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(54) **Titre : CONSTRUCTIONS IMMUNOGENES UTILES POUR LE TRAITEMENT D'INFECTION MYCOBACTERIUM AVIUM SOUS-ESPECE PARATUBERCULOSIS (MAP)**

(54) **Title: IMMUNOGENIC CONSTRUCTS USEFUL FOR TREATING MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) INFECTION**

(57) **Abrégé/Abstract:**

The present invention relates to molecules, which can be used to induce a therapeutic or prophylactic immune response against MAP. In particular, the present invention relates to polypeptides comprising an alipC polypeptide sequence, a gsd polypeptide sequence, a pl2 polypeptide sequence and an mpa polypeptide sequence, wherein said ahpC polypeptide comprises the sequence of SEQ ID NO: 2, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 2 across the full length of SEQ ID NO: 2, or a fragment of at least 8 amino acids of SEQ ID NO: 2 which comprises an epitope; said gsd polypeptide comprises the sequence of SEQ ID NO: 6, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 6 across the full length of SEQ ID NO: 6, or a fragment of at least 8 amino acids of SEQ ID NO: 6 which comprises an epitope; said pi 2 polypeptide comprises the sequence of SEQ ID NO: 10, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 10 across the full length of SEQ ID NO: 10, or a fragment of at least 8 amino acids of SEQ ID NO: 10 which comprises an epitope; and said mpa polypeptide comprises the sequence of SEQ ID NO: 14, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 14 across the full length of SEQ ID NO: 14, or a fragment of at least 8 amino acids of SEQ ID NO: 14 which comprises an epitope. Preferably such a variant maintains the ability to generate an immune response against the unmodified polypeptide.

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(54) Title: IMMUNOGENIC CONSTRUCTS

(57) Abstract: The present invention relates to molecules, which can be used to induce a therapeutic or prophylactic immune response against MAP. In particular, the present invention relates to polypeptides comprising an alipC polypeptide sequence, a gsd polypeptide sequence, a pl2 polypeptide sequence and an mpa polypeptide sequence, wherein said ahpC polypeptide comprises the sequence of SEQ ID NO: 2, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 2 across the full length of SEQ ID NO: 2, or a fragment of at least 8 amino acids of SEQ ID NO: 2 which comprises an epitope; said gsd polypeptide comprises the sequence of SEQ ID NO: 6, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 6 across the full length of SEQ ID NO: 6, or a fragment of at least 8 amino acids of SEQ ID NO: 6 which comprises an epitope; said pi 2 polypeptide comprises the sequence of SEQ ID NO: 10, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 10 across the full length of SEQ ID NO: 10, or a fragment of at least 8 amino acids of SEQ ID NO: 10 which comprises an epitope; and said mpa polypeptide comprises the sequence of SEQ ID NO: 14, a variant thereof having more than 70% amino acid sequence identity to SEQ ID NO: 14 across the full length of SEQ ID NO: 14, or a fragment of at least 8 amino acids of SEQ ID NO: 14 which comprises an epitope. Preferably such a variant maintains the ability to generate an immune response against the unmodified polypeptide.

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IMMUNOGENIC CONSTRUCTS USEFUL FOR TREATING *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* (MAP) INFECTION

Field of the Invention

The present invention relates to the treatment or prevention of infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and to the treatment or prevention of disorders associated with such infection.

Background to the Invention

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a member of the *Mycobacterium avium* complex MAC. Unlike other environmental MAC, MAP has the specific ability to cause chronic inflammation of the intestine of a range of histopathological types in many animals including primates. Despite its broad pathogenicity, MAP can live in animals for years without causing clinical disease. MAP is more thermotolerant than *M. bovis* and has been cultured from retail pasteurised milk in the UK, Czech Republic and the USA. Transmittal from livestock to humans by this route is therefore probable. There is also a high risk of transmittal from sources of environmental contamination such as rivers and surface waters used for domestic supply.

MAP can cause Crohn's disease in humans, in particular in people who have an inherited or acquired susceptibility. Recent studies have confirmed that the inflamed intestine of most people with Crohn's disease is infected with these chronic enteric pathogens. Further studies have reported that MAP can be cultured from the blood of 50% of patients with Crohn's disease showing that, as in animals, the infection is often systemic. Furthermore a high proportion of people with Irritable Bowel Syndrome are also infected with MAP.

The organisms in humans are very slow growing and exceedingly difficult to isolate and passage in conventional culture. They are present in low abundance and adopt a Ziehl Neelsen (ZN) staining negative form which cannot be seen in tissues by ordinary light microscopy. They appear to be able to minimise immune recognition and unlike conventional spheroplasts, their ZN negative form is highly resistant to chemical and enzymatic lysis procedures essential for reliable detection by PCR.

MAP infections are extremely difficult to eradicate. ZN-negative intracellular MAP are highly resistant *in vivo* to standard anti-TB drugs. However, a substantial proportion of patients with Crohn's disease who can take rifabutin/clarithromycin combinations, to which MAP are more sensitive, heal, sometimes dramatically.

Summary of the Invention

The present invention relates to molecules, in particular polypeptides and the polynucleotides which can be used to express them, which can be used to induce a therapeutic or prophylactic immune response against MAP in humans and animals.

In particular, the present invention provides a polypeptide comprising an ahpC polypeptide sequence, a gsd polypeptide sequence, a p12 polypeptide sequence and an mpa polypeptide sequence, wherein said ahpC polypeptide comprises

(ai) the sequence of SEQ ID NO: 2,

(aii) a variant of SEQ ID NO: 2 having more than 70% amino acid sequence identity to SEQ ID NO: 2 across the full length of SEQ ID NO: 2 which comprises at least one amino acid sequence selected from the group consisting of amino acids 48 to 56 of SEQ ID NO: 4, amino acids 90 to 101 of SEQ ID NO: 4 and amino acids 161 to 169 of SEQ ID NO: 4, wherein said variant of SEQ ID NO: 2 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 2, or

(aiii) a fragment of SEQ ID NO: 2 which comprises at least one amino acid sequence selected from the group consisting of amino acids 48 to 56 of SEQ ID NO: 4, amino acids 90 to 101 of SEQ ID NO: 4 and amino acids 161 to 169 of SEQ ID NO: 4, wherein said fragment of SEQ ID NO: 2 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 2;

said gsd polypeptide comprises

(bi) the sequence of SEQ ID NO: 6,

(bii) a variant of SEQ ID NO: 6 having more than 70% amino acid sequence identity to SEQ ID NO: 6 across the full length of SEQ ID NO: 6, which comprises at least one amino acid sequence selected from the group consisting of amino acids 1 to 32 of SEQ ID NO: 8, amino acids 58 to 68 of SEQ ID NO: 8, amino acids 99 to 119 of SEQ ID NO: 8, amino acids 123 to 147 of SEQ ID NO: 8, amino acids 159 to 169 of SEQ ID NO: 8, amino acids 180 to 194 of SEQ ID NO: 8, amino acids 200 to 231 of SEQ ID NO: 8, amino acids 64 to 76 of SEQ ID NO: 8, amino acids 95 to 110 of SEQ ID NO: 8, amino acids 192 to 206 of SEQ ID NO: 8 and amino acids 223 to 240 of SEQ ID NO: 8, wherein said variant of SEQ ID NO: 6 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 6, or

(biii) or a fragment of SEQ ID NO: 6 which comprises at least one amino acid sequence selected from the group consisting of amino acids 1 to 32 of SEQ ID NO: 8, amino acids 58 to 68 of SEQ ID NO: 8, amino acids 99 to 119 of SEQ ID NO: 8, amino acids 123 to 147 of SEQ ID NO: 8, amino acids 159 to 169 of SEQ ID NO: 8, amino acids 180 to 194 of SEQ ID NO: 8, amino acids 200 to 231 of SEQ ID NO: 8, amino acids 64 to 76 of SEQ ID NO: 8, amino acids 95 to 110 of SEQ ID NO: 8, amino acids 192 to 206 of SEQ ID NO: 8 and amino acids 223 to 240 of SEQ ID NO: 8, wherein said fragment of SEQ ID NO: 6 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 6;

said p12 polypeptide comprises

(ci) the sequence of SEQ ID NO: 10,

(cii) a variant of SEQ ID NO: 10 having more than 70% amino acid sequence identity to SEQ ID NO: 10 across the full length of SEQ ID NO: 10 which comprises at least one amino acid sequence selected from the group consisting of amino acids 33 to 56 of SEQ ID NO: 12, amino acids 98 to 117 of SEQ ID NO: 12 and amino acids 3 to 10 of SEQ ID NO: 12, wherein said variant of SEQ ID NO: 10 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 10, or

(ciii) a fragment of SEQ ID NO: 10 which comprises at least one amino acid sequence selected from the group consisting of amino acids 33 to 56 of SEQ ID NO: 12, amino acids 98 to 117 of SEQ ID NO: 12 and amino acids 3 to 10 of SEQ ID NO: 12, wherein said fragment of SEQ ID NO: 10 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 10;

and said mpa polypeptide comprises

(di) the sequence of SEQ ID NO: 14,

(dii) a variant of SEQ ID NO: 14 having more than 70% amino acid sequence identity to SEQ ID NO: 14 across the full length of SEQ ID NO: 14 which comprises at least one amino acid sequence selected from the group consisting of amino acids 130 to 160 of SEQ ID NO: 16, amino acids 56 to 64 of SEQ ID NO: 16, amino acids 150 to 160 of SEQ ID NO: 16 and amino acids 177 to 185 of SEQ ID NO: 16, wherein said variant of SEQ ID NO: 14 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 14 or

(diii) a fragment of SEQ ID NO: 14 which comprises at least one amino acid sequence selected from the group consisting of amino acids 130 to 160 of SEQ ID NO: 16, amino acids 56 to 64 of SEQ ID NO: 16, amino acids 150 to 160 of SEQ ID NO: 16 and amino acids 177 to 185 of SEQ ID NO: 16, wherein said fragment of of SEQ ID NO: 14 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 14.

Preferably such a variant maintains the ability to generate an immune response against the unmodified polypeptide. A preferred variant ahpC polypeptide has the amino acid sequence given in SEQ ID NO: 4. A preferred variant gsd polypeptide has the amino acid sequence given in SEQ ID NO: 8. A preferred variant pl2 polypeptide has the amino acid sequence given in SEQ ID NO: 12. A preferred variant mpa polypeptide has the amino acid sequence given in SEQ ID NO: 16. A preferred polypeptide of the invention comprises the amino acid sequence of SEQ ID Nos: 4, 8, 12 and 16. A particularly preferred amino acid sequence is given in SEQ ID NO: 24.

