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(54) Title: CHIMERIC POLYPEPTIDES

(57) **Abstract:** A chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide linked to a second polypeptide, wherein: A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*; wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), and wherein the second polypeptide: a) does not comprise: a sequence of a cleaved adhesin domain (CAD) or part thereof, b) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, c) comprises one or more cysteine amino acid substitutions in the adhesin domain, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or d) comprises one or more additional amino acid motif substitutions as defined herein.

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Chimeric polypeptides

Field of the invention

The invention relates to chimeric polypeptides that are useful for inducing an immune response to *P. gingivalis*, compositions comprising same and uses thereof for the prevention and treatment of *P. gingivalis*-related conditions and diseases.

Related application

This application claims priority from Australian provisional application AU 2022900101, the contents of which are incorporated herein by reference.

Background of the invention

If dental plaque is left to accumulate around the tooth at the gingival (gum) margin this causes gingival inflammation (gingivitis). Chronic gingivitis can allow the emergence of a periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) at the base of a periodontal pocket to result in a chronic infection and the development of severe disease. This severe form of periodontal disease is called periodontitis and can lead to tooth loss in an approach by the immune system to eliminate the infection.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth leading to resorption of alveolar bone and eventual tooth loss. The disease is a major public health problem in all societies and is estimated to affect up to 30% of the adult population with severe forms affecting 12-15% of the adult population.

One in three adults have moderate to severe periodontitis. From epidemiological surveys, periodontitis has been linked to an increased risk of inflammatory diseases including cardiovascular diseases, certain cancers, preterm birth, rheumatoid arthritis and dementia. More recent research has linked chronic infection by *P. gingivalis* with dementia and rheumatoid arthritis. For example, in one study, 96% of Alzheimer's disease (AD) brain samples showed the presence of *P. ginigvalis*. Another study shows that chronic oral infection of mice with *P. gingivalis* resulted in the brain plaques associated with AD in humans and that the *P. gingivalis* proteases could cleave amyloid precursor and tau proteins to form the plaques and tangles associated with AD.

A number of virulence factors have been reported to contribute to the pathogenicity of *P. gingivalis* including; LPS, fimbriae, hemagglutinin, hemolysin and extracellular hydrolytic enzymes (especially the Arg-X and Lys-X specific proteinases), otherwise known as "*P. gingivalis* gingipains".

The magnitude of the public health problem is such that there is a need for a vaccine that provides a strong protective response to *P. gingivalis* infection and means for providing same.

One problem has been that it is not clear how to obtain a strong protective response to *P. gingivalis* infection where there are a plethora of virulence factors to select from.

There is currently no commercially approved vaccine for use in preventing or reducing the incidence and/or severity of *P. gingivalis* infection or for treating *P. gingivalis* infection and disease in subjects.

There is therefore a need for alternative and/or improved approaches for the design and manufacture of *P. gingivalis* vaccines, and alternative and/or improved vaccines produced from *P. gingivalis*.

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

Summary of the invention

The present invention provides a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20, (ABM2) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2) SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18, or 22 or 27 or sequences at least 80% identical thereto;

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD) or part thereof, preferably a sequence of a CAD having a sequence set forth in SEQ ID NOs: 12 or 13 or sequences at least 80% identical thereto; and/orEreic

b) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, preferably as set forth in SEQ ID NO: 23, or a sequence at least 80% identical thereto;

comprises one or more amino acid substitutions selected from:

c) one or more cysteine amino acid substitutions in the adhesin domain, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

d) one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide or C-terminally to the second polypeptide. In certain embodiments, at least two further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto may be present. In such embodiments, the two further polypeptides may be located N-terminally to the second polypeptide, C-terminally to the second polypeptide, or N and C terminally to the second polypeptide.

The one or more further polypeptides may be linked to the first or second polypeptide of the chimeric or fusion protein, preferably via a linker of no more than 50 amino acids, or directly linked to the first or second polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In a first aspect, the present invention provides a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18, or 22 or 27 or a sequence at least 80% identical thereto;

and wherein the second polypeptide:

a) does not comprise a sequence of a cleaved adhesin domain (CAD), the CAD preferably having the sequence set forth in SEQ ID NO: 12 or 13 or sequences at least 80% identical thereto;

preferably, wherein the chimeric of fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto.

The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide. The one or more further polypeptides may be linked to the first or second polypeptide of the chimeric or fusion protein, preferably via a linker of no more than 50 amino acids, or directly linked to the first or second polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment of the first aspect of the invention, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In a second aspect, the present invention provides a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide linked to a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM2) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto;

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD) or part thereof, the CAD preferably having a sequence set forth in SEQ ID NOs: 12 or 13 or sequences at least 80% identical thereto; and

b) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, preferably as set forth in SEQ ID NO: 23, or a sequence at least 80% identical thereto.

As used, herein, an amino acid sequence corresponding substantially to the full length of the DUF2436 domain, or a sequence at least 80% identical thereto, refers to a sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the length of a DUF2436 domain of an Arg- or Lys-gingipain.

In a preferred embodiment, the amino acid sequence of the DUF2436 domain of an Arg- or Lys-gingipain is the sequence set forth in SEQ ID NO: 23 or is a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Most preferably, the second polypeptide comprises or consists of the sequence set forth in SEQ ID NO: 34 or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto. In alternative embodiments, the second polypeptide comprises the sequence set forth in SEQ ID NO: 76, or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide. The one or more further polypeptides may be linked to the first or second polypeptide of the chimeric or fusion protein, preferably via a linker of no more than 50 amino acids, or directly linked to the first polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In particularly preferred embodiments of the second aspect of the invention, the chimeric or fusion protein comprises or consists of an amino acid sequence as set forth in any one of SEQ ID NOs: 28, 50, 51, 52, 53 or 54, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In a third aspect of the invention, the present invention provides a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain,

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2) SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or SEQ ID NO: 27 or a sequence at least 80% identical thereto;

and wherein the second polypeptide comprises one or more amino acid substitutions selected from:

a) one or more cysteine amino acid substitutions in the adhesin domain, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

b) one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

The one or more cysteine amino acid substitutions may be a substitution to a serine residue or to a valine residue. Preferably, the one or more cysteine substitutions may comprise one or more substitutions to a serine residue.

In certain embodiments, only one cysteine residue may be substituted. In other embodiments, two or three cysteine residues may be substituted. In particularly preferred embodiments, the cysteine residues are substituted to a combination of valine and serine residues. In other embodiments, all substituted cysteine residues are substituted to serine or all substitute cysteine residues are substituted to valine.

In particularly preferred embodiments, the adhesin domain comprises the DUF2436 domain or part thereof, and the cysteine residue in the DUF2436 domain is substituted to a serine or valine, preferably a serine. In other embodiments, the adhesin domain comprises the DUF2436 domain, and the cysteine residue in the DUF2436 domain is not substituted and preferably, one or more cysteine residues in the remainder of the adhesin domain are substituted.

Optionally, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 23 or a sequence at least 80% identical thereto, wherein the cysteine residue at position 115 is substituted to a serine or valine residue.

Optionally, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 25 or a sequence at least 80% identical thereto, wherein the cysteine residue at position 77 is substituted to a serine or valine residue.

Preferably, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 34 or SEQ ID NO: 76 or a sequence at least 80% identical thereto, wherein one or more cysteine residues are substituted to a serine or valine residue.

In particularly preferred embodiments of the third aspect of the invention, there is provided a chimeric or fusion protein, for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain,

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or SEQ ID NO: 27 or a sequence at least 80% identical thereto;

and wherein the second polypeptide

c) comprises one or more cysteine to serine amino acid substitutions compared to the naturally occurring adhesin domain sequence; and

d) comprises a proline substitution and/or an asparagine substitution, in the sequence PxxN corresponding, or at a position equivalent to, residues at positions 6 to 9 of the sequence of SEQ ID NO: 14 or 19.

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

In a preferred embodiment, the second polypeptide comprises an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*, wherein the adhesin domain comprises the sequence set forth in SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto; and wherein one or both cysteine residues in SEQ ID NO: 18 or 22 are substituted to a serine residue and wherein the proline and/or asparagine residues in the sequence PxxN at positions 63 to 66 of SEQ ID NO: 18 or SEQ ID NO: 22 (corresponding to positions 6 to 9 of SEQ ID NOs: 14 or 19), or positions equivalent to, are substituted. Optionally, the proline residue is substituted to an alanine residue and/or the asparagine residue is substituted to a proline or alanine residue, preferably wherein the proline is substitute to alanine, and the asparagine is substituted to proline, such that the sequence at positions 63 to 66 of SEQ ID NO: 18 or SEQ ID NO: 22 is AxxP (eg AVQP).

In a particularly preferred embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34 or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues. Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions

208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues.

In a particularly preferred embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34, or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues and wherein the proline and asparagine residues in the sequence PxxN at positions 235 to 238, or positions equivalent to, are substituted. Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues, and wherein the proline residue at position 235, or position equivalent thereto, is substituted to an alanine residue and the asparagine residue at position 238, or a position equivalent thereto, is substituted to a proline.

In particularly preferred embodiments, the second polypeptide comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 35 to 49, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%,

83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In preferred embodiments of the third aspect of the invention, the chimeric or fusion protein comprises or consists of the amino acid sequence as set forth in any of SEQ ID NO: 55, 56, or 57, or sequences at 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In a fourth aspect of the invention, the present invention provides: a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to some or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto;

and wherein the second polypeptide:

a) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, preferably as set forth in SEQ ID NO: 23, or a sequence at least 80% identical thereto; and

one or more amino acid substitutions selected from:

b) one or more cysteine amino acid substitutions in the adhesin domain, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

d) one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

The one or more cysteine amino acid substitutions may be a substitution to a serine residue or a valine residue. Preferably, the one or more cysteine substitutions may comprise one or more substitutions to a serine residue.

In certain embodiments, only one cysteine residue is substituted. In other embodiments, two or three cysteine residues are substituted. In particularly preferred

embodiments, the cysteine residues are substituted to a combination of valine and serine residues. In other embodiments, all substituted cysteine residues are substituted to serine or all substitute cysteine residues are substituted to valine.

In particularly preferred embodiments, the adhesin domain comprises the DUF2436 domain, and the cysteine residue in the DUF2436 domain is substituted to a serine or valine, preferably a serine. In other embodiments, the adhesin domain comprises the DUF2436 domain, and the cysteine residue in the DUF2436 domain is not substituted and preferably, one or more cysteine residues in the remainder of the adhesin domain are substituted.

Preferably, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 34 or SEQ ID NO: 76, or a sequence at least 80% identical thereto, wherein one or more cysteine residues are substituted to a serine or valine residue.

In accordance with this aspect of the invention, the sequence PxxN, corresponding to or at a position equivalent to residues 235 to 238 of SEQ ID NO: 34, comprises a substitution of the proline and asparagine residues. Preferably, the substitution is from PxxN to AxxP.

In a particularly preferred embodiment of this aspect of the invention, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34, or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues. Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues.

In a particularly preferred embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34 or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues and wherein the proline and asparagine residues in the sequence PxxN at positions 235 to 238, or positions equivalent to, are substituted. Preferably the cysteine residue at

residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues, and wherein the proline residue at position 235, or position equivalent thereto, is substituted to an alanine residue and the asparagine residue at position 238, or a position equivalent thereto, is substituted to a proline.

In particularly preferred embodiments, the second polypeptide comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 35 to 49, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In especially preferred embodiments, the chimeric or fusion protein comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 62 to 63, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Preferably, the chimeric of fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least

80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In a fifth aspect of the invention, the present invention provides: a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide linked to a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to part or all of the ABMs between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto;

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD), or part thereof, preferably a sequence of a CAD having the amino acid sequence set forth in SEQ ID NO: 12 or 13 or sequences at least 80% identical thereto; and

b) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, preferably as set forth in SEQ ID NO: 23, or a sequence at least 80% identical thereto; and

comprises one or more amino acid substitutions:

c) one or more cysteine amino acid substitutions in the amino acid sequence of the DUF 2436 domain and ABMs compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or (preferably and)

d) one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of

the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

The one or more cysteine amino acid substitutions may be a substitution to a serine residue or a valine residue. Preferably, the one or more cysteine substitutions may comprise one or more substitutions to a serine residue.

In certain embodiments, only one cysteine residue is substituted. In other embodiments, two or three cysteine residues are substituted. In particularly preferred embodiments, the cysteine residues are substituted to a combination of valine and serine residues. In other embodiments, all substituted cysteine residues are substituted to serine or all substitute cysteine residues are substituted to valine.

In particularly preferred embodiments, the adhesin domain comprises the DUF2436 domain, and the cysteine residue in the DUF2436 domain is substituted to a serine or valine, preferably a serine. In other embodiments, the adhesin domain comprises the DUF2436 domain, and the cysteine residue in the DUF2436 domain is not substituted and preferably, one or more cysteine residues in the remainder of the adhesin domain are substituted.

Preferably, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 34 or a sequence at least 80% identical thereto, wherein one or more cysteine residues are substituted to a serine or valine residue.

In accordance with this aspect of the invention, the sequence PxxN, corresponding to or at a position equivalent to residues 235 to 238 of SEQ ID NO: 34, comprises a substitution of the proline and asparagine residues. Preferably, the substitution is from PxxN to AxxP.

In a particularly preferred embodiment of this aspect of the invention, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34 or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues. Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the

cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues.

In a particularly preferred embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 34, or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues and wherein the proline and asparagine residues in the sequence PxxN at positions 235 to 238, or positions equivalent to, are substituted. Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue. Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent thereto, are substituted to serine residues, and wherein the proline residue at position 235, or position equivalent thereto, is substituted to an alanine residue and the asparagine residue at position 238, or a position equivalent thereto, is substituted to a proline.

In particularly preferred embodiments, the second polypeptide comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 35 to 49, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In especially preferred embodiments, the chimeric or fusion protein comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 58 to 61, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In a sixth aspect of the invention, the present invention provides: a chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide linked to a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs), preferably wherein the ABMs correspond to part or all of the ABMs

between the DUF2436 domain and cleaved adhesin domain (CAD) of a *P. gingivalis* gingipain;

preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

more preferably wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20 (ABM2), SEQ ID NO: 14 or 19 (ABM1) and/or SEQ ID NO: 17 or 21 (ABM3) or sequences at least 80% identical thereto,

most preferably wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto;

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD), or part thereof, preferably a sequence of a CAD having the amino acid sequence set forth in SEQ ID NO: 12 or 13 or sequences at least 80% identical thereto; and

and comprises one or more amino acid substitutions selected from:

b) one or more cysteine amino acid substitutions in the amino acid sequence of the ABMs compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

c) one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN (eg PVQN) corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ (eg NEFA to SEYQ) in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

It will be appreciated that in this aspect of the invention, preferably the chimeric or fusion protein not comprise an amino acid sequence corresponding to or derived from a DUF2436 domain of an Arg- or Lys-gingipain, such as set forth in SEQ ID NO: 23, or a sequence at least 80% identical thereto.

In accordance with the 6th aspect of the invention, the one or more cysteine amino acid substitutions may be a substitution to a serine residue or a valine residue. Preferably, the one or more cysteine substitutions may comprise one or more substitutions to a serine residue.

Optionally, only one cysteine residue is substituted. In other embodiments, two cysteine residues are substituted. In particularly preferred embodiments, the cysteine residues are substituted to a combination of valine and serine residues. In other embodiments, all substituted cysteine residues are substituted to serine or all substitute cysteine residues are substituted to valine.

Preferably, the adhesin domain comprises or consists of the sequence set forth in SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto, wherein one or more cysteine residues are substituted to a serine or valine residue.

In accordance with this aspect of the invention, the sequence PxxN (eg PVQN), corresponding to or at a position equivalent to residues 63 to 66 of SEQ ID NO: 18, comprises a substitution of the proline and asparagine residues. Preferably, the substitution is from PxxN to AxxP, (eg AVQP).

In a particularly preferred embodiment of this 6th aspect of the invention, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 18 or a sequence at least 80% identical thereto, wherein one or two cysteine residues are substituted to serine residues.

In a particularly preferred embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 18, or a sequence at least 80% identical thereto, wherein one or two cysteine residues are substituted to serine residues and wherein the proline and asparagine residues in the sequence PxxN at positions 63 to 66, or positions equivalent to, are substituted.

In any embodiment of the 6th aspect of the invention, the first and second polypeptides may be joined directly, or joined with a linker. Preferably the linker comprises a series of amino acid sequences, preferably of between about 1 amino acid residue to about 20 amino acid residues, preferably no more than about 5 amino acids or no more than about 10 amino acids in length or no more than about 15 amino acids in length.

In preferred embodiments, the first and second polypeptides (with representative amino acid sequences of SEQ ID NOs: 1 and 18, respectively (ie the amino acid sequences without the amino acid substitutions described herein) may be linked via a peptide sequence having the amino acid sequence DMEVEDDSP.

In especially preferred embodiments, the chimeric or fusion protein comprises or consists of a sequence as set forth in any one of SEQ ID NOs: 80 or 81, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least

80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or wherein the amino acid sequences are identical. The first polypeptide and the further polypeptide may be derived from the active site of heterologous gingipains (e.g., from gingipains of different strains of *P. gingivalis*). The first polypeptide and the further polypeptide may have an amino acid sequence that is derived from different gingipains (e.g., wherein one of the polypeptides has an amino acid sequence of an active site from a Kgp and the other polypeptide has an amino acid sequence of an active site from an Rgp; or alternatively wherein one of the polypeptides has an amino acid sequence of an active site from a RgpA and the other polypeptide has an amino acid sequence of an active site from an RgpB).

In any embodiment of any aspect of the invention, the chimeric or fusion protein consists or consists essentially of the sequences of the first and second polypeptides as defined herein. It will be appreciated therefore that the chimeric or fusion proteins comprise an arrangement or configuration of domains that differs to the configuration of those domains in naturally occurring gingipain polyprotein sequences. In other words, the first and second polypeptides and domains therein have a differential spatial configuration to naturally occurring gingipain polyproteins.

In any embodiment of any aspect of the invention, the first and second polypeptides are linked. The first and second polypeptides may be linked directly, via a linker, or via a polypeptide sequence of no more than 100, preferably no more than 50 amino acids. Preferably the first and second polypeptides are directly linked, or linked by no more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids. Most preferably, the first and second polypeptides are directly linked.

In any embodiment of any aspect of the invention, the C-terminal residue of the first polypeptide may be linked to the N-terminal residue of the second polypeptide, directly, via a linker, or via a polypeptide sequence of no more than 50 amino acids.

Alternatively, the N-terminal residue of the first polypeptide may be linked to the C-terminal residue of the second polypeptide, directly, via a linker, or via a polypeptide sequence of no more than 50 amino acids.

In any of the 2nd to 5th aspects of the invention, the DUF2436 domain and ABM domains derived from an Arg- or Lys-gingipain, may be directly linked or joined via a linker, or via a polypeptide sequence. Preferably, the DUF2436 and ABM domains are linked via a short linker sequence comprising about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids. In a particularly preferred embodiment, the DUF and ABM domains are linked via a short linker sequence of no more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids.

In preferred embodiments, the DUF2436 and ABM domains (with representative amino acid sequences of SEQ ID NOs: 23 and 18, respectively (ie the amino acid sequences without the amino acid substitutions described herein) may be linked via a peptide sequence having the amino acid sequence EVEDDSP.

In any embodiment, the further polypeptide may be joined directly or via a linker to the chimeric or fusion protein comprising the first and second polypeptides. In embodiments where the further polypeptide is joined to the C terminal region of the fusion protein via the second polypeptide, preferably the C terminus of the second polypeptide is joined directly to the N terminus of the further polypeptide. (For example, the C terminus of the adhesin domain is preferably joined directly to the N terminus of the active site amino acid sequence).

In instances where more than one further polypeptide is included (eg as exemplified in SEQ ID NO: 54, the copies of the further polypeptide may be directly joined to each other or joined via a linker sequence.

In any embodiment of any aspect of the invention, the linker region is an amino acid sequence of no more than 15 amino acids, and preferably greater than 2 amino acids. Suitable linkers for use in protein constructs, including those with minimal impact on solubility are known in the art. Useful linkers include glycine-serine (GlySer) linkers, which are well-known in the art and comprise glycine and serine units combined in various orders. Examples include, but are not limited to, (GS), (GSGGS)n, (GGGS)n and

(GGGGS)n, where n is an integer of at least one, typically an integer between 1 and about 10, for example, between 1 and about 8, between 1 and about 6, or between 1 and about 5. Other useful linkers include DSSG, DSSGAS, KLDSSG or others described herein. In certain embodiments, the linker region may be derived from the native ginigipain polyprotein sequence.

The present invention also provides a nucleic acid encoding any chimeric or fusion protein as defined herein.

Preferably, the nucleic acid has a nucleotide sequence that encodes any one or more of the amino acid sequences define in Table 1 herein.

In any embodiment, such a nucleic acid is included in an expression construct in which the nucleic acid is operably linked to a promoter. Such an expression construct can be in a vector, e.g., a plasmid, or viral vector.

The present invention also provides a cell comprising a nucleic acid, or nucleic acid vector as herein described. Examples of cells of the present invention include bacterial cells, yeast cells, insect cells or mammalian cells. Preferably, the cell is isolated, substantially purified or recombinant.

The present invention also provides a composition comprising a chimeric or fusion protein as described herein, optionally in combination with a pharmaceutically acceptable carrier.

The composition may also comprise an adjuvant for potentiating an immune response to the chimeric or fusion protein.

The present invention accordingly further provides for a vaccine or immune stimulating composition for inducing an immune response to *P. gingivalis* in a subject, the composition comprising:

i) an immunogen in the form of a chimeric or fusion protein as described herein, and

ii) an adjuvant, for potentiating the immune response to the immunogen in the subject.

Preferably, the sole immunogen provided in the compositions, vaccines or immune stimulating compositions of the invention, is a chimeric or fusion protein as herein described.

The present invention also provides a method for inducing an immune response in a subject to *P. gingivalis*, the method comprising administering to a subject in need thereof, a chimeric or fusion protein, vaccine or immune stimulating composition as described herein.

The present invention also provides a method of inducing a humoural immune response to *P. gingivalis* in a subject, the method comprising administering to the subject, a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined.

Preferably the immune response that is induced comprises a switch from a Th1 to a Th2 immune response.

It will be appreciated that in any embodiment, the immune response elicited by administration of a chimeric or fusion protein described herein, or vaccine or other composition comprising the same, is preferably antigen-specific. Accordingly, in preferred embodiments, the methods and compositions and chimeric proteins described herein, are for inducing an immune response, preferably a protective immune response, to *P. gingivalis* gingipain antigens.

In any embodiment, the compositions, chimeric proteins and methods of the invention may be for strengthening an immune response (such as a protective immune response) of a subject to *P. gingivalis*.

The present invention also provides for methods of immunising a subject against *P. gingivalis* infection, the method comprising administering to the subject, a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined.

In any embodiment, a subject who has received or has been administered a chimeric or fusion protein of the invention, or composition or vaccine including the same, has an increased level of protection against infection with *P. gingivalis*, or severity of one

or more symptoms of *P. gingivalis* infection, compared to a subject who has not received the protein, composition or vaccine.

Further still, the invention provides a method of treating a *P. gingivalis* infection in a subject, the method comprising administering to a subject in need thereof, a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined, thereby treating the *P. gingivalis* infection in the subject.

The present invention provides a method for reducing or minimising the severity of a symptom associated with an infection with *P. gingivalis*, comprising administering to an individual in need thereof, a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined, wherein the symptoms are selected from the group consisting of swollen or puffy gums, gums that bleed easily, receding gums, periodontal pockets around the teeth, loss of tooth supporting tissues (periodontal ligament, cementum and/or alveolar bone) pus between gums and teeth, and gingivitis.

The present invention also provides a method for treating *P. gingivalis*-related disease in a subject, the method comprising administering to an individual in need thereof, a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined. Preferably the *P. gingivalis*-related disease comprises periodontal disease.

The present invention provides for a method of treatment comprising administration of a chimeric or fusion protein, composition, vaccine or immune stimulating composition of the invention may also comprise administration of one or more of: an antimicrobial compound, an anti-inflammatory agent.

The invention also provides use of a chimeric or fusion protein as herein defined, in the manufacture of a medicament for:

- inducing an immune response (preferably a protective immune response) in a subject to *P. gingivalis;*
- immunising a subject against *P. gingivalis* infection;
- treating a *P. gingivalis* infection in a subject;
- minimising or reducing the severity of one or more symptoms of *P. gingivalis* infection; or

- treating *P. gingivalis*-related disease in a subject.

The invention also provides a chimeric or fusion protein, composition, vaccine or immune stimulating composition as herein defined, for use in:

- inducing an immune response (preferably a protective immune response) in a subject to *P. gingivalis;*
- immunising a subject against *P. gingivalis* infection;
- treating a *P. gingivalis* infection in a subject;
- minimising or reducing the severity of one or more symptoms of *P. gingivalis* infection; or
- treating *P. gingivalis*-related disease in a subject.

In any method, use or protein, composition or vaccine for use according to the invention, the subject may be any subject that has or is at risk of having an infection with *P. gingivalis*. The subject may be a human. The subject may be a veterinary subject, such as a companion animal that has, or is at risk of having an infection with *P. gingivalis*.

The present invention also provides a method for obtaining an antibody directed to *P. gingivalis,* the method comprising administering a chimeric or fusion protein, composition, vaccine or immune stimulating composition of the invention, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal. Preferably the method further comprises isolating the antibody from the animal (eg, extracting from the blood of the animal) or from an egg thereof (in the case where the animal is an avian species, preferably chicken).

The present invention also provides an antibody preparation comprising an antibody directed to *P. gingivalis,* wherein the antibody preparation is obtained by administering a chimeric or fusion protein, composition, vaccine or immune stimulating composition of the invention, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal, and isolating the antibodies from the animal or egg thereof.

The antibody directed to *P. gingivalis* may be used therapeutically to eliminate or reduce *P. gingivalis* infection or prophylactically, to prevent or reduce the severity of *P. gingivalis* infection.

The present invention also provides a kit comprising a composition comprising a chimeric or fusion protein as herein defined, wherein optionally the kit comprises one or more cytokines and/or adjuvants in sealed containers.

Preferably, the kit comprises a label or package insert indicating that the composition is used for immunising an individual, optionally wherein the label or package insert includes instructions for use.

Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. Thus, use of the term "comprising" and the like indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements (e.g., 1, 2, 3, 4, 5, 6,7, 8,9 ,10, 11, 12 13, 14, 15, 16, 17,18, 19, 20, or more than 20 additional amino acid residues at the N-terminus or C-terminus of a polypeptide sequence) are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

Brief description of the drawings

Figure 1. A. Mouse periodontitis model – therapeutic vaccination. **B.** Schematic showing the domain structure of Kgp gingipain polyprotein and components derived therefrom for generating the chimeric or fusion proteins of the invention.

Figure 2. **A**. SDS-PAGE of *E coli* fractions expressing recombinant variants with or without a K1 domain and containing either a full length or truncated DUF domain. Low temperature and low IPTG conditions used. A full length DUF is essential for solubility. A full length K1 is detrimental to solubility. **B.** Native PAGE of chimera structures KDA and KDAK1 in the presence of absence of DTT. Addition of K1 domain to KDA reduces solubility but the proteins multimerise.

Figure 3. **Elimination of multimerisation A.** Diagrammatic representation of Kgp polyprotein showing positions of ABM1 and ABM2 domains. **B-D.** NATIVE_PAGE analysis of purified recombinant protein variants subject to electrophoresis in the presence or absence of DTT. **B:** Study of "PVQN" mutant variants. **C:** Study of "PVQN" mutant variants with additional Cys>Ser mutations. **D:** Study of mutations in loop preceeding "PVQN" motif (lanes 2-4) and highly conserved hydrophobic Tyr and Trp residues in ABM2 and ABM1 respectively (lanes 5-7). **E.** NATIVE PAGE analysis of purified chimera KDcAK1n and recombinant ABM21 with Cysteine and PVQP mutations. KDcAK1n was purified from Inclusion bodies and recombinant ABM21 was purified as a soluble protein from *E coli* expressing strains. **F.** NATIVE PAGE analysis of small scale purified recombinant variants. G. (i) 4-12% SDS gel and (ii) 3-12% Native gel of various vaccine candidates. Lanes 1 & 2: KDAK-3S-AVQP (1: unreduced; 2: reduced); Lanes 3 & 4: KDAK-1V-2S-AVQP(3: unreduced; 4:reduced); Lanes 5 & 6: KDAK-1V-AVQP (5: unreduced; 6: reduced); Lanes 7 & 8: KDAK-AVQP (7: unreduced; 8: reduced); Lanes 9 & 10: KDcAK1n (9: unreduced; 10: reduced). Gels were stained with Coomassie blue.

Figure 4. Bone loss study. Statistical analysis – One-way ANOVA and post-hoc Dunnet's T3. # significantly different from the Control Challenge group.

Figure 5. Antibody isotype response (individual sera) of antigens towards heat-killed *P. gingivalis* whole cells. Naïve = Control Non Challenge. Gingivalis = Control Challenge with *P. gingivalis*. Chimera = KDcAK1n antigen. Amox = amoxicillin.

Figure 6. Bone loss study. Statistical analysis – One-way ANOVA and post-hoc Dunnet's T3. # significantly different from the Control Challenge group.

Figure 7. Bone loss study Statistical analysis – One-way ANOVA and post-hoc Dunnet's T3. #p<0.01; ##p<0.05 (T test) significantly different from the Control Challenge group.

Figure 8. Bone loss study. S = soluble fraction; Urea = urea used in purification; AC = affinity column purification; IB = inclusion body; Batch = batch purification method. Statistical analysis: one-way ANOVA and post-hoc Dunnet's T3. #p<0.01 significantly different from the Control Challenge group.

Figure 9 Bone loss study study following immunisation of animals with various fusion protein constructs. Statistical analysis: one-way ANOVA and post hoc Dunnet's T3 Significantly different from the Control Challenge group # p<0.05 ## p<0.01 ### p<0.001 #### p<0.0001.

Figure 10 Bone loss study study following immunisation of animals with various fusion protein constructs. Statistical analysis: one-way ANOVA and post-hoc Dunnet's T3. ### (p<0.05); #### (p<0.01); significantly different from the Control Challenge group.

Figure 11 Bone loss study following immunisation of animals with various fusion protein constructs. Statistical analysis: one-way ANOVA and post-hoc Dunnet's T3. # (p<0.05) significantly different from the Control Challenge group.

Figure 12 Bone loss study following immunisation of animals with various fusion protein constructs. Other than KDcAK1n, all antigens tested also comprises the "AVQP" substitution; ie, KDAK-3S = KDAK-3S-AVQP; KDAK-1V2S = KDAK-1V2S-AVQP; KDAK-3S-Tag = KDAK-3S-AVQP-Tag; Statistical analysis: one-way ANOVA and post-hoc Dunnet's T3. * (p<0.05) significantly different from the naïve (ie Control Non-Challenged group).

Figure 13 Purity of the final products of the *P. gingivalis* antigens tested in Example 4. PAGE analysis of His-tagged antigens (A and B) and tagless KDAK-3S-AVQP (C). (A) SDS-PAGE; (B) Native PAGE or on native gels. (C) SDS- and native PAGE or on native gel with loaded samples being heated (95°C) or unheated. R: gel loaded

samples reduced; NR: gel loaded samples not reduced; M: protein standards. His-tagged KDAK-3S-AVQP was purified in parallel with the other His-tagged proteins.

Figure 14 Bone loss study following immunisation of animals with various fusion protein constructs. Statistical analysis – One-way ANOVA and post-hoc Dunnet's T3. # ($p<0.05$, compared to naïve control (ie Control Non-Challenge)); ## ($p<0.05$, compared to infected control (ie Control Challenge)).

Figure 15 Antibody titres in mouse sera (individual) towards heat-killed *P. gingivalis* whole cells. (A) Total IgG Titres; (B) IgG1 Subtype titres; (C) IgG2a Subtype titres.

Figure 16 Dose response trend of exemplary fusion protein of invention, with periodontal bone loss protection with Kgpcat cross-reactive Ab titre.

