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(57) Abstract: Mutant SpA with decreased affinity for the Fcγ portion of human IgG is provided. Immunisation with EsxA, EsxB, FhuD2, Sta011, Hla and said mutant SpA provides striking results in a renal abscess model of *S.aureus* infection.



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MUTANT STAPHYLOCOCCAL ANTIGENS

This application claim the benefit of European patent applications 14161861.1 (filed March 26 2014) and 14192913.3 (filed November 12 2014), the complete contents of both of which are hereby incorporated by reference for all purposes.

5 TECHNICAL FIELD

This invention relates to immunisation against infection by *S.aureus*.

BACKGROUND ART

Staphylococcus aureus is a Gram-positive spherical bacterium. Annual US mortality exceeds that of any other infectious disease, including HIV/AIDS, and *S.aureus* is the leading cause of bloodstream, lower respiratory tract, skin & soft tissue infections. It is also the predominant cause of bone infections worldwide, and these infections are painful, debilitating and difficult to treat.

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 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.

There is currently no authorised vaccine. A vaccine based on a mixture of surface polysaccharides from bacterial types 5 and 8, StaphVAX™, failed to reduce infections when compared to the placebo group in a phase III clinical trial. Similarly, the V710 vaccine [1], based on the IsdB antigen [2], failed to reduce the rate of post-operative *S.aureus* infections [3].

The need for a vaccine is particularly acute due to 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 Fc of immunoglobulin (Ig) and to the Fab portion of VH3-type B cell receptors. Interaction of SpA with B cell receptors leads to clonal expansion and subsequent cell death of B cell populations, leading to ablation of the adaptive immune response. SpA binding to Ig Fc interferes with opsonophagocytic clearance of staphylococci by polymorphonuclear leukocytes.

Mutant forms of SpA with reduced affinity to immunoglobulins have been developed. WO2011/005341 describes a SpA with point mutations in each of the five Ig-binding domains which reduce the ability of the protein to bind to IgG.

Reference 4 discloses various approaches to providing improved *S.aureus* vaccines, including two preferred immunogen combinations referred to as “Combo-1” and “Combo-2”. “Combo-1” included five antigens EsxA, EsxB, a mutant Hla, FhuD2, and Sta011, whereas “Combo-2” used a fragment of

IsdA in place of the mutant Hla. Both of these combinations were tested with aluminium hydroxide adjuvants and they increased median survival time in a mouse model of infection when compared to buffer alone or to the IsdB antigen. “Combo-1” has also been tested with an aluminium hydroxide adjuvant and a TLR7 agonist, and the addition of the TLR7 agonist improved responses [5].

- 5 Despite the positive results with “Combo-1” and “Combo-2”, there remains a need for further and improved compositions for immunising against *S.aureus*.

DISCLOSURE OF THE INVENTION

Protein A (SpA) (SEQ ID NO: 43), a cell wall anchored surface protein of *Staphylococcus aureus*, provides for bacterial evasion from innate and adaptive immune responses. Protein A binds immunoglobulins at their Fc portion, interacts with the VH3 domain of B cell receptors inappropriately stimulating B cell proliferation and apoptosis, binds to von Willebrand factor A1 domains to activate intracellular clotting, and also binds to the TNF Receptor- 1 to contribute to the pathogenesis of staphylococcal pneumonia. Due to the fact that Protein A captures immunoglobulin and displays toxic attributes, the possibility that this surface molecule may function as a vaccine in humans has not been rigorously pursued. Here the inventors demonstrate that Protein A variants no longer able to bind to immunoglobulins, which are thereby removed of their toxigenic potential, i.e., are non-toxicogenic, stimulate humoral immune responses that protect against staphylococcal disease.

The invention therefore provides mutant SpA antigens as described below. Said mutants preferably have decreased affinity for the Fc γ portion of human IgG relative to unmodified SpA. The mutants may also have decreased affinity, relative to unmodified SpA, for the Fab portion of VH3-containing human B cell receptors.

The inventors have found that the known “Combo-1” vaccine can be improved by adding a mutant staphylococcal protein A (SpA) which has been modified to decrease its affinity for the Fc γ portion of human IgG and for the Fab portion of VH3-containing B cell receptors. This extra antigen increases the combination’s protective efficacy and provides striking results in a renal abscess model. None of the antigen combinations tested in reference 4 included a SpA antigen.

The invention therefore further provides an immunogenic composition comprising EsxA, EsxB, FhuD2, Sta011, and Hla antigens, characterised in that the composition additionally includes a mutant SpA antigen, wherein the mutant has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG and for the Fab portion of VH3-containing human B cell receptors.

The invention also provides an immunogenic composition comprising: (i) at least one antigen selected from the group consisting of EsxA, EsxB, FhuD2, Sta011, and Hla antigens; and (ii) a mutant SpA antigen which has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG and for the Fab portion of VH3-containing human B cell receptors. Thus 1, 2, 3, 4 or preferably all 5 of EsxA, EsxB, FhuD2, Sta011, and Hla can be used in combination with the mutant SpA.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one skilled in the relevant art.

For convenience, the meaning of certain terms and phrases employed in the specification, examples and claims are provided.

5 *S.aureus* antigens

The invention relates inter alia to a mutant SpA antigen. The wild-type SpA (staphylococcal protein A) is a cell wall-anchored surface protein which is a crucial virulence factor for lung infections, septicemia, 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 leads to clonal expansion and subsequent cell death of B cell populations with effects on adaptive and innate immune responses, whereas its binding to the Fc γ 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 [6]. These domains display ~80% identity at the amino acid level, are 56 to 61 residues in length, and are organized as tandem repeats [7]. 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.

In the NCTC 8325 strain spa is SAOUHSC_00069 and has amino acid sequence SEQ ID NO: 43 (GI:88193885). In the Newman strain it is nwmn_0055 (GI:151220267). SpA antigens used with the invention can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 43 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: 43; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 43, 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: 43. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 43. 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: 43 while retaining at least one epitope of SEQ ID NO: 43. The final 35 C-terminal amino acids of SEQ ID NO: 43 can usefully be omitted. The first 36 N-terminal amino acids of SEQ ID NO: 43 can usefully be omitted. Reference 8 suggests that individual IgG-binding domains might be useful immunogens, alone or in combination.

A useful fragment of SEQ ID NO: 43 is amino acids 37 to 325. 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) 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 and then apoptosis which might occur if spa functions as a B cell superantigen. As reported in reference 18, 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, but ideally has 4 or fewer

Thus, another useful fragment of SpA comprises or consists of the amino acid sequence of SEQ ID NO: 50 wherein the amino acid doublet at positions 60 and 61 is not Gln-Gln. Said doublet may be mutated as described below, for example to Lys-Arg. Thus, said fragment may comprise or consist of SEQ ID NO: 51 or 52.

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(E) 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(E) domain, and another protein antigen, such as EsxA; EsxB; FhuD2; Sta011; and Hla.

SpA antigens of the invention are mutated relative to SEQ ID NO: 43, such that they have decreased affinity for the Fc γ portion of human IgG. For instance, the QQ dipeptide at residues 60-61 of SEQ ID NO: 43 can be mutated to reduce affinity for immunoglobulins. Useful dipeptide substitutions for a QQ dipeptide are discussed below, and a preferred substitution is KR dipeptide. Thus a useful SpA antigen can comprise SEQ ID NO: 49, in which one or more (preferably all) of the 11 XX dipeptides differ from the corresponding dipeptides within SEQ ID NO: 43. For instance, the SpA antigen can comprise SEQ ID NO: 46, and a preferred example of SEQ ID NO: 46 is SEQ ID NO: 47. When expressed with a N-terminal methionine, the SpA antigen comprising SEQ ID NO: 47 can consist of SEQ ID NO: 48.

SpA antigens used with the invention may be further mutated relative to SEQ ID NO: 43, such that they have decreased affinity for the Fc γ portion of human IgG and 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 reference 9. 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 underlined below, where dashes separate the five Ig-binding domains:

MKKKNIYSIRKLGVGIASVTLGTLISGGVTP-AANAAQHDEAQQNAFYQVLNMPNLNADQRNGF
 IQSLKDDPSQSANVLGEAQKLNDSSQAPK-ADAQQNNFNKDQQSAFYEILNMPNLNEAQRNGFIQS
 LKDDPSQSTNVLGEAKKLNESQAPK-ADNNFNKEQQNAFYEILNMPNLNEEQRNGFIQSLKDDPS
 QSANLLSEAKKLNESQAPK-ADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLL

AEAKKLNDQAQPK-ADNKFNKEQQNAFYEILHLPLNLTEEQRNGFIQSLKDDPSVSKEILAEAKKL
 NDAQAPK-EEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDGKNKPGKEDNKKPGK
 EDGKNKPGKEDNKKPGKEDGKNKPGKEDGKNKPGKEDGNGVHVVKPGD TVNDIAKANGTTADKIAADN
 KLADKNMIKPGQELVVDKKQPANHADANKAQAALPETGEENPFIGTTVFGLSLALGAALLAGRRL
 EL
 (SEQ ID NO: 43)

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: 43 (*e.g.* see above in relation to Gln-Gln and Asp-Asp sequences, but also see reference **Error! Bookmark not defined.** 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: 43, but will remove the antigen's binding to IgG and/or other human proteins (such as human blood proteins) as noted above. Particularly, the mutant SpA antigen is of sequence comprising or consisting of SEQ ID NO: 43 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 position 43, 44, 70, 71, 104, 105, 131, 132, 162, 163, 190, 191, 220, 221, 247, 248, 278, 279, 305 and/or 306 of SEQ ID NO: 43. Useful substitutions for these positions are mentioned above.

Furthermore, the native N-terminus can be removed, and the first 36 amino acids of SEQ ID NO: 43 can usefully be omitted. Similarly, the native C-terminal can be removed, and the sequence downstream of the fifth Ig-binding domain can usefully be omitted (*i.e.* downstream of Lys-327 in SEQ ID NO: 43). Thus a useful SpA antigen comprises SEQ ID NO: 44:

AQHDEAXXNAFYQVLNMPNLNADQQRNGFIQSLKXXPSQSANVLGEAQKLND SQAPKADAQQNNFN
 KDXXSFAFYEILNMPNLNEAQRNGFIQSLKXXPSQSTNVLGEAKKLNESQAPKADNNFNKEXXNAF
 YEILNMPNLNEEQRNGFIQSLKXXPSQSANLLSEAKKLNESQAPKADNKFNKEXXNAFYEILHLPL
 NLNEEQRNGFIQSLKXXPSQSANLLAEAKKLNDQAQPKADNKFNKEXXNAFYEILHLPLNLTEEQR
 NGFIQSLKXXPSVSKEILAEAKKLNDQAQPK

wherein the 10 underlined XX dipeptides differ from the corresponding dipeptides within SEQ ID NO: 43. Thus a QQ at these positions in SEQ ID NO: 43 will not be QQ in SEQ ID NO: 44, and ideally includes no glutamine residue *e.g.* it is KK. Similarly, a DD at these positions in SEQ ID NO: 43 will not be DD in SEQ ID NO: 44, and ideally includes no aspartate residue *e.g.* it is AA. The preferred form of SpA for use with the invention thus comprises SEQ ID NO: 45.

In addition to substitutions at the XX dipeptides in SEQ ID NO: 44 it is possible to modify the amino acid sequence with up to 5 single amino changes provided that the modified sequence can elicit antibodies which still bind to a polypeptide consisting of SEQ ID NO: 44. Thus, SEQ ID NO: 45 can be modified by 1, 2 or 3 substitutions at positions outside the XX dipeptides within SEQ ID NO: 44. For instance, the QQ dipeptide at residues 60-61 of SEQ ID NO: 44 can be mutated to further reduce affinity for immunoglobulins. Useful dipeptide substitutions for a QQ dipeptide are discussed above, and a preferred substitution is KR dipeptide. Thus a useful SpA antigen can comprise SEQ ID NO: 49, in which one or more (preferably all) of the 11 XX dipeptides differ from the corresponding dipeptides within SEQ ID NO: 43. For instance, the SpA antigen can comprise SEQ ID NO: 46, and

a preferred example of SEQ ID NO: 46 is SEQ ID NO: 47. When expressed with a N-terminal methionine, the SpA antigen comprising SEQ ID NO: 47 can consist of SEQ ID NO: 48.

As discussed above, a useful fragment of SpA 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(E) domain but not D, A B or C. Thus a SpA antigen useful with the invention may comprise only the SpA(E) domain mutated as described above, *i.e.* amino acid sequence comprising or consisting of SEQ ID NO: 54 mutated in at least 1 amino acid at positions 60 and 61 of SEQ ID NO: 54 amino acids 1 to 67 of SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 49, or amino acids 1 to 68 of SEQ ID NO: 48. For example, said antigen may comprise SEQ ID NO. 50, SEQ ID NO. 51, SEQ ID NO. 52 or SEQ ID NO. 53. Said antigen will preferably not include other sequence from SpA. It may include more than one copy of the SpA(E) domain, and/or additionally comprise another protein antigen such as an EsxA, EsxB, FhuD2, Sta011, or Hla antigen as described herein.

The antigen combinations of the invention use 1, 2, 3, 4, or all 5 of the following antigens: EsxA; EsxB; FhuD2; Sta011; and Hla. These five antigens are already known in the art (*e.g.* see references 4-14) and further details are given below. A particularly useful composition includes all five of these antigens (preferably where the Hla is a non-toxic (*i.e.* detoxified) mutant form).

The 'EsxA' antigen in the NCTC 8325 strain has amino acid sequence SEQ ID NO: 1 (GI:88194063). EsxA antigens used with the present invention can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 1 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: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 or more). These EsxA polypeptides include variants of SEQ ID NO: 1. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 1. 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: 1 while retaining at least one epitope of SEQ ID NO: 1.

The 'EsxB' antigen in the NCTC 8325 strain has amino acid sequence SEQ ID NO: 2 (GI:88194070). EsxB used with the present invention can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 2 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: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, 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 or more). These EsxB polypeptides include variants of SEQ ID NO: 2. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 2. 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: 2 while retaining at least one epitope of SEQ ID NO: 2. A useful EsxB antigen lacks the internal cysteine residue of SEQ ID NO: 2 *e.g.* it comprises SEQ ID NO: 35, wherein residue X at position 30 is either absent or is an amino acid residue without a free thiol group (under reducing conditions) *e.g.* is any natural amino acid except cysteine.

- 5 The 'FhuD2' antigen is annotated as 'ferrichrome-binding protein', and has also been studied in the literature [15]. It has also been known as 'Sta006' (*e.g.* in references 4-14). In the NCTC 8325 strain FhuD2 has amino acid sequence SEQ ID NO: 3 (GI:88196199). FhuD2 used with the present invention can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 3 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: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3, 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 FhuD2 polypeptides include variants of SEQ ID NO: 3. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 3. 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: 3 while retaining at least one epitope of SEQ ID NO: 3. The first 17 N-terminal amino acids of SEQ ID NO: 3 can usefully be omitted (to provide SEQ ID NO: 6). Mutant forms of FhuD2 are reported in reference 16. A useful FhuD2 antigen lacks the cysteine residue of SEQ ID NO: 3 *e.g.* it comprises SEQ ID NO: 34 and does not include any amino acid residue with a free thiol group (under reducing conditions) *e.g.* it is cysteine-free. A FhuD2 antigen may be lipidated *e.g.* with an acylated N-terminus cysteine. One useful FhuD2 sequence is SEQ ID NO: 7, which has a Met-Ala-Ser- sequence at the N-terminus; SEQ ID NO: 37 is another such sequence, but it lacks the cysteine present in SEQ ID NO: 7.
- 25 The 'Sta011' antigen has amino acid sequence SEQ ID NO: 4 (GI:88193872) in the NCTC 8325 strain. Sta011 antigens used with the invention can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 4 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: 4; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 4, 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 Sta011 polypeptides include variants of SEQ ID NO: 4. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 4. 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: 4 while retaining at least one epitope of SEQ ID NO: 4. The first 23 N-terminal amino acids of SEQ ID NO: 4 can usefully be omitted (to provide SEQ ID NO: 33). A useful Sta011 antigen lacks the cysteine residue of SEQ ID NO: 4 *e.g.* it comprises SEQ ID NO: 36 and does not include any amino acid residue with a free thiol group (under reducing

conditions) *e.g.* it is cysteine-free. A Sta011 antigen may be lipidated *e.g.* with an acylated N-terminus cysteine. One useful Sta011 sequence is SEQ ID NO: 8, which has a N-terminus methionine; SEQ ID NO: 39 is another such sequence, but it lacks the cysteine present in SEQ ID NO: 8. Variant forms of SEQ ID NO: 4 which may be used as or for preparing Sta011 antigens include, but are not limited to, SEQ ID NOs: 9, 10 and 11 with various Ile/Val/Leu substitutions (and Cys-free variants of these sequences can also be used with the invention). Sta011 can exist as a monomer or an oligomer, with Ca^{++} ions favouring oligomerisation. The invention can use monomers and/or oligomers of Sta011.

The 'Hla' antigen is the 'alpha-hemolysin precursor' also known as 'alpha toxin' or simply 'hemolysin'. In the NCTC 8325 strain Hla has amino acid sequence SEQ ID NO: 5 (GI:88194865). Hla is an important virulence determinant produced by most strains of *S.aureus*, having pore-forming and haemolytic activity. Anti-Hla antibodies can neutralise the detrimental effects of the toxin in animal models, and Hla is particularly useful for protecting against pneumonia.

Useful Hla antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 5 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: 5; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 5, 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 Hla antigens include variants of SEQ ID NO: 5. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 5. 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: 5 while retaining at least one epitope of SEQ ID NO: 5. The first 26 N-terminal amino acids of SEQ ID NO: 5 can usefully be omitted (*e.g.* to give SEQ ID NO: 12). Truncation at the C-terminus can also be used *e.g.* leaving only 50 amino acids (residues 27-76 of SEQ ID NO: 5) [17].

