

TITLE

IMMUNOGENIC PEPTIDE AGAINST GROUP A STREPTOCOCCUS

TECHNICAL FIELD

THIS INVENTION relates to an immunogenic peptide for prevention and
5 treatment of group A streptococcus streptococcus-associated diseases and
conditions.

BACKGROUND

Synthetic and recombinant peptides are being developed as vaccines to
prevent and protect against numerous infectious as well as non-infectious diseases.
10 Their utility resides in their ability to mimic native epitopes on foreign or host
proteins and to specifically activate or modulate protective or harmful immune
responses. Significant progress has been made for many non-infectious diseases
including cancer, allergies, multiple sclerosis and chronic diseases such as
hypertension, diabetes and Alzheimer's disease (1). However, their greatest
15 promise lies in vaccines to prevent infectious diseases. Here, their small size
greatly facilitates the design of multi-component vaccines incorporating epitopes
from multiple target antigens (2), vaccines that can prevent infections with
organisms that exist in multiple allelic forms (3), and vaccines that can target
hidden or cryptic epitopes resulting in the production of immune responses that
20 are not induced naturally by the organism but which are lethal to the organism
once induced by the vaccine (4).

Epitopes recognized by B and T cells are small and can, due of their size,
be easily and inexpensively synthesized. However, because they are small the
repertoire of B or T cells that they stimulate is very limited and it is primarily for
25 this reason that no peptide-based vaccines have yet been developed and licensed
for human use.

Vaccines against *Streptococcus pyogenes* (the Lancefield group A
streptococcus; GAS) have long been sought due to the debilitating diseases caused
by the bacterium, particularly rheumatic fever and rheumatic heart disease.
30 Rheumatic fever is an uncommon disease today in most developed countries but it
remains the major cause of acquired heart disease in children, adolescents and
young adults in the developing world. In addition, invasive GAS disease is a
frequent cause of sepsis in children and adults and has a high-case fatality rate.
Further adding to the burden of GAS disease is post-streptococcal

glomerulonephritis, which likely contributes to the high rates of end-stage renal failure in many GAS endemic regions. GAS pharyngitis and impetigo are responsible for the greatest absolute number of GAS infections each year. GAS pharyngitis affects approximately 8%–15% of school-aged children per year and
5 GAS impetigo is a very common infection in children prevalence of 10-50%. Not only are severe GAS-associated diseases a problem in developing countries, but even in developed countries particularly virulent GAS strains have emerged that are resistant to standard antibiotic therapies and cause debilitating diseases such as severe necrotizing fasciitis.

10 Peptide-based vaccines against group A streptococcus have focussed on M protein-derived peptides such as p145, J8 and J14 peptides. Currently, J8 peptide which comprises a twelve amino acid minimal epitope within p145 in embedded in a yeast-derived helical peptide (GCN4) amino acid sequence is a leading vaccine candidate (12). The GCN4 amino acid sequence assists maintenance of
15 peptide helicity so as to optimize display of the minimal epitope to the immune system. This vaccine candidate also requires three doses to induce protection against skin disease (19).

SUMMARY

20 Surprisingly, the present inventors have discovered that the immunogenicity of a p145 peptide can be substantially increased by targeted modification of the amino acid sequence of p145. Immunization with a single dose of the modified p145 peptide, rather than multiple immunizations, can protect against group A streptococcus infection even by hypervirulent strains such
25 as the CovR/S mutant strain. Unexpectedly, the modified p145 peptide, fragment or variant elicits a mucosal immune response upon administration to the mammal. The mucosal immune response is characterized by the production of IgG, while IgA production is substantially absent or at a relatively reduced level compared to the level of IgG produced.

30 The invention is broadly directed to a p145 peptide comprising one or more amino acid sequence modifications that substantially improve or enhance immunogenicity, particularly for eliciting mucosal immune responses. In a preferred form, the peptide comprises the amino acid sequence set forth in SEQ ID NO:1.

An aspect of the invention provides a method of eliciting an immune response to group A streptococcal bacteria in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby elicit the immune response.

Another aspect of the invention provides a method of inducing immunity against a group A streptococcal bacterial infection in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby induce immunity against the group A streptococcal bacterial infection in the mammal.

A further aspect of the invention provides a method of treating or preventing a group A streptococcal bacterial infection in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby prevent or treat the group A streptococcal bacterial infection in the mammal.

The modified p145 peptide, fragment or variant suitably comprises an asparagine (N) amino acid at residue 13 and an arginine (R) amino acid at residue 17.

In an embodiment, the modified p145 peptide, fragment or variant of comprises the amino acid sequence set forth in SEQ ID NO:1

In an embodiment, the modified p145 peptide fragment comprises the amino acid sequence set forth in SEQ ID NO:2.

Preferably, the modified p145 peptide, fragment or variant elicits a mucosal immune response upon administration to the mammal.

Suitably, the mucosal immune response is characterized by the production of IgG. Typically, IgA production is substantially absent or at a relatively reduced level compared to the level of IgG produced.

Preferably, the modified p145 peptide, fragment or variant elicits a mucosal immune response upon intramuscular administration to the mammal.

Preferably, the mammal of the aforementioned aspects is a human.

In a particular embodiment of the aforementioned aspects, the modified p145 peptide, fragment or variant may be administered in combination with an immunogenic fragment of a SpyCEP protein. Preferably, the fragment of SpyCEP is, or comprises an S2 peptide (SEQ ID NO:23) or a variant such as a K4S2 peptide (SEQ ID NO:24).

A related aspect of the invention therefore provides a composition comprising: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3, a fragment or variant thereof, an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; and an immunogenic fragment of a SpyCEP protein or variant thereof, an antibody or antibody fragment that binds or is raised against the SpyCEP immunogenic fragment or variant thereof or an isolated nucleic acid that encodes the SpyCEP immunogenic fragment or variant thereof.

Suitably, the modified p145 peptide amino acid sequence comprises an asparagine (N) amino acid at residue 13 and an arginine (R) amino acid at residue 17, preferably comprising the amino acid sequence set forth in SEQ ID NO:1. Preferably, the fragment of the modified p145 peptide comprises the amino acid sequence set forth in SEQ ID NO:2.

In a preferred embodiment, the fragment of SpyCEP is, or comprises an S2 peptide comprising the amino acid sequence set forth in SEQ ID NO:23, or a variant thereof. The variant may be a K4S2 peptide comprising the amino acid sequence set forth in SEQ ID NO:24.

As used herein, the indefinite articles ‘a’ and ‘an’ are used here to refer to or encompass singular or plural elements or features and should not be taken as meaning or defining “one” or a “single” element or feature.

Unless the context requires otherwise, the terms “comprise”, “comprises”
 5 and “comprising”, or similar terms are intended to mean a non-exclusive inclusion, such that a recited list of elements or features does not include those stated or listed elements solely, but may include other elements or features that are not listed or stated.

By “consisting essentially of” in the context of an amino acid sequence is
 10 meant the recited amino acid sequence together with an additional one, two or three amino acids at the N- or C-terminus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Designing of peptide libraries: (A) *Recognition of various p145 variant peptides by p145 antisera:* (A) A peptide library consisting of 86 variants
 15 of p145 was screened with p145 antisera. The p145 antiserum was raised in B10.D2 mice and the ability of p145 antiserum to bind each peptide was determined using ELISA. The minimal epitope sequence from within the p145 is at the top of the graph with numbers of peptide sequences undergoing mutation in
 20 each amino acid are represented in the parentheses below. The vertical lines separate the peptides based on mutation in the same position. The Peptide 87 is a non-specific control peptide (pNS). **(B) *Identification of immunodominant peptides within the p145 variants library:*** Peptide-inhibition ELISAs were performed to identify the immunodominant peptides within the pool of p145
 25 variants. Selected peptides at a concentration of 0.25 µg/ml were incubated with p145 antisera. The serum was then used in an ELISA against immobilised p145. Antigen (p145)-specific serum was collected from hyper-immunised B10.D2 mice. Data for each bar are Means ± SEM. **(C) *Designing of peptide library 2 (L2) with two alterations:*** The selected 7 peptides were utilised to design a new
 30 peptide library with two amino acids substitutions at positions that were identified to enhance immunogenicity of parent p145. The resulting peptides demonstrating mutations at various positions within the p145 minimal epitope are shown.

Figure 2: Immune responses to doubly mutated peptides: (A) *Immunogenicity of selected peptides in outbred Quackenbush mice showing*

ELISA titers to the immunising peptide: Cohorts of mice were subcutaneously immunized with peptide antigen formulated with adjuvant on days 0, 21 and 28. Mice in cohorts with antigen specific titers below 1000 were given an additional boost on day 35 (indicated as "). The mice were bled on days 20, 27, 34 and 41.

- 5 Control mice received the original p145 or PBS. Antigen-specific serum IgG titers were determined using ELISA and Mean $\pm$ SEM for peptides 1-5 (i), 6-12 (ii) and 13-18 (iii) are shown. **(B): Binding of anti-peptide IgG to immobilised p145:** The ability of affinity-purified peptide-specific serum IgG to bind increasing concentrations of p145 peptide was determined by ELISA. The peptide p145, 10 immobilised on ELISA plates, was incubated with different peptide specific purified serum IgG from outbred Quackenbush mice. The antibody binding at a concentration of 5 μ g/ml are shown for (i) peptides 1-5 and (ii) peptides 6-10 (iii) peptides 11-13 and (iv) peptides 15-18. Mean absorbance (450nm) $\pm$ SEM are shown. ANOVA with the Holm-Sidak's multiple comparison tests was used to 15 calculate significance in comparison to p145.

- Figure 3: Binding efficiency of peptide specific antibodies to the surface of GAS:** To determine the ability of the peptide-specific serum IgG to bind to whole bacteria, flow cytometry analysis was performed using two GAS strains (A) 2031 (*emm1*) and (B) 88/30 (*emm97*). Following incubation with anti-peptide 20 antibodies, FITC conjugated anti-mouse IgG was used to determine the binding of antibodies to GAS. The results are shown as Mean $\pm$ SEM of mean fluorescence intensity (MFI) for 3 replicates in each group. The horizontal line in the figure denotes relative binding of various peptide-specific IgG to p145 in comparison to p145 IgG. ANOVA with the Tukey's post hoc method was used to determine the 25 significance in comparison to p145. (C) Representative histograms demonstrating binding of p*17 -specific IgG to GAS 2031, 88/30 and JRS145 (an M-negative GAS strain) are shown.

- Figure 4: Immunogenicity and protective efficacy of p145-DT and its variant p*17-DT in protection against superficial skin infection with various 30 GAS strains:** Cohorts of BALB/c mice (n=10 mice each) were immunized subcutaneously with 30 μ g of p145-DT or p*17-DT or PBS–Alum formulations on day 0. Serum samples were collected at 1 day 20 post-immunisation to assess p145 specific IgG response. Data from some representative mice (n=5) are shown (A). On day 21, the mice were challenged via the skin route of infection with

GAS 2031 (B and C) or GAS 88/30 (D and E). On day 3 and 6 post-infection, 5 mice/group were sacrificed and samples collected to determine GAS bacterial burden in the skin (B and D) and the blood (C and E). The results are shown as mean $\pm$ SEM for 5 mice in each group at each time point. ANOVA with the Tukey's post hoc method was used to determine the significance between all the treated and control cohorts at each time point. * $p < 0.05$, *** $p < 0.001$.

