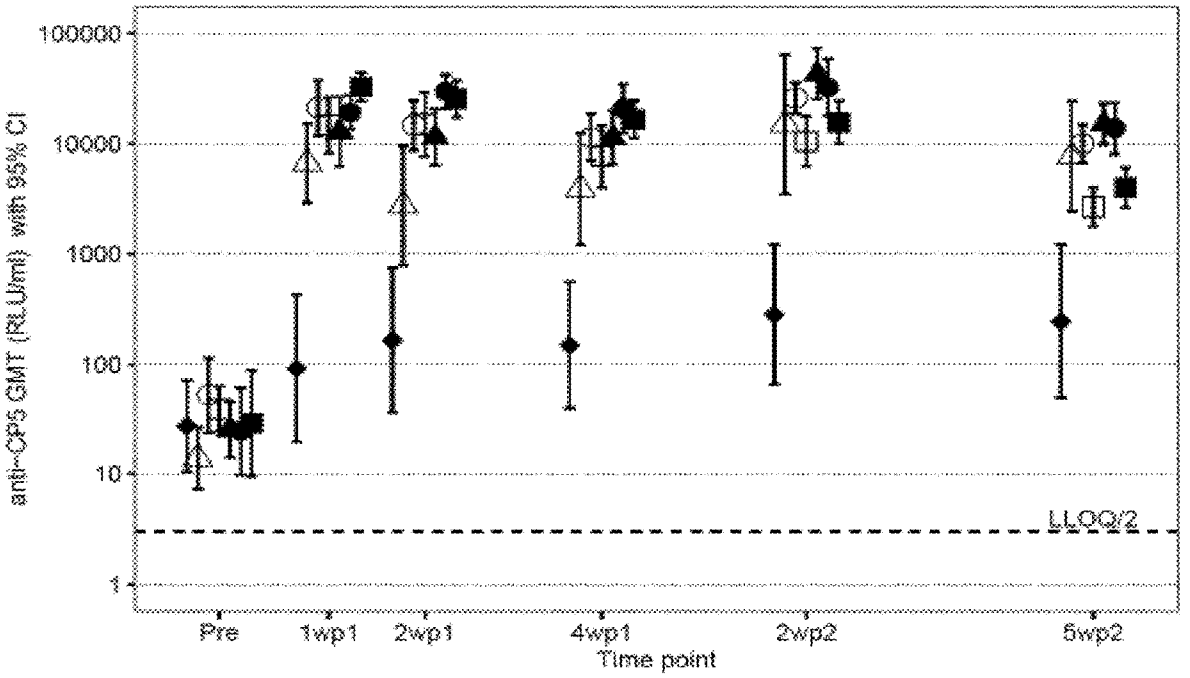

100000
10000
1000
100
10
1
anti-CP5 GMT (RLU/ml) with 95% CI
LLOQ/2
Pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time point

FIG. 3B

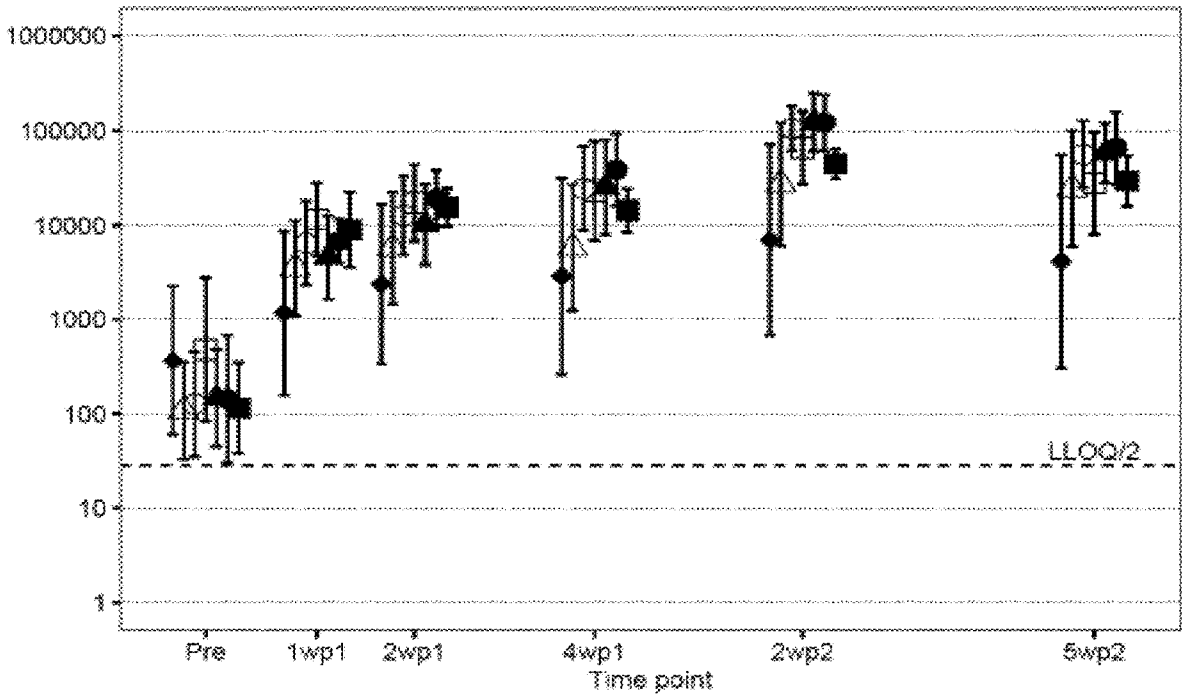

1000000
100000
10000
1000
100
10
1
LLOQ/2
Pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time point

FIG. 3C

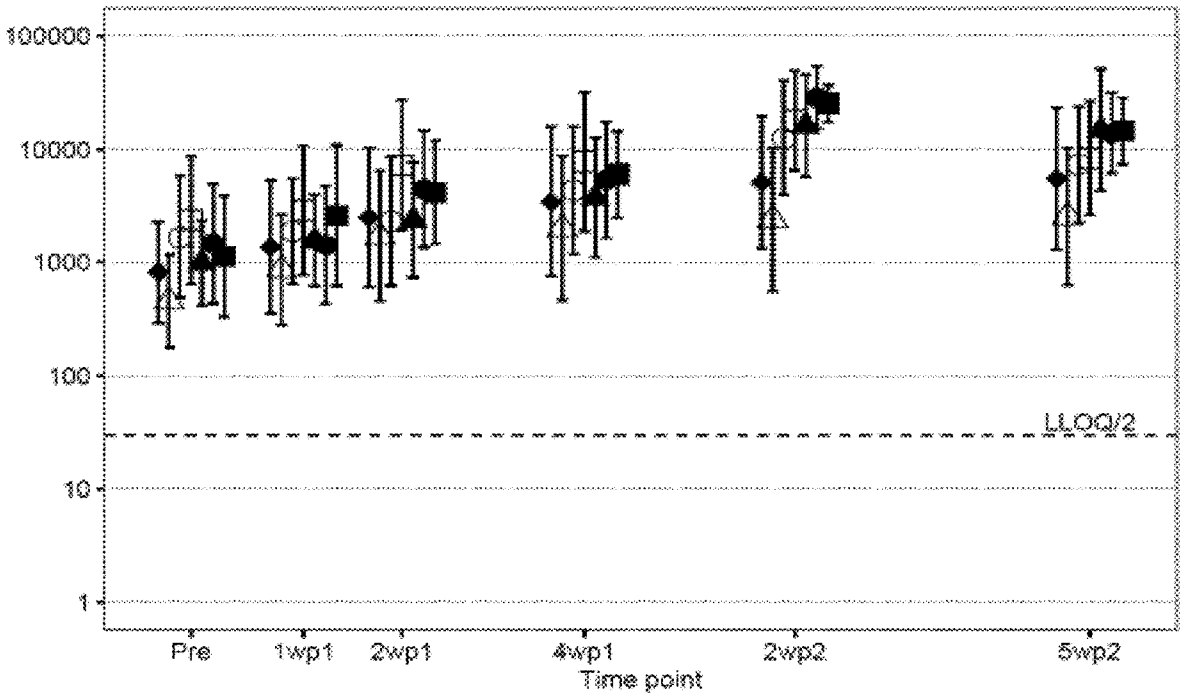

100000
10000
1000
100
10
1
LLOQ/2
Pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time point

FIG. 3D

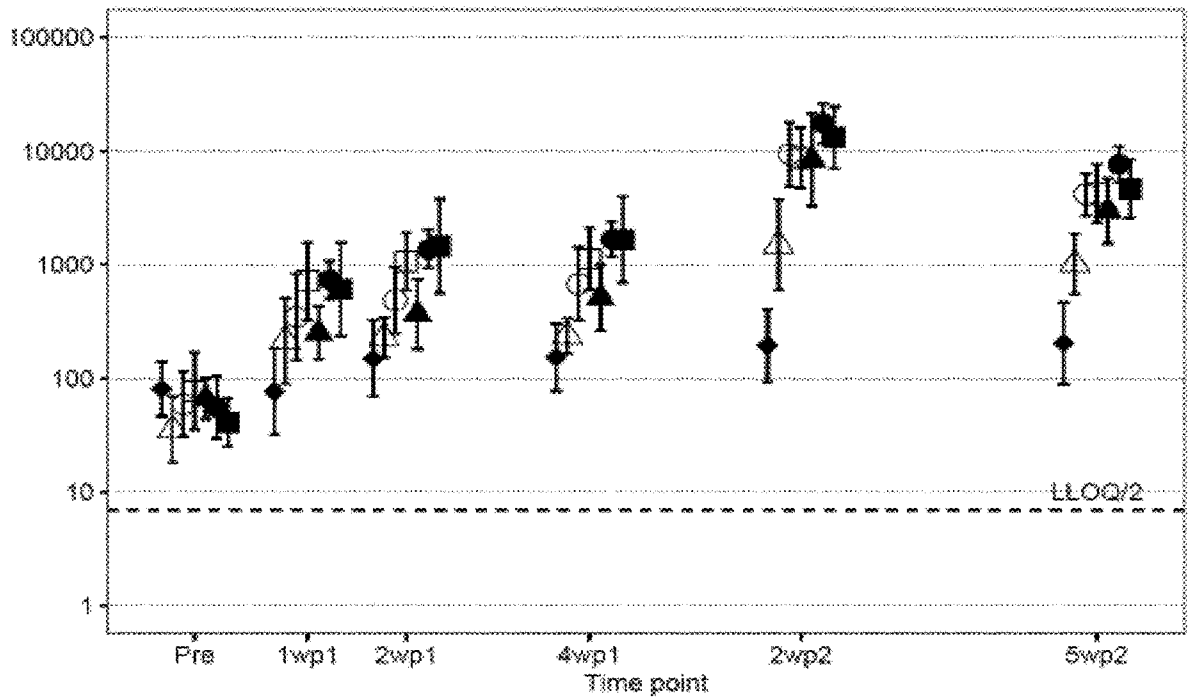

100000
10000
1000
100
10
1
LLOQ/2
Pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time point

FIG. 3E

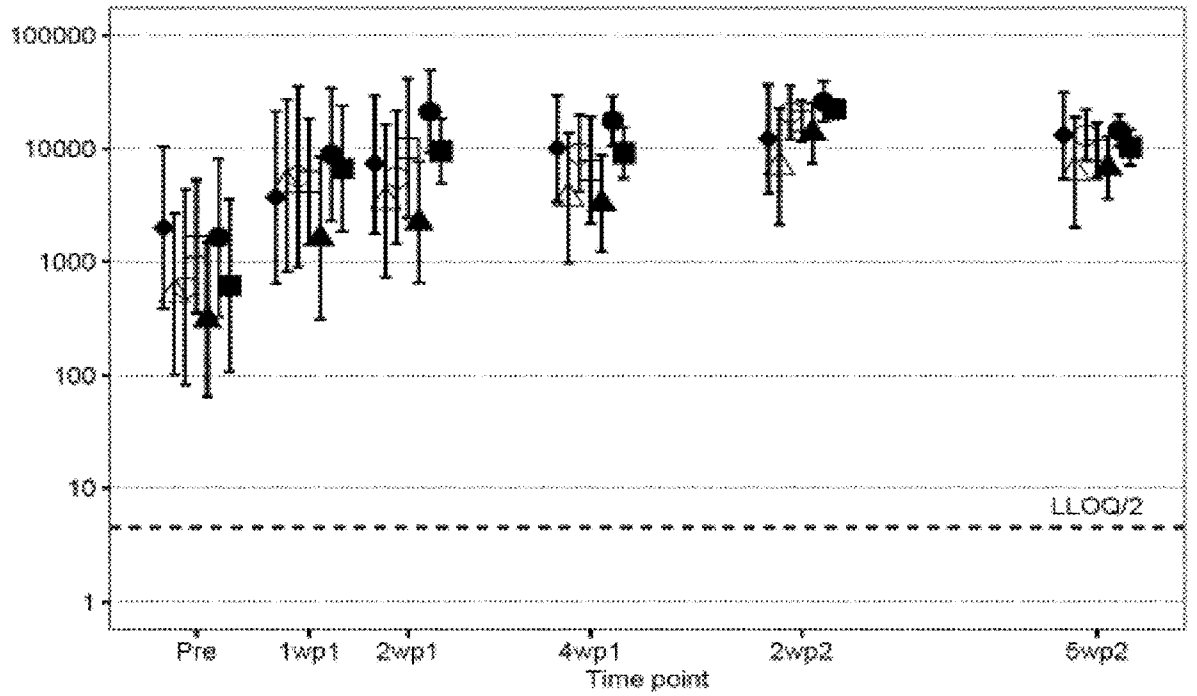

100000
10000
1000
100
10
1
LLOQ/2
Pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time point

FIG. 4A

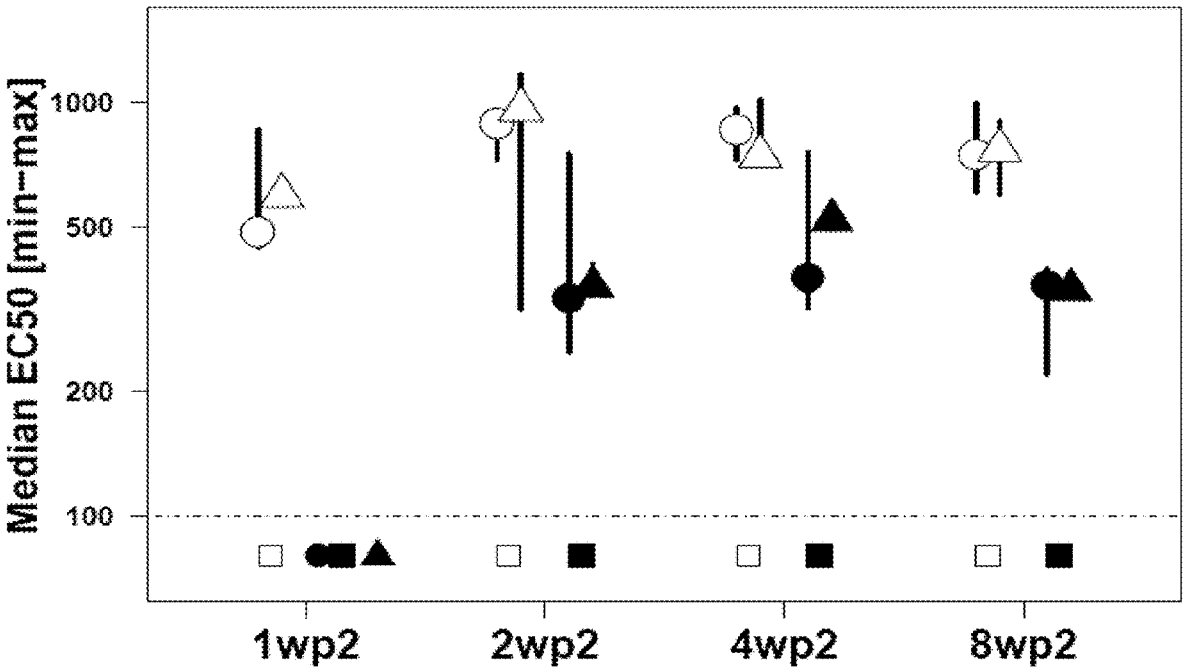


FIG. 4B

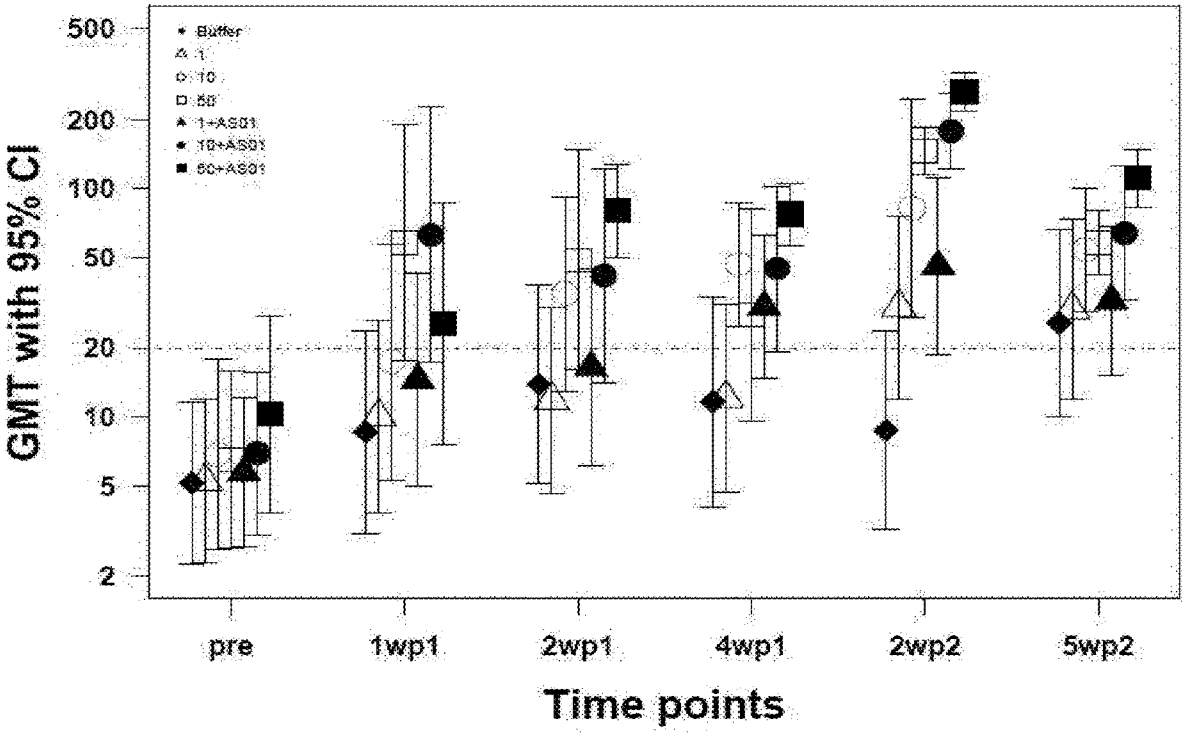

GMT with 95% CI
500
200
100
50
20
10
5
2
Buffer
1
10
50
1+AS01
10+AS01
50+AS01
pre
1wp1
2wp1
4wp1
2wp2
5wp2
Time points

FIG. 5A

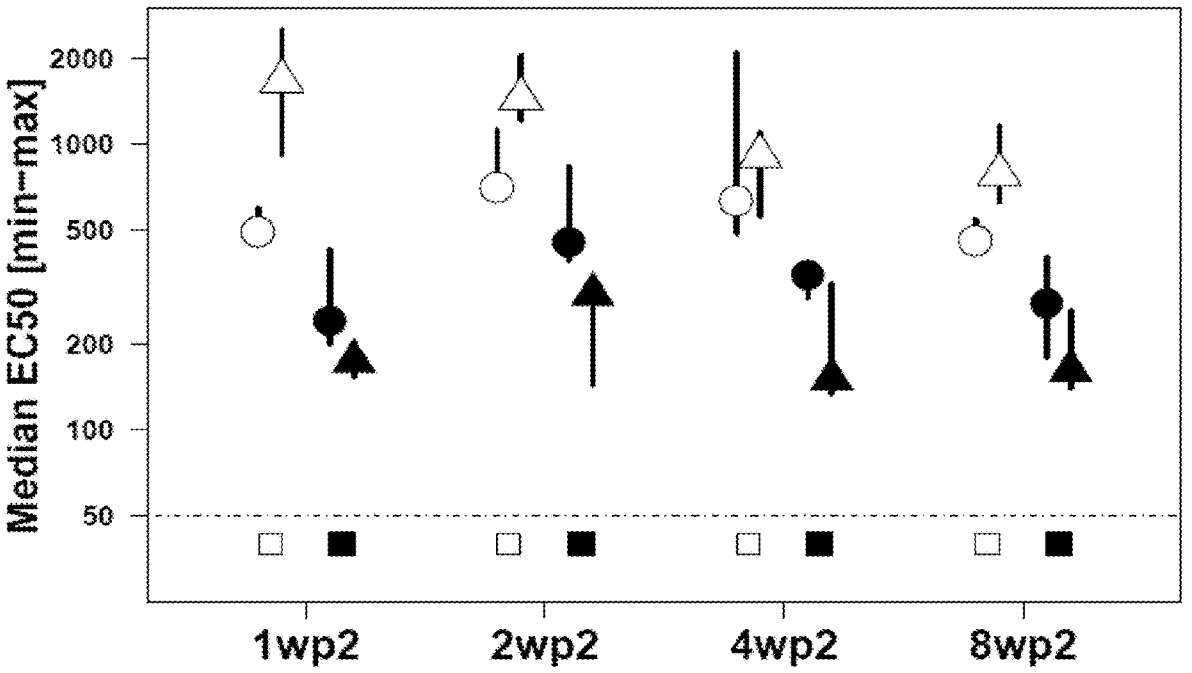

Median EC50 [min~max]
2000
1000
500
200
100
50
1wp2
2wp2
4wp2
8wp2

FIG. 5B

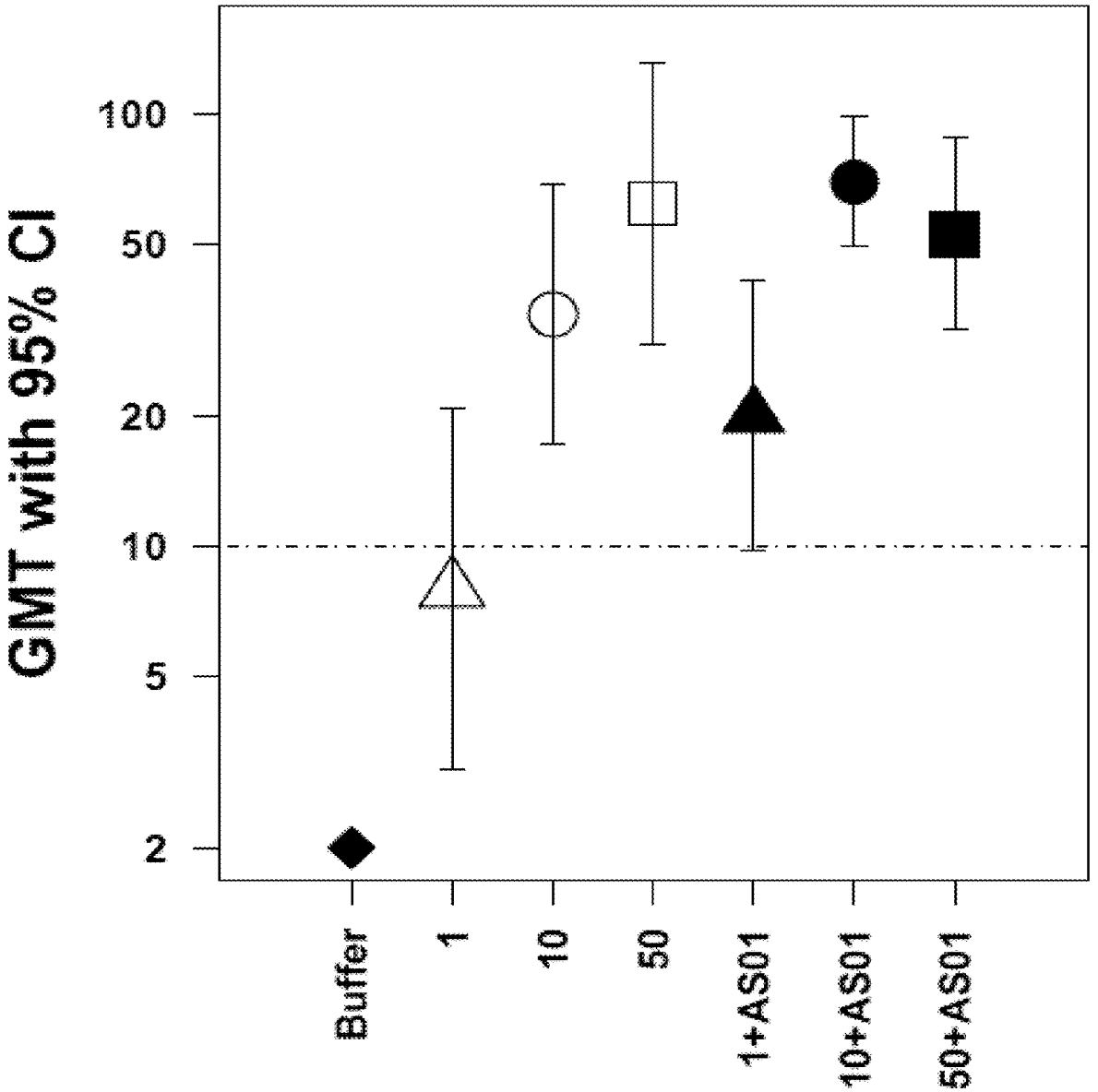

GMT with 95% CI
100
50
20
10
5
2
Buffer
1
10
50
1+AS01
10+AS01
50+AS01

FIG. 6A

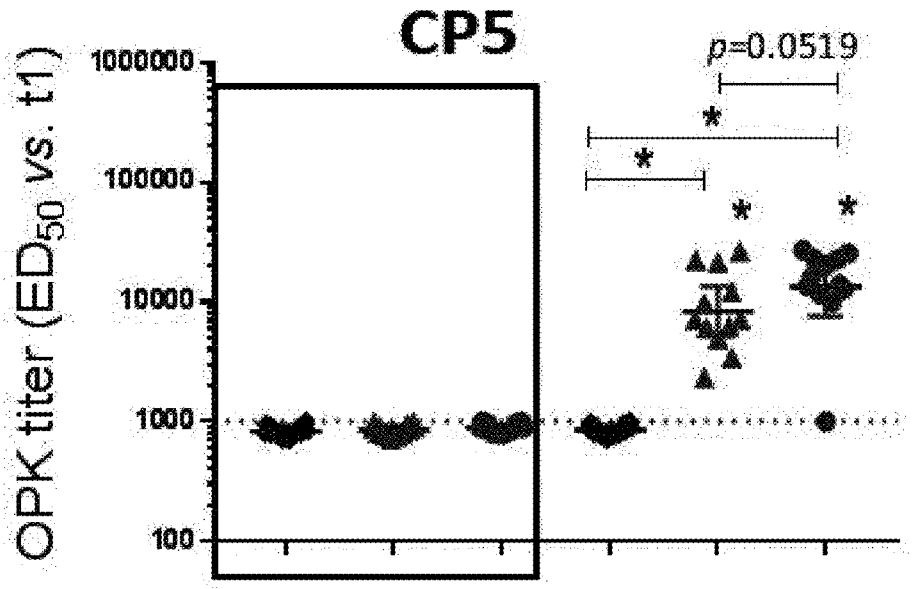

CP5
p=0.0519
OPK titer (ED50 vs. t1)
1000000
100000
10000
1000
100
*
*
*
*

FIG. 6B

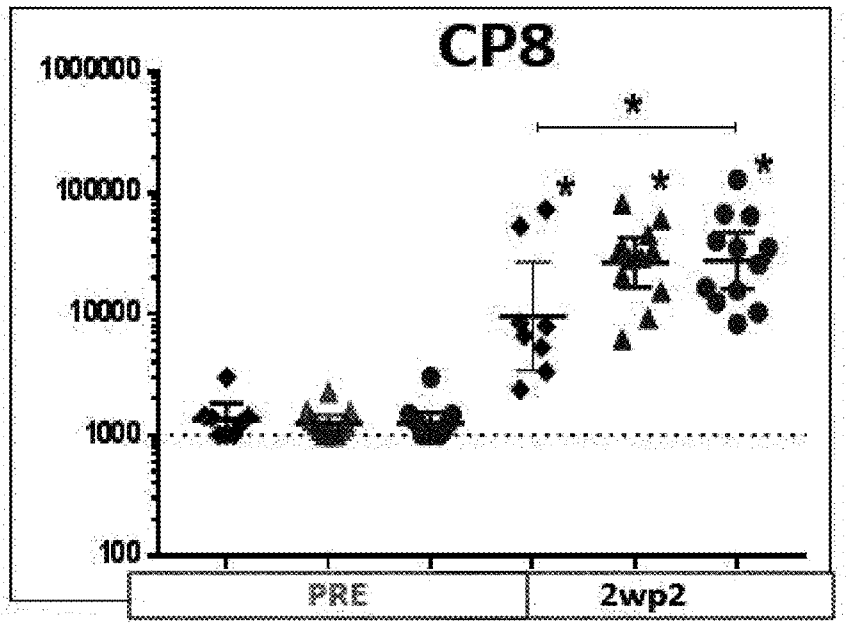


FIG. 7

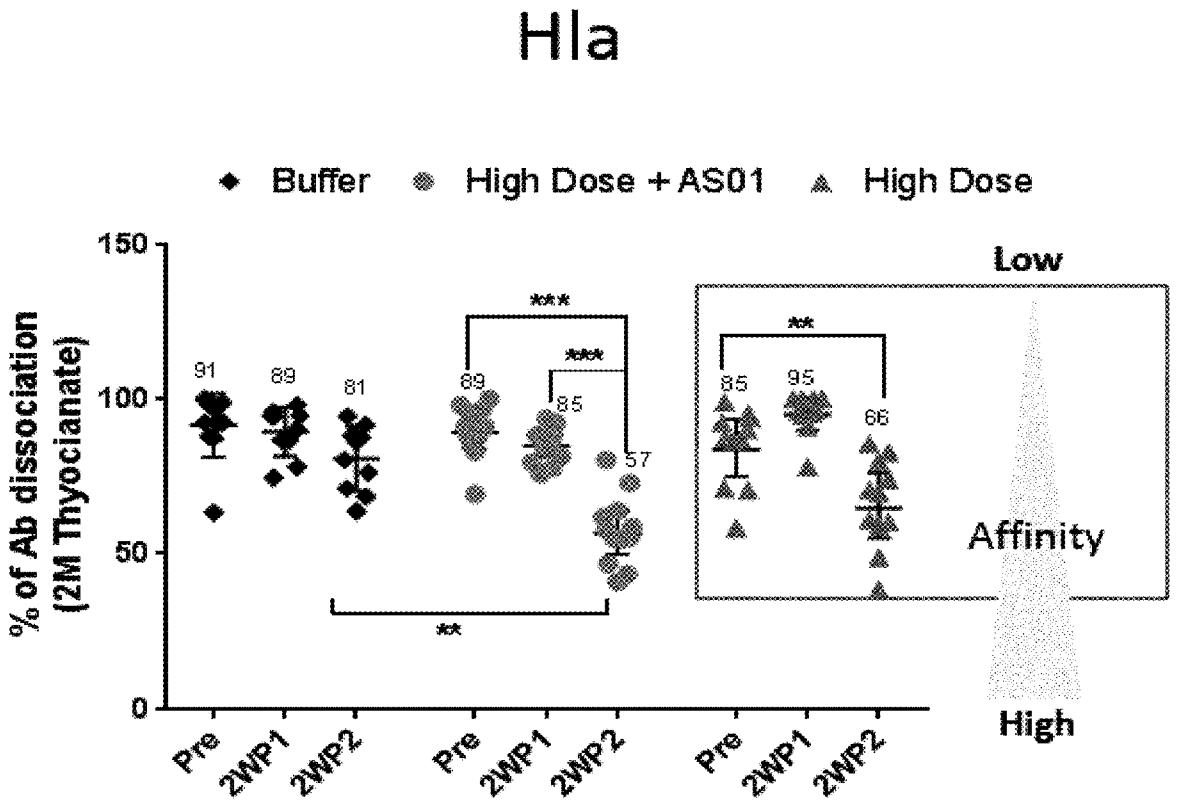

Hla
Buffer
High Dose + AS01
High Dose
% of Ab dissociation (2M Thyocianate)
150
100
50
0
91
89
81
89
85
57
85
95
66
***
***
**
**
Low
Affinity
High
Pre
2WP1
2WP2
Pre
2WP1
2WP2
Pre
2WP1
2WP2