Particularly, the Hla antigen is a detoxified Hla antigen, *i.e.* a mutant form of Hla, wherein Hla's natural toxicity has been removed. Hla's toxicity can be avoided by chemical inactivation (*e.g.* using formaldehyde, glutaraldehyde or other cross-linking reagents). Instead, however, it is preferred to use mutant forms of Hla which remove its toxic activity while retaining its immunogenicity. More particularly, the detoxified Hla antigen is a mutant form of Hla, wherein Hla's toxicity has been removed while Hla's immunogenicity has been retained. Such detoxified mutants are already known in the art. A preferred Hla antigen is a mutant *S.aureus* hemolysin having a mutation at residue 61 of SEQ ID NO: 5, which is residue 35 of the mature antigen (*i.e.* after omitting the first 26 N-terminal amino acids = residue 35 of SEQ ID NO: 12). Thus residue 61 may not be histidine, and may instead be *e.g.* Ile, Val or preferably Leu. A His-Arg mutation at this position can also be used. For example, SEQ ID NO: 13 is the sequence of the mature H35L mutant form of Hla (*i.e.* SEQ ID NO: 12 with a H35L mutation) and a useful detoxified Hla antigen is of sequence comprising or consisting of SEQ

ID NO: 13. Another useful mutation replaces a long loop with a short sequence *e.g.* to replace the 39mer at residues 136-174 of SEQ ID NO: 5 with a tetramer such as PSGS (SEQ ID NO: 14), as in SEQ ID NO: 15 (which also includes the H35L mutation) and SEQ ID NO: 16 (which does not include the H35L mutation). Another useful mutation replaces residue Y101 *e.g.* with a leucine (SEQ ID NO: 17). Another useful mutation replaces residue D152 *e.g.* with a leucine (SEQ ID NO: 18). Another useful mutant replaces residues H35 and Y101 *e.g.* with a leucine (SEQ ID NO: 19). Another useful mutant replaces residues H35 and D152 *e.g.* with a leucine (SEQ ID NO: 20).

Further useful Hla antigens are disclosed in references 18 and 19.

SEQ ID NOS: 21, 22 & 23 are three useful fragments of SEQ ID NO: 5 ('Hla₂₇₋₇₆', 'Hla₂₇₋₈₉' and 'Hla₂₇₋₇₉', respectively). SEQ ID NOS: 24, 25 & 26 are the corresponding fragments from SEQ ID NO: 13.

One useful Hla sequence is SEQ ID NO: 27. It has a N-terminal Met, then an Ala-Ser dipeptide from the expression vector, then SEQ ID NO: 13 (from NCTC8325 strain) including the H35L mutation.

Where a composition includes both EsxA and EsxB antigens, these may be present as a single polypeptide (*i.e.* as a fusion polypeptide comprising or consisting of both EsxA and EsxB). Thus a single polypeptide can elicit antibodies (*e.g.* when administered to a human) that recognise both SEQ ID NO: 1 and SEQ ID NO: 2. The single polypeptide can include: (i) a first polypeptide sequence 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: 1 and/or comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, as defined above for EsxA; and (ii) a second polypeptide sequence 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: 2 and/or comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, as defined above for EsxB. The first and second polypeptide sequences can be in either order, N- to C- terminus. SEQ ID NOS: 28 ('EsxAB') and 29 ('EsxBA') are examples of such polypeptides, both having hexapeptide linkers ASGGGS (SEQ ID NO: 30). Another 'EsxAB' hybrid comprises SEQ ID NO: 31, which may be provided with a N-terminus methionine (*e.g.* SEQ ID NO: 32). A useful variant of EsxAB lacks the internal cysteine residue of EsxB *e.g.* it comprises SEQ ID NO: 40 wherein residue X at position 132 is either absent or is an amino acid residue without a free thiol group (under reducing conditions) *e.g.* is any natural amino acid except cysteine. Thus a preferred EsxAB antigen for use with the invention has amino acid sequence SEQ ID NO: 38.

Thus a useful polypeptide comprises an amino acid sequence (a) having 80% or more identity (*e.g.* 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 31; and/or (b) comprising both a fragment of at least 'n' consecutive amino acids from amino acids 1-96 of SEQ ID NO: 31 and a fragment of at least 'n' consecutive amino acids from amino acids 103-205 of SEQ ID NO: 31, 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 polypeptides (*e.g.* SEQ ID NO: 32) can elicit antibodies (*e.g.* when administered to a human) which recognise both the wild-type staphylococcal protein comprising SEQ ID NO: 1 and the wild-type staphylococcal protein comprising SEQ ID NO: 2. Thus the immune response will recognise both of antigens EsxA and EsxB. Preferred fragments of
5 (b) provide an epitope from SEQ ID NO: 1 and an epitope from SEQ ID NO: 2.

The invention uses 1, 2, 3, 4, or all 5 of EsxA, EsxB, FhuD2, Sta011, and Hla (preferably a non-toxic mutant Hla). As mentioned above a particularly useful composition includes all five of these antigens, but in some embodiments the invention includes only 1, 2, 3 or 4 of these five antigens *i.e.* 1, 2, 3 or 4 of EsxA, EsxB, FhuD2, Sta011, and Hla is absent from the composition.

10 A preferred composition includes all four of: (i) a single polypeptide including both an EsxA antigen and an EsxB antigen *e.g.* comprising SEQ ID NO: 31; (ii) a FhuD2 antigen *e.g.* comprising SEQ ID NO: 6; (iii) a Sta011 antigen *e.g.* comprising SEQ ID NO: 33; and (iv) a H35L mutant form of Hla *e.g.* comprising SEQ ID NO: 13.

15 Although SEQ ID NOs: 31, 6, 33 and 13 are useful amino acid sequences in a combination, the invention is not limited to these precise sequences. Thus 1, 2, 3 or all 4 of these sequences can independently be modified by up to 5 single amino changes (*i.e.* 1, 2, 3, 4 or 5 single amino acid substitutions, deletions and/or insertions) provided that the modified sequence can elicit antibodies which still bind to a polypeptide consisting of the unmodified sequence.

20 Another useful composition includes all four of: (i) a first polypeptide having amino acid sequence SEQ ID NO: 32; (ii) a second polypeptide having amino acid sequence SEQ ID NO: 7; (iii) a third polypeptide having amino acid sequence SEQ ID NO: 8; and (iv) a fourth polypeptide having amino acid sequence SEQ ID NO: 27.

25 Although SEQ ID NOs: 32, 7, 8 and 27 are useful amino acid sequences in a combination, the invention is not limited to these precise sequences. Thus 1, 2, 3 or all 4 of these four sequences can independently be modified by 1, 2, 3, 4 or 5 single amino changes (*i.e.* 1, 2, 3, 4 or 5 single amino acid substitutions, deletions and/or insertions) provided that the modified sequence can elicit antibodies which still bind to a polypeptide consisting of the unmodified sequence. In a preferred embodiment, a composition thus includes these four specified polypeptides with 1, 2, 3 or all 4 of SEQ ID NO: 32, 7, 8 and 27 independently modified by 1 single amino acid substitution, deletion
30 and/or insertion.

For instance, wild-type FhuD2, Sta011 and EsxAB polypeptide sequences (*e.g.* SEQ ID NOs: 6, 31 and 33) each include a single cysteine residue which can lead to inter-polypeptide disulfide bridges, forming both homodimers and heterodimers. Such inter-linked polypeptides are undesirable and so Sta006, Sta011 and EsxB sequences can be modified to remove their natural cysteine residues, such
35 that they do not contain free thiol groups (under reducing conditions). The wild-type cysteine can be deleted or can be substituted with a different amino acid.

Thus: a FhuD2 antigen can comprise SEQ ID NO: 34; a Sta011 antigen can comprise SEQ ID NO: 36; and a EsxB antigen can comprise SEQ ID NO: 35 (*e.g.* as an EsxAB hybrid comprising SEQ ID NO: 40). Examples of such sequences include, but are not limited to, SEQ ID NOs: 37, 39, and 38. These sequences can be used singly as substitutes for the corresponding wild-type sequences, or in combination. Thus a particularly useful composition includes all four of: (i) a first polypeptide having amino acid sequence SEQ ID NO: 38; (ii) a second polypeptide having amino acid sequence SEQ ID NO: 37; (iii) a third polypeptide having amino acid sequence SEQ ID NO: 39; and (iv) a fourth polypeptide having amino acid sequence SEQ ID NO: 27.

Thus a preferred composition of the invention comprises all five of: (i) a single polypeptide including both an EsxA antigen and an EsxB antigen *e.g.* comprising SEQ ID NO: 31; (ii) a FhuD2 antigen *e.g.* comprising SEQ ID NO: 6; (iii) a Sta011 antigen *e.g.* comprising SEQ ID NO: 33; (iv) a H35L mutant form of Hla *e.g.* comprising SEQ ID NO: 13; and (v) a mutant SpA *e.g.* comprising SEQ ID NO: 45 or 47, or an antigen comprising a single domain thereof *e.g.* SEQ ID NO. 50, 51, 52 or 53. So, in particular the composition according to the invention comprises:

- (i) a single polypeptide including both an EsxA antigen and an EsxB antigen, particularly of sequence comprising or consisting of SEQ ID NO: 31;
- (ii) a FhuD2 antigen, particularly of sequence comprising or consisting of SEQ ID NO: 6;
- (iii) a Sta011 antigen, particularly of sequence comprising or consisting of SEQ ID NO: 33;
- (iv) a H35L mutant form of Hla, particularly of sequence comprising or consisting of SEQ ID NO: 13; and
- (v) a mutant SpA antigen, particularly of sequence comprising or consisting of SEQ ID NO: 45, SEQ ID NO: 47, or SEQ ID NO. 52.

Another preferred composition according to the invention comprises:

- (i) a first polypeptide of sequence comprising or consisting of SEQ ID NO: 32, more particularly consisting of SEQ ID NO: 32;
- (ii) a second polypeptide of sequence comprising or consisting of SEQ ID NO: 7, more particularly consisting of SEQ ID NO: 7;
- (iii) a third polypeptide of sequence comprising or consisting of SEQ ID NO: 8, more particularly consisting of SEQ ID NO: 8;
- (iv) a fourth polypeptide of sequence comprising or consisting of SEQ ID NO: 27, more particularly consisting of SEQ ID NO: 27; and
- (v) a fifth polypeptide of sequence comprising or consisting of SEQ ID NO. 52, more particularly comprising or consisting of comprising or consisting of SEQ ID NO: 45 modified by up to 3 amino acid substitutions (*e.g.* comprising SEQ ID NO: 47 or consisting of SEQ ID NO: 48).

Another preferred composition according to the invention comprises:

- (i) a first polypeptide of sequence comprising or consisting of SEQ ID NO: 38, more particularly consisting of SEQ ID NO: 38;

- (ii) a second polypeptide of sequence comprising or consisting of SEQ ID NO: 37, more particularly consisting of SEQ ID NO: 37;
- (iii) a third polypeptide of sequence comprising or consisting of SEQ ID NO: 39, more particularly consisting of SEQ ID NO: 39;
- (iv) a fourth polypeptide of sequence comprising or consisting of SEQ ID NO: 27, more particularly consisting of SEQ ID NO: 27; and
- (v) a fifth polypeptide of sequence comprising or consisting of SEQ ID NO: 52, more particularly SEQ ID NO: 45 modified by up to 3 amino acid substitutions (*e.g.* comprising SEQ ID NO: 47 or consisting of SEQ ID NO: 48).

Proteins (i) to (v) in these combinations can, as explained above, independently be modified by up to 5 single amino changes provided that the modified sequence can elicit antibodies which still bind to a polypeptide consisting of the unmodified sequence. In some embodiments, a composition may include one or more further polypeptides; in other embodiments the only polypeptides in a composition are these five specified polypeptides, and these polypeptides can even be the only immunogenic components in a composition.

When more than one polypeptide is present, they may be present at substantially equal masses *i.e.* the mass of each of them is within $\pm 5\%$ of the mean mass of all the polypeptides. Thus, when five polypeptides are present, they may be present at a mass ratio of a:b:c:d:e, where each of a-e is between 0.95 and 1.05.

Aside from EsxA, EsxB, Hla, FhuD2, Sta011, and SpA, other *S.aureus* antigens exist, and a composition can optionally include one or more further *S.aureus* antigens. For instance, both saccharide and polypeptide antigens are known for *S.aureus*. Thus a composition might include a *S.aureus* saccharide antigen *e.g.* known saccharide antigens include the exopolysaccharide of *S.aureus*, which is a poly-N-acetylglucosamine (PNAG), and the capsular saccharides of *S.aureus*, which can be *e.g.* from type 5, type 8 or type 336. A composition might also include a ClfA antigen, an IsdA antigen, an IsdB antigen, an IsdC antigen, and/or an IsdH antigen (each as defined on pages 15-17 of reference 5).

In some embodiments, a composition includes a *S.aureus* antigen as defined above, and also an antigen from a different organism (*e.g.* from a virus or from another bacterium).

Immunogenic compositions and medicaments

Immunogenic compositions according to the invention may be useful as vaccines. Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic.

Compositions may thus be pharmaceutically acceptable. They will usually include components in addition to the antigens *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in reference 121.

Compositions will generally be administered to a mammal in aqueous form. Prior to administration, however, the composition may have been in a non-aqueous form. For instance, although some vaccines are manufactured in aqueous form, then filled and distributed and administered also in aqueous form, other vaccines are lyophilised during manufacture and are reconstituted into an aqueous form at the time of use. Thus a composition may be dried, such as a lyophilised formulation. Reference 10 discloses the use of lyophilisation with *S.aureus* immunogenic compositions.

A composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5µg/ml) mercurial material *e.g.* thiomersal-free. Vaccines containing no mercury are more preferred. Preservative-free vaccines are particularly preferred.

To improve thermal stability, a composition may include a temperature protective agent (see below).

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml *e.g.* about 10±2mg/ml NaCl. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

Compositions may include a metal ion chelator, in particular a divalent metal ion chelator such as EDTA. Reference 10 discloses that inclusion of EDTA can improve stability of the compositions disclosed herein. The final concentration of EDTA in an immunogenic composition can be about 1-50 mM, about 1-10 mM or about 1-5 mM, preferably about 2.5 mM.

The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* 6.5 and 7.5, or between 7.0 and 7.8.

The composition is preferably sterile. The composition is preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten-free.

The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material.

S.aureus infections can affect various areas of the body and so a composition may be prepared in various forms. For example, a composition may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (*e.g.* a lyophilised composition or a spray-freeze dried composition). A composition may be prepared for topical administration *e.g.* as an ointment, cream or powder. A composition may be prepared for oral administration *e.g.* as a tablet or capsule, as a spray, or as a syrup (optionally flavoured). A composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. A composition may be prepared as a suppository or pessary. A composition may be prepared for nasal, aural or ocular administration *e.g.* as drops. A composition may be in kit form, designed such that a combined composition is reconstituted just prior to administration to a patient. Such kits may comprise one or more antigens in liquid form and one or more lyophilised antigens.

Where a composition is to be prepared extemporaneously prior to use (*e.g.* where a component is presented in lyophilised form) and is presented as a kit, the kit may comprise two vials, or it may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

Human vaccines are typically administered in a dosage volume of about 0.5ml, although a half volume (*i.e.* about 0.25ml) may also be useful *e.g.* for children.

Immunogenic compositions administered according to the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include one or more adjuvants (see below).

The compositions may elicit both a cell mediated immune response as well as a humoral immune response. This immune response will preferably induce long lasting (*e.g.* neutralising) antibodies and a cell mediated immunity that can quickly respond upon exposure to *S.aureus*.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Where more than one antigen is included in a composition then two antigens may be present at the same dose as each other or at different doses.

As mentioned above, a composition may include a temperature protective agent, and this component may be particularly useful in adjuvanted compositions (particularly those containing a mineral adjuvant, such as an aluminium salt). As described in reference 20, a liquid temperature protective agent may be added to an aqueous vaccine composition to lower its freezing point *e.g.* to reduce the freezing point to below 0°C. Thus the composition can be stored below 0°C, but above its freezing point, to inhibit thermal breakdown. The temperature protective agent also permits freezing of the composition while protecting mineral salt adjuvants against agglomeration or sedimentation after freezing and thawing, and may also protect the composition at elevated temperatures *e.g.* above 40°C. A starting aqueous vaccine and the liquid temperature protective agent may be mixed such that the liquid temperature protective agent forms from 1-80% by volume of the final mixture. Suitable temperature protective agents should be safe for human administration, readily miscible/soluble in water, and should not damage other components (*e.g.* antigen and adjuvant) in the composition. Examples include glycerin, propylene glycol, and/or polyethylene glycol (PEG). Suitable PEGs may have an average molecular weight ranging from 200-20,000 Da. In a preferred embodiment, the polyethylene glycol can have an average molecular weight of about 300 Da ('PEG-300').

Methods of treatment, and administration of an immunogenic composition

The invention relates to the immunogenic composition according to the invention for use as a medicament.

The invention also relates to the immunogenic composition according to the invention for use as a medicament in the prevention and/or treatment of a *S. aureus* infection.

The invention also provides a method for the prevention and/or treatment of a *S. aureus* infection in a mammal comprising the step of administering to a mammal in need thereof an immunologically effective amount of an immunogenic composition according to the invention as defined above. The advantageous embodiments are as defined above.

The invention also provides the use of (i) at least one antigen selected from the group consisting of EsxA, EsxB, FhuD2, Sta011, and Hla antigens, and (ii) a mutant SpA antigen which has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG and for the Fab portion of V_H3-containing human B cell receptors, in the manufacture of a medicament for preventing or treating *S. aureus* infection in a mammal. The advantageous embodiments are as defined above

The invention also provides (i) at least one antigen selected from the group consisting of EsxA, EsxB, FhuD2, Sta011, and Hla antigens, and (ii) a mutant SpA antigen which has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG and for the Fab portion of V_H3-containing human B cell receptors, for use in immunising a mammal to prevent or treat *S. aureus* infection. The advantageous embodiments are as defined above.