Sequence information

Table 1: sequence information

Descriptor	SEQ ID No	Sequence
KAS (active site domain)	1	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK
Kgp active site [432-468]	2	LNTGV[G/S]FANYTAHGSET[S/A]WADP[S/L][L/V]T[A/T][T/S]Q[V/L]KALTNK[D/N]K
RgpA active site [426-462]	3	FNGGISL[V/A]NYTGHGSETAWGTSHFGTTHVKQLTNSNQ
KAS1	4	LNTGV[G/S]FANYTAHGSET[S/A]WADP[S/L]
KAS2	5	NTGV[G/S]FANYTAHGSET[S/A]WADP[S/L][L/V]T[A/T][T/S]Q[V/L]KALTNK[D/N]K
KAS3	6	V[G/S]FANYTAHGSET[S/A]WADP[S/L][L/V]
PAS1K	7	LNTGVSFANYTAHGSETAWADP
RAS1	8	FNGGISL[V/A]NYTGHGSETAWGTSH
RAS2	9	NGGISL[V/A]NYTGHGSETAWGTSHFGTTHVKQLTNSNQ
RAS3	10	ISL[V/A]NYTGHGSETAWGTSHF

PAS1R	11	FNGGISLANYTGHGSETAWGT
K1 (CAD domain)	12	TLSESFENGIPASWKTIDADGDGHGWKPGNAPGIAGYNSNG CVYSESFGLGGIGVLTPDNYLITPALDLPNGGKLTFWVCAQDA NYASEHYAVYASSTGNDASNFTNALLEETITAKGVRSPKAIRG RIQGTWRQKTVDLPAGTKYVAFRHFQSTDMFYIDLDEVEIKAN GKR
K1n (truncated CAD domain K1)	13	TLSESFENGIPASWKTIDADGDGHGWKPGNAPGIAGYNSNG CVYSESFGLGGIGVLTPDNYLITPALDLPNGG
ABM1 Kgp [942-970]	14	SNEFAPVQNLTGSSVGQKVTLKWDAPNGT
ABM2 Kgp [885-941] Tyrosine site for substitution to alanine underlined	15	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYT AGVSPKVCKDVTVEG
ABM2+1 peptide [885-970] NEFA sequence motif bolded	16	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYT AGVSPKVCKDVTVEGS**NEFA**PVQNLTGSSVGQKVTLKWDAP NGT
ABM3 Kgp [971-983]	17	PNPNPNPNPNPGT
ABM2+1+3 peptide (Cys residues underlined) [885-983] NxFA sequence motif bolded; PxxN sequence motif italicised	18	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYT AGVSPKVCKDVTVEGS**NEFA***PVQN*LTGSSVGQKVTLKWDAP NGTPNPNPNPNPNPGT

ABM1 RgpA [921-949]	19	SNEFAPVQNLTGSAVGQKVTLKWDAPNGT
ABM2 RgpA [864-920]	20	SDYTYTVYRDGTKIKEGLTATTFEEDGVA(A/T)GNHEYCVEVK YTAGVSPKVCKDVTVEG
ABM3 Rgp [950-964]	21	PNPNPNPNPNPNPGT
ABM 2+1+3 Rgp [864+964]	22	SDYTYTVYRDGTKIKEGLTATTFEEDGVA(A/T)GNHEYCVEVK YTAGVSPKVCKDVTVEGSNEFAPVQNLTGSAVGQKVTLKWD APNGTPNPNPNPNPNPNPGT
DUF2436 (Cys underlined)	23	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDM
DUF2436 Cys to Ser/Val substitution	24	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[**S/V**]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDM
Dc (Truncated DUF2436) (Cys underlined)	25	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD M
Dc Cys to Ser substitution	26	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDY**S**ITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD M
Truncated DUF2436+AMB 2+1+3 Dc underlined	27	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD MEVEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHE YCVEVKYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQK VTLKWDAPNGTPNPNPNPNPNPGT
KDAK (ie K domain, full DUF	28	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV

domain, ABM2+1+3, K domain)		VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
AVQP ABM1	29	SNEFAAVQPLTGSSVGQKVTLKWDAPNGT
AVQP ABM213	30	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYT AGVSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAP NGTPNPNPNPNPNPGT
ABM213 Cys #1 to Ser	31	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTA GVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPN GTPNPNPNPNPNPGT
ABM213 Cys #2 to Ser	32	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYT AGVSPKVSKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAP NGTPNPNPNPNPNPGT
ABM213 Cys #1+ #2 to Ser	33	ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTA GVSPKVSKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPN GTPNPNPNPNPNPGT
DUF2436+ABM 213	34	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val (underlined) + ABM213	35	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYC VEVKYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT

DUF2436 + ABM213 Cys#1 to Ser (underlined)	36	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY<u>S</u>VEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val + ABM213 Cys #1 to Ser (underlined)	37	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY<u>[S/V]</u>ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY<u>S</u> VEVKYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT
DUF2436 + ABM213 Cys#2 to Ser (underlined)	38	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKV<u>S</u>KDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val + ABM213 Cys #2 to Ser (underlined)	39	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY<u>[S/V]</u>ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYC VEVKYTAGVSPKV<u>S</u>KDVTVEGSNEFAPVQNLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT
DUF2436 +ABM213 Cys #1 + #2 to Ser (underlined)	40	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY<u>**S**</u>VEV KYTAGVSPKV<u>**S**</u>KDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT

DUF2436 + ABM213 all 3 Cys residues to Ser or Val (underlined)	41	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY**[S/V]**ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY**S** VEVKYTAGVSPKV**S**KDVTVEGSNEFAPVQNLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT
DUF2436 + ABM213 AVQP (underlined)	42	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val + ABM213 AVQP	43	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYC VEVKYTAGVSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPNPGT
DUF2436 +ABM213 Cys#1 to Ser + AVQP	44	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEV KYTAGVSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val + ABM213 Cys#2 to Ser +AVQP (underlined)	45	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYC VEVKYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPNPGT

DUF2436 + ABM213 Cys#2 to Ser+ AVQP (underlined)	46	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT
DUF2436 Cys to Ser/Val + ABM213 Cys#2 to Ser + AVQP (underlined)	47	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYC VEVKYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT
DUF2436 +ABM213 Cys#1 + 2 to Ser + AVQP (underlined)	48	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEV KYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGT
DUF2436 +ABM213 All 3 Cys to Ser + AVQP (underlined)	49	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKM WIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDME VEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYS VEVKYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTL KWDAPNGTPNPNPNPNPNPGT
KDA	50	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG

		VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGT
KDAKK	51	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDKNTGVSFANYTAHGSETAWADPLLTTSQLKALTNK DK
KDAR	52	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNGGISL[V/A]NYTGHGSETAWGTSHFGT THVKQLTNSNQ
KKDA	53	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKKLNTGV SFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSREVKRI GDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFLLDAD HNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPVVTTQ NIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGNQPA RYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPASYTYT VYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAGVSPK VCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNGTPNP NPNPNPNPGT
KKDAKK (linkers underlined)	54	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK<u>DSSGAS</u> NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN

		QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDKKLDSSGNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KDcAK1nK-4S-AVQP	55	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEV KYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG HGWKPGNAPGIAGYNSNGSVYSESFGLGGIGVLTPDNYLITP ALDLPNGGNTGVSFANYTAHGSETAWADPLLTTSQLKALTNK DK
KDcAK1n-4S	56	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEV KYTAGVSPKVSKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG HGWKPGNAPGIAGYNSNGSVYSESFGLGGIGVLTPDNYLITP ALDLPNGG
KDcAK1n-4S-AVQP	57	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEV KYTAGVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG HGWKPGNAPGIAGYNSNGSVYSESFGLGGIGVLTPDNYLITP ALDLPNGG

KDA-1S/V-2S-AVQP (S/V option at Cys in D domain)	58	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY[S/V]ITNPEPASGKMWIAGDG GNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSP ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTA GVSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPN GTPNPNPNPNPNPGT
KDAK-1S/V-2S-AVQP (S/V option at Cys in D domain bold underline; cysteine to serine substitutions in ABM domains underlined; AVQP italicised)	59	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**[S/V]**ITNPEPASGKMWIAGDG GNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSP ASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTA GVSPKVSKDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKWDAPN GTPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTS QLKALTNKDK
KDA-2S-AVQP (Cys # 2 and 3 in ABM domains substituted to Ser)	60	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGT
KDAK-2S-AVQP (Cys in DUF domain unsubstituted – bold underline;	61	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**C**ITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS

Cys # 2 and 3 in ABM domains (underlined) substituted to Ser) AVQP italicised		YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KDAK1n-4S-AVQP	62	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDGHGWK PGNAPGIAGYNSNGSVYSESFGLGGIGVLTPDNYLITPALDLP NGG
KDAK1nK-4S-AVQP	63	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDGHGWK PGNAPGIAGYNSNGSVYSESFGLGGIGVLTPDNYLITPALDLP NGGNTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK
KDcAK1n	64	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG

		HGWKPGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITP ALDLPNGG
KDAK1n	65	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDGHGWK PGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITPALDLP NGG
KDAK1	66	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG HGWKPGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITP ALDLPNGGKLTFWVCAQDANYASEHYAVYASSTGNDASNFT NALLEETITAKGVRSPKAIRGRIQGTWRQKTVDLPAGTKYVAF RHFQSTDMFYIDLDEVEIKANGKR
KDcAK1nK Active site underlined	67	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKWGDNT GYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDG HGWKPGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITP ALDLPNGGNTGVSFANYTAHGSETAWADPLLTTSQLKALTNK DK

KDAK1nK Active site underlined	68	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDGHGWK PGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITPALDLP NGGNTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK
KDAK-3S-AVQP [active site underlined] 3 cysteine to S substitutions bold and underline; AVQP italicised	69	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**S**ITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY**S**VEVKYTAG VSPKV*S*KDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KDAK-1V-AVQP	70	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**V**ITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KDAK-AVQP	71	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG

		TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KDAR-1V-2S-AVQP Active sites underlined	72	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**V**ITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY**S**VEVKYTAG VSPKV**S**KDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNGGISLANYTGHGSETAWGTSHFGTTH VKQLTNSNQ
KDAR-1V-AVQP	73	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDY**V**ITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNGGISLANYTGHGSETAWGTSHFGTTH VKQLTNSNQ
KDAR-3S-AVQP (cysteine to serine substitutions underlined; AVQP italicized)	74	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNGGISLANYTGHGSETAWGTSHFGTTH VKQLTNSNQ
KDAR-AVQP	75	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS

		YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAAVQPLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNGGISLANYTGHGSETAWGTSHFGTTH VKQLTNSNQ
DUF2436 + ABM2+1	76	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGT
KDAK-3S K domain underlined	77	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYSITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYSVEVKYTAG VSPKVSKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPLLTTSQL KALTNKDK
KKDAK1nKK K domain underlined Linker in bold	78	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK**DSSGAS** NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKAEGSRE VKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDNTGYQFL LDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPANADPV VTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIAGDGGN QPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVEDDSPAS YTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEVKYTAG VSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKWDAPNG TPNPNPNPNPNPGTTLSESFENGIPASWKTIDADGDGHGWK PGNAPGIAGYNSNGCVYSESFGLGGIGVLTPDNYLITPALDLP NGGNTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK**KLD SSG**NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDK
DUF2436 + ABM2+3	79	AEGSREVKRIGDGLFVTIEPANDVRANEAKVVLAADNVWGDN TGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANFEYLIPA NADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASGKMWIA

		GDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGPNPNPNPNPNPGT
KAK	80	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEYCVEV KYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPL LTTSQLKALTNKDK
KAK-2S-AVQP (2 cysteines in ABM domains substituted to serine; underlined; AVQP italicized)	81	NTGVSFANYTAHGSETAWADPLLTTSQLKALTNKDKDMEVED DSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHEY<u>S</u>VEV KYTAGVSPKV<u>S</u>KDVTVEGSNEFA*AVQP*LTGSSVGQKVTLKW DAPNGTPNPNPNPNPNPGTNTGVSFANYTAHGSETAWADPL LTTSQLKALTNKDK
Dc+2+1+3	82	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD MEVEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHE YCVEVKYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQK VTLKWDAPNGTPNPNPNPNPNPGT
Dc+2+3	83	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD MEVEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHE YCVEVKYTAGVSPKVCKDVTVEGPNPNPNPNPNPGT
Dc+2+1	84	WGDNTGYQFLLDADHNTFGSVIPATGPLFTGTASSNLYSANF EYLIPANADPVVTTQNIIVTGQGEVVIPGGVYDYCITNPEPASG KMWIAGDGGNQPARYDDFTFEAGKKYTFTMRRAGMGDGTD MEVEDDSPASYTYTVYRDGTKIKEGLTATTFEEDGVAAGNHE YCVEVKYTAGVSPKVCKDVTVEGSNEFAPVQNLTGSSVGQK VTLKWDAPNGT

Detailed description of the embodiments

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described. It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

All of the patents and publications referred to herein are incorporated by reference in their entirety.

For purposes of interpreting this specification, terms used in the singular will also include the plural and vice versa.

In work leading to the present invention, the inventors investigated various chimeric or fusion proteins for use in inducing immune responses to *P. gingivalis* and methods for large-scale production of such chimeras for use as vaccine candidates.

The inventors identified various problems associated with large-scale manufacturing and production of chimeric or fusion proteins derived from components of *P. gingivalis* gingipains. Firstly, the chimeric or fusion proteins described in the prior art are difficult to produce at sufficiently high quantities, as soluble proteins. More specifically, the chimeric proteins of the prior art are typically produced in inclusion bodies in *E. coli*,

making it challenging to produce large amounts of soluble protein for downstream clinical product development.

Reduced solubility when produced in *E. coli* also substantially contributes to time and scale of manufacturing, as well as poor stability of the proteins once solubilised and refolded from inclusion bodies.

Further still, gingipain-derived chimeric proteins described in the prior art also suffer from multimerisation. The formation of beta-sheets between domains in the chimeric proteins makes an assessment of the final vaccine product challenging, from a regulatory perspective, as well as contributing to potentially reduced immune responses once administered.

The present invention is therefore concerned with improved design of chimeric or fusion proteins for use in inducing an immune response to *P. gingivalis*, and methods and uses comprising the same.

In any embodiment, the chimeric or fusion proteins of the present invention have improved solubility and/or stability compared to chimeric or fusion proteins described in the prior art. Improved solubility and/or stability provides for significant advantages when it comes to large-scale production of fusion proteins for use in a clinical setting.

In alternative embodiments, the chimeric or fusion proteins of the invention have a reduced propensity to aggregate and multimerise compared to chimeric or fusion proteins of the prior art. Aggregation and multimerisation may hinder large-scale manufacturing and production of therapeutic/prophylactic proteins. Consequently, reducing propensity for aggregation and/or multimerisation of the chimeric proteins of the invention provides for a significant improvement over prior art chimeric proteins for use in inducing immune responses to *P. gingivalis.*

Further still, in any embodiment, the chimeric or fusion proteins of the invention have increased immunogenicity compared to chimeric or fusion proteins of the prior art.

Thus, the inventors have identified various new approaches for obtaining a chimeric or fusion protein for inducing an immune response to *P. gingivalis* and which results in a chimeric or fusion protein with one or more advantages over the prior art.

The inventors believe that one or more of the new approaches for generating chimeric or fusion proteins for inducing an immune response to *P. gingivalis*, as described herein, may result in advantages including: increased ease of manufacturing of a suitable immunogenic composition for inducing an immune response to *P. gingivalis*; improved shelf-life and/or improved immunogenicity.

Gingipains

The pathogenicity of *P. gingivalis* is attributed to a number of surface-associated virulence factors that include cysteine proteinases (gingipains), fimbriae, haem-binding proteins, and outer membrane transport proteins amongst others. In particular, the extracellular Arg- and Lys-specific proteinases 'gingipains' (RgpA/B and Kgp) of *P. gingivalis* have been implicated as major virulence factors that are critical for colonisation, penetration into host tissue, dysregulation of the immune response, dysbiosis and disease.

The gingipains, in particular the Lys-specific proteinase Kgp are essential for *P. gingivalis* to induce alveolar bone resorption in the mouse periodontitis model. The gingipains have also been found in gingival tissue at sites of severe periodontitis at high concentrations proximal to the subgingival plaque and at lower concentrations at distal sites deeper into the gingival tissue. Lys-specific and Arg-specific proteinases have been shown to degrade a variety of host proteins *in vitro*, e.g., fibrinogen, fibronectin, and laminin. Plasma host defence and regulatory proteinase inhibitors α-trypsin, α2-macroglobulin, anti-chymotrypsin, antithrombin III and antiplasmin are also degraded by Lys- and Arg- proteinases from *P. gingivalis*. This has led to the development of a cogent mechanism to explain the keystone role played by *P. gingivalis* in the development of chronic periodontitis.

The RgpA, RgpB and Kgp genes all encode an N-terminal signal peptide of ~22 amino acids in length, an unusually long propeptide of ~200 amino acids in length, and a catalytic domain of ~480 amino acids. C-terminal to the catalytic domain is a large hemagglutinin-adhesin (HA) domain which is comprised of adhesin binding domains (ABMs, or which 5 distinct sequences have been described), a "Domain of Unknown Function" (termed DUF2436 which is defined as conserved Pfam Domain of Unknown Function; IPR018832) and C-terminal adhesin domains or cleaved adhesin domains (or

CADs). The particular arrangement of the ABMs, DUF and CADs varies between Kgp and RgpA/B.

The architecture of the domains in the Kgp polyprotein is illustrated in Figure 1B. For example, Kgp comprises (N terminus to C terminus): a catalytic domain, a first ABM (ABM1), DUF2436, a domain comprising ABM2, ABM1, ABM3, two CAD domains (termed K1 and K2), a further domain comprising ABM2 and ABM1, a further CAD domain (termed K3), ABM2, and a C-terminal domain.

As used herein, reference to ABMs 1, 2 and 3 will be understood to generally refer to the ABMs found in the order ABM2, ABM1 and ABM3 in the sequence immediately C terminal to DUF2436 of Kgp, as depicted in Figure 1B.

The catalytic domains of RgpB and RgpA share a high-degree of sequence homology. However, RgpB lacks the HA domains and is located in a monomeric form on the outer membrane. Some of the HA domains have been alternatively described as C-terminal adhesin domains or cleaved adhesin domains (CADs) and some are DUF ("Domain of Unknown Function") 2436 domains (conserved Pfam Domain of Unknown Function; IPR018832).

The RgpA and Kgp precursor proteins are cleaved into multiple domains that remain non-covalently associated forming large outer membrane protein complexes. *In vivo*, Arg- and Lys-specific proteinases are therefore found in a cell-associated complex of non-covalently associated proteinases and adhesins. One such complex has been designated the RgpA-Kgp proteinase-adhesin complex (previously referred to as the PrtR-PrtK proteinase-adhesin complex). The complex is composed of a 45kDa Arg-specific calcium-stabilised cysteine proteinase and seven sequence-related adhesin domains,

As used herein a Lys-gingipain catalytic domain may also be referred to a KAS domain or PAS domain. As used herein an Arg-gingipain catalytic domain may also be referred to as a RAS domain or PAS domain. Typically, the catalytic domain of the Lys-gingipain or Arg-gingipains is located in the N-terminal ~480 amino acid region of the protein. The active site within the catalytic domain is typically located at amino acid

residues 426-446 (for RgpA) and 432-453 (for Kgp). Exemplary active site peptides, as found within the catalytic domains are set out in in Table 1 as SEQ ID NOs: 1-11.

As used herein an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis* will be understood to typically refer to the region of an Arg- or Lys-gingipain that is C-terminal to the catalytic or active site domain. The adhesin domain (also referred to as the HA domain) typically comprise a Domain of Unknown Function (DUF) domain (especially DUF 2436 conserved Pfam Domain of Unknown Function; IPR018832) and several adhesin binding motifs (ABM) domains and a cleaved adhesin domain (CAD).

First polypeptide

The chimeric or fusion proteins of the present invention comprise a first polypeptide that comprises or consists of an amino acid sequence of the active site of an Arg-X or Lys-X proteinase (also referred to herein as Arg- or Lys-gingipain, respectively) of *P. gingivalis*, or a sequence that is at least 80% identical thereto.

In any embodiment, the first polypeptide comprises or consists of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

Improved immunogenicity

Preferably, the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. The one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* may be located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide or C-terminally to the second polypeptide. The one or more further polypeptides may be linked to the first or second polypeptide of the chimeric or fusion protein, preferably via a linker of no more than 50 amino acids, or directly linked to the first polypeptide.

The one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least

80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

In any embodiment, the first polypeptide and the further polypeptide that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* comprise or consist of an identical amino acid sequence, or sequences that are at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to each other.

In preferred embodiments, the chimeric or fusion proteins of the invention comprise no more than 2 or nor more than 3 or no more than 4 polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto. Preferably, the chimeric or fusion proteins of the invention have fewer than 5, more preferably fewer than 4, most preferably fewer than 3 polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto.

Second polypeptide

The chimeric or fusion proteins of the present invention comprise a second polypeptide that comprises or consists of an amino acid sequence of an adhesin domain of an ArgX or Lys-X proteinase of *P. gingivalis,* or a sequence that is at least 80% identical thereto.

It will be understood that the second polypeptide will typically comprise at least a sequence corresponding to one or more of the adhesin-binding motifs (ABM) that are recognised domains/motifs in the adhesin domains of ArgX or Lys-X proteinases of *P. gingivalis* (such as Kgp and RgpA).

There are five ABMs that have been defined for Kgp and RgpA (ABM1-5). Exemplary sequences for these ABMs are set forth in Table 1.

Typically, the second polypeptide comprises more than one ABM, preferably wherein the second polypeptide comprises at least ABM1 and ABM2, or a sequence at

least 80% identical to each of ABM1 or ABM2. The second polypeptide may also comprise the sequence of ABM3 or a sequence at least 80% identical thereto.

It will also be appreciated that the arrangement of the ABM peptides in the second polypeptide need not correspond to the arrangement of the ABM peptides as found in naturally occurring adhesin domains. For example, the ABM peptides may be arranged in the second polypeptide in the sequence (N-terminus to C-terminus) ABM1, ABM2, ABM3 etc. Alternatively, the ABM peptides may be arranged to reflect the arrangement in naturally occurring adhesin domains, such as ABM2, ABM1 and ABM3, such as occurs in Adhesin domain 1 of Kgp.

Further still, the ABMs in the second polypeptide may be arranged contiguously or may be separated from each other by an amino acid sequence of no more than 50 amino acids. It will be appreciated by the skilled person that the spacing between the ABMs is not critical in the design of the chimeric or fusion proteins of the invention.

In any embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 16 or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In any embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 18 or SEQ ID NO: 27 or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In any embodiment, the second polypeptide comprises an amino acid sequence as set forth in SEQ ID NO: 22 or SEQ ID NO: 27 or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

Improved solubility

The inventors have established that the methods of the prior art, which include simply selecting various domains found in naturally occurring gingipains for use in a chimeric or fusion protein typically result in the generation of a protein that is poorly

expressed in recombinant systems, and which has a propensity to be expressed as insoluble protein (for example in inclusion bodies in *E. coli*).

With reference to the examples of the present specification, the inventors recognised that the sequence set forth in SEQ ID NO: 13 (ie, a truncated form of the CAD domain), represents how the Kgp polyprotein is proteolytically processed and assembled on the cell surface. The inclusion of such a sequence was therefore thought to be critical to the induction of an immune response to *P. gingivalis*. However, the inventors surprisingly found that when present, this sequence contributed to poor solubility and formation of inclusion bodies of recombinant proteins when expressed in *E. coli*.

The inventors sought to rectify this issue by including the full sequence of the CAD in the fusion proteins. Surprisingly, this did not improve solubility and in fact contributed to lower solubility.

The present inventors have found therefore that the inclusion of a sequence corresponding to one or more cleaved adhesin domains (CADs), or part(s) thereof, contributes to reduced solubility of the proposed chimeric proteins and vaccine compositions derived from gingipain sequences.

As such, in particularly preferred embodiments of the present invention, the second polypeptide does not comprise the sequence set forth in SEQ ID NO: 13 or a sequence at least 80 % identical thereto.

Alternatively, the second polypeptide does not comprise a sequence as set forth in SEQ ID NO: 12 or a sequence at least 80 % identical thereto or part thereof.

The inventors have also found that the solubility of the chimeric or fusion proteins of the invention is reduced when the chimeric or fusion proteins of the invention comprise only part of the DUF2436 domain (ie an N-terminally truncated DUF2436 domain).

With reference to the examples of the present specification, the inventors recognised prior art approaches to *P. gingivalis* vaccine design included an N-terminally truncated form of the DUF2436 domain. Although the truncated DUF2436 domain represents how the Kgp polyprotein is proteolytically processed and assembled on the cell surface, the inventors found that providing the sequence of the full length of the

DUF2436 domain (ie including the N-terminal portion of the DUF2436 domain) substantially improved production of soluble recombinant protein.

As such, in a preferred embodiment, the second polypeptide in the chimeric or fusion proteins of the invention comprises residues 1 to 37 of a DUF2436 domain, preferably wherein the chimeric protein comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, or a sequence at least 80% identical thereto.

As used herein, an amino acid sequence corresponding substantially to the full length of the DUF2436 domain, or a sequence at least 80% identical thereto, refers to a sequence that is at least 80%, 81%, 82%, 83%, 84%,85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the length of a DUF2436 domain of an Arg- or Lys-gingipain.

Preferably the DUF2436 domain comprises the sequence set forth in SEQ ID NO: 23, or sequences at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

Reduced multimerisation

The inventors have also established that various domains in gingpains have a propensity to multimerise, contributing to reduced solubility of the chimeric or fusion proteins and reduced immunogenicity when multimerised or aggregated. Thus, the formation of multimers has implications for ease of large-scale production of chimeric proteins derived from ginigipain sequences and for use as vaccines, but may also affect the generation of protective responses.

In particular, the inventors have identified two mechanisms for multimer formation: the formation of disulphide bridges between cysteine residues and beta-strand exchange between ABM domains. With cross-reference to the schematic depicting the various domains of Kgp in Figure 1B, the inventors hypothesised that ABM1 may interact with its next available neighbour, ABM2, during folding of the Kgp polyprotein *in vivo*. Separately, the inventors show that co-expression of recombinant ABM1 and ABM2 as separate proteins, was able to form a stable beta sheet complexed structure.

Accordingly, in order to reduce mulitmerisation of the chimeric proteins proposed for use according to the present invention, the inventors targeted the cysteine residues of the DUF and ABM domains, in addition to specific motifs within ABMs 2 and 1, which may be responsible for beta sheet formation.

The inventors identified that one or more of following modifications contributes to reducing multimerisation of chimeric fusion proteins derived from Kgp and Rgp polyproteins:

c) comprises one or more cysteine amino acid substitutions in the amino acid sequence of the DUF 2436 domain and ABMs compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

d) comprises one or more amino acid motif substitutions selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

Accordingly, in particularly preferred embodiments of the invention, the second polypeptide comprises one or more cysteine amino acid substitutions compared to the naturally occurring adhesin domain sequence.

Cysteine residues are found (at least) in the DUF2436 domain, ABM 2 and in the K1 CAD sequence in the *P. gingivalis* Kgp sequence. As such, it will be appreciated that the present invention contemplates chimeric or fusion proteins in which one or more, two or more, or three or more cysteine residues are substituted so as to reduce, minimise or abolish formation of disulphide bridging.

The cysteine amino acid substitution may be a substitution to a serine residue or a valine residue. Preferably, the one or more cysteine substitutions comprise one or more substitutions to a serine residue.

In particularly preferred embodiments, the cysteine residue in the DUF2436 domain is not substituted, while one or more cysteine residues in the region of the adhesin domain between the C-terminus of the DUF2436 domain and N-terminal to the K1 CAD domain, may be substituted. An exemplary sequence of the region between the DUF domain and CAD domain is set forth in SEQ ID NO: 18. In preferred embodiments of the invention, one or both of the cysteine residues at positions 36 and 50, or positions equivalent thereto, are substituted, optionally to a serine or valine residue, preferably to a serine residue. An exemplary sequence of the DUF2436 domain plus ABM2+1+3 region is set forth in SEQ ID NO: 34. In preferred embodiments of the invention, one or both of the cysteine residues at positions 208 and 222, or positions equivalent thereto, are substituted, optionally to a serine or valine residue, preferably to a serine residue.

In alternative embodiments, the cysteine residue in the DUF2436 domain is substituted to a serine or valine residue, preferably a serine residue, and one or both cysteine residues in the region of the adhesin domain between the C-terminus of the DUF2436 domain and N-terminal to the K1 CAD domain, may be substituted. An exemplary sequence of the region between the DUF domain and the CAD domain is set forth in SEQ ID NO: 18. In preferred embodiments of the invention, one or both of the cysteine residues at positions 36 and 50, or positions equivalent thereto, are substituted, optionally to a serine or valine residue, preferably to a serine residue. An exemplary sequence of the DUF2436 domain plus ABM2+1+3 region is set forth in SEQ ID NO: 34. In preferred embodiments of the invention, the cysteine residue at position 115, and one or both of the cysteine residues at positions 208 and 222, or positions equivalent thereto, are substituted, optionally to a serine or valine residue, preferably to a serine residue.

Accordingly, in a particularly preferred embodiment, the chimeric or fusion protein of the invention comprises an amino acid sequence corresponding to the sequence as set forth in any one of SEQ ID NOs: 35 to 49, or sequences at least 80% identical thereto (but for the cysteine substitutions therein).

The inventors have further established that multimerisation is also reduced by the mutation of a conserved motifs present in the ABM domains 2, 1 and 3.

In one example, the inventors considered modification of the domain at the N terminus of ABM1 of the adhesin domain. More specifically, substitution of the motif PxxN (eg PVQN in ABM1 of *P. gingivalis* Kgp, as set forth in SEQ ID NO: 14), was found to substantially contribute to reduced multimerisation and reduction in beta-strand exchange between ABM domains.

In preferred embodiments, the chimeric or fusion proteins of the invention therefore comprise a modification of the ABM1 PxxN motif in the region of the chimeric or fusion protein corresponding to the adhesin domain of *P. gingivalis* gingipain. Accordingly, the second polypeptide preferably comprises a proline substitution and an asparagine substitution, in the sequence PxxN corresponding, or at a position equivalent to positions 6 to 9 of the sequence of SEQ ID NO: 14 or 19 defining ABM1.

The proline amino acid substitution is preferably a substitution to an alanine residue.

The asparagine amino acid substitution may be a substitution to a proline residue or an alanine residue. Preferably the asparagine residue is substituted to a proline residue. In other embodiments, the asparagine residue is not substituted.

In further examples, the inventors considered the motif NEFA in the sequence in ABM1 in the Kgp/Rgp polyprotein. This sequence is defined at residues 2 to 5 of SEQ ID NO: 14 and 19 herein. As demonstrated further in the examples, modification of the motif NEFA to SEQY, through substitution of the asparagine, phenylalanine and alanine residues to serine, glutamine and tyrosine, respectively, significantly reduces multimerisation.

In another example, the inventors determined that substitution of the tyrosine residue in ABM2 corresponding or at a position equivalent to residues at position 5 of SEQ ID NOs: 15 or 20, and of the tryptophan residue in ABM1, corresponding or at a position equivalent to residue at position 23 of SEQ ID NOs: 14 or 19, to alanine residues, also significantly reduced multimerisation.

Finally, the inventors found that multimerisation was practically eliminated through the combination of cysteine modifications and one or more of the substitutions:

i) substitution of the proline and/or an asparagine residues in the sequence PxxN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NxFA to SxYQ in the sequence, corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

In particularly preferred embodiments, the inventors found that the combination of one or more cysteine modifications, preferably at least 2 cysteine substitutions to serine, and modification of the PXXN motif of ABM1 to AXXP, eliminated multimerisation of the resulting recombinant chimeric protein.

Taken together, the cysteine modifications and the modification of the PXXN motif of the ABM1 domain, the present invention therefore provides for chimeric or fusion proteins as described herein, wherein the second polypeptide of the chimeric protein, corresponding to a region of an adhesin domain of a *P. gingivalis* Arg or Lys gingipain, comprises or consists of an amino acid sequence as set forth in SEQ ID NO: 34, or a sequence at least 80% identical thereto, wherein one, two or three cysteine residues are substituted to serine residues and/or wherein the proline and asparagine residues in the sequence PXXN at positions 235 to 238, or positions equivalent to, are substituted.