FIG. 8

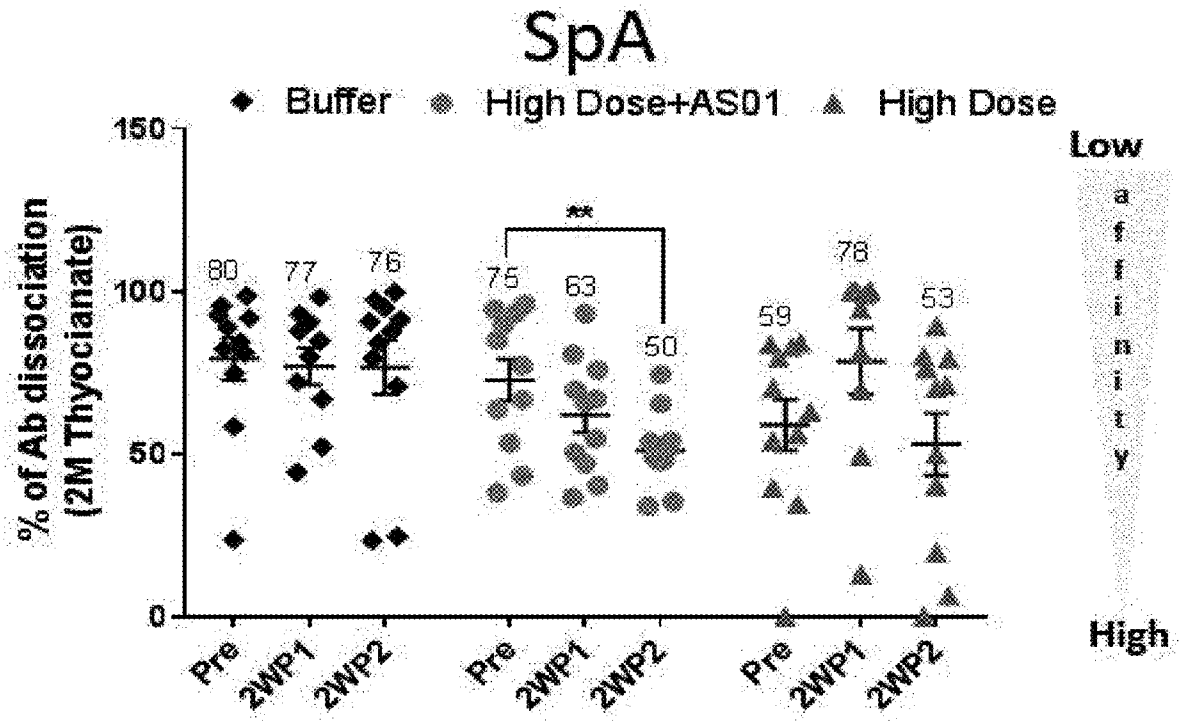

SpA
Buffer
High Dose+AS01
High Dose
% of Ab dissociation (2M Thyocianate)
150
100
50
0
80
77
76
75
63
50
59
78
53
**
Pre
2WP1
2WP2
Pre
2WP1
2WP2
Pre
2WP1
2WP2
Low
affinity
High

FIG. 9

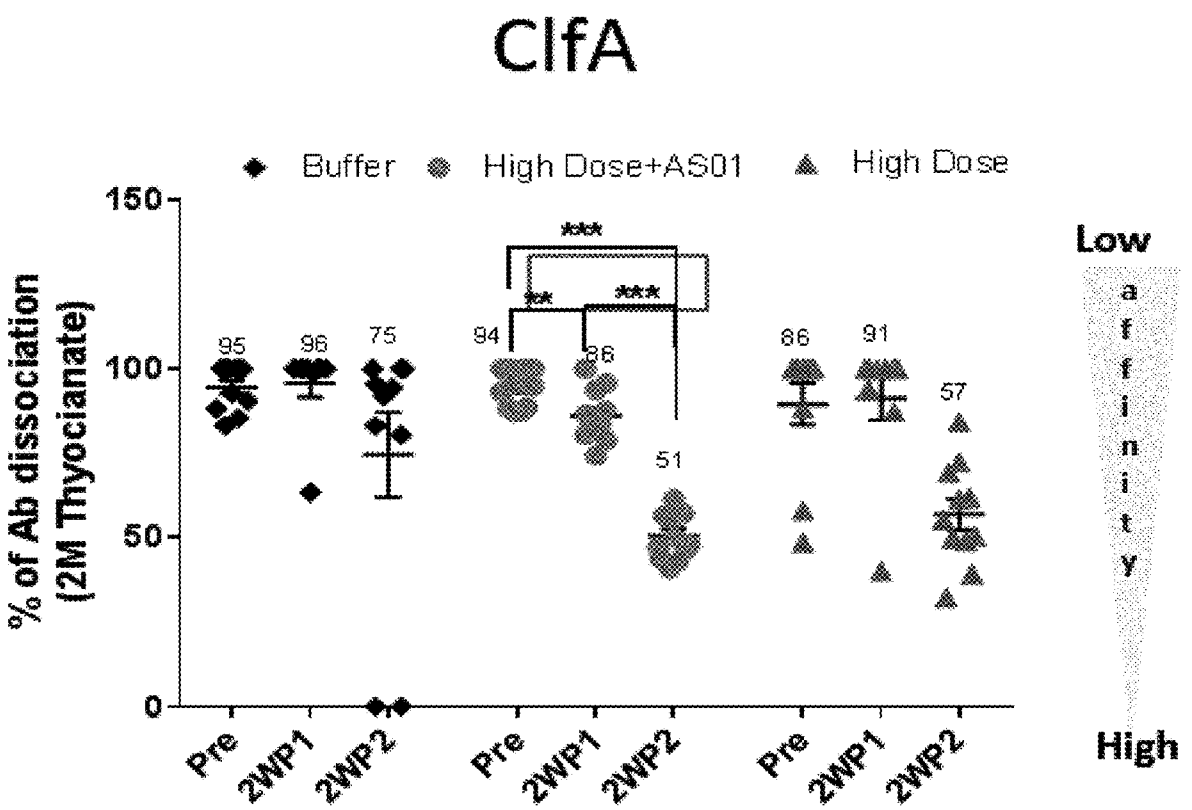
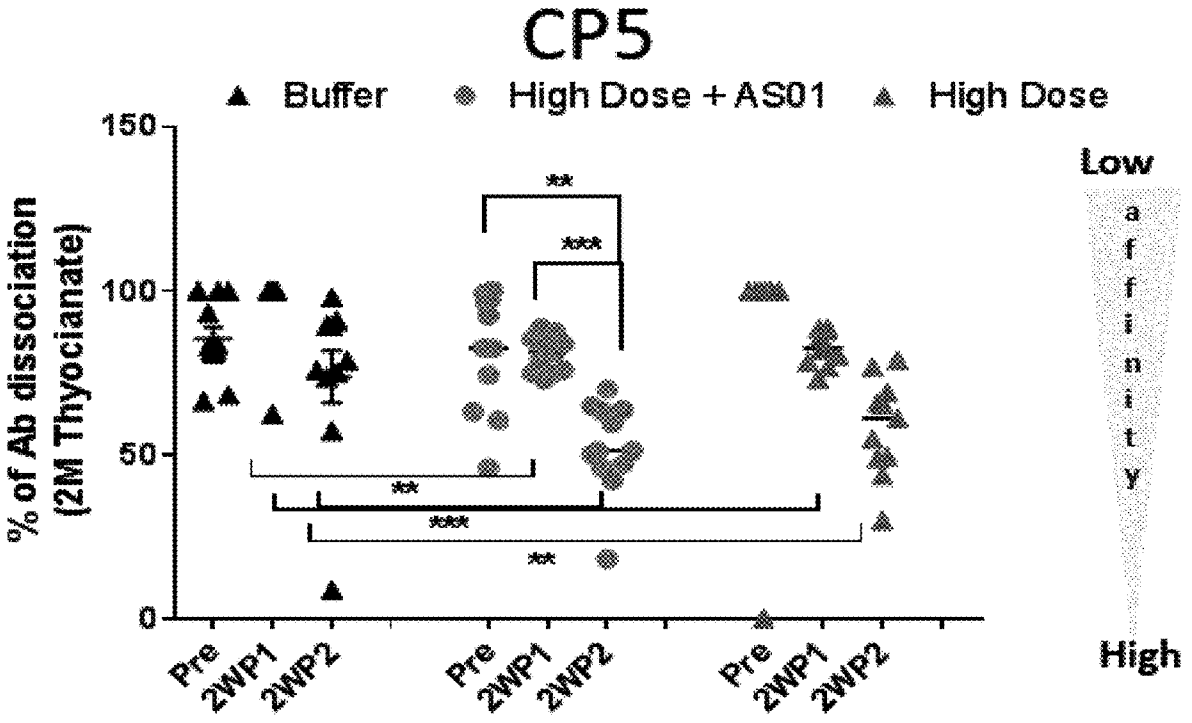

ClfA
Buffer
High Dose+AS01
High Dose
% of Ab dissociation (2M Thyocianate)
150
100
50
0
95
96
75
94
86
51
86
91
57
****
**
****
Pre
2WP1
2WP2
Low
affinity
High

FIG. 10


CP5
Buffer
High Dose + AS01
High Dose
% of Ab dissociation (2M Thyocianate)
150
100
50
0
**
***
**
***
**
Pre
2WP1
2WP2
Pre
2WP1
2WP2
Pre
2WP1
2WP2
Low
affinity
High

IMMUNOGENIC COMPOSITION COMPRISING STAPHYLOCOCCAL ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a Divisional of U.S. patent application Ser. No. 16/968,895, filed Aug. 11, 2020, which is a U.S. National Phase of International Patent Application No. PCT/EP2019/053463, filed Feb. 12, 2019, which claims priority to GB Application No. 1802339.0 filed Feb. 13, 2018, all of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML file format and is hereby incorporated by reference in its entirety. Said XML copy, created on Aug. 22, 2022, is named VB66439D1-US_SL.xml and is 37,858 bytes in size.

FIELD OF THE INVENTION

[0003] The invention relates to immunogenic compositions for the prevention and treatment of staphylococcal infection and disease, in particular *S. aureus* infection and disease. The invention provides an immunogenic composition comprising staphylococcal antigens, in particular Hla, ClfA, SpA and conjugates of capsular polysaccharides. Adjuvanted formulations are also provided, as are uses of such compositions in the prevention and treatment of staphylococcal infections.

BACKGROUND TO THE INVENTION

[0004] *S. aureus* is a Gram-positive spherical bacterium which is the leading cause of bloodstream, lower respiratory tract, skin & soft tissue infections in the US and Europe. It is also the predominant cause of bone infections worldwide, and these infections are painful, debilitating and difficult to treat.

[0005] Treatment of *S. aureus* is becoming increasingly challenging due to the development of antibiotic resistance by many strains of *S. aureus*. Methicillin-resistant *S. aureus* (MRSA) is endemic in hospitals, and community-associated MRSA strains are spreading worldwide, posing a major global challenge. MRSA is found in over half of all community and hospital infections. Recent years have seen the emergence of MRSA strains which are also resistant to vancomycin, the antibiotic of last resort, and which are essentially untreatable.

[0006] There is currently no authorised vaccine. The need for a vaccine is particularly acute due to the problem of antibiotic resistance and the fact that *S. aureus* infection does not provide immunity from future infection thanks to its well-developed immune evasion capabilities. The immune evasion properties of *S. aureus* in turn render the development of effective vaccines more difficult. The mechanisms of immune evasion are not fully understood, but are at least in part due to staphylococcal protein A (SpA), an *S. aureus* surface molecule that binds to Fcγ of immunoglobulin (Ig) and to the Fab portion of V_H3-type B cell receptors. Interaction of SpA with B cell receptors influences B cell development during infection, interfering with development of the adaptive immune response. SpA binding to Ig Fc interferes with opsonophagocytic clearance of staphylococci by polymorphonuclear leukocytes.

[0007] There is thus an urgent need for a vaccine to prevent staphylococcal disease. Several vaccines have been tested in clinical trials, including capsular polysaccharide (CPS) conjugates, individual protein antigens, and monoclonal antibodies (mAbs) to lipoteichoic acid. However, all have failed at various developmental stages, and to date there is no vaccine against *S. aureus* on the market.

[0008] A multicomponent vaccine comprising *S. aureus* CPS, ClfA and MntC (Anderson et al 2012, Hum Vaccine Immunother 8: 1585-1594) has been tested in Phl human trials. The vaccine induced opsonic anti-CP antibodies and inhibitory anti-ClfA antibodies in Phl, and was subsequently tested in Phllb efficacy trials for prophylactic use in elective spinal fusion surgery patients, but the Phllb trial was stopped for futility.

[0009] However, no vaccine against *S. aureus* has yet been found to have protective efficacy in PhIII trials. A vaccine containing conjugates of *S. aureus* Type 5 and Type 8 capsular polysaccharides conjugated to Pseudomonas exoprotein A (StaphVAX—Nabi Biopharmaceuticals) has been tested in clinical trials, where it demonstrated safety in Phl and II but failed to achieve the required endpoint in PhIII, as described in WO 03/61558.

[0010] Similarly, a vaccine based on the IsdB protein antigen (the V710 vaccine; Kuklin et al, Infect Immun, 2006, 74: 2215-23), failed to meet efficacy endpoints in a PhIII trial conducted in cardiothoracic *S. aureus* infections.

[0011] There is thus an ongoing need for an effective vaccine against staphylococcal infection, in particular *S. aureus* infection.

SUMMARY OF THE INVENTION

[0012] The present invention provides an immunogenic composition comprising staphylococcal antigens. In one aspect, the immunogenic composition comprises two or more of (a) a ClfA antigen;(b) a Hla antigen; (c) a SpA antigen; and/or (d) a staphylococcal capsular polysaccharide. The ClfA antigen, the Hla antigen and the SpA antigen are preferably staphylococcal antigens, suitably *S. aureus* antigens.

[0013] In an embodiment, the immunogenic composition comprises a ClfA antigen; a Hla antigen; a SpA antigen; and a staphylococcal capsular polysaccharide. The capsular polysaccharide may suitably be a *S. aureus* serotype 5 and/or type 8 capsular polysaccharide. In a preferred embodiment, the immunogenic composition comprises a ClfA antigen; a Hla antigen; a SpA antigen; a capsular polysaccharide from *S. aureus* serotype 5 and a capsular polysaccharide from *S. aureus* serotype 8.

[0014] In an embodiment, the capsular polysaccharide is conjugated to a carrier protein. The capsular polysaccharide protein may be conjugated to one of the antigens (a)-(c) above. In a preferred embodiment, the composition comprises a *S. aureus* serotype 5 capsular polysaccharide conjugated to a Hla antigen and/or a type 8 capsular polysaccharide conjugated to a ClfA antigen.

[0015] In an embodiment, the ClfA antigen is a ClfA protein comprising the amino acid sequence of SEQ ID NO. 2 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2, or immunogenic fragment thereof.

[0016] The ClfA antigen may comprise at least one amino acid substitution selected from P116 to S and Y118 to A with reference to the amino acid sequence of SEQ ID NO. 2 (or an equivalent position in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2), optionally comprising the sequence of any one of SEQ ID NOs 5-7 or 32.

[0017] The ClfA antigen may comprise one or more consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline. Said consensus sequence may been added at, or substituted for, one or more amino acids between amino acid residues 313-342 of SEQ ID NO: 2, optionally substituted for the amino acid at position 1337, or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 2. In an embodiment, X is Q (glutamine) and Z is A (alanine) (e.g. K-D-Q-N-A-T- K, SEQ ID NO: 31).

[0018] In a preferred embodiment, the ClfA antigen comprises or consists of the sequence of SEQ ID NO: 7 or SEQ ID NO: 32.

[0019] In an embodiment, the Hla antigen is a Hla protein having the amino acid sequence of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3 or immunogenic fragment thereof.

[0020] The Hla antigen may comprise an amino acid substitution at position H35 of SEQ ID NO. 3 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3. In an embodiment, said amino acid substitution is optionally H to L.

[0021] The Hla antigen may comprise one or more consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline. Said consensus sequence may be added at, or substituted for one or more amino acids of the amino acid sequence of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3. In an embodiment, said consensus sequence has been substituted for the amino acid at position K131 of SEQ ID NO. 3 of SEQ ID NO: 3, or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 3. In an embodiment, X is Q (glutamine) and Z is R (arginine) (e.g. K-D-Q-N-R-T-K (SEQ ID NO 30).

[0022] In a preferred embodiment, the Hla antigen comprises or consists of the sequence of SEQ ID NO: 11 or SEQ ID NO 12.

[0023] In an embodiment, the SpA antigen is a SpA protein comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 13, SEQ ID NO: 26 or SEQ ID NO: 27, or immunogenic fragment thereof.

[0024] The SpA antigen may comprise (a) one or more amino acid substitutions in a V_H3-binding sub-domain of domain E, D, A, B or C that disrupts or decreases binding to V_H3, and (b) one or more amino acid substitutions in an IgG Fc binding sub-domain of domain E, D, A, B or C that disrupts or decreases binding to IgG Fc.

[0025] In an embodiment, the SpA antigen comprises (i) a domain E with an amino acid substitution at the amino acid positions 34 and 35 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 39 and 40 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 36 and 37 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18; and/or (ii) comprises a domain E with an amino acid substitution at amino acid positions 7 and 8 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 12 and 13 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 9 and 10 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18. In an embodiment, said amino acid substitutions are substitution of lysine for glutamine and/or substitution of alanine for aspartic acid. Exemplary sequences are SEQ ID NOs: 19-23, 24 and 25.

[0026] In an embodiment, the SpA antigen comprises a domain D with an amino acid substitution at amino acid positions 4 and 5 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15. Said amino acid substitution may suitably be glutamine to lysine and/or glutamine to arginine, e.g. QQ to KR (e.g. SEQ ID NO 24 and SEQ ID NO: 25).

[0027] In an embodiment, the SpA antigen comprises an amino acid sequence of SEQ ID NOs: 19-23, 26 or 27, or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NOs: 19-23, 26 or 27. In a preferred embodiment, the SpA antigen comprises the amino acid sequence of SEQ ID NO: 27.

[0028] In an embodiment, the immunogenic composition comprises (i) a ClfA antigen comprising the amino acid

sequence of SEQ ID NO: 7 or SEQ ID NO: 32; (ii) a Hla antigen comprising the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 12; (iii) an SpA antigen comprising the amino acid sequence of SEQ ID NO: 27; (iv) a *S. aureus* serotype 5 capsular polysaccharide, and (v) a *S. aureus* serotype type 8 capsular polysaccharide. The ClfA antigen may be conjugated to the *S. aureus* serotype type 8 capsular polysaccharide, and the Hla antigen may be conjugated to the *S. aureus* serotype 5 capsular polysaccharide. Said ClfA-CP8 and Hla-CP5 conjugates may suitably be bioconjugates.

[0029] In an embodiment, the immunogenic composition comprises (i) a ClfA antigen comprising the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 32; (ii) a Hla antigen comprising the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 12; (iii) an SpA antigen comprising the amino acid sequence of SEQ ID NO: 27; (iv) a *S. aureus* serotype 5 capsular polysaccharide conjugated to the Hla antigen, and (v) a *S. aureus* serotype type 8 capsular polysaccharide conjugated to the ClfA antigen. Preferably, said ClfA-CP8 and Hla-CP5 conjugates are bioconjugates. The immunogenic composition may additionally comprise an adjuvant as described herein.

[0030] One aspect of the invention provides a vaccine comprising an immunogenic composition of the invention and a pharmaceutically acceptable excipient or carrier.

[0031] One aspect of the invention provides a kit comprising (i) a first container comprising an immunogenic composition ora vaccine of the invention; and (ii) a second container comprising an adjuvant.

[0032] One aspect of the invention provides an immunogenic composition or vaccine of the invention for use in a method of prevention or treatment of staphylococcal infection, for example *S. aureus* infection. In an embodiment, said immunogenic composition or vaccine is administered in combination with an adjuvant.

[0033] In one aspect of the invention, the immunogenic composition of the invention comprises an adjuvant. In another aspect, the immunogenic composition is for administration in combination with an adjuvant. The adjuvant may be administered concomitantly with the immunogenic composition, for example it may be mixed with the immunogenic composition before administration.

[0034] In an embodiment, the adjuvant comprises a saponin and a TLR4 agonist, suitably in liposomal formation. In an embodiment, the adjuvant further comprises a sterol. The saponin may be an immunologically active saponin fraction derived from the bark of Quillaja Saponaria Molina, preferably QS21. In an embodiment, the TLR4 agonist is a lipopolysaccharide. The lipopolysaccharide may be a lipid A derivative, preferably 3D-MPL. The sterol may be cholesterol. In an embodiment, the adjuvant comprises QS21 at a level of 25 μg per human dose and/or 3D-MPL at a level of 25 μg per human dose (e.g. $AS01_E$).

[0035] In one aspect, the invention provides a method of prevention or treatment of staphylococcal infection, for example *S. aureus* infection, comprising administering to a subject in need thereof an immunogenic composition or vaccine of the invention. The method may further comprise administering an adjuvant to said subject. The adjuvant may be administered concomitantly with the immunogenic composition, for example it may be mixed with the immunogenic composition before administration.

[0036] In a further aspect, the invention provides a method of making an immunogenic composition or vaccine according to any one of claims, comprising the steps of mixing antigens, and optionally an adjuvant, with a pharmaceutically acceptable excipient.

[0037] A further aspect of the invention provides a polynucleotide encoding an antigen of the immunogenic composition of the invention. Vectors comprising such a polynucleotide are also provided. Also provided is a host cell comprising a vector or polynucleotide of the invention.

DESCRIPTION OF THE FIGURES

[0038] FIG. **1**A, FIG. **1**B, FIG. **1**C, FIG. **1**D and FIG. **1**E. Vaccine-specific IgG in mice. Antigen-specific IgG titres (anti-CP5 (1A), anti-CP8 (1B), anti-Hla (1C), anti-ClfA (1D), and anti-SpA (1E) in naïve mice immunised with vaccine unadjuvanted and adjuvanted with Alum/TLR7 or $AS01_E$. Each symbol identifies a group of 30 mice, which were immunised at day 1 and 29 with 10 μg (open symbols) or 1 μg (filled symbols) with AS01 (triangles) Alum/TLR7 (circles) or no adjuvant (squares). Y-axis: GMT with 95% CI. Bleedings were taken at different time points after the first and the second immunisation. The GMT of IgG titres and the time points of the bleedings analysed are reported on the y and axis respectively. Dotted line=LLOQ/2: half of the lower limit of quantitation. 30 individual BALB/c sera for each timepoint were analysed by Luminex.

[0039] FIG. **2**A, FIG. **2**B and FIG. **2**C: Magnitude and quality of vaccine-specific CD4 T-cell responses in mice induced by vaccine unadjuvanted and adjuvanted with Alum/TLR7 or $AS01_E$. Splenocytes from single mice sacrificed 12 days after second immunisation were stimulated or not with vaccine proteins in vitro (2A: SpA_{mut}, 1 μg/ml; 2B: Hla_{mut}, 10 μg/ml; 2C: $ClfA_{mut}$, 10 μg/ml), stained and analysed by ICS. $CD4+CD44^{high}$ T cells producing IL-2, TNF, IL-4/IL-13, IFN-γ or IL-17A were identified. Upper graph: $CD4+CD44^{high}$≥CYT+. Lower graph: $CD4+CD44^{high}$≥IFN-γ. The response of unstimulated cells was subtracted from that of stimulated cells. Magnitude: Percentages of $CD4+CD44^{high}$ T cells producing at least one of the cytokines analysed in response to vaccine protein stimulation. Quality: Percentages of $CD4+CD44^{high}$ T cells producing at least: IFN-γ but not IL-4/IL-13. Two-way ANOVA of % Geometric Mean ratios, $p<0.05$ indicated: * Vaccine (with or without adjuvant) vs. control group; * Vaccine with adjuvant vs. Vaccine without adjuvant; * Vaccine with $AS01_E$ vs. Vaccine with Alum/TLR7; * Vaccine-10 μg vs. Vaccine-1 μg.

[0040] FIG. **3**A, FIG. **3**B, FIG. **3**C, FIG. **3**D and FIG. **3**E: Vaccine-specific IgG in pre-exposed rabbits. Geometric mean titres anti-CP5 (3A), anti-CP8 (3B), anti-Hla (3C), anti-ClfA (3D), and anti-SpA (3E) in pre-exposed rabbits that received two injections of vaccine with or without $AS01_E$ adjuvant or buffer. Pre: pre-dose 1, 1wp1: 1 week post-dose 1, 2wp1: 2 weeks post-dose 1, 4wp1: 4 weeks post-dose 1, 2wp2: 2 weeks post-dose 2, 4wp2: 4 weeks post-dose 2; 5wp2: 5 weeks post-dose 2. LLOQ2 dotted line: half of the lower limit of quantitation. Triangles: 1 μg dose; Circles: 10 μg dose; squares: 50 μg dose. Open symbols: no adjuvant, Filled symbols: $AS01_E$. Buffer control shown as filled diamonds. The grey dotted line indicates the reciprocal of the lowest dilution used in the assay.

[0041] FIG. **4**A: Vaccine induces functional IgG neutralising in vitro Hla activity in mice. Mice (10 mice/group)

were immunised with the vaccine at indicated dosages without adjuvant or in the presence of either Alum/TLR7 or $AS01_E$ adjuvant. Neutralisation titres of pooled sera were measured at each time point and expressed as median values (red and blue squares and stars) of the three independent studies. Upper and lower values of the bar represent the maximum and the minimum titres, respectively. Open symbols: 10 μg dose; Closed symbols: 1 μg dose. Squares: no adjuvant; Triangles: $AS01_E$ adjuvant; Circles: Alum/TLR7 adjuvant. The grey dotted line indicates the reciprocal of the lowest dilution used in the assay.

[0042] FIG. **4**B: Vaccine induces functional IgG neutralising in vitro Hla activity in pre-exposed rabbits. The Hla neutralising titers and the time points of the bleedings analyzed are reported on the y and x axis respectively. The grey dotted line indicates the reciprocal of the lowest dilution used in the assay. An arbitrary titre of 3 was assigned to non-responders. Triangles: 1 μg dose; Circles: 10 μg dose; squares: 50 μg dose. Open symbols: no adjuvant, Filled symbols: $AS01_E$. Buffer control shown as filled diamonds.

[0043] FIG. **5**A: Vaccine induces functional IgG neutralising in vitro ClfA activity in mice. Mice (10 mice/group) were immunised with the vaccine at indicated dosages without adjuvant or in the presence of either Alum/TLR7 or $AS01_E$ adjuvant in three independent in-vivo experiments. Neutralisation titres of pooled sera of the three in-vivo experiments were measured at each time point and expressed as median values. Upper and lower values of the bar represent the maximum and the minimum titres, respectively. Open symbols: 10 μg dose; Closed symbols: 1 μg dose. Squares: no adjuvant; Triangles: $AS01_E$ adjuvant; Circles: Alum/TLR7 adjuvant. The grey dotted line indicates the reciprocal of the lowest dilution used in the assay.

[0044] FIG. **5**B: Vaccine induces functional IgG neutralising in vitro ClfA activity in pre-exposed rabbits. Neutralisation titres of individual sera were measured pre-vaccination, at 4wp1 and 2wp2. Only at 2wp2 some neutralisation activity is expressed as geometric mean (GMTs) with 95% confidence intervals (CIs). The grey dotted line indicates the reciprocal of the lowest dilution used in the assay. An arbitrary titre of 2 was assigned to non-responders. Triangles: 10 μg dose; Circles: 10 μg dose; squares: 50 μg dose. Open symbols: no adjuvant, Filled symbols: $AS01_E$. Buffer control shown as filled diamonds.

[0045] FIG. **6**A and FIG. **6**B: Vaccine induces antibodies with OPK activity against serotype 5 (FIG. **6**A) and 8 (FIG. **6**B) *S. aureus* strains in pre-exposed rabbits. OPK titres of 12 individual sera were measured pre-vaccination and at 2wp2. GMTs expressed with 95% confidence intervals. Triangles: No adjuvant. Circles: $AS01_E$ adjuvant. Diamond: Buffer only.

[0046] FIG. **7**: Vaccination increases anti-Hla antibodies with greater affinity in pre-exposed rabbits. Percent antibody dissociation assayed in individual sera pre-vaccination, at 2wp1 and at 2wp2. GMTs expressed with 95% confidence intervals. Triangles: No adjuvant. Circles: $AS01_E$ adjuvant. Diamond: Buffer only.

[0047] FIG. **8**: Vaccination increases anti-SpAmut antibodies with greater affinity in pre-exposed rabbits. Percent antibody dissociation assayed in individual sera pre-vaccination, at 2wp1 and at 2wp2. GMTs expressed with 95% confidence intervals. Triangles: No adjuvant. Circles: $AS01_E$ adjuvant. Diamond: Buffer only.

[0048] FIG. **9**: Vaccination increases anti-ClfA antibodies with greater affinity in pre-exposed rabbits. Percent antibody dissociation assayed in individual sera pre-vaccination, at 2wp1 and at 2wp2. GMTs expressed with 95% confidence intervals. Triangles: No adjuvant. Circles: $AS01_E$ adjuvant. Diamond: Buffer only.

[0049] FIG. **10**: Vaccination increases anti-CP5 antibodies with greater affinity in pre-exposed rabbits. Percent antibody dissociation assayed in individual sera pre-vaccination, at 2wp1 and at 2wp2. GMTs expressed with 95% confidence intervals. Triangles: No adjuvant. Circles: $AS01_E$ adjuvant. Diamond: Buffer only.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0050] Terminology

[0051] Carrier protein: a protein covalently attached to an antigen (e.g. saccharide antigen) to create a conjugate (e.g. bioconjugate). A carrier protein activates T-cell mediated immunity in relation to the antigen to which it is conjugated.