Figure 5: Structures of p145 and p*17 peptides: Overlay of representative structures of p145 (magenta) and p*17 (green) obtained from 50 ns MD simulations with alignment based on CA of residues 3 to 13 (C-terminal amino acids shown in red, N-terminal amino acids in blue (A)). Lys-13 breaks the helical character in p145. The probability of the peptides to adopt a helical conformation are shown in (B) revealing that p145 is significantly less likely to form a helical conformation compared to p*17. Root mean square fluctuations (RMSF) of p145, and p*17 in comparison are shown in (C). p145 shows significantly higher fluctuations compared to p*17. The largest difference in flexibility can be seen for Lys-13 in p145 compared to Asn-13 in p*17.

Figure 6: Identification of immunodominant peptides within the p145 variants library: Peptide-inhibition ELISAs were performed to identify the immunodominant peptides within the pool of p145 variants. Selected peptides at a concentration of 2.5 $\mu\text{g/ml}$ were incubated with p145 antisera. The serum was then used in an ELISA against immobilised p145. Antigen (p145)-specific serum was collected from hyper-immunised B10.D2 mice. Data for each bar are means $\pm$ SEM.

Figure 7: Cross-reactivity of peptide antisera with human proteins: ELISA was used to determine cross-reactivity of the doubly mutated peptide (L2) anti-sera to human derived proteins (a) tropomyosin, (b) myosin and (c) keratin. Mean absorbance (450nm) $\pm$ SEM shown.

Figure 8: Protective efficacy of p145-DT in BALB/c mice following 1, 2 or 3 immunisations: Cohorts of BALB/c mice (10 mice/group) received 1, 2 or 3 subcutaneous injections of p145-DT or PBS with adjuvant on days 0, 21 and 28. Efficacy of the vaccine following skin challenge with 88/30 GAS was assessed. Bacterial burden in skin (A) and blood (B) for days 3 and 6 post-infection respectively for cohorts of mice is shown. *Protective efficacy of p*17-DT following single immunisation:* (C) Cohorts of BALB/c mice (10 mice/group)

received a single immunisation with p*17-DT, heat killed GAS (2031) or PBS with adjuvant on days 0. The antigen-specific IgG titers were determined on day-20 post-immunisation. The titers for mice immunised with heat-killed GAS 1 were tested against recombinant M1 protein immobilised on ELISA plate. Mann-Whitney test was used to determine significance between the two vaccinated groups where $**p < 0.01$. (D) On day 21 post immunisation, mice were challenged with CovR/S mutant 5448AP GAS via the skin route of infection. The skin samples were assessed for bacterial burden on day-3 and -6 post infection. Percent reduction in bacterial counts in p*17-DT or heat killed GAS immunised cohort in comparison to PBS control cohort are shown. ANOVA with the Tukey's post hoc method was used to determine the significance between all the vaccinated and control cohorts at each time point. $**p < 0.01$, $***p < 0.001$.

Figure 9: Intramuscular immunization schedule. Mice were immunized three (3) times with p*17-DT+K4S2-DT/Alum and intranasal challenge with 5448AP (CovR/S MT strain).

Figure 10: Immunogenicity following i.m. immunisation with p*17-based vaccines. BALB/c mice (n=15/group; female, 4-6 weeks old) were immunised i.m. with p*17-DT/Alum, p*17-DT+K4S2-DT/Alum and PBS/Alum on days 0, 21 and 42. One week post last boost serum and saliva samples were collected and p*17-specific IgG (A & C) and IgA (B & D) antibody responses were measured by ELISA and are represented as mean $\pm$ SEM. The titers are in comparison to the control cohort that received only PBS/Alum.

Figure 11: Protection following intranasal infection. Two weeks after last vaccine boost, the mice were infected intranasally with covR/S mutant GAS (5448AP). On days 1-3 nasal shedding (NS) were collected. Bacterial burden in NS were determined by pressing the nares of each mouse onto columbia blood agar (CBA) plates containing 5% defibrinated horse-blood and exhaled particles were streaked out. On days 1-3 NS were collected and represented as the log transformed mean $\pm$ SEM from each individual day (A) and as cumulative CFUs from all three days combined (B). o indicates mice culled on day 2 after 15% or more weight loss.

Figure 12: Percent reduction in bacterial burden the nasal shedding. Percent reduction was calculated in comparison to total mean of the PBS/Alum control from each individual day (A) and as cumulative reduction from all three

days combined (B). o indicates mice culled on day 2 after 15% or more weight loss.

Figure 13: Protection following intranasal infection. In addition to NS on days 1-3 throat swabs (TS) were collected. For throat swabs, swab applicators were placed in sterile PBS to dampen, mice were then immobilized and throats were swabbed. Swab applicator was then suspended in PBS, serially diluted and dot-plated in duplicate on CBA plates containing 5% defibrinated horse-blood. . On days 1-3 NS were collected and represented as the log transformed mean cfu/ml $\pm$ SEM from each individual day (A) and as cumulative CFUs from all three days combined (B). o indicates mice culled on day 2 after 15% or more weight loss.

Figure 14: Percent reduction in bacterial burden in throat swabs. Percent reduction was calculated in comparison to total mean of the PBS/Alum control from each individual day (A) and as cumulative reduction from all three days combined (B). o indicates mice culled on day 2 after 15% or more weight loss.

Figure 15: Protection following intranasal infection. On day 3 post-infection all surviving mice were euthanised and NALT, lungs, spleen and blood samples were collected to determine GAS bacterial burden, data represented as the log transformed mean cfu/ml $\pm$ SEM (A) and as percent reduction in comparison to PBS/Alum control (B). o indicates mice culled on day 2 after 15% or more weight loss.

Figure 16: Structural comparisons of J8 peptide, p145 and p*17. MD simulations comparing the structure of p*17 and J8 peptide in terms of (A) helicity percentage and (B) RMS fluctuation.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1	LRRDLASREAKNQVERALE
SEQ ID NO:2	SREAKNQVERAL
SEQ ID NO:3	LRRDLASREAKKQVEKALE
SEQ ID NO:4	SREAKKQVEKAL
SEQ ID NO:5	LRRDLDAENEAKKQVEKALE
SEQ ID NO:6	LRRDLDAEDEAKKQVEKALE
SEQ ID NO:7	LRRDLDAEREAKNQVEKALE
SEQ ID NO:8	LRRDLDAEREAKKQVERALE

	SEQ ID NO:9	LRRDLDAEREAKKQVEMALE
	SEQ ID NO:10	LRRDLDAVNEAKKQVEKALE
	SEQ ID NO:11	LRRDLDAVDEAKKQVEKALE
	SEQ ID NO:12	LRRDLDAVREAKKQVEKALE
5	SEQ ID NO:13	LRRDLDAVREAKKQVERALE
	SEQ ID NO:14	LRRDLDAVREAKKQVEMALE
	SEQ ID NO:15	LRRDLASNEAKKQVEKALE
	SEQ ID NO:16	LRRDLASNEAKKQVERALE
	SEQ ID NO:17	LRRDLASNEAKKQVEMALE
10	SEQ ID NO:18	LRRDLASDEAKKQVEKALE
	SEQ ID NO:19	LRRDLASDEAKKQVERALE
	SEQ ID NO:20	LRRDLASDEAKKQVEMALE
	SEQ ID NO:21	LRRDLASREAKKQVEMALE
	SEQ ID NO:22	LRRDLDA
15	SEQ ID NO:23	NSDNIKENQFEDFDEDWENF
	SEQ ID NO:24	KKKKNSDNIKENQFEDFDEDWENF
	SEQ ID NOS: 25-111 Table 3 peptide library in descending numerical order from peptide 1 to peptide 87, respectively.	
	SEQ ID NO:112:	SGSGLRRDLASREAKKQVEKALE
20		

DETAILED DESCRIPTION

The present invention is at least partly predicated on the discovery that particular amino acids of the p145 peptide may be modified to substantially improve immunogenicity against group A streptococci. A single immunization with the modified p145 peptide, rather than multiple immunizations, can protect against group A streptococcus infection even by hypervirulent strains such as the CovR/S mutant strain. More specifically, the modified p145 peptide can induce mucosal immunity, characterized by production of IgG rather than IgA. Mucosal immunity may be elicited after intramuscular administration of the peptide.

A broad aspect of the invention is directed to use of an isolated peptide comprising the amino acid sequence of SEQ ID NO:1, referred to herein as a "p*17 peptide" for inducing or eliciting immunity to group A streptococcal bacterial infection. Preferably, the isolated peptide induces mucosal immunity to group A streptococcal bacterial infection.

In some embodiments, the p*17 peptide may be administered in combination with a fragment of a SpyCEP protein. The SpyCEP fragment may be an "S2" peptide, such as comprising the amino acid sequence of SEQ ID NO:23, or a variant comprising an amino acid sequence such as set forth in SEQ ID
5 NO:24, as will be described in more detail hereinafter.

As used herein the terms "*group A streptococcus*", "*Group A Streptococci*", "*Group A Streptococcal*", "*Group A Strep*" and the abbreviation "*GAS*" refer to streptococcal bacteria of Lancefield serogroup A which are gram positive β -hemolytic bacteria of the species *Streptococcus pyogenes*. An
10 important virulence factor of GAS is M protein, which is strongly anti-phagocytic and binds to serum factor H, destroying C3-convertase and preventing opsonization by C3b. These also include virulent "mutants" such as CovR/S or CovRS mutants such as described in Graham *et al.*, 2002, PNAS USA **99** 13855, although without limitation thereto.

15 Diseases and conditions caused by group A streptococci include cellulitis, erysipelas, impetigo, scarlet fever, throat infections such as acute pharyngitis ("strep throat"), bacteremia, toxic shock syndrome, necrotizing fasciitis, acute rheumatic fever and acute glomerulonephritis, although without limitation thereto.

For the purposes of this invention, by "*isolated*" is meant material that has
20 been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native,
25 chemical synthetic or recombinant form.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L-amino acids as are well understood in the art.

The term "*protein*" includes and encompasses "*peptide*", which is
30 typically used to describe a protein having no more than fifty (50) amino acids and "*polypeptide*", which is typically used to describe a protein having more than fifty (50) amino acids.

The inventors have shown that modification of a “wild-type” or “unmodified” p145 peptide amino acid sequence substantially improves or enhances the immunogenicity of the peptide. Typically, modification includes substitution of one or more amino acids of a p145 peptide.