Preferably the cysteine residue at residue 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue.

Preferably, the cysteine residue at position 115 of SEQ ID NO: 34, or a position equivalent thereto, is not substituted to either a serine or a valine residue, while the cysteine residues at positions 208 and 222 of SEQ ID NO: 34, or at positions equivalent

thereto, are substituted to serine residues, and wherein the proline residue at position 235, or position equivalent thereto, is substituted to an alanine residue and the asparagine residue at position 238, or a position equivalent thereto, is substituted to a proline.

Linking of the first and second polypeptides

In the chimeric or fusion proteins of the present invention, the C-terminal residue of the first polypeptide may be covalently linked to the N-terminal residue of the second polypeptide (corresponding to an adhesin domain polypeptide) or the N-terminal residue of the first peptide may be covalently linked to the C-terminal residue of the second polypeptide (corresponding to an adhesin domain polypeptide). In this arrangement, the first peptide and adhesin domain polypeptide, are said to be "directly linked" or "adjacent".

In other embodiments, the chimeric or fusion protein includes a linker for linking the first peptide to an adhesin domain polypeptide. The linker may be any linker able to join a peptide to a polypeptide, including both amino acid and non-amino acid linkers.

Preferably, the linker is non-immunogenic. Typically, the linker is comprised of amino acids, and may therefore be termed a peptide linker.

A linker is usually a peptide having a length of up to 20 amino acids, although may be longer. The term “linked to” or “fused to” refers to a covalent bond, e.g., a peptide bond, formed between two moieties. Accordingly, in the context of the present invention the linker may have a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 or more amino acids. For example, the herein provided chimeric or fusion proteins may comprise a linker between the first polypeptide comprising or consisting of an amino acid sequence of a *P. gingivalis* gingipain active domain, and the second polypeptide corresponding to the adhesin domain of a *P. gingivalis* gingipain, such as between the N-terminus of the second polypeptide and the C-terminus of the first polypeptide. Such linkers have the advantage that they can make it more likely that the different polypeptides of the fusion protein fold independently and behave as expected. Suitable linkers may be up to 50 amino acids in length, although less than 20, less than 15 or less than five amino acids is preferred. The linker may function to bring the first peptide and adhesin domain polypeptide into a closer spatial arrangement than normally observed in a *P. gingivalis* trypsin-like enzyme. Alternatively, it may space the first

polypeptide and the second polypeptide (corresponding to an adhesin domain polypeptide) apart.

Suitable linkers for use in protein constructs, including those with minimal impact on solubility are known in the art. The linker may be any linker known in the art to the skilled person and may be a flexible linker (such as those comprising repeats of glycine and serine residues), a rigid linker (such as those comprising glutamic acid and lysine residues, flanking alanine repeats) and/or a cleavable linker (such as sequences that are susceptible by protease cleavage). Examples of such linkers are known to the skilled person and are described for example, in Chen et al., (2013) Advanced Drug Delivery Reviews, 65: 1357-1369.

Useful linkers include glycine-serine (GlySer) linkers, which are well-known in the art and comprise glycine and serine units combined in various orders. Examples include, but are not limited to, (GS), (GSGGS)n, (GGGS)n and (GGGGS)n, where n is an integer of at least one, typically an integer between 1 and about 10, for example, between 1 and about 8, between 1 and about 6, or between 1 and about 5.

In some embodiments, the peptide linker may include the amino acids glycine and serine in various lengths and combinations. In some aspects, the peptide linker can include the sequence Gly-Gly-Ser (GGS), Gly-Gly-Gly-Ser (GGGS) or Gly-Gly-Gly-Gly-Ser (GGGGS) and variations or repeats thereof. In some aspects, the peptide linker can include the amino acid sequence GGGGS (a linker of 6 amino acids in length) or even longer. The linker may a series of repeating glycine and serine residues (GS) of different lengths, i.e., (GS)n where n is any number from 1 to 15 or more. For example, the linker may be (GS)3 (i.e., GSGSGS) or longer (GS)11 or longer. It will be appreciated that n can be any number including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more. Fusion proteins having linkers of such length are included within the scope of the present invention. Similarly, the linker may be a series of repeating glycine residues separated by serine residues. For example (GGGGS)3 (i.e., the linker may comprise the amino acid sequence GGGGSGGGGSGGGGS (G4S)3.) and variations thereof.

In one embodiment, the peptide linker can include the amino acid sequence GGGGS (a linker of 6 amino acids in length) or even longer. The linker may a series of repeating glycine and serine residues (GS) of different lengths, i.e., (GS)n where n is any

number from 1 to 15 or more. For example, the linker may be (GS)3 (i.e., GSGSGS) or longer (GS)11 or longer. It will be appreciated that n can be any number including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more.

Other useful linkers include DSSG, DSSGAS, KLDSSG and variations thereof. Examples of other suitable linkers are described in Chen et al., (2013) Advanced Drug Delivery Reviews, 65: 1357-1369.

Chimeric or fusion proteins and recombinant proteins

The chimeric or fusion proteins of the invention can be prepared by any of a number of conventional techniques although typically, the polypeptides are made using recombinant technology.

In the case of recombinant polypeptides, a DNA fragment encoding a desired peptide can be subcloned into an appropriate vector using well-known molecular genetic techniques (see, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, 1982); Sambrook et al., Molecular Cloning A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, 1989). The fragment can be transcribed and the polypeptide subsequently translated *in vitro.* Commercially available kits also can be employed (e.g., such as manufactured by Clontech, Palo Alto, Calif.; Amersham Pharmacia Biotech Inc., Piscataway, N.J.; InVitrogen, Carlsbad, Calif., and the like). The polymerase chain reaction optionally can be employed in the manipulation of nucleic acids.

A "fragment" is a portion of a polypeptide of the present invention that retains substantially similar functional activity or substantially the same biological function or activity as the polypeptide, which can be determined using assays described herein.

“Percent (%) amino acid sequence identity” or “percent (%) identical” with respect to a polypeptide sequence, i.e. a polypeptide of the invention defined herein, is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide of the invention, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the

sequence identity. Reference herein to variants having “at least x% sequence identity” to a recited sequence, means that the variant is at least x% identical to the recited sequence.

In various aspects and embodiments of the invention, the defined polypeptides are described by reference to variants having at least 80% homology to a reference sequence or more. Percentage (%) homology generally refers to a polypeptide of the invention defined herein, defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide of the invention, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

The amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

Amino acid deletions or insertions can also be made relative to the native sequence of the *P. gingivalis* protein. Thus, for example, amino acids which do not have a substantial effect on the activity of the polypeptide, or at least which do not eliminate such activity, can be deleted. Such deletions can be advantageous, particularly with longer polypeptides since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining activity. This can enable the amount of polypeptide required for a particular purpose to be reduced - for example, dosage levels can be reduced.

Amino acid insertions relative to the sequence of the native polypeptide can also be made. This can be done to alter the properties of a polypeptide for use in the present invention (e.g. to enhance antigenicity).

Amino acid changes can be made using any suitable technique e.g. by using site-directed mutagenesis or solid-state synthesis.

It should be appreciated that amino acid substitutions or insertions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids. Whether or not natural or synthetic amino acids are used, it is preferred that only L-amino acids are present.

Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms (non-limiting examples described below) needed to achieve maximal alignment over the full-length of the sequences being compared. When amino acid sequences are aligned, the percent amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain percent amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as: percent amino acid sequence identity = X/Y100, where X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B and Y is the total number of amino acid residues in B. If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the percent amino acid sequence identity of A to B will not equal the percent amino acid sequence identity of B to A.

In calculating percent identity, typically exact matches are counted. The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTX programs of Altschul et al. (1990) J. Mol. Biol. 215:403. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389. Alternatively, PSI-Blast can be used to

perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., BLASTX and BLASTN) can be used. Alignment may also be performed manually by inspection. Another non- limiting example of a mathematical algorithm utilized for the comparison of sequences is the ClustalW algorithm (Higgins et al. (1994) Nucleic Acids Res. 22:4673-4680). ClustalW compares sequences and aligns the entirety of the amino acid or DNA sequence, and thus can provide data about the sequence conservation of the entire amino acid sequence. The ClustalW algorithm is used in several commercially available DNA/amino acid analysis software packages, such as the ALIGNX module of the Vector NTI Program Suite (Invitrogen Corporation, Carlsbad, CA). After alignment of amino acid sequences with ClustalW, the percent amino acid identity can be assessed. A non-limiting examples of a software program useful for analysis of ClustalW alignments is GENEDOC™ or JalView (http://www.jalview.org/). GENEDOC™ allows assessment of amino acid (or DNA) similarity and identity between multiple proteins. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) CABIOS 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys, Inc., 9685 Scranton Rd., San Diego, CA, USA). When utilizing the ALIGN program for comparing amino acid sequences, a PAM 120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The polypeptide desirably comprises an amino end and a carboxyl end. The polypeptide can comprise D-amino acids, L-amino acids or a mixture of D- and L-amino acids. The D-form of the amino acids, however, is particularly preferred since a polypeptide comprised of D-amino acids is expected to have a greater retention of its biological activity in vivo.

The term "conservative substitution" as used herein, refers to the replacement of an amino acid present in the native sequence in the peptide with a naturally or non-naturally occurring amino acid or a peptidomimetic having similar steric properties. Where the side-chain of the native amino acid to be replaced is either polar or hydrophobic, the conservative substitution should be with a naturally occurring amino acid, a non- naturally

occurring amino acid or with a peptidomimetic moiety which is also polar or hydrophobic (in addition to having the same steric properties as the side-chain of the replaced amino acid).

Conservative amino acid substitution tables providing functionally similar amino acids are well known to one of ordinary skill in the art. The following six groups are examples of amino acids that may be considered to be conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

As naturally occurring amino acids are typically grouped according to their properties, conservative substitutions by naturally occurring amino acids can be determined bearing in mind the fact that replacement of charged amino acids by sterically similar non-charged amino acids are considered as conservative substitutions. For producing conservative substitutions by non-naturally occurring amino acids it is also possible to use amino acid analogues (synthetic amino acids) well known in the art. A peptidomimetic of the naturally occurring amino acid is well documented in the literature known to the skilled person and non-natural or unnatural amino acids are described further below. When affecting conservative substitutions the substituting amino acid should have the same or a similar functional group in the side chain as the original amino acid.

The phrase "non-conservative substitution" or a “non-conservative residue” as used herein refers to replacement of the amino acid as present in the parent sequence by another naturally or non-naturally occurring amino acid, having different electrochemical and/or steric properties. Thus, the side chain of the substituting amino

acid can be significantly larger (or smaller) than the side chain of the native amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of non-conservative substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for glycine, or -NH-CH[(-CH2)5-COOH]-CO- for aspartic acid. Non-conservative substitution includes any mutation that is not considered conservative.

A non-conservative amino acid substitution can result from changes in: (a) the structure of the amino acid backbone in the area of the substitution; (b) the charge or hydrophobicity of the amino acid; or (c) the bulk of an amino acid side chain. Substitutions generally expected to produce the greatest changes in protein properties are those in which: (a) a hydrophilic residue is substituted for (or by) a hydrophobic residue; (b) a proline is substituted for (or by) any other residue; (c) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine; or (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl.

Alterations of the native amino acid sequence to produce mutant polypeptides, such as by insertion, deletion and/or substitution, can be done by a variety of means known to those skilled in the art. For instance, site-specific mutations can be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified site. Alternately, oligonucleotide-directed site-specific mutagenesis procedures can be used, such as disclosed in Walder et al., Gene 42: 133 (1986); Bauer et al., Gene 37: 73 (1985); Craik, Biotechniques, 12-19 (January 1995); and U.S. Pat. Nos. 4,518,584 and 4,737,462. A preferred means for introducing mutations is the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LaJolla, Calif.).

Any appropriate expression vector (e.g., as described in Pouwels et al., Cloning Vectors: A Laboratory Manual (Elsevier, N.Y.: 1985)) and corresponding suitable host can be employed for production of recombinant polypeptides. Expression hosts include, but are not limited to, bacterial species within the genera *Escherichia, Bacillus, Pseudomonas, Salmonella*, mammalian or insect host cell systems including baculovirus systems (e.g., as described by Luckow et al., Bio/Technology 6: 47 (1988)), and

established cell lines such as the COS-7, C127, 3T3, CHO, HeLa, and BHK cell lines, and the like. The skilled person is aware that the choice of expression host has ramifications for the type of polypeptide produced. For instance, the glycosylation of polypeptides produced in yeast or mammalian cells (e.g., COS-7 cells) will differ from that of polypeptides produced in bacterial cells, such as *Escherichia coli*.

Alternately, a polypeptide of the invention can be synthesized using standard peptide synthesizing techniques well-known to those of ordinary skill in the art (e.g., as summarized in Bodanszky, Principles of Peptide Synthesis (Springer-Verlag, Heidelberg: 1984)). In particular, the polypeptide can be synthesized using the procedure of solid-phase synthesis (see, e.g., Merrifield, J. Am. Chem. Soc. 85: 2149-54 (1963); Barany et al., Int. J. Peptide Protein Res. 30: 705-739 (1987); and U.S. Pat. No. 5,424,398). If desired, this can be done using an automated peptide synthesizer. Removal of the t-butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid blocking groups and separation of the polypeptide from the resin can be accomplished by, for example, acid treatment at reduced temperature. The polypeptide-containing mixture can then be extracted, for instance, with dimethyl ether, to remove non-peptidic organic compounds, and the synthesized polypeptide can be extracted from the resin powder (e.g., with about 25% w/v acetic acid). Following the synthesis of the polypeptide, further purification (e.g., using high performance liquid chromatography (HPLC)) optionally can be done in order to eliminate any incomplete polypeptides or free amino acids. Amino acid and/or HPLC analysis can be performed on the synthesized polypeptide to validate its identity. For other applications according to the invention, it may be preferable to produce the polypeptide as part of a larger fusion protein, such as by the methods described herein or other genetic means, or as part of a larger conjugate, such as through physical or chemical conjugation, as known to those of ordinary skill in the art and described herein.

In any embodiment of the invention, the chimeric or fusion protein of the invention may comprise additional amino acid residues to facilitate expression in a recombinant expression system and/or to facilitate purification of the protein. Thus, the proteins defined herein may include additional amino acids such as one, two, three, four, or five amino acids in the N-terminal region. Typically the additional amino acids will include an N-terminal methionine for facilitating expression in recombinant expression systems

although it will be appreciated that typically such N-terminal residues are cleaved following translation of the protein. In certain embodiments, the N-terminal amino acids include at least methionine and an alanine residue.

Further, a chimeric or fusion protein according to the invention may include additional amino acids such as one, two, three, four, or five amino acids in the N or C-terminal region, preferably to facilitate purification. It will be understood that such amino acid residues may facilitate the inclusion of a purification tag in the protein (such as histidine tags and the like). Such residues may not be included where untagged versions of the protein are produced.

A polypeptide of the invention may also be modified by, conjugated or fused to another moiety to facilitate purification, or increasing the *in vivo* half-life of the polypeptides, or for use in immunoassays using methods known in the art. For example, a polypeptide of the invention may be modified by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc.

Nucleic acids

Nucleic acid molecules that encode any of the chimeric or fusion proteins or polypeptides of the invention are also within the scope of the invention. The nucleic acids are useful, for example, in making the polypeptides of the present invention and as therapeutic agents. They may be administered to cells in culture or *in vivo* and may include a secretory signal that directs or facilitates secretion of the polypeptide of the invention from the cell. Also within the scope of the invention are expression vectors and host cells that contain or include nucleic acids of the invention (described further below). While the nucleic acids of the invention may be referred to as “isolated,” by definition, the polypeptides of the invention are not wild-type polypeptides and, as such, would not be encoded by naturally occurring nucleic acids. Thus, while the polypeptides and nucleic acids of the present invention may be “purified,” “substantially purified,” “isolated,” “recombinant” or “synthetic” they need not be so in order to be distinguished from naturally occurring materials.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes nucleic acid molecules contained in cells that ordinarily express Kgp where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The terms “nucleic acid molecule” and “polynucleotide” are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogues thereof. Non-limiting examples of polynucleotides include a gene, a gene fragment, messenger RNA (mRNA), cDNA, recombinant polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide of the invention may be provided in isolated or purified form. A nucleic acid sequence which “encodes” a selected polypeptide is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. For the purposes of the invention, such nucleic acid sequences can include, but are not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic sequences from viral or prokaryotic DNA or RNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3′ to the coding sequence.

Polynucleotides of the invention can be synthesised according to methods well known in the art, as described by way of example in Sambrook et al (1989, Molecular Cloning—a laboratory manual; Cold Spring Harbor Press).

The polynucleotide molecules of the present invention may be provided in the form of an expression cassette which includes control sequences operably linked to the inserted sequence, thus allowing for expression of the polypeptide of the invention in vivo in a targeted subject. These expression cassettes, in turn, are typically provided within

vectors (e.g., plasmids or recombinant viral vectors) which are suitable for use as reagents for nucleic acid immunization. Such an expression cassette may be administered directly to a host subject. Alternatively, a vector comprising a polynucleotide of the invention may be administered to a host subject. Preferably the polynucleotide is prepared and/or administered using a genetic vector. A suitable vector may be any vector which is capable of carrying a sufficient amount of genetic information and allowing expression of a polypeptide of the invention.

The present invention thus includes expression vectors that comprise such polynucleotide sequences. Thus, the present invention provides a vector for use in preventing or treating an inflammatory disease or condition comprising a polynucleotide sequence which encodes a polypeptide of the invention and optionally one or more further polynucleotide sequences which encode different polypeptides as defined herein.

Furthermore, it will be appreciated that the compositions and products of the invention may comprise a mixture of polypeptides and polynucleotides. Accordingly, the invention provides a composition or product as defined herein, wherein in place of any one of the polypeptide is a polynucleotide capable of expressing said polypeptide.

Expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for expression of a peptide of the invention. Other suitable vectors would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook et al.

Thus, a polypeptide of the invention may be provided by delivering such a vector to a cell and allowing transcription from the vector to occur. Preferably, a polynucleotide of the invention or for use in the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector.

“Operably linked” refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given regulatory

sequence, such as a promoter, operably linked to a nucleic acid sequence is capable of effecting the expression of that sequence when the proper enzymes are present. The promoter need not be contiguous with the sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the nucleic acid sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

A number of expression systems have been described in the art, each of which typically consists of a vector containing a gene or nucleotide sequence of interest operably linked to expression control sequences. These control sequences include transcriptional promoter sequences and transcriptional start and termination sequences. The vectors of the invention may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. A "plasmid" is a vector in the form of an extra-chromosomal genetic element. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell. The vectors may also be adapted to be used *in vivo*, for example to allow *in vivo* expression of the polypeptide.

A "promoter" is a nucleotide sequence which initiates and regulates transcription of a polypeptide-encoding polynucleotide. Promoters can include inducible promoters (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), repressible promoters (where expression of a polynucleotide sequence operably linked to the promoter is repressed by an analyte, cofactor, regulatory protein, etc.), and constitutive promoters. It is intended that the term "promoter" or "control element" includes full-length promoter regions and functional (e.g., controls transcription or translation) segments of these regions.

A polynucleotide, expression cassette or vector according to the present invention may additionally comprise a signal peptide sequence. The signal peptide sequence is generally inserted in operable linkage with the promoter such that the signal peptide is

expressed and facilitates secretion of a polypeptide encoded by coding sequence also in operable linkage with the promoter.

Typically a signal peptide sequence encodes a peptide of 10 to 30 amino acids for example 15 to 20 amino acids. Often the amino acids are predominantly hydrophobic. In a typical situation, a signal peptide targets a growing polypeptide chain bearing the signal peptide to the endoplasmic reticulum of the expressing cell. The signal peptide is cleaved off in the endoplasmic reticulum, allowing for secretion of the polypeptide via the Golgi apparatus.

Immunogenic and vaccine compositions

The invention further provides compositions comprising the chimeric or fusion proteins defined herein, and the use of such chimeric or fusion proteins in immunogenic or vaccine compositions in the treatment or prevention of *P. gingivalis* infection.

The term "vaccine composition" used herein is defined as a composition used to elicit an immune response against an antigen (immunogen) within the composition in order to protect or treat an organism against disease.

As used herein, the terms “immunostimulating composition”, “vaccine composition” and “immunogenic composition” may generally be used interchangeably.

The immunostimulating compositions or vaccines of the invention may suitably include a pharmaceutically acceptable carrier, excipient, diluent, adjuvant, vehicle, buffer or stabiliser in addition to one or more peptides of the invention as the therapeutically or prophylactically active ingredient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, liposomes, water, glycerol, polyethylene glycol, ethanol and combinations thereof.

The immunostimulating compositions or vaccine compositions can be adapted for administration by any appropriate route, for example by the parenteral (including subcutaneous, intramuscular, intravenous or intradermal or by injection into the cerebrospinal fluid), oral (including buccal or sublingual), nasal, topical (including buccal, sublingual or transdermal), vaginal or rectal route. Such compositions can be prepared by any method known in the art of pharmacy, for example by admixing peptides with the

carrier(s) or excipient(s) under sterile conditions. Typically, the vaccine composition is adapted for administration by the subcutaneous, intramuscular, intravenous or intradermal route, typically by injection. Alternatively, the vaccine composition may be adapted for oral or nasal administration.

An immunostimulating composition or vaccine composition adapted for parenteral administration may be an aqueous and non-aqueous sterile injection solution which can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. Excipients which can be used for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The composition can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carried, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets.

An immunostimulating or vaccine composition adapted for oral administration, can be presented as discrete units such as capsules or tablets or lozenges; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions).

Suitable excipients for tablets or hard gelatine capsules include lactose, maize starch or derivatives thereof, stearic acid or salts thereof. Suitable excipients for use with soft gelatine capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

For the preparation of solutions and syrups, excipients which can be used include for example water, polyols and sugars. For the preparation of suspensions, oils (e.g. vegetable oils) can be used to provide oil-in-water or water in oil suspensions.

An immunostimulating or vaccine composition adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns which is administered in the manner in which snuff is

taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. A suitable composition wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, may comprise an aqueous or oil solution of the active ingredient.

Compositions adapted for administration by inhalation include fine particle dusts or mists that can be generated by means of various types of metered dose pressurised aerosols, nebulizers or insufflators.

An immunostimulating or vaccine composition adapted for transdermal administration may be presented as a discrete patch intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient can be delivered from the patch by iontophoresis as generally described in Pharmaceutical Research. 3(6):318 (1986).

A composition adapted for topical administration may be formulated as an ointment, cream, suspension, lotion, powder, solution (eg mouth wash) paste, gel, spray, aerosol or oil. For infections of the eye or other external tissues, for example mouth and skin, the composition may be applied as a topical ointment or cream. When formulated in an ointment, the active ingredient can be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient can be formulated in a cream with an oil-in-water cream base or a water-in-oil base. A pharmaceutical composition adapted for topical administration to the eye may comprise eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. A pharmaceutical composition adapted for topical administration in the mouth may comprise lozenges, pastilles or mouth washes.

The immunostimulating or vaccine composition may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention can themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants.

The vaccine composition of the invention may also contain one or more other prophylactically or therapeutically active agents in addition to the chimeric or fusion protein as defined herein.

A chimeric or fusion protein for use in the vaccine compositions of the invention may or may not be lyophilised.

The vaccine compositions of the invention may also include a pharmaceutically acceptable adjuvant in addition to the peptide(s) as defined herein. Adjuvants are added in order to enhance the immunogenicity of the vaccine composition.

Suitable adjuvants for inclusion in a vaccine composition are known in the art and include incomplete Freund's adjuvant, complete Freund's adjuvant, Freund's adjuvant with MDP (muramyldipeptide), alum (aluminium hydroxide), alum plus Bordatella pertussis and immune stimulatory complexes (ISCOMs, typically a matrix of Quil A containing viral proteins), QS- 21, Detox-PC, MPL-SE, MoGM-CSF, TiterMax-G, CRL-1005, GERBU, TERamide, PSC97B, Adjumer, PG-026, GSK-I, GcMAF, B-alethine, MPC-026, Adjuvax, CpG ODN, Betafectin, and MF59.

The vaccine compositions of the invention may also include or be co-administered with one or more co-stimulatory molecules.

Dosages of the vaccine composition of the present invention can vary between wide limits, depending upon the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used.

This dosage can be repeated as often as appropriate. For example, an initial dose of the vaccine may be administered and then a booster administered at a later date.

For administration to mammals, and particularly humans, it is expected that the daily dosage of the active agent will be from 1 µg/kg to 10 mg/kg body weight, typically around 10 µg /kg to 1 mg/kg body weight. The physician in any event will determine the actual dosage which will be most suitable for an individual which will be dependent on factors including the age, weight, sex and response of the individual. The above dosages are exemplary of the average case. There can, of course, be instances where higher or lower dosages are merited, and such are within the scope of this invention.

The vaccine composition of the invention can be administered by any convenient route as described herein, such as via the intramuscular, intravenous, by inhalation, intraperitoneal or oral routes or by injection into the cerebrospinal fluid.

The vaccine composition of the invention can be provided in unit dosage form, will generally be provided in a sealed container and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It can include a plurality of said unit dosage forms.

Accordingly, in yet another aspect, the present invention provides a kit of parts comprising a vaccine composition of the invention and one or more cytokines and/or adjuvants in sealed containers.

Methods for immunising a subject using the subject chimeric or fusion proteins

The present invention provides methods and compositions for treating or preventing infection or minimising the likelihood of infection with *P. gingivalis*, in an individual in need thereof, the methods comprising administering a fusion or chimeric protein of the invention.

The present invention also provides for methods and compositions for inducing a a humoural immune response in a subject to *P. gingivalis*. The humoural response may be for the purposes of obtaining protective/therapeutic anti-*P. gingivalis* antibodies directly in the individual requiring protection/therapy. Alternatively, the humoural response may be for the purpose of generating antibodies which are then isolated from the subject (or egg thereof), such that the antibodies can then be directly administered to an individual requiring treatment/protection with the antibodies.

As such, the present invention includes methods and compositions for preventing infection with *P. gingivalis*, minimising the likelihood of infection and/or reducing the severity and duration of *P. gingivalis* infection in a subject.

The present invention also provides a method for obtaining an antibody directed to *P. gingivalis,* the method comprising administering a chimeric or fusion protein, composition, vaccine or immune stimulating composition of the invention, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal. Preferably

the method further comprises isolating the antibody from the animal (eg from the blood of the animal) or from an egg of the animal (eg in the case of generating IgY antibodies from chickens).

The present invention also provides an antibody preparation comprising an antibody directed to *P. gingivalis,* wherein the antibody preparation is obtained by administering a chimeric or fusion protein, composition, vaccine or immune stimulating composition of the invention, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal, and isolating the antibodies from the animal or egg thereof.

The antibody directed to *P. gingivalis* may be used therapeutically to eliminate or reduce *P. gingivalis* infection or prophylactically, to prevent or reduce the severity of *P. gingivalis* infection.

As used herein, the terms "treatment" or "treating" of a subject includes the application or administration of a composition of the invention to a subject (or application or administration of a compound of the invention to a cell or tissue from a subject) with the purpose of delaying, slowing, stabilizing, curing, healing, alleviating, relieving, altering, remedying, less worsening, ameliorating, improving, or affecting the disease or condition, the symptom of the disease or condition, or the risk of (or susceptibility to) the disease or condition. The term "treating" refers to any indication of success in the treatment or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; lessening of the rate of worsening; lessening severity of the disease; stabilization, diminishing of symptoms or making the injury, pathology or condition more tolerable to the subject; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; or improving a subject's physical or mental well-being.

As used herein, "preventing" or "prevention" is intended to refer to at least the reduction of likelihood of the risk of (or susceptibility to) acquiring a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop in a subject that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease). Biological and physiological parameters for identifying such subjects are provided herein and are also well known by physicians.

The vaccine compositions of the invention can be administered to subjects felt to be in greatest need thereof, for example in the context of human patients, to children or the elderly or individuals at risk of exposure to *P. gingivalis*. The vaccine compositions of the invention can also be administered to subjects suspected of having or diagnosed with having infection with *P. gingivalis.*

The compositions and methods of the present invention extend equally to uses in both human and/or veterinary medicine, generation of diagnostic agents or the generation of other treatment reagents.

As used herein, the term "subject" shall be taken to mean any animal including humans, for example a mammal. Exemplary subjects include but are not limited to humans and non-human primates. For example, the subject may be a human. In further examples, the subject may be a veterinary subject, such as a companion animal (cat, dog, guinea pig, and the like).

As used herein, the terms "subject", "individual" and "patient" may be used interchangeably.

The skilled person will be familiar with methods for determining successful vaccination/immunisation with a chimeric or fusion protein or composition as described herein. For example, the skilled person will be familiar with methods for quantifying the antibodies generated following immunisation and/or for quantifying the extent of the humoural (Th2) response induced following immunisation.

Kits

In another embodiment there is provided a kit or article of manufacture including one or more proteins, polypeptides or polynucleotides of the invention and/or immunogenic composition as described above.

In yet another aspect, the present invention provides a kit of parts comprising a vaccine composition of the invention and one or more adjuvants for separate, subsequent or simultaneous administration to a subject.

In other embodiments there is provided a kit for use in a therapeutic or prophylactic application mentioned above, the kit including:

- a container holding a protein, polypeptide, polynucleotide or immunogenic composition of the invention;

- a label or package insert with instructions for use.

In any embodiment the kit may contain one or more further active principles or ingredients for eliciting an immune response to *P. gingivalis* in a subject.

The kit or "article of manufacture" may comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, blister pack, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a therapeutic composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The label or package insert indicates that the therapeutic composition is used for treating the condition of choice. In one embodiment, the label or package insert includes instructions for use and indicates that the therapeutic or prophylactic composition can be used to treat an inflammatory disease or condition described herein.

The kit may comprise (a) a therapeutic or prophylactic composition; and (b) a second container with a second active principle or ingredient contained therein. The kit in this embodiment of the invention may further comprise a package insert indicating the composition and other active principle can be used to treat a disorder or prevent a complication stemming from an inflammatory disease or condition described herein. Alternatively, or additionally, the kit may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In any embodiment the therapeutic composition may be provided in the form of a device, disposable or reusable, including a receptacle for holding the therapeutic, prophylactic or immunogenic composition. In one embodiment, the device is a syringe, autoinjector or nanopatch. The device may hold between 0.1 to 2 mL of the therapeutic

or immunogenic composition. The therapeutic or prophylactic composition may be provided in the device in a state that is ready for use or in a state requiring mixing, dissolution or resuspension or addition of further components.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as limiting the broad scope of the invention. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the invention.

Examples

The following examples describe a series of *in vitro* and *in vivo* studies relating to the chimeric or fusion proteins of the invention. Example 1 describes the materials and methods used in the studies described in Examples 2 and 3. Example 2 describes the results of *in vitro* studies and Example 3 describes the results of *in vivo* studies.

Example 1: Materials and methods

1.1 Materials and methods for *in vitro* studies

Expression of recombinant proteins

Recombinant proteins (i.e., different chimeric or fusion proteins as described herein) were expressed from pET28 vector (or pDUET-1 for rABM1 + rABM2 co-expression from one vector) by induction with isopropyl β-D-thiogalactosidase (IPTG) essentially as follows.

Nucleic acids encoding different chimeras and components of the chimeras and fusion proteins (including active site and adhesin domains) were produced by standard PCR or DNA splicing via overlap extension ("SOEing") PCR using *P. gingivalis* W50 genomic DNA template and specifically designed oligonucleotide primers. PCR

fragments or SOEn PCR fragments were purified and ligated into cloning vectors pGEMTeasy or pBHA and transformed into chemically competent *E. coli* α-Gold cells (Bioline, New South Wales, Australia).