[0052] Conjugate: saccharide (such as a capsular polysaccharide) covalently linked to a carrier protein.

[0053] Bioconjugate: a conjugate which is produced recombinantly, by expressing the enzymes required for saccharide synthesis, the carrier protein and the enzymes required for conjugation in a host cell, resulting in a conjugate in which the saccharide is N-linked to the carrier protein via N-linked protein glycosylation—the addition of carbohydrate molecules to an asparagine residue in the polypeptide chain of the target protein by enzymatic action.

[0054] Any amino acid apart from proline (pro, P): refers to an amino acid selected from the group consisting of alanine (ala, A), arginine (arg, R), asparagine (asn, N) , aspartic acid (asp,D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile,l), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

[0055] ClfA: Clumping factor A from *S. aureus*

[0056] Hla: Alpha-haemolysin, also known as alpha-toxin, from *S. aureus*

[0057] ClfA: Staphylococcal protein A from *S. aureus*

[0058] CP: Capsular polysaccharide

[0059] LPS: lipopolysaccharide.

[0060] Reducing end: the reducing end of an polysaccharide is the monosaccharide with a free anomeric carbon that is not involved in a glycosidic bond and is thus capable of converting to the open-chain form.

[0061] As used herein, the term "bioconjugate" refers to conjugate between a protein (e.g. a carrier protein) and an antigen (e.g. a saccharide) prepared in a host cell background, wherein host cell machinery links the antigen to the protein (e.g. N-links).

[0062] As used herein, the term "effective amount," in the context of administering a therapy (e.g. an immunogenic composition or vaccine of the invention) to a subject refers to the amount of a therapy which has a prophylactic and/or therapeutic effect(s). In certain embodiments, an "effective amount" refers to the amount of a therapy which is sufficient to achieve one, two, three, four, or more of the following effects: (i) reduce or ameliorate the severity of a bacterial

infection or symptom associated therewith; (ii) reduce the duration of a bacterial infection or symptom associated therewith; (iii) prevent the progression of a bacterial infection or symptom associated therewith; (iv) cause regression of a bacterial infection or symptom associated therewith; (v) prevent the development or onset of a bacterial infection, or symptom associated therewith; (vi) prevent the recurrence of a bacterial infection or symptom associated therewith; (vii) reduce organ failure associated with a bacterial infection; (viii) reduce hospitalization of a subject having a bacterial infection; (ix) reduce hospitalization length of a subject having a bacterial infection; (x) increase the survival of a subject with a bacterial infection; (xi) eliminate a bacterial infection in a subject; (xii) inhibit or reduce a bacterial replication in a subject; and/or (xiii) enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

[0063] As used herein, the term "subject" refers to an animal, in particular a mammal such as a primate (e.g. human).

[0064] As used herein, the term "immunogenic fragment" is a portion of an antigen smaller than the whole, that is capable of eliciting a humoral and/or cellular immune response in a host animal, e.g. human, specific for that fragment. Fragments of a protein can be produced using techniques known in the art, e.g. recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Typically, fragments comprise at least 10, 20, 30, 40, or 50 contiguous amino acids of the full length sequence. However, fragments may also be 100 or more, 200 or more, 300 or more or 400 or more amino acids in length. Fragments may be readily modified by adding or removing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40 or 50 amino acids from either or both of the N and C termini.

[0065] As used herein, the term "conservative amino acid substitution" involves substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the size, polarity, charge, hydrophobicity, or hydrophilicity of the amino acid residue at that position, and without resulting in decreased immunogenicity. For example, these may be substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Conservative amino acid modifications to the sequence of a polypeptide (and the corresponding modifications to the encoding nucleotides) may produce polypeptides having functional and chemical characteristics similar to those of a parental polypeptide.

[0066] As used herein, the term "deletion" is the removal of one or more amino acid residues from the protein sequence.

[0067] As used herein, the term "insertion" is the addition of one or more non-native amino acid residues in the protein sequence..

[0068] As used herein, reference to "between amino acids . . . " (for example "between amino acids 313-342") is referring to the amino acid number counting consecutively from the N-terminus of the amino acid sequence, for example "between amino acids 313-342 . . . of SEQ ID NO. 2" refers to any position in the amino acid sequence between the $313^{rd\ and}$ 342^{nd} amino acid of SEQ ID NO. 2

[0069] Reference to "sequence identity" may be calculated over the full length of the reference sequence, or over the full length of the query sequence. Sequence alignment tools include, but are not limited to Clustal Omega (www(.)ebi(.)ac(.)ac(.)uk) MUSCLE (www(.)ebi(.)ac(.)uk), or T-coffee (www(.)tcoffee(.)org). In one aspect, the sequence alignment tool used is Clustal Omega (www(.)ebi(.)ac(.)ac(.)uk).

Statement of the Invention

SpA Antigen

[0070] The wild-type SpA (staphylococcal protein A) is a cell wall-anchored surface protein which is a crucial virulence factor for lung infections, septicaemia, and abscess development and is expressed by most clinical *S. aureus* isolates. Wild-type SpA binds to the Fc portion of human IgG, to V_H3-containing B cell receptors, to von Willebrand factor at its A1 domain, and to the TNF-α receptor 1. Interaction of SpA with B cell receptors affects B cell development with effects on adaptive and innate immune responses, whereas its binding to the Fcγ of IgG interferes with opsonophagocytic clearance of staphylococci by polymorphonuclear leukocytes. The N-terminal part of mature SpA is comprised of four or five 56-61-residue Ig-binding domains, which fold into triple helical bundles connected by short linkers, and are designated in order E, D, A, B, and C. These domains display ~80% identity at the amino acid level, are 56 to 61 residues in length, and are organized as tandem repeats. The C-terminal region is comprised of "Xr", a highly repetitive yet variable octapeptide, and "Xc", a domain which abuts the cell wall anchor structure of SpA.

[0071] In the NCTC 8325 strain SpA is SAOUHSC_00069 and has amino acid sequence SEQ ID NO: 4 (GI: 88193885). In the Newman strain it is nwmn_0055 (GI: 151220267). A useful fragment of SEQ ID NO: 4 is amino acids 37 to 325 (SEQ ID No: 13). This fragment contains all the five SpA Ig-binding domains (which are naturally arranged from N- to C-terminus in the order E, D, A, B, C, with sequence of SEQ ID NO: 14, 15, 16, 17 and 18 respectively) and includes the most exposed domain of SpA. It also reduces the antigen's similarity with human proteins. Other useful fragments may omit 1, 2, 3 or 4 of the natural A, B, C, D and/or E domains to prevent the excessive B cell expansion which might occur if SpA functions as a B cell superantigen. Other useful fragments may include only 1, 2, 3 or 4 of the natural A, B, C, D and/or E domains e.g. comprise only the SpA(A) domain but not B to E, or comprise only the SpA(D) domain but not A, B, C or E, etc. Thus a SpA antigen useful with the invention may include 1, 2, 3, 4 or 5 IgG-binding domains.

[0072] In an embodiment, a SpA antigen of the invention can elicit an antibody (e.g. when administered to a human) that recognises SEQ ID NO: 13 and/or may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 13; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 13 or amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO:,13 wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60,

70, 80, 90, 100, 150, 200, 250 or more). These SpA antigens include variants of SEQ ID NO: 13. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 13 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 13. Other preferred fragments lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 13 while retaining at least one epitope of SEQ ID NO: 13. Individual IgG-binding domains might be useful immunogens, alone or in combination.

[0073] If an antigen includes only one type of SpA domain (e.g. only the Spa(A), SpA(D) or Spa(E) domain), it may include more than one copy of this domain e.g. multiple SpA(D) domains in a single polypeptide chain. It may also include one type of SpA domain and another protein or polypeptide. Thus, an antigen of the invention may be a fusion protein comprising only one type of SpA domain, such as the SpA(D) domain.

[0074] SpA antigens used with the invention may be mutated relative to SEQ ID NO: 13, such that they have decreased affinity for the Fcy portion of human IgG and/or for the Fab portion of V_H3-containing human B cell receptors. This can be achieved and assessed by, for instance, following the guidance in WO2011/005341, WO12/003474 and WO2015/144653. Thus at least one Gln-Gln dipeptide in wild-type SpA can be mutated (e.g. to Lys-Lys; other possible mutations include Arg-Arg, Arg-Lys, Lys-Arg, Ala-Ala, Ser-Ser, Ser-Thr, Thr-Thr, etc.) and/or at least one Asp-Asp dipeptide in wild-type SpA can be mutated (e.g. to Ala-Ala; other possible mutations include Lys-Lys, Arg-Arg, Lys-Arg, Arg-Lys, His-His, Val-Val, etc.). These target sequences for mutation are the residues corresponding to amino acids 43, 44, 70, 71, 96, 97, 104, 105, 131, 132, 162, 163, 189, 190, 220, 221, 247, 248, 278, 279, 305 and/or 306 of SEQ ID NO: 4.

[0075] An individual domain within the antigen may be mutated at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids relative to SEQ ID NO: 4 (e.g. see above in relation to Gln-Gln and Asp-Asp sequences, but also see WO2011/005341 which discloses mutations at residues 3 and/or 24 of domain D, at residue 46 and/or 53 of domain A, etc.). Such mutations should not remove the antigen's ability to elicit an antibody that recognises SEQ ID NO: 13, but will reduce or remove the antigen's binding to IgG and/or other human proteins (such as human blood proteins) as noted above. In an embodiment, the mutant SpA antigen is of sequence comprising or consisting of SEQ ID NO: 13 mutated in at least 1, more particularly at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and even more particularly 20 amino acids at the amino acids corresponding to positions 43, 44, 70, 71, 96, 97, 104, 105, 131, 132, 162, 163, 189, 190, 220, 221, 247, 248, 278, 279, 305 and/or 306 of SEQ ID NO: 4. Useful substitutions for these positions are mentioned above. For example, a SpA Domain E may be mutated at positions corresponding to amino acid residues 43, 44, 70 and/or 71 of SEQ ID NO: 1 (egSEQ ID NO: 19). A SpA Domain D may be mutated at positions corresponding to amino acid residues 96, 97, 104, 105, 131 and/or 132 of SEQ ID NO: 4 (eg SEQ ID NO: 20 or 24). A SpA Domain A may be mutated at positions corresponding to amino acid residues 162, 163, 189 and/or 190 of SEQ ID NO: 4 (eg SEQ ID NO: 21). A SpA Domain B may be mutated at positions corresponding to amino acid residues 220, 221, 247 and/or 248 of SEQ ID NO: 4 (eg SEQ ID NO: 22). A SpA Domain C may be mutated at positions corresponding to amino acid residues 278, 279, 305 and/or 306 of SEQ ID NO: 4 (eg SEQ ID NO: 23). It is thought that the mutations at positions corresponding to positions 43, 44, 96, 97, 104, 105, 162, 163, 220, 221, 278 and/or 279 of SEQ ID NO: 4 decrease or eliminate binding to the Fcy portion of human IgG, while those at positions corresponding to positions 70, 71, 131, 132, 189, 190, 247, 248, 305 and/or 306 of SEQ ID NO: 4. decrease or eliminate binding to the Fab portion of V_H3-containing human B cell receptors.

[0076] Thus, the SpA antigen preferably comprises (a) one or more amino acid substitutions in a VH3-binding sub-domain of SpA domain E, D, A, B or C that disrupts or decreases binding to VH3, and (b) one or more amino acid substitutions in an IgG Fc binding sub-domain of SpA domain E, D, A, B or C that disrupts or decreases binding to IgG Fc. The E/D, D/A, A/B and B/C domain borders may be based on the domains described in Kim et al, 2014. Vaccine 32: 464-469.

[0077] In an embodiment, the SpA antigen comprises a domain E with an amino acid substitution at amino acid positions 7 and 8 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 12 and 13 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 9 and 10 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18. Preferably, said amino acid substitution is substitution of lysine for glutamine, as in for example SEQ ID NOs: 19-24. In a preferred embodiment, the SpA antigen further comprises a domain D with an amino acid substitution at amino acid positions 4 and 5 of SEQ ID NO: 15, for example a substitution of lysine or arginine for glutamine, eg KR (e.g. SEQ ID NO: 24 or SEQ ID NO: 25).

[0078] In an embodiment, the SpA antigen comprises a domain E with an amino acid substitution at the amino acid positions 34 and 35 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 39 and 40 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 36 and 37 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid

substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18. Preferably, said amino acid substitution is substitution of alanine for aspartic acid, as in for example SEQ ID NOs: 19-24.

[0079] Exemplary SpA antigens of the invention may comprise or consist of the sequence of SEQ ID NO: 26 or, more preferably, SEQ ID NO: 27. Other exemplary SpA antigens may have 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 26 or SEQ ID NO: 27; and/or may comprise a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 26 or SEQ ID NO: 27, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more).

ClfA Antigen

[0080] Adherence of bacteria to host extracellular matrix components is a critical initial phase in bacterial infection. Clumping factor A (ClfA) is an important *S. aureus* adhesin which is required for virulence and helps the bacteria evade host defence mechanisms. It binds to fibrinogen in the ECM, aiding in adherence and colonisation of host tissues and additionally causing cell clumping and coating of the bacterial cells in fibrinogen, which promotes immune evasion by impairing deposition of opsonins on the bacteria.

[0081] ClfA is present in nearly all *S. aureus* strains. It is an important virulence factor, contributing to the pathogenesis of septic arthritis and endocarditis. ClfA binds to the C-terminus of the γ-chain of fibrinogen, and is thereby able to induce clumping of bacteria in fibrinogen solution. Expression of ClfA on *S. aureus* hampers phagocytosis by both macrophages and neutrophils. In neutrophils this is due to both a fibrinogen-dependent and to a fibrinogen-independent mechanism. In contrast, platelets are activated by bacteria expressing ClfA through its interaction with GPIIb/IIIa leading to aggregation.

[0082] This is most efficiently executed when fibrinogen is present, but there is also a fibrinogen-independent pathway for platelet activation.

[0083] ClfA contains a 520 amino acid N-terminal A domain (the Fibrinogen Binding Region), which comprises three separately folded subdomains N1, N2 and N3. The A domain is followed by a serine-aspartate dipeptide repeat region and a cell wall- and membrane-spanning region, which contains the LPDTG-motif for sortase-promoted anchoring to the cell wall. The amino acid sequence of the A domain (subdomains N1-3) is shown in SEQ ID NO: 1. The amino acid sequence of the A domain subdomains N2N3 is shown in SEQ ID NO: 2.

[0084] In an embodiment, the ClfA antigen of the invention comprises an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 1 or SEQ ID NO: 2, or is an immunogenic fragment and/or a variant of SEQ ID NO. 1 or SEQ ID NO: 2 (e.g. SEQ ID NOs 5-7) or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 1 or SEQ ID NO: 2. In an embodiment, the ClfA antigen of the invention may comprise an immunogenic fragment of SEQ ID NO. 2 or SEQ ID NOs: 5-8 comprising at least about 15, at least about 20, at least about 40, at least about 60, at least about 100, at least about 300, or at least about 400 contiguous amino acid residues of the full length sequence, wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence.

[0085] In some embodiments, the ClfA antigen of the invention may comprise (or consist of) subdomains N1, N2 and N3 of ClfA (SEQ ID NO: 1) or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 1. In other embodiments, the modified ClfA antigen of the invention may comprise (or consist of) subdomains N2 and N3 (SEQ ID NO: 2) or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 2.

[0086] The present invention thus provides a ClfA antigen comprising (or consisting of) an amino acid sequence of SEQ ID NO. 2 or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2 (e.g. SEQ ID NO. 5). In an embodiment, the ClfA antigen comprises one or more consensus sequence(s) for a glycosyltransferase enzyme, e.g. PglB, selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline. In an embodiment, the consensus sequence is K-D/E-X-N-Z-S/T-K (SEQ ID NO. 28), wherein X is Q (glutamine) and Z is R (arginine), e.g. K-D-Q-N-R-T-K (SEQ ID NO. 30). In a preferred embodiment, the consensus sequence is K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X is Q (glutamine) and Z is A (alanine), e.g. K-D-Q-N-A-T-K (SEQ ID NO. 31). The ClfA antigen may be additionally modified by addition of an N-terminal serine for cloning purposes, e.g. SEQ ID NO: 7. The ClfA antigen may further be modified to contain mutations which abrogate fibrinogen binding, as described below, e.g. SEQ ID NOs 5-7.

[0087] In an embodiment, the ClfA antigen of the invention comprises an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2, which sequence is a variant of SEQ ID NO. 2 which has been modified by the deletion and/or addition and/or substitution of one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acids). Amino acid substitution may be conservative or non-conservative. In one aspect, amino acid substitution is conservative. Substitutions, deletions, additions or any combination thereof may be combined in a single variant so long as the variant is an immunogenic polypeptide. In an embodiment, the ClfA antigen of the present invention may be derived from a variant in which 1 to 10, 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1 amino acids are substituted, deleted, or added in any combination. For example, the ClfA antigen of the invention may be derived from an amino acid sequence which is a variant of SEQ ID NO. 2 in that it comprises an additional N-terminal serine (e.g. SEQ ID NO: 7).

[0088] In an embodiment, the present invention includes fragments and/or variants which comprise a B-cell or T-cell epitope. Such epitopes may be predicted using a combination of 2D-structure prediction, e.g. using the PSIPRED program (from David Jones, Brunel Bioinformatics Group,

Dept. Biological Sciences, Brunel University, Uxbridge UB8 3PH, UK) and antigenic index calculated on the basis of the method described by Jameson and Wolf (CABIOS 4:181-186 [1988]).

[0089] In an embodiment of the invention, one or more amino acids (e.g. 1-7 amino acids, e.g. one amino acid) of the ClfA amino acid sequence (for example, having an amino acid sequence of SEQ ID NO. 2 or a ClfA amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2) have been substituted by a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-A-T-K (SEQ ID NO. 31)) consensus sequence. For example, a single amino acid in the ClfA amino acid sequence (e.g. SEQ ID NO. 2 or 5) may be replaced with a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-A-T-K (SEQ ID NO. 31)) consensus sequence. Alternatively, 2, 3, 4, 5, 6 or 7 amino acids in the ClfA amino acid sequence (e.g. SEQ ID NO. 2 or a ClfA amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2) may be replaced with a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-A-T-K (SEQ ID NO. 31)) consensus sequence.

[0090] In an embodiment, a consensus sequence has been added or substituted for one or more amino acids residues 313-340 (e.g. in place of one or more amino acid residue(s) 330-340, or in place of amino acid residue Q327, D329, P331 or 1337, preferably 1337) of SEQ ID NO. 2 or in an equivalent position in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2 (e.g. SEQ ID NOS. 5-7).

[0091] Introduction of a consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) enables the ClfA antigen to be glycosylated. Thus, the present invention also provides a ClfA antigen of the invention wherein the ClfA antigen is glycosylated.

[0092] Because the fibrinogen-binding activity of ClfA is required for it to act as a virulence factor, the ClfA antigen may be modified to reduce or eliminate fibrinogen-binding activity in order that it may be administered in vivo. Such a modified ClfA antigen may have one of the mutations described in WO2011/007004, for example mutations at one or preferably both of the amino acids corresponding to residues P116 and Y118 of SEQ ID NO: 2, for example P116S and/or Y118A. Exemplary sequences are those of SEQ ID NOs: 5-7. In an embodiment, in one aspect, the ClfA antigen of the invention comprises (or consists of) an amino acid sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO. 2, said amino acid sequence comprising: the amino acid substitutions P116 to S and Y118 to A, e.g. SEQ ID NOS. 5-7 or 32.

[0093] In an embodiment, an additional amino acid residue (for example, serine or alanine) is added at the N-terminus of the mature antigen, as in for example SEQ ID NO: 7. Such a residue has the advantage of leading to more efficient cleavage of the leader sequence.

[0094] In one aspect, the ClfA antigen of the invention comprises (or consists of) an amino acid sequence which is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO. 2, said amino acid sequence comprising: the amino acid substitutions P116 to S and Y118 to A, a K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) consensus sequence wherein X and Z are independently any amino acid apart from proline (preferably K-D-Q-N-A-T-K (SEQ ID NO. 31), e.g. SEQ ID NO: 6) optionally with an additional serine residue at the N-terminus (e.g. SEQ ID NO: 7). In an embodiment, a ClfA antigen of the invention has an amino acid sequence at least 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NOs 1, 2 or 5-7. In another embodiment, the ClfA antigen of the invention has an amino acid sequence selected from SEQ ID NOs 1, 2 or 5-7.

Hla Antigen

[0095] Hla is an important secreted staphylococcal toxin. It creates a lipid-bilayer penetrating pore in the membrane of human erythrocytes and other cells, resulting in cell lysis.

[0096] In an embodiment, the Hla antigen of the invention comprises (or consists of) an amino acid sequence of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO.3.

[0097] In an embodiment, the Hla antigen comprises amino acid substitutions at positions H48 and G122 of SEQ ID NO. 3 or at equivalent positions within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3, wherein said substitutions are respectively H to C and G to C (e.g. H48C and G122C, for example SEQ ID NOs: 10-12). Said substitutions serve to introduce a disulphide bridge into the Hla antigen, which may improve stability and yield of the protein when produced recombinantly.

[0098] Hence, the Hla antigen of the invention may demonstrate a reduced tendency to aggregate compared to Hla lacking disulphide bridges, e.g. wild-type or detoxified Hla (for example, Hla H35L, e.g. SEQ ID NO: 8) For example, a suitable modified Hla antigen of the invention may be one that exhibits lower aggregation than wild-type Hla or HlaH35L (e.g. as detectable on Western blots or measured via chromatographic techniques, e.g IMAC or size exclusion chromatography), as described in the Examples. For instance, a suitable modified Hla antigen may show aggregation levels (as determined using any of the methods described herein) of 0%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, or 5%; about 0%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1% or 5%; less than 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1% or 5%; <10%, <20% , <30% , <40% , <50% , <60%, <70% , <80% or <90% of that the wild-type, detoxified (e.g. HlaH35L) Hla or other cross-linked Hla. For example, when using size exclusion chromatography or IMAC the peak representing monomeric Hla may be higher than wild-type Hla or HlaH35L or other Hla which has not been modified to reduce cross-linking, and/or the peak representing aggregated Hla may be lower.

[0099] The Hla antigen of the invention may be produced with a greater overall yield than Hla lacking disulphide bridges, e.g. wild-type or detoxified Hla (for example, Hla H35L, e.g. SEQ ID NO: 8). Where the overall yield is not greater, the modified Hla antigen may be produced with a greater yield of Hla monomer than Hla lacking disulphide bridges, e.g. wild-type or detoxified Hla (for example, Hla H35L, e.g. SEQ ID NO: 8). For instance, yield of the modified Hla antigen may be increased by 10%, 20%, 30% , 40% , 50% , 60%, 70% , 80% 90%, 110%, 120%, 150%,

200% or more, or about 10%, 20% , 30% , 40% , 50% , 60%, 70% , 80% 90%, 110%, 120%, 150%, 200% or more, compared to that of the wild-type, detoxified (e.g. HlaH35L) Hla or other cross-linked Hla. Protein yield may be determined as described below.

[0100] In an embodiment, the Hla antigen of the invention may be an immunogenic fragment and/or a variant of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO.3. In an embodiment, the Hla antigen of the invention may comprise an immunogenic fragment of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO.3 comprising at least about 15, at least about 20, at least about 40, or at least about 60 contiguous amino acid residues of the full length sequence, wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence.

[0101] In an embodiment, the Hla antigen of the invention may be derived from an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3 which is a variant of SEQ ID NO. 3 which has been modified by the deletion and/or addition and/or substitution of one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acids). Amino acid substitution may be conservative or non-conservative. In one aspect, amino acid substitution is conservative. Substitutions, deletions, additions or any combination thereof may be combined in a single variant so long as the variant is an immunogenic polypeptide. In an embodiment, the modified Hla antigen of the present invention may be derived from a variant in which 1 to 10, 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1 amino acids are substituted, deleted, or added in any combination. For example, the modified Hla antigen of the invention may be derived from an amino acid sequence which is a variant of any one of SEQ ID NOs. 3 or 8-11 in that it has one or two additional amino acids at the N terminus, for example an initial N-terminal S (e.g. SEQ ID NO. 12). The modified Hla antigen may additionally or alternatively have one or more additional amino acids at the C terminus, for example 1, 2, 3, 4, 5, or 6 amino acids. Such additional amino acids may include a peptide tag to assist in purification, and include for example GSHRHR (e.g. SEQ ID NO: 12).

[0102] In an embodiment, the present invention includes fragments and/or variants which comprise a B-cell or T-cell epitope. Such epitopes may be predicted using a combination of 2D-structure prediction, e.g. using the PSIPRED program (from David Jones, Brunel Bioinformatics Group, Dept. Biological Sciences, Brunel University, Uxbridge UB8 3PH, UK) and antigenic index calculated on the basis of the method described by Jameson and Wolf (CABIOS 4:181-186 [1988]).

[0103] Because Hla is a toxin, it needs to be detoxified (i.e. rendered non-toxic to a mammal, e.g. human, when provided at a dosage suitable for protection) before it can be administered in vivo. The cell lytic activity of Hla may be reduced by mutation of amino acid residues involved in pore formation, as described in Menzies and Kernodle (Menzies and Kernodle, 1994, Infect Immun 62, 1843-1847). A Hla antigen of the invention may be genetically detoxified (i.e. by mutation). Additionally and/or alternatively, the Hla antigen may be detoxified by conjugation, eg to a *S. aureus* capsular polysaccharide. The genetically detoxified sequences may remove undesirable activities such as the ability to form a lipid-bilayer penetrating pore, membrane permeation, cell lysis, and cytolytic activity against human erythrocytes and other cells, in order to reduce toxicity, whilst retaining the ability to induce anti-Hla protective and/or neutralising antibodies following administration to a human. For example, as described herein, a Hla antigen may be altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes.

[0104] The Hla antigens of the invention may be genetically detoxified by one or more point mutations. For example, residues involved in pore formation been implicated in the lytic activity of Hla. In one aspect, the modified Hla antigens of the invention may be detoxified by amino acid substitutions as described in Menzies and Kernodle (Menzies and Kernodle, 1994, Infect Immun 62, 1843-1847), for example substitution of H35, H48, H114 and/or H259 with another amino acid such as lysine. For example, the modified Hla antigens of the invention may comprise at least one amino acid substitution selected from H35L, H114L or H259L, with reference to the amino acid sequence of SEQ ID NO. 3 (or an equivalent position in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3). Preferably, the modified Hla antigen comprises the substitution H35L (e.g. SEQ ID NOs: 8-12).

[0105] The amino acid numbers referred to herein correspond to the amino acids in SEQ ID NO. 3 and as described above, a person skilled in the art can determine equivalent amino acid positions in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3 by alignment.

[0106] The haemolytic activity of the Hla antigen of the invention may be assayed and characterised by methods described for example in Menzies and Kernodle, 1994, Infect Immun 62, 1843-1847. An in vitro haemolysis assay may be used to measure the haemolytic (e.g. cytolytic) activity of modified Hla antigen relative to wild-type Hla. A haemolysis inhibition assay may be used to measure the ability of antisera raised against a modified Hla antigen of the invention to inhibit haemolysis by Hla, and (typically) comparing anti-(modified Hla) antisera to anti-(wild-type Hla) antisera. For example, a suitable modified Hla antigen of the invention may be one that exhibits lower haemolytic activity than wild-type Hla (e.g. measured via an in vitro haemolysis assay). For instance, a suitable modified Hla antigen may have a specific activity (as determined using the in vitro haemolysis assay) of about (referring to each of the following values independently) 0%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% or <10% the specific activity of the wild-type Hla. A suitable modified Hla antigen of the invention may also be one that, following administration to a host, causes the host to produce antibodies that inhibit haemolysis by wild-type Hla (e.g. via a haemolysis inhibition assay), is immunogenic (e.g. induces the production of antibodies against wild-type Hla), and/or protective (e.g. induces an immune response that protects the host against infection by or limits an already-existing infection). Assays may be used as described in the Examples.

[0107] In an embodiment of the invention, one or more amino acids (e.g. 1-7 amino acids, e.g. one amino acid) of the modified Hla amino acid sequence (for example, having an amino acid sequence of SEQ ID NO. 3 or a Hla amino

acid sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3, e.g. SEQ ID NO: 8 or SEQ ID NO: 10) have been substituted by a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-R-T-K (SEQ ID NO. 30)) consensus sequence for a glycosyltransferase enzyme, e.g. PglB. For example, a single amino acid in the Hla amino acid sequence (e.g. SEQ ID NO. 3) may be replaced with a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-R-T-K (SEQ ID NO. 30)) consensus sequence (e.g. SEQ ID NO: 9 or SEQ ID NOs: 11-12). Alternatively, 2, 3, 4, 5, 6 or 7 amino acids in the Hla amino acid sequence (e.g. SEQ ID NO. 3 or a Hla amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3) may be replaced with a D/E-X-N-Z-S/T (SEQ ID NO. 28) or K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-R-T-K (SEQ ID NO. 30)) consensus sequence.

[0108] In an embodiment, the consensus sequence(s) selected from D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) (e.g. K-D-Q-N-R-T-K (SEQ ID NO. 30)) is added or substituted at a position corresponding to amino acid K131 of SEQ ID NO. 3 or in an equivalent position in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 1 (e.g. SEQ ID NOs: 9 and 11-12). In a preferred embodiment, said consensus sequence is substituted for the amino acid corresponding to K131 of SEQ ID NO: 3.

[0109] Introduction of a consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) enables the Hla antigen to be glycosylated. Thus, the present invention also provides a Hla antigen of the invention which is glycosylated. In specific embodiments, the consensus sequences are introduced into specific regions of the Hla amino acid sequence, e.g. surface structures of the protein, at the N or C termini of the protein, and/or in loops that are stabilized by disulfide bridges. In an aspect of the invention, the position of the consensus sequence(s) provides improved glycosylation, for example increased yield.

[0110] Introduction of such glycosylation sites can be accomplished by, e.g. adding new amino acids to the primary structure of the antigen (i.e. the glycosylation sites are added, in full or in part), or by mutating existing amino acids in the antigen in order to generate the glycosylation sites (i.e. amino acids are not added to the antigen, but selected amino acids of the antigen are mutated so as to form glycosylation sites). Those of skill in the art will recognize that the amino acid sequence of an antigen can be readily modified using approaches known in the art, e.g. recombinant approaches that include modification of the nucleic acid sequence encoding the antigen.

[0111] In an embodiment, the Hla antigen may be further modified in that the amino acid sequence comprises one or more consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline (e.g. SEQ ID NO. 30). These sequences may be modified by insertion of an N-terminal serine and/or alanine for cloning purposes, as described herein. The sequences may further be modified to contain detoxifying mutations, such as any one or all of the detoxifying mutations described herein. A preferred detoxifying mutation is H35L of SEQ ID No 3.

[0112] Thus, in an embodiment, the present invention provides a Hla antigen having an amino acid sequence comprising one or more consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline, which have been recombinantly introduced into the Hla amino acid sequence of SEQ ID NO. 3 or a Hla amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3 (e.g. SEQ ID NOs 9 or 10).