5 This obviates the need to add a heterologous GCN4 amino acid sequence to the p145 peptide to maintain a desired alpha-helical structure of the peptide. Although not wishing to be bound by theory, molecular dynamic simulation shows that a modified p145 peptide disclosed herein does not have a “kink” in its structure, as opposed to wild-type or unmodified p145, which may be at least
10 partly responsible for its improved immunogenicity.

Suitably, a “wild-type” or unmodified p145 peptide comprises the amino acid sequence LRRDLASREAKKQVEKALE (SEQ ID NO:3). A minimal P145 epitope sequence is SREAKKQVEKAL (SEQ ID NO:4).

Preferably, a modified p145 peptide comprises an N residue corresponding
15 to residue 13 of SEQ ID NO:3 and an R amino acid at residue 17 of SEQ ID NO:3.

Preferably, a modified p145 minimal epitope comprises an N residue corresponding to residue 6 of SEQ ID NO:1 and an R amino acid at residue 10 of SEQ ID NO:1.

In one embodiment, a p*17 peptide comprises the amino acid sequence
20 LRRDLASREAKNQVERALE (SEQ ID NO:1).

In one embodiment, a modified minimal epitope of p*17 comprises the amino acid sequence SREAKNQVERAL (SEQ ID NO:2).

The invention also provides a fragment of a modified p145 peptide disclosed herein.

25 A “*fragment*” is a segment, domain, portion or region of a protein or peptide (such as SEQ ID NO:1, SEQ ID NO:2, SpyCEP, SEQ ID NO: 23 or SEQ ID NO:24) which constitutes less than 100% of the amino acid sequence of the protein. It will be appreciated that the fragment may be a single fragment or may be repeated alone or with other fragments.

30 In general, fragments may comprise, consist essentially of or consist of up to 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 contiguous amino acids (such as of SEQ ID NO:1, SEQ ID NO:2, SpyCEP, SEQ ID NO:23 or SEQ ID NO: 24). In one embodiment, the fragment comprises an N residue corresponding to residue 13 of SEQ ID NO:1 and an R amino acid at residue 17 of SEQ ID NO:1.

A non-limiting example of a fragment comprises the amino acid sequence SREAKNQVERAL (SEQ ID NO:2).

Suitably, the fragment is an immunogenic fragment. In the context of the present invention, the term “*immunogenic*” as used herein indicates the ability or potential to generate or elicit an immune response, such as to Group A strep or molecular components thereof, such as M protein or an M protein epitope corresponding to p145, upon administration of the immunogenic fragment to a mammal. Preferably, the immune response elicited by the immunogenic fragment is a mucosal immune response.

By “*elicit an immune response*” is meant generate or stimulate the production or activity of one or more elements of the immune system inclusive of the cellular immune system, antibodies and/or the native immune system. Suitably, the one or more elements of the immune system include B lymphocytes, antibodies and neutrophils. Preferably, the immune response is a mucosal immune response. Typically, mucosal immune responses are characterized by the production of IgA, with minimal production of other antibody isotypes such as IgG. Surprisingly, the mucosal immune response elicited by the p*17 peptide disclosed herein is characterized by the production of IgG. IgA production is relatively reduced compared to IgG production, or is substantially absent.

As used herein, a protein “*variant*” shares a definable nucleotide or amino acid sequence relationship with a reference amino acid sequence. The reference amino acid sequence may be the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:23, for example. The “*variant*” protein may have one or a plurality of amino acids of the reference amino acid sequence deleted or substituted by different amino acids. It is well understood in the art that some amino acids may be substituted or deleted without changing the activity of the immunogenic fragment and/or protein (conservative substitutions). Preferably, protein variants share at least 70% or 75%, preferably at least 80% or 85% or more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a reference amino acid sequence.

In particular embodiments, the variant may comprise: an N residue corresponding to residue 13 of SEQ ID NO:1 and an R amino acid corresponding to residue 17 of SEQ ID NO:1; or an N residue corresponding to residue 6 of SEQ ID NO:2 and an R amino acid corresponding to residue 10 of SEQ ID NO:2.

Accordingly, one or more of the other residues of SEQ ID NO:1 or SEQ ID NO: 2 may be conservatively modified (*e.g* by amino acid substitution or deletion) so that the variant substantially retains the immunogenicity of SEQ ID NO:1 or SEQ ID NO:2. In this regard, reference is made to Table 2 and the Examples which describe modifications of amino acids other than residues 13 and 17 of SEQ ID NO:1 and their effects upon immunogenicity.

In one particular embodiment, a variant protein or peptide may comprise one or a plurality of lysine residues at an N and/or C-terminus thereof. The plurality of lysine residues (*e.g* polylysine) may be a linear sequence of lysine residues or may be branched chain sequences of lysine residues. These additional lysine residues may facilitate increased peptide solubility. A non-limiting example of such a variant is designated "K4S2" (SEQ ID NO:24).

Terms used generally herein to describe sequence relationships between respective proteins and nucleic acids include "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". Because respective nucleic acids/proteins may each comprise (1) only one or more portions of a complete nucleic acid/protein sequence that are shared by the nucleic acids/proteins, and (2) one or more portions which are divergent between the nucleic acids/proteins, sequence comparisons are typically performed by comparing sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "*comparison window*" refers to a conceptual segment of typically 6, 9 or 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence for optimal alignment of the respective sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (Geneworks program by Intelligenetics; GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA, incorporated herein by reference) or by inspection and the best alignment (*i.e.* resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, Nucl. Acids Res. **25** 3389, which is incorporated herein by reference. A detailed

discussion of sequence analysis can be found in Unit 19.3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons Inc NY, 1995-1999).

The term “*sequence identity*” is used herein in its broadest sense to include
5 the number of exact nucleotide or amino acid matches having regard to an appropriate alignment using a standard algorithm, having regard to the extent that sequences are identical over a window of comparison. Thus, a “*percentage of sequence identity*” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the
10 identical nucleic acid base (*e.g.*, A, T, C, G, I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For example, “*sequence identity*” may be understood to mean the “match percentage”
15 calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA).

The invention also provides a derivative of a modified p145 peptide disclosed herein. Suitably, the modified p145 peptide comprises an amino acid
20 sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

As used herein, “*derivatives*” are molecules such as proteins, fragments or variants thereof that have been altered, for example by conjugation or complexing with other chemical moieties, by post-translational modification (*e.g.* phosphorylation, acetylation and the like), modification of glycosylation (*e.g.* adding, removing or altering glycosylation), lipidation and/or inclusion of
25 additional amino acid sequences as would be understood in the art. In one particular embodiment, an additional amino acid sequence may comprise one or a plurality of lysine residues at an N and/or C-terminus thereof. The plurality of lysine residues (*e.g.* polylysine) may be a linear sequence of lysine residues or may
30 be branched chain sequences of lysine residues. These additional lysine residues may facilitate increased peptide solubility. Another particular derivative is by conjugation of the peptide to diphtheria toxin (DT). This may be facilitated by addition of a C-terminal cysteine residue.

Additional amino acid sequences may include fusion partner amino acid sequences which create a fusion protein. By way of example, fusion partner amino acid sequences may assist in detection and/or purification of the isolated fusion protein. Non-limiting examples include metal-binding (*e.g.* polyhistidine) fusion
5 partners, maltose binding protein (MBP), Protein A, glutathione S-transferase (GST), fluorescent protein sequences (*e.g.* GFP), epitope tags such as myc, FLAG and haemagglutinin tags.

Other additional amino acid sequences may be of carrier proteins such as diphtheria toxoid (DT) or a fragment thereof, or a CRM protein fragment such as
10 described in International Publication WO2017/070735.

Other derivatives contemplated by the invention include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the
15 immunogenic proteins, fragments and variants of the invention.

In this regard, the skilled person is referred to Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE, Eds. Coligan *et al.* (John Wiley & Sons NY 1995-2008) for more extensive methodology relating to chemical modification of proteins.

20 The isolated immunogenic proteins, fragments and/or derivatives of the present invention may be produced by any means known in the art, including but not limited to, chemical synthesis, recombinant DNA technology and proteolytic cleavage to produce peptide fragments.

Chemical synthesis is inclusive of solid phase and solution phase synthesis.
25 Such methods are well known in the art, although reference is made to examples of chemical synthesis techniques as provided in Chapter 9 of SYNTHETIC VACCINES Ed. Nicholson (Blackwell Scientific Publications) and Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2008). In this regard, reference is also made to
30 International Publication WO 99/02550 and International Publication WO 97/45444.

Recombinant proteins may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press,

1989), in particular Sections 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2008), in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2008), in particular Chapters 1, 5 and 6. Typically, recombinant protein preparation includes expression of a nucleic acid encoding the protein in a suitable host cell.

A further aspect of the invention provides an antibody or antibody fragment that binds, or is raised against, an isolated immunogenic peptide comprising the amino acid sequence set forth in SEQ ID NO:1, or a fragment, variant or derivative thereof.

Suitably, the antibody or antibody fragment specifically binds an isolated immunogenic peptide comprising the amino acid sequence set forth in SEQ ID NO:1 or a fragment comprising the amino acid sequence of SEQ ID NO:2. In some embodiments, the antibody or antibody fragment binds a p145 peptide or minimal epitope sequence such as that set forth in SEQ ID NO:3 or SEQ ID NO:4, with a substantially higher affinity than an antibody raised against the p145 peptide. In this context, by “substantially higher affinity” is meant an affinity at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 fold higher at a particular concentration of p145 peptide.

Antibodies and antibody fragments may be polyclonal or monoclonal, native or recombinant. Antibody fragments include Fc, Fab or F(ab)₂ fragments and/or may comprise single chain Fv antibodies (scFvs). Such scFvs may be prepared, for example, in accordance with the methods described respectively in United States Patent No 5,091,513, European Patent No 239,400 or the article by Winter & Milstein, 1991, Nature 349:293. Antibodies may also include multivalent recombinant antibody fragments, such as diabodies, triabodies and/or tetrabodies, comprising a plurality of scFvs, as well as dimerisation-activated demibodies (*e.g.* WO/2007/062466). By way of example, such antibodies may be prepared in accordance with the methods described in Holliger *et al.*, 1993 Proc Natl Acad Sci USA **90** 6444; or in Kipriyanov, 2009 Methods Mol Biol **562** 177. Well-known protocols applicable to antibody production, purification and use may be found, for example, in Chapter 2 of Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY (John Wiley & Sons NY, 1991-1994) and

Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbour, Cold Spring Harbour Laboratory, 1988.

Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are described for example in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra*,
5 and in Harlow & Lane, 1988, *supra*. In a particular embodiment, anti-SpyCEP polyclonal antibodies may be obtained or purified from human sera from individuals exposed to, or infected by, Group A strep. Alternatively, polyclonal antibodies may be raised against purified or recombinant SpyCEP, or an
10 immunogenic fragment thereof, in production species such as horses and then subsequently purified prior to administration.