DNA sequences encoding single additional KAS (i.e. active site) residues containing either a "DSSG" amino acid linker region or no linker were sequentially added to the recombinant chimera and Kgp adhesin domains, one at a time, essentially as follows: restriction enzyme sites were introduced onto the ends of DNA insert fragments in chimera and adhesin variant motherclones by PCR with oligonucleotide primers containing nucleotides specific for restriction enzymes. Synthetic DNA fragments encoding an individual KAS sequence with corresponding restriction sequence were ligated onto the insert ends of DNA constructs from motherclones and recombinant clones were purified. Subsequently if a second or third additional KAS was added, further restriction sequences were introduced onto the ends of these cloned inserts and additional DNA fragments encoding KAS sequences with a different restriction site were ligated on one at a time. Motherclone constructs with single, dual or quadruple linear KAS encoding sequences were subject to specific restriction digestion and sub-fragments could then be interchanged via ligation to produce the numerous variants (including those exemplified in Table 1).

Residues within ABM1 and ABM2 and Cysteine residues were mutated using the QuickChange II Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions.

The integrity of every insert in the cloning vectors pGEMTeasy and pBH1 was confirmed by DNA sequencing (Applied Genetics and Diagnostics Facility, The University of Melbourne). Mutations were further verified by DNA sequencing of entire inserts. Verified constructs were then subject to restriction digest with select enzymes and the inserts were cloned into the relevant pET expression vector in *E. coli* α-select chemically competent cells and subsequently into the *E. coli* expression host, BL21-CodonPlus (DE3) RIPL (Stratagene, Australia).

Investigation into features of chimera which impact on solubility of recombinant proteins; small scale

Single colony transformants were used to inoculate 2 mL of Luria Bertani (LB) broth containing 30 µg/ml kanamycin at 37°C on an orbital shaker overnight. This inoculum was then used to inoculate (1:100) 2 ml of LB containing 30 µg/ml kanamycin. Cell cultures at OD600 = 0.6-1.0 were induced with 1mM IPTG at 37°C for 2 hours. Cell cultures were centrifuged and the pellet was resuspended in 250 µl PBS and briefly sonicated using a CPX750 Ultrasonic processor (Cole Parmer Instrument Company, USA) and centrifuged.

Total lysate, Soluble (supernatant) and Insoluble (Pellet) fractions were analyzed by SDS-PAGE to assess solubility of the recombinant proteins. Recombinant proteins that remained insoluble or exhibited low solubility under these initial pilot expression conditions were expressed under a variety of conditions with lower temperatures (16°C to 30°C) and lower IPTG (0.01 - 0.5 mM) to ascertain the optimal conditions for enhanced solubility. All recombinant protein inductions were scaled up (20 - 200 mls) under optimal conditions to test for solubility at medium-large scale. Cultures (200 mL) were subjected to IPTG induction at various temperatures (e.g., 5 h at 30°C or 16 h at 16°C).

Cells were harvested and resuspended in lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 0.5% v/v Triton X-100, 5mg/ml DNAseI, 1X proteinase inhibitor cocktail, 1mg/ml lysozyme, 10 mM imidazole, pH 8). Cell resuspensions were incubated at 4 °C for 1 h and cleared lysates were then centrifuged (8,000 g, 30 min, 4 °C) to collect the supernatant (soluble) and pellet (insoluble) fractions and analysed by SDS- and NATIVE-PAGE.

In-gel HIS staining of recombinant proteins

Recombinant proteins subject to SDS PAGE were subject to staining using Invision In Gel His Stain kit (Invitrogen) following manufacturers' instructions. This stain is highly specific for His-tagged proteins. This procedure was used to identify N-terminal degradation of recombinant proteins since all candidates contained a C-terminal His tag.

Large scale purification of chimera for study in animal models

The purification conditions for each recombinant protein are detailed in the following sections.

Protein expression and cell lysis

All recombinant proteins identified for use as vaccine candidates for animal models were expressed as recombinant C-terminally His-tagged fusion proteins or as "tagless" proteins, in *E. coli* BL21(DE3) as previously described. The cells were grown at 37°C in LB medium, TB medium or a modified M9 minimal medium supplemented with 50 µg/mL kanamycin. Cultures at OD600 = 0.8-1.0 were induced with 0.4 mM IPTG at 37°C or 34°C for 2-4 hours, 25°C for 12 hours, or 16°C for 16-20 hours. After harvest at 8000 g by centrifugation at 4°C, the cells were lysed for 1.5 h on-ice in lysis buffer [0.35 mg/mL lysozyme, 40 µg/mL DNAse I in TBS150 (50 mM Tris·Cl, 150 mM NaCl, pH 7.5], plus 1% Triton X-100 and EDTA-free protease inhibitors (Complete ULTRA Tablets, Sigma-Aldrich) unless indicated otherwise. The cell lysate was clarified by centrifugation for 40 min at 23,500 g at 4°C.

Initiator Met residue (aa 1) on all recombinant proteins denoted in Table 1, beginning with the translated sequence MA is removed *in vivo* by *E. coli* N terminal Methionine processing enzymes that cleave Methionine when the penultimate residue is a small residue. This was confirmed by mass spectrometric analyses of KDAK and KDAK-variant recombinant proteins.

Purification of His-tagged proteins from soluble fractions under non-denaturing conditions

1. Ni-affinity chromatography

Cell lysates were clarified by centrifugation at 20,000 g at 4 °C to remove the cell debris. After filtration through a 0.22 µm filter, lysates were loaded to a HisTrap Ni-affinity column (GE Healthcare) in loading buffer TBS300 (50 mM Tris·Cl, 300 mM NaCl, pH 7.5), plus 10 mM imidazole and 1% Triton X-100. Columns were washed extensively with the loading buffer and then 20 mM imidazole in TBS300. Bound proteins were eluted with a 20-350 mM imidazole gradient in TBS300 with the absorbance being monitored at 280

nm. Peak fractions were analysed with SDS- and/or native PAGE. Eluted target proteins were concentrated using the Amicon filter units with a molecular weight cutoff of 3 kDa or 10 kDa depending on the protein sizes. The resulting protein solutions were stored at -70 °C for further purification.

2. Anion exchange chromatography

The isoelectric points (pIs) of all candidate proteins were predicted to be in the acidic range using online analysing ProtParam tool – ExPASy (https://web.expasy.org/protparam/), thus anion exchange chromatography was applied for further purification when necessary. Briefly, the concentrated proteins from Ni-affinity purification were diluted into buffer A (50 mM Tris.Cl, 20 mM NaCl, pH 7.5) to reduce ionic strength and then loaded to an anion exchange HiTrap Q column (GE Healthcare) in buffer A. The proteins were eluted with a NaCl gradient from 20 to 400 mM in the Tris buffer with the absorbance at 280 nm monitored. Target proteins in the peak fractions were verified with SDS- and/or native PAGE and concentrated using the Amicon filter units of an appropriate molecular weight cut-off. The protein solutions were stored at -70 °C for further purification or buffer exchange using size exclusion chromatography or dialysis.

3. Size exclusion chromatography

Size exclusion chromatography was performed on a HiLoad Superdex 200 or a HiLoad Superdex 75 column (GE Healthcare) at 4°C. The proteins from Ni affinity purification or the further step of anion exchange chromatography were loaded to an appropriate column and eluted in the buffer of TBS150 or TBS100 (100 mM NaCl in 50 mM Tris.Cl, pH 7.5) with absorbance at 280 nm monitored. Target proteins in the peak fractions were verified with SDS- and/or native PAGE. For isolation of single oligomeric species such as a dimer, second or further rounds of size exclusion purification were performed. The fractions containing the best protein purity were pooled and concentrated. For preparation of a mixture of the protein homomultimers, the target protein fractions were simply pooled and concentrated. Protein identities were confirmed by determination of intact protein molar masses using liquid chromatography-electrospray ionisation spectrometry (LC-MS, Agilent) or by sequence analysis using liquid chromatography Orbitrap tandem mass spectrometry (LC-MS/MS, Agilent) where necessary. Protein

concentration was quantitated by determination of the absorbance at 280 nm using calculated extinction coefficients before submission for animal model experiments.

Purification of His-tagged proteins from inclusion bodies

1. Under oxidising conditions

Following lysis of cells (as described for purification from soluble fractions), the insoluble pellet was washed twice with TBS150. The proteins expressed as inclusion bodies were solubilised with 8 M urea at room temperature on a rolling platform for 1 hour in TBS300 or 20 mM NaPi, 500 mM NaCl, pH 7.4 (PBS500). The protein extracts were centrifuged at 20,000 g and filtered through a 0.22 µm filter unit and then loaded to the HisTrap affinity column in the urea containing buffer TBSU (8 M urea in TBS300 plus 20 mM imidazole) or PBSU (8 M urea, 20 mM NaPi pH 7.8, 500 mM NaCl, plus 20 mM imidazole). The column was extensively washed with the same 8 M urea buffer. A further wash with PBSU at pH 6.5 was applied for the columns with proteins loaded in PBSU. For removal of urea, the target proteins were eluted with a gradient of 20-500 mM imidazole in PBSU. When loose Ni-NTA resin (Thermofisher) was used, the urea extracts were mixed with the resin for 2 hours with gentle stirring in PBSU. After extensive wash with PBSU (pH 7.8 and then pH 6.5) and the bound proteins were eluted off the resin with 500 mM imidazole in the same buffer. The eluted proteins were stepwise dialysed into 6 M, 4 M and then 2 M urea phosphate buffers in a dialysis tube with molecular weight cut-off of 3.5 kDa (Fisher Biotec, Australia). The proteins in the 2 M urea buffer were further dialysed into saline only and kept at 4 °C before submission to animal model experiments or otherwise submitted in 2 M urea buffer without further dialysis.

2. Under reducing conditions

The proteins were extracted from inclusion bodies with 6 M urea buffer in the presence of 5 mM TCEP and loaded to a HisTrap column in the same buffer. After the column was washed with 4 M urea buffer containing 2 M TCEP, the target proteins were refolded on column with a decreasing concentration gradient of urea from 4 to 0 M in the presence of 2 M TCEP. The proteins were then eluted with an increasing concentration gradient of imidazole from 20-350 mM in TBS300 plus 2 mM TCEP. The eluted proteins were then concentrated for size exclusion chromatography in the reducing buffer (2 mM

TCEP in TBS150) the same way as described in the section for purification of the protein dimer or multimers from soluble fractions under nondenaturing conditions. After analysis with SDS- and native PAGE, mass spectrometry and quantitation, the samples were stored at -70 °C in the reducing buffer or submitted for animal model experiments.

Purification of His-tagged proteins from soluble fractions under oxidising and denaturing conditions

Cell lysates were clarified by centrifugation at 20,000 g to remove debris and insoluble material, then lysates were immediately denatured by addition of a urea solution to a final concentration of 8 M in PBS500 with stirring at room temperature for 1 hour. The resulting lysates were then centrifuged at 20,000 g at room temperature for 40 min. The target proteins were purified with the same approach using HisTrap Ni-affinity column or loose Ni-NTA resin as described in the section of Purification from inclusion bodies-Under oxidising conditions above. The purified antigens in saline were stored at 4 °C before submission to animal model experiments.

Purification of tagless candidates from soluble fractions

Anion exchange chromatography This is the first chromatographic step for purification of the tagless proteins. Like their His-tagged counterparts, pIs of the tagless candidates were predicted to also be in the acidic range using online analysing ProtParam tool – ExPASy (https://web.expasy.org/protparam/). Therefore, anion exchange chromatography was applied, and two rounds were performed. In the first round, the clarified cell lysates were filtered through a 0.22 µm filter disc and loaded to an anion exchange HiTrap Q column (GE Healthcare) in Buffer AA (20 mM NaPi, pH 6.5, supplemented with 0.05x protease inhibitor cocktails). After wash with 10 column volumes of Buffer AA, the bound proteins were eluted with a NaCl gradient of 0-100% Buffer BA (1M NaCl in 20 mM NaPi pH 6.5) and the absorbance at 280 nm was monitored. Target proteins in the peak fractions were verified with SDS- and/or native PAGE. In the second round, the best fractions from the first round of anion exchange step were pooled and diluted by 6 folds with Buffer AA and were then re-loaded to the anion exchange HiTrap Q column (GE Healthcare) in the same buffer. Bound proteins were eluted with a NaCl gradient of 0-50% Buffer BA with the absorbance at 280 nm being monitored. Target proteins in the peak fractions were verified with SDS- and/or native PAGE and the best

fractions were concentrated using the Amicon filter units of a 10 kDa molecular weight cut-off. The protein solutions were stored at -20 °C for further purification with size exclusion chromatography.

First-round size exclusion chromatography Size exclusion chromatography was performed on a HiLoad Superdex 200 column (GE Healthcare) at room temperature. The proteins from purification via anion exchange chromatography were loaded to the size exclusion column and eluted in the buffer of AS (1M $(NH_4)_2SO_4$, 50 mM NaPi pH 7.0, supplemented with 0.05x protease inhibitor cocktails) with absorbance at 280 nm monitored. Target proteins in the peak fractions were verified with SDS- and/or native PAGE. The fractions of the best protein purity were pooled for further purification with hydrophobic interaction chromatography.

Hydrophobic interaction chromatography The pooled fractions of target protein in Buffer AS from the first-round size exclusion chromatography was filtered through 0.22 µm membrane and loaded to a HiTrap Phenyl HP column pre-equilibrated in Buffer AH (1M (NH4)2SO4, 50 mM NaPi pH 7) at 4°C. The bound proteins were eluted with a gradient of decreasing ionic strength from 800 mM to 200 mM (NH4)2SO4 in Buffer BH (50 mM NaPi, pH 7) with absorbance at 280 nm monitored. Target protein in the peak fractions were verified with SDS- and/or native PAGE and the best fractions were pooled and concentrated using the Amicon filter units for the next purification or buffer exchange step of the second-round size exclusion chromatography.

Second-round size exclusion chromatography The concentrated samples from the purification step with hydrophobic interactions were loaded to the size exclusion column as used in first-round size exclusion chromatography and eluted in the buffer of BTS (20 mM Bis-Tris, 150 mM NaCl, pH 6.5). Peak fractions were analysed with SDS- and/or native PAGE. The fractions of best purity were pooled and concentrated. After identification with liquid chromatography-electrospray ionisation mass spectrometry (LC-MS, Agilent) and quantitation, the antigens were aliquoted and stored at -80 °C for future use.

Protein identification and quantitation of tagless candidates

In addition to SDS- or native PAGE verification, protein identities were confirmed by determination of intact protein molar masses using LC-MS or by sequence analysis using liquid chromatography Orbitrap tandem mass spectrometry (LC-MS/MS, Agilent) where necessary. Protein concentration was quantitated by determination of the absorbance at 280 nm using calculated extinction coefficients (Table 3). Where buffer exchange was necessary before submission for animal model experiments, Superdex 200 size exclusion column (GE Healthcare) or Zeba™ spin desalting columns (ThermoFisher Scientific) were used.

1.2 Materials and methods for *in vivo* studies

Culture of bacteria for mouse model of periodontitis

P. gingivalis strain; W50 (serotype C); was obtained from the culture collection of the Oral Health Cooperative Research Centre, The Melbourne Dental School, University of Melbourne, Australia. *P. gingivalis* W50 was grown on Horse Blood Agar (HBA) (20 g/L HBA; Oxoid Ltd., Hampshire, UK) supplemented with 10% v/v lysed horse blood (37 °C) in an anaerobic N_2 atmosphere containing 5% CO_2 in a MK3 Anaerobic Workstation (Don Whitley Scientific Ltd., Adelaide, Australia). Colonies were inoculated into starter culture comprised of 20 mL sterilised brain heart infusion (37 g/L BHI; Oxoid Ltd., Hamsphire, UK) medium supplemented with 5 mg/L hemin and 0.5 mg/L cysteine [McKee et al. (1986) *Infect Immun* 52: 349-355] and incubated anaerobically (24 h, 37 °C). Absorbance of batch cultures were monitored at OD650nm using a spectrophotometer (model 295E, Perkin-Elmer, Germany). Bacterial cells were harvested during late exponential growth by centrifugation (7,000 g, 20 min, 4 °C). Bacterial purity was routinely confirmed by Gram stain [Slots (1982). In: Host-Parasite Interaction in Periodontal Disease, Genco, R.J. and Merganhagan, S.E. (eds). Washington D.C.: American Society for Microbiology. pp. 27-45.].

Preparation of heat-killed bacteria

P. gingivalis W50 culture was harvested (6,500 g, 4 °C), washed once with phosphate buffered saline (PBS) (0.01 M Na2HPO4, 1.5 mM KH_2PO_4 and 0.15 M NaCl, pH 7.4) then pelleted by centrifugation (7,000 g, 20 min 4°C). Bacterial cells were

resuspended in PBS and heated to 65°C for 15 minutes. The suspension was centrifuged (7,000 g, 20 min 4ºC) and resuspended in sterile PBS and this was repeated once. After the second wash, the supernatant was discarded and the cell pellet was resuspended in sterile PBS to obtain a cell density of 2 x 10^{10} cells/mL, and protein concentration determined using Biorad Protein Assay Dye Reagent Concentrate (Life Science, NSW, Australia).

Mouse periodontitis model

The mouse periodontitis experiments were modified from Baker et al.'s mode (1994). *Arch Oral Biol* 39: 1035-1040) and performed as described previously by O'Brien-Simpson et al. (2005 *J Immunol* 175: 3980-3989). Mice (female BALB/c; 6-8 weeks old, 10 mice/group), on Day 0 were intra-orally inoculated with *P. gingivalis* consisting of four doses of *P. gingivalis* W50 [1 x 10^{10} viable *P. gingivalis* W50 cells suspended in 20 µL PG buffer (50 mM Tris-HCL, 150 mM NaCl, 10 mM $MgSO_4$ and 14.3 mM Mercaptoethanol, pH 7.4) containing 2% w/v carboxymethylcellulose (CMC, Sigma, New South Wales, Australia)], given two days apart. The inocula were prepared anaerobically and then immediately applied to the gingival margin of the maxillary molar teeth. The number of viable bacteria in each inoculum was verified by flow cytometry and CFU counts on blood agar. Groups of animals consisted of; *P. gingivalis* W50 orally inoculated (infected control), a non-bacterial inoculated control, and immunised groups. For the therapeutic vaccination periodontitis model (Figure 1) mice were immunised on day 19 after the first oral inoculation with 50 µg of vaccine candidate in saline/alum (Alhydrogel; 2% aluminium hydroxide wet gel suspension; Invivogen) via the intraperitoneal route. Mice received a second immunisation (50 µg in saline/alum) on day 40 via the subcutaneous route. On Day 62, mice were bled by cardiac puncture and killed. Maxillae were removed and halved through the midline, with 10 halves used to determine alveolar bone loss. Sera were used to determine the antibody profile using ELISA.

Measurement of alveolar bone loss in mouse maxillae

Maxillae to be examined for bone loss were boiled (1 min) in deionised water, mechanically defleshed, and immersed in 2% w/v potassium hydroxide (16 h, 25°C). Maxillae were washed twice with deionised water (25 °C), dried (1 h, 37 °C) and stained with 0.5% w/v aqueous methylene blue. Digital images of the buccal side of the maxillae

were captured with an Olympus DP12 digital camera mounted on a dissecting microscope, using OLYSIA BioReport software version 3.2 (Olympus Australia Pty Ltd, New South Wales, Australia) to assess horizontal bone loss. Maxillae were oriented so that the buccal and lingual molar cusps were superimposed. Images were captured with a micrometre in frame, so that measurements could be standardised for each image. Horizontal bone loss was defined as the loss occurring in a horizontal plane, perpendicular to the alveolar bone crest that resulted in a reduction of the crest height. The visible area from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) for each molar was measured using OLYSIA BioReport software version 3.2 imaging software to give the total visible CEJ-ABC area in mm^2. *P. gingivalis*-induced alveolar bone loss in mm^2 was calculated by subtracting the total visible CEJ-ABC area of the uninoculated (N-C) group from the total visible CEJ-ABC area of each experimental group. Alveolar bone loss measurements were determined twice in a random and blinded protocol. Data are expressed as the mean +/- standard deviation in mm^2 and were analysed using a one-way ANOVA and Dunnetts T3 post-hoc test.

Pre-screening of vaccine candidates by enzyme-linked immunosorbent assay (ELISA)

Several monoclonal antibodies to *P. gingivalis* were used to pre-screen vaccine candidates to determine whether major domains and epitopes present in the vaccine constructs were accessible to the antibodies, thus allowing the generation of an antibody response *in vivo*. Antigen to be screened was coated onto flat-bottom polyvinyl microtiter plates (Microtiter; Dynatech Laboratories, McLean, VA, US) in 0.1M PBS (pH 7.4) for 16 h, 4 ºC. In these experiments the following antibody dilution were used; a dilution of 1/4000 dilution of goat anti-mouse; IgG (M8642) antibody (Sigma, New South Wales, Australia). A 1/4000 dilution of a horseradish peroxidase-conjugated swine anti-goat IgG antibody (M5420; Sigma, New South Wales, Australia) was used to develop ELISA experiment.

Determination of subclass antibody in sera using ELISA

ELISAs were performed to evaluate subclass antibody in sera as described in Pathirana et al. (2007). *Infect Immun* 75: 1436-1442) using a solution (1 µg/mL) of either HK W50 cells, domain subunits or epitopes in 0.1M PBS (pH 7.4) to coat wells (16 h, 4

ºC) of flat-bottom polyvinyl microtiter plates (Microtiter; Dynatech Laboratories, McLean, VA, US). In these experiments the following antibody dilutions were used; a dilution of 1/4000 dilution of goat anti-mouse; IgG (M8642), IgG1 (M8770), IgG2a (M4434) antibodies (Sigma, New South Wales, Australia). A 1/4000 dilution of a horseradish peroxidase-conjugated swine anti-goat IgG antibody (M5420; Sigma, New South Wales, Australia) was used to develop ELISA experiment. For the epitope ELISAs biotinylated peptides were bound to pre-blocked streptavidin coated flat bottom plates (Pierce; Thermo-Fisher) at 10 µg/mL. Following incubation with sera, the ELISA was developed with 1/4000 goat anti-mouse IgG and 1/4000 horseradish peroxidase-conjugated swine anti-goat IgG antibody. All optical density measurements were conducted on a Wallac VICTOR3 1420 Multilabel counter (Perkin Elmer) at 405nm.

Example 2: Results of *in vitro* studies

Study 1: Chimera components impacting solubility of recombinant protein

One chimera previously reported (in WO 2010/022463) consists of a KAS (K) peptide to which is conjugated an N terminally truncated DUF2436 domain (Dc), an adhesin domain comprising ABMs213 (A) and a C terminally truncated CAD domain (truncated K1 domain, termed K1n). This chimera is termed “KDcAK1n” (SEQ ID NO: 64 and may also be referred to herein as “original chimera” or “chimera” in comparison to the chimeric and fusion proteins of the present invention). The KDcAK1n protein is produced as inclusion bodies *in E. coli* and is poorly soluble and poorly stable.

The truncated DUF and K1 domains of the A1 adhesin fragment represent how the Kgp polyprotein is naturally proteolytically processed and assembled on the *P. gingivalis* cell surface during infection. It follows that truncated DUF and K1 domains make obvious candidates for inclusion in a vaccine for generating immune responses to gingipains.

Extensive attempts to produce soluble KDcAK1n were undertaken. Variations in growth media, growth conditions, IPTG concentrations, *E. coli* expression strains, induction temperatures, cell stage of growth at induction, addition of growth stabilisers, extensive testing of lysis buffers and protein storage buffers were systematically investigated. An enhancement of soluble recombinant protein expression was observed in small scale expression under low temperatures and IPTG induction. At small scale,

KDcAK1n produced approximately 30 - 50% of total recombinant protein expression as soluble protein. Nevertheless, the soluble recombinant was unable to be scaled above 10 ml cultures resulting in inclusion body formation with any attempts to scale up.

Impact of K1 domain on solubility

All full DUF, ABM213, ABM21 and DUF-ABM213 domains of the Kgp polyprotein were expressed as highly soluble and stable recombinant proteins. The full K1 domain alone however was insoluble and expressed only as Inclusion Bodies (IB).

Expression of a KAS motif conjugated to a ABM213 domain (ie, "KA") or a KAS motif conjugated to both DUF and ABM213 domains (ie "KDA") produced highly soluble, stable recombinant proteins.

However, as shown in Figure 2, addition of a C-terminally truncated K1 domain (that is the K1n fragment) or a full K1 domain to recombinants KA or KDA (KAK1 or KDAK1) resulted in compromised solubility.

Both KDAK1n (SEQ ID NO: 65) and KDAK1 (SEQ ID NO: 66) proteins (ie comprising K domain, adhesin domain comprising DUF2436 and ABMs and a truncated or full length CAD domain) require more fastidious growth conditions for soluble expression, at significantly lower levels than for KDA (ie the same protein excluding truncated or full length CAD).

Together with the results for K1 domain expression it is apparent that the K1 (CAD) domain is a contributor to poor solubility of chimera-based recombinants.

Impact of DUF domain on protein solubility

Truncation of the DUF domain in chimeric protein constructs resulted in elimination of soluble protein expression. This result indicates that a full DUF domain containing the extra 38 N-terminal residues is desirable for optimising soluble expression and efficient stabilization of the recombinant protein.

Thus, a construct expressing a chimera variant with an extended Dc domain (chimera with Dc extended by 38 N terminal residues) was created. This recombinant was designated KDAK1n and results show that chimera solubility is significantly

enhanced with a full DUF domain. Expression of KDAK1n in large scale at 30 °C with 0.2-0.5 mM IPTG resulted in expression of a soluble recombinant protein of good yield.

Impact of K1 and full length DUF domains on solubility

Notwithstanding this soluble protein expression at this scale, the inclusion of K1n/K1 in KDAK1n/KDAK1 did appear to make preparation more problematic than that for the KDA variants lacking K1n/K1, such that these variants involving K1 may be less desirable for scale up and commercial development.

To summarise:

- A full length DUF domain is desirable for solubility of recombinant variants (Fig.2A)
- The K1 domain (whether full length or truncated) was largely responsible for aggregation and insolubility of variants.
- In the absence of a full length DUF domain (ie DUFc), K1 and K1n containing variants were totally insoluble (Fig. 2A).
- In the presence of a full length DUF domain, very low-level solubility of K1 and K1n – containing variants was evident under very gentle induction conditions. Nevertheless, this solubility was very low with approximately 80 – 90 % of all recombinant protein being expressed as Inclusion bodies.
- NATIVE PAGE gel analysis of mid-scale purifications of soluble KDAK1 recombinants revealed disordered aggregation of recombinant (ie not a neat even ladder as for KDA variants). Small scale purifications of these variants (at low protein concentrations) using mini-Nickle spin columns did not result in similar aggregation however small-scale purification of truncated DUF variants also revealed that characteristic ladders of proteins on NATIVE PAGE were uneven suggesting disordered aggregation.
- The K1 and K1n domain containing variants had an increased tendency for aggregation at higher concentrations and after freeze-thaw of purified samples. K1 domain was responsible for this instability. However, all variants lacking K1 or K1n were very stable upon freeze-thaw and at higher concentrations (eg concentrations above 30 mg/ml)

Variants containing a full length or truncated K1 domain were very insoluble and the small level of soluble recombinant protein produced was prone to instability and aggregation after purification. Efficient upscale of soluble K1 containing variants was not possible.

Extension of DUF to full length and/or removal of K1 (or K1n) domains, preferably both, are therefore important for solubility and stability of the recombinant protein.

Study 2: Factors impacting multimerisation

Native PAGE analysis of purified soluble DUF-ABM213 and ABM213 and ABM21 recombinant proteins results in extensive even laddering indicating multimerisation. Multimerisation is also apparent in the KDcAK1n chimera of the prior art, through intermolecular disulphide bond formation between denatured domains. This disulphide linked multimerisation was most evident in KDcAK1n chimera purified from inclusion bodies.

During the course of studies on the recombinant chimera and Kgp adhesins it became evident that the multimerization of recombinant proteins was occurring via an interaction between ABM1 and ABM2 motifs. A recombinant protein comprising ABM2(1), ABM2(2) and ABM3 domains (with reference to the schematic in Figure 3A) was readily able to form a multimer. Subsequent expression of a smaller recombinant protein encoding ABM2(1), ABM1(2) and ABM3 (designated rABM213) and a recombinant protein encoding ABM2(1) and ABM1(2) (designated rABM21) showed that both recombinants were readily able to multimerize. This multimerization was evident by the presence of a neat ladder of bands on Native PAGE.

It was proposed that during folding of the Kgp multidomain polyprotein, the ABM1 domains interact with the ABM2 domains to form an FnIII like stable beta sheet complexed structure. The inventors hypothesized that ABM1(1) will interact with its next available neighbour, ABM2(1), and ABM1(2) will interact with ABM2(2) and so on during folding of the Kgp polyprotein (as depicted schematically in Figure 3A).

In a separate study the inventors showed that co-expression of rABM1 and rABM2 as separate proteins were capable of interacting to form a stable beta sheet complexed structure. rABM2(1) was able to form a stable structure with either rABM1(1) or rABM1(2).

More specifically, NMR spectroscopic analysis of the folded ABM domain indicates that the domain multimerizes through beta-strand exchange involving the ABM1 and ABM2 motifs. Together these results provide evidence that the chimera and variants containing the ABM domain will multimerize through an interaction between the ABM1 and ABM2 motifs of a properly folded ABM domain.

Subsequently, the inventors aimed to mutate specific residues of ABM1 or ABM2 within a recombinant ABM21 (rABM21) protein that encompasses residues 878-968 on the Kgp W50 polyprotein.

BLAST analysis of Kgp ABM1 and ABM2 sequences in all available bacterial genomes revealed that submotifs within the ABM1 and ABM2 motifs were very highly conserved across all phyla. Subsequently an alignment of ABM1 and ABM2 sequences against all ORFs within the W83 genome revealed that these residues were similarly highly conserved *in P. gingivalis* genes. The "PVQN" motif with conserved Proline residue was highly conserved across Phyla, including *P. gingivalis.*

Molecular modelling of proposed tertiary folding using online programs revealed that this "PVQN" sequence was located at the start of a beta sheet structure and that the sequence immediately N terminal to this motif ("NEFA") was found to be most likely an unstructured "loop".

The inventors mutated residues within the PVQN motif of rABM21 as well as mutating residues within the loop immediately prior to the PVQN. The residues within the loop were targeted as it was believed that the size and shape of the loop preceding beta sheet structures can influence the strength of adjacent beta sheet folding. The number and location of Proline residues within such "loop" structures are reported to be relevant to adjacent beta sheet interaction.

In addition the inventors mutated highly conserved hydrophobic residues Y(878) and W(968) present in ABM2 and ABM1 respectively. The inventors also introduced substitutions into the sequence of the motif NxFA in ABM1, to SxYQ (see SSEYQ variant mentioned in Table 2 below).

The mutated rABM21 variants were purified and assessed for their ability to multimerise. The inventors sought to identify mutations that would result in a stable soluble recombinant molecule that could be purified as a stable monomer.

Table 2: Summary of recombinant protein solubility and ability to form multimers on NATIVE-PAGE in the presence or absence of DTT. (N/T = not tested)

Recombinant protein	Solubility of recombinant in expression strain induced in LB with 1M IPTG at 37 °C for 4h.	Ability to form Multimers (-DTT)	Ability to form Multimers in presence of DTT
rABM213	very high	+++++	+++++
rABM21	very high	+++++	+++++
rABM21-Cys>Ser	very high	+++++	+++++
rABM21-2XCys>Ser	very high	+++++	+++++
rABM21-AVQN	very high	+++++	+++++
rABM21-AVQA	very high	+++++	+++++
rABM21-AVQP	very high	++++	++
rABM21-AVQA-Cys>Ser	very high	++++	++++
rABM21-AVQA-2X Cys>Ser	very high	++++	++++
rABM21-AVQP-Cys>Ser	very high	+	-
rABM21-AVQP-2X Cys>Ser	very high	-	-
rABM21-PNPFA	very high	+++++	N/T
rABM21-PSPYQ	very high	+++++	N/T
rABM21-SSEYQ	very high	++	N/T
rABM21--Y>A	very high	+++++	N/T
rABM21-Y>A/W>A	very high	-	N/T

All recombinant proteins (WT rABM213, rABM21 and rABM21-mutant variants) produced very high level expression of highly soluble recombinant protein under harsh induction conditions (summarized in Table 2).

Purified recombinant proteins subject to Native Page analysis (Figure 3B-E) showed that rABM21 was able to form multimers via two mechanisms: (1) multimerization via ABM1 motif “PVQN” and (2) via disulfide bonds.