[0113] In a preferred embodiment, the Hla antigen of the invention comprises (or consists of) the amino acid sequence of SEQ ID NO. 11. In an embodiment, the modified Hla antigen of the invention comprises (or consists of) the amino acid sequence of any one of SEQ ID NOs. 3 or 8-11 with an N-terminal serine and/or alanine (i.e. S residues added at the N-terminus). In an embodiment, the modified Hla antigen of the invention comprises (or consists of) the amino acid sequence of SEQ ID NO. 12.

[0114] In an embodiment, the Hla antigen of the invention further comprises a "peptide tag" or "tag", i.e. a sequence of amino acids that allows for the isolation and/or identification of the modified Hla antigen. For example, adding a tag to a modified Hla antigen of the invention can be useful in the purification of that antigen and, hence, the purification of conjugate vaccines comprising the tagged modified Hla antigen. Exemplary tags that can be used herein include, without limitation, histidine (HIS) tags. In one embodiment, the tag is a hexa-histidine tag. In another embodiment, the tag is a HR tag, for example an HRHR tag. In certain embodiments, the tags used herein are removable, e.g. removal by chemical agents or by enzymatic means, once they are no longer needed, e.g. after the antigen has been purified. Optionally the peptide tag is located at the C-terminus of the amino acid sequence. Optionally the peptide tag comprises six histidine residues at the C-terminus of the amino acid sequence. Optionally the peptide tag comprises four HR residues (HRHR) at the C-terminus of the amino acid sequence. The peptide tag may comprise or be preceded by one, two or more additional amino acid residues, for example alanine, serine and/or glycine residues, e.g. GS. In one aspect, the modified Hla antigen of the invention comprises (or consists of) an amino acid sequence which is at least 80%, 85%, 90%, 92%, 95%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO. 3, said amino acid sequence comprising a D/E-X-N-Z-S/T (SEQ ID NO. 28) consensus sequence wherein X and Z are independently any amino acid apart from proline (e.g. K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) or K-D-Q-N-R-T-K (SEQ ID NO. 30)) and at least one amino acid substitution selected from H35L, H48C and G122C and a GSHRHR peptide tag at the C-terminus of the amino acid sequence. Optionally, the modified Hla antigen of the invention has an amino acid sequence at least 97%, 98%, 99% or 100% identical to SEQ ID NO. 3 or 9-12.

[0115] In an embodiment, a serine and/or alanine residue is added at the N-terminus of the mature protein, e.g. SA or S, preferably S (e.g. SEQ ID NO: 12). Such a residue or residues have the advantage of leading to more efficient cleavage of the leader sequence.

[0116] In one aspect, the Hla antigen of the invention comprises (or consists of) an amino acid sequence which is at least 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO. 3, said amino acid sequence comprising the amino acid substitutions G122 to C, H48 to C, and H35 to L, a K-D-Q-N-R-T-K (SEQ ID NO. 30)) substituted for the amino acid corresponding to position K131 of SEQ ID NO: 3, a HRHR tag (SEQ ID NO: 32) at the C-terminus of the amino acid sequence (E.g. SEQ ID NO: 11 or SEQ ID NO: 12).

[0117] In a preferred embodiment, said Hla antigen comprises (or consists of) the amino acid sequence of SEQ ID NO: 11. In a preferred embodiment, said Hla antigen comprises (or consists of) the amino acid sequence of SEQ ID NO: 12.

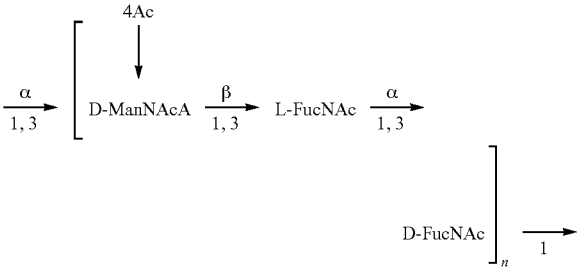
Polysaccharide Antigens

[0118] 75% of *S. aureus* strains express either Type 5 or Type 8 capsular polysaccharide, so a vaccine comprising CP5 and CP8 could potentially provide protection against the majority of circulating *S. aureus* strains.

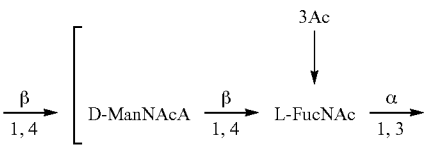
[0119] The compositions of the invention thus comprise a bacterial capsular saccharide from *S. aureus*. The bacterial capsular saccharide from *S. aureus* may be selected from a *S. aureus* serotype 5 or 8 capsular saccharide.

[0120] In an embodiment of the invention, the antigen is a repeating unit of a bacterial capsular saccharide from *S. aureus*. In an embodiment of the invention, the antigen comprises a repeat unit of a bacterial capsular saccharide from *S. aureus* serotype 5 or 8.

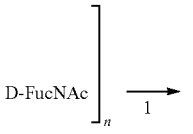
[0121] In an embodiment of the invention, the antigen comprises a repeat unit of a bacterial capsular saccharide from *S. aureus* serotype 8. In this embodiment, the antigen comprises:



[0122] In an embodiment of the invention, the antigen comprises a repeat unit of a bacterial capsular saccharide from *StaphylococcuS. aureus* serotype 5. In this embodiment of the invention, the antigen comprises:



-continued



[0123] In an embodiment, the antigen is a polysaccharide. In an embodiment, the antigen comprises two or more monosaccharides, for example 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20 or more monosaccharides. In an embodiment, the antigen is an polysaccharide containing no more than 20, 15, 12, 10, 9, or 8 monosaccharides.

[0124] In an embodiment, said capsular polysaccharide antigen is conjugated to a carrier protein. Conjugates of the invention are described below.

Conjugates

[0125] The present invention also provides a conjugate (e.g. bioconjugate) comprising (or consisting of) a capsular saccharide as described herein linked, e.g. covalently linked, to a carrier protein. In an embodiment, the carrier protein is a Hla protein of the invention. In an embodiment, the protein is a ClfA protein of the invention.

[0126] In an embodiment, the carrier protein is covalently linked to the polysaccharide antigen through a chemical linkage obtainable using a chemical conjugation method (i.e. the conjugate is produced by chemical conjugation).

[0127] In an embodiment, the chemical conjugation method is selected from the group consisting of carbodiimide chemistry, reductive animation, cyanylation chemistry (for example CDAP chemistry), maleimide chemistry, hydrazide chemistry, ester chemistry, and N-hydroysuccinimide chemistry. Conjugates can be prepared by direct reductive amination methods as described in, U.S. 200710184072 (Hausdorff) U.S. Pat. No. 4,365,170 (Jennings) and U.S. Pat No. 4,673,574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508. The conjugation method may alternatively rely on activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094. See also Chu C. et al Infect. Immunity, 1983 245 256.

[0128] In general the following types of chemical groups on a modified ClfA protein can be used for coupling/conjugation:

[0129] A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on saccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.

[0130] B) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on saccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC. In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on saccharides directly or to such groups on a linker; to saccharides or linkers having an aldehyde group; to saccharides or linkers having a succinimide ester group.

[0131] C) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro

acetylated saccharide or linker with maleimide chemistry. In one embodiment this group is activated/modified with bis diazobenzidine.

[0132] D) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.

[0133] E) Imidazolyl group (for instance via histidine). In one embodiment this group is activated/modified with bis diazobenzidine.

[0134] F) Guanidyl group (for instance via arginine).

[0135] G) Indolyl group (for instance via tryptophan).

[0136] On a saccharide, in general the following groups can be used for a coupling: OH, COOH or NH_2. Aldehyde groups can be generated after different treatments such as: periodate, acid hydrolysis, hydrogen peroxide, etc.

[0137] Direct Coupling Approaches:

[0138] Saccharide-OH+CNBr or CDAP----->cyanate ester+NH_2-Protein---->conjugate

[0139] Saccharide-aldehyde+NH_2-Protein---->Schiff base+NaCNBH3---->conjugate

[0140] Saccharide-COOH+NH_2-Protein+EDAC---->conjugate

[0141] Saccharide-NH_2+COOH-Protein+EDAC---->conjugate

[0142] Indirect Coupling Via Spacer (Linker) Approaches:

[0143] Saccharide-OH+CNBr or CDAP--->cyanate ester+NH_2----NH_2---->saccharide----NH_2+COOH-Protein+EDAC----->conjugate

[0144] Saccharide-OH+CNBr or CDAP---->cyanate ester+NH_2-----SH----->saccharide----SH+SH-Protein (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance)----->saccharide-S-S-Protein

[0145] Saccharide-OH+CNBr or CDAP--->cyanate ester+NH_2----SH------->saccharide----SH+maleimide-Protein (modification of amino groups)---->conjugate

[0146] Saccharide-OH+CNBr or CDAP--->cyanate ester+NH_2SH ----->Saccharide-SH+haloacetylated-Protein---->Conjugate

[0147] Saccharide-COOH+EDAC+NH2-----NH_2--->saccharide-------NH_2+EDAC +COOH-Protein---->conjugate

[0148] Saccharide-COOH+EDAC+NH_2----SH----->saccharide----SH+SH-Protein (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance)----->saccharide-S-S-Protein

[0149] Saccharide-COOH+EDAC+NH_2----SH----->saccharide----SH+maleimide-Protein (modification of amino groups)---->conjugate

[0150] Saccharide-COOH+EDAC+NH_2----SH--->Saccharide-SH+haloacetylated-Protein---->Conjugate

[0151] Saccharide-Aldehyde+NH_2 NH_2----->saccharide----NH2+EDAC+COOH-Protein---->conjugate

[0152] Note: instead of EDAC above, any suitable carbodiimide may be used.

[0153] In an embodiment, the antigen is directly linked to the carrier protein.

[0154] In an embodiment, the antigen is attached to the carrier protein via a linker. Optionally, the linker is selected from the group consisting of linkers with 4-12 carbon atoms, bifunctional linkers, linkers containing 1 or 2 reactive amino groups at the end, B-proprionamido, nitrophenyl-ethylamine, haloacyl halides, 6-aminocaproic acid and ADH. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS (4-Maleimidobutyric acid N-hydroxysuccinimide ester)) or a haloacetylated carrier protein (for example using SITAB (succinimidyl (4-iodoacetyl)aminobenzoate), or SIA (succinimidyl iodoacetate), or SBAP (succinimidyl-3-(bromoacetamide)propionate)). In an embodiment, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or ADH (adipic acid dihydrazide) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. 1-Ethyl-3-(3-dimethylarninopropyl)carbodiimide (EDAC or EDC)) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094.

[0155] In an embodiment, the amino acid residue on the carrier protein to which the antigen is linked is not an asparagine residue and in this case, the conjugate is typically produced by chemical conjugation. In an embodiment, the amino acid residue on the carrier protein to which the antigen is linked is selected from the group consisting of: Ala, Arg, Asp, Cys, Gly, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. Optionally, the amino acid is: an amino acid containing a terminal amine group, a lysine, an arginine, a glutaminic acid, an aspartic acid, a cysteine, a tyrosine, a histidine or a tryptophan. Optionally, the antigen is covalently linked to amino acid on the carrier protein selected from: aspartic acid, glutamic acid, lysine, cysteine, tyrosine, histidine, arginine or tryptophan.

[0156] In an embodiment, the antigen is linked to an amino acid on the carrier protein selected from asparagine, aspartic acid, glutamic acid, lysine, cysteine, tyrosine, histidine, arginine or tryptophan (e.g. asparagine). In an embodiment, the amino acid residue on the carrier protein to which the antigen is linked is an asparagine residue. In an embodiment, the amino acid residue on the carrier protein to which the antigen is linked is part of the D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) consensus sequence (e.g. the asparagine in the D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29) consensus sequence).

[0157] The invention provides a bioconjugate comprising a ClfA or Hla protein as described herein linked to a *StaphylococcuS. aureus* capsular saccharide (e.g. capsular polysaccharide). In a specific embodiment, the bioconjugate comprises a ClfA or Hla protein as described herein and an antigen selected from a capsular saccharide (e.g. capsular polysaccharide) of *StaphylococcuS. aureus* serotype CP5 or CP8. In an embodiment, the bioconjugate comprises a ClfA antigen of the invention and an antigen from a capsular saccharide (e.g. capsular polysaccharide) of *StaphylococcuS. aureus* serotype CP8. In an embodiment, the bioconjugate comprises a Hla antigen of the invention and an antigen from a capsular saccharide (e.g. capsular polysaccharide) of *StaphylococcuS. aureus* serotype CP5.

[0158] Methods of producing bioconjugates using host cells are described for example in WO 2003/074687, WO 2006/119987 and WO2011/138361.

[0159] Bioconjugates may be produced in a host cell comprising: one or more nucleic acids that encode glyco-

syltransferase(s); a nucleic acid that encodes an oligosaccharyl transferase; a nucleic acid that encodes a polypeptide of the invention; and optionally a nucleic acid that encodes a polymerase (e.g. wzy).

[0160] Host cells that can be used to produce the bioconjugates of the invention include archea, prokaryotic host cells, and eukaryotic host cells. Exemplary prokaryotic host cells for use in production of the bioconjugates of the invention, without limitation, *Escherichia* species, *Shigella* species, *Klebsiella* species, *Xhantomonas* species, *Salmonella* species, *Yersinia* species, *Lactococcus* species, *Lactobacillus* species, *Pseudomonas* species, *Corynebacterium* species, *Streptomyces* species, *Streptococcus* species, *Staphylococcus* species, *Bacillus* species, and *Clostridium* species. In a specific embodiment, the host cell is *E. coli.*

[0161] In an embodiment, the host cells used to produce the bioconjugates of the invention are engineered to comprise heterologous nucleic acids, e.g. heterologous nucleic acids that encode one or more carrier proteins and/or heterologous nucleic acids that encode one or more proteins, e.g. genes encoding one or more proteins. In a specific embodiment, heterologous nucleic acids that encode proteins involved in glycosylation pathways (e.g. prokaryotic and/or eukaryotic glycosylation pathways) may be introduced into the host cells. Such nucleic acids may encode proteins including, without limitation, oligosaccharyl transferases, epimerases, flippases, polymerases, and/or glycosyltransferases. Heterologous nucleic acids (e.g. nucleic acids that encode carrier proteins and/or nucleic acids that encode other proteins, e.g. proteins involved in glycosylation) can be introduced into the host cells using methods such as electroporation, chemical transformation by heat shock, natural transformation, phage transduction, and conjugation. In specific embodiments, heterologous nucleic acids are introduced into the host cells using a plasmid, e.g. the heterologous nucleic acids are expressed in the host cells by a plasmid (e.g. an expression vector). In another specific embodiment, heterologous nucleic acids are introduced into the host cells using the method of insertion described in International Patent application No. PCT/EP2013/068737 (published as WO 14/037585).

[0162] Additional modifications may be introduced (e.g. using recombinant techniques) into the host cells. For example, host cell nucleic acids (e.g. genes) that encode proteins that form part of a possibly competing or interfering glycosylation pathway (e.g. compete or interfere with one or more heterologous genes involved in glycosylation that are recombinantly introduced into the host cell) can be deleted or modified in the host cell background (genome) in a manner that makes them inactive/dysfunctional (i.e. the host cell nucleic acids that are deleted/modified do not encode a functional protein or do not encode a protein whatsoever). When nucleic acids are deleted from the genome of the host cells, they may be replaced by a desirable sequence, e.g. a sequence that is useful for glycoprotein production.

[0163] Exemplary genes that can be deleted in host cells (and, in some cases, replaced with other desired nucleic acid sequences) include genes of host cells involved in glycolipid biosynthesis, such as waaL (see, e.g. Feldman et al. 2005, PNAS USA 102:3016-3021), the lipid A core biosynthesis cluster (waa), galactose cluster (gal), arabinose cluster (ara), colonic acid cluster (wc), capsular polysaccharide cluster, undecaprenol-pyrophosphate biosynthesis genes (e.g. uppS (Undecaprenyl pyrophosphate synthase), uppP (Undecaprenyl diphosphatase)), Und-P recycling genes, metabolic enzymes involved in nucleotide activated sugar biosynthesis, enterobacterial common antigen cluster, and prophage O antigen modification clusters like the gtrABS cluster.

[0164] Such a modified prokaryotic host cell comprises nucleic acids encoding enzymes capable of producing a bioconjugate comprising an antigen, for example a saccharide antigen attached to a polypeptide of the invention. Such host cells may naturally express nucleic acids specific for production of a saccharide antigen, or the host cells may be made to express such nucleic acids, i.e. in certain embodiments said nucleic acids are heterologous to the host cells. One or or more of said nucleic acids specific for production of a saccharide antigen may be heterologous to the host cell and integrated into the genome of the host cell. The host cells may comprise nucleic acids encoding additional enzymes active in the N-glycosylation of proteins, e.g. the host cells further comprise a nucleic acid encoding an oligosaccharyl transferase and/or one or more nucleic acids encoding other glycosyltransferases.

[0165] Nucleic acid sequences comprising capsular polysaccharide gene clusters can be inserted into the host cells. For example, the capsular polysaccharide gene cluster inserted into a host cell may be a capsular polysaccharide gene cluster from a staphylococcal strain (e.g. *S. aureus*), as described below.

[0166] The host cells comprise, and/or can be modified to comprise, nucleic acids that encode genetic machinery (e.g. glycosyltransferases, flippases, polymerases, and/or oligosaccharyltransferases) capable of producing hybrid polysaccharides, as well as genetic machinery capable of linking antigens to the polypeptide of the invention.

[0167] *S. aureus* capsular polysaccharides are assembled on the bacterial membrane carrier lipid undecaprenyl pyrophosphate by a conserved pathway that shares homology to the polymerase-dependent pathway of O polysaccharide synthesis in Gram-negative bacteria. O antigen assembly is initiated by the transfer of a sugar phosphate from a DP-donor to undecaprenyl phosphate. The lipid linked O antigen is assembled at the cytoplasmic side of the inner membrane by sequential action of different glycosyltransferases. The glycolipid is then flipped to the periplasmic space and polymerised. By replacing the O antigen ligase WaaL with the oligosaccharyltransferase PglB, the polymerised O antigen can be transferred to a protein carrier rather than to the lipid A core.

[0168] The host cells further comprise nucleic acids that encode glycosyltransferases that produce a polysaccharide repeating unit. Preferably, said repeating unit does not comprise a hexose at the reducing end, and said polysaccharide repeat unit is derived from a donor polysaccharide repeat unit that comprises a hexose at the reducing end.

[0169] The host cells may comprise a nucleic acid that encodes a glycosyltransferase that assembles a hexose monosaccharide derivative onto undecaprenyl pyrophosphate (Und-PP). In one aspect, the glycosyltransferase that assembles a hexose monosaccharide derivative onto Und-PP is heterologous to the host cell and/or heterologous to one or more of the genes that encode glycosyltransferase(s). Said glycosyltransferase can be derived from, e.g. *Escherichia* species, *Shigella* species, *Klebsiella* species, *Xhantomonas* species, *Salmonella* species, *Yersinia* species, *Aeromonas* species, *Francisella* species, *Helicobacter* species, *Proteus* species, *Lactococcus* species, *Lactobacillus* species,

Pseudomonas species, *Corynebacterium* species, *Streptomyces* species, *Streptococcus* species, *Enterococcus* species, *Staphylococcus* species, *Bacillus* species, *Clostridium* species, *Listeria* species, or *Campylobacter* species. Usually, the glycosyltransferase that assembles a hexose monosaccharide derivative onto Und-PP is wecA, optionally from *E. coli* (wecA can assemble GlcNAc onto UndP from UDP-GlcNAc). The hexose monosaccharide may be selected from the group consisting of glucose, galactose, rhamnose, arabinotol, fucose and mannose (e.g. galactose).

[0170] The host cells may comprise nucleic acids that encode one or more glycosyltransferases capable of adding a monosaccharide to the hexose monosaccharide derivative assembled on Und-PP. For example, said one or more glycosyltransferases capable of adding a monosaccharide to the hexose monosaccharide derivative may be the galactosyltransferase (wfeD) from *Shigella boyedii*; the galactofuranosyltransferase (wbeY) from *E. coli* O28; or the galactofuranosyltransferase (wfdK) from *E. coli* O167. Galftransferases, such as wfdK and wbeY, can transfer Galf (Galactofuranose) from UDP-Galf to -GlcNAc-P-P-Undecaprenyl. Preferably, said one or more glycosyltransferases capable of adding a monosaccharide to the hexose monosaccharide derivative are the galactofuranosyltransferase (wbeY) from *E. coli* O28 and the galactofuranosyltransferase (wfdK) from *E. coli* O167.

[0171] The host cells may comprise nucleic acids that encode glycosyltransferases that assemble the donor polysaccharide repeating unit onto the hexose monosaccharide derivative. The glycosyltransferases that assemble the donor polysaccharide repeating unit onto the hexose monosaccharide derivative may comprise a glycosyltransferase that is capable of adding the hexose monosaccharide present at the reducing end of the first repeating unit of the donor polysaccharide to the hexose monosaccharide derivative. Exemplary glycosyltransferases include galactosyltransferases (wciP), e.g. wciP from *E. coli* O21.

[0172] The glycosyltransferases that assemble the donor polysaccharide repeating unit onto the hexose monosaccharide derivative comprise a glycosyltransferase that is capable of adding the monosaccharide that is adjacent to the hexose monosaccharide present at the reducing end of the first repeating unit of the donor polysaccharide to the hexose monosaccharide present at the reducing end of the first repeating unit of the donor polysaccharide. Exemplary glycosyltransferases include glucosyltransferase (wciQ), e.g. wciQ from *E. coli* O21.

[0173] The host cell may comprise glycosyltransferases for synthesis of the repeating units of an polysaccharide selected from the *StaphylococcuS. aureus* CP5 or CP8 gene cluster. *S. aureus* CP5 and CP8 have a similar structure to *P. aeruginosa* O11 antigen synthetic genes, so these genes may be combined with *E. coli* monosaccharide synthesis genes to synthesise an undecaprenyl pyrophosphate-linked CP5 or CP8 polymer consisting of repeatinging trisaccharide units.

[0174] Glycosyltransferases sufficient for synthesis of the repeating units of the CP5 or CP8 saccharide comprise capH, capl, capJ and/or capK from *S. aureus* CP5 or CP8. Optionally the host cell also comprises capD, capE, capF, capG, capL, capM, capN, capO, capP from *S. aureus* P5 or CP8. Alternatively, the host cell also comprises wbjB, wbjC, wbjD, wbjE, wbjF, wbjL, wbpM, wzz and/or wzx from *P. aeruginosa* O11 and wecB, wecC from *E. coli*O16.

[0175] Glycosyltransferases sufficient for synthesis of the repeating units of the CP5 saccharide comprise capH, capl, capJ and/or capK from *S. aureus* CP5. Optionally the host cell also comprises capD, capE, capF, capG, capL, capM, capN, capO, capP from *S. aureus* CP5. Alternatively, the host cell also comprises wbjB, wbjC, wbjD, wbjE, wbjF, wbjL, wbpM, wzz and/or wzx from *P. aeruginosa* O11 and wecB, wecC from *E. coli* O16.

[0176] The host cell may comprise glycosyltransferases that assemble the donor polysaccharide repeating unit onto the hexose monosaccharide derivative comprise a glycosyltransferase that is capable of adding the hexose monosaccharide present at the reducing end of the first repeating unit of the donor polysaccharide to the hexose monosaccharide derivative.

[0177] N-linked protein glycosylation—the addition of carbohydrate molecules to an asparagine residue in the polypeptide chain of the target protein—is accomplished by the enzymatic oligosaccharyltransferase complex (OST) responsible for the transfer of a preassembled oligosaccharide from a lipid carrier (dolichol phosphate) to an asparagine residue of a nascent protein within the conserved sequence Asn-X-Ser/Thr (where X is any amino acid except proline) in the Endoplasmic reticulum.

[0178] It has been shown that a bacterium, the food-borne pathogen *Campylobacter jejuni*, can also N-glycosylate its proteins (Wacker et al. Science. 2002; 298(5599):1790-3) due to the fact that it possesses its own glycosylation machinery. The machinery responsible of this reaction is encoded by a cluster called "pgl" (for protein glycosylation).

[0179] The *C. jejuni* glycosylation machinery can be transferred to *E. coli* to allow for the glycosylation of recombinant proteins expressed by the *E. coli* cells. Previous studies have demonstrated how to generate *E. coli* strains that can perform N-glycosylation (see, e.g. Wacker et al. Science. 2002; 298 (5599):1790-3; Nita-Lazar et al. Glycobiology. 2005; 15(4):361-7; Feldman et al. Proc Natl Acad Sci U S A. 2005; 102(8):3016-21; Kowarik et al. EMBO J. 2006; 25(9):1957-66; Wacker et al. Proc Nall Acad Sci U S A. 2006; 103(18):7088-93; International Patent Application Publication Nos. WO2003/074687, WO2006/119987, WO 2009/104074, and WO2011/06261, and WO2011/138361). PglB mutants having optimised properties are described in W02016/107818. A preferred mutant is $PglB_{cuo\ N311V\text{-}K482R\text{-}D483H\text{-}A669V}$.

[0180] Oligosaccharyl transferases transfer lipid-linked oligosaccharides to asparagine residues of nascent polypeptide chains that comprise a N-glycosylation consensus motif, e.g. Asn-X-Ser(Thr), wherein X can be any amino acid except Pro; or Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any natural amino acid except Pro (see WO 2006/119987). See, e.g. WO 2003/074687 and WO 2006/119987.

[0181] The host cells comprise a nucleic acid that encodes an oligosaccharyl transferase. The nucleic acid that encodes an oligosaccharyl transferase can be native to the host cell, or can be introduced into the host cell using genetic approaches, as described above. Preferably, the oligosaccharyl transferase is an oligosaccharyl transferase from *Campylobacter*, specifically *Campylobacter jejuni* (i.e. pglB; see, e.g. Wacker et al. 2002, Science 298:1790-1793; see also, e.g. NCBI Gene ID: 3231775, UniProt Accession No. 086154).

[0182] Hence, bioconjugates of *S. aureus* capsular polysaccharides may be produced in a prokaryotic host cell comprising (i) a glycosyltransferase derived from an capsular polysaccharide cluster from *S. aureus*, wherein said glycosyltransferase is integrated into the genome of said host cell; (ii) a nucleic acid encoding an oligosaccharyl transferase (e.g. pgIB from *Campylobacter jejuni*), wherein said nucleic acid encoding an oligosaccharyl transferase is plasmid-borne and/or integrated into the genome of the host cell; and (iii) a polypeptide of the invention, wherein said polypeptide is either plasmid-borne or integrated into the genome of the host cell. The waaL gene of the host cell may been functionally inactivated or deleted, e.g. replaced by a nucleic acid encoding an oligosaccharyltransferase, for example by *C. jejuni pglB*.

[0183] To produce poly- and oligosaccharides of capsular saccharides, a polymerase (e.g. wzy) is introduced into a host cell (i.e. the polymerase is heterologous to the host cell). For production of *S. aureus* CP5 and CP8, the polymerase introduced into the host cells is the wzy gene from a capsular polysaccharide gene cluster of *S. aureus* CP5 or CP8 (cap5J/cap81).

[0184] Finally, a flippase (wzx or homologue) is introduced into the host cell (i.e. the flippase is heterologous to the host cell). Flippases translocate wild type repeating units and/or their corresponding engineered (hybrid) repeating units from the cytoplasm into the periplasm of host cells (e.g. *E. coli*). Thus, a host cell may comprise a nucleic acid that encodes a flippase (wzx). Preferably, a flippase of a capsular polysaccharide biosynthetic pathway of *S. aureus* is introduced into a host cell. The flippase introduced into the host cells may be the *capK* gene from a capsular polysaccharide gene cluster of *S. aureus* CP5 or CP8. Other flippases that can be introduced into the host cells are for example from *Campylobacter jejuni* (e.g. *pglK*).

[0185] The bioconjugates of the invention can be purified for example, by chromatography (e.g. ion exchange, cationic exchange, anionic exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. See, e.g. Saraswat et al. 2013, Biomed. Res. Int. ID#312709 (p. 1-18); see also the methods described in WO 2009/104074. Further, the bioconjugates may be fused to heterologous polypeptide sequences described herein or otherwise known in the art to facilitate purification. For example, the Hla protein may incorporate a peptide tag such as a HRHR tag for purification by cationic exchange (e.g. SEQ ID NO: 12). The actual conditions used to purify a particular bioconjugate will depend, in part, on the synthesis strategy and on factors such as net charge, hydrophobicity, and/or hydrophilicity of the bioconjugate, and will be apparent to those having skill in the art.

[0186] A further aspect of the invention is a process for producing a bioconjugate that comprises (or consists of) a ClfA or Hla antigen linked to a saccharide, said method comprising (i) culturing the host cell of the invention under conditions suitable for the production of proteins (and optionally under conditions suitable for the production of saccharides) and (ii) isolating the bioconjugate produced by said host cell.

[0187] A further aspect of the invention is a bioconjugate produced by the process of the invention, wherein said bioconjugate comprises a *S. aureus* polysaccharide linked to a ClfA or Hla antigen.

Immunogenic Compositions and Vaccines

[0188] The immunogenic composition of the present invention may be formulated into pharmaceutical compositions prior to administration to a subject. According to one aspect, the invention provides a pharmaceutical composition comprising an immunogenic composition of the invention and a pharmaceutically acceptable excipient or carrier.

[0189] The present invention also provides a vaccine comprising an immunogenic composition of the invention and a pharmaceutically acceptable excipient or carrier.

[0190] Also provided is an adjuvant composition for use with the immunogenic compositions and vaccines of the invention as described herein, which composition comprises an adjuvant and a pharmaceutically acceptable excipient or carrier.

[0191] Pharmaceutically acceptable excipients and carriers can be selected by those of skill in the art. For example, the pharmaceutically acceptable excipient or carrier can include a buffer, such as Tris (trimethamine), phosphate (e.g. sodium phosphate), acetate, borate (e.g. sodium borate), citrate, glycine, histidine and succinate (e.g. sodium succinate), suitably sodium chloride, histidine, sodium phosphate or sodium succinate. The pharmaceutically acceptable excipient may include a salt, for example sodium chloride, potassium chloride or magnesium chloride. Optionally, the pharmaceutically acceptable excipient contains at least one component that stabilizes solubility and/or stability. Examples of solubilizing/stabilizing agents include detergents, for example, laurel sarcosine and/or polysorbate (e.g. TWEEN™ 80). Examples of stabilizing agents also include poloxamer (e.g. poloxamer 124, poloxamer 188, poloxamer 237, poloxamer 338 and poloxamer 407). The pharmaceutically acceptable excipient may include a non-ionic surfactant, for example polyoxyethylene sorbitan fatty acid esters, Polysorbate-80 (TWEEN™ 80), Polysorbate-60 (TWEEN™ 60), Polysorbate-40 (TWEEN™ 40) and Polysorbate-20 (TWEEN™ 20), or polyoxyethylene alkyl ethers (suitably polysorbate-80). Alternative solubilizing/stabilizing agents include arginine, and glass forming polyols (such as sucrose, trehalose and the like). The pharmaceutically excipient may be a preservative, for example phenol, 2-phenoxyethanol, or thiomersal. Other pharmaceutically acceptable excipients include sugars (e.g. lactose, sucrose), and proteins (e.g. gelatine and albumin). Pharmaceutically acceptable carriers include water, saline solutions, aqueous dextrose and glycerol solutions. Numerous pharmaceutically acceptable excipients and carriers are described, for example, in Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co. Easton, Pa., 5th Edition (975).