Monoclonal antibodies may be produced using the standard method as for example, originally described in an article by Köhler & Milstein, 1975, Nature **256**, 495, or by more recent modifications thereof as for example, described in
15 Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra* by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the isolated proteins, fragments, variants or derivatives of the invention. In certain embodiments, the monoclonal antibody or fragment thereof may be in recombinant form. This may
20 be particularly advantageous for “humanizing” the monoclonal antibody or fragment if the monoclonal antibody is initially produced by spleen cells of a non-human mammal.

In some embodiments, the antibody or antibody fragment may be administered to a mammal to provide “passive” immunity to group A
25 streptococcal infection.

Certain further aspects and embodiments of the invention relate to administration of one or more nucleic acids encoding an isolated immunogenic peptide comprising the amino acid sequence set forth in SEQ ID NO:1, or a fragment, variant or derivative thereof for:

- 30 (i) eliciting an immune response to group A streptococcal bacteria in a mammal;
(ii) immunizing a mammal against group A streptococcal bacteria;
and/or

- (iii) treating or preventing a group A streptococcal bacterial infection in a mammal.

The term “*nucleic acid*” as used herein designates single- or double-stranded DNA and RNA. DNA includes genomic DNA and cDNA. RNA includes mRNA, RNA, RNAi, siRNA, cRNA and autocatalytic RNA. Nucleic acids may also be DNA-RNA hybrids. A nucleic acid comprises a nucleotide sequence which typically includes nucleotides that comprise an A, G, C, T or U base. However, nucleotide sequences may include other bases such as modified purines (for example inosine, methylinosine and methyladenosine) and modified pyrimidines (for example thiouridine and methylcytosine).

In a preferred form, the isolated nucleic acid encoding the immunogenic peptide, fragment, variant or derivative thereof is in the form of a genetic construct suitable for administration to a mammal such as a human. In a preferred for, the genetic construct is suitable for DNA vaccination of a mammal such as a human.

Suitably, the genetic construct is in the form of, or comprises genetic components of, a plasmid, bacteriophage, a cosmid, a yeast or bacterial artificial chromosome as are well understood in the art. Genetic constructs may also be suitable for maintenance and propagation of the isolated nucleic acid in bacteria or other host cells, for manipulation by recombinant DNA technology.

For the purposes of protein expression, the genetic construct is an expression construct. Suitably, the expression construct comprises the one or more nucleic acids operably linked to one or more additional sequences in an expression vector. An “*expression vector*” may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome.

By “*operably linked or connected*” is meant that said additional nucleotide sequence(s) is/are positioned relative to the nucleic acid of the invention preferably to initiate, regulate or otherwise control transcription.

Regulatory nucleotide sequences will generally be appropriate for the host cell or tissue where expression is required. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells.

Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or
5 inducible promoters as known in the art are contemplated by the invention. The expression construct may also include an additional nucleotide sequence encoding a fusion partner (typically provided by the expression vector) so that the recombinant protein of the invention is expressed as a fusion protein, as hereinbefore described.

10 Suitably, DNA vaccination is by way of one or more plasmid DNA expression constructs. Plasmids typically comprise a viral promoter (such as SV40, RSV or CMV promoters). Intron A may be included to improve mRNA stability and thereby increase protein expression. Plasmids may further include a multiple cloning site, a strong polyadenylation/transcription termination signal,
15 such as bovine growth hormone or rabbit beta-globulin polyadenylation sequences. The plasmid may further comprise Mason-Pfizer monkey virus *cis*-acting transcriptional elements (MPV-CTE) with or without HIV rev increased envelope expression.. Additional modifications that may improve expression include the insertion of enhancer sequences, synthetic introns, adenovirus tripartite leader
20 (TPL) sequences and/or modifications to polyadenylation and/or transcription termination sequences. A non-limiting example of a DNA vaccine plasmid is pVAC which is commercially available from Invivogen.

A useful reference describing DNA vaccinology is DNA Vaccines, Methods and Protocols, Second Edition (Volume 127 of Methods in Molecular
25 Medicine series, Humana Press, 2006).

As hereinbefore described, the invention provides compositions, vaccines and/or methods of immunizing against, preventing or treating a Group A Strep-associated disease, disorder or condition in a mammal. Suitably, the compositions, vaccines and/or methods of immunizing against, preventing or treating a Group A
30 Strep-associated disease, disorder or condition elicit a mucosal immune response upon administration to a mammal. Preferably, the mucosal immune response is characterized by the production of IgG compared to a lower level of, or absent IgA.

As generally used herein the terms “immunize”, “vaccinate” and “vaccine” refer to methods and/or compositions that elicit a protective immune response against Group A Strep, whereby subsequent infection by Group A Strep is at least partly prevented or minimized.

5 As used herein, “treating”, “treat” or “treatment” refers to a therapeutic intervention that at least partly ameliorates, eliminates or reduces a symptom or pathological sign of a Group A strep-associated disease, disorder or condition after it has begun to develop. Treatment need not be absolute to be beneficial to the subject. The beneficial effect can be determined using any methods or
10 standards known to the ordinarily skilled artisan.

 As used herein, “preventing”, “prevent” or “prevention” refers to a course of action initiated prior to infection by, or exposure to, group A strep and/or before the onset of a symptom or pathological sign of a Group A strep-associated disease, disorder or condition, so as to prevent infection and/or reduce the
15 symptom or pathological sign. It is to be understood that such preventing need not be absolute to be beneficial to a subject. A “prophylactic” treatment is a treatment administered to a subject who does not exhibit signs of a group A strep-associated disease, disorder or condition, or exhibits only early signs for the purpose of decreasing the risk of developing a symptom or pathological sign of a
20 Group A strep-associated disease, disorder or condition.

 In the context of the present invention, by “group A-strep-associated disease, disorder or condition” is meant any clinical pathology resulting from infection by group A strep and includes cellulitis, erysipelas, impetigo, scarlet fever, throat infections such as acute pharyngitis (“strep throat”), bacteremia,
25 toxic shock syndrome, necrotizing fasciitis, acute rheumatic fever and acute glomerulonephritis, although without limitation thereto.

 In certain aspects and embodiments, the p*17 peptide, fragment, variant or derivative, isolated nucleic acids, genetic constructs, antibodies and/or antibody fragments as disclosed herein, may be administered to a mammal separately, or in
30 combination, in the form of a composition.

 In a preferred form, the composition comprises an acceptable carrier, diluent or excipient.

 By “acceptable carrier, diluent or excipient” is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic

administration. Depending upon the particular route of administration, a variety of carriers, diluent and excipients well known in the art may be used. These may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates, water and pyrogen-free water.

A useful reference describing acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Preferably, for the purposes of eliciting an immune response, certain immunological agents may be used in combination with the p*17 peptide, fragment, variant or derivative.

The term "*immunological agent*" includes within its scope carriers, delivery agents, immunostimulants and/or adjuvants as are well known in the art. As will be understood in the art, immunostimulants and adjuvants refer to or include one or more substances that enhance the immunogenicity and/or efficacy of a composition. Non-limiting examples of suitable immunostimulants and adjuvants include squalane and squalene (or other oils of plant or animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); *Bordetella pertussis* antigens; tetanus toxoid; diphtheria toxoid; surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyldioctadecylammonium bromide, *N,N*-dioctadecyl-*N'*, *N'*bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextran sulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminium phosphate, aluminium hydroxide or alum; interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon;

immunostimulatory DNA such as CpG DNA, combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes (*e.g.* see International Publication WO2017/070735); ISCOM® and ISCOMATRIX® adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran alone or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (*e.g.* U.S. Pat. No. 5,047,238); water in oil emulsifiers such as Montanide ISA 720; poliovirus, vaccinia or animal poxvirus proteins; or mixtures thereof.

Immunological agents may include carriers such as thyroglobulin and diphtheria toxoid (DT); albumins such as human serum albumin; toxins, toxoids or any mutant crossreactive material (CRM) of the toxin from tetanus, diphtheria, pertussis, *Pseudomonas*, *E. coli*, *Staphylococcus*, and *Streptococcus*; polyamino acids such as poly(lysine:glutamic acid); influenza; Rotavirus VP6, Parvovirus VP1 and VP2; hepatitis B virus core protein; hepatitis B virus recombinant vaccine and the like. Alternatively, a fragment or epitope of a carrier protein or other immunogenic protein may be used. For example, a T cell epitope of a bacterial toxin, toxoid or CRM may be used. In this regard, reference may be made to U.S. Patent No 5,785,973 which is incorporated herein by reference. Other immunological agents may include immunogenic peptides from Group A strep proteins other than M protein, for example a SpyCEP peptide as described in International Publication WO2015/157820.

Any suitable procedure is contemplated for producing vaccine compositions. Exemplary procedures include, for example, those described in New Generation Vaccines (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel, Hong Kong), which is incorporated herein by reference.

In some embodiments, compositions and vaccines may be administered to mammals in the form of attenuated or inactivated bacteria that may be genetically modified to express the p*17 peptide, fragment, variant or derivative and/or the agent that facilitates restoring or enhancing neutrophil activity. Non-limiting examples of attenuated bacteria include *Salmonella* species, for example *Salmonella enterica* var. Typhimurium or *Salmonella typhi*. Alternatively, other enteric pathogens such as *Shigella* species or *E. coli* may be used in attenuated

form. Attenuated *Salmonella* strains have been constructed by inactivating genes in the aromatic amino acid biosynthetic pathway (Alderton *et al.*, Avian Diseases 35 435), by introducing mutations into two genes in the aromatic amino acid biosynthetic pathway (such as described in U.S. patent 5,770,214) or in other
5 genes such as *htrA* (such as described in U.S. patent 5,980,907) or in genes encoding outer membrane proteins, such as *ompR* (such as described in U.S. patent 5,851,519).

Any safe route of administration may be employed, including oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-
10 dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, topical, mucosal and transdermal administration, although without limitation thereto.

In one particular embodiment, administration is intramuscular such as by way of one or more intramuscular injections. Typically, intranasal administration
15 is considered to be optimal for inducing mucosal immunity. Surprisingly, intramuscular administration of p*17 peptide elicits a mucosal immune response to group A streptococcal bacteria.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, nasal sprays, suppositories, aerosols,
20 transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release may be effected by coating with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and
25 certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Compositions may be presented as discrete units such as capsules, sachets, functional foods/feeds or tablets each containing a pre-determined amount of one
30 or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier

which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

5 The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of
10 the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

 In a particular embodiment of the aforementioned methods and compositions, the modified p145 peptide, fragment or variant may be administered in combination with an immunogenic fragment of a SpyCEP protein.
15 It will be appreciated that the SpyCEP protein fragment may act as an agent that facilitates restores or enhances neutrophil activity, by directly or indirectly at least partly increasing enhancing or restoring the production, migration and/or chemotaxis of neutrophils and/or one or more immunological activities of neutrophils. Typically, the SpyCEP fragment elicits an inhibitory immune
20 response to the SpyCEP serine protease that proteolytically cleaves interleukin 8. SpyCEP is a 170-kDa multidomain serine protease expressed on the surface of the human pathogen *Streptococcus pyogenes*, which plays an important role in infection by catalyzing cleavage and inactivation of the neutrophil chemoattractant interleukin-8. Non-limiting examples of SpyCEP amino acid
25 sequences may be found under accession numbers YP597949.1 and (*S. pyogenes* MGAS10270) and YP596076.1 (*S. pyogenes* MGAS9429). Preferably, the fragment of SpyCEP is, or comprises an S2 peptide (SEQ ID NO:23) or a variant such as a K4S2 peptide (SEQ ID NO:24).