The present invention also provides polynucleotides which encode such polypeptides. A polynucleotide of the invention may comprise

(a) the ahpC polynucleotide of SEQ ID NO: 1 or a variant thereof having at least 70% homology to SEQ ID NO: 1 across the full length of SEQ ID NO: 1 or a fragment of at least 24 nucleotides of SEQ ID NO: 1 which encodes an epitope;

(b) the gsd polynucleotide of SEQ ID NO: 5 or a variant thereof having at least 70% homology to SEQ ID NO: 5 across the full length of SEQ ID NO: 5 or a fragment of at least 24 nucleotides of SEQ ID NO: 5 which encodes an epitope;

(c) the pl2 polynucleotide of SEQ ID NO: 9 or a variant thereof having at least 70% homology to SEQ ID NO: 1 across the full length of SEQ ID NO: 9 or a fragment of at least 24 nucleotides of SEQ ID NO: 9 which encodes an epitope; and

(d) the mpa polynucleotide of SEQ ID NO: 13 or a variant thereof having at least 70% homology to SEQ ID NO: 1 across the full length of SEQ ID NO: 13 or a fragment of at least 24 nucleotides of SEQ ID NO: 13 which encodes an epitope.

A polynucleotide of the invention will encode a polypeptide of the invention. In one embodiment a polynucleotide variant as defined above will differ from the given SEQ ID NO by virtue of degeneracy in the genetic code. In one embodiment, a polynucleotide variant will be codon optimised for the species which it is desired to treat.

The present invention also provides:

A vector comprising a polynucleotide of the invention, or a vector expressing an ahpC polypeptide, a gsd polypeptide, a p12 polypeptide and an mpa polypeptide as defined above. Preferred vector types include poxvirus vectors, adenovirus vectors and plasmids.

A host cell comprising a polypeptide, polynucleotide or vector of the invention or a host cell which expresses a polypeptide of the invention.

A polypeptide, polynucleotide, vector or host cell of the invention for use in therapy. In particular, the present invention provides a polypeptide, polynucleotide, vector or host cell of the invention for treating or preventing MAP infection; or use of such a polypeptide, polynucleotide, vector or host cell for treating or preventing MAP infection or in the manufacture of a medicament for treating or preventing MAP infection.

- A method of treating or preventing MAP infection of a condition or symptom associated with MAP infection comprising administering to a subject in need thereof an effective amount of a polypeptide, polynucleotide, vector or host cell of the invention. The subject may also be administered with a further therapeutic agent.
- A kit for use in treating or preventing MAP infection or a condition or symptom associated with MAP infection, said kit comprising (i) at least one polypeptide, polynucleotide, vector or host cell of the invention and (ii) at least one other therapeutic agent, for simultaneous, sequential or separate use.

Brief Description of the Figures

Figure 1 shows the nucleotide and amino acid sequences of the Havilah construct. The amino acid sequence comprises a ubiquitin leader sequence followed by an ahpC sequence (italics), a gsd sequence (bold), a p12 sequence (plain text) and an mpa sequence (bold italics). The amino acid sequence ends with a pK tag.

Figure 2 shows the ahpC (A), gsd (B), p12 (C) and mpa (D) polypeptides included in the Havilah construct. Bold italics = predicted strong class II human epitope. Underlined = predicted class I epitope.

Figure 3. A. Shows the highly significantly increased antigen-specific ELISPOT responses to rec.AhpC and rec.Mpa, as well as to synthetic peptides from the Havilah polyprotein in pools B and F, resulting from vaccination of naïve uninfected C57/BL6 MICE with the recombinant plasmid pSG2.HAV followed by MVA.HAV when compared to vector-only controls. B. example of the identification of an epitope from ELISPOT responses to the synthetic peptides in pool F (see also Figure 4) in the pSG2.HAV/MVA.HAV vaccinated animals. The response in vaccinated, but not unvaccinated animals is seen to be due to the strong recognition of the peptide 9.1 with the amino acid sequence GFAEINPIA constituting a specific T cell epitope.

Figure 4. Summary of the sequences of the synthetic peptide antigens spanning the Havilah polyprotein used in the detection of epitope regions. AhpC peptides in italics, Mpa peptides in bold italics, p12 peptides in plain text, and gsd peptides in bold.

Figure 5. A. Diagram of the structure of the mpa antigen in the cell surface membrane of MAP showing intracellular, transmembrane and extracellular domains.

The strong T cell epitope GFAEINPIA in peptide 9.1 is seen to comprise the fifth and smallest extracellular loop of the protein. B. Highly significant reduction by 2-3 log units and elimination of MAP organisms in the spleen tissue of MAP-infected C57/BL6 mice in response to therapeutic vaccination, 26 weeks after infection with a slow growing laboratory strain of MAP compared to vector-only and sham vaccinated animals. * MAP not detected by sensitive qPCR in the spleens of 6 of the 8 mice.

Figure 6. Highly significant antigen-specific T cell (A. ELISPOT) and antibody (B. ELISA) responses to vaccination using Ad5.HAV to prime followed by MVA.HAV to boost in C57/BL6 mice. Prominent T cell recognition of the strong epitope in peptide 9.1 is again seen together with significant recognition of rec.Gsd and rec.Mpa proteins and peptide pools J and L, compared with animals vaccinated using vectors alone. By contrast none of the peptides including 9.1 are recognised by antibody in ELISA assays in response to vaccination but there is substantial recognition of rec.AhpC and rec.Mpa.

Figure 7. Highly significant reduction compared to vector-only controls, in the infective load of MAP organisms in the spleen tissue of C57/BL6 mice challenged i.p with low dose or high dose MAP in response to vaccination using Ad5.HAV followed by MVA.HAV given either before (prophylactic) or after (therapeutic) the MAP challenge. A = prophylactic treatment (E1 + G1). Low dose vector vs vaccine: $p = 0.0207$; high dose vector vs vaccine: $p = 0.0205$. B = therapeutic treatment (E2 +G2). Low dose vector vs vaccine: $p = 0.0207$; high dose vector vs vaccine: $p = 0.0023$.

Figure 8. The same experiment as in Figure 7 also showing significant reductions in the MAP infective load in the liver tissues of mice vaccinated with Ad5.HAV followed by MVA.HAV as described above. A = prophylactic treatment (E1 + G1). Low dose vector vs vaccine: $p = 0.0379$; high dose vector vs vaccine: $p = 0.0003$. B = therapeutic treatment (E2 +G2). Low dose vector vs vaccine: $p = 0.0499$.

Figure 9. Prominent recognition of the strong T cell epitope GFAEINPIA in mpa (peptide 9.1) and significant recognition of peptide pool J compared to vector-only controls, in the presence of MAP infection in C57/BL6 mice, in response to vaccination administered prophylactically (A) or therapeutically (B) as described for Figure 7 above. In the presence of established MAP infection (therapeutic panel B) there is also significant recognition of peptide pool L compared to vector-only controls such that pre-existing MAP infection was capable of priming the response to

vaccination. A = Pool J (p = 0.0006); peptide 9.1 (p = <0.0001). B = Pool J (p = <0.0001); Pool L (p = 0.0032); Peptide 9.1 (p = <0.0001).

Brief Description of the Sequences

SEQ ID Nos 1 and 2 are the polynucleotide and polypeptide sequences respectively for the MAP ahpC gene.

SEQ ID Nos 3 and 4 are a modified version of the MAP ahpC gene, in which the polynucleotide sequence has been codon optimised for human use.

SEQ ID Nos 5 and 6 are the polynucleotide and polypeptide sequences respectively for the MAP gsd gene.

SEQ ID Nos 7 and 8 are a modified version of the MAP gsd gene, in which the polynucleotide sequence has been codon optimised for human use and which is truncated at the N-terminal in order to remove the cysteine residue at position 22.

SEQ ID Nos 9 and 10 are the polynucleotide and polypeptide sequences respectively for the MAP p12 gene.

SEQ ID Nos 11 and 12 are a modified version of the MAP p12 gene, in which the polynucleotide sequence has been codon optimised for human use.

SEQ ID Nos 13 and 14 are the polynucleotide and polypeptide sequences respectively for the MAP mpa gene.

SEQ ID Nos 15 and 16 are a modified version of the MAP mpa gene, in which the polynucleotide sequence has been codon optimised for human use and a number of transmembrane regions have been removed.

SEQ ID NO: 17 is a ubiquitin leader sequence.

SEQ ID NO: 18 is a pK Tag sequence.

SEQ ID Nos 19 and 20 are a polynucleotide construct and the encoded peptide consisting of the modified ahpC, gsd, p12 and mpa sequences above.

SEQ ID Nos 21 and 22 are a polynucleotide construct and the encoded peptide consisting of the modified ahpC, gsd, p12 and mpa sequences above, together with a ubiquitin leader sequence and a pK tag.

SEQ ID NO: 23 is the polynucleotide sequence of the Havilah construct and SEQ ID NO: 24 is the polypeptide sequence encoded by Havilah.

SEQ ID NO: 25 is a T cell epitope from the mpa polypeptide sequence as identified in Figures 3 and 4.

Detailed Description of the Invention

The present invention relates to the use of a combination of four proteins deriving from MAP in the treatment or prevention of MAP infection or conditions associated with the presence of MAP in humans or animals. The four proteins are ahpC, gsd, p12 and mpa. AhpC and p12 are secreted components while gsd and mpa are both membrane bound molecules in MAP.

ahpC is a secreted component shared by many pathogenic mycobacteria. It is involved in the ability of MAP to survive within macrophages and is upregulated on entry into a state of microbial dormancy. The nucleic acid and amino acid sequences of the MAP ahpC gene and protein are given in SEQ ID Nos 1 and 2 respectively. For use in the present invention, this sequence, or a variant thereof as discussed below may be used. For example, the MAP ahpC gene sequence may be codon optimised as discussed further below to make it more suitable for mammalian, in particular human, use. A suitable modified ahpC sequence and encoded protein are given in SEQ ID Nos 3 and 4 respectively.

gsd is a glycosyl transferase encoded by the GS pathogenicity element with a predicted signal sequence and lipid acylation site. Microarray analysis shows that it is up-regulated in the intracellular environment. It is expressed on the microbial cell surface and is predicted to transfer GDP-fucose to sub-terminal rhamnose to cap surface glycopeptidolipid on MAP with derivatised fucose giving the pathogen in its ZN-negative state an inert, hydrophobic, and highly resistant cell surface. The nucleic acid and amino acid sequences of the MAP gsd gene and protein are given in SEQ ID Nos 5 and 6 respectively. For use in the present invention, this sequence, or a variant thereof as discussed below, may be used. For example, the MAP gsd gene sequence may be codon optimised as discussed further below to make it more suitable for mammalian, in particular human, use. Other modifications may be made, for example potential acylation sites may be removed. One suitable modified gsd sequence and encoded protein are given in SEQ ID Nos 7 and 8 respectively.

p12 is the carboxyterminal 17 kDa fragment of p43 encoded by IS900 which is also up-regulated intracellularly. It is strongly predicted on the cell surface and both in MAP and in p43.rec.E.coli it is the substrate for specific proteolytic cleavage and exodomain release. The nucleic acid and amino acid sequences of the MAP p12 gene and protein are given in SEQ ID Nos 9 and 10 respectively. For use in the present invention, this sequence, or a variant thereof as discussed below may be used. For example, the MAP p12 gene sequence may be codon optimised as discussed further below to make it more suitable for mammalian, in particular human, use. One suitable modified p12 sequence and encoded protein are given in SEQ ID Nos 11 and 12 respectively.

mpa is also expressed on the surface of MAP and is believed to be unique to the pathogen. It is both an acetylase and a predicted pore molecule with 10 transmembrane regions and a large extracellular peptide loop. The nucleic acid and amino acid sequences of the MAP mpa gene and protein are given in SEQ ID Nos 13 and 14 respectively. For use in the present invention, this sequence, or a variant thereof as discussed below may be used. For example, the MAP mpa gene sequence may be codon optimised as discussed further below to make it more suitable for mammalian, in particular human, use. Other modifications may be made, for example transmembrane regions may be removed to reduce the hydrophobicity of the protein. One suitable modified mpa sequence and encoded protein are given in SEQ ID Nos 15 and 16 respectively.