The invention also provides (i) at least one antigen selected from the group consisting of EsxA, EsxB, FhuD2, Sta011, and Hla antigens, and (ii) a mutant SpA antigen which has decreased affinity,

relative to unmodified SpA, for the Fc γ portion of human IgG and for the Fab portion of V_H3-containing human B cell receptors, for use in a method of immunising a mammal to prevent or treat *S.aureus* infection by administering a therapeutically effective amount of the antigens to the mammal. The advantageous embodiments are as defined above.

5 As noted above, 1, 2, 3, 4 or preferably all 5 of EsxA, EsxB, FhuD2, Sta011, and Hla can be used in combination with the mutant SpA. In this manner the methods, uses, compositions and antigen combinations of the invention elicit an immune response which is effective for preventing or treating *S.aureus* infections. The immune response can involve antibodies and/or cell-mediated immunity. By raising an immune response in the mammal by these uses and methods, the mammal can be protected
10 against *S.aureus* infection, including a nosocomial infection. More particularly, the mammal may be protected against a skin infection, pneumonia, meningitis, osteomyelitis endocarditis, toxic shock syndrome, and/or septicemia. The invention is also useful for protecting against *S.aureus* infection of a mammal's bones and joints (and thus for preventing disorders including, but not limited to, osteomyelitis, septic arthritis, and prosthetic joint infection). In many cases these disorders may be
15 associated with the formation of a *S.aureus* biofilm.

S.aureus infects various mammals (including cows, dogs, horses, and pigs), but the preferred mammal for use with the invention is a human. The human can be a child (e.g. a toddler or infant), a teenager, or an adult. In some embodiments the human may have a prosthetic bone or joint, or may be an intended recipient of such prostheses (e.g. a pre-operative orthopedic surgery patient). A
20 vaccine intended for children may also be administered to adults e.g. to assess safety, dosage, immunogenicity, etc. The vaccines are not suitable solely for these groups, however, and may be used more generally in a human population.

One way of checking efficacy of therapeutic treatment involves monitoring *S.aureus* infection after administration of the compositions or antigens according to the invention. One way of checking
25 efficacy of prophylactic treatment involves monitoring immune responses, systemically (such as monitoring the level of IgG1 and IgG2a production) and/or mucosally (such as monitoring the level of IgA production), against the antigens in the administered composition after its administration. Another way of assessing the immunogenicity of the compositions is to express the antigens recombinantly for screening patient sera or mucosal secretions by immunoblot and/or microarrays. A
30 positive reaction between the protein and the patient sample indicates that the patient has mounted an immune response to the protein in question.

The efficacy of vaccine compositions can also be determined *in vivo* by challenging animal models of *S.aureus* infection, e.g., guinea pigs or mice, with the vaccine compositions. There are three generally useful animal models for the study of *S.aureus* infectious disease, namely: (i) the murine
35 abscess model [21], (ii) the murine lethal infection model [21], and (iii) the murine pneumonia model [22]. The abscess model looks at abscesses in mouse kidneys after intravenous challenge. The lethal infection model looks at the number of mice which survive after being infected by a normally-lethal

dose of *S.aureus* by the intravenous or intraperitoneal route. The pneumonia model also looks at the survival rate, but uses intranasal infection. Further useful models are disclosed in reference 23 for studying both *S.aureus* disease in relation to biofilm-mediated implant infection, skin and soft tissue infection (SSTI), and sepsis. A useful vaccine may be effective in one or more of these models. For instance, for some clinical situations it may be desirable to protect against pneumonia, without needing to prevent hematic spread or to promote opsonisation; in other situations the main desire may be to prevent hematic spread or sepsis. Different antigens, and different antigen combinations, may contribute to different aspects of an effective vaccine.

Compositions will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or mucosally, such as by rectal, oral (*e.g.* tablet, spray), vaginal, topical, transdermal or transcutaneous, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular injection is the most typical route for administering compositions according to the invention.

The invention may be used to elicit systemic and/or mucosal immunity, preferably to elicit an enhanced systemic and/or mucosal immunity. Preferably the enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1 and/or TH2 immune response. Preferably, the enhanced immune response includes an increase in the production of IgG1 and/or IgG2a and/or IgA.

Dosage can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, *etc.*).

Immunogenic compositions may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines.

Immunogenic compositions may be administered to patients in combination with an antibiotic. For instance, they may be administered at substantially the same time as an antibiotic. Similarly, they may be administered to a subject who is receiving antibiotic therapy. Similarly, they may be administered as part of a co-therapy which involves administration of both a composition as discussed herein and an antibiotic. The antibiotic will be effective against a *S.aureus* bacterium, for instance a beta-lactam.

Strains and variants

Antigens are discussed above by reference to existing nomenclature (*e.g.* "EsxA") and exemplary sequences given as GI numbers and also in the sequence listing. The invention is not limited to these

precise sequences. Genome sequences of several strains of *S.aureus* are available, including those of MRSA strains N315 and Mu50 [24], MW2, N315, COL, MRSA252, MSSA476, RF122, USA300 (very virulent), JH1, JH9, NCTC 8325, and Newman. Standard search and alignment techniques can be used to identify in any of these (or other) further genome sequences the homolog of any particular sequence mentioned herein. Moreover, the specific sequences disclosed herein can be used to design primers for amplification of homologous sequences from other strains. Thus the invention encompasses such variants and homologs from any strain of *S.aureus*, as well as non-natural variants. In general, suitable variants of a particular SEQ ID NO include its allelic variants, its polymorphic forms, its homologs, its orthologs, its paralogs, its mutants, *etc.*

Thus, for instance, polypeptides used with the invention may, compared to the SEQ ID NO herein, include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) amino acid substitutions, such as conservative substitutions (*i.e.* substitutions of one amino acid with another which has a related side chain). Genetically-encoded amino acids are generally divided into four families: (1) acidic *i.e.* aspartate, glutamate; (2) basic *i.e.* lysine, arginine, histidine; (3) non-polar *i.e.* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar *i.e.* glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity. The polypeptides may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) single amino acid deletions relative to the SEQ ID NO sequences. The polypeptides may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) insertions (*e.g.* each of 1, 2, 3, 4 or 5 amino acids) relative to the SEQ ID NO sequences.

Similarly, a polypeptide used with the invention may comprise an amino acid sequence that:

- (a) is identical (*i.e.* 100% identical) to a sequence disclosed in the sequence listing;
- (b) shares sequence identity (*e.g.* 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) with a sequence disclosed in the sequence listing (ideally over the entire length of said sequence);
- (c) has 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 (or more) single amino acid alterations (deletions, insertions, substitutions), which may be at separate locations or may be contiguous, as compared to the sequences of (a) or (b); or
- (d) when aligned with a particular sequence from the sequence listing using a pairwise alignment algorithm, each moving window of x amino acids from N-terminus to C-terminus (such that for an alignment that extends to p amino acids, where $p > x$, there are $p-x+1$ such windows) has at least $x \cdot y$ identical aligned amino acids, where: x is selected from 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200; y is selected from 0.50, 0.60, 0.70, 0.75, 0.80, 0.85, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99; and if $x \cdot y$ is not an integer then it is rounded up to the nearest integer. The preferred pairwise alignment algorithm is the Needleman-Wunsch global alignment algorithm [25], using default parameters (*e.g.* with Gap

opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the *needle* tool in the EMBOSS package [26].

Where hybrid polypeptides are used, the individual antigens within the hybrid (*i.e.* individual -X-moieties) may be from one or more strains. Where $n=2$, for instance, X_2 may be from the same strain as X_1 or from a different strain. Where $n=3$, the strains might be (i) $X_1=X_2=X_3$ (ii) $X_1=X_2\neq X_3$ (iii) $X_1\neq X_2=X_3$ (iv) $X_1\neq X_2\neq X_3$ or (v) $X_1=X_3\neq X_2$, *etc.*

Within group (c), deletions or substitutions may be at the N-terminus and/or C-terminus, or may be between the two termini. Thus a truncation is an example of a deletion. Truncations may involve deletion of up to 40 (or more) amino acids at the N-terminus and/or C-terminus. N-terminus truncation can remove leader peptides *e.g.* to facilitate recombinant expression in a heterologous host. C-terminus truncation can remove anchor sequences *e.g.* to facilitate recombinant expression in a heterologous host.

In general, when an antigen comprises a sequence that is not identical to a complete *S.aureus* sequence from the sequence listing (*e.g.* when it comprises a sequence listing with <100% sequence identity thereto, or when it comprises a fragment thereof) it is preferred in each individual instance that the antigen can elicit an antibody which recognises the respective complete *S.aureus* sequence.

Polypeptides used with the invention

Polypeptides used with the invention can take various forms (*e.g.* native, fusions, glycosylated, non-glycosylated, lipidated, non-lipidated, phosphorylated, non-phosphorylated, myristoylated, non-myristoylated, monomeric, multimeric, *etc.*).

Polypeptides used with the invention can be prepared by various means (*e.g.* recombinant expression, purification from cell culture, chemical synthesis, *etc.*). Recombinantly-expressed proteins are preferred, particularly for hybrid polypeptides.

Polypeptides used with the invention are preferably provided in purified or substantially purified form *i.e.* substantially free from other polypeptides (*e.g.* free from naturally-occurring polypeptides), particularly from other staphylococcal or host cell polypeptides, and are generally at least about 50% pure (by weight), and usually at least about 90% pure *i.e.* less than about 50%, and more preferably less than about 10% (*e.g.* 5%) of a composition is made up of other expressed polypeptides. Thus the antigens in the compositions are separated from the whole organism with which the molecule is expressed.

The term “polypeptide” refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included

are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. Polypeptides can occur as single chains or associated chains.

Although expression of the polypeptides of the invention may take place in a *Staphylococcus*, the invention will usually use a heterologous host for expression (recombinant expression). The heterologous host may be prokaryotic (*e.g.* a bacterium) or eukaryotic. It may be *E.coli*, but other suitable hosts include *Bacillus subtilis*, *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Neisseria lactamica*, *Neisseria cinerea*, *Mycobacteria* (*e.g.* *M.tuberculosis*), yeasts, *etc.* Compared to the wild-type *S.aureus* genes encoding polypeptides of the invention, it is helpful to change codons to optimise expression efficiency in such hosts without affecting the encoded amino acids.

Adjuvants

As mentioned above, immunogenic compositions used according to the invention may include one or more adjuvants. Adjuvants which may be used with the invention include, but are not limited to:

A. Mineral-containing compositions

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the “CAP” particles disclosed in ref. 27). Aluminum salts include hydroxides and phosphates *etc.*, with the salts taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*). Adsorption to these salts is preferred (*e.g.* all antigens may be adsorbed). The mineral containing compositions may also be formulated as a particle of metal salt [28].

The adjuvants known as aluminum hydroxide and aluminum phosphate may be used. These names are conventional, but are used for convenience only, as neither is a precise description of the actual chemical compound which is present (*e.g.* see chapter 9 of reference 29). The invention can use any of the “hydroxide” or “phosphate” adjuvants that are in general use as adjuvants. The adjuvants known as “aluminium hydroxide” are typically aluminium oxyhydroxide salts, which are usually at least partially crystalline. The adjuvants known as “aluminium phosphate” are typically aluminium hydroxyphosphates, often also containing a small amount of sulfate (*i.e.* aluminium hydroxyphosphate sulfate). They may be obtained by precipitation, and the reaction conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt.

A fibrous morphology (*e.g.* as seen in transmission electron micrographs) is typical for aluminium hydroxide adjuvants. The pI of aluminium hydroxide adjuvants is typically about 11 *i.e.* the adjuvant itself has a positive surface charge at physiological pH. Adsorptive capacities of between 1.8-2.6 mg protein per mg Al⁺⁺⁺ at pH 7.4 have been reported for aluminium hydroxide adjuvants.

Aluminium phosphate adjuvants generally have a PO₄/Al molar ratio between 0.3 and 1.2, preferably between 0.8 and 1.2, and more preferably 0.95±0.1. The aluminium phosphate will generally be

amorphous, particularly for hydroxyphosphate salts. A typical adjuvant is amorphous aluminium hydroxyphosphate with PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al^{3+}/ml . The aluminium phosphate will generally be particulate (*e.g.* plate-like morphology as seen in transmission electron micrographs). Typical diameters of the particles are in the range 0.5-20 μm (*e.g.* about 5-10 μm) after any antigen adsorption. Adsorptive capacities of between 0.7-1.5 mg protein per mg Al^{+++} at pH 7.4 have been reported for aluminium phosphate adjuvants.

The point of zero charge (PZC) of aluminium phosphate is inversely related to the degree of substitution of phosphate for hydroxyl, and this degree of substitution can vary depending on reaction conditions and concentration of reactants used for preparing the salt by precipitation. PZC is also altered by changing the concentration of free phosphate ions in solution (more phosphate = more acidic PZC) or by adding a buffer such as a histidine buffer (makes PZC more basic). Aluminium phosphates used according to the invention will generally have a PZC of between 4.0 and 7.0, more preferably between 5.0 and 6.5 *e.g.* about 5.7.

Suspensions of aluminium salts used to prepare compositions of the invention may contain a buffer (*e.g.* a phosphate or a histidine or a Tris buffer), but this is not always necessary. The suspensions are preferably sterile and pyrogen-free. A suspension may include free aqueous phosphate ions *e.g.* present at a concentration between 1.0 and 20 mM, preferably between 5 and 15 mM, and more preferably about 10 mM. The suspensions may also comprise sodium chloride.

The invention can use a mixture of both an aluminium hydroxide and an aluminium phosphate. In this case there may be more aluminium phosphate than hydroxide *e.g.* a weight ratio of at least 2:1 *e.g.* $\geq 5:1$, $\geq 6:1$, $\geq 7:1$, $\geq 8:1$, $\geq 9:1$, *etc.*

The concentration of Al^{+++} in a composition for administration to a patient is preferably less than 10mg/ml *e.g.* ≤ 5 mg/ml, ≤ 4 mg/ml, ≤ 3 mg/ml, ≤ 2 mg/ml, ≤ 1 mg/ml, *etc.* A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

B. Oil-in-water emulsions

Oil-in-water emulsion compositions suitable for use as adjuvants in the invention include squalene-in-water emulsions, such as MF59 (see Chapter 10 of ref. 29; see also ref. 30) and AS03 [31].

Various oil-in-water emulsion adjuvants are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The emulsion will include submicron oil droplets, and emulsions with droplets having a diameter less than 220nm are preferred as they can be subjected to filter sterilization.

The emulsion comprises one or more oils. Suitable oil(s) include those from, for example, an animal (such as fish) or a vegetable source. The oil is ideally biodegradable (metabolisable) and biocompatible. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be

used *e.g.* obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolisable and so may be used. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art.

Most fish contain metabolisable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Preferred emulsions comprise squalene, a shark liver oil which is a branched, unsaturated terpenoid. Squalane, the saturated analog to squalene, can also be used. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art.

Other useful oils are the tocopherols, particularly in combination with squalene. Where the oil phase of an emulsion includes a tocopherol, any of the α , β , γ , δ , ϵ or ξ tocopherols can be used, but α -tocopherols are preferred. D- α -tocopherol and DL- α -tocopherol can both be used. A preferred α -tocopherol is DL- α -tocopherol. An oil combination comprising squalene and a tocopherol (*e.g.* DL- α -tocopherol) can be used.

The oil in the emulsion may comprise a combination of oils *e.g.* squalene and at least one other oil.

The aqueous component of the emulsion can be plain water (*e.g.* w.f.i.) or can include further components *e.g.* solutes. For instance, it may include salts to form a buffer *e.g.* citrate or phosphate salts, such as sodium salts. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer. A buffered aqueous phase is preferred, and buffers will typically be included in the 5-20mM range.

In addition to the oil and cationic lipid, an emulsion can include a non-ionic surfactant and/or a zwitterionic surfactant. Such surfactants include, but are not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); polyoxyethylene-9-lauryl ether; and sorbitan esters

(commonly known as the Spans), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Preferred surfactants for including in the emulsion are polysorbate 80 (Tween 80; polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of surfactants can be used *e.g.* Tween 80/Span 85 mixtures, or Tween 80/Triton-X100 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80) and an octoxynol such as t-octylphenoxy-polyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol. Useful mixtures can comprise a surfactant with a HLB value in the range of 10-20 (*e.g.* polysorbate 80, with a HLB of 15.0) and a surfactant with a HLB value in the range of 1-10 (*e.g.* sorbitan trioleate, with a HLB of 1.8).

Preferred amounts of oil (% by volume) in the final emulsion are between 2-20% *e.g.* 5-15%, 6-14%, 7-13%, 8-12%. A squalene content of about 4-6% or about 9-11% is particularly useful.

Preferred amounts of surfactants (% by weight) in the final emulsion are between 0.001% and 8%. For example: polyoxyethylene sorbitan esters (such as polysorbate 80) 0.2 to 4%, in particular between 0.4-0.6%, between 0.45-0.55%, about 0.5% or between 1.5-2%, between 1.8-2.2%, between 1.9-2.1%, about 2%, or 0.85-0.95%, or about 1%; sorbitan esters (such as sorbitan trioleate) 0.02 to 2%, in particular about 0.5% or about 1%; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100) 0.001 to 0.1%, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 8%, preferably 0.1 to 10% and in particular 0.1 to 1% or about 0.5%.

The absolute amounts of oil and surfactant, and their ratio, can be varied within wide limits while still forming an emulsion. A skilled person can easily vary the relative proportions of the components to obtain a desired emulsion, but a weight ratio of between 4:1 and 5:1 for oil and surfactant is typical (excess oil).