 In some embodiments, the modified p145 peptide, fragment or variant and
30 the S2 peptide, fragment or variant may be provided as a single, chimeric peptide. In this embodiment, the modified p145 peptide may be N-terminal or C-terminal of the S2 peptide.

 As generally used herein, the terms “*patient*”, “*individual*” and “*subject*” are used in the context of any mammalian recipient of a treatment or composition

disclosed herein. Accordingly, the methods and compositions disclosed herein may have medical and/or veterinary applications. In a preferred form, the mammal is a human.

So that the invention may be fully understood and put into practical effect,
5 reference is made to the following non-limiting Examples.

EXAMPLES

Introduction

The ability to enhance the immunogenicity of small peptide epitopes
10 would greatly facilitate vaccine development. The approaches that have been undertaken so far to improve immunogenicity for antibody production have centred on novel folding technologies that result in the epitope more closely resembling native structure (e.g. (5)) or strategies to incorporate additional molecules with the peptide that will activate antigen presenting cells (e.g. (6, 7))
15 or helper T cells (8, 9).

Approaches to alter the sequence of the peptide to improve its immunogenicity are also being considered. Heteroclitic peptides have been tested for enhancing immunogenicity for T cells (10). However, nearly all vaccines are reliant on the production of protective antibodies and here modified peptides have
20 been largely ignored, but amino acid modifications outside of a minimal 4-amino acid epitope were shown to be able to improve peptide recognition by a monoclonal antibody suggesting that this approach may be useful for vaccine development (11). Sequence modifications of complex epitopes and modifications within minimal epitopes to improve immunogenicity have not been tested as a
25 mode to enhance efficacy.

A conserved peptide epitope from the M protein of *Streptococcus pyogenes* provides an ideal focus and opportunity to develop a strategy to rationally design and test an enhanced complex peptide immunogen for inducing protective antibodies. The M protein has a conserved carboxyl terminal region and
30 a 20-amino acid peptide, 'p145', which has an alpha helical structure, was described that can induce broad strain-protective antibodies (reviewed in (12)). A minimal B cell epitope of 12 amino acids exists within p145. However, the entire 20 amino acids are required to maintain the helical folding of the minimal epitope. This epitope is cryptic for the organism.

Exposure to *S. pyogenes* does not usually result in the production of antibodies to p145 (13), (14); however, antibodies induced to the minimal epitope by vaccination can kill the organism. Immunity requires multiple rounds of vaccination and protection correlates with the magnitude of the immune response (15).

In the present study, iterative rounds of targeted amino acid substitutions within the minimal B cell epitope were performed to create p145 variants. As a result the immunogenicity and ability of the peptide-antisera to bind p145 and streptococci were significantly increased and vaccine efficacy was enhanced such that only a single immunization was required to induce immunity. We observed a 10,000-fold enhanced level of protection from invasive streptococcal disease compared to control mice vaccinated with the parent peptide.

Materials & Methods

Peptides

The peptides were obtained from Mimotopes Pty Ltd (Victoria, Australia). The peptide library (87 peptides including p145) was purchased as a cleaved PepSet with a biotin coupled to the N5 terminus and a linker of four amino acids (SGSG) was added between the peptide and the biotin. The peptides were biotinylated via the N-terminus to minimize interference with the ligand binding. The p*1-p*18 peptides were obtained as custom peptides with minimal purity of >90 %. An additional cysteine residue was added in the N-terminal end to each peptide to enable coupling to Gel Spin columns (see below). The p145 used for coating in ELISA was synthesized at the QIMR Berghofer Medical Research Institute, DNA and Peptide Unit or at China peptides. The p*17 peptide with C-terminal cysteine residue, was synthesized at China peptides and conjugated to DT as described elsewhere (16).

Antisera

Antisera used for peptide screening were from J8-immunised B10.BR, J8-DT-immunised BALB/c, or p145-immunised BALB/c, Quackenbush and B10.D2 mice.

Bacteria

- GAS strains, 2031 (*emm1*) is an isolate from Prague reference center and 8830 (*emm97*) is a clinical isolate from the Northern Territory of Australia (17). Both strains were obtained from Menzies School of Health Research (MSHR); mouse-adapted for *in vivo* studies and made streptomycin resistant (200 µg/mL).
- 5 JRS145 is an M-protein negative mutant.

Enzyme-linked Immunosorbent Assay (ELISA)

- ELISA with biotinylated peptides was performed as recommended by the manufacture (Mimotopes, Australia). Briefly, 100 µl aliquots of streptavidin
- 10 (Sigma-Aldrich, Australia) at a final concentration of 5 µg/ml in water were added to Oltrack activated 96-well microtiter plates (Concentrack, Netherlands) and incubated at 37°C overnight. The wells were blocked for 1 hr at RT with 200 µl PBS, pH 7.2, containing 0.05% Tween 20 and 1 % Sodium caseinate and thereafter washed with PBS/Tween 20. The peptides were diluted in PBS/Tween
- 15 20 with 0.1 % sodium azide to a working concentration of 20 µg/ml and 100 µl of each was added to the wells and incubated for 1 h at RT. After thorough washings, murine p145 or J8 antisera were added. The plates were incubated for 1 h at RT, washed and HRP-conjugated goat anti-mouse-IgG (Bio-Rad, Australia) (1/3000) was added for another 20 min at RT. After additional washings, OPD substrate
- 20 (Sigma-Aldrich) was added and measured at OD⁴⁵⁰. Titre was defined as the highest dilution that gave an optical density (OD) reading of more than three standard deviations (SD) above the mean OD of control wells containing normal mouse sera at the same dilution (19).

- For the inhibition assays, the microtiter plates were coated with 100 µl of
- 25 purified p145 at a final concentration of 5 µg/ml in 75 mM sodium carbonate, pH 9.6, at 4°C overnight. Plates were washed with washing buffer (PBS with 0.05 % Tween 20, pH 7.2) and blocked for 90 min at 37°C with washing buffer supplied with 5% skim milk (blocking buffer). Anti-p145 sera were incubated with varying concentrations (0.25 µg/ml - 2.5 µg/ml) of selected peptides from the peptide
- 30 library for 30 min at RT. Test antisera were thereafter added to the blocked plates and incubated for 90 min at 37°C. The plates were washed four times and HRP-conjugated goat anti-mouse IgG (Biorad, Australia) (1/3000) was added and

incubated for another 90 min at 37°C. After additional washing, the plates were developed as above and measured at OD⁴⁵⁰.

In the analysis for cross-reactivity with human heart proteins, 5 µg/ml of tropomyosin, myosin or keratin (Sigma-Aldrich, Australia) were immobilised in microtiter plates. After blocking and washings as above, the plates were incubated for 90 min at 37°C with triplets of anti-peptide (p*1-p*18) sera (1/100), respectively. Thereafter, the secondary antibody and the substrate were added as above.

10 *Immunization of mice*

All protocols were approved by the QIMR Berghofer Medical Research Institute's Animal Ethics Committee, in compliance with Australian National Health and Medical Research (NHMRC) guidelines. On day 0, Quackenbush mice (Animal Resources Centre, Australia) ($n = 3$ per group) were inoculated subcutaneously (s.c.) at the tail base with 30 µg of the different peptides emulsified 1:1 with complete Freund's adjuvant (CFA) in a total volume of 50 µl. Control mice received sterile-filtered PBS in CFA. Three weeks after primary immunization, mice received 2 booster immunizations of immunogen (30 µg each in sterile-filtered PBS) given at weekly intervals (days 21 and 28). Groups of mice with antibody titers of less than 1000 received a third boost emulsified 1:1 with incomplete Freund's adjuvant. Heat-killed GAS 2031 (emm1) was prepared as previously described (18). For the skin challenge studies, BALB/c mice ($n=10$ /group) were immunised s.c. once at the tail base with 30 µg of p145-DT or p*17-DT or heat killed 2031 GAS preparation formulated in Alum. For the p145 dose-ranging study, following primary immunisation with p145-DT/Alum on day 0, specific cohorts were administered 1 or 2 boosts on days 21 and 28. Control mice received adjuvant alone. Blood was collected from each mouse 1-day prior to boosts and one week after the final immunization. The blood was allowed to clot at 4°C for at least 4 h and serum was collected after centrifugation at 1000 g for 10 minutes. Sera were stored at -20 °C.

Affinity purification of sera

For purification of the peptide specific sera, Microlink peptide coupling kit (Pierce, USA) was used according to the manufacturer's instructions. Briefly, each peptide was diluted to 0.6 mg/ml in coupling buffer and immobilised to an Ultralink iodoacetyl gel spin column (Pierce, USA). The columns were
5 thoroughly washed with coupling buffer and blocked with L-Cysteine HCl (10 mg/ml). Thereafter, the columns were washed with wash buffer followed by PBS. For affinity purification, 300 µl of the different Quackenbush sera were added to the respective columns and incubated end over end at 4°C overnight. The columns
10 were washed three times with PBS and antibodies were eluted with 100 µl elution buffer. Thereafter, the columns were washed and blocked again as above and additional sera were added. The eluted immunoglobulin (Ig) concentrations were estimated by using the Nanodrop (Thermo Scientific, USA). The purified IgG preparations at a concentration 5 µg/ml, followed by two-fold serial dilutions, were assessed for their binding to varying concentration of p145.

15

Flow cytometry

The binding of the peptide antibodies to the *S. pyogenes* was analysed by flow cytometry. Bacteria were grown in Todd-Hewitt broth (THB) with 1% neopeptone overnight and washed twice in PBS. Thereafter, bacteria (3×10^7
20 colony forming units; cfu) were pre-incubated with 100 µl of Fc blocker for 15 min at RT followed by addition of the peptide antibodies at a concentration of 10 mg/ml. After 30 min incubation at RT, the bacteria were washed twice in PBS followed by incubation with a FITC-conjugated anti-mouse IgG (diluted 1/50 in PBS with 2% BSA), total volume of 100 µl, was added for another 30 minutes at
25 RT. The bacteria were washed and incubated in 300 µl of 1 % formaldehyde (in PBS) and for 15 minutes at RT and then transferred to ice until reading in a FACS Calibur Flow Cytometer 1 (Becton Dickinson, USA).

The FITC-conjugated anti-mouse IgG was added separately as a negative control for each strain analysed and in addition, a non-specific mouse IgG was
30 included as a control.