According to the present invention each of these four proteins, or variants of any thereof, are provided in combination.

Polypeptides

The invention relates to the provision of an ahpC polypeptide, a gsd polypeptide, a p12 polypeptide and an mpa polypeptide in combination.

A "polypeptide" is used herein in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or other peptidomimetics. The term "polypeptide" thus includes short peptide sequences and also longer polypeptides and proteins. As used herein, the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

A suitable ahpC polypeptide may have the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. A suitable gsd polypeptide may have the amino acid sequence of SEQ ID NO: 6 or SEQ ID NO: 8. A suitable p12 polypeptide may have the amino acid sequence of SEQ ID NO: 10 or SEQ ID NO: 12. A suitable mpa polypeptide may have the amino acid sequence of SEQ ID NO: 14 or SEQ ID NO: 16.

A suitable ahpC, gsd, p12 or mpa sequence may alternatively be a variant of one of these specific sequences. For example, a variant may be a substitution, deletion or addition variant of any of the above amino acid sequences, or may be a fragment of any thereof as described further below.

A variant of one of the four polypeptide may comprise 1, 2, 3, 4, 5, up to 10, up to 20, up to 30 or more amino acid substitutions and/or deletions from the sequences given in the sequence listing. "Deletion" variants may comprise the deletion of individual amino acids, deletion of small groups of amino acids such as 2, 3, 4 or 5 amino acids, or deletion of larger amino acid regions, such as the deletion of specific amino acid domains or other features. "Substitution" variants preferably involve the replacement of one or more amino acids with the same number of amino acids and making conservative amino acid substitutions. For example, an amino acid may be substituted with an alternative amino acid having similar properties, for example, another basic amino acid, another acidic amino acid, another neutral amino acid, another charged amino acid, another hydrophilic amino acid, another hydrophobic amino acid, another polar amino acid, another aromatic amino acid or another aliphatic amino acid. Some properties of the 20 main amino acids which can be used to select suitable substituents are as follows:

Ala	aliphatic, hydrophobic, neutral	Met	hydrophobic, neutral
Cys	polar, hydrophobic, neutral	Asn	polar, hydrophilic, neutral
Asp	polar, hydrophilic, charged (-)	Pro	hydrophobic, neutral
Glu	polar, hydrophilic, charged (-)	Gln	polar, hydrophilic, neutral
Phe	aromatic, hydrophobic, neutral	Arg	polar, hydrophilic, charged (+)
Gly	aliphatic, neutral	Ser	polar, hydrophilic, neutral
His	aromatic, polar, hydrophilic, charged (+)	Thr	polar, hydrophilic, neutral
Ile	aliphatic, hydrophobic, neutral	Val	aliphatic, hydrophobic, neutral
Lys	polar, hydrophilic, charged(+)	Trp	aromatic, hydrophobic, neutral
Leu	aliphatic, hydrophobic, neutral	Tyr	aromatic, polar, hydrophobic

Preferred "derivatives" or "variants" include those in which instead of the naturally occurring amino acid the amino acid which appears in the sequence is a structural analog thereof. Amino acids used in the sequences may also be derivatized or modified, e.g. labelled, providing the function of the peptide is not significantly adversely affected.

Derivatives and variants as described above may be prepared during synthesis of the peptide or by post- production modification, or when the peptide is in recombinant form using the known techniques of site- directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

Suitable variants may also be naturally occurring polypeptides from mycobacteria other than MAP. For example, a variant ahpC polypeptide sequence may derive from a different mycobacterial strain to MAP. Such naturally occurring variants preferably maintain the ability to stimulate an immune response which is capable of acting against MAP. That is, the immune response to the variant polypeptide will react against MAP polypeptides as well as the variant polypeptide used.

Preferably variants according to the invention have an amino acid sequence which has more than 60%, or more than 70%, e.g. 75 or 80%, preferably more than 85%, e.g. more than 90 or 95% amino acid identity to, for example, SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16, (according to the test described hereinafter). This level of amino acid identity may be seen across the full length of the sequence or over a part of the sequence, such as 20, 30, 50, 75, 100, 150, 200 or more amino acids, depending on the size of the full length polypeptide.

In connection with amino acid sequences, "sequence identity" refers to sequences which have the stated value when assessed using ClustalW (Thompson et al., 1994, supra) with the following parameters:

Pairwise alignment parameters -Method: accurate, Matrix: PAM, Gap open penalty: 10.00, Gap extension penalty: 0.10;

Multiple alignment parameters -Matrix: PAM, Gap open penalty: 10.00, % identity for delay: 30, Penalize end gaps: on, Gap separation distance: 0, Negative matrix: no, Gap extension penalty: 0.20, Residue-specific gap penalties: on, Hydrophilic gap penalties: on, Hydrophilic residues: GPSNDQEKR. Sequence identity at a particular residue is intended to include identical residues which have simply been derivatized.

Particular modifications can be made to any of the wild type MAP proteins sequences given in SEQ ID Nos: 2, 6, 10 and 14. For example, modification can be made to try to improve the overall properties of the variant protein as an immunogen.

In one embodiment, a wild-type protein may be modified by deletion or substitution to remove an acylation site. Such an acylation site might affect the overall conformation of the protein. By omitting acylation sites, for example by excluding or substituting a cysteine residue, the presentation of effective epitopes within the protein may be optimised. For example, the wild type MAP gsd sequence given in SEQ ID NO: 6 includes a cysteine residue at position 22. In the variant of SEQ ID NO: 8, the amino acid sequence has been modified by truncation at the N-terminal such that this cysteine residue is no longer present. Such a modification may be made to a wild type protein or to any of the variant or fragment sequences, such as the codon optimised sequences, described herein.

In another embodiment, a wild type MAP protein may be modified to disable or remove potential cross-reacting epitopes. For example, where a polypeptide of the invention is intended for use in a human, the polypeptide sequence may be modified to disable or remove potential cross-reacting human epitopes, such as sequences which generate antibodies in human patients which may cross-react with similar sequences in human proteins. Modifications may thus be made to the MAP sequences to avoid such cross-reactivity but to maintain the ability to generate an anti-MAP immune response.

For example within the wild type MAP gsd sequence the lysine residues at positions 239 and 241 (see SEQ ID NO: 6) may each be substituted with asparagine. An equivalent substitution may be made in any of the variant or fragment gsd sequences described herein. For example in the variant sequence of SEQ ID NO: 8, the lysine residues at positions 216 and 218 may be replaced with asparagines. This may be achieved by modifying the nucleic acid sequence which encodes the gsd polypeptide. For example, in the gsd polynucleotide sequence of SEQ ID NO: 7, the AAG codons at positions 646 to 648 and 651 to 654 may be replaced by AAT. This maintains the optimised human codon usage of SEQ ID NO: 7 and further removes potentially cross-reacting human epitopes.

Similarly, modifications mat be made to the MAP ahpC sequence. In the wild-type ahpC sequence of SEQ ID NO: 2, the lysine at position 29 may be replaced with threonine and the proline at position 31 may be replaced with leucine. An equivalent substitution may be made in any of the variant or fragment ahpC sequences described herein. For example, in the modified variant sequence of SEQ ID NO: 4, the same substitutions may be made at the position 28 lysine and the position 30 proline. This may be achieved by modifying the nucleic acid sequence which encodes the ahpC polypeptide. For example, in the ahpC polynucleotide sequence of SEQ ID NO: 3, the AAA codon at positions 82 to 84 may be replaced by ACA and the CCC codon at positions 88 to 90 may be replaced by CTC. This maintains the optimised human codon usage of SEQ ID NO: 3 and further removes potentially cross-reacting human epitopes.

Similarly, modifications may be made to reduce the hydrophobicity of the protein and thus to help optimise the surface presentation of epitopes. For example, the wild-type mpa sequence of SEQ ID NO: 14 includes ten transmembrane regions. In order to reduce the hydrophobicity of the protein, one or more of these regions, or parts of these regions, may be omitted or substituted. For example, one, more or all of the transmembrane regions may be deleted. Such regions may be deleted totally or partially, optionally leaving none, one, two or more amino acid residues from the ends of the transmembrane sequence in the protein. Thus one modification may be the deletion or substitution of one or more hydrophobic amino acids. An example of this is seen in SEQ ID NO: 16 which is a variant of MAP mpa in which most of the transmembrane sequences have been deleted, leaving only one or two amino acids from the transmembrane regions in the variant polypeptide.

Polypeptide "fragments" according to the invention may be made by truncation, e.g. by removal of one or more amino acids from the N and/or C-terminal ends of a polypeptide. Up to 10, up to 20, up to 30, up to 40 or more amino acids may be removed from the N and/or C terminal in this way. Fragments may also be generated by one or more internal deletions. For example, a variant of the invention may consist of or comprise two or more epitope regions from a full length polypeptide of the region in the absence of non-epitope amino acids. Preferably a fragment of an ahpC, gsd, p12 or mpa polypeptide comprises at least one epitope capable of inducing an immune response against the unmodified MAP polypeptide. Such fragments may be derived from a sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16 or may be derived from a variant peptide as described herein. Preferably such fragments are between 8 and 150 residues in length, e.g. 8 to 50 or 8 to 30 residues. Alternatively, fragments of the invention may be longer sequences, for example comprising at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of a full length polypeptide of the invention.