Mutation of highly conserved “PVQN” motif to AVQN or AVQA in rABM21 did not eliminate multimerization.

Mutation of "PVQN" to AVQP resulted in significant reduction in multimerization in the presence of DTT and a slight reduction in multimerization in the absence of DTT, suggesting that the change from PVQN to AVQP alone has some impact on reducing multimerization.

The AVQA mutation in combination with substitution of one or both cysteine residues in the ABM domain to serine resulted in a slight reduction in multimerization. Mutation of "PVQN" to AVQP together with mutation of ***one or both*** Cysteine residues in the ABM domain to Serine resulted in near or complete elimination of multimerization of rABM21.

Manipulation and mutation of residues in the modelled "loop" region directly preceding the ABM1 "PVQN" motif of the modelled "beta sheet region" were also able to reduce multimerization but not completely eliminate it. More specifically, the "SSEYQ" substitution in ABM1 (eg modification of the sequence SNEFA to SSEYQ immediately N terminal to the highly conserved PVQN motif) resulted in a significant reduction in multimerization.

Mutation of Tyr-889 and Trp-964 residues (in ABM2 and 1 respectively) together also resulted in elimination of multimerization of rABM21.

Manipulation of "PVQN" motif to AVQP together with substitution of cysteine residues within the ABM domains enabled expression and purification of a very high soluble monomeric protein using the *E. coli* pET expression system.

All the chimeric candidates composed of the original sequences with the two Cys residues within the ABM21 domain being intact demonstrated extensive laddering multimerisation (Figure 3B and C). However, the substitution of these two Cys residues did not result in complete elimination of laddering multimerisation (Figure 3C lanes 15 and 16) although substitution of one Cys residue in combination with the AVQP substitution provided a significant reduction in multimerisation. Complete elimination of the laddering multimerisation was only achieved by substitution of the two ABM cysteine residues plus the variation of the motif PVQN to AVQP.

Interestingly, KDAK-1V-AVQP (SEQ ID NO: 70) and KDAK-AVQP (SEQ ID NO: 71) with the two ABM cysteine residues remaining and with or without variation of the only

DUF domain cysteine residue had significantly reduced multimerisation under reducing conditions. The two proteins shared essentially the same profiles on the native gel either reduced or unreduced (Figure 3G). Apparently, the DUF cysteine residue did not contribute to the laddering multimerisation but the presence of the two ABM cysteine residues was critical for the formation of the laddering multimers in these two variants. This suggests that the DUF domain cysteine residue is not a significant contributor to multimerisation.

Although the laddering multimerisation were disabled by variation of cysteine residues and the PVQN motif, the equilibrium between the monomeric and oligomeric states still existed under certain conditions. This kind of equilibrium was found to be temperature, pH, and concentration dependent from analysis with size exclusion chromatography. The increase in temperature, decrease in pH and reduced concentration were found to be favourable for the monomeric state. The presence of His-tag was not responsible for the equilibrium of the protein states. SEC-MALS analysis exhibited the overwhelming existence of monomer in solution at 2 mg/mL or a lower concentration with high stability for the leading candidates.

Study 3: Active site (KAS) Motifs

Additional KAS motifs were engineered onto recombinant chimera variants to determine if this would have an impact on immunogenicity. Single KAS or two successive KAS were added to variants at their N terminus or at both termini. Further, a linear sequence of 4 KAS residues containing DSSG linker sequence between each KAS motif was also added to the N terminus of selected variants.

Purification profiles and His gel stain analysis of recombinants revealed that additional KAS residues caused some instability and degradation of the recombinant proteins. Degradation and instability was greatest for the 4x linear KAS variants, suggesting that proteolytic processing was occurring within the additional KAS sequences. Subsequent removal of the DSSG linker between adjacent KAS sequences did not improve stability.

Thus, it was concluded that these variants were prone to proteolytic processing when successive, multiple KAS sequences were expressed in a linear sequence. Soluble

variants with a single KAS at one or both termini were relatively stable and less prone to degradation and less prone to compromised purification yields.

Study 4: Large scale protein yields and stability

Most recombinant proteins expressed as soluble proteins and purified under non-denaturing conditions had relatively high yields of their final products (> 10 mg/L culture). Some of them even reached a yield of over 20 mg/L culture. In particular, the His-tagged KDAK-1V2S-AVQP protein (SEQ ID NO: 59, excluding His tag) had a yield of 45 mg/L culture when expressed in 2 YT rich medium. The most remarkable yields were seen for the purified final products of the tagless KDAK-1V2S-AVQP (SEQ ID NO: 59) and KDAK-3S-AVQP (SEQ ID NO: 69) which were expressed in Terrific Broth (TB) and had production of over 80 and 60 mg/L culture, respectively, multiple times as those with cells grown in LB or 2YT medium. This significant improvement in target protein production was owing to the high growth rates of the cells and the high cell density when the cells were grown in TB.

The yields of these same recombinant proteins when purified from inclusion bodies or from soluble fractions under conditions to deliberately denature them (e.g. urea) were much lower (2.1-3.6 mg/L culture). It was noted that denatured proteins had low binding affinity to the Ni-affinity resin under denaturing conditions and low solubility when finally equilibrated in non-denaturing buffers. In addition, the variants purified from their denatured forms were unstable in unbuffered saline, regardless of whether the protein was initially expressed as a soluble protein or insoluble form and was purified using a prepacked column or using loose Ni-NTA resin. These results suggest that where denaturing agents are not required, it may be desirable to reduce or avoid their usage.

Low expression temperature at 16 °C proved to be effective for improving soluble expression of poorly soluble proteins such as KDAK1 (SEQ ID NO: 66). Nevertheless, enrichment on column in a buffer of low ionic strength during anion exchange purification remarkably compromised yield due to aggregation or precipitation on column, even at the low temperature of 4 °C. For these proteins, purification bypassing the anion exchange step resulted in a higher yield, e.g., the yield of KDAK1 was doubled when produced this way. It was also found that KDAK1n (SEQ ID NO: 65) was expressed as a soluble protein,

but it did not appear to be stable in solution in later purification process, with a yield of only 2.3 mg/L culture.

Given that K1 is insoluble and that the addition of K1 or K1n to KDA or DA decreases solubility of recombinant variants on large scale purification, as stated above it seems likely that the K1 region may be responsible for instability of K1-containing chimera variants such that exclusion of K1 would be preferred if the domain is not necessary for protection.

Although His staining of gels confirmed that there was degradation within the regions containing multi KAS (K) residues, a negative effect on yield by Ni-affinity chromatography was not observed. For example, the three protective antigens, KKDAK1nKK (SEQ ID NO: 78), KKDAKK (SEQ ID NO: 54) and KDAK1nK-4S-AVQP (SEQ ID NO: 63), with over 90% of the expected full-length protein species in their final products had yields in a range of 12-21 mg/L culture.

Reducing conditions were found to be favourable for extraction of insoluble KDcAK1n from inclusion bodies using urea. A higher concentration of urea was needed under non-reducing conditions than that under reducing conditions. Apparently, the formation of disulphide bonds under denaturing conditions had adverse effects on the protein solubility. Interestingly, Native PAGE gel analysis of KDcAK1n chimera versus chimera with all four Cysteine residues mutated to Serine (SEQ ID NO: 56) showed that elimination of disulphide bonds in the mutated KDcAK1n chimera resulted in a clearer ladder formation on Native PAGE consistent with only beta-strand exchange multimerization occurring. Furthermore, the chimera mutant KDcAK1n PVQN>AVQP / 4 Cys> Ser variant (SEQ ID NO: 57) exhibited a higher solubility than KDcAK1n under identical non-reducing denaturing conditions in urea.

The concentrations of some soluble proteins such as His-tagged KDAK-1V2S-AVQP were able to reach at least 40 mg/mL. The two lead tagless candidates SEQ ID NO: 59 and SEQ ID NO: 69 (KDAK-1V2S-AVQP and KDAK-3S-AVQP) have been concentrated up to over 30 mg/mL and 16 mg/mL in well buffering solutions at pH 6.5, respectively, and these concentrations are not yet the maximum.

In contrast, the C-term His-tagged original chimera (KDcAK1n) and its variant KDcAK1n-4S which were purified from inclusion bodies were the least stable in unbuffered saline. The highest reachable concentrations of KDcAK1n and KDcAK1n-4S in saline were 1.35 mg/mL and 2.3 mg/mL, respectively. Apparently, non-specific disulphide bond formation was one of the factors for significant protein aggregation and low solubility of the Chimera. The removal of Cys residues in KDcAK1n-4S disabled the formation of intermolecular disulfide bonds and improved the protein solubility.

The C-term His-tag was not seen to have considerable effects on protein production and solubility when the proteins were expressed under the same conditions.

In purification of tagless candidates, although anion exchange chromatography enriched the target proteins, this step was not able to efficiently separate the target proteins from those of the *E. coli* host due to close binding affinities to the resin as most *E. coli* proteins are acidic in a pI range of 4-7 with many of them having close pI to the target proteins. However, interestingly, the size exclusion chromatography at room temperature with the buffer of 1M $(NH_4)_2SO_4$ appeared to be a critical step and provided a highly efficient separation of target proteins from those of *E. coli*. After further purification with hydrophobic interaction and the second round of size exclusion chromatography, both lead tagless candidates were purified to a purity of 99% with a high homogeneity, which were evidenced by SDS-/native PAGE and SEC-MALS analyses.

Table 3: Summary of recombinant Kgp chimera and fragments expressed in *E. coli*

Name	Comments	Multimer [yes/no]	(Solubility in 20ml to 200ml)		His Staining degradations
			37°C, 0.2 to 0.5 mM IPTG	16°C-30°C, 0.2 - 0.5 mM IPTG	
K1	CAD domain SEQ ID 12	NA	(-)	(-)	Not tested
DUF	DUF2436 domain (SEQ ID NO: 23)	No	(+++)		Not tested
DUF-ABM213	DUF2436 domain plus ABMs (SEQ ID N): 34)	Yes	(+++)		Not tested

ABM213	ABM2+1+3 (SEQ ID NO: 18)	Yes	(+++)		Not tested
ABM21	SEQ ID NO: 16	Yes	(+++)		Not tested
ABM21-2S-AVQP	SEQ ID NO: 16 with cysteine to serine modification plus AVPQ	No	(+++)		Not tested
KDcAK1n	SEQ ID NO: 64 (truncated DUF, truncated CAD domain, no cysteine or AVPQ substitution)	Yes	(-)	(-)	(-)
KDcAK1n-4S	truncated DUF, truncated CAD domain (4C>4S)	Yes	(-)	(-)	Not tested
KDcAK1n-4S-AVQP	truncated DUF, truncated CAD domain (4C>4S) +PVQN>AVQP	No	(-)	(-)	(-)
KA	KAS domain plus ABMs 2+1+3, No DUF domain	Not tested	(+++)	(+++)	(+/-)
KDA	KAS domain, Full length DUF, ABMs 2+1+3, no CAD domain	Yes	(+++)	(+++)	(+/-)
KDAK1n	Full DUF domain and including truncated CAD domain	Yes	(+/-)	(+++) {30°C}	(-/+)
KDAK1	Full DUF domain and full length CAD domain	Yes	(-)	(++) {16°C}	(+/-)
KDA-3S-AVQP	KDA mutant	No	(+++)		(+/-)
KDAK1n-4S-AVQP	KDAK1n mutant	No	(++)	(+++) {30°C}	(-/+)

KKA	double KAS on N-terminus with no linker	not tested	(+++)		(+++)
KKDA	“	(+)	(+++)		(++)
KKDAK1n	“	(+)	(+/-)	(+++) {30°C}	(+)
KDAK	KAS on both termini with no linker	Yes	(+++)		(+)
KDcAK1nK	“	Yes	(-)	(-)	(+)
KDAK1nK	“	Yes	(+/-)	(+++) {30°C}	(+)
KDAK-3S-AVQP	“	No	(+++)		(+)
KDAK-3S	N/A	Yes	(+++)	N/A	N/A
KDAK-2S-AVQP	N/A	No	(+++)	N/A	N/A
KDAK-1V2S-AVQP	N/A	No	(+++)	N/A	N/A
KDAK-1V-AVQP	N/A	No	(+++)	N/A	N/A
KDAK-AVQP	N/A	Minimal	(+++)	N/A	(N/A)
KDcAK1nK-4S-AVQP	“	No	(-)	(-)	(+)
KDAK1nK-4S-AVQP	“	No	(++)	(+++) {30°C}	(+)
KKAKK	Double KAS on N & C termini with DSSG linkers	Yes	(+++)		(+++)
KKDAKK	“	Yes	(+++)		(++)
KKDcAK1nKK	“	Yes	(-)	(-)	(+)
KKDAK1nKK	“	Yes	(+/-)	(+++) {30°C}	(+)
KKDAK1KK	“	Yes	(-)	(++) {16°C}	(+)
KKKKA	4XKAS on N-terminus with DSSG linkers	Yes	(+++)		(++++)
KKKKDA	“	Yes	(+++)		(+++)
KKKKDcAK1n	“	Yes	(-)	(-)	(+++)
KKKKDAK1n	“	Yes	(+/-)	(+++) {30°C}	(+++)
KKKKDAK1	“	Yes	(-)	(++) {16°C}	(++)

Study 5: Assessing importance of DUF domain and KAS domain

In this study, the inventors tested various constructs (antigens) to determine the importance of the DUF domain in the chimeric fusion proteins, and to compare it to the solubility/stability of a construct comprising an active site sequence derived from an Arginine-dependent gingipain. The constructs produced were: KDAK-3S-AVQP ; KDAK-2S-AVQP (His tag), KAK-2S-AVQP (His tag) and KDAR-3S-AVQP (His tag).

Protein production and yields

All antigens expressed well. The remaining one cysteine residue within the DUF domain in the KDAK-2S-AVQP construct did not appear to affect the protein solubility.

The RAS containing candidate KDAR-3S-AVQP was less soluble than the other antigens, indicating an interference of the RAS sequence with the protein structure, but was still able to be used in subsequent mouse model experiments and could be purified under non-reducing conditions from soluble fractions.

All the antigens were purified under non-reducing conditions from soluble fractions. The three His-tagged proteins were purified through Ni affinity, anion exchange and size exclusion chromatography. KDAK-3S-AVQP (untagged) was purified through anion exchange, hydrophobic interaction and size exclusion chromatography. The yields of purified antigens were high. Among the three His-tagged proteins, KDAK-2S-AVQP-His6 had the highest yield (>160 mg/L culture, 20 mg/g wet cells).

The C-term His-tag was not seen to have effects on protein production and solubility when the proteins were expressed under the same conditions, suggesting that the presence of His-tag did not interfere with the protein core structures.

In contrast, the KDAR-3S-AVQP-His6 antigen had a lower yield (55 mg/L culture, 7.2 mg/g wet cells) due to its lower solubility. The antigen KAK-2S-AVQP-His6 with the smallest molecular weight, had a yield of 106 mg/L culture (14 mg/g wet cells). Identities of all the antigens were confirmed by intact protein MS spectrometry, with the first Met residue missing.

As for the His-tagged proteins, the higher solubility of KAS only antigens relative to the RAS containing antigen was reflected from the peak intensities in the elution

profiles of the His-tagged proteins at the Ni-affinity purification step. Nevertheless, when the RAS antigen was pure, it was also highly soluble, able to be concentrated up to at least 15 mg/mL.

Although these proteins were concentrated so far to a range of 15-33 mg/mL their high solubility may allow them to be concentrated even further to a higher concentration.

Oligomerisation

Some minor bands could be seen below each major full-length target protein on the SDS gels of the anion exchange fractions of KAK-2S-AVQP-His6 and KDAR-3S-AVQP-His6, suggesting occurrence of minor degradation in these two proteins. Most degradants were essentially removed by size exclusion chromatography and a high quality of final product for each protein was obtained (Figure 13). Nevertheless, the construct that did not contain the DUF domain (KAK-2S-AVQP-His6) had more serious degradation, persistent to its final product (Figure 13), suggesting that DUF domain may provide a level of protection from degradation.

Two species in KAK-2S-AVQP-His6 and the species of laddering molecular weights below the monomeric form in KDAR-3S-AVQP-His6 co-eluted in one predominant peak in AIEX as also seen from previous Ni-affinity chromatography.

The slightly asymmetrical major peaks of both KAK-2S-AVQP-His6 and KDAR-3S-AVQP-His6 in their size exclusion tailing profiles may also be an indication of the presence of degradants.

Such degradation was not clearly seen for KDAK-2S-AVQP-His6 (eg Figure 13). It appeared that the presence of RAS destabilised the protein and DUF domain made the protein more stable.

Although minor degradation may occur, all the final products except KAK-2S-AVQP-His6 had a high purity and high homogeneity, which was also supported by SEC-MALS analysis. SEC-MALS exhibited predominant existence of monomer in solution at 2 mg/mL for all these proteins (data not shown). KDAK-3S-AVQP and KDAK-2S-AVQP-His6 appeared to have a higher quality among the four antigens and KAK-2S-AVQP-His6

seemed to be the least homogeneous according to the SDS-PAGE analysis although the minor species was not resolved in SEC-MALS analysis (eg Figure 13).

Example 3: Animal studies

Prevention of *P. gingivalis* bone loss in mouse model of periodontitis

1. Experiment 1

Antigens tested: KDcAK1n 50 µg; KDcAK1n 0.5 µg; KDA 50 µg; KDA 0.5 µg; KDAK1 50 µg; KDAK1 0.5 µg. All antigens absorbed onto alum in PBS (pH 7.4).

KDcAK1n: from inclusion bodies, batch purified with Ni-NTA resin followed by dialysis into 2 M urea-PBS under non-reducing conditions.

KDA and KDAK1: from soluble fractions, Ni-affinity (prepacked column) and anion exchange chromatography with gradient elution followed by dialysis into PBS, under non-reducing conditions.

P. gingivalis-induced alveolar bone loss in mouse maxillae

KDcAK1n and KDA protected against bone loss in the animal model. KDcAK1n protected against bone loss at both concentrations tested while KDA only protected at 50 µg (Figure 4). Data for KDAK1 not shown.

Antibody response

Serum antibody subclass responses of immunised mice in the periodontitis model were examined by ELISA. Antisera was used to probe heat killed *P. gingivalis* strain W50 as the absorbed antigen. Antibody responses are expressed as the ELISA titre obtained minus triple the background level, with each titre representing the mean ± s.d. of the 10 individual mice (Figure 5). KDcAK1n (referred to in Figure 5 as “chimera”) and KDA induced the strongest total IgG and IgG1 responses against *P. gingivalis* whole cells followed by KDAK1. There was no significant difference in IgG and IgG1 antibody responses at 50 µg between the antigens tested.

2. Experiment 2

Antigens tested: KDcAK1n 50 µg; KDcAK1n 0.5 µg; KDA 50 µg; KDA 0.5 µg; KDAK1 50 µg; KDAK1 0.5 µg. All antigens absorbed onto alum in saline (pH 7.4).

Antigens were purified in the same way as for Experiment 1.

P. gingivalis-*induced alveolar bone loss in mouse maxillae*

This experiment examined the same antigens as Experiment 1; however, they were absorbed onto alum using saline rather than PBS. Bone loss results mirrored Experiment 1 and in Figure 4, (data not shown) where only KDcAK1n and KDA showed protection, indicating using PBS or saline for alum preparation had no effect on the experimental outcome.

Antibody response

All antigens tested induced similar total IgG and IgG1 responses against whole cell *P. gingivalis*. KDA and KDAK1 induced stronger IgG2a responses compared to KDcAK1n (not shown).

3. Experiment 3

Antigens tested: all antigens except KDcAK1n were fractionated as described: KDcAK1n (dimer); KDcAK1n (multimer); KDcAK1n (Chimera, original); KDcAK1n-4S-AVQP; KDA (dimer); KDA (multimer).

KDcAK1n-4S-AVQP, KDcAK1n dimer and multimers: urea extracted from inclusion bodies, Ni-affinity column purified followed by gel filtration, with the two KDcAK1n samples under reducing conditions.

KDA dimer and multimers: from soluble fractions, Ni-affinity column purified followed by anion exchange and gel filtration, under non-reducing conditions.

KDcAK1n : purified as in Experiment 1.

Pre-screen of vaccine candidates with mAbs to P. gingivalis *epitopes*

In an attempt to develop a pre-screening assay to determine antigen suitability for animal models, antigens were screened by ELISA using mAbs against KAS2, ABM2, ABM3 and EP1. All vaccine candidates were able to bind the KAS2, ABM3 and EP1 mAbs, as seen by the large titration curve compared to the negative control muBM4 mAb. While KDcAK1n and the KDcAK1n-4S-AVQP bound the ABM2 mAb, KDcAK1n dimer, KDcAK1n multimer, KDA dimer and KDA multimer did not – indicating that the epitope this mAb recognises is not accessible in these constructs (not shown).

P. gingivalis-*induced alveolar bone loss in mouse maxillae*

Apart from KDcAK1n with 2x His tags, none of the fractionated antigens including the KDcAK1n with a single His tag protected against *P. gingivalis*-induced bone loss (Figure 6), although there was a trend (not significant) for protection by the purified dimer species of the single His tag chimera (KDcAK1n).

Antibody response

Serum antibody subclass responses of immunised mice in the periodontitis model were examined by ELISA. Antisera was used to probe heat killed *P. gingivalis* strain W50 as the adsorbed antigen. All antigens tested generated an IgG response to varying degrees. KDcAK1n multimer and KDA multimer generated strong IgG1 isotype responses.

Pooled serum samples were used to probe *P. gingivalis* domains adsorbed onto ELISA plates (not shown). Total IgG responses against ABM21 (multimer and dimer) and ABM213 (multimer and dimer) were generated by all antigens except KDcAK1n-4S-AVQP and KDA dimer.

An IgG response to DUF2436 was only evident in KDA (dimer and multimer) – the only antigen tested in the model with the full DUF domain. Pooled serum samples were also used to probe *P. gingivalis* epitope peptides (not shown). No clear pattern between protective and non-protective sera could be observed.

Analysis of the unprotective purified dimer and multimer antigens

Analysis of the anion exchange and gel filtration purified, single KDA dimer and multimer species that did not provide protection in Experiment 3 using reducing and non-

reducing Native and SDS PAGE revealed that the purified single species in both cases were disulphide cross-linked denatured domains that were disulphide locked into a stable species. This analysis explains why these molecular weight species were stable and could be purified by anion exchange and gel filtration chromatography.

4. Experiment 4

Antigens tested: KKDA1nKK; KDAK1n; KDAK1nK-4S-AVQP; KKDAKK.

All the antigens were purified from soluble fractions under non-reducing conditions with Ni-affinity column, anion exchange and gel filtration chromatography except KDAK1nK-4S-AVQP without using anion exchange chromatography.

Alum absorption

The ability of Alhydrogel 2% to absorb antigens was tested by incubating antigens with alum for 60 minutes at 4°C with gentle mixing. Alum was pelleted and a Bradford protein assay was then conducted on the antigens pre- and post- alum adsorption. All antigens bound to alum at percentages of 91.7% to 99.3%. An SDS-PAGE gel was also run on samples pre- and post- alum absorption and was in agreeance with the Bradford Assay protein determination.

Pre-screen of vaccine candidates with mAbs to P. gingivalis epitopes

All vaccine candidates were able to bind the KAS2, ABM3 and EP1 mAb, as seen by the large titration curve compared to the negative control muBM4 mAb. While KDAK1nK-4S-AVQP bound the ABM2 mAb, KKDA1nKK, KDAK1n and KKDAKK did not – indicating that the epitope this mAb recognises is not accessible in these constructs (not shown).

P. gingivalis *induced alveolar bone loss in mouse maxillae*

KKDAK1nKK, KDAK1nK-4S-AVQP and KKDAKK protected against *P. gingivalis*-induced bone loss (Figure 7).

Antibody response

Serum antibody subclass responses of immunised mice in the periodontitis model were examined by ELISA. Antisera was used to probe heat killed *P. gingivalis* strain W50 as the adsorbed antigen. All antigens tested generated an IgG response to varying degrees. The protective KKDAK1nKK and KKDAKK induced robust total IgG and IgG1 responses against heat-killed *P. gingivalis*. Interestingly, KDAK1nK-4S-AVQP, which protected against bone loss, had a lower antibody response to the whole cell *P. gingivalis* than some of the less protective antigens (KDAK1n).

Pooled serum samples were used to probe *P. gingivalis* domains adsorbed onto ELISA plates. Total IgG responses against ABM21(multimer and dimer) and ABM213 (multimer and dimer) were generated by all antigens to varying degrees. An IgG response to DUF2436 was only evident in the antigens that contain the full DUF domain (KKDAK1nKK, KDAK1n, KDAK1nK-4S-AVQP and KKDAKK). Pooled sera samples were also used to probe *P. gingivalis* epitope peptides and again no clear pattern between protection and non-protection could be observed, although there was a definite tendency for a high KAS titre in the protective antisera.

5. Experiment 5

Antigens tested

All the antigens were purified under non-reducing conditions using Ni-affinity chromatography followed by dialysis into saline.

KDcAK1n - from inclusion body, using urea and affinity column purified (IB, Urea, AC); KDA – from soluble fraction and affinity column purified (S, AC); KDA – from soluble fraction, using urea and affinity column purified (S, Urea, AC); KDA – from soluble fraction, using urea and batch purification method (S, Urea, Batch); KDAK1 – from soluble fraction, using urea and affinity column purified (S, Urea, AC); KDAK1 – from inclusion body, using urea and affinity column purified (IB, Urea, AC); KDAK1 – from inclusion body, using urea and batch purification method (IB, Urea, Batch).

Pre-screen of vaccine candidates with mAbs to P. gingivalis *epitopes.*

All candidates were able to bind the KAS2, ABM3 and EP1 mAb, as seen by the large titration curve compared to the negative control muBM4 mAb. None of the antigens bound the ABM2 mAb, indicating that the epitope this mAb recognises is not accessible in these constructs (not shown).

P. gingivalis *induced alveolar bone loss in mouse maxillae*

KDcAK1n (IB, Urea, AC), KDA (S, AC), KDAK1 (S, Urea, AC), KDAK1 (IB, Urea, AC) KDAK1 (IB, Urea, Batch) and KDA (S, Urea, Batch) showed significant protection against bone loss with KDcAK1n (IB, Urea, AC), KDA (S, AC), and KDAK1 (IB, Urea, Batch) providing the best protection. It should be noted that the significant protection observed with KDA (S, AC) was lost on denaturation with urea, e.g. KDA (S, Urea, AC) (Figure 8).

It is noteworthy that unfractionated affinity-purified antigens provided protection of bone loss, in particular good protection was observed with affinity purified soluble KDA but this protection was partially lost on treatment with urea under oxidizing conditions that would promote disulphide cross links of denatured D and A domains. Again these results suggest that disulphide cross linking of denatured domains may destroy antigen-induced protection, such that urea should be avoided and preparation of soluble, folded domains should be preferred.

6. Experiment 6

Antigens tested

All the antigens were purified under non-reducing conditions using Ni-affinity chromatography followed by dialysis into saline. Antigens tested were: KDcAK1n - from inclusion body, using urea and affinity column purified (IB, Urea, AC); KDAK-3S-AVQP, KDAK1nK, KDAK1n-4S-AVQP, KDAK, KKDAKK, KKDAK1nKK.

P. gingivalis *induced alveolar bone loss in mouse maxillae*

All antigens tested protected against *P. gingivalis*-induced bone loss (Figure 9).

7. Experiment 7

Antigens tested

In this experiment, the inventors tested several new constructs based on the 4S, 3S, 4S-AVQP and 3S-AVQP mutations: KDcAK1n (prior art chimera), KDcAK1n-4S, KDAK, KDAK-3S, KDAK-3S-AVQP, KDAK1nK-4S-AVQP.

All the antigens possessed a His-tag and were purified using affinity chromatography. Antigens were absorbed onto alum by the addition of equal volumes of Alum to protein on a weight-to-weight ratio. The Alum:Protein compound was adjusted to the appropriate concentration for IP or SC injection (0.5mg/mL), prior to incubation at 4 °C with constant mixing. Mice were then injected IP (Vaccination 1) or SC (Vaccination 2). All the antigens were purified under non-reducing conditions using Ni-affinity chromatography followed by dialysis into saline.

P. gingivalis *induced alveolar bone loss in mouse maxillae*

Compared to the naïve (non-challenge) control, animals injected with the KDcAK1n, KDAK-3S-AVQP and KDAK did not develop significant bone loss (Figure 10). KDAK1nK-4S-AVQP provided partial protection. The infected control mice developed significant levels of alveolar bone loss (Figure 10).

Results were statistically different to the Control Challenge (###p <0.05, #### p< 0.01, ###), one-way ANOVA and post hoc Dunnet's T3) (Figure 10).

8. Experiment 8

Antigens tested

In this experiment, the inventors tested several new constructs based on different permutations of the 1V, 1V-2S, 3S and AVQP mutations: KDcAK1n (prior art chimera), KDAK-3S-AVQP, KDAK-1V-2S-AVQP, KDAK-1V-AVQP and KDAK-AVQP.

All the antigens possessed a His-tag and were purified using affinity chromatography. Antigens were absorbed onto alum by the addition of equal volumes of Alum to protein on a weight-to-weight ratio. The Alum:Protein compound was adjusted to

the appropriate concentration for IP or SC injection (0.5mg/mL), prior to incubation at 4°C with constant mixing. Mice were then injected IP (Vaccination 1) or SC (Vaccination 2).

P. gingivalis *induced alveolar bone loss in mouse maxillae*

The mice immunised with Chimera (KDcAK1n), KDAK-3S-AVQP, KDAK-1V-2S-AVQP, KDAK-1V-AVQP nor KDAK-AVQP developed significantly less bone loss than the mice in the infected control group (Figure 11). The infected control mice developed significant levels of alveolar bone loss compared to the naïve mice. (Statistical analysis was by one-way ANOVA and post-hoc Dunnet's T3. * = $p<0.05$; in comparison to the Control Challenge Group).

9. Experiment 9

Antigens tested

In this experiment, the inventors tested several constructs with different permutations of the 1V-2S, 3S and AVQP mutations, at different concentrations, as well as introducing antigens purified without the use of a His-tag.

Groups were also included to determine the effect of alum on the antigenicity of the proteins. All the antigens that possessed a His-tag and were purified using affinity chromatography, and those without a His-Tag were purified using a combination of anion exchange-, size exclusion- and hydrophobic interaction-chromatography (Figure 12), as described in the methods section. When appropriate, antigens were absorbed onto alum using a constant concentration of Alum (100µg/dose) regardless of the concentration of protein (100 µg, 50 µg and 25 µg). The Alum:Protein compound was adjusted to the appropriate antigen concentration for IP and SC injection (1.0 mg/mL, 0.5mg/mL or 0.25mg/mL), prior to incubation at 4°C with constant mixing. Mice were then injected IP (Vaccination 1) and SC (Vaccination 2).

Antigens tested were: KDcAK1n – His-tagged, from inclusion body, using urea and affinity column purified (IB, Urea, AC); KDAK-3S-AVQP untagged (100 µg, 50 µg and 25 µg doses) KDAK-1V-2S-AVQP untagged (100 µg, 50 µg and 25 µg doses), KDAK-3S-AVQP His-tagged (100 µg dose), KDAK-3S-AVQP untagged and without alum.

P. gingivalis *induced alveolar bone loss in mouse maxillae*

As shown in Figure 12, compared to the naïve control, mice treated with the prior art chimera (KDcAK1n) at 100 µg did not develop significant bone loss. Furthermore, mice treated with all concentration of the KDAK-3S-AVQP (100 µg, 50 µg and 25 µg) also did not develop significant levels of alveolar bone loss compared to the naïve control mice.

Mice that received the KDAK-1V-2S-AVQP vaccination were protected from alveolar bone loss at the highest dose of 100 µg but did develop significant bone loss when vaccinated with 50 µg and 25 µg or protein.

The removal of the His-tag from the KDAK-3S-AVQP did not significantly affect the efficacy of protection. However, the removal of alum from the vaccination resulted in higher levels of bone loss for both vaccines.