An important parameter for ensuring immunostimulatory activity of an emulsion, particularly in large animals, is the oil droplet size (diameter). The most effective emulsions have a droplet size in the submicron range. Suitably the droplet sizes will be in the range 50-750nm. Most usefully the average droplet size is less than 250nm *e.g.* less than 200nm, less than 150nm. The average droplet size is usefully in the range of 80-180nm. Ideally, at least 80% (by number) of the emulsion's oil droplets are less than 250 nm in diameter, and preferably at least 90%. These droplet sizes can conveniently be achieved by techniques such as microfluidisation. Apparatuses for determining the average droplet size in an emulsion, and the size distribution, are commercially available. These typically use the techniques of dynamic light scattering and/or single-particle optical sensing *e.g.* the Accusizer™ and Nicomp™ series of instruments available from Particle Sizing Systems (Santa Barbara, USA), or the Zetasizer™ instruments from Malvern Instruments (UK), or the Particle Size Distribution Analyzer instruments from Horiba (Kyoto, Japan).

Ideally, the distribution of droplet sizes (by number) has only one maximum *i.e.* there is a single population of droplets distributed around an average (mode), rather than having two maxima. Preferred emulsions have a polydispersity of <0.4 *e.g.* 0.3, 0.2, or less.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- 5 • A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' [32-34], as described in more detail in Chapter 10 of ref. 35 and chapter 12 of ref. 36. The MF59 emulsion advantageously includes citrate ions *e.g.* 10mM sodium citrate buffer.
10
- An emulsion comprising squalene, a tocopherol, and polysorbate 80. The emulsion may include phosphate buffered saline. These emulsions may have by volume from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% polysorbate 80, and the weight ratio of squalene:tocopherol is preferably <1 (*e.g.* 0.90) as this can provide a more stable emulsion.
15 Squalene and polysorbate 80 may be present volume ratio of about 5:2 or at a weight ratio of about 11:5. Thus the three components (squalene, tocopherol, polysorbate 80) may be present at a weight ratio of 1068:1186:485 or around 55:61:25. One such emulsion ('AS03') can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL α tocopherol and 5ml squalene), then microfluidising the mixture.
20 The resulting emulsion may have submicron oil droplets *e.g.* with an average diameter of between 100 and 250nm, preferably about 180nm. The emulsion may also include a 3-de-O-acylated monophosphoryl lipid A (3d MPL). Another useful emulsion of this type may comprise, per human dose, 0.5-10 mg squalene, 0.5-11 mg tocopherol, and 0.1-4 mg polysorbate 80 [37] *e.g.* in the ratios discussed above.
- 25 • An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.* Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.
- An emulsion comprising a polysorbate (*e.g.* polysorbate 80), a Triton detergent (*e.g.* Triton X-100) and a tocopherol (*e.g.* an α -tocopherol succinate). The emulsion may include these
30 three components at a mass ratio of about 75:11:10 (*e.g.* 750 μ g/ml polysorbate 80, 110 μ g/ml Triton X-100 and 100 μ g/ml α -tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.
- 35 • An emulsion of squalene, polysorbate 80 and poloxamer 401 ("Pluronic™ L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the

“SAF-1” adjuvant [38] (0.05-1% Thr-MDP, 5% squalene, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the “AF” adjuvant [39] (5% squalene, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monoleate or ‘Span 80’). The emulsion is preferably thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm [40]. The emulsion may also include one or more of: alditol; a cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. The emulsion may include a TLR4 agonist [41]. Such emulsions may be lyophilized.
- An emulsion of squalene, poloxamer 105 and Abil-Care [42]. The final concentration (weight) of these components in adjuvanted vaccines are 5% squalene, 4% poloxamer 105 (pluronic polyol) and 2% Abil-Care 85 (Bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone; caprylic/capric triglyceride).
- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 43, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.
- A submicron oil-in-water emulsion of a non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 44, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyldioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.
- An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [45].
- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [46].
- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty alcohol, and a non-ionic lipophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [46].

In some embodiments an emulsion may be mixed with antigen(s) extemporaneously, at the time of delivery, and thus the adjuvant and antigen(s) may be kept separately in a packaged or distributed vaccine, ready for final formulation at the time of use. In other embodiments an emulsion is mixed

with antigen during manufacture, and thus the composition is packaged in a liquid adjuvanted form. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.* between 5:1 and 1:5) but is generally about 1:1. Where concentrations of components are given in the above descriptions of specific emulsions, these concentrations are typically for an undiluted composition, and the concentration after mixing with an antigen solution will thus decrease.

C. *Saponin formulations [chapter 22 of ref. 29]*

Saponin formulations may also be used as adjuvants in the invention. Saponins are a heterogeneous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsapilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™.

Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 47. Saponin formulations may also comprise a sterol, such as cholesterol [48].

Combinations of saponins and cholesterol can be used to form particles called ISCOMs (chapter 23 of ref. 29). ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA & QHC. ISCOMs are further described in refs. 48-50. Optionally, the ISCOMS may be devoid of additional detergent [51].

A review of the development of saponin based adjuvants can be found in refs. 52 & 53.

D. *Bacterial or microbial derivatives*

Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylating toxins and detoxified derivatives thereof.

Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred “small particle” form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 54. Such “small particles” of 3dMPL are small enough to be sterile filtered through a 0.22µm membrane [54]. Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 (see below).

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in refs. 55 & 56.

Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogues such as phosphorothioate modifications and can be double-stranded or single-stranded. References 57, 58 and 59 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 60-65.

The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [66]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such as a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 67-69. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, refs. 66 & 70-72.

A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.). Another is CpG1826. As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [73], and these oligonucleotides may be free from unmethylated CpG motifs. The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (*e.g.* TTTT, as disclosed in ref. 73), and/or it may have a nucleotide composition with >25% thymidine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). For example, it may comprise more than one consecutive cytosine nucleotide (*e.g.* CCCC, as disclosed in ref. 73), and/or it may have a nucleotide composition with >25% cytosine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). These oligonucleotides may be free from unmethylated CpG motifs. Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

A particularly useful adjuvant based around immunostimulatory oligonucleotides is known as IC-31™ [74]. Thus an adjuvant used with the invention may comprise a mixture of (i) an oligonucleotide (*e.g.* between 15-40 nucleotides) including at least one (and preferably multiple) CpI motifs (*i.e.* a cytosine linked to an inosine to form a dinucleotide), and (ii) a polycationic polymer, such as an oligopeptide (*e.g.* between 5-20 amino acids) including at least one (and preferably multiple) Lys-Arg-Lys tripeptide sequence(s). The oligonucleotide may be a deoxynucleotide comprising 26-mer sequence 5'-(IC)₁₃-3' (SEQ ID NO: 41). The polycationic polymer may be a

peptide comprising 11-mer amino acid sequence KKLKLLLLKLK (SEQ ID NO: 42). The oligonucleotide and polymer can form complexes *e.g.* as disclosed in references 75 & 76.

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E.coli* (*E.coli* heat labile enterotoxin “LT”), cholera (“CT”), or pertussis (“PT”). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 77 and as parenteral adjuvants in ref. 78. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in refs. 79-86. A useful CT mutant is or CT-E29H [87]. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in ref. 88, specifically incorporated herein by reference in its entirety.

E. TLR agonists

Compositions can include a TLR agonist *i.e.* a compound which can agonise a Toll-like receptor. Most preferably, a TLR agonist is an agonist of a human TLR. The TLR agonist can activate any of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 or TLR11; preferably it can activate human TLR4 or human TLR7.

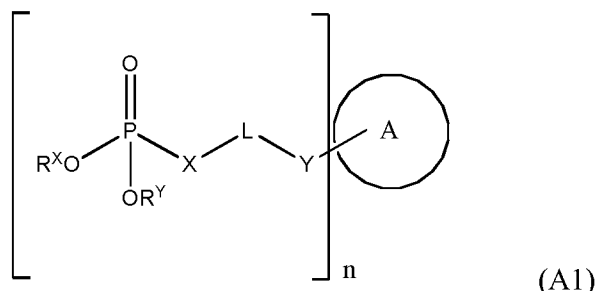
Agonist activity of a compound against any particular Toll-like receptor can be determined by standard assays. Companies such as Imgenex and Invivogen supply cell lines which are stably co-transfected with human TLR genes and NFκB, plus suitable reporter genes, for measuring TLR activation pathways. They are designed for sensitivity, broad working range dynamics and can be used for high-throughput screening. Constitutive expression of one or two specific TLRs is typical in such cell lines. See also reference 89. Many TLR agonists are known in the art *e.g.* reference 90 describes certain lipopeptide molecules that are TLR2 agonists, references 91 to 94 each describe classes of small molecule agonists of TLR7, and references 95 & 96 describe TLR7 and TLR8 agonists for treatment of diseases.

A TLR agonist used with the invention ideally includes at least one adsorptive moiety. The inclusion of such moieties in TLR agonists allows them to adsorb to insoluble aluminium salts (*e.g.* by ligand exchange or any other suitable mechanism) and improves their immunological behaviour [97]. Phosphorus-containing adsorptive moieties are particularly useful, and so an adsorptive moiety may comprise a phosphate, a phosphonate, a phosphinate, a phosphonite, a phosphinite, *etc.* Preferably the TLR agonist includes at least one phosphonate group.

Thus, in preferred embodiments, a composition includes a TLR agonist (more preferably a TLR7 agonist) which includes a phosphonate group. This phosphonate group can allow adsorption of the agonist to an insoluble aluminium salt [97].

TLR agonists useful with the invention may include a single adsorptive moiety, or may include more than one *e.g.* between 2 and 15 adsorptive moieties. Typically a compound will include 1, 2 or 3 adsorptive moieties.

Useful phosphorus-containing TLR agonists can be represented by formula (A1):



wherein:

R^{X} and R^{Y} are independently selected from H and $\text{C}_1\text{-C}_6$ alkyl;

X is selected from a covalent bond, O and NH;

Y is selected from a covalent bond, O, C(O), S and NH;

L is a linker *e.g.* selected from, $\text{C}_1\text{-C}_6$ alkylene, $\text{C}_1\text{-C}_6$ alkenylene, arylene, heteroarylene, $\text{C}_1\text{-C}_6$ alkyleneoxy and $-(\text{CH}_2)_p\text{O})_q(\text{CH}_2)_p-$ each optionally substituted with 1 to 4 substituents independently selected from halo, OH, $\text{C}_1\text{-C}_4$ alkyl, $-\text{OP}(\text{O})(\text{OH})_2$ and $-\text{P}(\text{O})(\text{OH})_2$;

each p is independently selected from 1, 2, 3, 4, 5 and 6;

q is selected from 1, 2, 3 and 4;

n is selected from 1, 2 and 3; and

A is a TLR agonist moiety.

In one embodiment, the TLR agonist according to formula (A1) is as follows: R^{X} and R^{Y} are H; X is O; L is selected from $\text{C}_1\text{-C}_6$ alkylene and $-(\text{CH}_2)_p\text{O})_q(\text{CH}_2)_p-$ each optionally substituted with 1 to 2 halogen atoms; p is selected from 1, 2 and 3; q is selected from 1 and 2; and n is 1. Thus in these embodiments the adsorptive moiety comprises a phosphate group.

Other useful TLR agonists of formula (A1) are disclosed on pages 6-13 of reference 98.

Compositions can include an imidazoquinolone compound, such as Imiquimod ("R-837") [99,100], Resiquimod ("R-848") [101], and their analogs; and salts thereof (*e.g.* the hydrochloride salts).

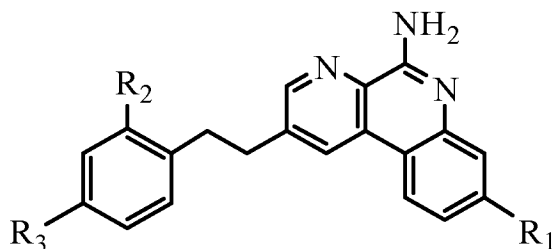
Further details about immunostimulatory imidazoquinolines can be found in references 102 to 106.

Compositions can include a TLR4 agonist, and most preferably an agonist of human TLR4. TLR4 is expressed by cells of the innate immune system, including conventional dendritic cells and macrophages [107]. Triggering via TLR4 induces a signalling cascade that utilizes both the MyD88- and TRIF-dependent pathways, leading to NF- κ B and IRF3/7 activation, respectively. TLR4

activation typically induces robust IL-12p70 production and strongly enhances Th1-type cellular and humoral immune responses.

Various useful TLR4 agonists are known in the art, many of which are analogs of endotoxin or lipopolysaccharide (LPS). For instance, the TLR4 agonist can be: 3d-MPL (*i.e.* 3-O-deacylated monophosphoryl lipid A; present in GSK's 'AS04' adjuvant, with further details in references 108 to 111 glucopyranosyl lipid A (GLA) [112] or its ammonium salt; an aminoalkyl glucosaminide phosphate, such as RC-529 or CRX-524 [113-115]; E5564 [116,117]; or a compound of formula I, II or III as defined in reference 118, or a salt thereof, such as compounds 'ER 803058', 'ER 803732', 'ER 804053', 'ER 804058', 'ER 804059', 'ER 804442', 'ER 804680', 'ER 803022', 'ER 804764' or 'ER 804057' (also known as E6020).

The invention is particularly useful when using human TLR7 agonists, such as a compound of formula (K). These agonists are discussed in detail in reference 119:



(K)

wherein:

R^1 is H, C_1 - C_6 alkyl, $-C(R^5)_2OH$, $-L^1R^5$, $-L^1R^6$, $-L^2R^5$, $-L^2R^6$, $-OL^2R^5$, or $-OL^2R^6$;

L^1 is $-C(O)-$ or $-O-$;

L^2 is C_1 - C_6 alkylene, C_2 - C_6 alkenylene, arylene, heteroarylene or $-((CR^4R^4)_pO)_q(CH_2)_p-$, wherein the C_1 - C_6 alkylene and C_2 - C_6 alkenylene of L^2 are optionally substituted with 1 to 4 fluoro groups;

each L^3 is independently selected from C_1 - C_6 alkylene and $-((CR^4R^4)_pO)_q(CH_2)_p-$, wherein the C_1 - C_6 alkylene of L^3 is optionally substituted with 1 to 4 fluoro groups;

L^4 is arylene or heteroarylene;

R^2 is H or C_1 - C_6 alkyl;

R^3 is selected from C_1 - C_4 alkyl, $-L^3R^5$, $-L^1R^5$, $-L^3R^7$, $-L^3L^4L^3R^7$, $-L^3L^4R^5$, $-L^3L^4L^3R^5$, $-OL^3R^5$, $-OL^3R^7$, $-OL^3L^4R^7$, $-OL^3L^4L^3R^7$, $-OR^8$, $-OL^3L^4R^5$, $-OL^3L^4L^3R^5$ and $-C(R^5)_2OH$;

each R^4 is independently selected from H and fluoro;

R^5 is $-P(O)(OR^9)_2$,

R^6 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^7 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^8 is H or C_1 - C_4 alkyl;

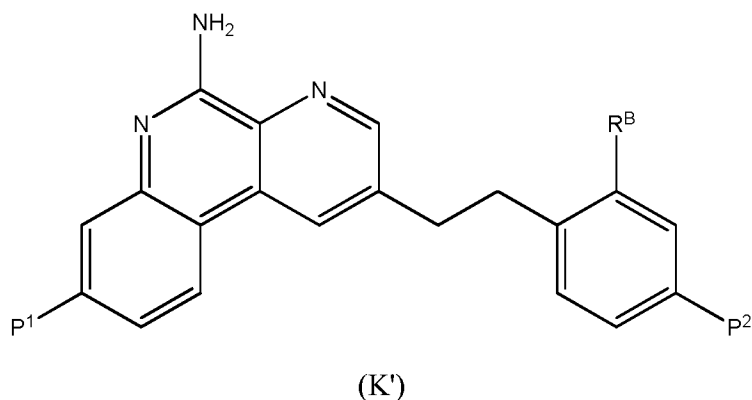
each R^9 is independently selected from H and C_1 - C_6 alkyl;

R^{10} is H or C_1 - C_4 alkyl;

each p is independently selected from 1, 2, 3, 4, 5 and 6, and

5 q is 1, 2, 3 or 4.

The compound of formula (K) is preferably of formula (K'):



wherein:

10 P^1 is selected from H, C_1 - C_6 alkyl optionally substituted with COOH and $-Y-L-X-P(O)(OR^X)(OR^Y)$;

P^2 is selected from H, C_1 - C_6 alkyl, C_1 - C_6 alkoxy and $-Y-L-X-P(O)(OR^X)(OR^Y)$;

with the proviso that at least one of P^1 and P^2 is $-Y-L-X-P(O)(OR^X)(OR^Y)$;

R^B is selected from H and C_1 - C_6 alkyl;

15 R^X and R^Y are independently selected from H and C_1 - C_6 alkyl;

X is selected from a covalent bond, O and NH;

Y is selected from a covalent bond, O, C(O), S and NH;

20 L is selected from, a covalent bond C_1 - C_6 alkylene, C_1 - C_6 alkenylene, arylene, heteroarylene, C_1 - C_6 alkyleneoxy and $-((CH_2)_pO)_q(CH_2)_p-$ each optionally substituted with 1 to 4 substituents independently selected from halo, OH, C_1 - C_4 alkyl, $-OP(O)(OH)_2$ and $-P(O)(OH)_2$;

each p is independently selected from 1, 2, 3, 4, 5 and 6; and

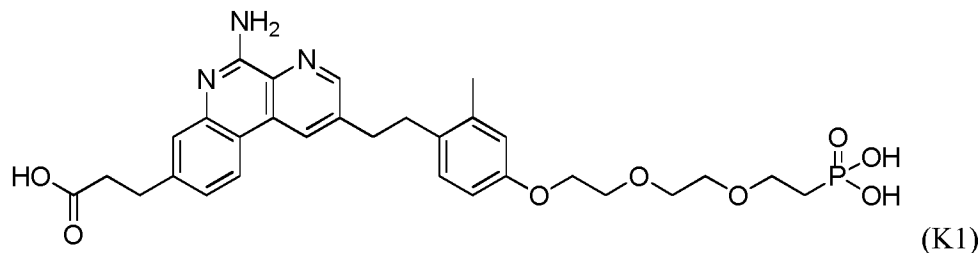
q is selected from 1, 2, 3 and 4.