[0192] In an embodiment, the compositions comprises a buffer. The pH of a liquid preparation is adjusted in view of the components of the composition and necessary suitability for administration to the subject. Suitably, the pH of a liquid mixture is at least 4, at least 5, at least 5.5, at least 5.8, at least 6. The pH of the liquid mixture may be less than 9, less than 8, less than 7.5 or less than 7. In other embodiments, pH of the liquid mixture is between 4 and 9, between 5 and 8, such as between 5.5 and 8.

[0193] An appropriate buffer may be selected from acetate, citrate, histidine, maleate, phosphate, succinate, tartrate and TRIS. In one embodiment, the buffer is a phosphate buffer such as Na/Na_2PO_4, Na/K_2PO_4 or K/K_2PO_4.

[0194] The buffer can be present in the liquid mixture in an amount of at least 6 mM, at least 10 mM or at least 40 mM. The buffer can be present in the liquid mixture in an amount of less than 100 mM, less than 60 mM or less than 40 mM.

[0195] In an embodiment, the compositions of the invention have a pharmaceutically acceptable osmolality to avoid cell distortion or lysis. A pharmaceutically acceptable osmolality will generally mean that solutions will have an osmolality which is approximately isotonic or mildly hypertonic. Suitably the compositions of the present invention when reconstituted will have an osmolality in the range of 250 to 750 mOsm/kg, for example, the osmolality may be in the range of 250 to 550 mOsm/kg, such as in the range of 280 to 500 mOsm/kg.

[0196] Osmolality may be measured according to techniques known in the art, such as by the use of a commercially available osmometer, for example the Advanced® Model 2020 available from Advanced Instruments Inc. (USA).

[0197] An "isotonicity agent" is a compound that is physiologically tolerated and imparts a suitable tonicity to a formulation to prevent the net flow of water across cell membranes that are in contact with the formulation. In some embodiments, the isotonicity agent used for the composition is a salt (or mixtures of salts). In other embodiments, however, the composition comprises a non-ionic isotonicity agent and the concentration of sodium chloride in the composition is less than 100 mM, such as less than 80 mM, e.g. less than 50 mM, such as less 40 mM, less than 30 mM and especially less than 20 mM. The ionic strength in the composition may be less than 100 mM, such as less than 80 mM, e.g. less than 50 mM, such as less 40 mM or less than 30 mM.

[0198] In a particular embodiment, the non-ionic isotonicity agent is a polyol, such as sorbitol. The concentration of sorbitol may e.g. between about 3% and about 15% (w/v), such as between about 4% and about 10% (w/v). Adjuvants comprising an immunologically active saponin fraction and a TLR4 agonist wherein the isotonicity agent is salt or a polyol have been described in WO2012/080369.

[0199] In an embodiment, the compositions of the invention additionally comprise one or more salts, e.g. sodium chloride, calcium chloride, sodium phosphate, monosodium glutamate, and Aluminum salts (e.g. Aluminum hydroxide, Aluminum phosphate, Alum (potassium Aluminum sulfate), or a mixture of such Aluminum salts). In other embodiments, the compositions of the invention does not comprise a salt.

[0200] The invention also provides a method of making the immunogenic composition or vaccine of the invention comprising the step of mixing antigens of the invention with a pharmaceutically acceptable excipient or carrier.

[0201] Immunogenic compositions comprise an immunologically effective amount of the protein or conjugate (e.g. bioconjugate) of the invention, as well as any other components. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either as a single dose or as part of a series is effective for treatment or prevention. This amount varies depending on the health and physical condition of the individual to be treated, age, the degree of protection desired, the formulation of the vaccine and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

[0202] Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M. F. & Newman M. J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, U.S. Pat. No. 4,235,877.

[0203] The immunogenic compositions and vaccines of the invention can be included in a container, pack, or dispenser together with instructions for administration. The invention provides a kit comprising (i) a first container comprising an immunogenic composition ora vaccine of the invention; and (ii) a second container comprising an adjuvant as described herein.

Adjuvants

[0204] In an embodiment, the immunogenic compositions of the invention comprise, or are administered in combination with, an adjuvant. The adjuvant for administration in combination with an immunogenic composition of the invention may be administered before, concomitantly with, or after administration of said immunogenic composition or vaccine. In an embodiment, said adjuvant is mixed with the immunogenic composition before administration.

[0205] Adjuvants can enhance an immune response by several mechanisms including, e.g. lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages. In an embodiment, the adjuvant is selected to be a preferential inducer of either a TH1 or a TH2 type of response, preferably a TH1 type response. High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to a given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen. It is important to remember that 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 +ve T cell clones by Mosmann and Coffman (Mosmann, T. R. and Coffman, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). 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 II-4, IL-5, IL-6, IL-10. Suitable adjuvant systems which promote a predominantly Th1 response include: Monophosphoryl lipid A or a derivative thereof, particularly 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (for its preparation see GB 2220211 A); MPL, e.g. 3D-MPL and the saponin QS21 in a liposome, for example a liposome comprising cholesterol and DPOC; and a combination of monophosphoryl lipid A, for example 3-de-O-acylated monophosphoryl lipid A, together with either an Aluminium salt (for instance Aluminium phosphate or Aluminium hydroxide) or an oil-in-water emulsion. In such combinations, the antigen and 3D-MPL may be contained in the same particulate structures, allowing for more efficient delivery of antigenic and immunostimulatory signals. Studies have shown that

3D-MPL is able to further enhance the immunogenicity of an Alum-adsorbed antigen (Thoelen et al. Vaccine (1998) 16:708-14; EP 689454- B1).

[0206] In one embodiment the adjuvant comprises both a TLR4 agonist and immunologically active saponin. In an embodiment, the TLR4 agonist is a lipopolysaccharide. Suitably the saponin comprises an active fraction of the saponin derived from the bark of Quillaja Saponaria Molina, such as QS21. Suitably the lipopolysaccharide is a Lipid-A derivative such as 3D-MPL. In a specific embodiment, the lipopolysaccharide is 3D-MPL and the immunologically active saponin is QS21. In an embodiment, said adjuvant composition comprises a lipopolysaccharide and immunologically active saponin in a liposomal formulation. Suitably in one form of this embodiment, the adjuvant consists essentially of 3D-MPL and QS21, with optionally sterol which is preferably cholesterol.

[0207] Liposome size may vary from 30 nm to several um depending on the phospholipid composition and the method used for their preparation. In particular embodiments of the invention, the liposome size will be in the range of 50 nm to 500 nm and in further embodiments 50 nm to 200 nm. Optimally, the liposomes should be stable and have a diameter of ~100 nm to allow sterilisation by filtration.

[0208] Other TLR4 agonists which may be of use in the present invention include Glucopyranosyl Lipid Adjuvant (GLA) such as described in WO2008/153541 or WO2009/143457 or the literature articles Coler RN et al. (2011) Development and Characterization of Synthetic Glucopyranosyl Lipid Adjuvant System as a Vaccine Adjuvant. PLoS ONE 6(1): e16333. doi:10.1371/journal.pone.0016333 and Arias MA et al. (2012) Glucopyranosyl Lipid Adjuvant (GLA), a Synthetic TLR4 Agonist, Promotes Potent Systemic and Mucosal Responses to Intranasal Immunization with HIVgp140. PLoS ONE 7(7): e41144. doi:10.1371/journal.pone.0041144. WO2008/153541 or WO2009/143457 are incorporated herein by reference for the purpose of defining TLR4 agonists which may be of use in the present invention.

[0209] 4'-monophosporyl lipid A (MPL), which may be obtained by the acid hydrolysis of LPS extracted from a deep rough mutant strain of gram-negative bacteria, retains the adjuvant properties of LPS while demonstrating a toxicity which is reduced by a factor of more than 1000 (as measured by lethal dose in chick embryo eggs) (Johnson et al. 1987 *Rev. Infect. Dis.* 9 Suppl:S512-S516). LPS is typically refluxed in mineral acid solutions of moderate strength (e.g. 0.1 M HCl) fora period of approximately 30 minutes. This process results in dephosphorylation at the 1 position, and decarbohydration at the 6' position, yielding MPL.

[0210] 3-O-deacylated monophosphoryl lipid A (3D-MPL), which may be obtained by mild alkaline hydrolysis of MPL, has a further reduced toxicity while again maintaining adjuvanticity, see U.S. Pat. No. 4,912,094 (Ribi Immunochemicals). Alkaline hydrolysis is typically performed in organic solvent, such as a mixture of chloroform/methanol, by saturation with an aqueous solution of weak base, such as 0.5 M sodium carbonate at pH 10.5. Further information on the preparation of 3D-MPL is available in, for example, U.S. Pat. No. 4,912,094 and WO02/078637 (Corixa Corporation).

[0211] Quillaja saponins are a mixture of triterpene glycosides extracted from the bark of the tree *Quillaja saponaria*. Crude saponins have been extensively employed as veterinary adjuvants. Quil-A is a partially purified aqueous extract of the Quillaja saponin material. Quil A is a saponin preparation isolated from the South American tree *Quillaja Saponaria Molina* and was first described by Dalsgaard et al. in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254) to have adjuvant activity. Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of *Quillaja saponaria Molina*, which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response and is a preferred saponin in the context of the present invention. QS21 is a HPLC purified non toxic fraction of Quil A and its method of production is disclosed (as QA21) in U.S. Pat. No. 5,057,540. Preferably the adjuvant contains QS21 in substantially pure form, that is to say, the QS21 is at least 90% pure, for example at least 95% pure, or at least 98% pure.

[0212] Adjuvants containing combinations of lipopolysaccharide and Quillaja saponins have been disclosed previously, for example in EP0671948. This patent demonstrated a strong synergy when a lipopolysaccharide (3D-MPL) was combined with a Quillaja saponin (QS21). Good adjuvant properties may be achieved with combinations of lipopolysaccharide and quillaja saponin as immunostimulants in an adjuvant composition even when the immunostimulants are present at low amounts in a human dose, as described in WO2007/068907.

[0213] In a specific embodiment, QS21 is provided in its less reactogenic composition where it is quenched with an exogenous sterol, such as cholesterol for example. Several particular forms of less reactogenic compositions wherein QS21 is quenched with an exogenous cholesterol exist. In a specific embodiment, the saponin /sterol is in the form of a liposome structure (WO 96/33739, Example 1). In this embodiment the liposomes suitably contain a neutral lipid, for example phosphatidylcholine, which is suitably non-crystalline at room temperature, for example egg yolk phosphatidylcholine, dioleoyl phosphatidylcholine (DOPC) or dilauryl phosphatidylcholine. The liposomes may also contain a limited amount of a charged lipid which increases the stability of the liposome-saponin structure for liposomes composed of saturated lipids. In these cases the amount of charged lipid is suitably 1-20% w/w, preferably 5-10% w/w of the liposome composition. Suitable examples of such charged lipids include phosphatidylglycerol and phosphatidylserine. Suitably, the neutral liposomes will contain less than 5% w/w charged lipid, such as less than 3% w/w or less than 1% w/w. The ratio of sterol to phospholipid is 1-50% (mol/mol), suitably 20-25%.

[0214] Suitable sterols include β-sitosterol, stigmasterol, ergosterol, ergocalciferol and cholesterol. In one particular embodiment, the adjuvant composition comprises cholesterol as sterol. These sterols are well known in the art, for example cholesterol is disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat.

[0215] Where the active saponin fraction is QS21, the ratio of QS21:sterol will typically be in the order of 1:100 to 1:1 (w/w), suitably between 1:10 to 1:1 (w/w), and preferably 1:5 to 1:1 (w/w). Suitably excess sterol is present, the

ratio of QS21:sterol being at least 1:2 (w/w). In one embodiment, the ratio of QS21:sterol is 1:5 (w/w). The sterol is suitably cholesterol.

[0216] 3D-MPL is sold under the name MPL by GlaxoSmithKline Biologicals S.A. and is referred throughout the document as MPL or 3D-MPL. see, for example, U.S. Pat. Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094. 3D-MPL primarily promotes CD4+T cell responses with an IFN-g (Th1) phenotype. 3D-MPL can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle 3D-MPL is used. Small particle 3D-MPL has a particle size such that it may be sterile-filtered through a 0.22μm filter. Such preparations are described in WO 94/21292.

[0217] Suitable adjuvant compositions are those wherein liposomes are initially prepared without MPL (as described in WO 96/33739), and MPL is then added, suitably as small particles of below 100 nm particles or particles that are susceptible to sterile filtration through a 0.22 μm membrane. The MPL is therefore not contained within the vesicle membrane (known as MPL out). Compositions where the MPL is contained within the vesicle membrane (known as MPL in) also form an aspect of the invention. The antigen can be contained within the vesicle membrane or contained outside the vesicle membrane.

[0218] For a maximum batch size of 12 g, MPL liquid bulk preparation is carried over in sterile glass containers. The dispersion of MPL consists of the following steps: suspend the MPL powder in water for injection: disaggregate any big aggregates by heating (thermal treatment; reduce the particle size between 100 nm and 200 nm by microfluidisation; prefilter the preparation on a Sartoclean Pre-filter unit, 0.8/0.65 μm; sterile filter the preparation at room temperature (Sartobran P unit, 0.22 μm)

[0219] MPL powder is lyophilised by microfluidisation resulting in a stable colloidal aqueous dispersion (MPL particles of a size susceptible to sterile filtration). The MPL lyophilised powder is dispersed in water for injection in order to obtain a coarse 10 mg/ml suspension. The suspension then undergoes a thermal treatment under stirring. After cooling to room temperature, the microfluidisation process is started in order to decrease the particle size. Microfluidisation is conducted using Microfluidics apparatus M110EH, by continuously circulating the dispersion through a microfluidisation interaction chamber, at a defined pressure for a minimum amount of passages (number of cycles: n_{min}). The microfluidisation duration, representing the number of cycles, is calculated on basis of the measured flow rate and the dispersion volume. On a given equipment at a given pressure, the resulting flow rate may vary from one interaction chamber to another, and throughout the lifecycle of a particular interaction chamber. In the present example the interaction chamber used is of the type F20Y Microfluidics. As the microfluidisation efficiency is linked to the couple pressure—flow rate, the processing time may vary from one batch to another. The time required for 1 cycle is calculated on basis of the flow rate. The flow rate to be considered is the flow rate measured with water for injection just before introduction of MPL into the apparatus. One cycle is defined as the time (in minutes) needed for the total volume of MPL to pass once through the apparatus. The time needed to obtain n cycles is calculated as follows:

n x Quantity of MPL to treat (ml)/Flow Rate (ml/min)

[0220] The number of cycles is thus adapted accordingly. Minimum amount of cycles to perform (n_{min}) are described for the preferred equipment and interaction chambers used. The total amount of cycles to run is determined by the result of a particle size measurement performed after n_{min} cycles. A particle size limit (d_{lim}) is defined, based on historical data. The measurement is realized by photon correlation spectroscopy (PCS) technique, and d_{lim} is expressed as an unimodal result ($Z_{average}$). Under this limit, the microfluidisation can be stopped after n_{min} cycles. Above this limit, microfluidisation is continued until satisfactory size reduction is obtained, for maximum another 50 cycles.

[0221] If the filtration does not take place immediately after microfluidisation, the dispersed MPL is stored at +2 to +8° C. awaiting transfer to the filtration area.

[0222] After microfluidisation, the dispersion is diluted with water for injection, and sterile filtered through a 0.22 μm filter under laminal flow. The final MPL concentration is 1 mg/ml (0.80-1.20 mg/ml).

[0223] In the process of production of liposomes containing MPL the DOPC (Dioleyl phosphatidylcholine), cholesterol and MPL are dissolved in ethanol. A lipid film is formed by solvent evaporation under vacuum. Phosphate Buffer Saline (9 mM Na_2HPO_4, 41 mM KH_2PO_4, 100 mM NaCl) at pH 6.1 is added and the mixture is submitted to prehomogenization followed by high pressure homogenisation at 15,000 psi (around 15 to 20 cycles). This leads to the production of liposomes which are sterile filtered through a 0.22 μm membrane in an aseptic (class 100) area. The sterile product is then distributed in sterile glass containers and stored in a cold room (+2 to +8° C.).

[0224] In this way the liposomes produced contain MPL in the membrane (the "MPL in" embodiment of WO 96/33739).

[0225] QS21 is added in aqueous solution to the desired concentration.

[0226] The adjuvant AS01 comprises 3D-MPL and QS21 in a quenched form with cholesterol, and was made as described in WO 96/33739. In particular the AS01 adjuvant was prepared essentially as Example 1.1 of WO 96/33739. The $AS01_E$ adjuvant comprises: liposomes, which in turn comprise dioleoyl phosphatidylcholine (DOPC), cholesterol and 3D MPL [in an amount of 1000 μg DOPC, 250 μg cholesterol and 50 μg D-MPL, each value given approximately per vaccine dose], QS21 [50 μg/dose], phosphate NaCl buffer and water to a volume of 0.5 ml.

[0227] The $AS01_E$ adjuvant comprises the same ingredients as $AS01_B$ but at a lower concentration in an amount of 500 μg DOPC, 125 μg cholesterol, 25 μg 3D-MPL and 25 μg QS21, phosphate NaCl buffer and water to a volume of 0.5 ml.

[0228] In a preferred embodiment, the adjuvant used in the present invention is $AS01_E$.

Method of Administration

[0229] In one aspect, the immunogenic composition or vaccine of the invention is administered by the intramuscular delivery route. Intramuscular administration may be to the thigh or the upper arm. Injection is typically via a needle (e.g. a hypodermic needle). A typical intramuscular dose is 0.5 ml, as described below.

Dosage

[0230] The amount of conjugate antigen in each immunogenic composition or vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. The content of each protein antigen will typically be in the range 1-200 μg, suitably 1-100 μg, suitably 5-50 μg. The content of each saccharide antigen will typically be in the range 0.1-50 μg, suitably 0.1-10 μg, suitably 1-5 μg.

[0231] A dose which is in a volume suitable for human use is generally between 0.25 and 1.5 ml, although, for administration to the skin a lower volume of between 0.05 ml and 0.2 ml may be used. In one embodiment, a human dose is 0.5 ml. In a further embodiment, a human dose is higher than 0.5 ml, for example 0.6, 0.7, 0.8, 0.9 or 1 ml. In a further embodiment, a human dose is between 1 ml and 1.5 ml. In another embodiment, in particular when the immunogenic composition is for the paediatric population, a human dose may be less than 0.5 ml such as between 0.25 and 0.5 ml.

[0232] Where the immunogenic composition comprises an adjuvant comprising 3D-MPL and QS21, QS21 and 3D-MPL are preferably present in the same final concentration per human dose of the immunogenic composition. In an embodiment, the human dose of the immunogenic composition comprises 3D-MPL at a level of around 25 μg, for example between 20-30 μg, suitably between 21- 29 μg or between 22 and 28 μg or between 23 and 27 μg or between 24 and 26 μg, or 25 μg. In an embodiment, the human dose of the immunogenic composition comprises QS21 at a level of around 25 μg, for example between 20-30 μg, suitably between 21-29 μg or between 22 and 28 μg or between 23 and 27 μg or between 24 and 26 μg, or 25 μg. In a preferred embodiment, a human dose of immunogenic composition comprises a final level of 25 μg of 3D-MPL and 25 μg of QS2. In another embodiment, a human dose of immunogenic composition comprises a final level of 50 μg of 3D-MPL and 50 μg of QS21.

[0233] Where the immunogenic composition is for use in combination with an adjuvant composition comprising 3D-MPL and QS21, QS21 and 3D-MPL are preferably present in the same final concentration per human dose of the adjuvant composition. In an embodiment, the human dose of the adjuvant composition comprises 3D-MPL at a level of around 25 μg, for example between 20-30 μg, suitably between 21-29 μg or between 22 and 28 μg or between 23 and 27 μg or between 24 and 26 μg, or 25 μg. In an embodiment, an adjuvant composition in a volume which is suitable for a human dose which human dose of the adjuvant composition comprises QS21 at a level of around 25 μg, for example between 20-30 μg, suitably between 21-29 μg or between 22 and 28 μg or between 23 and 27 μg or between 24 and 26 μg, or 25 μg. In a preferred embodiment, a human dose of adjuvant composition comprises a final level of 25 μg of 3D-MPL and 25 μg of QS21. In another embodiment, a human dose of adjuvant composition comprises a final level of 50 μg of 3D-MPL and 50 μg of QS21.

[0234] Where the adjuvant is in a liquid form to be combined with a liquid form of an antigenic composition, the adjuvant composition will be in a human dose suitable volume which is approximately half of the intended final volume of the human dose, for example a 360 μl volume for an intended human dose of 0.7 ml, or a 250 μl volume for an intended human dose of 0.5 ml. The adjuvant composition is diluted when combined with the antigen composition to provide the final human dose of vaccine. The final volume of such dose will of course vary dependent on the initial volume of the adjuvant composition and the volume of antigen composition added to the adjuvant composition. In an alternative embodiment, liquid adjuvant is used to reconstitute a lyophilised antigen composition. In this embodiment, the human dose suitable volume of the adjuvant composition is approximately equal to the final volume of the human dose. The liquid adjuvant composition is added to the vial containing the lyophilised antigen composition. The final human dose can vary between 0.5 and 1.5 ml. In a particular embodiment the human dose is 0.5 ml.

Prophylactic and Therapeutic Uses

[0235] The present invention provides methods of treating and/or preventing bacterial infections of a subject comprising administering to the subject an immunogenic composition or vaccine of the invention. In a specific embodiment, the immunogenic composition of the invention is used in the prevention of infection of a subject (e.g. human subjects) by a staphylococcal bacterium. *S. aureus* infects various mammals (including cows, dogs, horses, and pigs), but the preferred subject for use with the invention is a human.

[0236] In a specific embodiment, the immunogenic composition of the invention is used to treat or prevent an infection by *Staphylococcus* species, in particular *S. aureus*. For example, the immunogenic composition of the invention may be used to prevent against *S. aureus* infection, including a nosocomial infection.

[0237] Also provided are methods of inducing an immune response in a subject against a staphylococcal bacterium, in particular *S. aureus*, comprising administering to the subject an immunogenic composition or vaccine of the invention. In one embodiment, said subject has bacterial infection at the time of administration. In another embodiment, said subject does not have a bacterial infection at the time of administration. The immunogenic composition or vaccine of the invention can be used to induce an immune response against *Staphylococcus* species, in particular *S. aureus.*

[0238] Also provided are methods of inducing the production of opsonophagocytic antibodies in a subject against a staphylococcal bacterium, in particular *S. aureus*, comprising administering to the subject an immunogenic composition or vaccine of the invention. In one embodiment, said subject has bacterial infection at the time of administration. In another embodiment, said subject does not have a bacterial infection at the time of administration.

[0239] Also provided are methods of inducing the production of antibodies able to neutralise or reduce the activity of staphylococcal Hla, ClfA and/or SpA in a subject, comprising administering to the subject an immunogenic composition or vaccine of the invention. Said Hla activity may be ability to lyse human erythrocytes (haemolysis). Said ClfA activity may be ability to bind to human fibrinogen. Said SpA activity may be ability to bind to Fcγ of immunoglobulin (Ig) and to the Fab portion of V_H3-type B cell receptors.

[0240] Also provided is a method of immunising a human host against staphylococcal infection, particularly *S. aureus* infection,comprising administering to the host an immunogenic composition or vaccine of the invention.

[0241] Also provided is a method of inducing an immune response to a staphylococcal bacterium, in particular *S. aureus*, in a subject, the method comprising administering a therapeutically or prophylactically effective amount of the an imunogenic composition or vaccine of the invention.

[0242] Also provided is an immunogenic composition or vaccine of the invention for use in a method of treatment and/or prevention of disease, for example for use a method of treatment or prevention of a disease caused by staphylococcal infection, particularly *S. aureus* infection.

[0243] Also provided is an immunogenic composition or vaccine of the invention in the manufacture of a medicament for the treatment or prevention of a disease caused by staphylococcal infection, particularly *S. aureus* infection.

[0244] Also provided is the use of an immunogenic composition or vaccine of the invention for the manufacture of a medicament for use in a method of treatment and/or prevention of disease, for example for use a method of treatment or prevention of a disease caused by staphylococcal infection, particularly *S. aureus* infection.

[0245] Also provided is a pharmaceutical for treatment or prevention of staphylococcal infection, particularly *S. aureus* infection, comprising an immunogenic composition or vaccine of the invention.

[0246] Also provided is an immunogenic composition or vaccine of the invention for use in a method of inducing an immune response in a subject against a staphylococcal bacterium, in particular *S. aureus*.

[0247] Also provided is an immunogenic composition or vaccine of the invention for use in a method of inducing the production of opsonophagocytic antibodies in a subject against a staphylococcal bacterium, in particular *S. aureus*.

[0248] Also provided is an immunogenic composition or vaccine of the invention for use in a method of inducing the production of antibodies able to neutralise or reduce the activity of staphylococcal Hla, ClfA and/or SpA in a subject, comprising administering to the subject an immunogenic composition or vaccine of the invention. Said Hla activity may be ability to lyse human erythrocytes (haemolysis). Said ClfA activity may be ability to bind to human fibrinogen. Said SpA activity may be ability to bind to Fcγ of immunoglobulin (Ig) and to the Fab portion of V_H3-type B cell receptors.

[0249] All references or patent applications cited within this patent specification are incorporated by reference herein.

[0250] Aspects of the invention are summarised in the subsequent numbered paragraphs:

[0251] 1. An immunogenic composition comprising

[0252] a. a ClfA antigen;

[0253] b. a Hla antigen;

[0254] c. a SpA antigen; and

[0255] d. a staphylococcal capsular polysaccharide.

[0256] 2. An immunogenic composition according to paragraph 1, wherein the capsular polysaccharide is conjugated to a carrier protein.

[0257] 3. An immunogenic composition according to paragraph 1 or paragraph 2, wherein the capsular polysaccharide is a *S. aureus* serotype 5 and/or type 8 capsular polysaccharide.

[0258] 4. An immunogenic composition according to any one of paragraphs 1 to 3, wherein the capsular polysaccharide is conjugated to one of the antigens (a)-(c) of paragraph 1.

[0259] 5. An immunogenic composition according to any one of paragraphs 1 to 4, wherein the composition comprises a *S. aureus* serotype 5 capsular polysaccharide and a type 8 capsular polysaccharide.

[0260] 6. An immunogenic composition according to any one of paragraphs 1 to 5, wherein the composition comprises a *S. aureus* serotype 5 capsular polysaccharide conjugated to a Hla antigen and/or a type 8 capsular polysaccharide conjugated to a ClfA antigen.

[0261] 7. An immunogenic composition according to any one of paragraphs 1 to 6, wherein

[0262] a. the ClfA antigen is a ClfA protein comprising the amino acid sequence of SEQ ID NO. 2 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2, or immunogenic fragment thereof;

[0263] b. the Hla antigen is a Hla protein having the amino acid sequence of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3 or immunogenic fragment thereof; and/or

[0264] c. the SpA antigen is a SpA protein having an amino acid sequence of SEQ ID NO. 13 or SEQ ID NO: 26or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 13 or SEQ ID NO: 26, or immunogenic fragment thereof.

[0265] 8. A composition according to any of paragraphs 1 to 7, wherein the ClfA antigen comprises at least one amino acid substitution selected from P116 to S and Y118 to A with reference to the amino acid sequence of SEQ ID NO. 2 (or an equivalent position in an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 2), optionally comprising the sequence of any one of SEQ ID NOs 5-7 or 32.

[0266] 9. An immunogenic composition according to paragraph 7 or paragraph 8, wherein the ClfA antigen comprises one or more PglB consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline.

[0267] 10. An immunogenic composition according to paragraph 9, wherein said consensus sequence has been added at, or substituted for, one or more amino acids between amino acid residues 313-342 of SEQ ID NO: 2, optionally substituted for the amino acid at position 1337, or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 2.

[0268] 11. An immunogenic composition according to paragraph 9 or paragraph 10, wherein X is Q (glutamine) and Z is A (alanine) (e.g. K-D-Q-N-A-T-K, SEQ ID NO: 31.

[0269] 12. An immunogenic composition according to paragraph 11, wherein the ClfA antigen comprises or consists of the sequence of SEQ ID NO: 7 or SEQ ID NO: 32.

[0270] 13. An immunogenic composition according to any one of paragraphs 1 to 12, wherein the Hla antigen comprises an amino acid substitution at position H35 of SEQ ID NO. 3 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%,

95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3, wherein said amino acid substitution is optionally H to L.

[0271] 14. An immunogenic composition according to any one of paragraphs 1 to 13, wherein the Hla antigen comprises one or more PglB consensus sequence(s) selected from: D/E-X-N-Z-S/T (SEQ ID NO. 28) and K-D/E-X-N-Z-S/T-K (SEQ ID NO. 29), wherein X and Z are independently any amino acid apart from proline.

[0272] 15. An immunogenic composition according to paragraph 14, wherein said consensus sequence has been added at, or substituted for, one or more amino acids of the amino acid sequence of SEQ ID NO. 3 or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO. 3.

[0273] 16. An immunogenic composition according to paragraph 14, wherein said consensus sequence has been substituted for the amino acid at position K131 of SEQ ID NO. 3 of SEQ ID NO: 3, or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 3.

[0274] 17. An immunogenic composition according to any one of paragraphs 14 to 16, wherein X is Q (glutamine) and Z is R (arginine) (e.g. K-D-Q-N-R-T-K (SEQ ID NO 30)

[0275] 18. An immunogenic composition according to paragraph 17, wherein the Hla antigen comprises or consists of the sequence of SEQ ID NO: 11 or SEQ ID NO 12.

[0276] 19. The immunogenic composition according to any one of paragraphs 1 to 18, wherein the SpA antigen comprises (a) one or more amino acid substitutions in a V_H3-binding sub-domain of domain E, D, A, B or C that disrupts or decreases binding to V_H3, and (b) one or more amino acid substitutions in an IgG Fc binding sub-domain of domain E, D, A, B or C that disrupts or decreases binding to IgG Fc.

[0277] 20. An immunogenic composition according to any one of paragraphs 1 to 19, wherein the SpA antigen comprises (i) a domain E with an amino acid substitution at the amino acid positions 34 and 35 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 39 and 40 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 36 and 37 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 36 and 37 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18; and/or (ii) comprises a domain E with an amino acid substitution at amino acid positions 7 and 8 of SEQ ID NO: 14 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 14; a domain D with an amino acid substitution at amino acid positions 12 and 13 of SEQ ID NO: 15 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15; a domain A with an amino acid substitution at positions 9 and 10 of SEQ ID NO: 16 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 16; a domain B with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 17 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 17, and/or a domain C with an amino acid substitution at positions amino acid positions 9 and 10 of SEQ ID NO: 18 or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 18.

[0278] 21. An immunogenic composition according to paragraph 20, wherein said amino acid substitutions are substitution of lysine for glutamine and/or substitution of alanine for aspartic acid.