Antibody response

Serum antibody subclass responses of immunised mice in the periodontitis model were examined by ELISA. Antisera was used to probe heat killed *P. gingivalis* strain W50 as the adsorbed antigen. All antigens tested generated a total IgG response towards *P. gingivalis,* with KDA (S, Urea, AC), followed by KDAK1 (IB, Urea, Batch) and KDcAK1n (IB, Urea, AC) having strong IgG1 responses to *P. gingivalis* whole cells. Only KDAK1 (IB, Urea, AC) induced a strong IgG2a response to *P. gingivalis* whole cells.

Pooled serum samples were used to probe *P. gingivalis* domains adsorbed onto ELISA plates. Total IgG responses against ABM21 (multimer and dimer) and ABM213 (multimer and dimer) were generated by all antigens to varying degrees, with KDA (S, Urea, AC), KDcAK1n-1His (IB, Urea, AC) and KDAK1 (IB, Urea, Batch) consistently inducing the strongest response, while KDA (S, AC) induced the weakest response to the ABM21 or ABM213 (multimer and dimer). Interestingly, KDA (S, Urea, AC), KDA (S, AC) and KDAK1 (IB, Urea, AC) induced the strongest response to the DUF2436 domain.

Pooled serum samples were also used to probe *P. gingivalis* epitope peptides. KDA (S, Urea, AC), KDAK1 (IB, Urea, Batch) and KDAK1 (IB, Urea, AC) induced a strong IgG response to the KAS and ABM3 epitopes. KDA (S, Urea, AC) also induced IgG

antibodies to the EP1 and ABM2a epitopes. All antigen groups with the exception of KDAK1 (S, Urea, AC) induced a response to the EP1 epitope. None of the protective antigens generated antisera that bound to the K1 epitope ABM5.

10. Experiment 10

Antigens tested

In this experiment, the inventors further tested the *in vivo* activity of constructs previously tested in Examples 6, 7, 8 and 9, and compared the activity with a construct that did not comprise the DUF domain and a construct comprising an active site sequence derived from an Arginine-dependent gingipain.

The constructs tested were: KDAK-3S-AVQP (previously tested in Experiments 6, 7, 8 and 9); KDAK-2S-AVQP (His tag), KAK-2S-AVQP (His tag) and KDAR-3S-AVQP (His tag).

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The antigen KDAK-3S-AVQP was used at two concentrations in this experiment: the previously tested 100 µg/mouse and a two fold increase at 200 µg/mouse. There was a dose dependent increase in protection against alveolar bone loss, with the mice that received the 200 µg dose developing less bone loss than the 100 µg group (Figure 14).

Restoration of one cysteine, the difference between KDAK-3S-AVQP and KDAK-2S-AVQP, did not result in a significant difference in alveolar bone loss between these two variants (Figure 14).

Similarly, removal of the D domain, the difference between KDAK-2S-AVQP and KAK-2S-AVQP, did not significantly affect the observed alveolar bone loss (Figure 14), however, the mean value for the KAK-2S-AVQP variant was higher and may have reached significance with greater power in the study (eg more animals).

Changing the C terminal KAS to a RAS group, the difference between KDAK-3S-AVQP and KDAR-3S-AVQP, significantly increased the mean alveolar bone loss even further when compared with the other variants at an equivalent vaccine dose (Figure 14). This emphasises the importance of the KAS sequence for providing protection against

bone loss in this model. Nonetheless, the KDAR-3S-AVQP antigen still produced lower mean bone loss than the infected control animals and therefore still provides evidence that the RAS domain can be utilised in the context of a chimeric fusion protein antigen as described herein.

Antibody response

Serum antibody subclass responses of immunised mice in the periodontitis model were examined by ELISA against several different antigens, including whole *P. gingivalis*, purified RgpA/Kgp complex, the purified catalytic domain Kgpcat as well as several different protective epitopes including KAS, and the protective epitopes of the ABM domain, ABM3 and ABM2. (The RgpA/Kgp complex and purified catalytic domain Kgpcat were purified from *P. gingivalis*, and confirmed to be folded and enzymatically active when assayed).

Initially antisera were used to probe heat killed *P. gingivalis* as the adsorbed antigen. Antibody responses are expressed as the ELISA titre obtained minus double the background level, with each titre representing the mean ± s.d. of the 10 individual mice. All antigens tested generated IgG responses towards *P. gingivalis,* with the strongest response observed after vaccination with KDAR-3S-AVQP and the KDAK-3S-AVQP 200 µg vaccinations. The immune response induced was predominately an IgG1 immune response, which is associated with a protective immune response (Figure 15).

Antibody titres against *P. gingivalis* purified RgpA/Kgp protease complex (data not shown) and recombinant Kgpcat from *P. gingivalis* (data not shown) were also measured. In both cases the KDAK-3S-AVQP vaccine gave a strong, dose dependent, again, predominately IgG1 immune response. The KDAK-2S-AVQP vaccine induced higher IgG1 antibodies against RgpA/Kgp protease complex and recombinant Kgpcat compared to the other two vaccines tested, KAK-2S-AVQP and KDAR-3S-AVQP.

To further define the reactivity to protective epitopes, serum samples were pooled and used to probe *P. gingivalis* specific biotinylated peptides bound to streptdavidin plates. Very high titres were observed against the KAS2 peptide for every antigen tested. However, KDAK-3S-AVQP induced the highest anti-KAS2 antibodies in a dose dependent, IgG1 dominant manner. Of the three previously untested vaccines, similar to

the Kgpcat and RgpA/Kgp complex responses, vaccination with KDAK-2S-AVQP induced the highest amount of anti-KAS2 antibodies.

Lower levels of anti-ABM2 antibodies were induced after vaccination with all proteins compared to anti-KAS2 antibodies, however the immune responses were still strong and predominantly IgG1. Vaccination with KDAK-3S-AVQP induced a dose dependent IgG1 dominant immune response, however KAK-2S-AVQP induced the strongest immune response observed against ABM2.

Finally the dose response data from Experiments 9 and 10 for KDAK-3S-AVQP protection of *P gingivalis*-induced bone loss was closely associated with antibody titre cross-reactive with the purified, native Kgpcat protease (Figure 16).

Summary and discussion of results presented in Examples 2 and 3

The work described in this report compares production and efficacy of various vaccine candidates in order to identify the components of a chimera vaccine that promote ease of production (such as components contributing to improved solubility, stability and reduced propensity to multimerise) and which are most effective in eliciting immune responses to *P. gingivalis* and/or in reducing *P. gingivalis*-induced alveolar bone loss.

While efficacious in preventing periodontal bone loss in the animal periodontitis model, a prior art vaccine, KDcAK1n, is expressed by *E. coli* as inclusion bodies and exhibits variable solubility and stability.

KDcAK1n was based on a fusion between the active site sequence (KAS or K) of the Lys-specific gingipain Kgp and the processed adhesin fragment (A1) of the Kgp polyprotein. From structural analysis of the Kgp polyprotein domains it is now clear that the A1 adhesin fragment found on the cell surface comprises three discrete structural domains, the DUF domain (D), the ABM domain (A) and the K1 domain (K1). The processing of the Kgp polyprotein on the surface of *P. gingivalis* to release the proteinase catalytic domain and adhesins involves N-terminal truncation of the DUF domain by 38 amino acid residues. Adding these extra N-terminal 38 residues to the construct to produce the full DUF domain instead of the truncated domain (Dc) produced a highly soluble recombinant protein (e.g. DUF2436, KDA and KKDAKK).

KDcAK1n also included a C-terminally truncated K1 domain (K1n) again based on the cell surface processing of the Kgp polyprotein to produce the A1 adhesin fragment. The full K1 domain with the addition of the missing C-terminal sequence to K1n was not improved in terms of solubility as the protein was still expressed largely as inclusion bodies by *E. coli*. As most constructs with partial or complete K1 domains exhibited some solubility issues it was concluded that the full DUF domain was the critical domain for expression of constructs as soluble proteins by *E. coli* and that inclusion of the K1 domain in the chimera variants should be avoided if possible. Furthermore, from periodontal bone loss and epitope analysis data from the animal experiments it appeared that the K1 domain may not add significantly to protection, particularly where none of the protective antigens generated anti-sera that bound to the K1 epitope ABM5.

KDcAK1n contains four cysteine residues, one in DUF, two in ABM and one in K1. These cysteine residues are thought to contribute to the problem with the formation of inclusion bodies. *P. gingivalis* is an obligate anaerobe and requires a highly reduced environment to be pathogenic. The known or modelled structures of the Kgp domains indicate that the cysteine residues are reduced on the cell surface and not involved in disulphide bridges. Expression in *E coli* under more oxidizing conditions results in disulphide bridge formation between denatured domains and inclusion body formation. The disulphide bridges between denatured domains of the chimera can be seen on non-reducing Native-PAGE which changes upon reduction.

The results presented herein shown that the folded ABM domain of Kgp multimerizes by beta-strand exchange to form a uniform ladder which is independent of reducing agent (Figure 3, lanes 9 and 10). This novel beta-strand exchange multimerization is an important mechanism that *P. gingivalis* uses to form a surface layer of the gingipains, not only on cells but also on outer membrane vesicles that are released into the host during disease progression. This work characterising the prior art chimera KDcAK1n and domains clearly shows there are two forms of multimers present in the original chimera; disulphide bridged denatured domain-based multimers and beta-strand exchange native ABM domain-based multimers. Attempts to separate and purify these different forms into a specific dimer and larger molecular weight multimer were unsuccessful. Furthermore, use of these different purified single species, did not result in protection against periodontal bone loss in the animal model due to the single species

being disulphide-locked denatured structures (Figure 6). This work suggests that soluble, defined and homogeneous recombinants with folded D and A domains without disulphide cross links would be superior to the mixtures of denatured and native forms that are produced by solubilization of the chimera from inclusion bodies in 8M urea with low yields (Figures 6, 8 and 9).

These results suggest that the presence of cysteine residues in the antigen may be problematic for expression and purification of the soluble and defined candidate for commercial development. Hence the possibility of mutating those cysteine resides to serines was explored. Mutation of the 4 cysteine residues to 4 serine residues did not stop ABM (A) native domain beta-strand exchange multimerization indicating that the ABM domain was still properly folded in the “4C” to “4S” mutant (Figure 3).

Furthermore, these results show that mutation in the critical sequence of the ABM domain (PVQN > AVQP) which is predicted to be the Pro hinge elbow to allow beta-strand flipping, eliminated beta-strand exchange multimerization (Figure 3). Hence the combination of mutating the four Cys residues to Ser and hinge region PVQN to AVQP eliminates multimerization under both oxidizing and reducing conditions (Figure 3). In this regard, the mutant KDAK1nK-4S-AVQP protected against periodontal bone loss and produced good antibody titres against the predicted protective epitopes (KAS, DUF, ABM) in the animal periodontitis model (Figure 7).

An examination of antibody responses to the different segments of the chimera (K, D, A and K1) and suspected important epitopes (KAS, ABM2, ABM3, EP1) using protective and non-protective sera did not provide any obvious patterns, although it was clear that those chimera variants that protected tended to show strong (IgG/IgG1) responses against whole *P. gingivalis* cells as well as against the known protective epitopes (KAS, DUF, ABM) such that it appeared that protection may not be related to any one epitope but more to a combination of epitopes being required to ensure full protection of periodontal bone loss. However, there was a tendency for one epitope to stand out involving the active site sequence KAS for chimera variants that provided protection.

Although there was no clear indication of any one epitope being more important such that the response against that epitope could be used as a surrogate or biomarker of

protection induced by the vaccine, it was clear that the protective chimera variants tended to produce a good antibody response against the protease active site sequence (KAS or K). Antibodies generated to the active site sequence have been shown previously to neutralize the proteolytic activity of the gingipains (Kgp & RgpA/B), the major virulence factors of *P ginigvalis*. Hence in an approach to enhance the antibody titre against the active site and therefore enhance protection the inventors added extra copies of the active site sequence (K) to KDA and KDAK1n and both constructs exhibited improved protection in the animal periodontitis model (Figure 8). The soluble, non-multimerising mutant KDAK1nK-4S-AVQP with two copies of the KAS (K) motif gave good protection against periodontal bone loss induced by *P. gingivalis*. (Figure 9).

Other soluble, non-multimerising mutants, including KDAK-3S-AVQP, KDAK-1V-2S-AVQP and KDAK-2S-AVQP also gave good protection against periodontal bone loss induced by *P. gingivalis* (Figures 9 to 13).

CLAIMS

1. A chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs),

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD); and

b) comprises an amino acid sequence corresponding substantially to the full length of the DUF2436 domain of an Arg- or Lys-gingipain, or a sequence at least 80% identical thereto;

and comprises one or more amino acid substitutions selected from:

c) one or more cysteine amino acid substitutions in the ABMs, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

d) an amino acid motif substitution selected from:

i) substitution of the proline and/or an asparagine residues in the sequence PVQN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

ii) substitution of the motif NEFA to SEYQ in the sequence corresponding to, or at a position equivalent to residues 2 to 5 of the sequence of SEQ ID NO: 14 or 19 (ABM1);

iii) substitution of the second tyrosine residue, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20 (ABM2), and of the tryptophan residue, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19 (ABM1) to alanine residues.

2. A chimeric or fusion protein according to claim 1, wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20, SEQ ID NO: 14 or 19 and/or SEQ ID NO: 17 or 21 or sequences at least 80% identical thereto.

3. A chimeric or fusion protein according to claim 1 or 2, wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto.

4. A chimeric or fusion protein according to any one of claims 1 to 3 wherein the chimeric or fusion protein does not comprise a sequence of a CAD, or part thereof, having the amino acid sequence set forth in SEQ ID NO: 12 or 13 or a sequence at least 80% identical thereto.

5. A chimeric or fusion protein according to any one of claims 1 to 4, wherein an amino acid sequence corresponding substantially to the full length of the DUF2436 domain, of an Arg- or Lys-gingipain, is a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the length of a DUF2436 domain of an Arg- or Lys-gingipain.

6. A chimeric or fusion protein according to claim 5, wherein the amino acid sequence of the DUF2436 domain of an Arg- or Lys-gingipain is a sequence set forth in SEQ ID NO: 23 or is a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

7. A chimeric or fusion protein according to any one of claims 1 to 6, wherein the second polypeptide comprises the sequence set forth in SEQ ID NO: 34 or a sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

8. A chimeric or fusion protein according to any one of the preceding claims wherein the second polypeptide comprises a substitution of the proline and/or asparagine

residues in the sequence PVQN of ABM1 of a *P. gingivalis* adhesin domain, corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NOs: 14 or 19.

9. A chimeric or fusion protein according to claim 8, wherein the second polypeptide comprises the sequence AVQP, in ABM1, corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19.

10. A chimeric or fusion protein according to any one of claims 1 to 7, wherein the second polypeptide comprises a substitution of the motif NEFA to SEYQ in the ABM1 of a *P. gingivalis* adhesin domain, corresponding to, or at a position equivalent to residues 2 to 5 of SEQ ID NO: 14 or 19.

11. A chimeric or fusion protein according to any one of claims 1 to 7, wherein the second polypeptide comprises a substitution of the second tyrosine residue of ABM2, corresponding to or at a position equivalent to residues at position 5 of SEQ ID NO: 15 or 20, and of the tryptophan residue of ABM1, corresponding to or at a position equivalent to residue at position 23 of SEQ ID NO: 14 or 19, to alanine residues.

12. A chimeric or fusion protein according to any one of claims 8 to 11, wherein the second polypeptide does not comprise a substitution of the cysteine residue in the DUF2436 domain.

13. A chimeric or fusion protein according to any one of claims 8 to 11, wherein the second polypeptide comprises a substitution of the cysteine residue in the DUF2436 domain.

14. A chimeric or fusion protein according to any one of claims 8 to 13, wherein the second polypeptide comprises a substitution of one or more of the cysteine residues in the ABM domains.

15. A chimeric or fusion protein according to claim 13 or 14, wherein the substitution of the one or more cysteine residues is a substitution to a serine residue or a valine residue.

16. A chimeric or fusion protein according to claim 14, wherein the substitution of the one or more cysteine residues is a substitution to a serine residue.

17. A chimeric or fusion protein according to any one of claims 13 to 16, wherein the sequence of the one or more ABM domains is set forth in SEQ ID NO: 18 and wherein the one or more cysteine residues correspond to the residues at positions 36 and 50 of SEQ ID NO: 18 or 22, or positions equivalent thereto.

18. A chimeric or fusion protein according to any one of the preceding claims, wherein the second polypeptide comprises or consists of the amino acid sequence as set forth in any one of SEQ ID NOs: 35 to 49.

19. A chimeric or fusion protein according to any one of claims 8 to 18, wherein the second polypeptide comprises or consists of the amino acid sequence as set forth in a SEQ ID NO: 42, or 44 to 49.

20. A chimeric or fusion protein according to any one of claims 1 to 19, wherein the first polypeptide is linked to the second polypeptide.

21. A chimeric or fusion protein according to any one of claims 1 to 20, wherein the first polypeptide is located N-terminally to the second polypeptide.

22. A chimeric or fusion protein according to any one of claims 1 to 21, wherein the first polypeptide is joined directly to the second polypeptide.

23. A chimeric or fusion protein according to any one of claims 1 to 21, wherein the first polypeptide is linked to the second polypeptide via a linker.

24. A chimeric or fusion protein according to claim 23, wherein the linker is a peptide linker.

25. A chimeric or fusion protein according to claim 24, wherein the peptide linker comprises at least 2 amino acids, at least 5 amino acids, at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, preferably wherein the linker comprises no more than 50 amino acids.

26. A chimeric or fusion protein according to claim 24 or 25, wherein the peptide linker is no more than 25 amino acids, no more than 20 amino acids, no more than 15 amino acid or no more than 10 amino acids.

27. A chimeric or fusion protein according to any one of claims 1 to 26, wherein the first polypeptide comprises or consists of the amino acid sequence as set forth in any one of SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

28. A chimeric or fusion protein according to claim 27, wherein the first polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1 or 2, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

29. A chimeric or fusion protein according to any one of claims 1 to 28, wherein the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto.

30. A chimeric or fusion protein according to claim 29, wherein the one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* is located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

31. A chimeric or fusion protein according to claim 30, wherein the one or more further polypeptides is directly linked to the first or second polypeptide of the chimeric or fusion protein.

32. A chimeric or fusion protein according to claim 31, wherein the one or more further polypeptides is linked to the first or second polypeptide by a peptide linker.

33. A chimeric or fusion protein according to claim 32, wherein the peptide linker comprises at least 2 amino acids, at least 5 amino acids, at least 10 amino acids,

at least 15 amino acids, at least 20 amino acids, preferably wherein the linker comprises no more than 50 amino acids.

34. A chimeric or fusion protein according to claim 32 or 33, wherein the peptide linker is no more than 25 amino acids, no more than 20 amino acids, no more than 15 amino acid or no more than 10 amino acids.

35. A chimeric or fusion protein according to any one of claims 29 to 34, wherein the one or more further polypeptides preferably comprise or consist of an amino acid sequence selected from the group of: SEQ ID NOs: 1 to 11, or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical thereto.

36. A chimeric or fusion protein according to claim 34, wherein the first polypeptide and the one or more further polypeptides, are identical or at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to each other.

37. A chimeric or fusion protein according to claim 35, wherein the first polypeptide and the one or more further polypeptide comprise an amino acid sequence of the active site of heterologous gingipains of *P. gingivalis*.

38. A chimeric or fusion protein according to claim 35, wherein the first polypeptide and the one or more further polypeptide comprise the amino acid sequences of the active site of a Lys-gingipain.

39. A chimeric or fusion protein according to claim 35, wherein the first polypeptide comprises an amino acid sequence of the active site of a Lys-gingipain, and the one or more further polypeptides comprise the amino acid sequence of the active site of an Arg-gingipain.

40. A chimeric or fusion protein according to any one of claims 1 to 39, wherein the chimeric or fusion protein comprises or consists of an amino acid sequence as set forth in any one of SEQ ID NOs: 58, 59, 60, 61, 69, 70, 71, 72, 73, 74 or 75 or sequences at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% thereto.

41. A chimeric or fusion protein according to any one of claims 1 to 39, wherein the chimeric or fusion protein comprises or consists of the amino acid sequence as set forth in SEQ ID NO: 58 or 59.

42. A chimeric or fusion protein according to any one of claims 1 to 39, wherein the chimeric or fusion protein comprises or consists of the amino acid sequence as set forth in SEQ ID NO: 60 or 61.

43. A chimeric or fusion protein according to any one of claims 1 to 39, wherein the chimeric or fusion protein comprises or consists of the amino acid sequence as set forth in SEQ ID NO: 69.

44. A chimeric or fusion protein according to any one of claims 1 to 39, wherein the chimeric or fusion protein comprises or consists of the amino acid sequence as set forth in SEQ ID NO: 72 or 74.

45. A chimeric or fusion protein for inducing an immune response to *P. gingivalis*, the protein comprising a first polypeptide and a second polypeptide, wherein:

A) the first polypeptide comprises or consists of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis*, or a sequence that is at least 80% identical thereto; and

B) the second polypeptide comprises or consists of an amino acid sequence of an adhesin domain of an Arg- or Lys-gingipain of *P. gingivalis*;

wherein the second polypeptide comprises the sequence of one or more adhesin binding motifs (ABMs)

and wherein the second polypeptide:

a) does not comprise: a sequence of a cleaved adhesin domain (CAD); and

comprises one or more amino acid substitutions selected from:

b) one or more cysteine amino acid substitutions in the ABMs, compared to the naturally occurring Arg- or Lys-gingipain sequences in corresponding regions; and/or

c) substitution of the proline and/or an asparagine residues in the sequence PVQN corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19 (ABM1).

46. A chimeric or fusion protein according to claim 45, wherein the one or more ABMs comprise the sequence as set forth in SEQ ID NO: 15 or 20, SEQ ID NO: 14 or 19 and/or SEQ ID NO: 17 or 21 or sequences at least 80% identical thereto.

47. A chimeric or fusion protein according to claim 45 or 46, wherein the one or more ABMs comprise the sequence set forth in any of SEQ ID NO: 16 or SEQ ID NO: 18 or 22 or a sequence at least 80% identical thereto.

48. A chimeric or fusion protein according to any one of claims 45 to 47 wherein the chimeric or fusion protein does not comprise a sequence of a CAD, or part thereof, having the amino acid sequence set forth in SEQ ID NO: 12 or 13 or a sequence at least 80% identical thereto.

49. A chimeric or fusion protein according to any one of claims 47 or 48, wherein the second polypeptide comprises the sequence AVQP, in ABM1, corresponding to, or at a position equivalent to, residues 6 to 9 of the sequence of SEQ ID NO: 14 or 19.

50. A chimeric or fusion protein according to claim 50, wherein the second polypeptide comprises the amino acid sequence substitution of one or more of the cysteine residues in the ABM domains.

51. A chimeric or fusion protein according to any one of claims 45 to 50, wherein the chimeric or fusion protein comprises one or more further polypeptides that comprise or consist of an amino acid sequence of the active site of an Arg- or Lys-gingipain of *P. gingivalis,* or sequences that are at least 80% identical thereto.

52. A chimeric or fusion protein according to claim 51, wherein the one or more further polypeptides comprising or consisting of the active site of an Arg- or Lys-gingipain of *P. gingivalis* is located N-terminally to the first polypeptide, C-terminally to the first polypeptide, N-terminally to the second polypeptide of C-terminally to the second polypeptide.

53. A chimeric or fusion protein according to any one of claims 45 to 52, wherein the protein comprises or consists of the amino acid sequence set forth in SEQ ID NO: 80 or 81.

54. A chimeric or fusion protein according to any one of claims 1 to 53, wherein the protein comprises additional amino acid residues to facilitate expression in a recombinant expression system and/or to facilitate purification of the protein.

55. A chimeric or fusion protein according to claim 54, wherein the additional amino acids comprise one, two, three, four, or five amino acids in the N-terminal region, optionally including an N-terminal methionine and/or alanine residue.

56. A chimeric or fusion protein according to claim 54 or 55, wherein the additional amino acids comprise one, two, three, four, or five amino acids in the C-terminal region, preferably to facilitate purification.

57. A nucleic acid encoding a chimeric or fusion protein according to any one of claims 1 to 56.

58. A vector or construct comprising a nucleic acid according to claim 57.

59. A host cell comprising a nucleic acid according to claim 57, or a vector or construct according to claim 58.

60. A composition comprising a chimeric or fusion protein according to any one of claims 1 to 56.

61. A composition according to claim 60, wherein the composition is a vaccine composition and comprises one or more adjuvants for potentiating an immune response to the chimeric or fusion protein.

62. A vaccine or immune stimulating composition comprising a chimeric or fusion protein according to any one of claims 1 to 56, and optionally, an adjuvant for potentiating an immune response to the chimeric or fusion protein.

63. A method for inducing an immune response in a subject to *P. gingivalis*, the method comprising administering to a subject in need thereof, a chimeric or

fusion protein according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62.

64. A method of inducing a humoural immune response to *P. gingivalis* in a subject, the method comprising administering to the subject, a chimeric or fusion protein, according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62.

65. A method according to claim 64 wherein the immune response that is induced comprises a switch from a Th1 to a Th2 immune response.

66. A method of immunising a subject against *P. gingivalis* infection, the method comprising administering to the subject, a chimeric or fusion protein according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62.

67. A method of treating a *P. gingivalis* infection in a subject, the method comprising administering to a subject in need thereof, a chimeric or fusion protein, according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62.

68. A method for reducing or minimising the severity of a symptom associated with an infection with *P. gingivalis*, comprising administering to an individual in need thereof, a chimeric or fusion protein, according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62,

wherein the symptoms are selected from the group consisting of swollen or puffy gums, gums that bleed easily, receding gums, periodontal pockets around the teeth, loss of tooth supporting tissues (periodontal ligament, cementum and/or alveolar bone) pus between gums and teeth, and gingivitis.

69. A method according to claim 67 or 68, wherein the method further comprises administration of one or more of: an antimicrobial compound, an anti-inflammatory agent.

70. Use of a chimeric or fusion protein according to any one of claims 1 to 56, in the manufacture of a medicament for:

- inducing an immune response in a subject to *P. gingivalis;*
- immunising a subject against *P. gingivalis* infection;
- treating a *P. gingivalis* infection in a subject; or
- minimising or reducing the severity of one or more symptoms of *P. gingivalis* infection in a subject.

71. A chimeric or fusion protein according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62, for use in:

- inducing an immune response in a subject to *P. gingivalis;*
- immunising a subject against *P. gingivalis* infection;
- treating a *P. gingivalis* infection in a subject; or
- minimising or reducing the severity of one or more symptoms of *P. gingivalis* infection in a subject.

72. A method for obtaining an antibody directed to *P. gingivalis,* the method comprising administering a chimeric or fusion protein according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal.

73. A method according to claim 72, wherein the method further comprises isolating the antibody from the animal or an egg of the animal.

74. An antibody preparation comprising an antibody directed to *P. gingivalis,* wherein the antibody preparation is obtained by administering a chimeric or fusion protein, according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62, to a non-human animal, thereby generating antibodies directed to *P. gingivalis* in the animal, and isolating the antibodies from the animal or an egg thereof.

75. A kit comprising a composition comprising a chimeric or fusion protein according to any one of claims 1 to 56, a composition according to claim 60 or 61, or a vaccine or immune stimulating composition according to claim 62, wherein optionally the kit comprises one or more cytokines and/or adjuvants in sealed containers, preferably wherein the kit comprises a label or package insert indicating that the composition is used for immunising an individual, optionally wherein the label or package insert includes instructions for use.

Figure 1

A

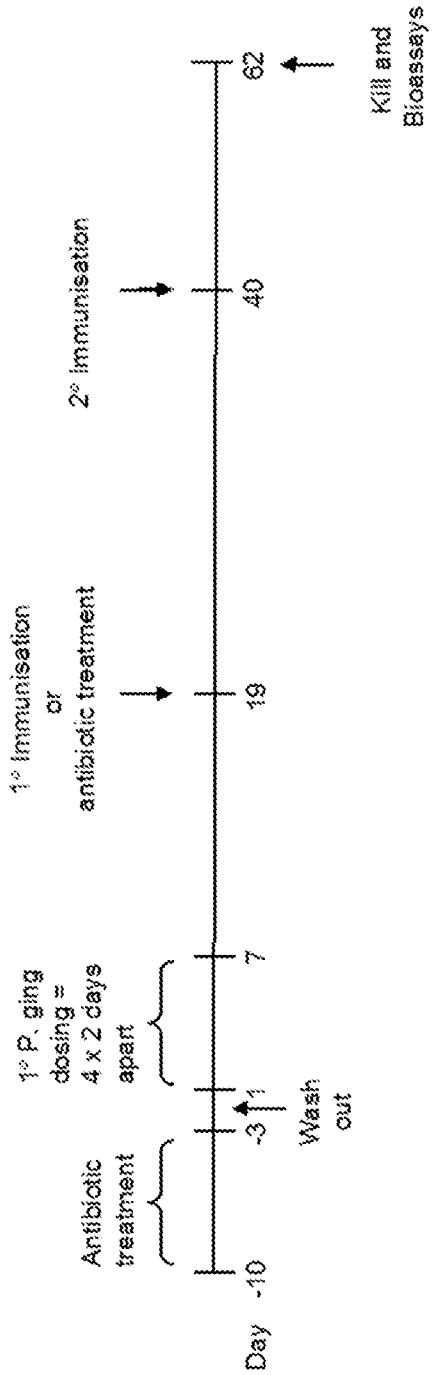

Day -10
-3
1
7
19
40
62
Antibiotic treatment
1° P. ging dosing = 4 x 2 days apart
Wash out
1° Immunisation or antibiotic treatment
2° Immunisation
Kill and Bioassays

Figure 1 continued

B

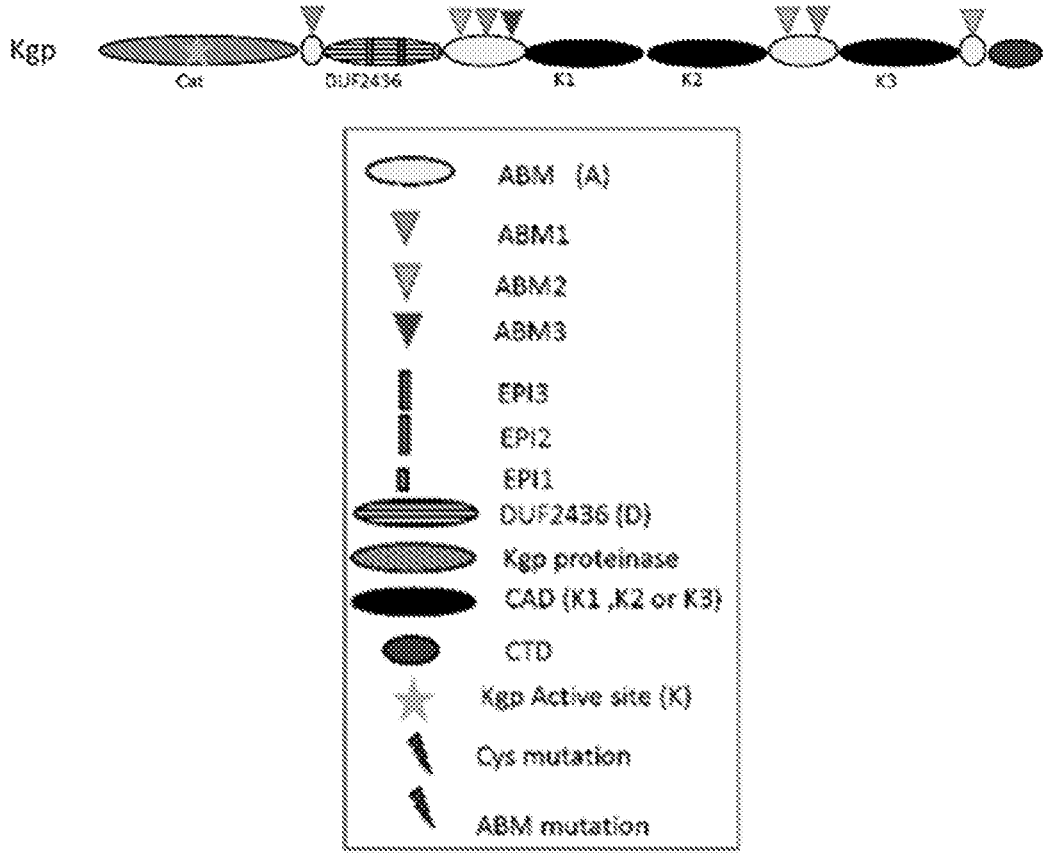
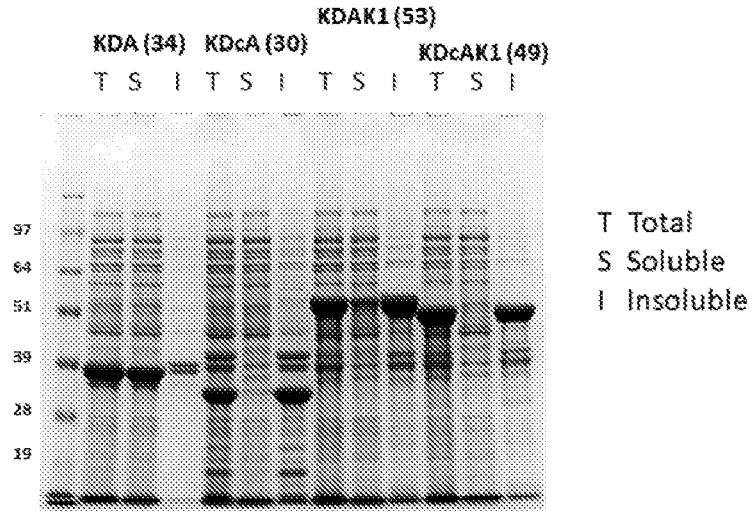


Figure 2

A.