Procedure for GAS skin challenge

S. pyogenes 2031 (*emm1*), 88/30 (*emm97*) and 5448AP (*emm1*) were cultured overnight in THB with 1% Neopeptone (THBN), washed twice in THBN

and resuspended in 25% of the original volume. The inoculum dose (CFU/ml) was determined by optical density at 600 nm and plating out 10-fold dilutions of bacterial suspensions on 2% horse blood THB agar. Following overnight incubation at 37°C, colony counts were determined. Two weeks post final immunization, immunized and control mice were challenged with selected GAS strain via the skin route of infection, as described previously (19).

Molecular dynamics (MD) simulations

Molecular dynamics (MD) simulations of peptides p*17 and P145 were performed to examine possible molecular basis of their immunogenicity. Their sequences, with a C-terminal Cysteine attached, were submitted to PEP-FOLD server (20) to construct initial structural models. These models were used as starting points for MD simulations, which were carried out in GROMACS 5 (21). All residues were parameterized with Amber ff14SB force field (22). Each peptide was placed in a dodecahedral box with a minimal distance of 1.2 nm between the solute and box edge, followed by solvation with TIP3P water molecules (23). Salt ions were then added to a concentration of 0.15 M to balance ionic charge in the system. Energy minimizations were carried out with steepest descent integrator and conjugate gradient algorithm sequentially to achieve a maximum force of less than 500 kJ mol⁻¹ nm⁻¹ on any atom. The Verlet cutoff scheme (24) was used to evaluate short-range, non-bonded interactions, with both van der Waals and electrostatic interactions truncated at 0.8 nm. Long-range electrostatic interactions were treated by the particle mesh Ewald (PME) method (25, 26). The temperature was maintained at 298 K using a velocity rescaling thermostat (27) with a coupling constant of 0.1 ps, while the pressure was maintained at 1.0 atm using a Berendsen barostat (28), with a coupling constant of 1 ps.

Simulations were performed with a time step of 2 fs, and all bonds involving hydrogen atoms were constrained by a parallel linear constraint solver (P-LINCS) (29). Each system was equilibrated under a constant volume (NVT) ensemble for 100 ps and a constant pressure (NPT) ensemble for 100 ps. A harmonic position restraint with a force constant of 1000 kJ mol⁻¹ nm⁻² was applied to all the heavy atoms of non-solvent molecules. After equilibration, production MD simulations were conducted for 100 ns for each system without

any constraints. Three replicate MD runs were performed for each system by varying the random seed for initial velocity generation. Analyses of MD trajectories were performed by inbuilt programs of GROMACS 5 on the final 40 ns of each 100-ns run to allow for equilibrium.

5

Statistical analysis.

The mean and standard error was calculated using standard formulae. Mann-Whitney tests were used to compare the antibody titers between two groups. Unless stated otherwise, *P* values were corrected using the Bonferroni method for multiple comparisons. ANOVA with the Tukey's post hoc method was used to determine the significance between all the treated and control cohorts at each time point.

Results

Our goal was to design a superior peptide immunogen against GAS as compared to p145 using iterative rounds of selection of mutant sequences within the minimal 12 amino-acid epitope within p145 (Table 1). Initially, we designed a mutant peptide library (L1) to ask whether the antigenicity of p145 could be increased with single mutations. We sought to identify mutations that would enhance antigenicity but without creating sequences that would resemble known human proteins.

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In L1, one amino acid was altered at a time and the amino acid substitutions were selected based on their physico-chemical properties relevant to keeping the peptide folded as an alpha helix (5,11 30). The resulting library consisted of 86 variants of p145 plus the original p145 (Table 3). In addition, a non-specific peptide (pNS [# 87],) was included as control. The binding of p145 antisera to each peptide was analysed using ELISA. In these studies, to ensure that the same amount of each peptide was bound to the plate, we used biotinylated peptides, which were attached to the plates via streptavidin. A four amino acid peptide linker was placed between the mutant peptide and biotin to ensure sufficient space for antibody recognition. The antisera were obtained from p145-immunized B10.D2, BALB/c and outbred Quackenbush mice. For each of the three different mouse strains, the binding of antisera to the variant peptides ranged from being minimal to being slightly enhanced compared with their binding to

30

p145 (Fig. 1A). Most substitutions resulted in reduced binding. Moreover, similar results were obtained with J8 or J8-DT antisera taken from B10.BR or BALB/c mice respectively (data not shown).

The non-specific peptide was recognized least well of all peptides. We identified 17 peptides to which the p145-specific antisera showed similar or better binding than to p145 and asked whether these peptides in solution could inhibit the binding of p145-specific antiserum to the parent p145 peptide. These peptides when tested in inhibition ELISA, at two different concentrations (0.25 µg/ml and 2.5 µg/ml), demonstrated a dose dependent response (Fig. 1 B and Fig. 6). At both the concentrations, peptides inhibited the binding of anti-p145 antibodies in solution to a comparable extent to p145, probably due to having retained folding similar to p145, however none were clearly superior (Fig. 1B and Fig. 6).

To avoid induction of human specific antibodies, we analysed whether the amino acid substitutions resulted in sequences that were closely related to human proteins using bioinformatics programs (BLASTP and Clustal Omega). Three peptides (2, 9 and 41) were eliminated from further consideration due to similarities (>50% sequence identity) with human proteins including rootletin (Ciliary rootlet coiled-coil protein), Dynactin 1, B-cell receptor associated protein 29, microtubule-actin cross linking factor 1 and myosin (heavy polypeptide 7B, cardiac muscle, beta, Isoform CRA_c). Hence, seven peptides emerged from the primary screen (5, 6, 11, 13, 40, 66, and 72), which involved mutations at four positions: S1E, S1V, R2N, R2D, K6N, K10R and K10M within the p145 minimal epitope (Fig. 1C).

Reasoning that single mutations in those peptides resulted in slightly enhanced recognition by p145-antisera, we then designed a second library, L2, which contained 18 peptides involving every possible combination of two mutations from the seven amino acid mutations identified above. This resulted in peptides designated p*1-18 (Table 2).

We investigated the immunogenicity of these doubly mutated peptides in outbred (Quackenbush) mice. The immunogenicity of the peptides varied but all except p*14 and p*16 generated antibodies to the immunizing peptide with most giving titers between 1 10⁵ and 10⁶ after three immunizations (Fig. 2A). All peptides, except p*14, p*15 and p*16 were significantly more immunogenic than

p145. The most immunogenic peptide was p*17. Antibodies to all peptides, except p*14 and p*16 were then affinity-purified using the immunizing peptide and we ascertained the relative binding of each of the antibodies to titrating concentrations of p145 peptide immobilized on plastic. Each plate was incubated
5 with purified antibodies at 5 µg/ml.

Antibodies induced by seven peptides (p*2, p*4, p*6, p*10, p*13, p*17, and p*18) showed higher affinity, binding significantly more strongly to immobilised p145 than did p145-induced antibodies (Fig. 2B). We noted that five of these seven peptides (p*4, p*10, p*13, p*17 and p*18) carried a K10 mutation.
10 We then used flow cytometry to investigate the binding of affinity purified antibodies, each at the same concentration, to the surface of streptococci expressing different *emm* proteins - 2031 (*emm*1) and 88/30 (*emm*97), both containing the exact same p145 sequence.

Antibodies to p*2, p*4, p*17, and p*18 bound to both GAS strains to a
15 much higher extent as compared to p145-induced antibodies (Fig. 3A-B), with anti-p*17 IgG demonstrating the highest binding. For most peptide antisera there was a correlation between binding to p145 and binding to the bacterial surface, with antisera to p*17 showing the strongest binding to both p145 and streptococci (Figs. 2B and 3). This antiserum was also tested against an *emm*-negative strain
20 (JRS145). No binding was observed against this strain (Fig. 3C) confirming that the antibodies were recognizing an epitope within the M protein.

Out of the most effective peptides (p*2, p*4, p*17, and p*18), peptides *2 and *4 were not considered further because antibodies to both these peptides recognised alpha helical coil human proteins (Fig. 7). Thus two peptides, p*17
25 and p*18 remained for further consideration and we focused on p*17 due to its superior immunogenicity.

Having shown that p*17 was more immunogenic than p145, that antibodies to p*17 demonstrated a higher affinity to p145 than antibodies induced by p145 itself and that they bound different strains of streptococci more strongly
30 than antibodies induced by p145, we asked whether p*17 would be a superior vaccine to p145. p145 conjugated to diphtheria toxoid (p145-DT) can protect against streptococcal skin challenge, but three doses of vaccine are required (Supplementary Fig. 3A and B). p*17 was thus conjugated to diphtheria toxoid and compared in a head-to-head study with p145-DT, but using only a single dose

of vaccine. We used conjugated peptides to provide a similar degree of T-cell help for the anti-peptide responses, enabling us to directly compare the immunogenicities of the peptides for B cells.

BALB/c mice were immunized subcutaneously with p*17-DT, p145-DT or PBS each formulated in Alum. Even though the immunogens were conjugated to DT, we noted that the p145-specific antibody titers induced by p*17-DT were significantly higher than those induced by p145-DT when measured three weeks post-vaccination (Fig. 4 A). Mice were then challenged via the skin route with the 2031 (*emm1*) or 88/30 (*emm97*) strains of GAS to determine vaccine efficacy. Again, no protection was afforded by vaccination with a single dose of p145-DT against 2031 or 88/30 GAS (Fig. 4B and D). However, vaccination with p*17-DT resulted in 100-1000 fold reduction in skin bacterial burden six days after challenge as compared to mice vaccinated with p145-DT (Fig. 4 B and D). Moreover, p*17-DT vaccination resulted in sterile protection from bacteraemia due to 2031 GAS in mice (>10,000-fold reduction in bacterial blood burden compared to mice vaccinated with p145-DT) (Fig. 4C). In addition, the p*17-DT vaccinated cohort completely resolved the systemic infection, due to 88/30, by day 6 post infection, whereas the p145 and PBS immunised cohorts still carried significantly higher bacteremia (Fig. 4E).

Protective ability of p*17-DT was further demonstrated in 1 another study utilising a CovR/S mutant strain. Single vaccination with p*17-DT induced high peptide specific titers (Fig. 8C) and resulted in >90% reduction in skin bacterial burden in comparison to PBS control cohort (Fig. 8D). Moreover, the level of protection was significantly better compared to that offered by heat-killed GAS (Fig. 8D).

Thus, p*17, a non-natural derivative of p145, can induce superior antibodies reactive against p145 in terms of titers and affinity than can p145 itself, and is a significantly superior immunogen to p145 in terms of inducing antibodies that recognize streptococci and protect against them. To ask whether significant structural differences existed between p145 and p*17 that may explain this discrepancy, we used molecular dynamic (MD) simulations to analyse the peptides. The simulations revealed that the helix in p145 breaks between position 12 and 14, probably due to the disruptive effect of the positively charged Ks at positions 12 and 13 (Fig. 5A). p*17 has a non-charged asparagine (N) at position

13 facilitating the folding of the helix. The data also demonstrate that p145 peptide was less likely to adopt a helical structure than p*17 (helicity percentage of <50 vs <80; Fig. 5B) and also showed larger flexibility (RMS fluctuation; Fig. 5 C).