In some embodiments of formula (K'): P^1 is selected from C_1 - C_6 alkyl optionally substituted with COOH and $-Y-L-X-P(O)(OR^X)(OR^Y)$; P^2 is selected from C_1 - C_6 alkoxy and $-Y-L-X-P(O)(OR^X)(OR^Y)$; R^B is C_1 - C_6 alkyl; X is a covalent bond; L is selected from C_1 - C_6 alkylene and $-((CH_2)_pO)_q(CH_2)_p-$ each optionally substituted with 1 to 4 substituents independently selected from

25

halo, OH, C₁-C₄alkyl, -OP(O)(OH)₂ and -P(O)(OH)₂; each p is independently selected from 1, 2 and 3; q is selected from 1 and 2.

A preferred compound of formula (K) for use with the invention is 3-(5-amino-2-(2-methyl-4-(2-(2-(2-phosphonoethoxy)ethoxy)ethoxy)phenethyl)benzo[f][1,7]naphthyridin-8-yl)propanoic acid, or compound 'K1':



This compound can be used as free base or in the form of a pharmaceutically acceptable salt *e.g.* an arginine salt [120].

F. Microparticles

- 10 Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150µm in diameter, more preferably ~200nm to ~30µm in diameter, and most preferably ~500nm to ~10µm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*), with poly(lactide-co-glycolide) are preferred, optionally treated to have a
- 15 negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

Adjuvant combinations

- The individual adjuvants listed above may also be included in combinations. For instance, a combination of an aluminium hydroxide and an aluminium phosphate adjuvant can be used.
- 20 Similarly, a combination of aluminium phosphate and 3dMPL may be used.

A particularly preferred adjuvant combination is an insoluble metal salt (*e.g.* an aluminium salt, such as an aluminium hydroxide) and a TLR agonist (*e.g.* a human TLR7 agonist, such as compound 'K1' identified above), as disclosed in references 5 and 97. So, in particular, said adjuvant is selected from the group consisting of:

- aluminium salts, particularly aluminium hydroxides and aluminium phosphates;
- human TLR agonists, particularly TLR7 agonists; and
- a mixture thereof.

- 25 The TLR agonist is preferably adsorbed to the metal salt, and the *S.aureus* antigen(s) can also be adsorbed to the metal salt.

A composition including a TLR agonist of the invention adsorbed to a metal salt can also include a buffer (*e.g.* a phosphate or a histidine or a Tris buffer). When such a composition includes a

phosphate buffer, however, it is preferred that the concentration of phosphate ions in the buffer should be less than 50mM *e.g.* <40mM, <30mM, <20mM, <10mM, or <5mM, or between 1-15mM. A histidine buffer is preferred *e.g.* between 1-50mM, between 5-25mM, or about 10mM.

5 A composition can include a mixture of both an aluminium oxyhydroxide and an aluminium hydroxyphosphate, and a TLR agonist may be adsorbed to one or both of these salts.

As mentioned above, a maximum of 0.85mg/dose Al^{+++} is preferred. Because the inclusion of a TLR agonist can improve the adjuvant effect of aluminium salts then the invention advantageously permits lower amounts of Al^{+++} per dose, and so a composition can usefully include between 10 and 250µg of Al^{+++} per unit dose. Current pediatric vaccines typically include at least 300µg Al^{+++} . In
10 concentration terms, a composition may have an Al^{+++} concentration between 10 and 500 µg/ml *e.g.* between 10-300µg/ml, between 10-200µg/ml, or between 10-100µg/ml.

In general, when a composition includes both a TLR agonist and an aluminium salt, the weight ratio of agonist to Al^{+++} will be less than 5:1 *e.g.* less than 4:1, less than 3:1, less than 2:1, or less than 1:1. Thus, for example, with an Al^{+++} concentration of 0.5mg/ml the maximum concentration of TLR
15 agonist would be 1.5mg/ml. But higher or lower levels can be used.

Where a composition includes a TLR agonist and an insoluble metal salt, it is preferred that at least 50% (by mass) of the agonist in the composition is adsorbed to the metal salt *e.g.* ≥60%, ≥70%, ≥80%, ≥85%, ≥90%, ≥92%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99%, or even 100%.

Thus, in one embodiment, the invention uses an immunogenic composition comprising:

- 20 ▪ an aluminium hydroxide adjuvant;
- a TLR7 agonist of formula (K), such as compound K1;
- a first polypeptide comprising SEQ ID NO: 6, or a modified amino acid sequence which differs from SEQ ID NO: 6 by up to 5 single amino changes provided that the modified sequence can elicit antibodies which bind to a polypeptide consisting of SEQ ID NO: 6;
- 25 ▪ a second polypeptide comprising SEQ ID NO: 13, or a modified amino acid sequence which differs from SEQ ID NO: 13 by up to 5 single amino changes provided that the modified sequence can elicit antibodies which bind to a polypeptide consisting of SEQ ID NO: 13;
- a third polypeptide comprising SEQ ID NO: 31, or a modified amino acid sequence which differs from SEQ ID NO: 31 by up to 5 single amino changes provided that the modified
30 sequence can elicit antibodies which bind to a polypeptide consisting of SEQ ID NO: 31;
- a fourth polypeptide comprising SEQ ID NO: 33, or a modified amino acid sequence which differs from SEQ ID NO: 33 by up to 5 single amino changes provided that the modified sequence can elicit antibodies which bind to a polypeptide consisting of SEQ ID NO: 33; and
- 35 ▪ a fifth polypeptide comprising SEQ ID NO: 45, or a modified amino acid sequence which differs from SEQ ID NO: 45 by up to 5 single amino changes provided that the modified sequence can elicit antibodies which bind to a polypeptide consisting of SEQ ID NO: 43; and,

in which the TLR7 agonist and/or at least one of the polypeptides is/are adsorbed to the aluminium hydroxide adjuvant.

For example, as explained in more detail elsewhere herein: the first polypeptide can comprise SEQ ID NO: 34; the second polypeptide can comprise SEQ ID NO: 13; the third polypeptide can comprise SEQ ID NO: 40; the fourth polypeptide can comprise SEQ ID NO: 36; and the fifth polypeptide can comprise SEQ ID NO: 45, optionally modified by up to 3 amino acid substitutions (other than at positions which are X in SEQ ID NO: 44). Thus the composition can use a mixture of five polypeptides having SEQ ID NOs: 37, 27, 38, 39, and 45 (except that SEQ ID NO: 45 can be modified by up to 3 amino acid substitutions as discussed above).

10 **Chemical groups**

Unless specifically defined elsewhere, the chemical groups discussed herein have the following meaning when used in present specification:

The term "alkyl" includes saturated hydrocarbon residues including:

- linear groups up to 10 atoms (C₁-C₁₀), or of up to 6 atoms (C₁-C₆), or of up to 4 atoms (C₁-C₄).
Examples of such alkyl groups include, but are not limited, to C₁ - methyl, C₂ - ethyl, C₃ - propyl and C₄- n-butyl.
 - branched groups of between 3 and 10 atoms (C₃-C₁₀), or of up to 7 atoms (C₃-C₇), or of up to 4 atoms (C₃-C₄). Examples of such alkyl groups include, but are not limited to, C₃ - iso-propyl, C₄ - sec-butyl, C₄ - iso-butyl, C₄ - tert-butyl and C₅ - neo-pentyl.
- The term "alkylene" refers to the divalent hydrocarbon radical derived from an alkyl group, and shall be construed in accordance with the definition above.

The term "alkenyl" includes monounsaturated hydrocarbon residues including:

- linear groups of between 2 and 6 atoms (C₂-C₆). Examples of such alkenyl groups include, but are not limited to, C₂ - vinyl, C₃ - 1-propenyl, C₃ - allyl, C₄ - 2-butenyl
- branched groups of between 3 and 8 atoms (C₃-C₈). Examples of such alkenyl groups include, but are not limited to, C₄ - 2-methyl-2-propenyl and C₆ - 2,3-dimethyl-2-butenyl.

The term alkenylene refers to the divalent hydrocarbon radical derived from an alkenyl group, and shall be construed in accordance with the definition above.

The term "alkoxy" includes O-linked hydrocarbon residues including:

- linear groups of between 1 and 6 atoms (C₁-C₆), or of between 1 and 4 atoms (C₁-C₄). Examples of such alkoxy groups include, but are not limited to, C₁ - methoxy, C₂ - ethoxy, C₃ - n-propoxy and C₄ - n-butoxy.
- branched groups of between 3 and 6 atoms (C₃-C₆) or of between 3 and 4 atoms (C₃-C₄). Examples of such alkoxy groups include, but are not limited to, C₃ - iso-propoxy, and C₄ - sec-butoxy and tert-butoxy.

Halo is selected from Cl, F, Br and I. Halo is preferably F.

The term "aryl" includes a single or fused aromatic ring system containing 6 or 10 carbon atoms; wherein, unless otherwise stated, each occurrence of aryl may be optionally substituted with up to 5 substituents independently selected from (C₁-C₆)alkyl, (C₁-C₆)alkoxy, OH, halo, CN, COOR¹⁴, CF₃ and NR¹⁴R¹⁵; as defined above. Typically, aryl will be optionally substituted with 1, 2 or 3 substituents. Optional substituents are selected from those stated above. Examples of suitable aryl groups include phenyl and naphthyl (each optionally substituted as stated above). Arylene refers the divalent radical derived from an aryl group, and shall be construed in accordance with the definition above.

The term "heteroaryl" includes a 5, 6, 9 or 10 membered mono- or bi-cyclic aromatic ring, containing 1 or 2 N atoms and, optionally, an NR¹⁴ atom, or one NR¹⁴ atom and an S or an O atom, or one S atom, or one O atom; wherein, unless otherwise stated, said heteroaryl may be optionally substituted with 1, 2 or 3 substituents independently selected from (C₁-C₆)alkyl, (C₁-C₆)alkoxy, OH, halo, CN, COOR¹⁴, CF₃ and NR¹⁴R¹⁵; as defined below. Examples of suitable heteroaryl groups include thienyl, furanyl, pyrrolyl, pyrazolyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, oxadiazolyl, thiadiazolyl, tetrazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, indolyl, benzimidazolyl, benzotriazolyl, quinoliny and isoquinoliny (optionally substituted as stated above). Heteroarylene refers the divalent radical derived from heteroaryl, and shall be construed in accordance with the definition above.

The term "heterocyclyl" is a C-linked or N-linked 3 to 10 membered non-aromatic, mono- or bi-cyclic ring, wherein said heterocycloalkyl ring contains, where possible, 1, 2 or 3 heteroatoms independently selected from N, NR¹⁴, S(O)_q and O; and said heterocycloalkyl ring optionally contains, where possible, 1 or 2 double bonds, and is optionally substituted on carbon with 1 or 2 substituents independently selected from (C₁-C₆)alkyl, (C₁-C₆)alkoxy, OH, CN, CF₃, halo, COOR¹⁴, NR¹⁴R¹⁵ and aryl.

In the above definitions R¹⁴ and R¹⁵ are independently selected from H and (C₁-C₆)alkyl.

When a structural formula is defined with a substituent attached to the core of the molecule by an unspecified, or "floating" bond, for example, as for the group P³ in the case of formula (C), this definition encompasses the cases where the unspecified substituent is attached to any of the atoms on the ring in which the floating bond is located, whilst complying with the allowable valence for that atom.

In the case of compounds of the invention which may exist in tautomeric forms (*i.e.* in keto or enol forms), for example the compounds of formula (C) or (H), reference to a particular compound optionally includes all such tautomeric forms.

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, references 121-128, *etc.*

“GI” numbering is used above. A GI number, or “GenInfo Identifier”, is a series of digits assigned consecutively to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence is updated (*e.g.* for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed.

Where the invention concerns an “epitope”, this epitope may be a B-cell epitope and/or a T-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [129,130] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [131], matrix-based approaches [132], MAPITOPE [133], TEPITOPE [134,135], neural networks [136], OptiMer & EpiMer [137, 138], ADEPT [139], Tsites [140], hydrophilicity [141], antigenic index [142] or the methods disclosed in references 143-147, *etc.*). Epitopes are the parts of an antigen that are recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as “antigenic determinants”.

Where an antigen “domain” is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

The term “comprising” encompasses “including” as well as “consisting” *e.g.* a composition “comprising” X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term “about” in relation to a numerical value x is optional and means, for example, $x \pm 10\%$.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 148. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 149. Percentage identity to any particular sequence (*e.g.* to a particular SEQ ID) is ideally calculated over the entire length of that sequence.

Binding affinity may be determined by any method known in the art, including surface plasmon resistance, isothermal titration calorimetry, competitive binding assays, thermal shift assay, *etc.* Although the absolute figures obtained using different methods may vary, it is envisaged that the determination relative binding affinity of one protein compared to another should not depend on the method used.

Phosphorous-containing adjuvants used with the invention may exist in a number of protonated and deprotonated forms depending on the pH of the surrounding environment, for example the pH of the solvent in which they are dissolved. Therefore, although a particular form may be illustrated, it is intended that these illustrations are merely representative and not limiting to a specific protonated or

deprotonated form. For example, in the case of a phosphate group, this has been illustrated as $-\text{OP}(\text{O})(\text{OH})_2$ but the definition includes the protonated forms $[\text{OP}(\text{O})(\text{OH}_2)(\text{OH})]^+$ and $[\text{OP}(\text{O})(\text{OH})_2]^{2+}$ that may exist in acidic conditions and the deprotonated forms $[\text{OP}(\text{O})(\text{OH})(\text{O})]^-$ and $[\text{OP}(\text{O})(\text{O})_2]^{2-}$ that may exist in basic conditions.

- 5 Compounds can exist as pharmaceutically acceptable salts. Thus, compounds (*e.g.* adjuvants) may be used in the form of their pharmaceutically acceptable salts *i.e.* physiologically or toxicologically tolerable salt (which includes, when appropriate, pharmaceutically acceptable base addition salts and pharmaceutically acceptable acid addition salts).

10 The word “substantially” does not exclude “completely” *e.g.* a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

MODES FOR CARRYING OUT THE INVENTION

SpAkR mutant

15 SpA is a crucial virulence factor in *S. aureus* and it acts by interfering with opsonophagocytic clearance of the bacterium and by ablating adaptive immune responses. The wild-type sequence SEQ ID NO: 43 includes five Ig-binding domains (IgBD), as shown above, and it is known to mutate amino acid residues within these domains to abolish Fc/Fab binding activity while retaining immunogenicity *e.g.* see reference **Error! Bookmark not defined.**, which replaces Gln-Gln dipeptides with Lys-Lys and/or replaces Asp-Asp dipeptides with Ala-Ala.

20 A new mutant SpA has been produced in which a further Gln-Gln dipeptide is mutated to Lys-Arg. This dipeptide was identified based on bioinformatics analysis and predictions of an additional site involved in Ig binding. In particular, residues 96 & 97 of SEQ ID NO: 43 were hypothesised to be involved in Ig binding. This site was overlooked in earlier work because it is outside the conserved IgBD and is not part of the solved structures. The new mutant is referred to as ‘SpAkR’ and it has the following amino acid sequence, where the extra QQ/KR substitution relative to the previous
25 ‘SpAkAA’ mutant is boxed:

```
MAQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLNDQAPKADAKRNNF
NKDKKSAFYEILNMPNLNEAQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNA
FYEILNMPNLNEEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHL
PNLNEEQRNGFIQSLKAAPSQSANLLAEAKKLNDQAPKADNKFNKEKKNAFYEILHLPNLTEEQ
RNGFIQSLKAAPSVSKEILAEAKKLNDQAPK (SEQ ID NO: 48)
```

The SpAkR mutant has been confirmed to have reduced affinity for immunoglobulins relative to known mutants, while retaining immunogenicity.

30 CD and DSC analysis showed that the introduction of the KR mutant did not materially affect the structure of SpA. The CD spectra of SpAkAA and SpAKR were identical. T_{m1} and T_{m2} from DSC analysis were as follows. T_{m1} : SpAkAA 48.9°C; SpAKR 51.1°C. T_{m2} : SpAkAA 68.1°C; SpAKR 68.0°C.

Affinity of wild-type SpA, and of the SpAkkAA and SpAkR mutants, for human IgG, IgA and IgM was tested using surface plasmon resonance. The proteins were immobilized on a sensor chip and human IgG, IgA and IgM were used as analytes. Both mutants displayed a greatly reduced binding capacity compared to wild-type, but while SpAkkAA showed residual binding to IgG and IgM, no detectable interactions with any immunoglobulins were seen with SpAkR. Residual binding activities of SpAkkAA towards IgG and IgM were very low (11-12-fold lower than wild-type), but reproducible and concentration-dependent.

S. aureus survival in the presence of SpA (WT, SpAkkAA and SpAkR) was assessed through whole blood survival assay (WBA). 1ml whole blood from healthy donors, supplemented with 50mg/l lepirudin anticoagulant (10µl/ml blood), was incubated with 0.15µM SpA or with PBS, for 15 min at 37°C, before adding approximately 2.5×10^5 CFU of *S. aureus* USA300 LAC (OD₆₀₀, 0.4) diluted in BHI. Aliquots of this culture were plated on BHI-agar to determine the input CFU. After incubation at 37°C for 2h with shaking (180 RPM), neutrophils were lysed at 0.5% saponin-PBS for 3 min on ice. The number of viable bacteria was determined by serial tenfold dilutions in BHI and plating on nutrient agar plates. Colonies were counted after incubation of the plates at 37°C for 18h. Control was the blood sample preincubated with PBS. Relative survival was calculated based on the input CFU compared to CFU post-incubation. Experiments were performed in triplicate; there was little variation between the experiments.

In the WBA, relative survival of *S. aureus* in human whole blood was considerably lower when incubated with SpAKR than with wild type SpA ($p < 0.001$, Mann-Whitney) or with SpAkkAA ($p < 0.05$). Incubation with SpAwt resulted in increased *S. aureus* survival compared to control ($p < 0.001$), whereas survival in WBA incubated with SpAkkAA was comparable to control. Incubation with SpAkR reduced survival to about half of that of the control.