Preferably, a variant of one of the four polypeptides is a functional variant thereof. In particular, a variant polypeptide should retain the ability to stimulate an immune response against the unmodified MAP polypeptide. In one embodiment, a functional variant polypeptide should be capable of acting as an antigen and should include at least one functional epitope from the original polypeptide.

An "antigen" refers to any agent, generally a macromolecule, which can elicit an immunological response in an individual. As used herein, "antigen" is generally used to refer to a polypeptide molecule or portion thereof which contains one or more epitopes. Furthermore, for the purposes of the present invention, an "antigen" includes a polypeptide having modifications, such as deletions, additions and substitutions (generally conservative in nature) to the native sequence, so long as the polypeptide maintains sufficient immunogenicity. These modifications may be deliberate, for example through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immune response" against an antigen of interest is the development in an individual of a humoral and/or a cellular immune response to that antigen. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells.

As used herein, the term "epitope" generally refers to the site on a target antigen which is recognised by an immune receptor such as a T-cell receptor and/or an antibody. Preferably it is a short peptide derived from or as part of a protein. However the term is also intended to include peptides with glycopeptides and carbohydrate epitopes. A single antigenic molecule, such as one of the four proteins described herein, may comprise several different epitopes. The term "epitope" also includes modified sequences of amino acids or carbohydrates which stimulate responses which recognise the whole organism.

It is advantageous if the selected epitope is specific to MAP, or involved in the pathogenicity of MAP. For example, it is advantageous if the immune receptor and/or antibody which recognises the epitope will only recognise this epitope from MAP, and not epitopes in other unrelated proteins, in particular proteins from unrelated organisms or host proteins. If the epitope is involved in pathogenicity of MAP, then an immune response against such an epitope may be used to target pathogenic MAP infections.

An epitope may also be related to equivalent epitopes on other mycobacteria. For example, many individuals suffering from MAP infection are also infected by *M. avium* as a secondary co-pathogen. Other *M. avium* complexes may be present or involved in Crohn's disease. Many of the proteins expressed in MAP such as AhpC are very similar to those expressed in *M. avium*. If the polypeptide of the invention includes one or more epitopes which are capable of stimulating an immune response which acts against *M. avium* in addition to MAP, a further, secondary, therapeutic effect may be achieved.

Epitopes can be identified from knowledge of the amino acid and corresponding DNA sequences of the peptide or polypeptide, as well as from the nature of particular amino acids (e.g., size, charge, etc.) and the codon dictionary, without undue experimentation. See, e.g., Ivan Roitt, Essential Immunology, 1988; Janis Kuby, Immunology, 1992 e.g., pp. 79-81. Some guidelines in determining whether a protein or an epitope of interest will stimulate a response, include: peptide length--the peptide should be at least 8 or 9 amino acids long to fit into the MHC class I complex and at least 8-25, such at least as 13-25 amino acids long to fit into a class II MHC complex. These lengths are the minimum for the peptide to bind to the respective MHC complex. It is preferred for the peptides to be longer than these lengths because cells may cut peptides. The peptide should contain an appropriate anchor motif which will enable it to bind to the various class I or class II molecules with high enough specificity to generate an immune response. This can be done, without undue experimentation, by comparing the sequence of the protein of interest with published structures of peptides associated with the MHC molecules. Thus, the skilled artisan can ascertain an epitope of interest by comparing the protein sequence with sequences listed in the protein database.

Suitable epitopes may thus be identified by routinely used methods such as those demonstrated in Figures 3 and 4 for identifying the strong T cell epitope GFAEINPIA (peptide 9.1) in the 5th extracellular loop of mpa. In such a method, a library of short peptides which are fragments of the polypeptide sequence of interested may be generated and each of these peptides assessed separately for their ability to identify an immune response against the full length polypeptide. Members of the library may be screened in groups or pools or individual members of the library, such as individual members of a single pool, may be assessed separately.

In a further example, epitope scanning of the individual proteins of SEQ ID Nos 4, 8, 12 and 16 revealed a number of predicted class I and class II epitopes.

In the ahpC variant sequence of SEQ ID NO: 4, predicted strong class II epitopes were identified at amino acids 48 to 56, 90 to 101 and 161 to 169. An ahpC polypeptide of the invention, such as an ahpC variant or fragment polypeptide, preferably comprises at least one, for example one, two or all three of these epitopes.

In the gsd variant sequence of SEQ ID NO: 8, predicted class I epitopes were identified at amino acids 1 to 32, 58 to 68, 99 to 119, 123 to 147, 159 to 169, 180 to 194 and 200 to 231, and predicted strong class II epitopes were identified at amino acids 64 to 76, 95 to 110, 192 to 206 and 223 to 240. A gsd polypeptide of the invention, such as a gsd variant or fragment polypeptide, preferably comprises at least one, for example one, two, three, four, five, six, seven, eight, nine, ten or all of these epitopes.

In the p12 variant sequence of SEQ ID NO 12, predicted class I epitopes were identified at amino acids 33 to 56 and 98 to 117 and a predicted strong class II epitope was identified at amino acids 3 to 10. A p12 polypeptide of the invention, such as a p12 variant or fragment polypeptide, preferably comprises at least one, for example one, two or all three of these epitopes.

In the mpa variant sequence of SEQ ID NO: 16, a predicted class I epitope was identified at amino acids 130 to 160, and predicted strong class II epitopes were identified at amino acids 56 to 64 and 150 to 160. An mpa polypeptide of the invention, such as an mpa variant or fragment polypeptide, preferably comprises at least one, for example one, two or all three of these epitopes.

As shown in the Examples, a particular strong T cell epitope has been identified in the mpa polypeptide sequence. This epitope has the amino acid sequence

GFAEINPIA (SEQ ID NO: 25) and is located at amino acids 357 to 365 of SEQ ID NO: 14 and amino acids 177 to 185 of SEQ ID NO: 16. This sequence is found in the construct of SEQ ID NO: 24 at amino acids 761 to 769. A preferred mpa polypeptide sequence is a sequence which comprises GFAEINPIA. Such a sequence may also comprise one, two or all three of the predicted class I and class II epitopes mentioned above.

This epitope is believed to be located in the fifth extracellular loop of mpa (Figure 5A). A preferred mpa polypeptide may therefore maintain the sequence of the fifth extracellular loop. An mpa polypeptide may therefore comprise the amino acid sequence GFAEINPIA and also adjacent amino acids from the fifth extracellular loop of mpa. Preferably, this fifth extracellular loop will be present in a polypeptide of the invention in a suitable form and conformation for it to be recognised by the immune system.

Preferably, the four polypeptides ahpC, gsd, p12 and mpa are provided together in a single fusion protein. The four polypeptide sequences in such a fusion protein may be any of the polypeptides or variants described herein. The four polypeptides may be provided in any order in the fusion protein. In one embodiment they are provided in the order ahpC – gsd – p12 – mpa.

In one embodiment, the four polypeptides present in a fusion protein are those given in SEQ ID Nos 4, 8, 12 and 16. For example, these four proteins may be provided in a fusion protein in this order as shown in SEQ ID NO: 20.

In an alternative embodiment, the polypeptides may be present in two or more separate polypeptide molecules, which may or may not be linked by non-covalent linkages. For example, the four polypeptides may be provided separately, or may be provided in two or three separate fusion protein polypeptide molecules. For example, three of the polypeptides may be provided in a single polypeptide molecule and the fourth provided separately, two may be provided in one molecule and the other two provided separately, or the four polypeptides may be provided in two polypeptide molecules, each comprising two of the four polypeptides.

In a fusion protein of the invention, linker sequences may separate the required polypeptide sequences and/or there may or may not be additional sequences present at the N terminal or C terminal of the peptide. Typically the fusion protein comprises 1, 2, 3, or more such linkers. The linkers are typically 1, 2, 3, 4 or more amino acids in length. Thus in the peptide 1, 2, 3 or all of the polypeptide sequences may be contiguous with each other or may be separated from each other, for example by such linkers.

A polypeptide of the invention may comprise further additional sequences, for example those encoded by the polynucleotides and vectors described below. For example, it may comprise additional epitopes, therapeutic polypeptides, adjuvants or immunomodulatory molecules.

The polypeptide may comprise a leader sequence, i.e. a sequence at or near the amino terminus of the polypeptide that functions in targeting or regulation of the polypeptide. For example a sequence may be included in the polypeptide that targets it to particular tissues in the body, or which helps the processing or folding of the polypeptide upon expression. Various such sequences are well known in the art and could be selected by the skilled reader depending upon, for example, the desired properties and production method of the polypeptide. One example of such a leader is the ubiquitin leader sequence given in SEQ ID NO: 17.

A polypeptide may further comprise a tag or label to identify or screen for the polypeptide, or for expression of the polypeptide. Suitable labels include radioisotopes such as ^{125}I, ^{32}P or ^{35}S, fluorescent labels, enzyme labels, or other protein labels such as biotin. Suitable tags may be short amino acid sequences that can be identified by routine screening methods. For example, a short amino acid sequence may be included that is recognised by a particular monoclonal antibody. One such tag of the pK tag given in SEQ ID NO: 18.

In one embodiment, a polypeptide of the invention has the sequence given in SEQ ID NO: 24. This is referred to herein as the Havilah polypeptide sequence and comprises the four modified polypeptides of SEQ ID Nos: 4, 8, 12 and 16, and additional sequences such as a ubiquitin leader sequence and a pK tag.

Peptides of the invention, as defined herein, may be chemically modified, for example, post-translationally modified. For example they may be glycosylated or comprise modified amino acid residues. They can be in a variety of forms of polypeptide derivatives, including amides and conjugates with polypeptides.

Chemically modified peptides also include those having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized side groups include those which have been derivatized to form amine hydrochlorides, ptoluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups and formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Peptides may also be modified by phosphorylation, for example 3 amino phosphorylation and by glycosylation for example mannosylation.

Also included as chemically modified peptides are those which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline or homoserine may be substituted for serine.

Polynucleotides

The invention also relates to polynucleotide constructs comprising nucleic acid sequences which encode the four polypeptides or variants thereof. For example, a single nucleic acid molecule may be provided which encodes an ahpC polypeptide, a gsd polypeptide, a p12 polypeptide and an mpa polypeptide. These four polypeptides may be encoded in any order in the nucleic acid molecule, but are preferably provided in the order ahpC – gsd – p12 – mpa. For example, a polynucleotide of the invention may encode any of the polypeptides or fusion proteins described above.

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 analogs 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.