KDA (34)
KDcA (30)
KDAK1 (53)
KDcAK1 (49)
T S I T S I T S I T S I
97
64
51
39
28
19
T Total
S Soluble
I Insoluble

B,

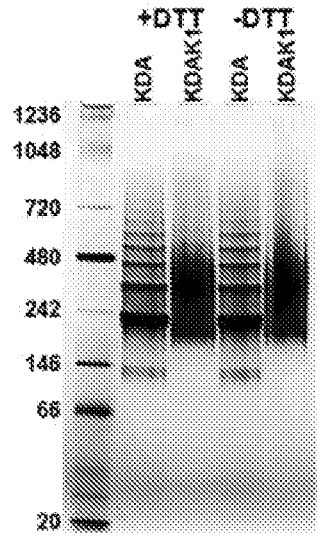
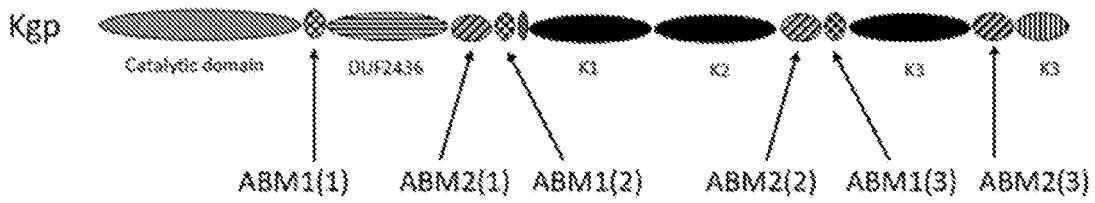
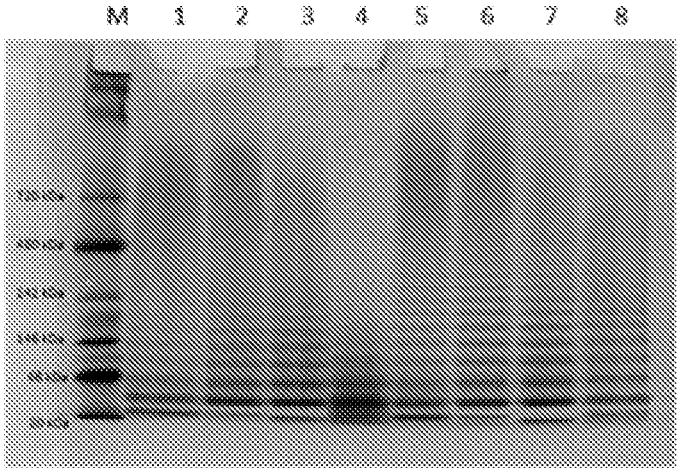

+DTT
-DTT
KDA
KDAK1
KDA
KDAK1
1236
1048
720
480
242
146
66
20

Figure 3

A



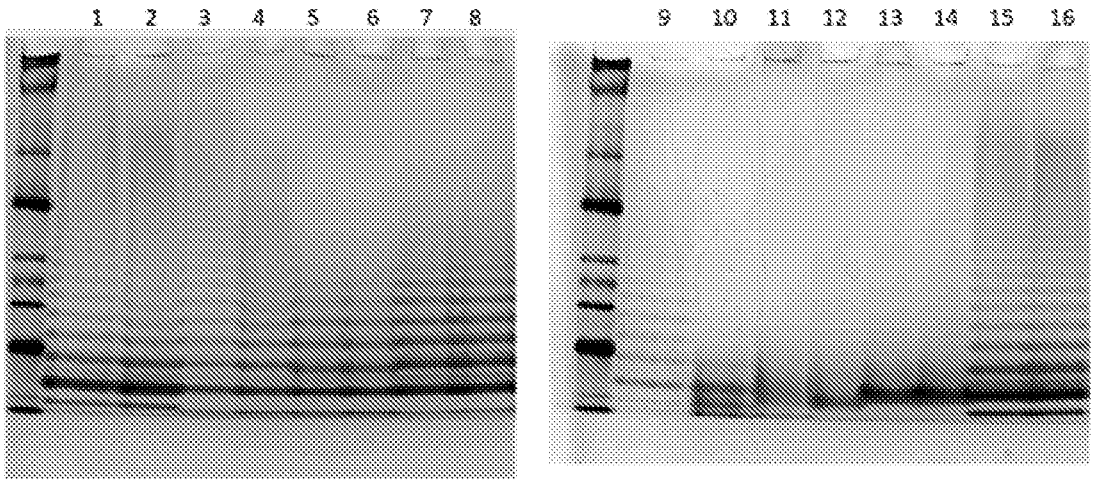
B



M Native Marker
1 rABM21 (+ DTT)
2 rABM21-AVQN (+ DTT)
3 rABM21-AVQA (+ DTT)
4 rABM21-AVQP (+ DTT)
5 rABM21 (- DTT)
6 rABM21-AVQN (- DTT)
7 rABM21-AVQA (- DTT)
8 rABM21-AVQP (- DTT)

Figure 3 continued

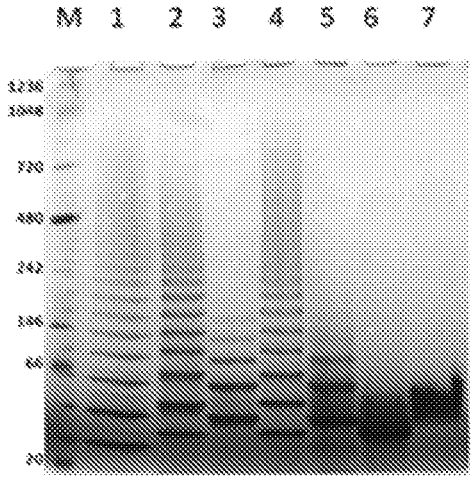
C



1: rABM12 (– DTT)
2: rABM12 (+ DTT)
3: rABM21-AVQA (– DTT)
4: rABM21-AVQA (+ DTT)
5: rABM21-AVQA-Cys>Ser (– DTT)
6: rABM21-AVQA-Cys>Ser (+ DTT)
7: rABM21-AVQA-2X Cys>Ser (– DTT)
8: rABM21-AVQA-2X Cys>Ser (+ DTT)

9: rABM21-AVQP (– DTT)
10: rABM21-AVQP (+ DTT)
11: rABM21-AVQP-Cys>Ser (– DTT)
12: rABM21-AVQP-Cys>Ser (+ DTT)
13: rABM21-AVQP-2X Cys>Ser (– DTT)
14: rABM21-AVQP-2X Cys>Ser (+ DTT)
15: rABM21-2X Cys>Ser (– DTT)
16: rABM21-2X Cys>Ser (+ DTT)

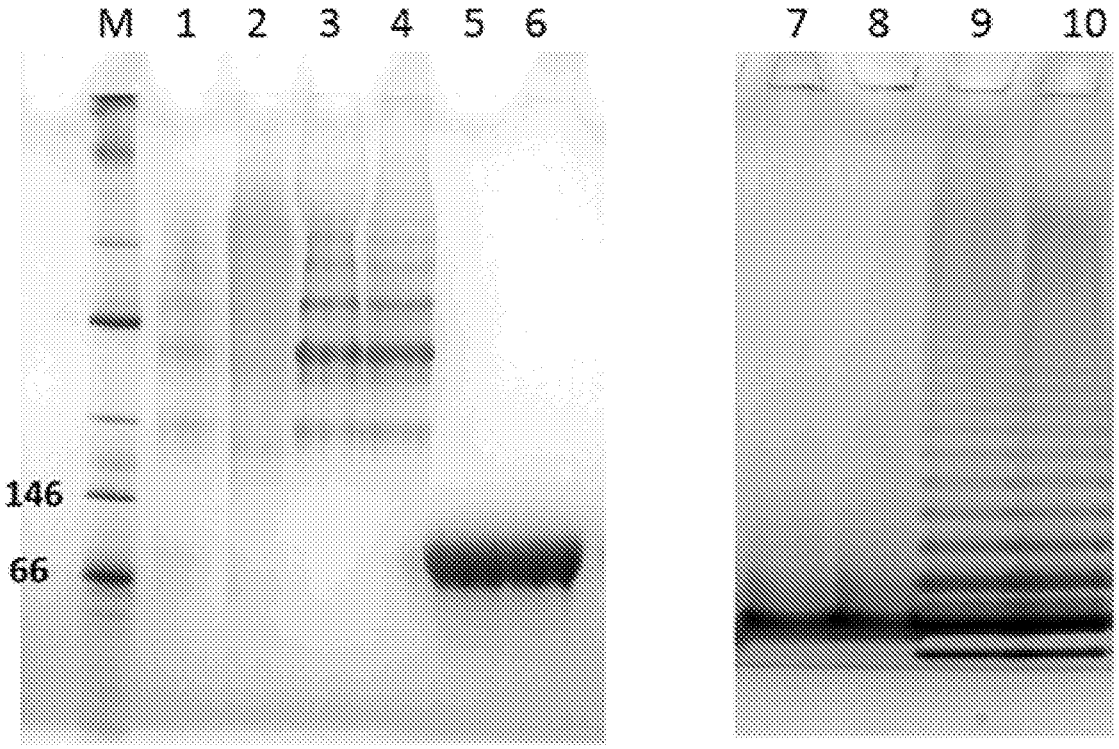
D



1. rABM21
2. rABM21-PNPFA
3. rABM21-SSEYQ
4. rABM21-PSPY
5. rABM21-Y>A
6. rABM21-Y>A/W>A
7. rABM21-AVQP-2X Cys>Ser

Figure 3 continued

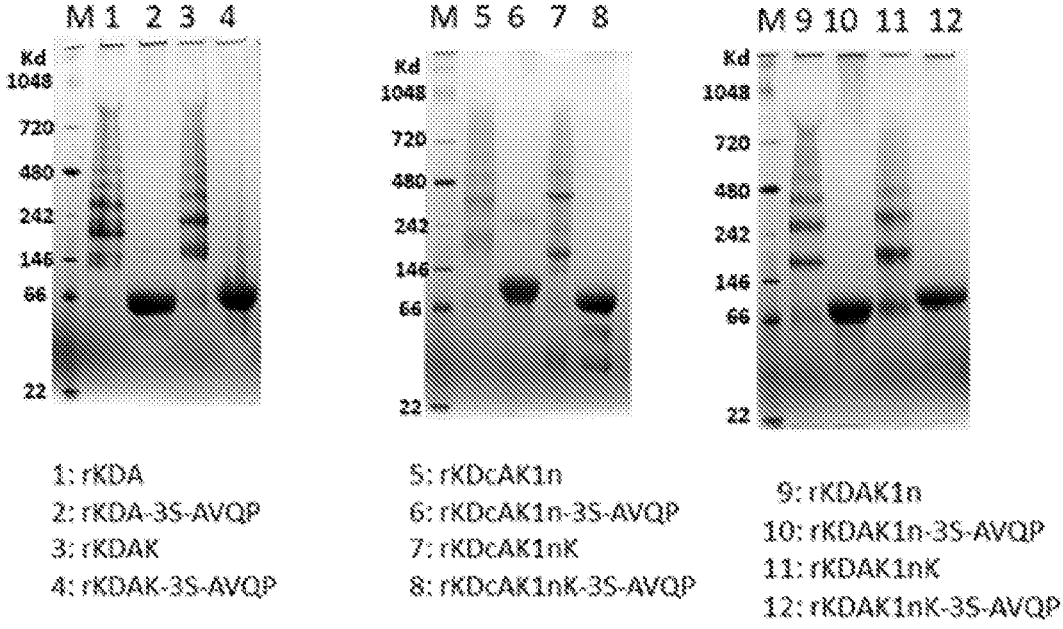
E



1- KDcAK1n + DTT
2- KDcAK1n - DTT
3- KDcAK1n 4XCys>Ser + DTT
4- KDcAK1n 4XCys>Ser - DTT
5- KDcAK1n 4XCys>Ser/PVQN>AVQP + DTT
6- KDcAK1n 4XCys>Ser/PVQN>AVQP - DTT
7- ABM21 2XCys>Ser/PVQN>AVQP - DTT
8- ABM21- 2XCys>Ser/PVQN>AVQP + DTT
9- ABM21 2X Cys>Ser - DTT
10-ABM21 2XCys>Ser + DTT

Figure 3 Continued

F



1: rKDA
2: rKDA-3S-AVQP
3: rKDAK
4: rKDAK-3S-AVQP

5: rKDcAK1n
6: rKDcAK1n-3S-AVQP
7: rKDcAK1nK
8: rKDcAK1nK-3S-AVQP

9: rKDAK1n
10: rKDAK1n-3S-AVQP
11: rKDAK1nK
12: rKDAK1nK-3S-AVQP

G

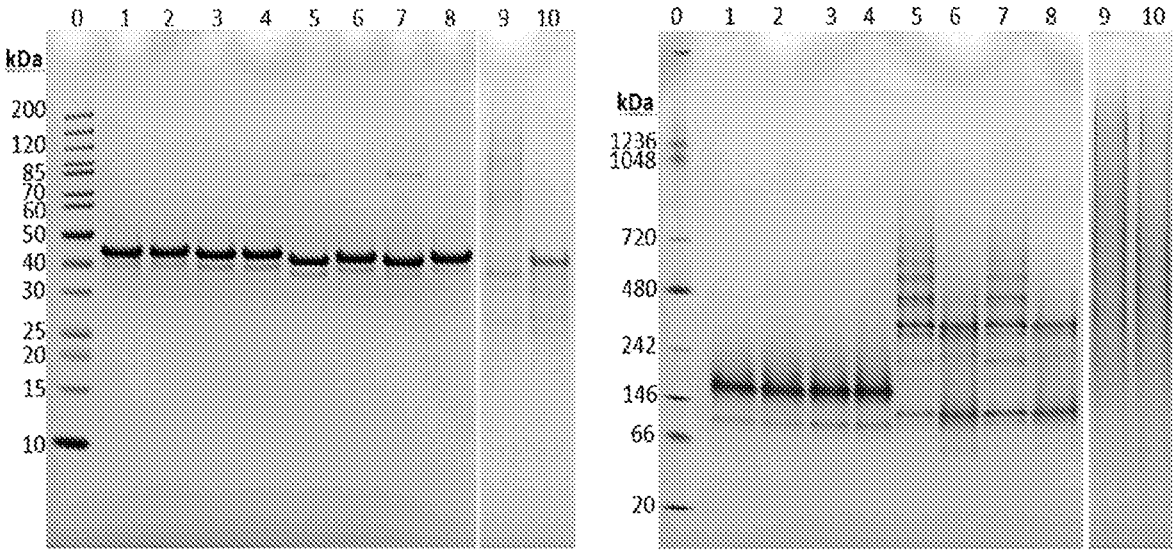


Figure 4

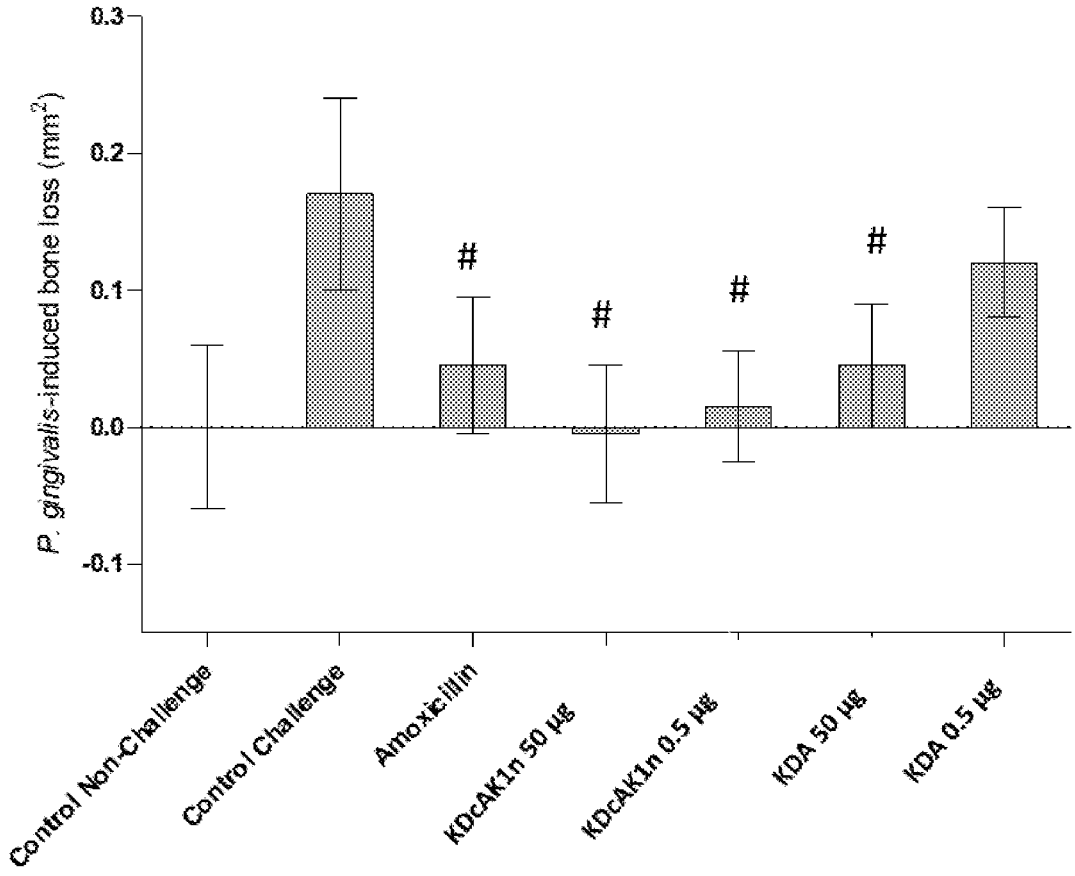

0.3
0.2
0.1
0.0
-0.1
P. gingivalis-induced bone loss (mm²)
#
#
#
#
Control Non-Challenge
Control Challenge
Amoxicillin
KDcAK1n 50 µg
KDcAK1n 0.5 µg
KDA 50 µg
KDA 0.5 µg

Figure 5

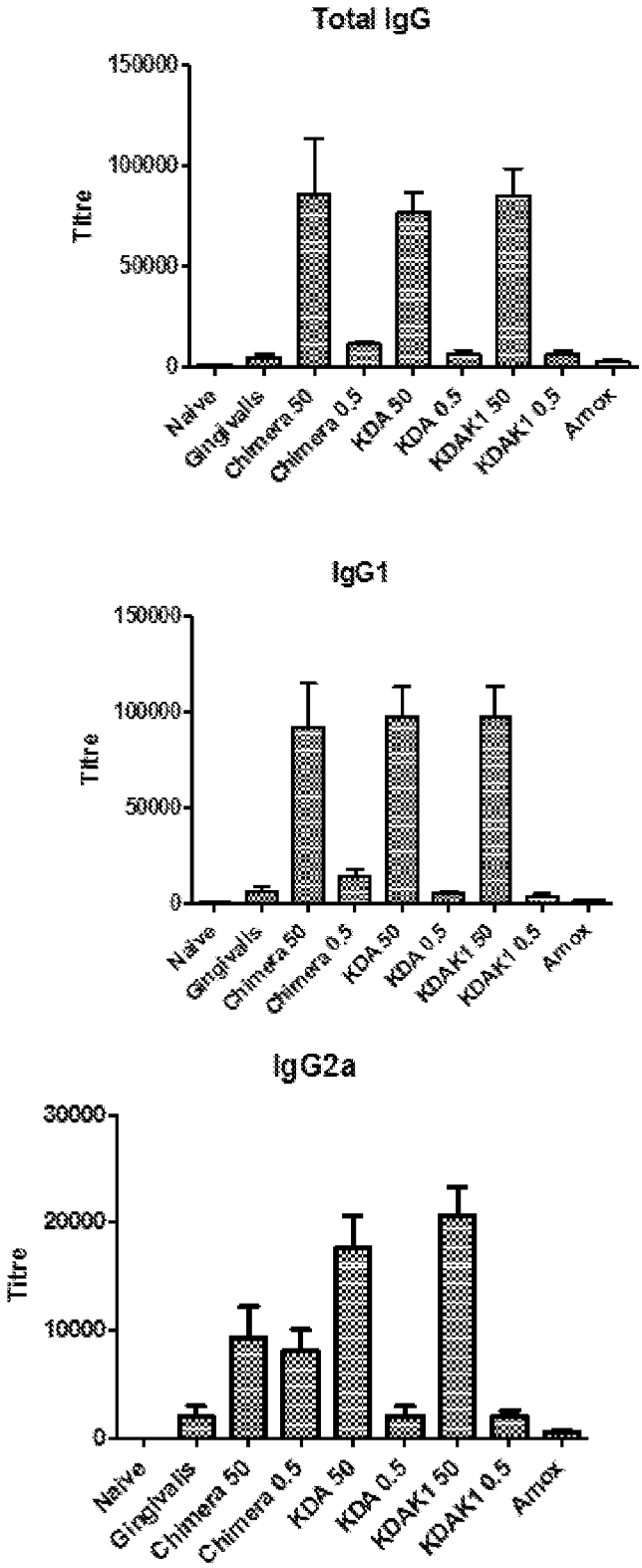

Total IgG
Titre
150000
100000
50000
0
Naive
Gingivalis
Chimera 50
Chimera 0.5
KDA 50
KDA 0.5
KDAK1 50
KDAK1 0.5
Amox
IgG1
Titre
150000
100000
50000
0
Naive
Gingivalis
Chimera 50
Chimera 0.5
KDA 50
KDA 0.5
KDAK1 50
KDAK1 0.5
Amox
IgG2a
Titre
30000
20000
10000
0
Naive
Gingivalis
Chimera 50
Chimera 0.5
KDA 50
KDA 0.5
KDAK1 50
KDAK1 0.5
Amox

Figure 6

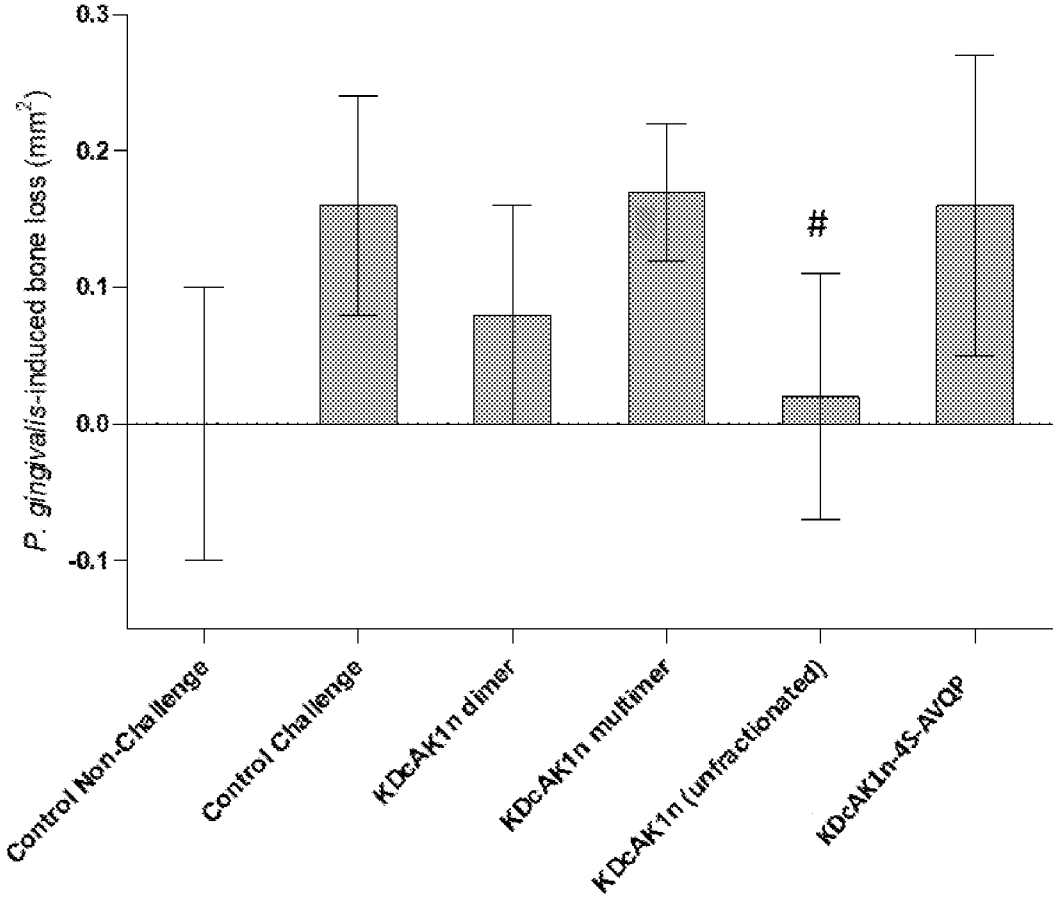

0.3
0.2
0.1
0.0
-0.1
P. gingivalis-induced bone loss (mm²)
#
Control Non-Challenge
Control Challenge
KDcAK1n dimer
KDcAK1n multimer
KDcAK1n (unfractionated)
KDcAK1n-4S-AVQP

Figure 7

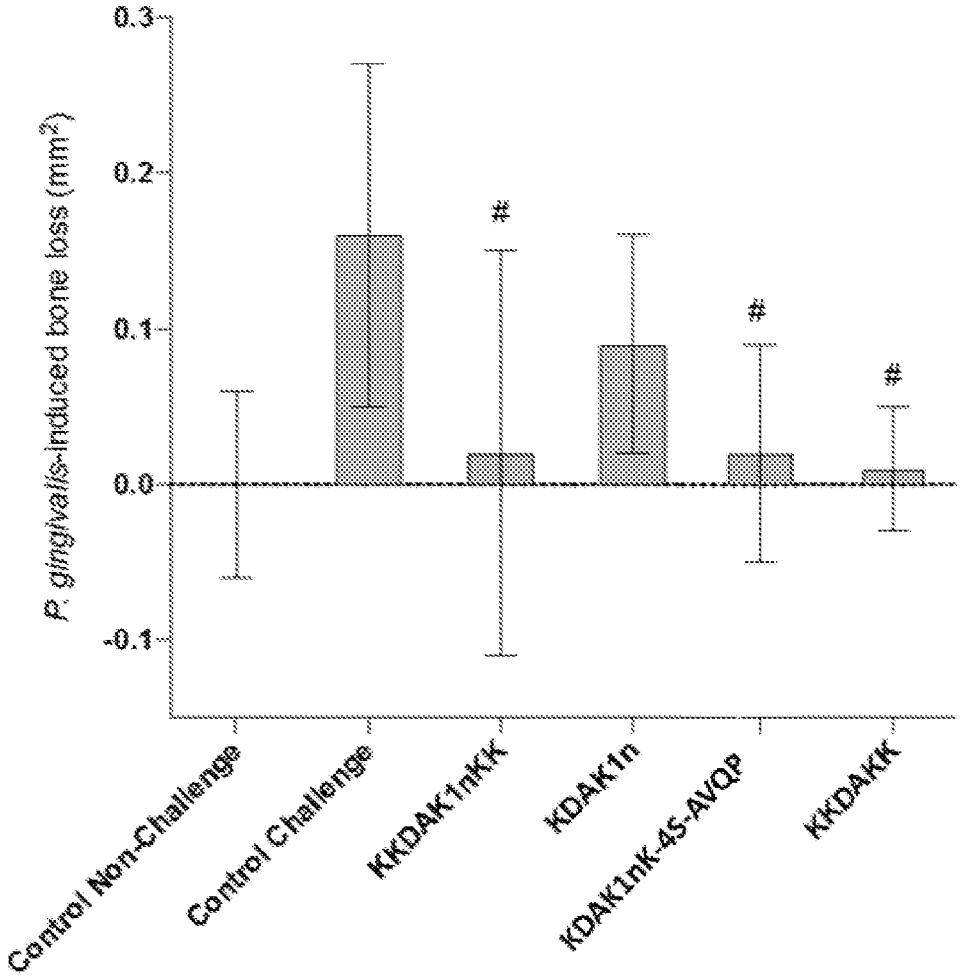

0.3
0.2
0.1
0.0
-0.1
P. gingivalis-induced bone loss (mm²)
#
#
#
Control Non-Challenge
Control Challenge
KKDAK1nKK
KDAK1n
KDAK1nK-4S-AVQP
KKDAKK

Figure 8

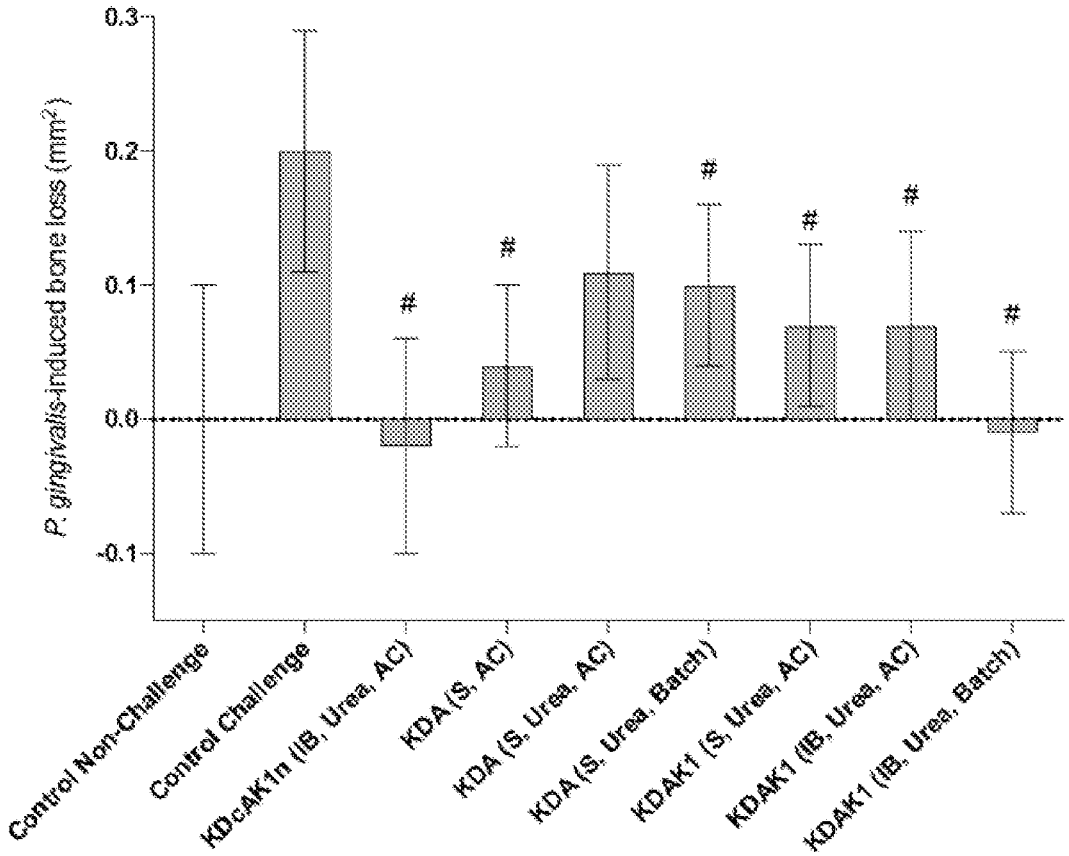

P. gingivalis-induced bone loss (mm²)
0.3
0.2
0.1
0.0
-0.1
#
Control Non-Challenge
Control Challenge
KDcAK1n (IB, Urea, AC)
KDA (S, AC)
KDA (S, Urea, AC)
KDA (S, Urea, Batch)
KDAK1 (S, Urea, AC)
KDAK1 (IB, Urea, AC)
KDAK1 (IB, Urea, Batch)