SpAkkAA or SpAkR formulated with aluminium hydroxide adjuvant were found to be weakly immunogenic in mice on their own, but inclusion of adsorbed TLR7 agonist 'K1' significantly increased antibody titres. Because the mechanism of action associated with SpA immunisation appears to be mainly driven by antibodies, this is an important improvement.

***S. aureus* vaccines**

SpAkR, SpAkkAA, SpAkR E domain alone and SpAkR E domain fused to H1aH35L were tested in the renal abscess model, adjuvanted with aluminium hydroxide (Al-H) at 2mg/ml (total salt). Antigens were each present at 10µg in a 100µL dose for intramuscular injection.

Four or five-week old mice (CD1) were immunized intramuscularly (IM) with prime-booster injections with a 14-day interval. Control mice received equal amounts of adjuvants alone. Serum was collected from mice both pre- and post-vaccination to document serum antibody titers to each protein component in the combination vaccine. These titers were measured by Luminex technology using the recombinant vaccine antigens conjugated to microspheres.

Renal abscess model: Immunized animals were challenged on day 24 by intravenous injection of a sublethal dose of *S.aureus* Newman strain ($\sim 2-6 \times 10^7$ CFU). On day 28, mice were euthanized and kidneys were removed and homogenized in 2 mL of PBS and plated on agar media in duplicate for determination of colony forming units (CFU).

- 5 A comparable reduction in $\log_{10}$ CFU/ml (around 1 log reduction) was obtained on vaccination with SpAKR, SpAkkAA, SpAkR E domain alone and SpAkR E domain fused to HlaH35L, compared to adjuvant alone.

Combination S.aureus vaccines

- 10 A 5-valent and a 6-valent vaccine were prepared. The 5-valent vaccine included antigens consisting of SEQ ID NOs: 7, 8, 27 and 32 (FhuD2, Sta011, Hla-H35L, and EsxAB); the 6-valent vaccine also included the SpAkR mutant. The vaccines were adjuvanted with: (i) aluminium hydroxide, Al-H; (ii) Al-H + adsorbed TLR7 agonist K1; or (iii) the oil-in-water emulsion MF59. Al-H was used at 2mg/ml (total salt), K1 was present at 50 μ g per dose, and MF59 was mixed with the antigens at a 1:1 volume ratio. Antigens were each present at 10 μ g in a 100 μ L dose for intramuscular injection.

- 15 Four or five-week old mice (CD1) were immunized with prime-booster injections with a 14-day interval. Control mice received equal amounts of adjuvants alone. Serum was collected from mice both pre- and post-vaccination to document serum antibody titers to each protein component in the combination vaccine. These titers were measured by Luminex technology using the recombinant vaccine antigens conjugated to microspheres.

- 20 *Renal abscess model:* Immunized animals were challenged on day 24 by intravenous injection of a sublethal dose of *S.aureus* ($\sim 2-6 \times 10^7$ CFU, where the specific inoculum varied depending on the challenge strain). On day 28, mice were euthanized and kidneys were removed and homogenized in 2 mL of PBS and plated on agar media in duplicate for determination of colony forming units (CFU). Kidneys were also processed for histopathology.

- 25 *Peritonitis model:* Separately, immunized animals were challenged on day 24 by intraperitoneal injection of a lethal dose of *S.aureus*. ($\sim 2-5 \times 10^8$ CFU) and then monitored daily for 14 days.

- 30 *Skin infection model:* Immunized mice were inoculated by subcutaneous injection in the shaved right flank with 2×10^7 CFU *S.aureus* LAC strain (USA300 clone, which is one of the most important clones worldwide and highly associated to community-acquired cutaneous infections). Mass and abscess formation (size and dermonecrosis) were monitored at 24-hour intervals over a course of 7 days. The size of an abscess and associated overlying dermonecrotic lesion was determined using image analysis software. Mouse skin and abscesses were harvested on day 7 post inoculation for CFU enumeration. This model was used only with the Al-H/K1 adjuvant.

Results were as follows:

	Adjuvant alone	5-valent	6-valent
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	Al-H	Al-H/K1	MF59	Al-H	Al-H/K1	MF59	Al-H	Al-H/K1	MF59
<i>Renal abscess</i> — log reduction in median cfu/ml									
A	-	-	-	1.5**	1.3**	0.93**	1.57**	2.31**	1.07**
<i>Peritonitis</i> — % survival									
B	29	42	33	67*	88**	67*	83**	92**	71**
<i>Skin infection</i> — log reduction in median cfu/ml									
C	N/A	-	-	-	3.35	-	-	4.39	-

* $p \leq 0.05$ compared to control; ** $p < 0.01$ compared to control

These results demonstrate that the mutant SpAkR improves the 5-valent Combo-1 product in both models. Furthermore, the best results were seen using the Al-H/K1 adjuvant. Strikingly, results in the renal abscess model the 6-valent vaccine with Al-H/K1 approximated to sterility.

- 5 In the peritonitis model, the 6-valent vaccine with Al-H/K1 was statistically superior when compared to the negative control (see table above), and also when compared to the 5-valent Al-H vaccine. In the abscess model, the 6-valent vaccine with Al-H/K1 was statistically superior when compared to the negative control (see table above), to the 5-valent Al-H vaccine, and to the 5-valent Al-H/K1 vaccine, thus showing that the contribution of the mutant SpA goes beyond the enhancement which
- 10 was due solely to the K1 agonist.

In the skin infection model, the 5-valent and 6-valent vaccines both significantly reduced abscess formation and CFU counts (see table above). Dermonecrosis was absent in the vaccinated mice while it was observed in all mice that received adjuvant alone ('N/A' in table). Furthermore, CFU reduction was significantly improved by the inclusion of SpAkR in the vaccine, and fewer mice were

15 observed with distinguishable abscesses at a macroscopic level (71% vs. 88%).

Anti-SpA titers were also compared for the three adjuvants. Median titers using Al-H or MF59 were not significantly different, but the titer using Al-H/K1 was significantly higher than with Al-H alone ($p=0.0047$, Mann-Whitney test) and than with MF59 ($p=0.01$). Thus the formulation which performed best in the functional assays was the one which elicited the highest anti-SpA antibody

20 titres, in line with the hypothesis that SpA's activity as a vaccine antigen depends on antibodies, which can bind it and inhibit its immune-evasion activity.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES

- [1] Sheridan (2009) *Nature Biotechnology* 27:499-501.
 [2] Kuklin *et al.* (2006) *Infect Immun.* 74(4):2215-23.

- [3] Fowler *et al.* (2013) *JAMA* 309(13):1368-78.
- [4] WO2010/119343.
- [5] WO2013/030378.
- [6] Sjodahl (1977) *J. Biochem.* 73:343-351.
- [7] Uhlen *et al.* (1984) *J. Biol. Chem.* 259:1695-1702 & 13628 (Corr.).
- [8] WO2007/071692.
- [9] Kim *et al.* (2010) *J Exp Med* 207(9):1863-70.
- [10] WO2013/092985.
- [11] WO2014/033190.
- [12] WO2014/033191.
- [13] WO2014/033192.
- [14] WO2014/033193.
- [15] Sebulsky & Heinrichs (2001) *J Bacteriol* 183:4994-5000.
- [16] Sebulsky *et al.* (2003) *J Biol Chem* 278:49890-900.
- [17] Rable & Wardenburg (2009) *Infect Immun* 77:2712-8.
- [18] WO2007/145689.
- [19] WO2009/029831.
- [20] WO2006/110603.
- [21] Stranger-Jones *et al.* (2006) *PNAS USA* 103:16942-7.
- [22] Wardenburg *et al.* (2007) *Infect Immun* 75:1040-4.
- [23] Prabhakara *et al.* (2011) *J Immunol* 186:155.29
- [24] Kuroda *et al.* (2001) *Lancet* 357:1225-1240.
- [25] Needleman & Wunsch (1970) *J. Mol. Biol.* 48, 443-453.
- [26] Rice *et al.* (2000) *Trends Genet* 16:276-277.
- [27] US patent 6355271.
- [28] WO00/23105.
- [29] *Vaccine Design...* (1995) eds. Powell & Newman. ISBN: 030644867X. Plenum.
- [30] WO90/14837.
- [31] Garçon *et al.* (2012) *Expert Rev Vaccines* 11:349-66.
- [32] WO90/14837.
- [33] Podda & Del Giudice (2003) *Expert Rev Vaccines* 2:197-203.
- [34] Podda (2001) *Vaccine* 19: 2673-2680.
- [35] *Vaccine Design: The Subunit and Adjuvant Approach* (eds. Powell & Newman) Plenum Press 1995 (ISBN 0-306-44867-X).
- [36] *Vaccine Adjuvants: Preparation Methods and Research Protocols* (Volume 42 of *Methods in Molecular Medicine* series). ISBN: 1-59259-083-7. Ed. O'Hagan.
- [37] WO2008/043774.
- [38] Allison & Byars (1992) *Res Immunol* 143:519-25.
- [39] Hariharan *et al.* (1995) *Cancer Res* 55:3486-9.
- [40] US-2007/014805.
- [41] US-2007/0191314.
- [42] Suli *et al.* (2004) *Vaccine* 22(25-26):3464-9.
- [43] WO95/11700.
- [44] US patent 6,080,725.
- [45] WO2005/097181.
- [46] WO2006/113373.
- [47] US 5,057,540.
- [48] WO96/33739.
- [49] EP-A-0109942.
- [50] WO96/11711.

- [51] WO00/07621.
- [52] Barr *et al.* (1998) *Advanced Drug Delivery Reviews* 32:247-271.
- [53] Sjolanderet *et al.* (1998) *Advanced Drug Delivery Reviews* 32:321-338.
- [54] EP-A-0689454.
- [55] Meraldi *et al.* (2003) *Vaccine* 21:2485-2491.
- [56] Pajak *et al.* (2003) *Vaccine* 21:836-842.
- [57] Kandimalla *et al.* (2003) *Nucleic Acids Research* 31:2393-2400.
- [58] WO02/26757.
- [59] WO99/62923.
- [60] Krieg (2003) *Nature Medicine* 9:831-835.
- [61] McCluskie *et al.* (2002) *FEMS Immunology and Medical Microbiology* 32:179-185.
- [62] WO98/40100.
- [63] US 6,207,646.
- [64] US 6,239,116.
- [65] US 6,429,199.
- [66] Kandimalla *et al.* (2003) *Biochemical Society Transactions* 31 (part 3):654-658.
- [67] Blackwell *et al.* (2003) *J Immunol* 170:4061-4068.
- [68] Krieg (2002) *Trends Immunol* 23:64-65.
- [69] WO01/95935.
- [70] Kandimalla *et al.* (2003) *BBRC* 306:948-953.
- [71] Bhagat *et al.* (2003) *BBRC* 300:853-861.
- [72] WO03/035836.
- [73] WO01/22972.
- [74] Schellack *et al.* (2006) *Vaccine* 24:5461-72.
- [75] Kamath *et al.* (2008) *Eur J Immunol* 38:1247-56.
- [76] Riedl *et al.* (2008) *Vaccine* 26:3461-8.
- [77] WO95/17211.
- [78] WO98/42375.
- [79] Beignon *et al.* (2002) *Infect Immun* 70:3012-3019.
- [80] Pizza *et al.* (2001) *Vaccine* 19:2534-2541.
- [81] Pizza *et al.* (2000) *Int J Med Microbiol* 290:455-461.
- [82] Scharton-Kersten *et al.* (2000) *Infect Immun* 68:5306-5313.
- [83] Ryan *et al.* (1999) *Infect Immun* 67:6270-6280.
- [84] Partidos *et al.* (1999) *Immunol Lett* 67:209-216.
- [85] Peppoloni *et al.* (2003) *Expert Rev Vaccines* 2:285-293.
- [86] Pine *et al.* (2002) *J Control Release* 85:263-270.
- [87] Tebbey *et al.* (2000) *Vaccine* 18:2723-34.
- [88] Domenighini *et al.* (1995) *Mol Microbiol* 15:1165-1167.
- [89] Rosenberg *et al.* (2010) *J Immunol* 184:136.20.
- [90] US-4,666,886.
- [91] WO2009/118296.
- [92] WO2008/005555.
- [93] WO2009/111337.
- [94] WO2009/067081.
- [95] WO2007/040840.
- [96] WO2010/014913.
- [97] WO2012/031140.
- [98] WO2013/132041.
- [99] US 4,680,338.
- [100] US 4,988,815.

- [101] WO92/15582.
- [102] Stanley (2002) *Clin Exp Dermatol* 27:571-577.
- [103] Wu *et al.* (2004) *Antiviral Res.* 64(2):79-83.
- [104] Vasilakos *et al.* (2000) *Cell Immunol.* 204(1):64-74.
- [105] US patents 4689338, 4929624, 5238944, 5266575, 5268376, 5346905, 5352784, 5389640, 5395937, 5482936, 5494916, 5525612, 6083505, 6440992, 6627640, 6656938, 6660735, 6660747, 6664260, 6664264, 6664265, 6667312, 6670372, 6677347, 6677348, 6677349, 6683088, 6703402, 6743920, 6800624, 6809203, 6888000 and 6924293.
- [106] Jones (2003) *Curr Opin Investig Drugs* 4:214-218.
- [107] Steinhagen *et al.* (2011) *Vaccine* 29:3341-55.
- [108] Myers *et al.* (1990) pages 145-156 of *Cellular and molecular aspects of endotoxin reactions*.
- [109] Ulrich (2000) Chapter 16 (pages 273-282) of reference 36.
- [110] Johnson *et al.* (1999) *J Med Chem* 42:4640-9.
- [111] Baldrick *et al.* (2002) *Regulatory Toxicol Pharmacol* 35:398-413.
- [112] Coler *et al.* (2011) *PLoS ONE* 6(1):e16333.
- [113] Johnson *et al.* (1999) *Bioorg Med Chem Lett* 9:2273-2278.
- [114] Evans *et al.* (2003) *Expert Rev Vaccines* 2:219-229.
- [115] Bazin *et al.* (2006) *Tetrahedron Lett* 47:2087-92.
- [116] Wong *et al.* (2003) *J Clin Pharmacol* 43(7):735-42.
- [117] US2005/0215517.
- [118] WO03/011223.
- [119] WO2011/027222.
- [120] WO2013/131985
- [121] Gennaro (2000) *Remington: The Science and Practice of Pharmacy*. 20th edition, ISBN: 0683306472.
- [122] *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.)
- [123] *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds, 1986, Blackwell Scientific Publications)
- [124] Sambrook *et al.* (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition (Cold Spring Harbor Laboratory Press).
- [125] *Handbook of Surface and Colloidal Chemistry* (Birdi, K.S. ed., CRC Press, 1997)
- [126] Ausubel *et al.* (eds) (2002) *Short protocols in molecular biology*, 5th edition (Current Protocols).
- [127] *Molecular Biology Techniques: An Intensive Laboratory Course*, (Ream *et al.*, eds., 1998, Academic Press)
- [128] *PCR (Introduction to Biotechniques Series)*, 2nd ed. (Newton & Graham eds., 1997, Springer Verlag)
- [129] Geysen *et al.* (1984) *PNAS USA* 81:3998-4002.
- [130] Carter (1994) *Methods Mol Biol* 36:207-23.
- [131] Jameson, BA *et al.* 1988, *CABIOS* 4(1):181-186.
- [132] Raddrizzani & Hammer (2000) *Brief Bioinform* 1(2):179-89.
- [133] Bublil *et al.* (2007) *Proteins* 68(1):294-304.
- [134] De Lalla *et al.* (1999) *J. Immunol.* 163:1725-29.
- [135] Kwok *et al.* (2001) *Trends Immunol* 22:583-88.
- [136] Brusic *et al.* (1998) *Bioinformatics* 14(2):121-30
- [137] Meister *et al.* (1995) *Vaccine* 13(6):581-91.
- [138] Roberts *et al.* (1996) *AIDS Res Hum Retroviruses* 12(7):593-610.
- [139] Maksyutov & Zagrebelskaya (1993) *Comput Appl Biosci* 9(3):291-7.
- [140] Feller & de la Cruz (1991) *Nature* 349(6311):720-1.
- [141] Hopp (1993) *Peptide Research* 6:183-190.
- [142] Welling *et al.* (1985) *FEBS Lett.* 188:215-218.

- [143] Davenport *et al.* (1995) *Immunogenetics* 42:392-297.
- [144] Tsurui & Takahashi (2007) *J Pharmacol Sci.* 105(4):299-316.
- [145] Tong *et al.* (2007) *Brief Bioinform.* 8(2):96-108.
- [146] Schirle *et al.* (2001) *J Immunol Methods.* 257(1-2):1-16.
- [147] Chen *et al.* (2007) *Amino Acids* 33(3):423-8.
- [148] *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987) Supplement 30
- [149] Smith & Waterman (1981) *Adv. Appl. Math.* 2: 482-489.