In one embodiment, therefore, a polynucleotide of the invention comprises an ahpC gene sequence, a gsd gene sequence, a p12 gene sequence and an mpa gene sequence. Suitable gene sequences are provided in SEQ ID Nos 1, 3, 5, 7, 9, 11, 13 and 15. A suitable ahpC, gsd, p12 or mpa sequence may alternatively be a variant of one of these specific sequences. For example, a variant may be a substitution, deletion or addition variant of any of the above nucleic acid sequences. A variant of one of the four genes may comprise 1, 2, 3, 4, 5, up to 10, up to 20, up to 30, up to 40, up to 50, up to 75 or more nucleic acid substitutions and/or deletions from the sequences given in the sequence listing.

Suitable variants may be at least 70% homologous to a MAP polynucleotide of the invention, preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto. Methods of measuring homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of nucleic acid identity. Such homology may exist over a region of at least 15, preferably at least 30, for instance at least 40, 60, 100, 200 or more contiguous nucleotides. Such homology may exist over the entire length of the unmodified MAP polynucleotide sequence.

Methods of measuring polynucleotide homology or identity are known in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (e.g. used on its default settings) (Devereux et al (1984) Nucleic Acids Research 12, p387-395).

The PILEUP and BLAST algorithms can also be used to calculate homology or line up sequences (typically on their default settings), for example as described in Altschul S.F. (1993) J Mol Evol 36:290-300; Altschul, S, F et al (1990) J Mol Biol 215:403-10.

Software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul et al, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The homologues typically hybridize with the relevant polynucleotide at a level significantly above background. The signal level generated by the interaction between the homologue and the polynucleotide is typically at least 10 fold, preferably at least 100 fold, as intense as "background hybridisation". The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P. Selective hybridisation is typically achieved using conditions of medium to high stringency, (for example, 0.03M sodium chloride and 0.003M sodium citrate at from about 50°C to about 60°C.

Stringent hybridization conditions can include 50% formamide, 5x Denhardt's Solution, 5x SSC, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA and the washing conditions can include 2x SSC, 0.1% SDS at 37°C followed by 1x SSC, 0.1%

SDS at 68°C. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra.

The homologue may differ from a sequence in the relevant polynucleotide by less than 3, 5, 10, 15, 20 or more mutations (each of which may be a substitution, deletion or insertion). These mutations may be measured over a region of at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides of the homologue.

In one embodiment, a variant sequence may vary from the specific sequences given in the sequence listing by virtue of the redundancy in the genetic code. The DNA code has 4 primary nucleic acid residues (A, T, C and G) and uses these to "spell" three letter codons which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons. A variant polynucleotide of the invention may therefore encode the same polypeptide sequence as another polynucleotide of the invention, but may have a different nucleic acid sequence due to the use of different codons to encode the same amino acids.

In one embodiment the coding sequence of the polynucleotide construct may be optimised to more closely resemble the codon usage of highly expressed genes in mammalian cells. Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilization of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others.

For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, it is possible that, for example a mycobacterial gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons which are rarely observed in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

In the polynucleotide of the invention, the codon usage pattern may therefore be altered from that found naturally in MAP to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura et.al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are “frequently used codons”. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

According to the present invention, the codon usage pattern of the polynucleotide of the invention will preferably exclude codons with a relative synonymous codon usage (RSCU) value of less than 0.2 in highly expressed genes of the target organism. A RSCU value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. The polynucleotide of the invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. Codon usage tables for human can also be found in GenBank.

It can thus be seen that the particular polynucleotide sequence which encodes a polypeptide of the invention may be altered to optimise the codons based on the species to be treated. As an example of this, the MAP sequences given in SEQ ID Nos: 1, 5, 9 and 13 have been codon optimised for human use in the polynucleotides of SEQ ID Nos: 3, 7, 11 and 17. Such modifications may improve the ability of such polynucleotides to express their encoded proteins in a human cell.

As explained above in relation to polypeptides, the polynucleotides of the invention may also be modified to disable or remove potential cross-reacting epitopes in the encoded polypeptide.

Polynucleotide "fragments" according to the invention may be made by truncation, e.g. by removal of one or more nucleotides from one or both ends of a polynucleotide. Up to 10, up to 20, up to 30, up to 40, up to 50, up to 75, up to 100, up to 200 or more amino acids may be removed from the 3' and/or 5' end of the polynucleotide in this way. Fragments may also be generated by one or more internal deletions. For example, a variant of the invention may encode a polypeptide that consists of or comprises two or more epitope regions from a full length polypeptide of the invention in the absence of non-epitope amino acids. Preferably a fragment of an ahpC, gsd, p12 or mpa polynucleotide sequence comprises at least one region encoding an epitope capable of inducing an immune response against the unmodified MAP polypeptide. Such fragments may be derived from a sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15 or may be derived from a variant polynucleotide as described herein. Preferably such fragments are between 24 and 500 residues in length, e.g. 24 to 400, 24 to 300, 24 to 100, 100 to 200 or 200 to 400 residues. Alternatively, fragments of the invention may be longer sequences, for example comprising at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of a full length polynucleotide of the invention.

A peptide of the invention may thus be produced from or delivered in the form of a polynucleotide which encodes, and is capable of expressing, it. 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). Substantially pure antigen preparations can be obtained using standard molecular biological tools. That is, polynucleotide sequences coding for the above-described moieties can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing an antigen, or by deriving the coding sequence for a polypeptide from a vector known to include the same. Furthermore, the desired sequences can be isolated directly from cells and tissues containing the same, using standard techniques, such as phenol extraction and PCR of cDNA or genomic DNA. See, e.g., Sambrook et al., supra, for a description of techniques used to obtain and isolate DNA. Polynucleotide sequences can also be produced synthetically, rather than cloned.

Yet another convenient method for isolating specific nucleic acid molecules is by the polymerase chain reaction (PCR). Mullis et al. (1987) Methods Enzymol. 155:335-350. This technique uses DNA polymerase, usually a thermostable DNA polymerase, to replicate a desired region of DNA. The region of DNA to be replicated is identified by oligonucleotides of specified sequence complementary to opposite ends and opposite strands of the desired DNA to prime the replication reaction. The product of the first round of replication is itself a template for subsequent replication, thus repeated successive cycles of replication result in geometric amplification of the DNA fragment delimited by the primer pair used.

Once the sequences have been obtained, they may be linked together to provide a nucleic acid molecule using standard cloning or molecular biology techniques. Alternatively, the sequences can be produced synthetically, rather than cloned. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. As explained herein, one will generally select preferred codons for the intended host in which the sequence will be expressed. The complete sequence can then be assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence.

Vectors

The nucleic acid 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 species. 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. Such 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 extrachromosomal genetic element. The vectors may contain one or more selectable marker genes, for example an ampicillin resistence 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.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which expression is designed. For example, yeast promoters include S. cerevisiae GAL4 and ADH promoters, S. pombe nmt1 and adh promoter. Mammalian promoters, such as β-actin promoters, may be used. Tissue-specific promoters are especially preferred. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium.

In one embodiment a viral promoter is used to drive expression from the polynucleotide. Typical viral promoters for mammalian cell expression include the SV40 large T antigen promoter, adenovirus promoters, the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the mouse mammary tumor virus LTR promoter, the rous sarcoma virus (RSV) LTR promoter, the SV40 early promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, including the adenovirus major late promoter (Ad MLP), HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). All these promoters are readily available in the art.

In one embodiment, the promoter is a Cytomegalovirus (CMV) promoter. A preferred promoter element is the CMV immediate early (IE) promoter devoid of intron A, but including exon 1. Thus the expression from the polynucleotide may be under the control of hCMV IE early promoter. Expression vectors using the hCMV immediate early promoter include for example, pWRG7128, and pBC12/CMV and pJW4303. A hCMV immediate early promoter sequence can be obtained using known methods. A native hCMV immediate early promoter can be isolated directly from a sample of the virus, using standard techniques. US 5385839, for example, describes the cloning of a hCMV promoter region. The sequence of a hCMV immediate early promoter is available at Genbank #M60321 (hCMV Towne strain) and X17403 (hCMV Ad169 strain). A native sequence could therefore be isolated by PCR using PCR primers based on the known sequence. See e.g Sambrook et al, supra, for a description of techniques used to obtain and isolate DNA. A suitable hCMV promoter sequence could also be isolated from an existing plasmid vector. Promoter sequences can also be produced synthetically.

A polynucleotide, expression cassette or vector of the invention may comprise an untranslated leader sequence. In general the untranslated leader sequence has a length of from about 10 to about 200 nucleotides, for example from about 15 to 150 nucleotides, preferably 15 to about 130 nucleotides. Leader sequences comprising, for example, 15, 50, 75 or 100 nucleotides may be used. Generally a functional untranslated leader sequence is one which is able to provide a translational start site for expression of a coding sequence in operable linkage with the leader sequence.

Typically, transcription termination and polyadenylation sequences will also be present, located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those derived from SV40, as described in Sambrook et al., supra, as well as a bovine growth hormone terminator sequence. Introns, containing splice donor and acceptor sites, may also be designed into the expression cassette or vector.

Expression systems often include transcriptional modulator elements, referred to as “enhancers”. Enhancers are broadly defined as a cis-acting agent, which when operably linked to a promoter/gene sequence, will increase transcription of that gene sequence. Enhancers can function from positions that are much further away from a sequence of interest than other expression control elements (e.g. promoters), and may operate when positioned in either orientation relative to the sequence of interest. Enhancers have been identified from a number of viral sources, including polyoma virus, BK virus, cytomegalovirus (CMV), adenovirus, simian virus 40 (SV40), Moloney sarcoma virus, bovine papilloma virus and Rous sarcoma virus. Examples of suitable enhancers include the SV40 early gene enhancer, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, and elements derived from human or murine CMV, for example, elements included in the CMV intron A sequence.

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.

Nucleic acids encoding for polypeptides known to display antiviral or antibacterial activity, immunomodulatory molecules such as cytokines (e.g. TNF-alpha, interferons such as IL-6, and IL-2, interferons, colony stimulating factors such as GM-CSF), adjuvants and co-stimulatory and accessory molecules (B7-1, B7-2) may be included in a polynucleotide, expression cassette or vector of the invention. Alternatively, such polypeptides may be provided separately, for example in a formulation comprising a molecule of the invention, or may be administered simultaneously, sequentially or separately with a composition of the invention. Concurrent provision of an immunomodulatory molecule and a polypeptide of the invention at a site *in vivo* may enhance the generation of specific effectors which may help to enhance the immune response. The degree of enhancement of the immune response may be dependent upon the specific immunostimulatory molecules and/or adjuvants used because different immunostimulatory molecules may elicit different mechanisms for enhancing and/or modulating the immune response. By way of example, the different effector mechanisms/immunomodulatory molecules include but are not limited to augmentation of help signal (IL-2), recruitment of professional APC (GM-CSF), increase in T cell frequency (IL-2), effect on antigen processing pathway and MHC expression (IFN-gamma and TNF-alpha) and diversion of immune response away from the Th1 response and towards a Th2 response. Unmethylated CpG containing oligonucleotides are also preferential inducers of a Th1 response and are suitable for use in the present invention.