[0279] 22. An immunogenic composition of any one of paragraphs 1 to 21, wherein the SpA antigen comprises a domain D with an amino acid substitution at amino acid positions 4 and 5 of SEQ ID NO: 15. or at an equivalent position within an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 15

[0280] 23. An immunogenic composition according to paragraph 22, wherein said amino acid substitution is glutamine to lysine and/or glutamine to arginine, e.g. QQ to KR.

[0281] 24. An immunogenic composition according to any one of paragraphs 1 to 23, wherein said SpA antigen comprises an amino acid sequence of SEQ ID NOs: 19-23, 26 or 27, or an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NOs: 19-23, 26 or 27.

[0282] 25. An immunogenic composition according to any one of paragraphs 1 to 24, wherein the immunogenic composition comprises (i) a ClfA antigen comprising the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 32; (ii) a Hla antigen comprising the amino acid sequence sequence of SEQ ID NO: 11 or SEQ ID NO: 12; (iii) an SpA antigen comprising the amino acid sequence of SEQ ID NO: 27; (iv) a *S. aureus* serotype 5 capsular polysaccharide, and (v) a *S. aureus* serotype type 8 capsular polysaccharide.

[0283] 26. An immunogenic composition according to paragraph 25, wherein the ClfA antigen is conjugated to the *S. aureus* serotype type 8 capsular polysaccharide, and the Hla antigen is conjugated to the *S. aureus* serotype 5 capsular polysaccharide.

[0284] 27. An immunogenic composition according to paragraph 26, wherein said ClfA-CP8 and Hla-CP5 conjugates are bioconjugates.

[0285] 28. An immunogenic composition according to any one of paragraphs 1 to 27, which composition comprises an adjuvant.

[0286] 29. An immunogenic composition according to paragraph 28, wherein the adjuvant comprises a saponin and a lipopolysaccharide.

[0287] 30. An immunogenic composition according to paragraph 29, wherein the adjuvant comprises a saponin, a lipopolysaccharide in a liposomal formation.

[0288] 31. An immunogenic composition according to paragraph 29 or paragraph 30, further comprising a sterol.

[0289] 32. An immunogenic composition according to paragraph any one of paragraphs 29 to 31, wherein the saponin is an immunologically active saponin fraction derived from the bark of *Quillaja Saponaria Molina.*

[0290] 33. An immunogenic composition according to paragraph 32, wherein the saponin is QS21.

[0291] 34. An immunogenic composition according to any one of paragraphs 29 to 33, wherein the lipopolysaccharide is a lipid A derivative.

[0292] 35. An immunogenic composition according to paragraph 34, wherein the lipopolysaccharide is 3D-MPL.

[0293] 36. An immunogenic composition according to any one of paragraphs 31 to 35, wherein the sterol is cholesterol.

[0294] 37. An immunogenic composition according to any one of paragraphs 29 to 36, wherein the immunogenic composition comprises QS21 at a level of 25 μg per human dose.

[0295] 38. An immunogenic composition according to any one of paragraphs 29 to 37, wherein the immunogenic composition comprises 3D-MPL at a level of 25 μg per human dose.

[0296] 39. A vaccine comprising an immunogenic composition according to any one of paragraphs 1 to 27 and a pharmaceutically acceptable excipient or carrier.

[0297] 40. A vaccine comprising an immunogenic composition according to any one of paragraphs 28 to 38 and a pharmaceutically acceptable excipient or carrier.

[0298] 41. An immunogenic composition according to any one of paragraphs 1 to 27, or a vaccine according to paragraph 39, for use in a method of prevention or treatment of staphylococcal infection, wherein said composition is administered in combination with an adjuvant.

[0299] 42. An immunogenic composition or vaccine for use according to paragraph 41, wherein the immunogenic composition is mixed with the adjuvant before administration to a subject.

[0300] 43. An immunogenic composition or vaccine for use according to paragraph 41 or paragraph 42, wherein the adjuvant is as defined in any one of paragraphs 29 to 36.

[0301] 44. An immunogenic composition or vaccine for use according to paragraph 43, wherein the adjuvant comprises 3D-MPL at a level of 25 pg per human dose and QS21 at a level of 25 μg per human dose.

[0302] 45. A kit comprising (i) a first container comprising an immunogenic composition of any one of paragraphs 1 to 27 or a vaccine according to paragraph 39; and (ii) a second container comprising an adjuvant.

[0303] 46. A kit according to paragraph 45, wherein the adjuvant is as defined in any one of paragraphs 29 to 36.

[0304] 47. A kit according to paragraph 46, wherein the adjuvant comprises 3D-MPL at a level of 25 μg per human dose and QS21 at a level of 25 μg per human dose.

[0305] 48. A method of prevention or treatment of staphylococcal infection, comprising administering to a subject in need thereof an immunogenic composition according to any one of paragraphs 1 to 27 or a vaccine according to paragraph 39.

[0306] 49. A method according to paragraph 48, further comprising administering an adjuvant to said subject.

[0307] 50. A method according to paragraph 49, wherein said adjuvant is administered concomitantly with the immunogenic composition.

[0308] 51. A method according to paragraph 49 or paragraph 50, wherein the adjuvant is as defined in any one of paragraphs 29 to 36.

[0309] 52. A method of prevention or treatment of staphylococcal infection, comprising administering to a subject in need thereof an immunogenic composition according to any one of paragraphs 28 to 38 or a vaccine according to paragraph 40.

[0310] 53. A method of making an immunogenic composition or vaccine according to any one of paragraphs, comprising the steps of mixing antigens, and optionally an adjuvant, with a pharmaceutically acceptable excipient.

EXAMPLES

Example 1: Vaccine Composition and Formulation

[0311] Vaccine Components

[0312] Bioconjugates of CPS, containing 6 to 20 repeating units, linked to Hla_{mut} containing one glycosylation site, H35L/H48C/G122C mutations and a C-terminal HRHR tag (SEQ ID NO: 12, expressed using a FIgL signal sequence) were produced using fed-batch fermentation of *E coli* cells transformed with the plasmids encoding the *S. aureus* capsular polysaccharide CP5, the *S. aureus* carrier protein $Hla_{H35L-H48C-G122C}$ carrying a glycosylation site at position 131 and a C-terminal histidine-arginine-histidine-arginine tag, and *Campylobacter jejuni* oligosaccharyltransferase $PglB_{cuo\ N311V-K482R-D483H-A669V}$.

[0313] Bioconjugates of CP8, 5 to 16 repeating units, linked to $ClfA_{mut}$ comprising the ClfA N2/N3 domains with one glycosylation site and P116S/Y118A mutations (SEQ ID NO. 7) were produced by fed-batch fermentation of *E coli* cells expressing $PglB_{cuo\ N311V-K482R-D483H-A669V}$ and *S. aureus* CP8 (W3110 waaL::$pglB_{cuo\ N311V-K482R-D483H-A669V}$; O16::O11_wbjB-wbpM; ΔrmlB-wecG; wecA-wzzE::CP8_p2636(CCW)_Cat) and transformed with the plasmid encoding $ClfA_{mut}$.

[0314] Bioconjugates were tested for stability in different formulations The stability of three different formulations for each batch was tested at −80° C. (long term), +4° C. (intermediate) and +25° C. up to 3 months by applying part of the analytical panel reported in the table above to evaluate content, purity, aggregation, degradation with regards to protein and polysaccharide stability.

[0315] CP5-Hla and CP8-ClfA bioconjugates were stable at 4° C. and −80° C. for at least 3 months and up to 3 months at 25° C.

[0316] A SpA variant ($SpA_{KR\text{-}KKAA}$, herein indicated as SpA_{mut} and comprising the sequence of SEQ ID No: 27, described in WO2015/144653) comprising the IgG binding portion (IgG binding domains EDABC) harbouring amino acid substitutions at four key residues in each of the five IgBD (glutamine and aspartate changed into lysine and alanine respectively), which highly impaired IgG and IgM binding, as well as two additional immunoglobulin binding residues corresponding to Q70 and Q71 of the IgG binding portion (Q96 and Q97 of the full-length protein) which were mutated into lysine 70 and arginine 71 respectively (K70 and R71). SpA_{mut} was shown to have no detectable affinity to human IgG and IgM by surface plasmon resonance experiments, making the molecule safer for vaccine usage. SpA_{mut} was expressed in recombinant form by bacterial batch fermentation using a commercial *Escherichia coli* BL21 (DE3) strain.

[0317] Accelerated stability studies were performed by using SDS-PAGE and SE-UPLC for the analysis of the protein after storage for 3, 10 and 30 days at 4° C., at RT and at 37° C. in four suitable formulation buffers. SpA_{mut} was stable at 4° C. up to 3 months, stable at 25° C. up to 10 days, and stable at 37° C. up to 4 days.

[0318] Vaccine Formulation

[0319] Formulations for mouse immunisation studies were prepared unadjuvanted and adjuvanted with $AS01_E$ (3D-MPL +QS21 in a liposome composition, as described above) and Alum/TLR7 (TLR7 agonist adsorbed to AlOH, as described in WO2011/027222, Example 20, WO2012/031140 and Bagnoli et al, PNAS, 2015, 112: 3680-3685). The three components (CP5-Hla, CP8-ClfA, SpA_{mut}) were formulated in the same vial at a concentration of 200 μg/ml for each component (two fold concentrated, here referred as 2X, with respect to final high dose), based on protein content. To obtain the final different formulations, vial 2X with the three mixed components were reconstituted with formulation buffer (10 mM NaH_2PO_4, 150 mM NaCl pH 6.5) or adjuvants (two fold concentrated, with respect to adjuvant final dose) through a bed-side mixing approach prior to immunisation. High protein dosage was reached by reconstituting one volume of vial 2X with an equivalent volume of $AS01_B$ (two fold concentrated compared to $AS01_E$), or Alum/TLR7 (two fold concentrated, Alum /TLR7 at 20 μg/dose) or, alternatively, formulation buffer for the non-adjuvanted group in order to have the desired final protein concentration of 100 μg/ml (equivalent to 10 μg/100 μl). Medium and low protein dosage were instead obtained through a pre-dilution step of vial 2X, respectively one to ten and one to twenty with formulation buffer, prior to final reconstitution with an equivalent volume of $AS01_B$ (2X) or Alum /TLR7 (2X) to reach final concentration of 10 μg/ml (equivalent to 1 μg/100μl) and 1 μg/ml (equivalent to 0.1 μg/100μl). Formulation buffer was used as diluent for control group. Compatibility studies were conducted in order to evaluate the short term stability of the formulation (up to 24 hours at 2-8° C. storage condition), both for vial with mix 2X and after the reconstitution process with adjuvants or placebo. Extended analytical panel was applied to characterise the formulations, through evaluation of the following parameters:

[0320] 1. Physico-chemical (visual inspection, pH and osmolality)

[0321] 2. Antigen identity (Western Blot for each single component, both protein and saccharide)

[0322] 3. Total protein content (μBCA)

[0323] 4. Sterility (microbial contamination through plate growth)

[0324] 5. AS01 particle size (DLS)

[0325] 6. Alum/TLR7 recovery (RP-HPLC)

[0326] No major incompatibilities were observed for the antigens/adjuvant or placebo combinations considered for evaluation in pre-clinical studies.

Example 2: Immunogenicity in Mice and Pre-Exposed Rabbits to Vaccine Adjuvanted with $AS01_E$ or ALUM/TLR7

[0327] The vaccine formulation was used to immunise different animal species:

[0328] i) Naïve mice, to investigate in vivo induction of both IgG and T cell response specific for the vaccine components. In these studies the vaccine was tested either non adjuvanted or adjuvanted with $AS01_E$ or Alum/TLR7 at different immunisation doses.

[0329] ii) *S. aureus* pre-exposed rabbits, to evaluate the response to the vaccine in a model in which pre-existing levels of IgG specific for the vaccine components are present. In this study the vaccine was tested at different doses either adjuvanted with $AS01_E$ or non adjuvanted. Alum /TLR7 adjuvant could not be used in this model due to the lack of the agonist receptor on rabbit cells.

[0330] The specific immune response was assessed by:

[0331] a) Measuring antigen-specific IgG by Luminex technology, both in mouse and rabbit immune sera at different time points after vaccination.

[0332] b) Evaluating the presence of CD4+T cells specific for the vaccine antigens in spleens of immunised mice.

[0333] c) Measuring the capability of antibodies present in mouse and rabbit sera to neutralise the biological activities of two of the protein components, the wild-type Hla and ClfA antigens.

[0334] An overview of the studies is presented in Table 1.

TABLE 1

Overview of vaccine in vivo preclinical studies in mice and rabbits

Species, Sex/Age	Objectives	Antigen	Number of animals	Regimen	Dose protein (ug)	Dose polysaccharides (ug)*	Adjuvant#	Injection Volume/Route
BALB/c mice/female/ 5-week old	IgG quantitation, adjuvants comparison and sera testing in	1-Vaccine	30	2 injections 4 weeks apart	10	2	none	100 μl/IM
		2-Vaccine	30		1	0.2	none	100 μl/IM
		3-Vaccine	30		0.1	0.02	none	100 μl/IM
		4-Vaccine	30		10	2	Alum/TLR7	100 μl/IM
		5-Vaccine	30		1	0.2	Alum/TLR7	100 μl/IM
		6-Vaccine	30		0.1	0.02	Alum/TLR7	100 μl/IM

TABLE 1-continued

Overview of vaccine in vivo preclinical studies in mice and rabbits

Species, Sex/Age	Objectives	Antigen	Number of animals	Regimen	Dose protein (ug)	Dose polysaccharides (ug)*	Adjuvant#	Injection Volume/Route
	in vitro	7-Vaccine	30		10	2	$AS01_E$	100 μl/IM
	assays	8-Vaccine	30		1	0.2	$AS01_E$	100 μl/IM
		9-Vaccine	30		0.1	0.02	$AS01_E$	100 μl/IM
		10-Buffer	15		—	—	none	100 μl/IM
		11-Buffer	15		—	—	Alum/TLR7	100 μl/IM
		12 Buffer	15		—	—	$AS01_E$	100 μl/IM
Rabbit (New Zealand)/male/ 12-14-week old	IgG quantitation, adjuvants comparison and sera testing in in vitro assays	1-Buffer	12	2 injections 4 weeks apart	buffer	—	none	500 μl/IM
		2-Vaccine	12		1 μg	0.2	none	500 μl/IM
		3-Vaccine	12		10 μg	2	none	500 μl/IM
		4-Vaccine	12		50 μg	10	none	500 μl/IM
		5-Vaccine	12		1 μg	0.2	$AS01_E$	500 μl/IM
		6-Vaccine	12		10 μg	2	$AS01_E$	500 μl/IM
		7-Vaccine	12		50 μg	10	$AS01_E$	500 μl/IM
BALB/C Mice/female/ 5-week old	T cell response analysis	1-Vaccine	15	2 injections 4 weeks apart	10 μg	2	none	100 μl/IM
		2-Vaccine	15		10 μg	2	ALUM/TLR7	100 μl/IM
BALB/C Mice/female/ 5-week old	T cell response analysis	1-Vaccine	15	2 injections 4 weeks apart	10 μg	2	none	100 μl/IM
		3-Vaccine	15		10 μg	2	$AS01_E$	100 μl/IM
		4-Vaccine	15		1 μg	0.2	none	100 μl/IM
		5-Vaccine	15		1 μg	0.2	ALUM/TLR7	100 μl/IM
		6-Vaccine	15		1 μg	0.2	$AS01_E$	100 μl/IM
		7-buffer	6		—	—	none	100 μl/IM
		8-buffer	6		—	—	ALUM/TLR7	100 μl/IM
		9-buffer	6		—	—	$AS01_E$	100 μl/IM

*Polysaccharide in the bioconjugates content is about $1/5^{th}$ as compared to protein.

#Alum/TLR7 adjuvant dose used is 10 μg of TLR7 agonist adsorbed to Aluminum hydroxide.

$AS01_E$ adjuvant used is 1/20 of the human dose in mice (2.5 μg of MPL and 2.5 μg of QS-21 *Quillaja saponaria* Molina, fraction 21) and 1/2 of the human dose in rabbits (12.5 μg of MPL and 12.5 μg of QS-21).

[0335] Immunisation of Mice

[0336] Five week-old female mice were given two doses of 100 μl of either the buffer or the tested formulations (50 μl in each hind leg quadriceps) 28 days apart by the IM route. Blood samples were taken before 1^{st} injection (day 0), 1 week, 2 and 4 weeks after the first injection (1wp1, 2wp1, 4wp1), and 1, 2, 4, 8, 12 and 16 weeks after the second injection (1wp2, 2wp2, 4wp2, 8wp2, 12wp2, 6wp2). The experiment was repeated 3 times in order to assess experimental variability. The statistical analysis was conducted on the pooled sample for each group. An overview of the study with the total number of mice used in the three experiments is shown in Table 2.

TABLE 2

Study Design for determination of IgG titre in immunised mice

Group	Total Number of mice	Antigen	Protein Dose (μg)	Polysaccharides Dose (μg)	Adjuvant#	Injection Volume/Route
1	30	Vaccine	10	2	none	100 μl/IM
2	30	Vaccine	1	0.2	none	100 μl/IM
3	30	Vaccine	0.1	0.02	none	100 μl/IM
4	30	Vaccine	10	2	Alum/TLR7	100 μl/IM
5	30	Vaccine	1	0.2	Alum/TLR7	100 μl/IM
6	30	Vaccine	0.1	0.02	Alum/TLR7	100 μl/IM
7	30	Vaccine	10	2	$AS01_E$	100 μl/IM
8	30	Vaccine	1	0.2	$AS01_E$	100 μl/IM
9	30	Vaccine	0.1	0.02	$AS01_E$	100 μl/IM
10	15	Buffer	—	—	none	100 μl/IM
11	15	Buffer	—	—	Alum/TLR7	100 μl/IM
12	15	Buffer	—	—	$AS01_E$	100 μl/IM

[0337] Evaluation of Vaccine-Specific IgG

[0338] The serological analysis to evaluate vaccine-specific IgG in mice and rabbits was based on a multiplex assay by the Luminex technology. This technology has been used to measure antibodies in human sera from subjects immunised with a vaccine against *StaphylococcuS. aureus* (Raedler et al, Clin Vaccine Immuno 2009, 16: 739-49).

[0339] The assay analyses five antigens simultaneously (5-plex) using magnetic beads coated with the three SA recombinant proteins (SPA_{mut}, $ClfA_{mut}$, Hla_{mut}) and the two capsular polysaccharides of different serotypes (CP5 and CP8). Protein antigens are covalently conjugated to the free carboxyl groups of microspheres using an N-hydroxysulfosuccinimide-enhanced carbodiimide (EDC)-mediated conjugation chemistry. CP5 and CP8 are biotinylated using Biotin-Hydrazide (BH) and EDC, purified and subsequently conjugated to Streptavidin beads.

[0340] A pentavalent standard serum was prepared by pooling hyperimmune sera collected at D12 Post 2 from 5 mice immunised with Hla_{H35R}+$ClfA_{mut}$+CP5–TT+CP8–TT+SpA_{mut}-10 μg/Alum/TLR7. An arbitrary titre of 100 RLU/ml was assigned to the pentavalent standard.

[0341] The assay was set up accordingly to the following criteria:

[0342] No cross-reactivity between the five antigens observed by comparing signals obtained by monoplex versus multiplex setting.

[0343] Specificity of the assay confirmed by multiplex pre-adsorption studies.

[0344] Intra and inter-assay reproducibility of the assay (%CV<10% and 20% respectively).

[0345] Linearity assessment in the linear range of the 5PL MFI curve obtained with standard and test sera: R^2 values >0.9.

[0346] Lower Limits of Detection (LLOD, see table below) for each antigen were determined as mean RLU from black samples.

[0347] Lower Limits of Quantification (LLOQ, see table below): the lower titres that could be determined with acceptable accuracy (CV<20% in spiking experiments) using a Minimum Required dilution (MDR) of 1:1000.

	Hla	CP8	CIFA	SPA	CP5
	RLU/ml (MDR 1:1000)				
LOD = Mean + 3SD	0.03	0.01	0.04	0.05	0.02
LLOQ (RLU/ml) preiim based	0.7	0.18	3.09	0.96	0.77

[0348] For sample testing, up to five increasing dilutions were independently prepared and loaded onto 96 wells plates with an adequate amount of coupled microspheres. Two replicates of each dilution were tested. Antigen specific antibodies were revealed by an anti-mouse IgG Phycoerithrin-labelled secondary antibody and MFI were measured using the Luminex 200 Reader.

[0349] The analysis was performed using three experiments for each group at different time-points. In order to assess the biological variability among experiments for each antigen and dose, a 2-way mixed ANOVA was applied ("group" and "experiment" as fixed and "individual mice" as random effects). No significant group-experiment interaction was observed, except for $ClfA_{mut}$ and Hla_{mut} at dose 0.1 μg with p-value=0.001.

[0350] The overall results on mouse studies (FIG. **1**, data not shown for 0.1 μg dose) indicated that:

[0351] All the vaccine components were immunogenic in mice. Control groups (10, 11 and 12) showed no detectable antibodies against any of the five antigens. Similarly, no detectable IgGs were measured in pre-immune sera, except for Hla_{mut} antigen, for which 14% of estimated titres were slightly over the LLOQ.

[0352] Responses for vaccine without adjuvants were always inferior to responses to vaccine with adjuvants.

[0353] A significant dose effect was observed in all groups with and without adjuvant (1-way ANOVA was applied and the adjustments were made for multiple comparisons, using Tukey's "Honest Significant Difference."). In particular:

[0354] Without adjuvant: 0.1 μg<1 μg<10 μg for all antigens at post 2^{nd} immunisation (p-value **21** 0.01 Tukey's post-test).

[0355] $AS01_E$: 0.1 μg<1 μg<10 μg for $ClfA_{mut}$, HLA_{mut} and SpA_{mut} at any time points (p-value <0.05 Tukey's post-test); 0.02 μg<0.2 μg 5 2 μg (polysaccharide-based dose) for CP8 (p-value <0.05 Tukey's post-test). For CP5 0.02 μg=0.2 μg >2 μg at post 2^{nd} immunisation (p-value <0.0001 Tukey's post-test).

[0356] Alum/TLR7: 0.1 μg<1 μg 10 μg for $ClfA_{mut}$ and SpA_{mut} at any time points (p-value <0.05 Tukey's post-test). While for CP5 0.02 μg=0.2 μg>2 μg (polysaccharide-based) at post 2^{nd} immunisation (p-value <0.05 Tukey's post-test), for CP8 0.02 μg<0.2 μg>2 μg at any time points (p-value<0.05 Tukey's post-test), finally 0.1 μg<1 μg 5 10 μg for HLA_{mut}.

[0357] Two immunisations were required to obtain the highest IgG titres (p-value <0.01 Tukey's post-test).

[0358] Selection of Pre-Exposed Rabbits and Study Design

[0359] A total of 350 rabbits were screened by ELISA to measure in the sera pre-existing antibodies against the three protein antigens and the two polysaccharides present in the vaccine. The ELISA analysis showed that absorbance A_{450nm}=0.2 was the limit of detection to measure IgG response at 1/100 dilutions, and that all rabbits were positive to at least one antigen.

[0360] In order to assemble the rabbit groups according to the study design shown in Table 3, a selection of 84 rabbits was undertaken.

TABLE 3

Rabbit Study Design

Group	Number of Rabbits	Antigen	Dose protein (μg)	Dose polysaccharides (μg)	Adjuvant[#]	Injection Volume/Route
1	12	buffer	—	—	none	500 μl/IM
2	12	Vaccine	1 μg	0.2	none	500 μl/IM

TABLE 3-continued

Rabbit Study Design

Group	Number of Rabbits	Antigen	Dose protein (μg)	Dose polysaccharides (μg)	Adjuvant[#]	Injection Volume/Route
3	12	Vaccine	10 μg	2	none	500 μl/IM
4	12	Vaccine	50 μg	10	none	500 μl/IM
5	12	Vaccine	1 μg	0.2	$AS01_E$	500 μl/IM
6	12	Vaccine	10 μg	2	$AS01_E$	500 μl/IM
7	12	Vaccine	50 μg	10	$AS01_E$	500 μl/IM

[#]$AS01_E$ adjuvant is composed of 12.5 μg of MPL, a TLR4 activator, and 12.5 μg of QS- 21 (*Quillaja saponaria* Molina, fraction 21), which increases antigen presentation to APCs.

[0361] The 350 rabbits were divided into 5 subgroups, or strata, based on number of antigens with an ELISA result >0.2 A_{450nm} (i.e., ELISA >0.2 for 5, 4, 3, 2 or ≤antigens). All rabbits in each of the two strata 'positive for 5' or 'positive for 4' antigens (total n=35) were selected to be included in the study and randomly allocated to the 7 experimental group (total n=5 to each group). The other required rabbits were taken from the 'positive for 3 antigen' strata (n=102) in which a further selection criteria was applied. In the 'positive for 3 antigens' strata, only rabbits positive for ClfA (n=64) were considered eligible for the study and among these, only rabbits with ELISA result, for both Hla and SpA antigens, within the interquartile range were considered eligible for the study (n=51). Forty-nine of the 51 rabbits were randomly selected and allocated to one of the 7 study groups (total n=7 to each group).

[0362] Immunisation of Rabbits

[0363] The same bed-side mix approach described above was used for preparing the vaccine formulations to be tested in pre-exposed rabbits, using appropriate concentration and dilution step according to antigens final dose and injection volume.

[0364] Pre-exposed rabbits (12-14 week old male) were given two doses of either formulation buffer only (control groups) or one of the six tested vaccine formulations 28 days apart, at day 1 and day 29, by the IM route. Blood samples were taken before 1^{st} injection (day 0), 1 week, 2 and 4 weeks after the first injection (1wp1, 2wp1, 4wp1), and 2 and 5 weeks after the second injection (2wp2, 5wp2).

[0365] Evaluation of Vaccine-Specific IgG

[0366] The assay used for determination of vaccine-specific IgG in rabbits was the Luminex 5-plex described above. The rabbit assay was developed in a 96 well format.

[0367] A pentavalent standard serum was prepared by pooling hyperimmune monovalent rabbit polyclonal sera against all 5 antigens. An arbitrary titre of 50000 RLU/ml for all antigens was assigned to the pentavalent standard.

[0368] The assay was set up according to the criteria reported above. Inter-assay reproducibility was determined (% CV<15%). Linearity assessment yielded R^2 values >0,9. LOQ and LOQ are reported below.

	CLFA	CP 5	CP 8	HLA	SpA_{mut}
	RLU/ml (MDR 1:100)				
LOD (mean + 3SD)	0.01	0.01	0.01	0.01	0.01
LLOQ (dilution correct)	14	6	57	9	60

[0369] For sample testing, 8 serial 3 fold dilutions (starting from 1:100) were automatically prepared and loaded onto single 96 wells plate with an adequate amount of coupled microspheres. Antigen specific antibodies were revealed by an anti-rabbit Fab2 IgG. Phycoeritrin-labelled secondary antibody and MFI were measured using the Luminex Flexmap 3D Reader. IgG titres of each sample/antigen were determined by estimating the median of all valid individual titres obtained from the 5PL curve.

[0370] Geometric mean titres (GMTs) for anti-$ClfA_{mut}$, -Hla_{mut}, -CP5, -CP8 and -SpA_{mut}, at 4 weeks after 1^{st} vaccination and 2 weeks after 2^{nd} vaccination, showed a statistically significant increase from pre-vaccination in all seven groups (FIG. **3**). Antibody titres at 2 weeks post 2^{nd} vaccination were higher than after 1st vaccination. At 2 weeks after 2^{nd} vaccination, all vaccinated groups showed a statistically significant higher antibody response as compared to buffer control group for all antigens except for Hla_{mut} for which antibody titres were similar to buffer control group at any time point. After the 2^{nd} vaccination, the GMTs between the adjuvanted and non-adjuvanted did not show a significant difference.

Example 3: Analysis of Functional Antibodies

[0371] Sera obtained from the immunised mice and rabbits were tested for the presence of functional antibodies capable of neutralising wild-type Hla and ClfA activities in vitro.

[0372] Inhibition of Hla Activity—Mice

[0373] The ability of vaccine-specific antibodies to inhibit Hla-induced hemolysis was evaluated in an in vitro red blood cell (RBC)-based hemolysis neutralisation assay, carried out modifying a previously described method (Bagnoli et al, PNAS 2015, 112: 3680-3685). Alpha hemolysin neutralisation assay was performed using three pools of 10 sera each from three different repeated experiments for each immunisation group. Neutralisation titres were determined calculating the effective dilution (ED_{50}) defined as the dilution of the serum which neutralises the toxicity of Hla by 50%.

[0374] For each group at each time point, the ED_{50} was evaluated by estimation of the inflection point parameter of a four-parameter logistic (4PL) by a non-linear regression of neutralisation curves.

[0375] The results obtained are shown in FIG. **4**A and indicated that:

[0376] No functional antibodies were detected in absence of adjuvant and in 0.1 μg adjuvanted groups (data not shown).

[0377] Hla neutralisation was observed after two immunisations, using both adjuvants with 1 or 10 μg doses.

[0378] Formulation with 10 μg induced statistically significant higher neutralisation titres as compared to formulation with 1 μg (p 5 0.05 Wilcoxon test).

[0379] Inhibition of ClfA Activity—Mice

[0380] A ClfA binding inhibition assay was developed to evaluate whether the vaccine elicited functional antibodies able to inhibit ClfA activity. The assay is an ELISA-based measurement of the ability of antibodies to inhibit the binding of ClfA to fibrinogen. If the antibodies efficiently bind to ClfA, interaction with fibrinogen is inhibited. The sera titres are defined as the reciprocal serum dilution giving 50% reduction of fibrinogen binding (ED_{50}). A four-parameter logistic (4PL) non-linear regression model is used for curve—fitting analysis of inhibition curves and the ED_{50} has been evaluated by estimation of the 4PL inflection point.

[0381] ClfA binding assay has been applied to sera elicited by vaccine formulation in naïve mice.

[0382] Sera collected at the following immunisation time-points were analysed: 4 weeks after the first immunisation (4wp1); and 1-2-4-8-12-16 weeks after the second immunisation (1wp2, 2wp2, . . .)

[0383] The results obtained are shown in FIG. **5**A.

[0384] Sera from each group were pooled (10 mice each) and neutralisation titres (ED_{50}) were measured for each pool at different time-points. The analysis was performed on three independent mouse immunisation experiments for each group at different time-points. Results are shown in FIG. **5**A where each dot represents the median value of the three independent experiments; the upper and the lower ends of the bar represent the maximum and the minimum value respectively.

[0385] The results obtained indicated that:

[0386] No ClfA neutralisation was measured at 0.1 μg dosage and in absence of adjuvants (data not shown).

[0387] ClfA neutralisation was observed using both adjuvants with 1 or 10 μg

[0388] Formulation with 10 μg dosage (referred to ClfA content) induced statistically significant higher neutralisation titres as compared to the formulation with 1 μg. (p value Wilcoxon test 5≤0.05)

[0389] At 10 μg dosage $AS01_E$ outperformed Alum/TLR7 at 1w and 2wp2 (p value Wilcoxon test≤5 0.05)

[0390] Sera obtained from the immunised rabbits were tested for the presence of functional antibodies capable of neutralising wild-type Hla and ClfA activities in vitro, using the assays previously described for mouse sera.