Figure 9

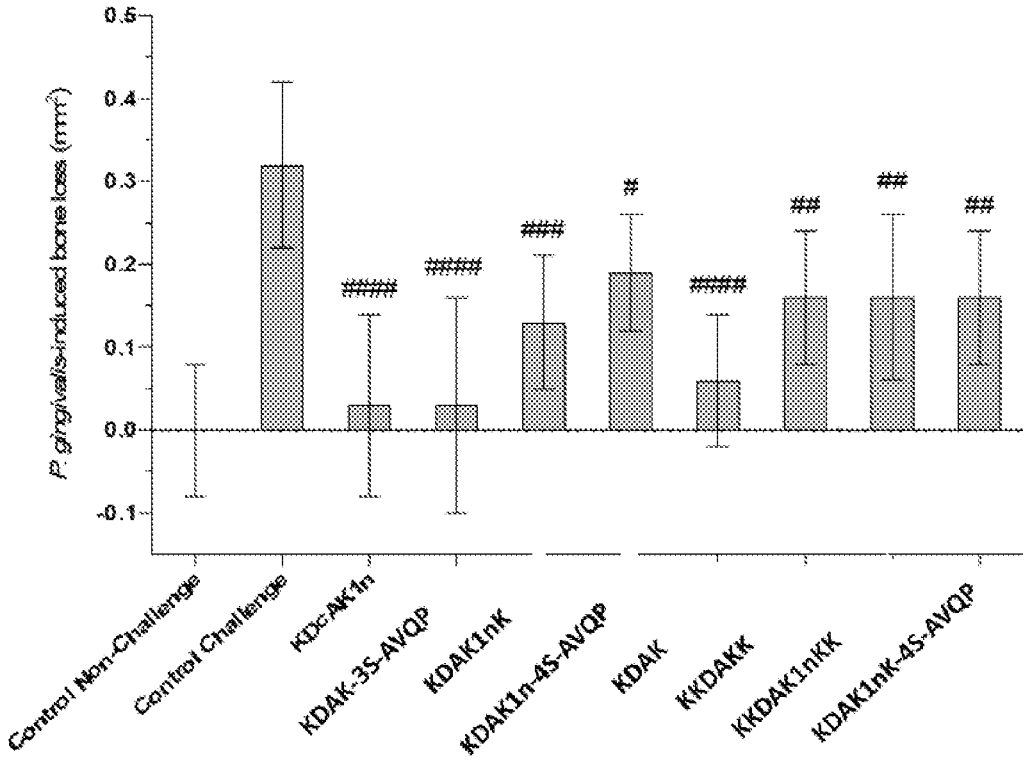

P. gingivalis-induced bone loss (mm²)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
####
####
###
#
####
##
##
##
Control Non-Challenge
Control Challenge
KDcAK1n
KDAK-3S-AVQP
KDAK1nK
KDAK1n-4S-AVQP
KDAK
KKDAKK
KKDAK1nKK
KDAK1nK-4S-AVQP

Figure 10

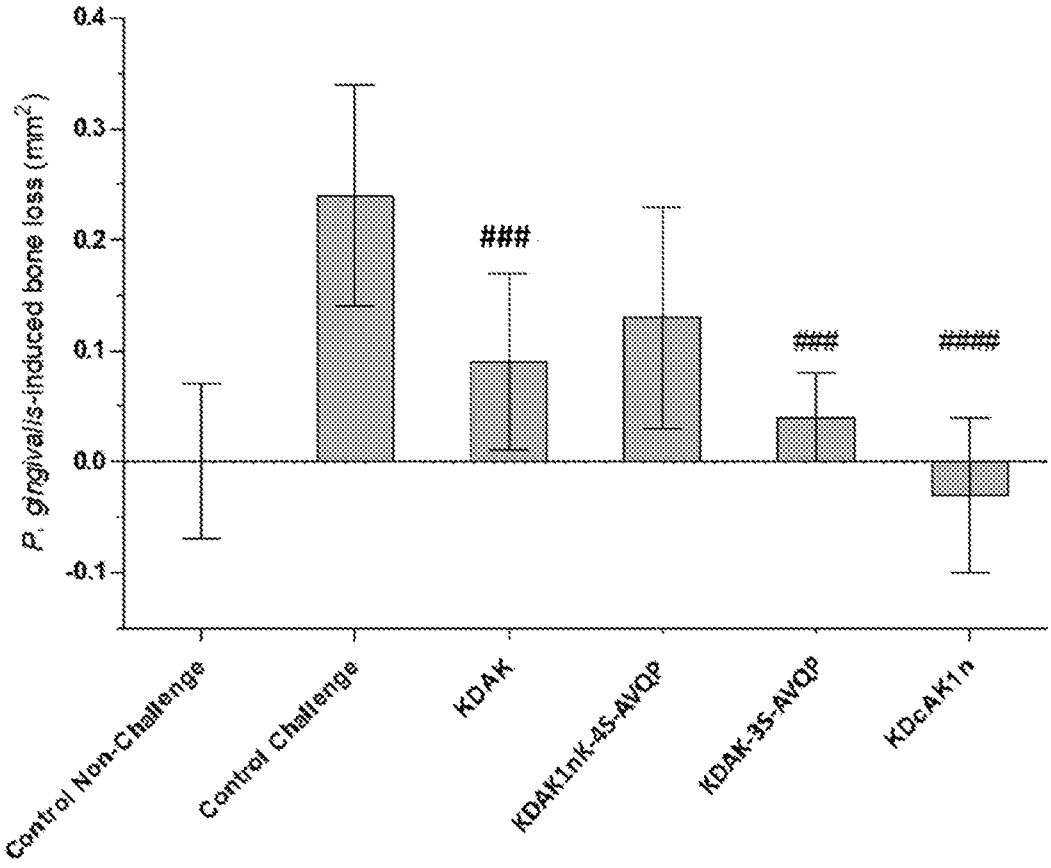

P. gingivalis-induced bone loss (mm²)
0.4
0.3
0.2
0.1
0.0
-0.1
###
###
####
Control Non-Challenge
Control Challenge
KDAK
KDAK1nK-45-AVQP
KDAK-35-AVQP
KDcAK1n

Figure 11

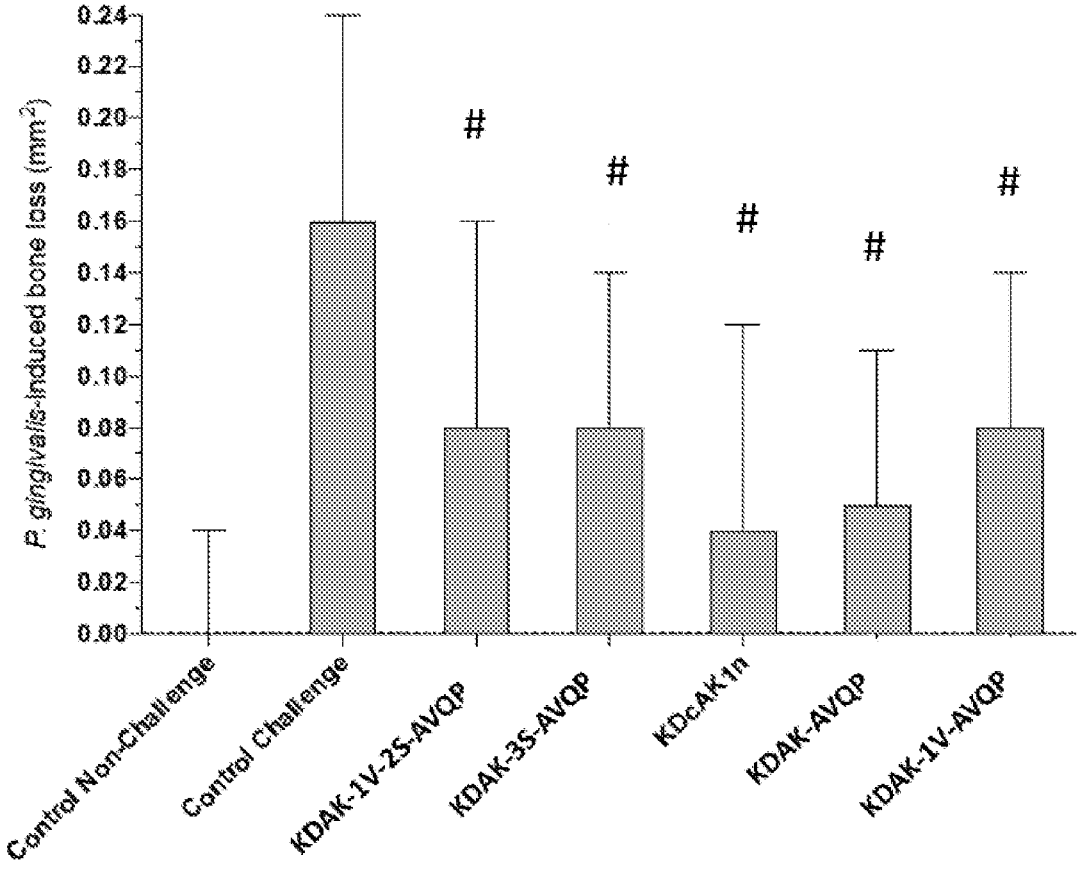

P. gingivalis-induced bone loss (mm²)
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
#
#
#
#
#
Control Non-Challenge
Control Challenge
KDAK-1V-2S-AVQP
KDAK-3S-AVQP
KDcAK1n
KDAK-AVQP
KDAK-1V-AVQP

Figure 12

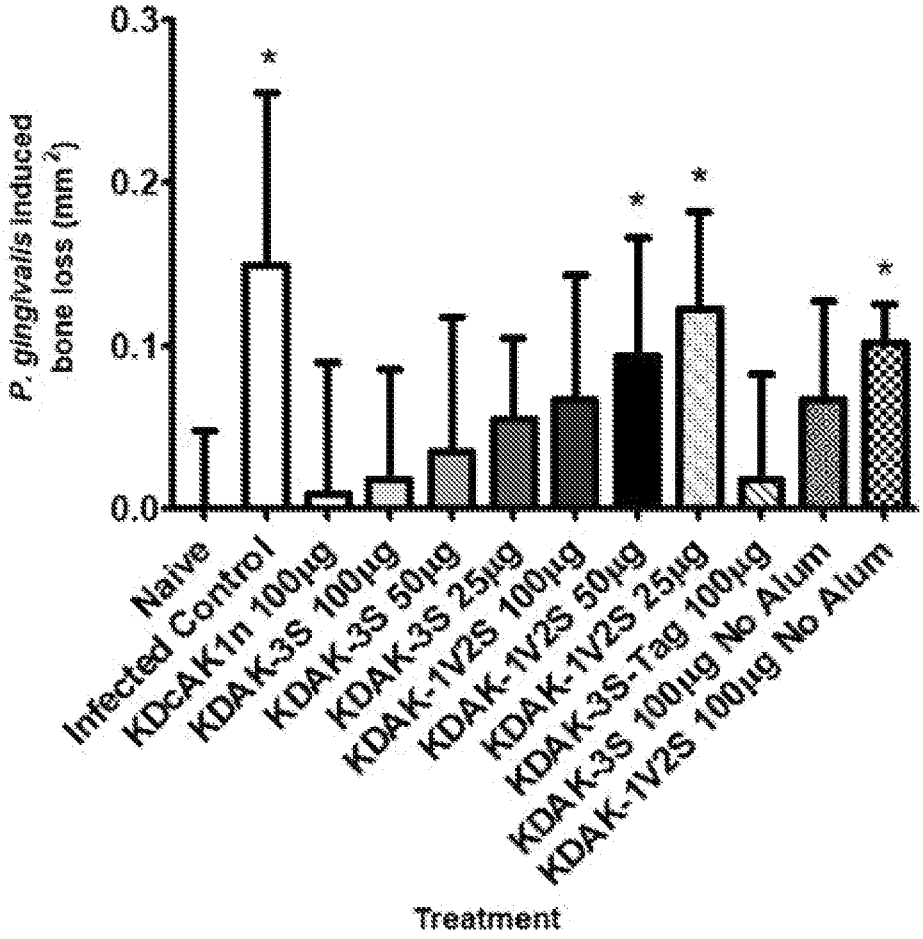

P. gingivalis induced bone loss (mm²)
0.3
0.2
0.1
0.0
*
*
*
*
Naive
Infected Control
KDcAK1n 100µg
KDAK-3S 100µg
KDAK-3S 50µg
KDAK-3S 25µg
KDAK-1V2S 100µg
KDAK-1V2S 50µg
KDAK-1V2S 25µg
KDAK-3S-Tag 100µg
KDAK-3S 100µg No Alum
KDAK-1V2S 100µg No Alum
Treatment

Figure 13

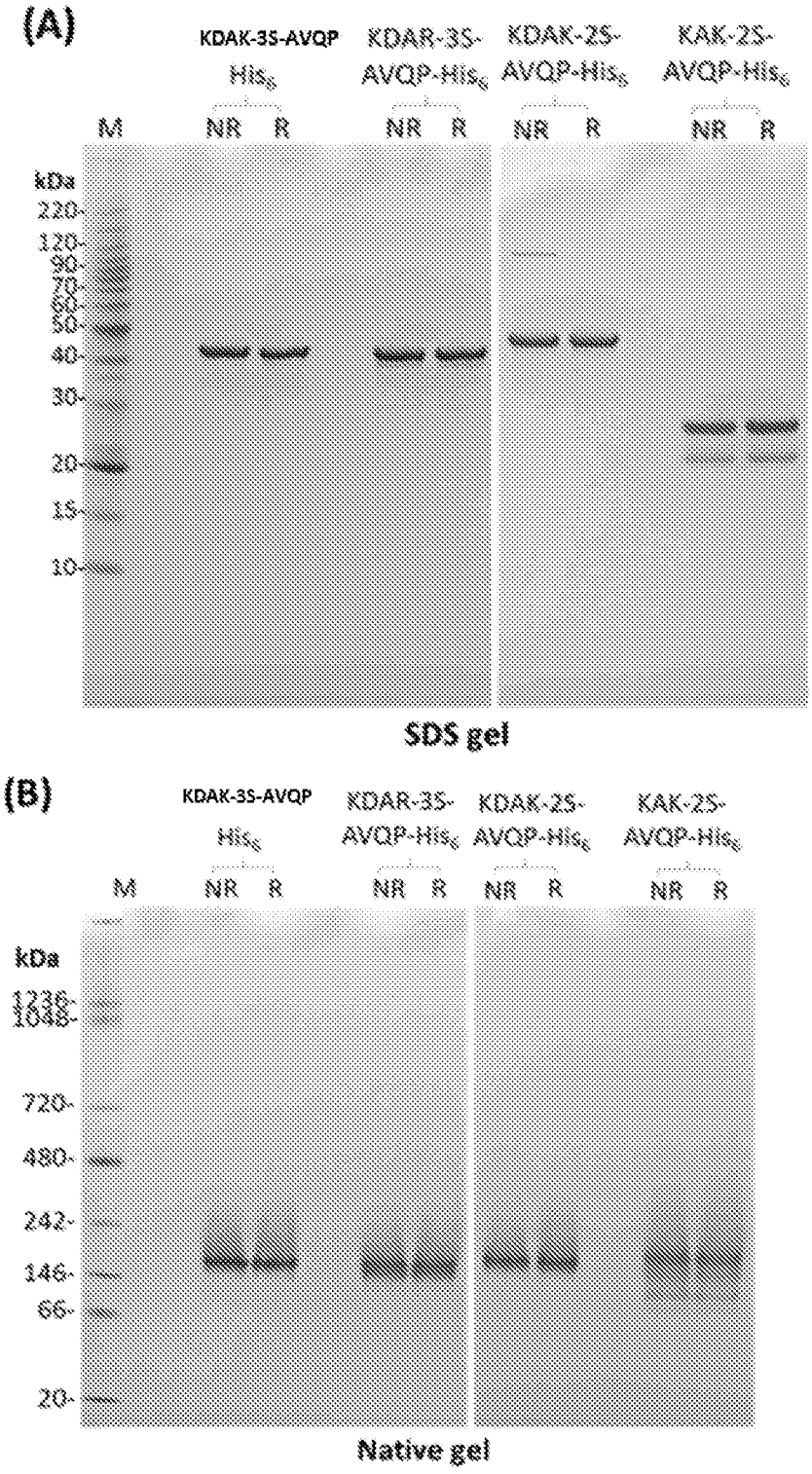

(A)
KDAK-3S-AVQP-His6
KDAR-3S-AVQP-His6
KDAK-2S-AVQP-His6
KAK-2S-AVQP-His6
M
NR R
NR R
NR R
NR R
kDa
220
120
90
70
60
50
40
30
20
15
10
SDS gel
(B)
KDAK-3S-AVQP-His6
KDAR-3S-AVQP-His6
KDAK-2S-AVQP-His6
KAK-2S-AVQP-His6
M
NR R
NR R
NR R
NR R
kDa
1236
1048
720
480
242
146
66
20
Native gel

Figure 13 continued

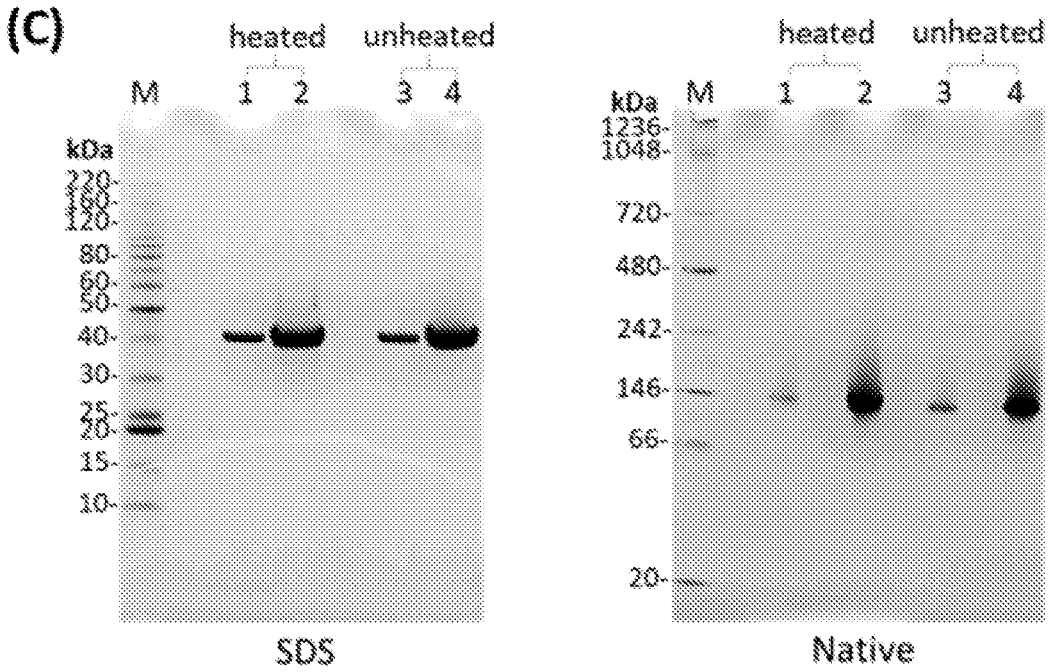


Figure 14

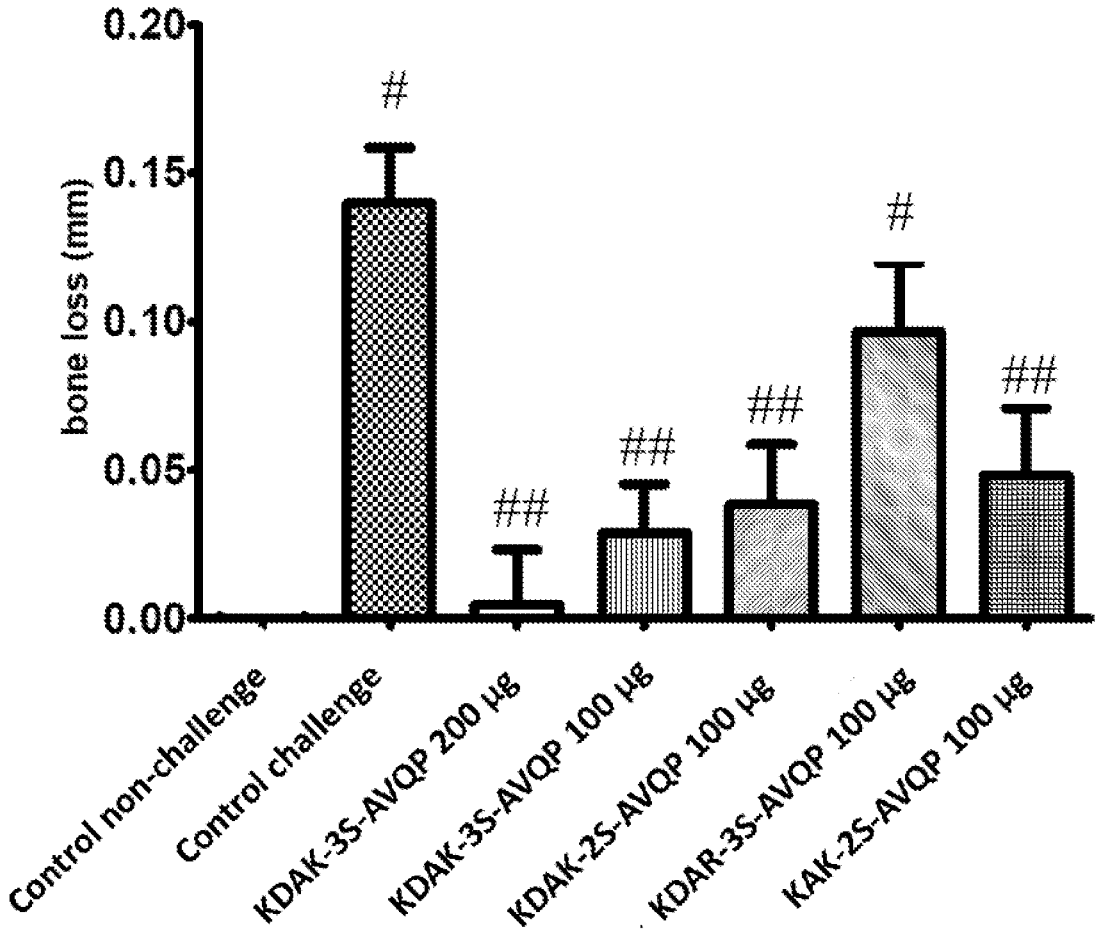

0.20
0.15
0.10
0.05
0.00
bone loss (mm)
#
##
##
##
#
##
Control non-challenge
Control challenge
KDAK-3S-AVQP 200 µg
KDAK-3S-AVQP 100 µg
KDAK-2S-AVQP 100 µg
KDAR-3S-AVQP 100 µg
KAK-2S-AVQP 100 µg

Figure 15

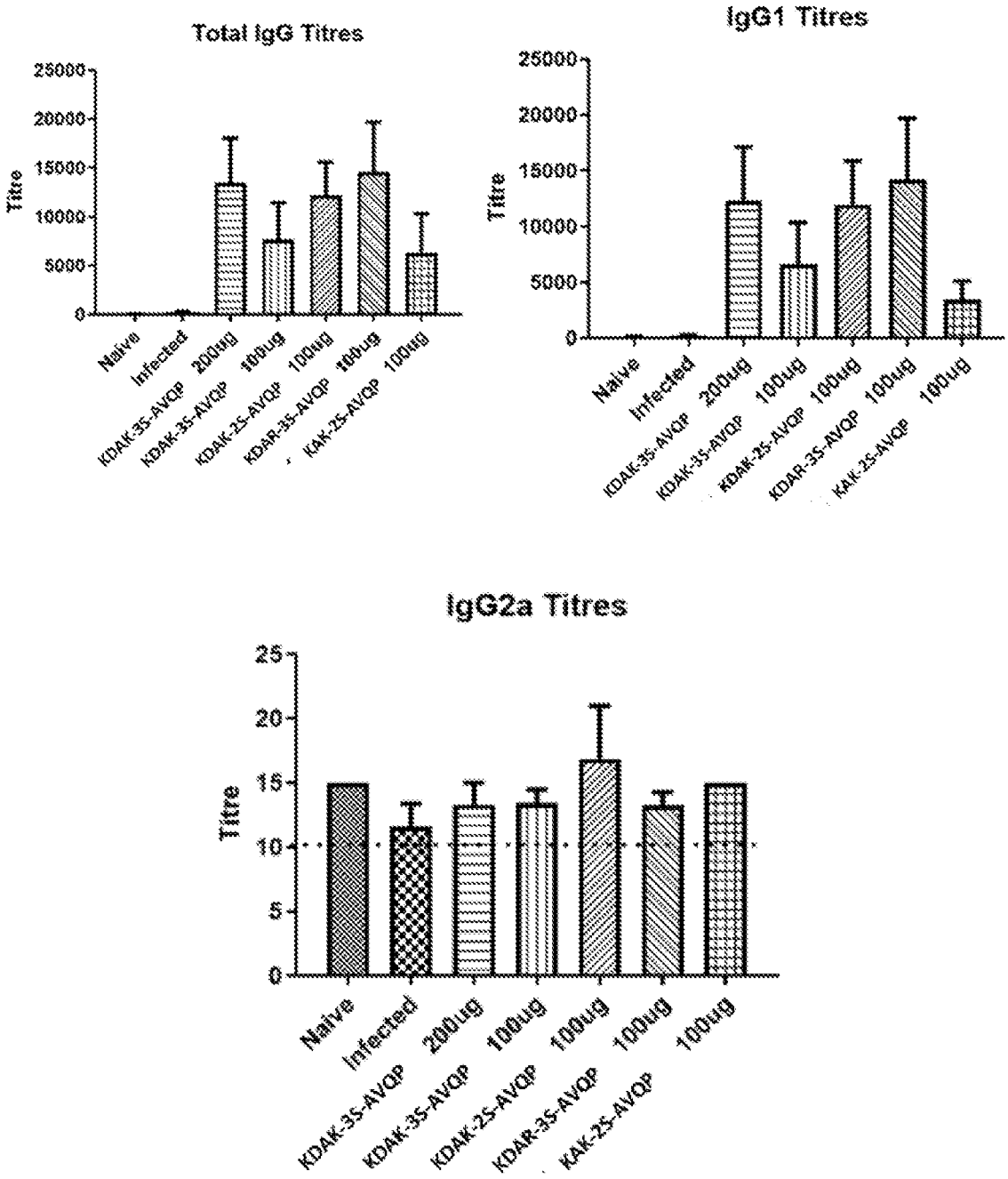
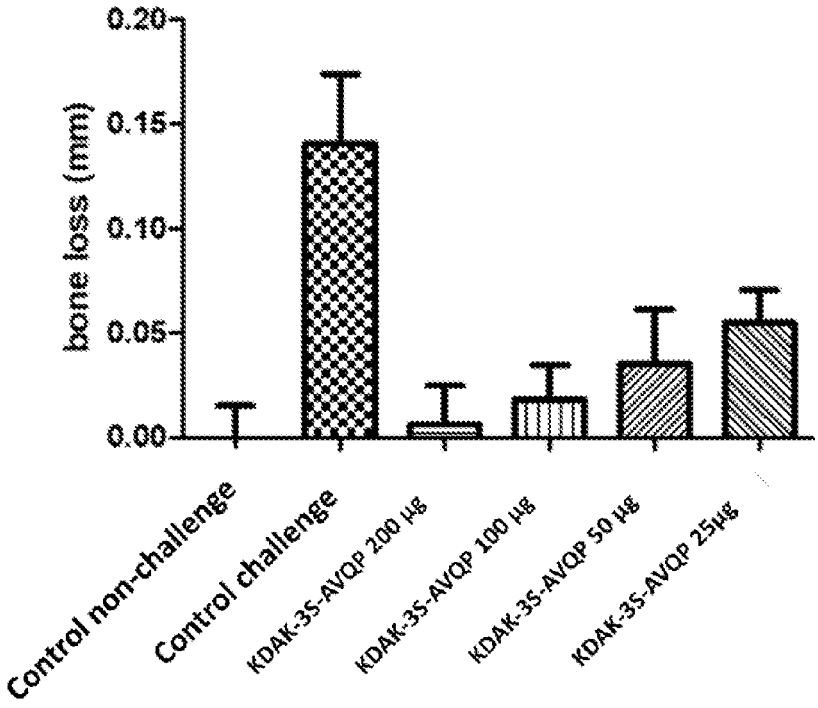
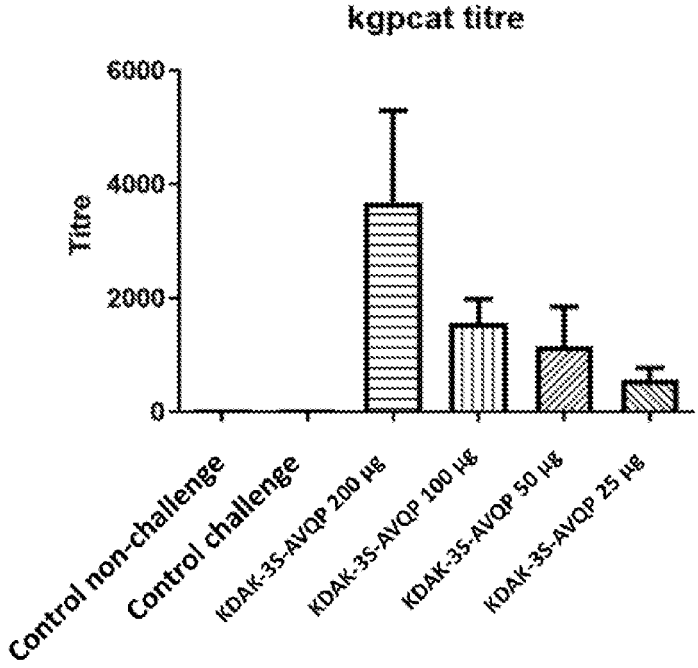

Total IgG Titres
Titre
25000
20000
15000
10000
5000
0
Naive
Infected
200ug
100ug
100ug
100ug
100ug
KDAK-3S-AVQP
KDAK-3S-AVQP
KDAK-2S-AVQP
KDAR-3S-AVQP
KAK-2S-AVQP
IgG1 Titres
Titre
25000
20000
15000
10000
5000
0
Naive
Infected
200ug
100ug
100ug
100ug
100ug
KDAK-3S-AVQP
KDAK-3S-AVQP
KDAK-2S-AVQP
KDAR-3S-AVQP
KAK-2S-AVQP
IgG2a Titres
Titre
25
20
15
10
5
0
Naive
Infected
200ug
100ug
100ug
100ug
100ug
KDAK-3S-AVQP
KDAK-3S-AVQP
KDAK-2S-AVQP
KDAR-3S-AVQP
KAK-2S-AVQP

Figure 16


bone loss (mm)
0.20
0.15
0.10
0.05
0.00
Control non-challenge
Control challenge
KDAK-3S-AVQP 200 µg
KDAK-3S-AVQP 100 µg
KDAK-3S-AVQP 50 µg
KDAK-3S-AVQP 25µg


kgpcat titre
Titre
6000
4000
2000
0
Control non-challenge
Control challenge
KDAK-3S-AVQP 200 µg
KDAK-3S-AVQP 100 µg
KDAK-3S-AVQP 50 µg
KDAK-3S-AVQP 25 µg