SEQUENCE LISTING***SEQ ID NO: 1 (EsxA)***

MAMIKMSPEEIRAKSQSYGQGSQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLE
EIKQQLNSTADAVQEQQQLSNNFGLQ

SEQ ID NO: 2 (EsxB)

MGGYKGIKADGGKVDQAKQLAAKTAKDIEACQKQTQQLAEYIEGSDWEGQFANKVKDVLIMAKFQEELV
QPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 3 (FhuD2)

MKKLLLLPLIIMLLVLAACGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGGLKKLGANIVA
VNQQVDQSKVLKDKFKGVTKIGDGDVEKVAKEKPDLIIVYSTDKDIKKYQKVAPT VVVDYNKHKYLEQQE
MLGKIVGKEDKVKAWKKDWEETTAKDGEIKKAIGQDATVSLFDEFDCKLYTYGDNWGRGGEVLYQAFGL
KMQPEQQKLTAKAGWAEVKQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNL KATKEGHIVKVDAGTYWY
NDPYTLDFMRKDLKEKLIKAAK

SEQ ID NO: 4 (Sta011)

MMKRLNKLVLGIIIFLFLVISITAGCGIGKEAEVKKSFECTLSMYP IKNLEDLYDKEGYRDDQFDKNDKGT
WIINSEMV IQPNNEDMVAKGMVLYMNRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKI IPTKE
IKDEKIKKEIENFKFFVQYGDFKNLKNYKGDGDISYNPEVPSYSAKYQLTND DYNVKQLRKRYDIPTSKAP
KLLLKGSGNLKGSSVGYKDIEFTFVEKKEENIYFSDSLDYKKS GDV

SEQ ID NO: 5 (Hla)

MKTRIVSSVTTTLLLSILMNPVANAADSDINIKTGTTDIGSNTTVKTGDLV TYDKENG MHKKVFYSFID
DKNHNKLLVIRTKGTIAGQYRVYSEEGANKSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMS
TLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQPDFKTI LESPTDKKVGWKVI FNNMVNQNWGPYDR
DSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFS PDFATVITMDRKASKQQT NIDVIYERVRD
DYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

SEQ ID NO: 6 (N-terminally truncated FhuD2 of SEQ ID NO: 3)

CGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGGLKKLGANIVAVNQQVDQSKVLKDKFKG
VTKIGDGDVEKVAKEKPDLIIVYSTDKDIKKYQKVAPT VVVDYNKHKYLEQQEMLGKIVGKEDKVKAWKK
DWEETTAKDGEIKKAIGQDATVSLFDEFDCKLYTYGDNWGRGGEVLYQAFGLKMQPEQQKLTAKAGWAE
VKQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNL KATKEGHIVKVDAGTYWYNDPYTLDFMRKDLKEKL
IKAAK

SEQ ID NO: 7 (Example FhuD2 sequence)

MASCGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGGLKKLGANIVAVNQQVDQSKVLKDK
FKGVTKIGDGDVEKVAKEKPDLIIVYSTDKDIKKYQKVAPT VVVDYNKHKYLEQQEMLGKIVGKEDKVKAW
WKKDWEETTAKDGEIKKAIGQDATVSLFDEFDCKLYTYGDNWGRGGEVLYQAFGLKMQPEQQKLTAKAG
WAEVKQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNL KATKEGHIVKVDAGTYWYNDPYTLDFMRKDLK
EKLIKAAK

SEQ ID NO: 8 (Example Sta011 sequence)

MGCGIGKEAEVKKSFECTLSMYP IKNLEDLYDKEGYRDDQFDKNDKGTWIINSEMV IQPNNEDMVAKGMV
LYMNRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKI IPTKEIKDEKIKKEIENFKFFVQYGDF
KNLKNYKGDGDISYNPEVPSYSAKYQLTND DYNVKQLRKRYDIPTSKAPKLLLKGSGNLKGSSVGYKDIEF
TFVEKKEENIYFSDSLDYKKS GDV

SEQ ID NO: 9 (Example Sta011 sequence)

MMKRLNKLVLGIIIFLFLVISITAGCGIGKEAEVKKSFECTLSMYP IKNLEDLYDKEGYRDDQFDKNDKGT
WIINSEMV IQPNNEDMVAKGMVLYMNRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKI IPTKE
IKDEKLKKEIENFKFFVQYGDFKNLKNYKGDGDISYNPEVPSYSAKYQLTND DYNVKQLRKRYDIPTSKAP
KLLLKGSGNLKGSSVGYKDIEFTFVEKKEENIYFSDSLDYKKS GDV

SEQ ID NO: 10 (Example Sta011 sequence)

MMKRLNKLVLGIIIFLFLVISITAGCGIGKEAEVKKSFEKTLSPYPIKNLEDLYDKEGYRDDQFDKNDKGT
 WIINSEMVIIQPNNEDMVAAGMVLYMNRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKIIPTKE
 IKDEKVKKEIENFKFFVQYGDGFKNIKNYKDGDISYNPEVPSYSAKYQLTNDYDYNVKQLRKRYDIPTSKAP
 KLLLLKSGSNLKGSSVGYKDIEFTFVEKKEENIYFSDSLDYKKS GDV

SEQ ID NO: 11 (Example Sta011 sequence)

MMKRLNKLVLGIIIFLFLVISITAGCGIGKEAEVKKSFEKTLSPYPIKNLEDLYDKEGYRDDQFDKNDKGT
 WIINSEMVIIQPNNEDMVAAGMVLYMNRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKIIPTKE
 IKDEKLKKEIENFKFFVQYGDGFKNVKNYKDGDISYNPEVPSYSAKYQLTNDYDYNVKQLRKRYDIPTSKAP
 KLLLLKSGSNLKGSSVGYKDIEFTFVEKKEENIYFSDSLDYKKS GDV

SEQ ID NO: 12 (N-terminally truncated Hla SEQ ID NO: 5)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMMHKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTKEYMSTLTYGFGNGVTGDDTGKIGGLIGANV
 SIGHTLKYVQPDFKTIESTPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
 LDPNKASSLLSSGFS PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
 RYKIDWEKEEMTN

SEQ ID NO: 13 (Mature Hla-H35L)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTKEYMSTLTYGFGNGVTGDDTGKIGGLIGANV
 SIGHTLKYVQPDFKTIESTPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
 LDPNKASSLLSSGFS PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
 RYKIDWEKEEMTN

SEQ ID NO: 14 (tetramer)

PSGS

SEQ ID NO: 15 (Hla-H35L with PSGS substitution)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTPSGSVQPDFKTIESTPTDKKVGWKVIFNNMV
 NQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF LDPNKASSLLSSGFS PDFATVITMDRKASKQQTN
 IDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

SEQ ID NO: 16 (Hla with PSGS substitution)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMMHKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTPSGSVQPDFKTIESTPTDKKVGWKVIFNNMV
 NQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF LDPNKASSLLSSGFS PDFATVITMDRKASKQQTN
 IDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSERYKIDWEKEEMTN

SEQ ID NO: 17 (Hla with Y101 mutation)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMMHKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDLYPRNSIDTKEYMSTLTYGFGNGVTGDDTGKIGGLIGANV
 SIGHTLKYVQPDFKTIESTPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
 LDPNKASSLLSSGFS PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
 RYKIDWEKEEMTN

SEQ ID NO: 18 (Hla with D152 mutation)

ADSDINIKTGTTDIGSNTTVKTGDLVITYDKENGMMHKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
 EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTKEYMSTLTYGFGNGVTGDDTGKIGGLIGANV
 SIGHTLKYVQPLFKTIESTPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
 LDPNKASSLLSSGFS PDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
 RYKIDWEKEEMTN

SEQ ID NO: 19 (Hla with H35 and Y101 mutations)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
EGANKSGLAWPSAFKVQLQLPDNEVAQISDLYPRNSIDTKEYMSTLTYGFGNVTGDDTGKIGGLIGANV
SIGHTLKYVQPDFKTI¹LESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
LDPNKASSLLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
RYKIDWEKEEMTN

SEQ ID NO: 20 (Hla with H53 and D152 mutations)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSE
EGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTKEYMSTLTYGFGNVTGDDTGKIGGLIGANV
SIGHTLKYVQPLFKTI¹LESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNF
LDPNKASSLLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDRSSE
RYKIDWEKEEMTN

SEQ ID NO: 21 (Hla fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNK

SEQ ID NO: 22 (Hla fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAG

SEQ ID NO: 23 (Hla fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLL

SEQ ID NO: 24 (Hla-H35L fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNK

SEQ ID NO: 25 (Hla-H35L fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAG

SEQ ID NO: 26 (Hla-H35L fragment)

ADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLL

SEQ ID NO: 27 (Useful HLA sequence with H35L mutation)

MASADSDINIKTGTTDIGSNTTVKTDGLVITYDKENGMLKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRV
YSEEGANKSGLAWPSAFKVQLQLPDNEVAQISDYPRNSIDTKEYMSTLTYGFGNVTGDDTGKIGGLIG
ANVSIGHTLKYVQPDFKTI¹LESPTDKKVGWKVIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAA
DNFLDPNKASSLLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWIDR
SSERYKIDWEKEEMTN

SEQ ID NO: 28 (EsxAB example)

MAMIKMSPEEIRAKSQSYGQGS¹DQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLE
EIKQQLNSTADAVQE¹QDQQLSNNFGLQASGGGSMGGYKG¹IKADGGKVDQAKQLAAKTAKDIEACQKQTQ
LAEYIEGSDWEGQFANKVKDVLLIMAKFQEELVQPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 29 (EsxBA example)

MGGYKG¹IKADGGKVDQAKQLAAKTAKDIEACQKQTQQLAEYIEGSDWEGQFANKVKDVLLIMAKFQEELV
QPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNPASGGGSMAMIKMSPEEIRAKSQSYGQGS¹DQIRQILS
DLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLEEIKQQLNSTADAVQE¹QDQQLSNNFGLQ

SEQ ID NO: 30 (linker)

ASGGGS

SEQ ID NO: 31 (EsxAB example)

AMIKMSPEEIRAKSQSYGQGS¹DQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLEE
IKQQLNSTADAVQE¹QDQQLSNNFGLQASGGGSMGGYKG¹IKADGGKVDQAKQLAAKTAKDIEACQKQTQQLA
EYIEGSDWEGQFANKVKDVLLIMAKFQEELVQPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 32 (EsxAB example)

MAMIKMSPEEIRAKSQSYGQGSQDQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLE
EIKQQLNSTADAVQEQQQLSNNFGLQASGGGSGGYKGIKADGGKVDQAKQLAAKTAKDIEACQKQTQQL
AEYIEGSDWEGQFANKVKDVLLIMAKFQEELVQPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 33 (N-terminally truncated SEQ ID NO: 4)

GCGIGKEAEVKKSFEKTLSPYPIKNLEDLYDKEGYRDDQFDKNDKGTWIINSEMVIQPNNEDMVAKGMVL
YMNRRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKIIPTKEIKDEKIKKEIENFKFFVQYGDFF
NLKNYKDGDISYNPEVPSYSAKYQLTNDYDYNVKQLRKRYDIPTSKAPKLLLLKGSGNLKGSSVGYKDIEFT
FVEKKEENIYFSDSLDYKKSQDV

SEQ ID NO: 34 (FhuD2)

GNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGGLKKLGANIVAVNQVDQSKVLKDKFKGV
TKIGDGDVEKVAKEKPDIIIVYSTDKDIKKYQKVAPTIVVDYNKHKYLEQQEMLGKIVGKEDKVKAWKDD
WEETAKDGKEIKKAIGQDATVSLFDEFDKKLYTYGDNWGRGGEVLYQAFGLKMQPEQQKLTAKAGWAEV
KQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNLTKATKEGHIVKVDAGTYWYNDPYTLDFMRKDLKEKLI
KAAK

SEQ ID NO: 35 (Cys-free EsxB sequence)

GGYKGIKADGGKVDQAKQLAAKTAKDIEAXQKQTQQLAEYIEGSDWEGQFANKVKDVLLIMAKFQEELVQ
PMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 36 (Cys-free version of Sta011 SEQ ID NO: 4)

GIGKEAEVKKSFEKTLSPYPIKNLEDLYDKEGYRDDQFDKNDKGTWIINSEMVIQPNNEDMVAKGMVLYM
NRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKIIPTKEIKDEKIKKEIENFKFFVQYGDFFNL
KNYKDGDISYNPEVPSYSAKYQLTNDYDYNVKQLRKRYDIPTSKAPKLLLLKGSGNLKGSSVGYKDIEFTFV
EKKEENIYFSDSLDYKKSQDV

SEQ ID NO: 37 (Cys-free version of FhuD2 SEQ ID NO: 7)

MASGNQGEKNNKAETKSYKMDDGKTVDIPKDPKRIAVVAPTYAGGLKKLGANIVAVNQVDQSKVLKDKF
KGVTKIGDGDVEKVAKEKPDIIIVYSTDKDIKKYQKVAPTIVVDYNKHKYLEQQEMLGKIVGKEDKVKAW
KKDWEETAKDGKEIKKAIGQDATVSLFDEFDKKLYTYGDNWGRGGEVLYQAFGLKMQPEQQKLTAKAGW
AEVKQEEIEKYAGDYIVSTSEGKPTPGYESTNMWKNLTKATKEGHIVKVDAGTYWYNDPYTLDFMRKDLKE
KLIKAAK

SEQ ID NO: 38 (EsxAB Cys-Ala mutant)

MAMIKMSPEEIRAKSQSYGQGSQDQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLE
EIKQQLNSTADAVQEQQQLSNNFGLQASGGGSGGYKGIKADGGKVDQAKQLAAKTAKDIEAXQKQTQQL
AEYIEGSDWEGQFANKVKDVLLIMAKFQEELVQPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 39 (useful Sta011 sequence)

MGSGIGKEAEVKKSFEKTLSPYPIKNLEDLYDKEGYRDDQFDKNDKGTWIINSEMVIQPNNEDMVAKGMV
LYMNRRNTKTTNGYYYVDVTKDEDEGKPHDNEKRYPVKMVDNKIIPTKEIKDEKIKKEIENFKFFVQYGDFF
KNLKNYKDGDISYNPEVPSYSAKYQLTNDYDYNVKQLRKRYDIPTSKAPKLLLLKGSGNLKGSSVGYKDIEF
TFVEKKEENIYFSDSLDYKKSQDV

SEQ ID NO: 40 (Cys-free EsxAB example)

AMIKMSPEEIRAKSQSYGQGSQDQIRQILSDLTRAQGEIAANWEGQAFSRFEEQFQQLSPKVEKFAQLLEE
IKQQLNSTADAVQEQQQLSNNFGLQASGGGSGGYKGIKADGGKVDQAKQLAAKTAKDIEAXQKQTQQLA
EYIEGSDWEGQFANKVKDVLLIMAKFQEELVQPMADHQKAIDNLSQNLAKYDTLSIKQGLDRVNP

SEQ ID NO: 41

icicicicicicicicicicicic

SEQ ID NO: 42

KLKLLLLLLK

SEQ ID NO: 43 (SpA)

MKKKNIYSIRKLGVGIASVTLTLLISGGVTPAANAAQHDEAQQNAFYQVLNMPNLNADQQRNGFIQSLKD
 DPSQSANVLGEAQKLND SQAPKADAQQNNFNKDQQSAFYEILNMPNLNEAQQRNGFIQSLKDDPSQSTNVL
 GEAKKLNESQAPKADNNFNKEQQNAFYEILNMPNLNEEQQRNGFIQSLKDDPSQSANLLSEAKKLNESQAP
 KADNKFNKEQQNAFYEILHLPNLNEEQQRNGFIQSLKDDPSQSANLLAEAKKLND AQAPKADNKFNKEQQN
 AFYEILHLPNLTEEQRNGFIQSLKDDPSVSKEILAEAKKLND AQAPKEEDNNKPGKEDNNKPGKEDNNKPG
 GKEDNNKPGKEDNNKPGKEDGNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDNNK
 GVHVVKPGD TVNDIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQFANHADANKAQAALPETGEENP
 FIGTTVFGGLSLALGAALLAGRRREL

SEQ ID NO: 44 (SpAkkAA)

AQHDEAXXNAFYQVLNMPNLNADQQRNGFIQSLKXXPSQSANVLGEAQKLND SQAPKADAQQNNFNKDXXS
 AFYEILNMPNLNEAQQRNGFIQSLKXXPSQSTNVLGEAKKLNESQAPKADNNFNKEXXNAFYEILNMPNLN
 EEQRNGFIQSLKXXPSQSANLLSEAKKLNESQAPKADNKFNKEXXNAFYEILHLPNLNEEQQRNGFIQSLK
 XXPSQSANLLAEAKKLND AQAPKADNKFNKEXXNAFYEILHLPNLTEEQRNGFIQSLKXXPSVSKEILAE
 AKKLND AQAPK

SEQ ID NO: 45 (SpAkkAA)

AQHDEAKKNAFYQVLNMPNLNADQQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAQQNNFNKDKKS
 AFYEILNMPNLNEAQQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLN
 EEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQQRNGFIQSLK
 AAPSQSANLLAEAKKLND AQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAE
 AKKLND AQAPK

SEQ ID NO: 46 (SpAkR)

AQHDEAKKNAFYQVLNMPNLNADQQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAXNNFNKDKKS
 AFYEILNMPNLNEAQQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLN
 EEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQQRNGFIQSLK
 AAPSQSANLLAEAKKLND AQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAE
 AKKLND AQAPK

SEQ ID NO: 47 (SpAkR)

AQHDEAKKNAFYQVLNMPNLNADQQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAKRNNFNKDKKS
 AFYEILNMPNLNEAQQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLN
 EEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQQRNGFIQSLK
 AAPSQSANLLAEAKKLND AQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILAE
 AKKLND AQAPK

SEQ ID NO: 48 (SpAkR used in example)

MAQHDEAKKNAFYQVLNMPNLNADQQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAKRNNFNKDKK
 SAFYEILNMPNLNEAQQRNGFIQSLKAAPSQSTNVLGEAKKLNESQAPKADNNFNKEKKNAFYEILNMPNLN
 NEEQRNGFIQSLKAAPSQSANLLSEAKKLNESQAPKADNKFNKEKKNAFYEILHLPNLNEEQQRNGFIQSL
 KAAPSQSANLLAEAKKLND AQAPKADNKFNKEKKNAFYEILHLPNLTEEQRNGFIQSLKAAPSVSKEILA
 EAKKLND AQAPK

SEQ ID NO: 49 (SpAkR)

AQHDEAXXNAFYQVLNMPNLNADQQRNGFIQSLKXXPSQSANVLGEAQKLND SQAPKADAXNNFNKDXXS
 AFYEILNMPNLNEAQQRNGFIQSLKXXPSQSTNVLGEAKKLNESQAPKADNNFNKEXXNAFYEILNMPNLN
 EEQRNGFIQSLKXXPSQSANLLSEAKKLNESQAPKADNKFNKEXXNAFYEILHLPNLNEEQQRNGFIQSLK
 XXPSQSANLLAEAKKLND AQAPKADNKFNKEXXNAFYEILHLPNLTEEQRNGFIQSLKXXPSVSKEILAE
 AKKLND AQAPK

SEQ ID NO: 50 (SpAkR E domain)

AQHDEAXXNAFYQVLNMPNLNADQQRNGFIQSLKXXPSQSANVLGEAQKLND SQAPKADAXNNFNKD

SEQ ID NO: 51 (SpAkR E domain)

AQHDEAXXNAFYQVLNMPNLNADQQRNGFIQSLKXXPSQSANVLGEAQKLND SQAPKADAKRNNFNKD

SEQ ID NO: 52 (*SpAkR E domain*)

AQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAKRNNFNKD

SEQ ID NO: 53 (*E domain of SpAkR used in example*)

MAQHDEAKKNAFYQVLNMPNLNADQRNGFIQSLKAAPSQSANVLGEAQKLND SQAPKADAKRNNFNKD

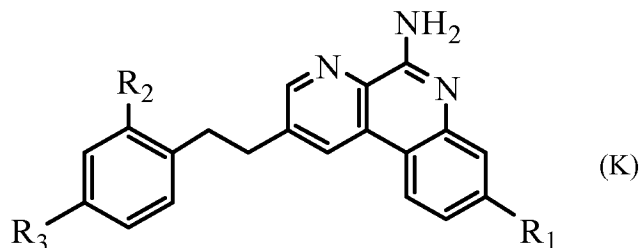
SEQ ID NO: 54 (*SpA E domain*)AQHDEAQQNAFYQVLNMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLND SQAPKADAQQNNFNKD

CLAIMS.