[0391] Inhibition of Hla Activity—Rabbits

[0392] Rabbits were treated as reported in table 3. All animals were bled before 1st injection (day 0), 1 week, 2 and 4 weeks after the first injection (1wp1, 2wp1, 4wp1), and 2 and 5 weeks after the second injection (2wp2, 5wp2). Neutralisation assays were performed on single sample sera at the different time points described above.

[0393] Geometric mean titres, at each time point were descriptively summarised, including 95% confidence intervals (CIs). The GMTs and 95% CIs were calculated by back transformations of the confidence limits computed for the mean of the log-transformed titres based on Student's t-distribution (FIG. **4**B). The dashed line represents the minimum required dilution established for measurement of each rabbit serum.

[0394] Titres Vs. Pre-Immune

[0395] The geometric mean fold rise from pre-vaccination at each post-vaccination time point was calculated based on the log-transformed titres. Student's paired t test was used to analyse the differences between pre and post log-transformed titres.

[0396] At 2wp1 all groups with a protein content ug induce higher titres as compared to pre-immune (all pvalues <0.017)

[0397] At 2wp2: all immunised groups induce higher titres as compared to pre-immune (all p values <0.005)

[0398] Titres Vs. Buffer Group

[0399] Pair-wise comparisons between immunised groups and buffer at each time-point were performed using Welch's Student's t-test.

[0400] At 2wp1 only adjuvanted 50 μg dosage induces higher titre as compared to buffer group (all p values <0.003)

[0401] At 2wp2 all immunised groups, excluded no-adjuvanted 1 μg dosage, induce higher titre as compared to buffer (all p values <0.013)

[0402] Post-1 Vs. Post-2

[0403] Significant differences (p value Welch's Student t-test <0.025) where observed between 2wp1 and 2wp2 in the 50 μg, 10 μg+$AS01_E$, 50 μg+$AS01_E$.

[0404] Inhibition of ClfA Activity—Rabbits

[0405] The ClfA binding inhibition assay was applied to sera elicited by vaccine formulation in pre-exposed rabbits. Titres were determined by estimation of the inflection point parameter of a four-parameter logistic (4PL) by a non-linear regression of the inhibition curves.

[0406] Sera collected at the following immunisation time-points were analysed: Pre-immunisation, 4 weeks after the first immunisation (4wp1), 2 weeks after the second immunisation (2wp2).

[0407] Sera from each group (12 rabbits each) were collected and neutralisation titres (ED5O) were measured for each individual rabbit at different time-points.

[0408] Geometric mean titres, at each time point were descriptively summarised, including 95% confidence intervals (CIs). The GMTs and 95% Cls were calculated by back transformations of the confidence limits computed for the mean of the log-transformed titres based on Student's t-distribution (FIG. **5**B)

[0409] The neutralisation results obtained with rabbit sera are shown in FIG. **5**B and indicated that:

[0410] No anti-ClfA functional activity in pre-immune, 4wp1 and Buffer group sera.

[0411] Two doses are required to induce ClfA neutralising activity.

[0412] The 10 μg dose with adjuvant performs as well as the 50 μg dose without or with adjuvant

[0413] Overall neutralisation titres are much lower than those measured in mouse sera.

Example 4: CD4 T-Cell response Versus Vaccine Proteins in Vaccinated Mice

[0414] The potential role of pathogen-specific CD4+T cells in triggering the host immune response against SA is well described. Therefore, the capacity of the vaccine, adjuvanted either with Alum/TLR7 or $AS01_E$, to induce a T cell response in mice was also tested.

[0415] In particular the aims of the study were:

[0416] See whether immunisation of mice with the vaccine could elicit a T-cell response against the vaccine vaccine proteins (magnitude).

[0417] Investigate the Th1, Th2 or Th17 polarization of the response (quality).

[0418] Evaluate whether inclusion of an adjuvant in the vaccine formulation improves the magnitude and/or influences the quality of the T cell response.

[0419] Compare AS01 and Alum/TLR7 adjuvants for the magnitude and quality of the T-cell response.

[0420] To this purpose, five week-old BALB/c female mice were given two immunisations (50 μl in each hind leg quadriceps) at day 1 and 29 by IM route. Two doses of the formulation, vaccine-10 and vaccine-1, consisting of 10 pg or 1 μg (protein-based) of each component were given either alone or with Alum/TLR7 or $AS01_E$ adjuvant. Control groups were injected with PBS, Alum/TLR7 or $AS01_E$ alone. The experiment was repeated 3 times in order to assess experimental variability. An overview of the study with the total number of mice used in the 3 experiments is shown in Table 4.

TABLE 4

Study Design for determination of T cell response in immunised mice

Group	Total Number of mice	Antigen	Dose protein (μg)	Dose polysaccharides (μg)	Adjuvant#	Injection Volume/Route
1	15	Vaccine	10	2	none	100 μl/IM
2	15	Vaccine	10	2	Alum/TLR7	100 μl/IM
3	15	Vaccine	10	2	$AS01_E$	100 μl/IM
4	15	Vaccine	1	0.2	none	100 μl/IM
5	15	Vaccine	1	0.2	Alum/TLR7	100 μl/IM
6	15	Vaccine	1	0.2	$AS01_E$	100 μl/IM
7	6	Vaccine	—	—	none	100 μl/IM
8	6	Vaccine	—	—	Alum/TLR7	100 μl/IM
9	6	Vaccine	—	—	$AS01_E$	100 μl/IM

[0421] The induction of vaccine-specific CD4 T-cell response was evaluated by intracellular cytokine staining (ICS) of splenocytes isolated from single mice 12 days after the second immunisation (d12p2) and then treated in vitro with anti-CD28 and anti-CD49d mAb (2 μg/ml each) alone or together with each vaccine protein (Hla_{mut} and $ClfA_{mut}$, at 10 μg/ml; SpA_{mut}, at 1 μg/ml) at 37° C. for 16 h. Brefeldin A, 5 μg/ml, was added for the last 4 h of incubation. Cells were then stained with Live/Dead Nearlr (Invitrogen), fixed and permeabilized with Cytofix/Cytoperm (BD), washed in Perm/Wash buffer (BD), incubated with anti-CD16/CD32 Fc block (BD) for 20 min at RT, and stained with the following mAbs anti:

[0422] CD3-BV605, CD4-BV510, CD8-PE-CF594, CD44-V450, IFNγ-BV785, IL-2-PE Cy5, TNF-AF488, IL-17A-PE Cy7, IL-4-PerCP eFluor710, and IL-13-PerCP eFluor710 for 20 min at RT, washed twice in Perm/Wash buffer, and suspended in PBS.

[0423] Samples were acquired on a LSRII flow cytometer (BD Biosciences) and CD4 T-cell responses were analysed using FlowJo software (TreeStar).

[0424] $CD4^+CD44^{high}$ T cells producing IL-2, TNF, IL-4IL-13, IFN-γ or IL-17A were identified according to a gating strategy.

[0425] The magnitude of the T cell response was calculated measuring the frequencies (%) of $CD4^+CD44^{high}$ T cells producing at least one of the cytokines analysed in response to in vitro stimulation with vaccine proteins ($CD4^+$ $CD44^{high}$ T cells≥1 $cytokine^+$).

[0426] The quality of the CD4 T cell response (Th1, Th2 or Th17 polarization) was evaluated measuring the frequencies (%) of $CD4^+CD44^{high}$ T cells producing: IFN-γ but not IL-4/IL-13 (≥IFN-γ^+, Th1), IL-4/IL-13 but not IFN-γ (≥IL-$4/IL\text{-}13^+$, Th2) or at least IL-17A (≥$IL\text{-}17A^+$, Th17).

[0427] Boolean gates analysis was applied and the response of medium-treated cells was subtracted from that of stimulated cells.

[0428] For the analysis of results, a two-way ANOVA was fitted on $\log_{10}$ $CD4^+CD44^{high}$ percentages including group (9 groups), experiment (3 experiments) and interaction group*experiment as fixed effects and using a heterogeneous variance model (identical variances were not assumed between groups). This model was used to estimate the group geometric means and their 95% CIs as well as geometric mean ratios and their 95% CIs.

[0429] Geometric mean ratios above 2-fold were considered as significantly different ($p<0.05$) if their 95% CIs did not include 1.

[0430] The results obtained are illustrated in FIG. **2** in which statistically significant differences are indicated. They can be summarised as follows:

[0431] Inclusion of an adjuvant in the vaccine formulation was required to obtain significantly higher frequencies of vaccine-specific T cells as compared to control groups.

[0432] AS01 outperformed Alum/TLR7 with each protein antigen.

[0433] Only the vaccine adjuvanted with AS01 induced $CD4^+$ T-cells producing IFN-γ (indicating Th1 polarization) specific for each antigen, while Vaccine adjuvanted with Alum/TLR7 induced only SpA_{mut}-specific Th1, at significantly lower frequencies.

[0434] Very low frequencies of Th2 and Th17 were measured only with AS01 adjuvanted Vaccine.

[0435] No major differences in the magnitude and quality of the T-cell response were observed after immunisation either with 1 or 10 μg of unadjuvanted vaccine.

Example 5: Protection in Animal Model: Skin Infection Model

[0436] Experiments were carried out in mouse models of *S. aureus* infection to evaluate the efficacy of the 5Ag/AS01 E vaccine (comprising SpA_{mut}, $ClfA_{mut}$-CP8 bioconjugate and Hla_{mut}- CP5 bioconjugate adjuvanted with $AS01_E$) in

protecting against *S. aureus* infection in vivo. The experiments also compared the 5Ag/AS01$_E$ vaccine to a 4Ag/AS01$_E$ vaccine lacking SpA, and the effect of the adjuvant AS01$_E$ in the 5Ag vaccine compared to the 5Ag vaccine adjuvanted with aluminum hydroxide (Al(OH)$_3$). The mouse models used were a skin infection model and a kidney abscess model.

[0437] Study Design and Protocol

[0438] CD1 mice female 5-week old were used.

[0439] For each model three experiments were performed sequentially. A total of 6 experiments were performed, 3 identical studies in the abscess model (Sa-5Ag-7, Sa-5Ag-8, Sa-5Ag-9) and 3 in the skin model (Sa-5Ag-10, Sa-5Ag-11, Sa-5Ag-12). In the skin model, each group was composed of 10 mice, so a total of 30 mice per group was tested in each model.

[0440] Mice received two injections given one month apart (at day 0 and day 30) intramuscularly (IM, 30 μl each paw) of one of the different vaccine formulations or PBS.

[0441] At 3 weeks after last injection (day 51) mice were infected with an appropriate sub-lethal dose of USA300 bacteria, using the subcutaneous (SC, skin model) infection route. 50 μl of SA USA 300 (theoretical 3×10^7 CFU/mouse) was inoculated.

[0442] In the skin model, the relevant lesion area was measured 5 days after infection, before being collected 7 days after infection, homogenized and CFU values determined.

TABLE 5

Study design of skin model with *S. aureus* strain USA 300

	Immunization			Infection		Readout Skin area (days post infection)	
Group (mouse number)	Antigen and dose (μg/Ag/mouse)	Adjuvant	Route and volume	Route and volume	Dose (CFU)	Sizing	Collection
1 (1-10)	PBS	None	IM 60 μl	SC 50 μl	3×10^7	5	7
2 (11-20)	Sa-5Ag (10 μg*)	AS01$_E$	IM 60 μl	SC 50 μl	3×10^7	5	7
3 (21-30)	Sa-5Ag (10 μg*)	Al(OH)$_3$	IM 60 μl	SC 50 μl	3×10^7	5	7
4 (31-40)	Sa-4Ag (10 μg*)	AS01$_E$	IM 60 μl	SC 50 μl	3×10^7	5	7

IM: intramuscular; SC: subcutaneous; AS01$_E$ contains 2.5 μg/dose of MPL and 2.5 μg/dose of QS21; Al(OH)$_3$: Aluminum hydroxide.
*Protein based.

TABLE 6

Schedule of treatments of skin model

Day	Procedures
−1	Pre-vaccination blood sample collection
0	1st vaccination
30	2nd vaccination
50	Post-2nd vaccination blood sample collection
51	Subcutaneous infection (USA300)
56	Lesion area quantitation
58	Mice sacrifice, collection of the skin lesion area and CFU determination

[0443] Materials and Methods

[0444] Bacterial Aliquot Preparation for Storing at −80° C.

[0445] 20 ml fresh Tryptic Soy Broth (TSB) (prewarmed at 37° C. overnight or at least 1 h before use) was mixed with 0.3 ml of thawed bacteria (old stock) in 50 ml disposable tube. Initial OD_{600nm} was 0.03 and bacteria were grown until OD_{600nm}=0.55. 2 ml of this bacterial suspension was aliquoted in cryovials and stored at −80° C.

[0446] Growth Conditions

[0447] A stock of bacteria (*S. aureus* USA300) from -80° C. freezer, prepared as described above, was thawed in a water bath at 37° C. for 10 min. 20 ml fresh Tryptic Soy Broth (TSB) (prewarmed at 37° C. overnight or at least 1 h before to use) was mixed with 0.3 ml of thawed bacteria. Initial OD_{600nm} was 0.03 and bacteria were grown in 50 ml disposable tube. Bacteria were incubated about for 2.0 hours at 37° C., 150 rpm agitation until a final OD_{600nm} of 0.6, then centrifuged at 4500 rpm, 10 minutes, 4° C. Supernatant was removed and the pellet resuspended in equal volume of PBS and again centrifuged at 4500 rpm, 10 minutes, 4° C. The supernatant was again removed and the bacterial pellet resuspended in 2 ml of PBS (around 5×10^9 cfu/ml). At this point, a final dilution was made to reach a bacterial concentration of 3×10^7 cfu/mouse in 50 μl. 100 μl of final bacterial suspension was diluted and 10^{-5} and 10^{-6} dilutions plated onto Tryptic Soy Agar (TSA) plates to count CFU.

[0448] In Vivo Model of Subcutaneous Infection with *S. Aureus*

[0449] The day before infection, anaesthetised mice (Zolazepam+Tiletamina/Xylazine) were shaved in dorsal position using electric razor and depilatory cream. The depilatory cream was removed gently by water at 37° C. Bacteria, prepared as described before, were inoculated subcutaneously (50 μl/animal) in mice anaesthetised with Zolazepam +Tiletamina/Xylazine. Animals were followed every day using a dedicated score sheet for clinical symptoms of disease. Pictures of lesions to determine dimension were taken at days 5 post infection with a digital camera. Seven days after infection, mice were sacrificed and the skin with lesion removed using circular scalpel (8 mm); for larger lesions scissors were used to ensure entire lesion recovered. The removed skin was homogenised for CFU counts. Decimal dilutions were prepared up to 10^{-8} and 10 μl spots (in duplicate) plated onto TSA plates.

[0450] Readouts of the Model

[0451] Skin lesion area at days 5: expressed in mm^2 measured by ImageJ software.

[0452] Clinical score registered every day after infection.

[0453] CFU counts (expressed as CFU/sample) at day 7 post infection.

[0454] Results

[0455] The 5Ag+AS01$_E$ vaccine was effective in preventing skin lesions and reducing CFU counts.

[0456] At 5 days after the skin infection none of the mice vaccinated with 5Ag+AS01$_E$ exhibited a skin lesion. 17% of the mice (5/30) vaccinated with 5Ag+Al(OH)$_3$ exhibited a skin lesion while 60% (18/30) and 100% (30/30) of mice vaccinated with 4Ag+AS01$_E$ and PBS respectively, showed a skin lesion.

[0457] Results are shown in Tables 7-9.

[0458] The CFU GM at 7 days after the infection, in the SA vaccine formulation without SpA antigen (Sa-4Ag+AS01$_E$) was almost 3-fold higher (2.84-fold) than in the Sa vaccine formulation with SpA (Sa-5Ag+AS01$_E$) and the difference was statistically significant (the 95% CI of CFU GMs vaccine group ratio between the two vaccine groups didn't include the value 1) (Tables 7 and 8).

[0459] There was a difference in the level of viable bacteria in skin between the two Sa-5Ag vaccine formulation adjuvanted with AS01$_E$ or with Al(OH)$_3$, but the difference was not statistically significant. The CFU GM, was 1.6-fold higher in the Sa-5Ag vaccine formulation with Al(OH)$_3$ as adjuvant [Sa-5Ag+Al(OH)$_3$] than the vaccine Sa-5Ag vaccine formulation with AS01 $_E$ (Sa-5Ag+AS01 $_E$) as adjuvant and the 95% CI of CFU GMs vaccine group ratio included the value 1 (Tables 7 and 8).

[0460] In the PBS alone group the level of viable bacteria in skin at 7 days after infection was significantly higher than the level observed in any of the vaccine formulations evaluated. The CFU GM in the PBS group was 40.32- to 114.48-fold higher than the CFU GMs observed in the vaccine formulations. (Tables 7 and 8).

[0461] Explorative pairwise comparisons of the median of two groups, performed using a two-sample median test, showed a significative difference between the vaccine formulations with and without SpA ($p<0.0001$) and between the Sa-5Ag vaccine formulations with AS01$_E$ and Al(OH)$_3$ ($p=0.0206$).

TABLE 7

Skin model - CFU Geometric mean and 95% CI at 4 days after infection with *S. aureus* strain USA 300

Group Label	Group	N	CFU Geometric Mean	95% CI - Lower Limit	95% CI - Upper Limit
PBS	1	30	144660225	85736457	244080306
Sa-5Ag + AS01$_E$	2	30	1263656	748937	2132125
Sa-5Ag + Al(OH)$_3$	3	30	2037202	1207398	3437302
Sa-4Ag + AS01$_E$	4	30	3587442	2126186	6052970

TABLE 8

Skin model - CFU GM vaccine group ratio and 95% CI at 4 days after infection with *S. aureus* strain USA 300

Comparison	Ratio of Geometric Mean	95% CI - Lower Limit	95% CI - Upper Limit	Statistically significant*
Gr1 PBS/Gr2 Sa-5Ag + AS01$_E$	114.48	54.63	239.89	√
Gr1 PBS/Gr3 Sa-5Ag + Al(OH)$_3$	71.01	33.89	148.80	√
Gr1 PBS/Gr4 Sa-4Ag + AS01$_E$	40.32	19.24	84.50	√
Gr4 Sa-4Ag + AS01$_E$/Gr2 Sa-5Ag + AS01$_E$	2.84	1.35	5.95	√
Gr3 Sa-5Ag + Al(OH)$_3$/Gr2 Sa-5Ag + AS01$_E$	1.61	0.77	3.38	—

*When the 95% CI of the GM Ratio does not include the value 1 (i.e., equal GMs) then the difference is statistically significant. In particular, if the 95% CI-Lower limit of the ratio of GM between two groups is >1, the first component is considered statistically significantly higher than the second component of the comparison. Vice versa, if the 95% CI-Upper limit of the ratio of GM between two groups is <1, the first component is considered statistically significantly lower than the second component of the comparison.

The symbol √ indicates a statistically significant comparison.

TABLE 9

Skin model - Mean, Median, Min, Max of lesion area by group*

Group	N	Mean lesion area	Median lesion area	Min lesion area	Max lesion area
Gr1 PBS	30	19.07	14.00	2.30	66.00
Gr2 Sa-5Ag + AS01	30	0.10	0.10	0.10	0.10
Gr3 Sa-5Ag + AlOH	30	0.39	0.10	0.10	2.30
Gr4 Sa-4Ag + AS01	30	1.08	0.80	0.10	3.70

*No lesion area was set to 0.1 for the purpose of the analysis.

[0462] CFU could be measured also in mice who did not show any skin lesion, since bacteria are also present in subcutaneous abscesses. In the two vaccine formulations where a subset of mice showed no lesion and the remaining mice in the group showed a lesion (i.e., groups 5Ag+Al $(OH)_3$ and 4Ag+$AS01_E$) the mean and median CFU count in the skin was lower in the mice with no lesion area than in the mice with a lesion. For each vaccine group, descriptive statistics (mean, median, min, max) of CFU count by presence or absence of lesion area are shown in Table 10.

TABLE 10

Skin model - Mean, Median, Min, Max of CFU count

Group	Presence of lesion	N	Mean CFU count	Median CFU count	Min CFU count	Max CFU count
Gr1 PBS	Yes Lesion	30	578366667	107500000	5500000	7000000000
Gr2 Sa-5Ag + AS01	No Lesion	30	2764000	1675000	85000	10500000
Gr3 Sa-5Ag + AlOH	No Lesion	25	2776600	2150000	80000	11500000
Gr3 Sa-5Ag + AlOH	Yes Lesion	5	9500000	10500000	4000000	14500000
Gr4 Sa-4Ag + AS01	No Lesion	12	2584583	2025000	365000	8500000
Gr4 Sa-4Ag + AS01	Yes Lesion	18	17522222	6950000	750000	195000000

Example 6: Protection in Animal Models: Kidney Abscess Model

[0463] Experiments were carried out in a kidney abscess mouse model, as a model of systemic infection, to evaluate the efficacy of the 5Ag/$ASO1_E$ vaccine in protecting against *S. aureus* infection in vivo, as described at Example 5 above.

Protocol and Study Design

[0464] CD1 mice female 5-week old were used.

[0465] For each model three experiments were performed sequentially. A total of 6 experiments were performed, 3 identical studies in the abscess model (Sa-5Ag-7, Sa-5Ag-8, Sa-5Ag-9). Each group was composed of 12 mice, so a total of 36 mice was tested in each model.

[0466] Mice received two injections given one month apart (at day 0 and day 30) intramuscularly (IM, 30 μI each paw) of one of the different vaccine formulations or PBS.

[0467] At 3 weeks after last injection (day 51) mice were infected with an appropriate sub-lethal dose of USA300 bacteria, using the intravenous (IV, kidney abscess model) infection route.

[0468] 100 μl of SA USA 300 (theoretical 1×10^7 CFU/mouse) strain was inocuated.

[0469] The infected mice were sacrificed 4 days post-infection, kidneys were collected, homogenised and CFU number was counted.

TABLE 11

Study design of abscess model with S. aureus strain USA 300

	Immunization			Infection route and volume		
Group (mouse number)	Antigen dose (μg/Ag/mouse)	Adjuvant	Route and volume	Route and volume	Infection dose (CFU)**	Kidney collection (days post-infection)
1 (1-12)	PBS	None	IM 60 μl	IV 100 μl	1×10^7	4
2 (13-24)	Sa-5Ag (10 μg*)	$AS01_E$	IM 60 μl	IV 100 μl	1×10^7	4
3 (25-36)	Sa-5Ag (10 μg*)	$Al(OH)_3$	IM 60 μl	IV 100 μl	1×10^7	4
4 (37-48)	Sa-4Ag (10 μg*)	$AS01_E$	IM 60 μl	IV 100 μl	1×10^7	4

IM: intramuscular; IV: intravenous; $AS01_E$ contains 2.5 μg/dose of MPL and 2.5 μg/dose of QS21; $Al(OH)_3$: Aluminum hydroxide
*Protein based.

TABLE 12

Schedule of treatments of abscess model

Day	Procedures
−1	Pre-vaccination blood sample collection
0	1st vaccination
30	2nd vaccination
50	Post-2nd vaccination blood sample collection
51	Intravenous infection (1×10^7)
55	Mice sacrificed 4 days after infection, with collection of kidneys and CFU count

[0470] Materials and Methods

[0471] Bacterial Aliquot Preparation for Storing at −80° C.

[0472] 20 ml fresh Tryptic Soy Broth (TSB) (prewarmed at 37° C. overnight or at least 1h before use) was mixed with 0.3 ml of thawed bacteria (old stock) in 50 ml disposable tube. Initial OD_{600nm} is 0.03 and bacteria were grown until OD_{600nm}=0.55. 2 ml of this bacterial suspension was aliquoted in cryovials and stored at −80° C.

[0473] Growth Conditions

[0474] A stock of bacteria (*S. aureus* USA300) from −80° C. freezer, prepared as described above, was thawed in a water bath at 37° C. for 10 min. 20 ml fresh Tryptic Soy Broth (TSB) (prewarmed at 37° C. overnight or at least 1h before use) was mixed with 0.3 ml of thawed bacteria. Initial OD_{600nm} was 0.03 and bacteria grown in 50 ml disposable tube. Bacteria were incubated about for 2.0 hours at 37° C., 150rpm agitation to reach a final OD_{600nm} of 0.6. Bacteria were centrifuged at 4500 rpm, 10 minutes, 4° C. Supernatant was removed and the pellet resuspended in egual volume of PBS and again centrifuged at 4500 rpm, 10 minutes, 4° C. Supernatant was again removed and the bacterial pellet resuspended in 2 ml of PBS (around 5×10^9cfu/ml). At this point, a final dilution was made to reach a bacterial concentration of 1×10^7 cfu/mouse in 100 μl. 100 μl of final bacterial suspension was diluted and 10^{-5} and 10^{-8} dilutions plated onto Tryptic Soy Agar (TSA) plates to count CFU.

[0475] In Vivo Model of Subcutaneous Infection with *S. Aureus*

[0476] Mice were warmed using an infrared lamp in order to inflate the tail veins. Bacteria, prepared as described above, were inoculated intravenously (100 μl/animal) into the tail vein. Animals were followed every day using a dedicated score sheet for clinical symptoms of disease. Four days after infection, mice were sacrificed and the kidneys recovered. The removed kidneys were homogenised for CFU counts (2ml PBS). Decimal dilutions were prepared up to 10^{-8} and 10 μl spots (in duplicate) are plated onto TSA plates.

[0477] Readouts of the Model

[0478] Clinical score registered every day after infection.

[0479] CFU counts (expressed as CFU/sample) at day 4 post infection.

[0480] Results

[0481] The 5Ag+AS01$_E$ vaccine was highly effective in reducing bacterial infection compared to control. The level of viable bacteria in kidney collected at 4 days after the infection, as measured by CFU geometric mean (GM), in the vaccine formulation without the SpA antigen (Sa-4Ag+AS01$_E$) was 6.6-fold higher than in the SA vaccine formulation with SpA (Sa-5Ag+AS01$_E$) and the difference was statistically significant. The CFU GM, was almost 2-fold higher (1.78-fold) in the Sa-5Ag vaccine formulation with $Al(OH)_3$ as adjuvant [Sa-5Ag+$Al(OH)_3$] than in the vaccine Sa-5Ag vaccine formulation with AS01$_E$ as adjuvant (Sa-5Ag+AS01$_E$), but this difference was not statistically significant. In the PBS alone group the level of viable bacteria in kidney at 4 days after infection was at least 4-fold higher than the level observed in any GSK Sa vaccine formulation evaluated in the studies. The results are shown in Table 13.

TABLE 13

Kidney abscess model - CFU Geometric mean and 95% CI at 4 days after infection with *S. aureus* strain USA 300

Group Label	Group	Number	CFU Geometric Mean*	95% CI - Lower Limit	95% CI - Upper Limit
PBS	1	36	93010566	60102858	143936005
Sa-5Ag + AS01$_E$	2	36	3257105	1563051	6787196
Sa-5Ag + $Al(OH)_3$	3	36	5811332	3119367	10826419
Sa-4Ag + AS01$_E$	4	36	21491537	12673064	36446289

*CFU GMs are unadjusted GMs.

SEQUENCE LISTING

[0482]

Wild-type ClfAN1N2N3

SEQ ID NO: 1

ASENSVTQSDSASNESKSNDSSSVSAAPKTDDTNVSDTKTSSNTNNGETSVAQNPAQQETTQSSSTNATTEETPV

TGEATTTTTNQANTPATTQSSNTNAEELVNQTSNETTFNDTNTVSSVNSPQNSTNAENVSTTQDTSTEATPSNNE

SAPQSTDASNKDVVNQAVNTSAPRMRAFSLAAVAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGF

SVPNSAVKGDTFKITVPKELNLNGVTSTAKVPPIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMPAY

IDPENVKKTGNVTLATGIGSTTANKTVLVDYEKYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLT

-continued

GNLKPNTDSNALIDQQNTSIKVYKVDNAADLSESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYI

VVVNGHIDPNSKGDLALRSTLYGYNSNIIWRSMSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEPIPED

Wild-type ClfAN2N3

SEQ ID NO: 2

VAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKVP

PIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMPAYIDPENVKKTGNVTLATGIGSTTANKTVLVDYE

KYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADLS

ESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWRS

MSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEPIPED

Mature wild-type Hla

SEQ ID NO: 3

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMHKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEGANK

SGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQ

PDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFS

PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

WILD-TYPE SpA. Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH3 binding respectively

SEQ ID NO: 4

MKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVLNMPNLNADQRNGFIQSLKDDPSQS

ANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQ

APKADNNFNKEQQNAFYEILNMPNLNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFY

EILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFI

QSLKDDPSVSKEILAEAKKLNDAQAPKEEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDGNKPG

KEDNKKPGKEDGNKPGKEDNKKPGKEDGNKPGKEDGNKPGKEDGNGVHVVKPGDTVNDIAKANGTTADKIAADNK

LADKNMIKPGQELVVDKKQPANHADANKAQALPETGEENPFIGTTVFGGLSLALGAALLAGRRREL

ClfA N2N3 P116S/Y118A

SEQ ID NO: 5

VAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKVP

PIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMSAAIDPENVKKTGNVTLATGIGSTTANKTVLVDYE

KYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADLS

ESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWRS

MSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEP**I**PED

ClfA N2N3 P116S/Y118A with PglB consensus sequence (KDQNATK, underlined) substituted for residue I337

SEQ ID NO: 6

VAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKVP

PIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMSAAIDPENVKKTGNVTLATGIGSTTANKTVLVDYE

KYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADLS

ESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWRS

MSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEP**KDQNATK**PED

ClfA N2N3 P116S/Y118A with PglB consensus sequence (KDQNATK, underlined) substituted for residue I337 and N-terminal S

SEQ ID NO: 7

SVAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKV

PPIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMSAAIDPENVKKTGNVTLATGIGSTTANKTVLVDY

EKYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADL

-continued

SESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWR

SMSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEP**KDQNATK**PED

Mature Hla H35L

SEQ ID NO: 8

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMLKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEGANK

SGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQ

PDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFS

PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

Mature Hla H35L with PglB consensus sequence (KDQNRTK) substituted for residue K131

SEQ ID NO: 9

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMLKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEGANK

SGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKDQNRTKIGGLIGANVSIGH

TLKYVQPDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSL

LSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

Mature Hla H35L/H48C/G122C

SEQ ID NO: 10

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMLKKVFYSFIDDKNCNKKLLVIRTKGTIAGQYRVYSEEGANK

SGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNCNVTGDDTGKIGGLIGANVSIGHTLKYVQ

PDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFS

PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

Mature Hla H35L/H48C/G122C with PglB consensus sequence (KDQNRTK) substituted for residue K131

SEQ ID NO: 11

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMLKKVFYSFIDDKNCNKKLLVIRTKGTIAGQYRVYSEEGANK

SGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNCNVTGDDTG**KDQNATK**IGGLIGANVSIGH

TLKYVQPDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSL

LSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

Hla H35L/H48C/G122C with N-terminal S, C-terminal GSHRHR, and KDQNRTK substituted for residue K131

SEQ ID NO: 12

SADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMLKKVFYSFIDDKNCNKKLLVIRTKGTIAGQYRVYSEEGAN

KSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNCNVTGDDTG**KDQNATK**IGGLIGANVSIG

HTLKYVQPDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASS

LLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

GSHRHR

SpA IgG binding portion (domains E, D, A, B, C). Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH3 binding respectively.