5 In further experiments according to the schedule in Figure 9, intramuscular immunization with p*17 alone or together with S2 peptide was investigated. Mice were immunized three (3) times with p*17-DT+K4S2-DT/Alum and intranasal challenge with 5448AP (CovR/S MT strain). Figure 10 shows immunogenicity following i.m. immunisation with p*17-based vaccines. BALB/c mice
10 (n=15/group; female, 4-6 weeks old) were immunised i.m. with p*17-DT/Alum, p*17-DT+K4S2-DT/Alum and PBS/Alum on days 0, 21 and 42. One week post last boost serum and saliva samples were collected and p*17-specific IgG (A & C) and IgA (B & D) antibody responses were measured by ELISA and are represented as mean $\pm$ SEM. The titers are in comparison to the control cohort
15 that received only PBS/Alum.

Figure 11 shows protection following intranasal infection. Two weeks after last vaccine boost, the mice were infected intranasally with covR/S mutant GAS (5448AP). On days 1-3 nasal shedding (NS) were collected. Bacterial burden in NS were determined by pressing the nares of each mouse onto columbia
20 blood agar (CBA) plates containing 5% defibrinated horse-blood and exhaled particles were streaked out. On days 1-3 NS were collected and represented as the log transformed mean $\pm$ SEM from each individual day (A) and as cumulative CFUs from all three days combined (B). The percent reduction in bacterial burden the nasal shedding is shown in Figure 12.

25 Figure 13 shows protection following intranasal infection. In addition to NS on days 1-3 throat swabs (TS) were collected. For throat swabs, swab applicators were placed in sterile PBS to dampen, mice were then immobilized and throats were swabbed. Swab applicator was then suspended in PBS, serially diluted and dot-plated in duplicate on CBA plates containing 5% defibrinated
30 horse-blood. . On days 1-3 NS were collected and represented as the log transformed mean cfu/ml $\pm$ SEM from each individual day (A) and as cumulative CFUs from all three days combined (B). Figure 14 shows the reduction in bacterial burden in throat swabs.

Figure 15 shows protection following intranasal infection. On day 3 post-infection all surviving mice were euthanised and NALT, lungs, spleen and blood samples were collected to determine GAS bacterial burden, data represented as the log transformed mean cfu/ml $\pm$ SEM (A) and as percent reduction in comparison to PBS/Alum control (B).

We further observed that 3x i.m. immunisations with p*17-DT/Alum protected against intranasal challenge with GAS. There was 70%-90% reduction in bacterial load in nasal sheddings and throat swabs respectively. In addition there was greater than 70% reduction in the systemic bacterial burden. This was despite the fact that these mice did not induce serum or salivary IgA. Interestingly we saw an induction of salivary IgG following i.m. immunisation. For determination of antibody responses we performed semi-quantitative analysis (ELISA) which were defined as the end point titers. In the experiments that utilised intramuscular vaccination with p*17-DT (with or without K4S2), the mucosal IgA responses were undetectable whereas the mucosal IgG responses were at least 10 times higher.

Structural comparisons of J8 peptide, p145 and p*17 were performed that suggest differences between J8 and p*17 peptides that further support the enhanced immunogenicity of p*17. J8 is 28-mer whereas p*17 is a 20-mer peptide. The two peptides share 10 amino acids, which are derived from native peptide p145 (Fig 16), streptococcal amino acids shown in bold. The MD simulation studies comparing J8 and p*17 demonstrated that both peptides fold as a helix, however, J8 is more flexible in comparison to p*17. We believe that the overall tertiary structure of p*17 renders it more stable, facilitating a better interaction with host molecules. Moreover, the amino acid composition of p*17 (more similar to the strep sequence than J8) likely induces antibodies recognising the native structure of the organism better than J8.

Discussion

Development of peptide vaccines would be enormously facilitated by technologies that increase the immunogenicity of peptides. Very few studies have used selective amino acid substitutions to modify immunogenicity and where tested these have focused on epitope modifications to augment stimulation of

CD8+ T cells, where the results have been mixed. Here, we describe a strategy to significantly enhance the immunogenicity of a complex 12-amino acid helical synthetic peptide from *S. pyogenes*. A number of doubly modified peptides demonstrated significantly enhanced immunogenicity in terms of inducing
5 antibodies to the immunizing peptide and to the parent peptide with some antisera also showing enhanced binding to the surface of streptococci. The peptide with the antiserum that bound most strongly to both the parent peptide and the organism (anti-p*17) was tested as a vaccine to prevent streptococcal infection where we observed that a single immunization resulted in significantly enhanced
10 protection from skin and invasive disease (more than 100-fold reduction in the bio-burden of skin streptococci and a >10,000-fold reduction to blood bacterial-burden).

We designed p*17 based on our initial observations of antigenicity of a peptide library of 86 peptides with one amino acid change in each peptide. Four
15 amino acids from the 12 amino acids of the minimal helical epitope were relevant to the construction of peptides that were recognized at least as well as the parent sequence. Even though these singly mutated peptides showed minimal improved recognition over the parent peptide, p145, by anti-p145 antisera, new peptides with two mutations were far superior as immunogens than p145. But more
20 surprisingly, the antibodies generated by these doubly mutated peptides recognized p145 with significantly higher affinity than antibodies induced by p145 itself. Furthermore, a number of peptide antisera recognized the surface of streptococci more avidly than antibodies induced by p145 vaccination.

Even more so, the protective efficacy of one such peptide, p*17, shows
25 that this peptide is a superior vaccine candidate. Single immunisation with p*17 induced high levels of self and p145-specific antibodies, which were protective against multiple GAS strains. Additionally, the peptide also protected against a hypervirulent GAS strain that is responsible for invasive infections globally. Currently, the minimal epitope within p145, displayed within a yeast helical
30 peptide and referred to as J8, is a leading vaccine candidate for streptococci (12). This vaccine candidate also requires three doses to induce protection against skin disease (19).

MD simulations revealed that p*17 is more likely to fold as a helix than p145 and has greater stability. The enhanced stability is likely to have made it

more immunogenic than p145 and more likely to induce antibodies that recognize the native structure of the organism, where the epitope is held as a helix. The conformational binding of p*17 antibodies to other epitopes along the M protein that are distinct from the p145 sequence could also, in part, account for the enhanced efficacy of p*17. However, it is curious that the anti-p*17 antibodies recognize p145 better than antibodies raised to p145 itself. It is possible that anti-p*17 antibodies can fold p145 into a helix.

We have found that of the seven variant peptides that induced anti-p145 antibodies of both higher titer and higher affinity, four (p*10, p*13, p*17 and p*18) contained mutations at positions and/or of the minimal epitope such that the two positions were not both occupied by charged amino acids. These positions in the minimal epitope correspond to positions f and c in the heptad motif which come together on the hydrophilic side of the helical peptide; this is likely to render these structures more stable. We have not performed MD simulations for the other peptides to confirm this as our focus was on the most immunogenic one that did not share sequence homology with human proteins (p*17).

We also hypothesize that protection at the mucosal surface is due to induction of salivary IgG. IgG at the mucosal surface has been shown to prevent colonisation by agglutination and thereby clearance of bacteria (Roche et. al., Mucosal Immunol, 2015). Furthermore p*17-DT when combined with K4S2-DT was also able to induce salivary IgG and the combination vaccine was able to protect against hypervirulent CovR/S mutant GAS 5448AP significantly better than p*17-DT alone.

The MD simulation studies comparing J8 and p*17 demonstrated that both peptides fold as a helix, however, J8 is more flexible in comparison to p*17. We believe that the overall tertiary structure of p*17 renders it more stable, facilitating a better interaction with host molecules. Moreover, the amino acid composition of p*17 (more similar to the strep sequence than J8) likely induces antibodies recognising the native structure of the organism better than J8.

In conclusion we have defined a strategy to design peptides with significantly enhanced immunogenicity via a mechanism that may relate not to modifications of the interacting residues of the epitope with the immunoglobulin receptor, but with non-specific ionic forces between the epitope and B cell Ig

receptor. The generic strategy that we describe could improve the immunogenicity of many complex peptide epitopes.

Throughout this specification, the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one
5 embodiment or specific collection of features. Various changes and modifications may be made to the embodiments described and illustrated herein without departing from the broad spirit and scope of the invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference in their entirety

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Table 1. p145 and the minimal epitope

Peptide	Sequence	Peptide length
p145	LRRDLASREAKKQVEKALE	20 amino acids
Minimal epitope*	SREAKKQVEKAL	12 amino acids

*A 12-mer minimal B-cell epitope from within p145 (31).

Table 2. The L2* library with the sequence of individual peptides

p145	LRRDLDA SREAKKQVEKAL E
p*1.	LRRDLDA <u>E</u> NEAKKQVEKAL E
p*2.	LRRDLDA <u>E</u> DEAKKQVEKAL E
p*3.	LRRDLDA <u>E</u> REAK <u>N</u> QVEKAL E
p*4.	LRRDLDA <u>E</u> REAKKQVE <u>R</u> AL E
p*5.	LRRDLDA <u>E</u> REAKKQVE <u>M</u> AL E
p*6.	LRRDLDA <u>V</u> NEAKKQVEKAL E
p*7.	LRRDLDA <u>V</u> DEAKKQVEKAL E
p*8.	LRRDLDA <u>V</u> REAK <u>N</u> QVEKAL E
p*9.	LRRDLDA <u>V</u> REAKKQVE <u>R</u> AL E
p*10.	LRRDLDA <u>V</u> REAKKQVE <u>M</u> AL E
p*11.	LRRDLDA S <u>N</u> EAK <u>N</u> QVEKAL E
p*12.	LRRDLDA S <u>N</u> EAKKQVE <u>R</u> AL E
p*13.	LRRDLDA S <u>N</u> EAKKQVE <u>M</u> AL E
p*14.	LRRDLDA S <u>D</u> EAK <u>N</u> QVEKAL E
p*15.	LRRDLDA S <u>D</u> EAKKQVE <u>R</u> AL E
p*16.	LRRDLDA S <u>D</u> EAKKQVE <u>M</u> AL E
p*17	LRRDLDA SREAK <u>N</u> QVE <u>R</u> AL E
p*18.	LRRDLDA SREAK <u>N</u> QVE <u>M</u> AL E

*Each peptide in L2 library was designed with two amino acid alterations. The amino acids substituted in the original p145 sequence are underlined.