In some embodiments, the polynucleotide, expression cassette or vector will encode an adjuvant, or an adjuvant will otherwise be provided. As used herein, the term "adjuvant" refers to any material or composition capable of specifically or non-specifically altering, enhancing, directing, redirecting, potentiating or initiating an antigen-specific immune response.

A suitable adjuvant may be an ADP-ribosylating bacterial toxin. These include diphtheria toxin (DT), pertussis toxin (PT), cholera toxin (CT), the E.coli heat labile toxins (LT1 and LT2), Pseudomonas endotoxin A, Pseudomonas exotoxin S, B. cereus exoenzyme, B. sphaericus toxin, C. botulinum C2 and C3 toxins, C. limosum exoenzyme, as well as toxins from C. perfringens, C. spiriforma and C. difficile and Staphylococcus aureus EDIN. Most ADP-ribosylating bacterial toxins contain A and B subunits.

Polynucleotides of interest may be used *in vitro* or *in vivo* in the production of a peptide of the invention. Such polynucleotides may be administered or used in the manufacture of a medicament for the treatment of Crohn's disease or another disease or condition characterised by the expression of MAP.

Gene therapy and nucleic acid immunization are approaches which provide for the introduction of a nucleic acid molecule encoding one or more selected antigens into a host cell for the *in vivo* expression of the antigen or antigens. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859 and 5,589,466. The nucleic acid molecule can be introduced directly into the recipient subject, such as by standard intramuscular or intradermal injection; transdermal particle delivery; inhalation; topically, or by oral, intranasal or mucosal modes of administration. The molecule alternatively can be introduced *ex vivo* into cells which have been removed from a subject. In this latter case, cells containing the nucleic acid molecule of interest are re-introduced into the subject such that an immune response can be mounted against the antigen encoded by the nucleic acid molecule. The nucleic acid molecules used in such immunization are generally referred to herein as "nucleic acid vaccines."

Each of these delivery techniques requires efficient expression of the nucleic acid in the transfected cell, to provide a sufficient amount of the therapeutic or antigenic gene product. Several factors are known to affect the levels of expression obtained, including transfection efficiency, and the efficiency with which the gene or sequence of interest is transcribed and the mRNA translated.

The agent produced by a host cell may be secreted or may be contained intracellularly depending on the polynucleotide and/or the vector used. As will be understood by those of skill in the art, expression vectors containing the polynucleotides of the invention can be designed with signal sequences which direct secretion of the polypeptide expressed from the vector through a particular prokaryotic or eukaryotic cell membrane.

The vectors and expression cassettes of the present invention may be administered directly as "a naked nucleic acid construct", preferably further comprising flanking sequences homologous to the host cell genome. As used herein, the term "naked DNA" refers to a vector such as a plasmid comprising a polynucleotide of the present invention together with a short promoter region to control its production. It is called "naked" DNA because the vectors are not carried in any delivery vehicle. When such a vector enters a host cell, such as a eukaryotic cell, the proteins it encodes are transcribed and translated within the cell.

The vector of the invention may thus be a plasmid vector, that is, an autonomously replicating, extrachromosomal circular or linear DNA molecule. The plasmid may include additional elements, such as an origin of replication, or selector genes. Such elements are known in the art and can be included using standard techniques. Numerous suitable expression plasmids are known in the art. For example, one suitable plasmid is pSG2. This plasmid was originally isolated from *Streptomyces ghanaensis*. The length of 13.8 kb, single restriction sites for HindIII, EcoRV and PvuII and the possibility of deleting non-essential regions of the plasmid make pSG2 a suitable basic replicon for vector development.

Alternatively, the vectors of the present invention may be introduced into suitable host cells using a variety of viral techniques which are known in the art, such as for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses.

In one embodiment, the vector itself may be a recombinant viral vector. Suitable recombinant viral vectors include but are not limited to adenovirus vectors, adeno-associated viral (AAV) vectors, herpes-virus vectors, a retroviral vector, lentiviral vectors, baculoviral vectors, pox viral vectors or parvovirus vectors. In the case of viral vectors, administration of the polynucleotide is mediated by viral infection of a target cell.

A number of viral based systems have been developed for transfecting mammalian cells.

For example, a selected recombinant nucleic acid molecule can be inserted into a vector and packaged as retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. Retroviral vectors may be based upon the Moloney murine leukaemia virus (Mo-MLV). In a retroviral vector, one or more of the viral genes (gag, pol & env) are generally replaced with the gene of interest.

A number of adenovirus vectors are known. Adenovirus subgroup C serotypes 2 and 5 are commonly used as vectors. The wild type adenovirus genome is approximately 35kb of which up to 30kb can be replaced with foreign DNA. There are four early transcriptional units (E1, E2, E3 & E4), which have regulatory functions, & a late transcript, which codes for structural proteins. Adenovirus vectors may have the E1 and/or E3 gene inactivated. The missing gene(s) may then be supplied in trans either by a helper virus, plasmid or integrated into a helper cell genome. Adenovirus vectors may use an E2a temperature sensitive mutant or an E4 deletion. Minimal adenovirus vectors may contain only the inverted terminal repeats (ITRs) & a packaging sequence around the transgene, all the necessary viral genes being provided in trans by a helper virus. Suitable adenoviral vectors thus include Ad5 vectors and simian adenovirus vectors.

Viral vectors may also be derived from the pox family of viruses, including vaccinia viruses and avian poxvirus such as fowlpox vaccines. For example, modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. A recombinant MVA vector may therefore be used to deliver the polypeptide of the invention.

Addition types of virus such as adeno-associated virus (AAV) and herpes simplex virus (HSV) may also be used to develop suitable vector systems.

As an alternative to viral vectors, liposomal preparations can alternatively be used to deliver the nucleic acid molecules of the invention. Useful liposomal preparations include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes may mediate intracellular delivery of plasmid DNA and mRNA.

As another alternative to viral vector systems, the nucleic acid molecules of the present invention may be encapsulated, adsorbed to, or associated with, particulate carriers. Suitable particulate carriers include those derived from polymethyl methacrylate polymers, as well as PLG microparticles derived from poly(lactides) and poly(lactide-co-glycolides). Other particulate systems and polymers can also be used, for example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules.

In one embodiment, the vector may be a targeted vector, that is a vector whose ability to infect or transfect or transduce a cell or to be expressed in a host and/or target cell is restricted to certain cell types within the host subject, usually cells having a common or similar phenotype.

Preferably, a vector of the invention encodes an ahpC, a gsd, a p12 and an mpa polypeptide. As explained above, these four polypeptides may be expressed together as a single fusion protein molecule, or may be expressed in two or more separate polypeptides, each comprising one or more of the individual components. The vector of the invention may thus comprise a single expression cassette, from which a single polypeptide sequence can be expressed. Alternatively, a vector of the invention may comprise two or more expression cassettes each capable of expressing a different polypeptide, such that the vector as a whole is capable of expressing all four required polypeptides. In one embodiment, a vector of the invention may express all four required polypeptides separately as separate polypeptide molecules. Where the polypeptides are expressed from more than one locus in the vector, or are expressed as multiple separate molecules, the expression of the multiple sequences is preferably coordinated such that all four polypeptides are expressed together. For example, the same or similar promoters may be used to control expression of the various components. Inducible promoters may be used so that expression of the various polypeptide components can be coordinated.

Cell lines

The invention also includes cells that have been modified to express a peptide of the invention. Such cells include transient, or preferably stable higher eukaryotic cell lines, such as mammalian cells or insect cells, lower eukaryotic cells, such as yeast or prokaryotic cells such as bacterial cells. Particular examples of cells which may be modified by insertion of vectors or expression cassettes encoding for a peptide of the invention include mammalian HEK293T, CHO, HeLa and COS cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of a polypeptide. Expression may be achieved in transformed oocytes. A suitable peptide may be expressed in cells of a transgenic non-human animal, preferably a mouse. A transgenic non-human animal expressing a peptide of the invention is included within the scope of the invention. A peptide of the invention may also be expressed in Xenopus laevis oocytes or melanophores.

Such cell lines of the invention may be cultured using routine methods to produce a polypeptide of the invention, or may be used therapeutically or prophylactically to deliver polypeptides of the invention to a subject. For example, cell lines capable of secreting a polypeptide of the invention may be administered to a subject. Alternatively, polynucleotides, expression cassettes or vectors of the invention may be administered to a cell from a subject *ex vivo* and the cell then returned to the body of the subject.

For example, methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known (e.g., dextran-mediated transfection, calcium phosphate precipitation, electroporation, and direct microinjection into nuclei).

Antibodies

The present invention also extends to antibodies (monoclonal or polyclonal) and their antigen-binding fragments (e.g. F(ab)2, Fab and Fv fragments i.e. fragments of the "variable" region of the antibody, which comprises the antigen binding site) directed to peptides as defined hereinbefore, i.e. which bind to epitopes present on the peptides and thus bind selectively and specifically to such peptides, and which may be used in the methods of the invention. For example, a polyclonal antibody may be produced which has a broad spectrum effect against a variety of epitopes on a polypeptide of the invention.

Pharmaceutical compositions

Formulation of a composition comprising a molecule of the invention, such as a polynucleotide, expression cassette, vector, polypeptide, cell or antibody as described above, can be carried out using standard pharmaceutical formulation chemistries and methodologies all of which are readily available to the reasonably skilled artisan. For example, compositions containing one or more molecules of the invention can be combined with one or more pharmaceutically acceptable excipients or vehicles. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances and the like, may be present in the excipient or vehicle. These excipients, vehicles and auxiliary substances are generally pharmaceutical agents that do not induce an immune response in the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, polyethyleneglycol, hyaluronic acid, glycerol and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients, vehicles and auxiliary substances is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

Such compositions may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable compositions may be prepared, packaged, or sold in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Compositions include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such compositions may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a composition for parenteral administration, the active ingredient is provided in dry (for e.g., a powder or granules) form for reconstitution with a suitable vehicle (e. g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono-or di-glycerides.