1. A mutant SpA antigen, wherein the antigen comprises an amino acid sequence comprising or consisting of SEQ ID NO: 50 wherein the dipeptide at positions 60 and 61 is not QQ; wherein said dipeptide is optionally KR; wherein said sequence is optionally SEQ ID NO: 51 or SEQ ID NO: 52.
2. A mutant SpA antigen according to claim 1, which antigen comprises an amino acid sequence comprising or consisting of SEQ ID NO: 49 wherein the dipeptide at position 96 and 97 is not QQ; wherein said dipeptide is optionally KR; wherein said sequence is optionally SEQ ID NO: 47.
3. A mutant SpA antigen according to claim 1, which comprises more than one copy of said amino acid sequence comprising or consisting of SEQ ID NO: 50 wherein the dipeptide at positions 60 and 61 is not QQ.
4. A mutant SpA antigen according to any of claims 1 to 3, wherein the antigen elicits antibodies in a mammal that recognise SEQ ID NO: 43 and/or SEQ ID NO: 54.
5. A mutant SpA antigen according to any one of claims 1 to 4 which has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG.
6. A mutant SpA antigen according to claim 5 which has decreased affinity, relative to unmodified SpA, for the Fab portion of V_H3-containing human B cell receptors.
7. A mutant SpA antigen according to any one of claims 1 to 6, wherein the mutant SpA antigen is of sequence comprising or consisting of SEQ ID NO: 43 mutated in at least 1, particularly at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and more particularly 20 amino acids at position 43, 44, 70, 71, 96, 97, 104, 105, 131, 132, 162, 163, 190, 191, 220, 221, 247, 248, 278, 279, 305 and/or 306 of SEQ ID NO: 43; even more particularly is of sequence comprising or consisting of SEQ ID NO: 47 or SEQ ID NO: 49.
8. A mutant SpA antigen according to any one of claims 1 to 7, particularly of sequence comprising SEQ ID NO: 52, SEQ ID NO: 47 or SEQ ID NO: 48.
9. A fusion protein comprising a mutant SpA antigen according to any one of claims 1 to 8.
10. An immunogenic composition comprising a mutant SpA antigen according to any one of claims 1 to 8 or a fusion protein according to claim 9.
11. The composition according to any one of Claims 1 to 10, wherein the composition also comprises an adjuvant.
12. The composition according to Claim 11, wherein said adjuvant is selected from the group consisting of:
 - aluminium salts, particularly aluminium hydroxides and aluminium phosphates;

- human TLR agonists, particularly TLR7 agonists; and
- a mixture thereof.

13. The composition according to Claim 12, wherein said TLR7 agonist is a compound of the following formula (K):



wherein:

R^1 is H, C_1 - C_6 alkyl, $-C(R^5)_2OH$, $-L^1R^5$, $-L^1R^6$, $-L^2R^5$, $-L^2R^6$, $-OL^2R^5$, or $-OL^2R^6$;

L^1 is $-C(O)-$ or $-O-$;

L^2 is C_1 - C_6 alkylene, C_2 - C_6 alkenylene, arylene, heteroarylene or $-((CR^4R^4)_pO)_q(CH_2)_p-$,

5 wherein the C_1 - C_6 alkylene and C_2 - C_6 alkenylene of L^2 are optionally substituted with 1 to 4 fluoro groups;

each L^3 is independently selected from C_1 - C_6 alkylene and $-((CR^4R^4)_pO)_q(CH_2)_p-$, wherein the C_1 - C_6 alkylene of L^3 is optionally substituted with 1 to 4 fluoro groups;

L^4 is arylene or heteroarylene;

10 R^2 is H or C_1 - C_6 alkyl;

R^3 is selected from C_1 - C_4 alkyl, $-L^3R^5$, $-L^1R^5$, $-L^3R^7$, $-L^3L^4L^3R^7$, $-L^3L^4R^5$, $-L^3L^4L^3R^5$, $-OL^3R^5$, $-OL^3R^7$, $-OL^3L^4R^7$, $-OL^3L^4L^3R^7$, $-OR^8$, $-OL^3L^4R^5$, $-OL^3L^4L^3R^5$ and $-C(R^5)_2OH$;

each R^4 is independently selected from H and fluoro;

R^5 is $-P(O)(OR^9)_2$,

15 R^6 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^7 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^8 is H or C_1 - C_4 alkyl;

each R^9 is independently selected from H and C_1 - C_6 alkyl;

R^{10} is H or C_1 - C_4 alkyl;

20 each p is independently selected from 1, 2, 3, 4, 5 and 6, and

q is 1, 2, 3 or 4;

particularly 3-(5-amino-2-(2-methyl-4-(2-(2-(2-phosphonoethoxy)ethoxy)ethoxy)phenethyl)benzo[f][1,7] naphthyridin-8-yl)propanoic acid (K1).

14. The composition according to Claim 12 or 13, wherein the adjuvant comprises a human TLR agonist adsorbed to an aluminium salt.
15. The immunogenic composition according to any one of Claims 1 to 14, for use as a medicament.
16. The immunogenic composition according to any one of Claims 1 to 15, for use as a medicament in the prevention and/or treatment of a *S. aureus* infection, optionally in a human.

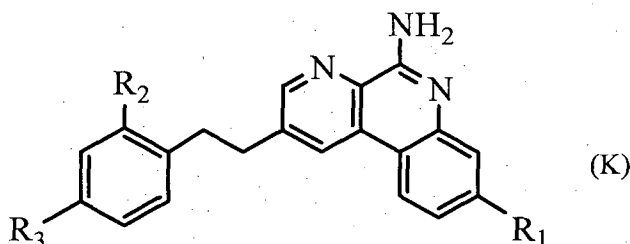
AMENDED CLAIMS
received by the International Bureau on 06 August 2015 (06.08.2015)

CLAIMS.

1. A mutant SpA antigen, wherein the antigen comprises an amino acid sequence comprising or consisting of SEQ ID NO: 50 wherein the dipeptide at positions 60 and 61 is not QQ; wherein said dipeptide is optionally KR; wherein said sequence is optionally SEQ ID NO: 51 or SEQ ID NO: 52.
2. A mutant SpA antigen according to claim 1, which antigen comprises an amino acid sequence comprising or consisting of SEQ ID NO: 49 wherein the dipeptide at positions 60 and 61 is not QQ; wherein said dipeptide is optionally KR; wherein said sequence is optionally SEQ ID NO: 47.
3. A mutant SpA antigen according to claim 1, which comprises more than one copy of said amino acid sequence comprising or consisting of SEQ ID NO: 50 wherein the dipeptide at positions 60 and 61 is not QQ.
4. A mutant SpA antigen according to any of claims 1 to 3, wherein the antigen elicits antibodies in a mammal that recognise SEQ ID NO: 43 and/or SEQ ID NO: 54.
5. A mutant SpA antigen according to any one of claims 1 to 4 which has decreased affinity, relative to unmodified SpA, for the Fc γ portion of human IgG.
6. A mutant SpA antigen according to claim 5 which has decreased affinity, relative to unmodified SpA, for the Fab portion of V_H3-containing human B cell receptors.
7. A mutant SpA antigen according to any one of claims 1 to 6, wherein the mutant SpA antigen is of sequence comprising or consisting of SEQ ID NO: 43 mutated in at least 1, particularly at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and more particularly 20 amino acids at position 43, 44, 70, 71, 96, 97, 104, 105, 131, 132, 162, 163, 190, 191, 220, 221, 247, 248, 278, 279, 305 and/or 306 of SEQ ID NO: 43; even more particularly is of sequence comprising or consisting of SEQ ID NO: 47 or SEQ ID NO: 49.
8. A mutant SpA antigen according to any one of claims 1 to 7, particularly of sequence comprising SEQ ID NO: 52, SEQ ID NO: 47 or SEQ ID NO: 48.
9. A fusion protein comprising a mutant SpA antigen according to any one of claims 1 to 8.
10. An immunogenic composition comprising a mutant SpA antigen according to any one of claims 1 to 8 or a fusion protein according to claim 9.
11. The composition according to claim 10, wherein the composition also comprises an adjuvant.
12. The composition according to Claim 11, wherein said adjuvant is selected from the group consisting of:
 - aluminium salts, particularly aluminium hydroxides and aluminium phosphates;
 - human TLR agonists, particularly TLR7 agonists; and

- a mixture thereof.

13. The composition according to Claim 12, wherein said TLR7 agonist is a compound of the following formula (K):



wherein:

R^1 is H, C_1 - C_6 alkyl, $-C(R^5)_2OH$, $-L^1R^5$, $-L^1R^6$, $-L^2R^5$, $-L^2R^6$, $-OL^2R^5$, or $-OL^2R^6$;

L^1 is $-C(O)-$ or $-O-$;

L^2 is C_1 - C_6 alkylene, C_2 - C_6 alkenylene, arylene, heteroarylene or $-((CR^4R^4)_pO)_q(CH_2)_p-$, wherein the C_1 - C_6 alkylene and C_2 - C_6 alkenylene of L^2 are optionally substituted with 1 to 4 fluoro groups;

each L^3 is independently selected from C_1 - C_6 alkylene and $-((CR^4R^4)_pO)_q(CH_2)_p-$, wherein the C_1 - C_6 alkylene of L^3 is optionally substituted with 1 to 4 fluoro groups;

L^4 is arylene or heteroarylene;

R^2 is H or C_1 - C_6 alkyl;

R^3 is selected from C_1 - C_4 alkyl, $-L^3R^5$, $-L^1R^5$, $-L^3R^7$, $-L^3L^4L^3R^7$, $-L^3L^4R^5$, $-L^3L^4L^3R^5$, $-OL^3R^5$, $-OL^3R^7$, $-OL^3L^4R^7$, $-OL^3L^4L^3R^7$, $-OR^8$, $-OL^3L^4R^5$, $-OL^3L^4L^3R^5$ and $-C(R^5)_2OH$;

each R^4 is independently selected from H and fluoro;

R^5 is $-P(O)(OR^9)_2$,

R^6 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^7 is $-CF_2P(O)(OR^9)_2$ or $-C(O)OR^{10}$;

R^8 is H or C_1 - C_4 alkyl;

each R^9 is independently selected from H and C_1 - C_6 alkyl;

R^{10} is H or C_1 - C_4 alkyl;

each p is independently selected from 1, 2, 3, 4, 5 and 6, and

q is 1, 2, 3 or 4;

particularly 3-(5-amino-2-(2-methyl-4-(2-(2-(2-phosphonoethoxy)ethoxy)phenethyl)benzo[f][1,7]naphthyridin-8-yl)propanoic acid (K1).

14. The composition according to Claim 12 or 13, wherein the adjuvant comprises a human TLR agonist adsorbed to an aluminium salt.
15. The immunogenic composition according to any one of Claims 1 to 14, for use as a medicament.
16. The immunogenic composition according to any one of Claims 1 to 15, for use as a medicament in the prevention and/or treatment of a *S. aureus* infection, optionally in a human.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2015/056175

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/056175

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/085 A61K39/39 C07K14/31 A61K39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM H K ET AL: "Nontoxigenic protein A vaccine for methicillin-resistant Staphylococcus aureus infections in mice", THE JOURNAL OF EXPERIMENTAL MEDICINE, ROCKEFELLER UNIVERSITY PRESS, US, vol. 207, no. 9, 30 August 2010 (2010-08-30), pages 1863-1870, XP002737099, ISSN: 0022-1007, DOI: 10.1084/JEM.20092514 [retrieved on 2010-08-16]	1-12,15, 16
Y	abstract figure 1 page 1864, right-hand column, paragraph 2 page 1866, right-hand column, paragraph 2 - page 1869, column f, paragraph 1 ----- -/--	13,14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 May 2015

Date of mailing of the international search report

08/06/2015

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/056175

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/127032 A1 (UNIV CHICAGO [US]; SCHNEEWIND OLAF [US]; CHENG ALICE [US]; MISSIAKAS D) 13 October 2011 (2011-10-13) paragraphs [0401] - [0405] claim 37 example 1 claim 39 paragraph [0376] paragraph [0515] paragraphs [0529] - [0539] -----	1-12,15, 16
X	WO 2011/005341 A2 (UNIV CHICAGO [US]; SCHNEEWIND OLAF [US]; CHENG ALICE [US]; MISSIAKAS D) 13 January 2011 (2011-01-13)	1-12,15, 16
Y	paragraph [0015] figure 1 paragraph [0104] paragraphs [0330] - [0332]; example 2 claims 12,20,22,23,42-44 paragraphs [0259] - [0265] -----	13,14
X	WO 2012/034067 A1 (UNIV CHICAGO [US]; SCHNEEWIND OLAF [US]; CHENG ALICE G [US]; MISSIAKAS) 15 March 2012 (2012-03-15) pages 106-110; example 3 page 107, paragraph 2 page 75, paragraph 1 - page 76, paragraph 4 -----	1-12,15, 16
X	DATABASE WPI Week 201437 Thomson Scientific, London, GB; AN 2014-J07484 XP002739612, -& CN 103 645 318 A (CHONGQING YUANLUN BIOLOGICAL TECHNOLOGY) 19 March 2014 (2014-03-19)	1-12,15, 16
Y	abstract; sequence 1 -----	13,14
X,P	DATABASE WPI Week 201440 Thomson Scientific, London, GB; AN 2014-K04482 XP002739613, -& CN 103 694 322 A (CHONGQING YUANLUN BIOLOGICAL TECHNOLOGY) 2 April 2014 (2014-04-02)	1-12,15, 16
Y,P	abstract; sequences 1-4 paragraphs [0268], [0270], [0299] -----	13,14
Y	WO 2013/030378 A1 (NOVARTIS AG [CH]; BAGNOLI FABIO [IT]; BAUDNER BARBARA [IT]; BUFALI SIM) 7 March 2013 (2013-03-07) page 37, lines 10-13 claim 24 -----	13,14
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/056175

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010/119343 A2 (NOVARTIS AG [CH]; BAGNOLI FABIO [IT]; BIAGINI MASSIMILLIANO [IT]; FIAS) 21 October 2010 (2010-10-21) page 127, lines 16-25 page 5, lines 1-5 page 98, line 1 - page 109, line 24 page 121, line 10 - page 122, line 32 page 123, lines 24-26 -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/056175

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011127032 A1	13-10-2011	EP 2555794 A1 JP 2013523818 A US 2013136746 A1 US 2014335095 A1 WO 2011127032 A1	13-02-2013 17-06-2013 30-05-2013 13-11-2014 13-10-2011
WO 2011005341 A2	13-01-2011	AU 2010271116 A1 CA 2757543 A1 CN 102612523 A EP 2414387 A2 JP 2012522808 A KR 20110138397 A SG 174968 A1 US 2012114686 A1 WO 2011005341 A2	27-10-2011 13-01-2011 25-07-2012 08-02-2012 27-09-2012 27-12-2011 28-11-2011 10-05-2012 13-01-2011
WO 2012034067 A1	15-03-2012	CN 103314008 A EP 2614074 A1 JP 2013542919 A US 2013230550 A1 WO 2012034067 A1	18-09-2013 17-07-2013 28-11-2013 05-09-2013 15-03-2012
CN 103645318 A	19-03-2014	NONE	
CN 103694322 A	02-04-2014	NONE	
WO 2013030378 A1	07-03-2013	AU 2012300765 A1 CA 2847204 A1 CN 104093418 A EP 2763695 A1 JP 2014525429 A KR 20140066212 A US 2014363461 A1 WO 2013030378 A1	13-03-2014 07-03-2013 08-10-2014 13-08-2014 29-09-2014 30-05-2014 11-12-2014 07-03-2013
WO 2010119343 A2	21-10-2010	AU 2010238255 A1 CA 2758490 A1 CN 102647999 A CO 6440592 A2 EP 2419129 A2 EP 2510947 A1 JP 2012523246 A KR 20120034612 A NZ 595689 A NZ 612315 A RU 2011146016 A SG 175092 A1 US 2012093850 A1 US 2012107340 A1 US 2014178425 A1 WO 2010119343 A2	03-11-2011 21-10-2010 22-08-2012 15-05-2012 22-02-2012 17-10-2012 04-10-2012 12-04-2012 28-03-2014 31-10-2014 20-05-2013 28-11-2011 19-04-2012 03-05-2012 26-06-2014 21-10-2010