Table 3. The L1* peptide library including 86 variants of p145**P145.** Biotin-SGSGLRRLDAS**S**REAKKQVEKAL E (SEQ ID NO:113)

1. Biotin-SGSGLRRLDA **L**REAKKQVEKAL E
2. Biotin-SGSGLRRLDA **A**REAKKQVEKAL E
3. Biotin-SGSGLRRLDA **Y**REAKKQVEKAL E
4. Biotin-SGSGLRRLDA **M**REAKKQVEKAL E
5. Biotin-SGSGLRRLDA **E**REAKKQVEKAL E
6. Biotin-SGSGLRRLDA **V**REAKKQVEKAL E
7. Biotin-SGSGLRRLDA **I**REAKKQVEKAL E
8. Biotin-SGSGLRRLDA **S****E**AKKQVEKAL E
9. Biotin-SGSGLRRLDA **S****Q**EAKKQVEKAL E
10. Biotin-SGSGLRRLDA **S****K**EAKKQVEKAL E
11. Biotin-SGSGLRRLDA **S****N**EAKKQVEKAL E
12. Biotin-SGSGLRRLDA **S****T**EAKKQVEKAL E
13. Biotin-SGSGLRRLDA **S****D**EAKKQVEKAL E
14. Biotin-SGSGLRRLDA **S****R****N**AKKQVEKAL E
15. Biotin-SGSGLRRLDA **S****R****K**AKKQVEKAL E
16. Biotin-SGSGLRRLDA **S****R****R**AKKQVEKAL E
17. Biotin-SGSGLRRLDA **S****R****D**AKKQVEKAL E
18. Biotin-SGSGLRRLDA **S****R****Q**AKKQVEKAL E
19. Biotin-SGSGLRRLDA **S****R****A**AKKQVEKAL E
20. Biotin-SGSGLRRLDA **S****R****S**AKKQVEKAL E
21. Biotin-SGSGLRRLDA **S****R****T**AKKQVEKAL E
22. Biotin-SGSGLRRLDA **S****R****E****E**KKQVEKAL E
23. Biotin-SGSGLRRLDA **S****R****E****K**KKQVEKAL E
24. Biotin-SGSGLRRLDA **S****R****E****Q**KKQVEKAL E

25. Biotin-SGSGLRRLDLDA SRENKKQVEKAL E
26. Biotin-SGSGLRRLDLDA SRERKKQVEKAL E
27. Biotin-SGSGLRRLDLDA SREDKKQVEKAL E
28. Biotin-SGSGLRRLDLDA SREALKQVEKAL E
29. Biotin-SGSGLRRLDLDA SREAIKQVEKAL E
30. Biotin-SGSGLRRLDLDA SREAMKQVEKAL E
31. Biotin-SGSGLRRLDLDA SREAVKQVEKAL E
32. Biotin-SGSGLRRLDLDA SREAYKQVEKAL E
33. Biotin-SGSGLRRLDLDA SREAAKQVEKAL E
34. Biotin-SGSGLRRLDLDA SREANKQVEKAL E
35. Biotin-SGSGLRRLDLDA SREARKQVEKAL E
36. Biotin-SGSGLRRLDLDA SREAKEQVEKAL E
37. Biotin-SGSGLRRLDLDA SREAKDQVEKAL E
38. Biotin-SGSGLRRLDLDA SREAKQQVEKAL E
39. Biotin-SGSGLRRLDLDA SREAKAQVEKAL E
40. Biotin-SGSGLRRLDLDA SREAKNQVEKAL E
41. Biotin-SGSGLRRLDLDA SREAKRQVEKAL E
42. Biotin-SGSGLRRLDLDA SREAKTQVEKAL E
43. Biotin-SGSGLRRLDLDA SREAKKEVEKAL E
44. Biotin-SGSGLRRLDLDA SREAKKDVEKAL E
45. Biotin-SGSGLRRLDLDA SREAKKKVEKAL E
46. Biotin-SGSGLRRLDLDA SREAKKNVEKAL E
47. Biotin-SGSGLRRLDLDA SREAKKRVEKAL E
48. Biotin-SGSGLRRLDLDA SREAKKAVEKAL E
49. Biotin-SGSGLRRLDLDA SREAKKSVEKAL E
50. Biotin-SGSGLRRLDLDA SREAKKTVEKAL E
51. Biotin-SGSGLRRLDLDA SREAKKHVEKAL E
52. Biotin-SGSGLRRLDLDA SREAKKQLEKAL E
53. Biotin-SGSGLRRLDLDA SREAKKQAEKAL E
54. Biotin-SGSGLRRLDLDA SREAKKQYEKAL E

55. Biotin-SGSGLRRLDLDA SREAKKQ**M**EKAL E
56. Biotin-SGSGLRRLDLDA SREAKKQ**E**EKAL E
57. Biotin-SGSGLRRLDLDA SREAKKQ**I**EKAL E
58. Biotin-SGSGLRRLDLDA SREAKKQ**V**QKAL E
59. Biotin-SGSGLRRLDLDA SREAKKQ**V**KKAL E
60. Biotin-SGSGLRRLDLDA SREAKKQ**V**NKAL E
61. Biotin-SGSGLRRLDLDA SREAKKQ**V**RKAL E
62. Biotin-SGSGLRRLDLDA SREAKKQ**V**TKAL E
63. Biotin-SGSGLRRLDLDA SREAKKQ**V**DKAL E
64. Biotin-SGSGLRRLDLDA SREAKKQVE**E**AL E
65. Biotin-SGSGLRRLDLDA SREAKKQVE**N**AL E
66. Biotin-SGSGLRRLDLDA SREAKKQVE**R**AL E
67. Biotin-SGSGLRRLDLDA SREAKKQVE**D**AL E
68. Biotin-SGSGLRRLDLDA SREAKKQVE**Q**AL E
69. Biotin-SGSGLRRLDLDA SREAKKQVE**A**AL E
70. Biotin-SGSGLRRLDLDA SREAKKQVE**S**AL E
71. Biotin-SGSGLRRLDLDA SREAKKQVE**T**AL E
72. Biotin-SGSGLRRLDLDA SREAKKQVE**M**AL E
73. Biotin-SGSGLRRLDLDA SREAKKQVE**K**EL E
74. Biotin-SGSGLRRLDLDA SREAKKQVE**K**KL E
75. Biotin-SGSGLRRLDLDA SREAKKQVE**K**QL E
76. Biotin-SGSGLRRLDLDA SREAKKQVE**N**L E
77. Biotin-SGSGLRRLDLDA SREAKKQVE**R**L E
78. Biotin-SGSGLRRLDLDA SREAKKQVE**D**L E
79. Biotin-SGSGLRRLDLDA SREAKKQVEKA**I**E
80. Biotin-SGSGLRRLDLDA SREAKKQVEKAM**E**
81. Biotin-SGSGLRRLDLDA SREAKKQVEKAV**E**
82. Biotin-SGSGLRRLDLDA SREAKKQVEKAY**E**
83. Biotin-SGSGLRRLDLDA SREAKKQVEKAK**E**
84. Biotin-SGSGLRRLDLDA SREAKKQVEKAA**E**

85. Biotin-SGSGLRRLDA SREAKKQVEKANE

86. Biotin-SGSGLRRLDA SREAKKQVEKARE

87. Biotin-SGSGEGKVSTLPLDIQIIAATMSK

*The L1 library was designed with one amino acid substitution in each of the 86 variants of p145. The substituted amino acid is underlined. Biotin and the extra amino acids SGSG were included at the N-terminal end. Peptide 87 is a non-specific peptide (pNS) from *Schistosoma*.

CLAIMS

1. A method of eliciting an immune response to group A streptococcal bacteria in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby elicit the immune response.
2. A method of inducing immunity against a group A streptococcal bacterial infection in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby induce immunity against the group A streptococcal bacterial infection in the mammal.
3. A method of treating or preventing a group A streptococcal bacterial infection in a mammal, said method including the step of administering: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; to the mammal to thereby prevent or treat the group A streptococcal bacterial infection in the mammal.
4. The method of any preceding claim, wherein the administering: an isolated protein comprising the amino acid sequence of SEQ ID NO:1, or a fragment or variant thereof; an antibody or antibody fragment that binds or is raised against the isolated protein or fragment or variant thereof; or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; is administered to the mammal intramuscularly.

5. The method of any preceding claim, wherein the immune response is a mucosal immune response.

6. The method of Claim 5, wherein the mucosal immune response is characterized by the production of mucosal IgG.

5 7. The method of Claim 5 or Claim 6, wherein the mucosal immune response is characterized by the substantial absence of mucosal IgA, or at least reduced production of IgA.

8. The method of any preceding claim, wherein the modified p145 peptide amino acid sequence comprises an asparagine (N) amino acid at
10 residue 13 and an arginine (R) amino acid at residue 17.

9. The method of Claim 8, wherein the modified p145 peptide amino acid sequence is set forth in SEQ ID NO:1.

10. The method of Claim 8, wherein the fragment comprises the amino acid sequence set forth in SEQ ID NO:2.

15 11. The method of any preceding claim, wherein the modified p145 peptide, variant or fragment is conjugated, coupled or otherwise linked to a carrier protein.

12. The method of Claim 11, wherein the carrier protein is diphtheria toxin, CRM protein or a fragment thereof.

20 13. The method of any preceding claim, which further includes administering an immunogenic fragment of a SpyCEP protein.

14. The method of Claim 13, wherein the fragment of SpyCEP is, or comprises an S2 peptide (SEQ ID NO:23) or a variant thereof.

25 15. The method of Claim 14, wherein the variant is a K4S2 peptide (SEQ ID NO:24).

16. A composition comprising: an isolated protein comprising: an isolated protein comprising a modified p145 peptide amino acid sequence comprising an amino acid substitution of residues 13 and 17 of SEQ ID NO:3, a fragment or variant thereof, an antibody or antibody fragment that binds or is
30 raised against the isolated protein or fragment or variant thereof or an isolated nucleic acid encoding the isolated protein or fragment or variant thereof; and an immunogenic fragment of a SpyCEP protein or variant thereof, an antibody or antibody fragment that binds or is raised against the SpyCEP immunogenic

fragment or variant thereof or an isolated nucleic acid that encodes the SpyCEP immunogenic fragment or variant thereof.

17. The composition of Claim 16, wherein the modified p145 peptide amino acid sequence comprises an asparagine (N) amino acid at residue 13
5 and an arginine (R) amino acid at residue 17.

18. The composition of Claim 17 wherein the modified p145 peptide amino acid sequence is set forth in SEQ ID NO:1.

19. The composition of Claim 18, wherein the fragment of the modified p145 peptide comprises the amino acid sequence set forth in SEQ ID
10 NO:2.

20. The composition of any one of Claims 16-19, wherein the modified p145 peptide, variant or fragment is conjugated, coupled or otherwise linked to a carrier protein.

21. The composition of Claim 20, wherein the carrier protein is diphtheria toxin, CRM protein or a fragment thereof.
15

22. The composition of any one of Claims 16-21, wherein the fragment of SpyCEP is, or comprises an S2 peptide (SEQ ID NO:23) or a variant thereof.

23. The composition of Claim 22, wherein the variant is a K4S2 peptide (SEQ ID NO:24).
20

24. The composition of any one of Claims 16-23 which further comprises an adjuvant.

25. The composition of any one of Claims 16-24, which is suitable for intramuscular administration.

25 26. The composition of any one of Claims 16-25, for use according to the method of any one of Claims 1-15.