SEQ ID NO: 13

AQHDEA<u>QQ</u>NAFYQVLNMPNLNADQRNGFIQSLK<u>DD</u>PSQSANVLGEAQKLNDSQAPKADA<u>QQ</u>NNFNKD<u>QQ</u>SAFYEI

LNMPNLNEAQRNGFIQSLK<u>DD</u>PSQSTNVLGEAKKLNESQAPKADNNFNKE<u>QQ</u>NAFYEILNMPNLNEEQRNGFIQS

LK<u>DD</u>PSQSANLLSEAKKLNESQAPKADNKFNKE<u>QQ</u>NAFYEILHLPNLNEEQRNGFIQSLK<u>DD</u>PSQSANLLAEAKK

LNDAQAPKADNKFNKE<u>QQ</u>NAFYEILHLPNLTEEQRNGFIQSLK<u>DD</u>PSVSKEILAEAKKLNDAQAPK

SpA E domain . Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH_3 binding respectively

-continued

SEQ ID NO: 14

AQHDEAQQNAFYQVLNMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPK

SpA D domain. Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH_3 binding respectively

SEQ ID NO: 15

ADAQQNNFNKDQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPK

SpA A domain. Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH_3 binding respectively

SEQ ID NO: 16

ADNNFNKEQQNAFYEILNMPNLNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPK

SpA B domain. Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH_3 binding respectively

SEQ ID NO: 17

ADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPK

SpA C domain. Underlined are the QQ and DD residues which may be mutated to reduce Fey and VH_3 binding respectively

SEQ ID NO: 18

ADNKFNKEQQNAFYEILHLPNLTEEQRNGFIQSLKDDPSVSKEILAEAKKLNDAQAPK

SpA E domain with QQ-KK substitutions at positions 7 and 8 and DD-AA substitutions at positions 34 and 35

SEQ ID NO: 19

AQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLNDSQAPK

SpA D domain with QQ-KK substitutions at positions 12 and 13 and DD-AA substitutions at positions 39 and 40

SEQ ID NO: 20

ADAQQNNFNKDKKSAFYEILNMPNLNEAQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPK

SpA A domain with QQ-KK substitutions at positions 9 and 108 and DD-AA substitutions at positions 36 and 37

SEQ ID NO: 21

ADNNFNKEKKNAFYEILNMPNLNEEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPK

SpA B domain with QQ-KK substitutions at positions 9 and 10 and DD-AA substitutions at positions 36 and 37

SEQ ID NO: 22

ADNKFNKEKKNAFYEILHLPNLNEEQRNGFIQSLKAAPSQSANLLAEAKKLNDAQAPK

SpA C domain with QQ-KK substitutions at positions 9 and 10 and DD-AA substitutions at positions 36 and 37

SEQ ID NO: 23

ADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAEAKKLNDAQAPK

SpA D domain with QQ-KR substitutions at positions 4 and 5, QQ-KK substitutions at positions 12 and 13 and DD-AA substitutions at positions 39 and 40

SEQ ID NO: 24

ADAKRNNFNKDKKSAFYEILNMPNLNEAQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPK

SpA D domain with QQ-KR substitutions at positions 4 and 5

SEQ ID NO: 25

ADAKRNNFNKDQQSAFYEILNMPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPK

SpA IgG binding portion (EDABC domains) with the substitutions of SEQ ID NOs 19-23 (SpA_{KKAA})

SEQ ID NO: 26

AQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDKKSAFYEI

LNMPNLNEAQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLNEEQRNGFIQS

LKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQRNGFIQSLKAAPSQSANLLAEAKK

LNDAQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAEAKKLNDAQAPK

SpA IgG binding portion (EDABC domains) with the substitutions of SEQ ID NOs 19 and 21-24 ($SpA_{KKAA-KR}$)

SEQ ID NO: 27

AQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLNDSQAPKADAKRNNFNKDKKSAFYEI

LNMPNLNEAQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLNEEQRNGFIQS

-continued

LKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQRNGFIQSLKAAPSQSANLLAEAKK

LNDAQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAEAKKLNDAQAPK

PglB consensus sequence

SEQ ID NO: 28

D/E-X-N-Z-S/T

PglB consensus sequence

SEQ ID NO: 29

K-D/E-X-N-Z-S/T-K

PglB consensus sequence

SEQ ID NO: 30

K-D-Q-N-R-T-K

PglB consensus sequence

SEQ ID NO: 1

K-D-Q-N-A-T-K

ClfAN2N3P116S/Y118A with (underlined) at I337, N-terminal S,
C-terminal GS

SEQ ID NO: 32

SVAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKV

PPIMAGDQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMSAAIDPENVKKTGNVTLATGIGSTTANKTVLVDY

EKYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADL

SESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWR

SMSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEP**KDQNATK**PEDGS

SEQUENCE LISTING

Sequence total quantity: 32
SEQ ID NO: 1 moltype = AA length = 522
FEATURE Location/Qualifiers
source 1..522
mol_type = protein
organism = Staphylococcus aureus
SEQUENCE: 1
ASENSVTQSD SASNESKSND SSSVSAAPKT DDTNVSDTKT SSNTNNGETS VAQNPAQQET 60
TQSSSTNATT EETPVTGEAT TTTTNQANTP ATTQSSNTNA EELVNQTSNE TTFNDTNTVS 120
SVNSPQNSTN AENVSTTQDT STEATPSNNE SAPQSTDASN KDVVNQAVNT SAPRMRAFSL 180
AAVAADAPAA GTDITNQLTN VTVGIDSGTT VYPHQAGYVK LNYGFSVPNS AVKGDTFKIT 240
VPKELNLNGV TSTAKVPPIM AGDQVLANGV IDSDGNVIYT FTDYVNTKDD VKATLTMPAY 300
IDPENVKKTG NVTLATGIGS TTANKTVLVD YEKYGKFYNL SIKGTIDQID KTNNTYRQTI 360
YVNPSGDNVI APVLTGNLKP NTDSNALIDQ QNTSIKVYKV DNAADLSESY FVNPENFEDV 420
TNSVNITFPN PNQYKVEFNT PDDQITTPYI VVVNGHIDPN SKGDLALRST LYGYNSNIIW 480
RSMSWDNEVA FNNGSGSGDG IDKPVVPEQP DEPGEIEPIP ED 522

SEQ ID NO: 2 moltype = AA length = 340
FEATURE Location/Qualifiers
source 1..340
mol_type = protein
organism = Staphylococcus aureus
SEQUENCE: 2
VAADAPAAGT DITNQLTNVT VGIDSGTTVY PHQAGYVKLN YGFSVPNSAV KGDTFKITVP 60
KELNLNGVTS TAKVPPIMAG DQVLANGVID SDGNVIYTFT DYVNTKDDVK ATLTMPAYID 120
PENVKKTGNV TLATGIGSTT ANKTVLVDYE KYGKFYNLSI KGTIDQIDKT NNTYRQTIYV 180
NPSGDNVIAP VLTGNLKPNT DSNALIDQQN TSIKVYKVDN AADLSESYFV NPENFEDVTN 240
SVNITFPNPN QYKVEFNTPD DQITTPYIVV VNGHIDPNSK GDLALRSTLY GYNSNIIWRS 300
MSWDNEVAFN NGSGSGDGID KPVVPEQPDE PGEIEPIPED 340

SEQ ID NO: 3 moltype = AA length = 293
FEATURE Location/Qualifiers
source 1..293
mol_type = protein
organism = Staphylococcus aureus
SEQUENCE: 3
ADSDINIKTG TTDIGSNTTV KTGDLVTYDK ENGMHKKVFY SFIDDKNHNK KLLVIRTKGT 60
IAGQYRVYSE EGANKSGLAW PSAFKVQLQL PDNEVAQISD YYPRNSIDTK EYMSTLTYGF 120

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NGNVTGDDTG KIGGLIGANV SIGHTLKYVQ PDFKTILESP TDKKVGWKVI FNNMVNQNWG  180
PYDRDSWNPV YGNQLFMKTR NGSMKAADNF LDPNKASSLL SSGFSPDFAT VITMDRKASK  240
QQTNIDVIYE RVRDDYQLHW TSTNWKGTNT KDKWIDRSSE RYKIDWEKEE MTN         293

SEQ ID NO: 4           moltype = AA  length = 516
FEATURE                Location/Qualifiers
source                 1..516
                       mol_type = protein
                       organism = Staphylococcus aureus
SEQUENCE: 4
MKKKNIYSIR KLGVGIASVT LGTLLISGGV TPAANAAQHD EAQQNAFYQV LNMPNLNADQ  60
RNGFIQSLKD DPSQSANVLG EAQKLNDSQA PKADAQQNNF NKDQQSAFYE ILNMPNLNEA  120
QRNGFIQSLK DDPSQSTNVL GEAKKLNESQ APKADNNFNK EQQNAFYEIL NMPNLNEEQR  180
NGFIQSLKDD PSQSANLLSE AKKLNESQAP KADNKFNKEQ QNAFYEILHL PNLNEEQRNG  240
FIQSLKDDPS QSANLLAEAK KLNDAQAPKA DNKFNKEQQN AFYEILHLPN LTEEQRNGFI  300
QSLKDDPSVS KEILAEAKKL NDAQAPKEED NNKPGKEDNN KPGKEDNNKP GKEDNNKPGK  360
EDNNKPGKED GNKPGKEDNK KPGKEDGNKP GKEDNKKPGK EDGNKPGKED GNKPGKEDGN  420
GVHVVKPGDT VNDIAKANGT TADKIAADNK LADKNMIKPG QELVVDKKQP ANHADANKAQ  480
ALPETGEENP FIGTTVFGGL SLALGAALLA GRRREL                            516

SEQ ID NO: 5           moltype = AA  length = 340
FEATURE                Location/Qualifiers
source                 1..340
                       mol_type = protein
                       organism = synthetic construct
                       note = ClfA N2N3 P116S/Y118A
SEQUENCE: 5
VAADAPAAGT DITNQLTNVT VGIDSGTTVY PHQAGYVKLN YGFSVPNSAV KGDTFKITVP  60
KELNLNGVTS TAKVPPIMAG DQVLANGVID SDGNVIYTFT DYVNTKDDVK ATLTMSAAID  120
PENVKKTGNV TLATGIGSTT ANKTVLVDYE KYGKFYNLSI KGTIDQIDKT NNTYRQTIYV  180
NPSGDNVIAP VLTGNLKPNT DSNALIDQQN TSIKVYKVDN AADLSESYFV NPENFEDVTN  240
SVNITFPNPN QYKVEFNTPD DQITTPYIVV VNGHIDPNSK GDLALRSTLY GYNSNIIWRS  300
MSWDNEVAFN NGSGSGDGID KPVVPEQPDE PGEIEPIPED                        340

SEQ ID NO: 6           moltype = AA  length = 346
FEATURE                Location/Qualifiers
source                 1..346
                       mol_type = protein
                       organism = synthetic construct
                       note = ClfA N2N3 P116S/Y118A  with PglB
SEQUENCE: 6
VAADAPAAGT DITNQLTNVT VGIDSGTTVY PHQAGYVKLN YGFSVPNSAV KGDTFKITVP  60
KELNLNGVTS TAKVPPIMAG DQVLANGVID SDGNVIYTFT DYVNTKDDVK ATLTMSAAID  120
PENVKKTGNV TLATGIGSTT ANKTVLVDYE KYGKFYNLSI KGTIDQIDKT NNTYRQTIYV  180
NPSGDNVIAP VLTGNLKPNT DSNALIDQQN TSIKVYKVDN AADLSESYFV NPENFEDVTN  240
SVNITFPNPN QYKVEFNTPD DQITTPYIVV VNGHIDPNSK GDLALRSTLY GYNSNIIWRS  300
MSWDNEVAFN NGSGSGDGID KPVVPEQPDE PGEIEPKDQN ATKPED                 346

SEQ ID NO: 7           moltype = AA  length = 347
FEATURE                Location/Qualifiers
source                 1..347
                       mol_type = protein
                       organism = synthetic construct
                       note = ClfA N2N3 P116S/Y118A
SEQUENCE: 7
SVAADAPAAG TDITNQLTNV TVGIDSGTTV YPHQAGYVKL NYGFSVPNSA VKGDTFKITV  60
PKELNLNGVT STAKVPPIMA GDQVLANGVI DSDGNVIYTF TDYVNTKDDV KATLTMSAAI  120
DPENVKKTGN VTLATGIGST TANKTVLVDY EKYGKFYNLS IKGTIDQIDK TNNTYRQTIY  180
VNPSGDNVIA PVLTGNLKPN TDSNALIDQQ NTSIKVYKVD NAADLSESYF VNPENFEDVT  240
NSVNITFPNP NQYKVEFNTP DDQITTPYIV VVNGHIDPNS KGDLALRSTL YGYNSNIIWR  300
SMSWDNEVAF NNGSGSGDGI DKPVVPEQPD EPGEIEPKDQ NATKPED               347

SEQ ID NO: 8           moltype = AA  length = 293
FEATURE                Location/Qualifiers
source                 1..293
                       mol_type = protein
                       organism = synthetic construct
                       note = Mature Hla H35L
SEQUENCE: 8
ADSDINIKTG TTDIGSNTTV KTGDLVTYDK ENGMLKKVFY SFIDDKNHNK KLLVIRTKGT  60
IAGQYRVYSE EGANKSGLAW PSAFKVQLQL PDNEVAQISD YYPRNSIDTK EYMSTLTYGF  120
NGNVTGDDTG KIGGLIGANV SIGHTLKYVQ PDFKTILESP TDKKVGWKVI FNNMVNQNWG  180
PYDRDSWNPV YGNQLFMKTR NGSMKAADNF LDPNKASSLL SSGFSPDFAT VITMDRKASK  240
QQTNIDVIYE RVRDDYQLHW TSTNWKGTNT KDKWIDRSSE RYKIDWEKEE MTN         293
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SEQ ID NO: 9          moltype = AA  length = 299
FEATURE               Location/Qualifiers
source                1..299
                      mol_type = protein
                      organism = synthetic construct
                      note = Mature Hla H35L with PglB consensus sequence
                       substituted for residue K131
SEQUENCE: 9
ADSDINIKTG TTDIGSNTTV KTGDLVTYDK ENGMLKKVFY SFIDDKNHNK KLLVIRTKGT  60
IAGQYRVYSE EGANKSGLAW PSAFKVQLQL PDNEVAQISD YYPRNSIDTK EYMSTLTYGF  120
NGNVTGDDTG KDQNRTKIGG LIGANVSIGH TLKYVQPDFK TILESPTDKK VGWKVIFNNM  180
VNQNWGPYDR DSWNPVYGNQ LFMKTRNGSM KAADNFLDPN KASSLLSSGF SPDFATVITM  240
DRKASKQQTN IDVIYERVRD DYQLHWTSTN WKGTNTKDKW IDRSSERYKI DWEKEEMTN   299

SEQ ID NO: 10         moltype = AA  length = 293
FEATURE               Location/Qualifiers
source                1..293
                      mol_type = protein
                      organism = synthetic construct
                      note = Mature Hla H35L/H48C/G122C
SEQUENCE: 10
ADSDINIKTG TTDIGSNTTV KTGDLVTYDK ENGMLKKVFY SFIDDKNCNK KLLVIRTKGT  60
IAGQYRVYSE EGANKSGLAW PSAFKVQLQL PDNEVAQISD YYPRNSIDTK EYMSTLTYGF  120
NCNVTGDDTG KIGGLIGANV SIGHTLKYVQ PDFKTILESP TDKKVGWKVI FNNMVNQNWG  180
PYDRDSWNPV YGNQLFMKTR NGSMKAADNF LDPNKASSLL SSGFSPDFAT VITMDRKASK  240
QQTNIDVIYE RVRDDYQLHW TSTNWKGTNT KDKWIDRSSE RYKIDWEKEE MTN         293

SEQ ID NO: 11         moltype = AA  length = 299
FEATURE               Location/Qualifiers
source                1..299
                      mol_type = protein
                      organism = synthetic construct
                      note = Mature Hla H35L/H48C/G122C with PglB consensus
                       sequence substituted for residue K131
SEQUENCE: 11
ADSDINIKTG TTDIGSNTTV KTGDLVTYDK ENGMLKKVFY SFIDDKNCNK KLLVIRTKGT  60
IAGQYRVYSE EGANKSGLAW PSAFKVQLQL PDNEVAQISD YYPRNSIDTK EYMSTLTYGF  120
NCNVTGDDTG KDQNRTKIGG LIGANVSIGH TLKYVQPDFK TILESPTDKK VGWKVIFNNM  180
VNQNWGPYDR DSWNPVYGNQ LFMKTRNGSM KAADNFLDPN KASSLLSSGF SPDFATVITM  240
DRKASKQQTN IDVIYERVRD DYQLHWTSTN WKGTNTKDKW IDRSSERYKI DWEKEEMTN   299

SEQ ID NO: 12         moltype = AA  length = 306
FEATURE               Location/Qualifiers
source                1..306
                      mol_type = protein
                      organism = synthetic construct
                      note = Hla H35L/H48C/G122C with N-terminal S, C-terminal
                       GSHRHR, and KDQNRTK substituted for residue K131
SEQUENCE: 12
SADSDINIKT GTTDIGSNTT VKTGDLVTYD KENGMLKKVF YSFIDDKNCN KKLLVIRTKG  60
TIAGQYRVYS EEGANKSGLA WPSAFKVQLQ LPDNEVAQIS DYYPRNSIDT KEYMSTLTYG  120
FNCNVTGDDT GKDQNRTKIG GLIGANVSIG HTLKYVQPDF KTILESPTDK KVGWKVIFNN  180
MVNQNWGPYD RDSWNPVYGN QLFMKTRNGS MKAADNFLDP NKASSLLSSG FSPDFATVIT  240
MDRKASKQQT NIDVIYERVR DDYQLHWTST NWKGTNTKDK WIDRSSERYK IDWEKEEMTN  300
GSHRHR                                                             306

SEQ ID NO: 13         moltype = AA  length = 291
FEATURE               Location/Qualifiers
source                1..291
                      mol_type = protein
                      organism = Staphylococcus aureus
SEQUENCE: 13
AQHDEAQQNA FYQVLNMPNL NADQRNGFIQ SLKDDPSQSA NVLGEAQKLN DSQAPKADAQ  60
QNNFNKDQQS AFYEILNMPN LNEAQRNGFI QSLKDDPSQS TNVLGEAKKL NESQAPKADN  120
NFNKEQQNAF YEILNMPNLN EEQRNGFIQS LKDDPSQSAN LLSEAKKLNE SQAPKADNKF  180
NKEQQNAFYE ILHLPNLNEE QRNGFIQSLK DDPSQSANLL AEAKKLNDAQ APKADNKFNK  240
EQQNAFYEIL HLPNLTEEQR NGFIQSLKDD PSVSKEILAE AKKLNDAQAP K           291

SEQ ID NO: 14         moltype = AA  length = 56
FEATURE               Location/Qualifiers
source                1..56
                      mol_type = protein
                      organism = Staphylococcus aureus
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SEQUENCE: 14
AQHDEAQQNA FYQVLNMPNL NADQRNGFIQ SLKDDPSQSA NVLGEAQKLN DSQAPK      56

SEQ ID NO: 15          moltype = AA  length = 61
FEATURE                Location/Qualifiers
source                 1..61
                       mol_type = protein
                       organism = Staphylococcus aureus
SEQUENCE: 15
ADAQQNNFNK DQQSAFYEIL NMPNLNEAQR NGFIQSLKDD PSQSTNVLGE AKKLNESQAP  60
K                                                                  61

SEQ ID NO: 16          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = Staphylococcus aureus
SEQUENCE: 16
ADNNFNKEQQ NAFYEILNMP NLNEEQRNGF IQSLKDDPSQ SANLLSEAKK LNESQAPK    58

SEQ ID NO: 17          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = Staphylococcus aureus
SEQUENCE: 17
ADNNFNKEQQ NAFYEILNMP NLNEEQRNGF IQSLKDDPSQ SANLLSEAKK LNESQAPK    58

SEQ ID NO: 18          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = Staphylococcus aureus
SEQUENCE: 18
ADNKFNKEQQ NAFYEILHLP NLTEEQRNGF IQSLKDDPSV SKEILAEAKK LNDAQAPK    58

SEQ ID NO: 19          moltype = AA  length = 56
FEATURE                Location/Qualifiers
source                 1..56
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA E domain with QQ-KK substitutions at positions 7
                        and 8 and DD-AA substitutions at positions 34 and 35
SEQUENCE: 19
AQHDEAKKNA FYQVLNMPNL NADQRNGFIQ SLKAAPSQSA NVLGEAQKLN DSQAPK      56

SEQ ID NO: 20          moltype = AA  length = 61
FEATURE                Location/Qualifiers
source                 1..61
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA D domain with QQ-KK substitutions at positions
                        12 and 13 and DD-AA substitutions at positions 39 and 40
SEQUENCE: 20
ADAQQNNFNK DKKSAFYEIL NMPNLNEAQR NGFIQSLKAA PSQSTNVLGE AKKLNESQAP  60
K                                                                  61

SEQ ID NO: 21          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA A domain with QQ-KK substitutions at positions 9
                        and 108 and DD-AA substitutions at positions 36 and 37
SEQUENCE: 21
ADNNFNKEKK NAFYEILNMP NLNEEQRNGF IQSLKAAPSQ SANLLSEAKK LNESQAPK    58

SEQ ID NO: 22          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA B domain with QQ-KK substitutions at positions 9
                        and 10 and DD-AA substitutions at positions 36 and 37
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SEQUENCE: 22
ADNKFNKEKK NAFYEILHLP NLNEEQRNGF IQSLKAAPSQ SANLLAEAKK LNDAQAPK    58

SEQ ID NO: 23          moltype = AA  length = 58
FEATURE                Location/Qualifiers
source                 1..58
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA C domain with QQ-KK substitutions at positions 9
                        and 10 and DD-AA substitutions at positions 36 and 37
SEQUENCE: 23
ADNKFNKEKK NAFYEILHLP NLTEEQRNGF IQSLKAAPSV SKEILAEAKK LNDAQAPK    58

SEQ ID NO: 24          moltype = AA  length = 61
FEATURE                Location/Qualifiers
source                 1..61
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA D domain with QQ-KR substitutions at positions 4
                        and 5, QQ-KK substitutions at positions 12 and 13 and
                        DD-AA substitutions at positions 39 and 40
SEQUENCE: 24
ADAKRNNFNK DKKSAFYEIL NMPNLNEAQR NGFIQSLKAA PSQSTNVLGE AKKLNESQAP  60
K                                                                  61

SEQ ID NO: 25          moltype = AA  length = 61
FEATURE                Location/Qualifiers
source                 1..61
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA D domain with QQ-KR substitutions at positions  4
                         and 5
SEQUENCE: 25
ADAKRNNFNK DQQSAFYEIL NMPNLNEAQR NGFIQSLKDD PSQSTNVLGE AKKLNESQAP  60
K                                                                  61

SEQ ID NO: 26          moltype = AA  length = 291
FEATURE                Location/Qualifiers
source                 1..291
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA IgG binding portion with the substitutions of
                        SEQ ID NOs 19-23
SEQUENCE: 26
AQHDEAKKNA FYQVLNMPNL NADQRNGFIQ SLKAAPSQSA NVLGEAQKLN DSQAPKADAQ  60
QNNFNKDKKS AFYEILNMPN LNEAQRNGFI QSLKAAPSQS TNVLGEAKKL NESQAPKADN  120
NFNKEKKNAF YEILNMPNLN EEQRNGFIQS LKAAPSQSAN LLSEAKKLNE SQAPKADNKF  180
NKEKKNAFYE ILHLPNLNEE QRNGFIQSLK AAPSQSANLL AEAKKLNDAQ APKADNKFNK  240
EKKNAFYEIL HLPNLTEEQR NGFIQSLKAA PSVSKEILAE AKKLNDAQAP K           291

SEQ ID NO: 27          moltype = AA  length = 291
FEATURE                Location/Qualifiers
source                 1..291
                       mol_type = protein
                       organism = synthetic construct
                       note = SpA IgG binding portion with the substitutions of
                        SEQ ID NOs 19 and 21 24
SEQUENCE: 27
AQHDEAKKNA FYQVLNMPNL NADQRNGFIQ SLKAAPSQSA NVLGEAQKLN DSQAPKADAK  60
RNNFNKDKKS AFYEILNMPN LNEAQRNGFI QSLKAAPSQS TNVLGEAKKL NESQAPKADN  120
NFNKEKKNAF YEILNMPNLN EEQRNGFIQS LKAAPSQSAN LLSEAKKLNE SQAPKADNKF  180
NKEKKNAFYE ILHLPNLNEE QRNGFIQSLK AAPSQSANLL AEAKKLNDAQ APKADNKFNK  240
EKKNAFYEIL HLPNLTEEQR NGFIQSLKAA PSVSKEILAE AKKLNDAQAP K           291

SEQ ID NO: 28          moltype =   length =
SEQUENCE: 28
000

SEQ ID NO: 29          moltype =   length =
SEQUENCE: 29
000
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SEQ ID NO: 30          moltype = AA  length = 7
FEATURE                Location/Qualifiers
source                 1..7
                       mol_type = protein
                       organism = synthetic construct
                       note = PglB consensus sequence
SEQUENCE: 30
KDQNRTK                                                        7

SEQ ID NO: 31          moltype = AA  length = 7
FEATURE                Location/Qualifiers
source                 1..7
                       mol_type = protein
                       organism = synthetic construct
                       note = PglB consensus sequence
SEQUENCE: 31
KDQNATK                                                        7

SEQ ID NO: 32          moltype = AA  length = 349
FEATURE                Location/Qualifiers
source                 1..349
                       mol_type = protein
                       organism = synthetic construct
                       note = ClfAN2N3P116S/Y118A at I337, N-terminal S,
                        C-terminal  GS
SEQUENCE: 32
SVAADAPAAG TDITNQLTNV TVGIDSGTTV YPHQAGYVKL NYGFSVPNSA VKGDTFKITV  60
PKELNLNGVT STAKVPPIMA GDQVLANGVI DSDGNVIYTF TDYVNTKDDV KATLTMSAAI  120
DPENVKKTGN VTLATGIGST TANKTVLVDY EKYGKFYNLS IKGTIDQIDK TNNTYRQTIY  180
VNPSGDNVIA PVLTGNLKPN TDSNALIDQQ NTSIKVYKVD NAADLSESYF VNPENFEDVT  240
NSVNITFPNP NQYKVEFNTP DDQITTPYIV VVNGHIDPNS KGDLALRSTL YGYNSNIIWR  300
SMSWDNEVAF NNGSGSGDGI DKPVVPEQPD EPGEIEPKDQ NATKPEDGS               349
```

1-47. (canceled)

48. A method of prevention or treatment of staphylococcal infection, comprising administering to a subject in need thereof an immunogenic composition comprising:

a. a clumping factor A (ClfA) antigen comprising an amino acid sequence at least 95% identical to SEQ ID NO: 32, wherein the ClfA antigen comprises a serine at a position equivalent to P116 of SEQ ID NO: 2; an alanine at a position equivalent to Y118 of SEQ ID NO: 2, and the sequence KDQNATK (SEQ ID NO: 31) beginning at a position equivalent to 1337 of SEQ ID NO: 2;

b. an alpha-haemolysin (Hla) antigen comprising an amino acid sequence at least 95% identical to SEQ ID NO: 12, wherein the Hla antigen comprises a leucine at a position equivalent to H35 of SEQ ID NO: 3, a cysteine at a position equivalent to H48 of SEQ ID NO: 3, a cysteine at a position equivalent to G122 of SEQ ID NO: 3, and the sequence KDQNRTK (SEQ ID NO: 30) beginning at a position equivalent to K131 of SEQ ID NO: 3; and

c. a Staphylococcal protein A (SpA) antigen comprising an amino acid sequence at least 95% identical to SEQ ID NO: 27, wherein the SpA antigen comprises a lysine at ositions equivalent to positions Q43, Q44, Q96, Q104, Q105, Q162, Q163, Q220, Q221, Q278, and Q279 of SEQ ID NO: 4; an arginine at a position equivalent to position Q97 of SEQ ID NO: 4; and an alanine at positions equivalent to positions D70, D71, D131, D132, D189, D190, D247, D248, D305, and D306;

wherein the ClfA antigen is conjugated to a *Staphylococcus* aureus serotype type 8 capsular polysaccharide, and the Hla antigen is conjugated to a *S. aureus* serotype 5 capsular polysaccharide.

49-53. (canceled)

54. The method according to claim **48**, wherein:

a. the amino acid sequence of part (a) is at least 96%, 97%, 98% or 99% identical to SEQ ID NO. 32;

b. the amino acid sequence of part (b) is at least 96%, 97%, 98% or 99% identical to SEQ ID NO. 12; and/or

c. the amino acid sequence of part (c) is at least 96%, 97%, 98% or 99% identical to SEQ ID NO. 27.

55. The method according to claim **54**, wherein the ClfA antigen comprises or consists of the sequence of SEQ ID NO: 7 or SEQ ID NO: 32.

56. The method according to claim **54**, wherein the Hla antigen comprises or consists of the sequence of SEQ ID NO: 11 or SEQ ID NO: 12.

57. The method according to claim **54**, wherein the SpA antigen comprises or consists of the sequence of SEQ ID NO: 26 or SEQ ID NO: 27.

58. The method according to claim **54**, wherein (i) the ClfA antigen comprises the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 32; (ii) the Hla antigen comprises the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 12; and (iii) the SpA antigen comprises the amino acid sequence of SEQ ID NO: 26 or SEQ ID NO: 27.

59. The method according to claim **48**, wherein said ClfA-CP8 and Hla-CP5 conjugates are bioconjugates.

60. The method according to claim **48**, the immunogenic composition further comprising an adjuvant.

61. The method of claim **60**, wherein the adjuvant comprises a saponin and a Toll Like Receptor 4 (TLR4) agonist.

62. The method according to claim **61**, wherein the TLR4 agonist is a lipopolysaccharide.

63. The method according to claim **62**, wherein the lipopolysaccharide is 3-de-O-acylated monophosphoryl lipid A (3D-MPL).

64. The method according to claim **61**, wherein the saponin comprises an active fraction of the saponin derived from the bark of *Quillaja Saponaria Molina*.

65. The method according to claim **64**, wherein the active fraction of the saponin derived from the bark of *Quillaja Saponaria Molina* is QS21.

66. The method according to claim **60**, wherein the adjuvant comprises 3D-ML and QS21 in a liposome.

67. The method according to claim **60**, wherein the adjuvant comprises a Toll Like Receptor 7 (TLR7) agonist adsorbed to aluminum hydroxide (AlOH).

68. The method of claim **66**, wherein the adjuvant comprises 3D-MPL at a level of 25 μg per human dose and QS21 at a level of 25 μg per human dose.

69. The method of claim **60**, wherein said adjuvant is administered concomitantly with the immunogenic composition.

70. The method according to claim **60**, wherein the adjuvant is mixed with the immunogenic composition before administering to the subject.

71. The method of claim **69**, wherein the adjuvant comprises a saponin and a TLR4 agonist.

72. The method of claim **69**, wherein the adjuvant comprises 3D-MPL and QS21 in a liposome.

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