Other parentally-administrable compositions which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Certain facilitators of nucleic acid uptake and/or expression ("transfection facilitating agents") can also be included in the compositions, for example, facilitators such as bupivacaine, cardiotoxin and sucrose, and transfection facilitating vehicles such as liposomal or lipid preparations that are routinely used to deliver nucleic acid molecules. Anionic and neutral liposomes are widely available and well known for delivering nucleic acid molecules (see, e.g., Liposomes: A Practical Approach, (1990) RPC New Ed., IRL Press). Cationic lipid preparations are also well known vehicles for use in delivery of nucleic acid molecules. Suitable lipid preparations include DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), available under the tradename Lipofectin™, and DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio)propane), see, e.g., Felgner et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413-7416; Malone et al. (1989) Proc. Natl. Acad. Sci. USA 86:6077-6081; US Patent Nos 5,283,185 and 5,527,928, and International Publication Nos WO

90/11092, WO 91/15501 and WO 95/26356. These cationic lipids may preferably be used in association with a neutral lipid, for example DOPE (dioleyl phosphatidylethanolamine). Still further transfection-facilitating compositions that can be added to the above lipid or liposome preparations include spermine derivatives (see, e.g., International Publication No. WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine S and cationic bile salts (see, e.g., International Publication No. WO 93/19768).

Alternatively, the nucleic acid molecules of the present invention may be encapsulated, adsorbed to, or associated with, particulate carriers. Suitable particulate carriers include those derived from polymethyl methacrylate polymers, as well as PLG microparticles derived from poly(lactides) and poly(lactide-co-glycolides). See, e.g., Jeffery et al. (1993) Pharm. Res. 10:362-368. Other particulate systems and polymers can also be used, for example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules.

The formulated compositions will include an amount of the molecule (e.g. vector) of interest which is sufficient to mount an immunological response. An appropriate effective amount can be readily determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials. The compositions may contain from about 0.1% to about 99.9% of the vector and can be administered directly to the subject or, alternatively, delivered *ex vivo*, to cells derived from the subject, using methods known to those skilled in the art.

Therapeutic methods

The present invention relates to immunogenic molecules which are intended to direct an immune response against MAP. The compositions of the invention can thus be used in the treatment or prevention of infection by MAP, or in the treatment or prevention of any disease, condition or symptom which is associated with MAP infection, that is any disease condition or symptom which is a direct or indirect result of MAP infection, or which results from a disease or condition to which the presence of MAP contributes. MAP is known to be linked to numerous specific medical conditions, such as chronic inflammation of the intestine, including inflammatory bowel disease and as well as Irritable Bowel Syndrome. For example, MAP infection can cause chronic enteritis, such as Johne's disease (paratuberculosis) in livestock and

Crohn's disease and Irritable Bowel Syndrome in humans. The compositions of the invention may therefore be used in the prevention or treatment of any of these specific conditions.

Accordingly, the present invention relates to a polypeptide, polynucleotide, expression cassette, vector, cell, antibody or composition of the invention for use in a method of therapy, in particular in a method or treating or preventing a disease, disorder or symptoms associated with or caused by a MAP infection. These molecules of the invention may thus also be used in the manufacture of a medicament for treating or preventing such a disease, disorder or condition. In particular, the molecules of the invention are proposed for the treatment or prevention of a chronic inflammation of the intestine, preferably in a mammal such as a human, cow, sheep or goat. The invention thus also provides a method of treating or preventing any such disease, disorder or symptom comprising administering to a subject in need thereof a polypeptide, polynucleotide, expression cassette, vector, cell, antibody or composition of the invention.

The present invention is broadly applicable to vaccination methods and is relevant to the development of prophylactic and/or therapeutic vaccines (including immunotherapeutic vaccines). It is to be appreciated that all references herein to treatment include curative, palliative and prophylactic treatment.

According to the present invention, a polynucleotide, vector, polypeptide or other molecule of the invention may be employed alone as part of a composition, such as but not limited to a pharmaceutical composition or a vaccine composition or an immunotherapeutic composition to prevent and/or treat a condition associated with MAP infection. The administration of the composition may be for either "prophylactic" or "therapeutic" purpose. As used herein, the term "therapeutic" or "treatment" includes any of following: the prevention of infection or reinfection; the reduction or elimination of symptoms; and the reduction or complete elimination of a pathogen. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

Prophylaxis or therapy includes but is not limited to eliciting an effective immune response to a polypeptide of the invention and/or alleviating, reducing, curing or at least partially arresting symptoms and/or complications resulting from or associated with a MAP infection. When provided prophylactically, the composition of the present invention is typically provided in advance of any symptom. The prophylactic administration of the composition of the present invention is to prevent or ameliorate any subsequent infection or disease. When provided therapeutically, the composition of the present invention is typically provided at or shortly after the onset of a symptom of infection or disease. Thus the composition of the present invention may be provided either prior to the anticipated exposure to MAP or onset of the associated disease state or after the initiation of an infection or disease.

Subject to be treated

The present invention relates in particular to the treatment or prevention of diseases or other conditions which are associated with infection by MAP. These treatments may be used on any animal which is susceptible to infection by MAP.

The subject to be treated may be any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The terms do not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The methods described herein are intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly. If a mammal, the subject will preferably be a human, but may also be a domestic livestock, laboratory subject or pet animal.

The subject to be treated may thus be any vertebrate that is susceptible to infection by MAP. Numerous animals have been shown in the art to be capable of such infection, including livestock such as cattle, goat and sheep, primates such as macaques and humans, other mammals including alpaca, antelope, ass, elk, horses, deer, dogs, gerbils and rabbits, and birds including the chicken. The compositions of the present invention may thus be used in the treatment of any such species.

Combined therapy

In one instance, a molecule of the invention may be used in combination with another molecule, such as another polynucleotide, vector or polypeptide, preferably another therapeutic agent. The therapeutic agent may be, for example an agent which has activity against MAP, or an agent used in the treatment of a condition which is associated with MAP infection. The molecule of the invention is preferably administered in an amount which is sufficient to augment the anti-MAP effects of the other therapeutic agent or vice versa. Numerous other agents may be used in the treatment of MAP or conditions which are associated with MAP infection. These include the rifamycins such as rifabutin and rifaximin, clarithromycin and other macrolides. Various anti tuberculosis drugs may also be used.

The other therapeutic agent may be an agent which potentiates the effects of the molecule of the invention. For example, the other agent may be an immunomodulatory molecule or an adjuvant which enhances the immune response to the polypeptide of the invention. Alternatively, the other molecule may increase the susceptibility of MAP present in the subject to attack, such as attack from the immune system.

In one embodiment, therefore, a molecule of the invention is used for therapy in combination with one or more other therapeutic agents.

The two molecules may be administered separately, simultaneously or sequentially. The two may be administered in the same or different compositions. Accordingly, in a method of the invention, the subject may also be treated with a further therapeutic agent.

A composition may therefore be formulated which comprises a molecule of the invention and also one or more other therapeutic molecules. For example, a vector of the invention may be formulated with another vector which encodes one or more other antigens or therapeutic molecules. A vector of the invention may alternatively be formulated with one or more therapeutic proteins.

A composition of the invention may alternatively by used simultaneously, sequentially or separately with one or more other therapeutic conditions as part of a combined treatment. Thus the invention also provides the use of a molecule, such as a polypeptide, polynucleotide, vector or host cell of the invention, in the manufacture of one or more medicament(s) for the treatment or prevention of MAP infection or a disease, condition or symptom associated with MAP infection as described herein.

Delivery methods

Once formulated the compositions can be delivered to a subject *in vivo* using a variety of known routes and techniques. For example, a composition can be provided as an injectable solution, suspension or emulsion and administered via parenteral, subcutaneous, epidermal, intradermal, intramuscular, intraarterial, intraperitoneal, intravenous injection using a conventional needle and syringe, or using a liquid jet injection system. Compositions can also be administered topically to skin or mucosal tissue, such as nasally, intratracheally, intestinal, rectally or vaginally, or provided as a finely divided spray suitable for respiratory or pulmonary administration. Other modes of administration include oral administration, suppositories, and active or passive transdermal delivery techniques. Particularly in relation to the present invention, compositions may be administered directly to the gastrointestinal tract.

Alternatively, the compositions can be administered *ex vivo*, for example delivery and reimplantation of transformed cells into a subject are known (e.g., dextran-mediated transfection, calcium phosphate precipitation, electroporation, and direct microinjection into nuclei).

Delivery regimes

The compositions are administered to a subject in an amount that is compatible with the dosage formulation and that will be prophylactically and/or therapeutically effective. An appropriate effective amount will fall in a relatively broad range but can be readily determined by one of skill in the art by routine trials. The "Physicians Desk Reference" and "Goodman and Gilman's The Pharmacological Basis of Therapeutics" are useful for the purpose of determining the amount needed.

As used herein, the term "prophylactically or therapeutically effective dose" means a dose in an amount sufficient to elicit an immune response to one or more epitopes of a polypeptide of the invention and/or to alleviate, reduce, cure or at least partially arrest symptoms and/or complications from a disease, such as an inflammatory bowel disorder, which is associated with a MAP infection.

Prophylaxis or therapy can be accomplished by a single direct administration at a single time point or by multiple administrations, optionally at multiple time points. Administration can also be delivered to a single or to multiple sites. Those skilled in the art can adjust the dosage and concentration to suit the particular route of delivery. In one embodiment, a single dose is administered on a single occasion. In an alternative embodiment, a number of doses are administered to a subject on the same occasion but, for example, at different sites. In a further embodiment, multiple doses are administered on multiple occasions. Such multiple doses may be administered in batches, i.e. with multiple administrations at different sites on the same occasion, or may be administered individually, with one administration on each of multiple occasions (optionally at multiple sites). Any combination of such administration regimes may be used.

In one embodiment, different compositions of the invention may be administered at different sites or on different occasions as part of the same treatment regime. It is known that improved immune responses may be generated to an antigen by varying the vectors used to deliver the antigen. There is evidence that in some instances antibody and/or cellular immune responses may be improved by using two different vectors administered sequentially as a "prime" and a "boost".

For example, the same polynucleotide of the invention may be administered as a "prime" in one composition, and then subsequently administered as a "boost" in a different composition. The two vaccine compositions may differ in the choice of vector comprising the polynucleotide. For example, two or more of different vectors each selected from plasmid vectors, poxvirus vectors, adenovirus vectors or other vectors as described herein may be administered sequentially.

In one embodiment, a "prime" is effected by administering a polynucleotide of the invention, such as the Havilah polynucleotide of SEQ ID NO: 23, in a plasmid vector such as pSG2. A "boost" is then effected at a later time using a polynucleotide of the invention, such as the Havilah polynucleotide of SEQ ID NO: 23 in a poxvirus vector such as MVA.

In an alternative embodiment a "prime" is effected by administering a polynucleotide of the invention, such as the Havilah polynucleotide of SEQ ID NO: 23, in an adenovirus vector such as Ad5. A "boost" is then effected at a later time using a polynucleotide of the invention, such as the Havilah polynucleotide of SEQ ID NO: 23 in a poxvirus vector such as MVA.

In such a prime-boost protocol, one or more administrations of the prime and/or the boost may be performed. For example, the prime and/or boost step may be achieved using a single administration or using two or more administrations at different sites and/or on different occasions. In one embodiment, two administrations on different occasions are given for the prime step and a single administration on a later occasion is given for the boost step.

Different administrations may be performed on the same occasion, on the same day, one, two, three, four, five or six days apart, one, two, three, four or more weeks apart. Preferably, administrations are 1 to 5 weeks apart, more preferably 2 to 4 weeks apart, such as 2 weeks, 3 weeks or 4 weeks apart. The schedule and timing of such multiple administrations can be optimised for a particular composition or compositions by one of skill in the art by routine trials.

Dosages for administration will depend upon a number of factors including the nature of the composition, the route of administration and the schedule and timing of the administration regime. Suitable doses of a molecule of the invention may be in the order of up to 15μg, up to 20μg, up to 25μg, up to 30μg, up to 50μg, up to 100μg, up to 500 μg or more per administration. For some molecules of the invention, such as plasmids, the dose used may be higher, for example, up to 1 mg, up to 2 mg, up to 3 mg, up to 4 mg, up to 5 mg or higher. Such doses may be provided in a liquid formulation, at a concentration suitable to allow an appropriate volume for administration by the selected route. In the case of a viral vector, a dose of about 10^6, 10^7, 10^8, 10^9, 10^{10} or more pfu may be given per administration. For example, a dose of 10^9 pfu or 25μg of a vector of the invention may be administered in a 50μl dose at multiple sites and/or on multiple occasions.

Kits

The invention also relates to a combination of components described herein suitable for use in a treatment of the invention which are packaged in the form of a kit in a container. Such kits may comprise a series of components to allow for a treatment of the invention. For example, a kit may comprise two or more different vectors of the invention, or one or more vectors of the invention and one or more additional therapeutic agents suitable for simultaneous administration, or for sequential or separate administration such as using a prime and boost protocol. The kit may optionally contain other suitable reagent(s), control(s) or instructions and the like.

Examples

1. Production of Havilah (HAV) construct and recombinant vectors expressing HAV.

A targeted bioinformatic analysis of the MAP genome was carried out and two secreted and two membrane bound components each related to the pathogenic phenotype were selected. These are AhpC, gsd, p12 and mpa. Three of these 4 components are 'seen' by antibody in sera from Crohn's disease patients as well as by sera from MAP infected C57/BL6 mice. Detailed epitope scans using the available databases revealed multiple predicted human class I and class II epitopes in the selected vaccine components. The peptides comprising these epitopes, when matched against the available human genome sequence, did not reveal any potential cross reacting antigens as potential targets for autoimmunity.

A construct consisting of a fusion of these four antigens was assembled from 40mer oligonucleotide precursors synthesized with optimal mammalian codon usage. Functional domains including potential cross-reacting human epitopes, lipid acylation sites and hydrophobic transmembrane regions were excluded. A monoclonal antibody recognition peptide was added to the C-terminus and a short human ubiquitin leader sequence to the N-terminus. This construct is referred to herein as Havilah and has the nucleic acid sequence given in SEQ ID NO: 23.

The Havilah (HAV) construct was cloned into the pSG2 expression vector and inserted by homologous recombination into the Modified Vaccinia Ankara (MVA) vector pMVA-GFP2 which carried a fluorescent marker. pMVA-GFP2 was used to transform MVA carrying a red marker at the target site for insertion, so that successful recombinants changed from red to green enabling them to be isolated using a fluorescence activated cell sorter. HAV was also inserted into the vector comprising replication defective human adenovirus 5. The rec.pSG2.HAV plasmid, rec.MVA.HAV, and rec.Ad5.hav were all shown to express the predicted 95 kDa

HAV encoded polyprotein. The successful rec.pSG2.HAV, rec.MVA.HAV and rec.Ad5.HAV and corresponding control vectors were prepared in bulk, purified and stored ready for testing *in vivo*. *E.coli* were transformed with the individual components AhpC and mpa representing the upstream and downstream ends of HAV and the His-tagged recombinant proteins were purified. Libraries of synthetic 15 residue peptides spanning the entire amino acid sequences of Havilah were obtained (Figure 4). The recombinant proteins and pools of the synthetic peptides were used to develop the ELISPOT and ELISA assays required to monitor the immune responses to the vaccine.

2. Safety and immunogenicity of vaccination using pSG2.HAV then MVA.HAV.

Two groups of 6 naïve 5 week old C57/BL6 female mice were isolator maintained. Their physical condition was monitored daily and body weights recorded twice weekly and at the end of the study. After an initial settle-in period of 7 days control Group 1 mice were prime vaccinated with 25μg of pSG2 expression plasmid in 50μl sterile buffered saline i.m. into each thigh. The experimental group received the same vaccination using recombinant pSG2.Hav plasmid expressing the Havilah construct. Ten days later Group 1 mice were boost vaccinated i.v. with 10^6 pfu Modified Vaccinia Ankara.GFP (MVA) vector alone. Group 2 mice received the same dose i.v. of recombinant MVA.Hav expressing the Havilah construct. At the end of the study 10 days later the mice were killed using a humane procedure. Spleen weights were recorded and spleen cells were obtained.

Stimulation of splenocytes and ELISPOT assay.

Spleen cells were harvested into 5 ml of RPMI (Sigma, UK) supplemented with 2mM glutamine, 1x penicillin-streptomycin (from a 100x stock, Life Technologies, UK) and 10% FCS (Life Technologies, UK). Cells were strained using 70μM cell strainer (BD biosciences) and pelleted by centrifugation 200g for 5 minutes at 4^0C. Erythrocytes were lysed using 1 ml of Red Cell Lysis Buffer (Sigma, UK) for 1m at room temperature and neutralised with 14 ml of RPMI. Cells were washed with RPMI twice by centrifugation as described and resuspended in 2ml of RPMI with supplements. 50μl of each pool of previously prepared recombinant AhpC or MPA antigen diluted in RPMI and supplements were added to the wells of 96 well PVDF membrane filter plates (cat # S2EM04M99, Millipore, UK), which were previously coated with capture antibody. 50 μl of splenocytes adjusted to a concentration of 2.5 x 10^7 cells/ml were added to the wells containing antigen and incubated. Final concentration of antigens in each well was 2.5μg/ml of recombinant protein. The following materials were obtained from BD Biosciences Pharmingen, UK. BD™ ELISPOT Horseradish peroxidase, BD™ AEC substrate set, and BD™ mouse gamma interferon cytokine ELISPOT pair consisting of a capture and detection antibody. The ELISPOT procedure followed was that described in BD Pharmingen Technical data sheet TDS 03/24/03. Spots were enumerated manually. Statistical analysis was performed using the Mann-Whitney (two tailed) test.

Results.

No adverse effects were seen in either control Group 1 mice or the experimental Group 2 mice during the course of the study or at autopsy. No significant difference was found in spleen weights between the two Groups. Mean ELISPOT responses (Figure 3 A) by spleen cells to purified rec.AhpC antigen were 83.3 in sham vaccinated Group 1 and 903.8 in the test vaccinated Group 2 ($p < 0.015$). Mean ELISPOT responses by spleen cells to purified rec.MPA antigen were 91.0 in sham vaccinated Group 1 and 900.7 in the test vaccinated Group 2 ($p < 0.004$) (Figure 3 A). Highly significant ELISPOT responses were also seen with peptide groups B and F compared with vector-only controls (Figure 3 A). In a further experiment (Figure 3 B) ELISPOT responses were determined for the individual peptides within pool F. The entire response is seen to be due to prominent recognition of peptide 9.1. This has the sequence GFAEINPIA (Figure 4) and comprises a strong T cell epitope corresponding to the 5^{th} extracellular loop in mpa (Figure 5A) and within Havilah Seq ID. No 24 residues 761-769 and Figure 1.

Conclusion.

Prime boost vaccination of female C57/BL6 mice using plasmid and MVA vectors expressing Havilah are highly immunogenic resulting in significant populations of antigen specific spleen cells against both AhpC at the amino terminus, and MPA at the carboxy terminus and with synthetic peptide epitopes of the Havilah polyprotein. The high level of antigen specific immunity to Havilah polyprotein and peptide epitopes following vaccination as described is not associated with any adverse effect.

3. Safety and efficacy of therapeutic and protective vaccination against a slow growing laboratory strain of MAP using pSG2.HAV then MVA.HAV.

A first study was carried out to test the Havilah vaccine as a treatment for pre-existing *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection as occurs in Johne's disease in animals and Crohn's disease and Irritable Bowel Syndrome in humans. Four groups of 8 naïve 4-6 week old C57/BL6 female mice were isolator maintained. Their physical condition was monitored daily and body weights recorded twice weekly and at the end of the study. After an initial settle-in period of 7 days all 32 mice were infected with 4 x 10^7 of a slow growing attenuated laboratory strain of bovine MAP i.p. in 200 µl sterile saline. Four weeks later control Group 1 mice were given 25µg of pSG2 expression plasmid in 50µl sterile endotoxin free TE buffer i.m. into each thigh. Experimental group 2 mice were prime vaccinated with 25µg of rec.pSG2.Hav in 50µl sterile endotoxin free TE buffer i.m. into each thigh. Control Group 3 mice received 50µl of sterile endotoxin free TE buffer i.m. into each thigh. This priming procedure was repeated after 1 week. Nine days later control Group 1 mice were given 5 x 10^6 pfu Modified Vaccinia Ankara.GFP (MVA) vector alone i.v. into the tail vein in 100 µl sterile endotoxin free TE buffer. Experimental Group 2 mice were boost vaccinated with the same dose i.v. of recombinant MVA.Hav expressing the Havilah construct. Control Group 3 mice were given 100 µl sterile endotoxin free TE buffer i.v. into the tail vein. Pairs of naïve mice from Group 4 were autopsied at intervals throughout the study to monitor the progress of the MAP infection. The study was terminated 26 weeks after the original MAP infection. The infective load of MAP organisms was measured in the spleens and livers of all the mice using a sensitive and specific quantitative real-time IS900 PCR.

A second study was carried out to test the ability of the Havilah vaccine to confer some protection against subsequent MAP infection. Three groups of 8 naïve 4-6 week old C57/BL6 female mice were isolator maintained. Their physical condition was monitored daily and body weights recorded twice weekly and at the end of the study. After an initial settle-in period of 7 days control Group 1 mice were given 25µg of pSG2 expression plasmid in 50μl sterile endotoxin free TE buffer i.m. into each thigh. Experimental group 2 mice were prime vaccinated with 25μg of rec.pSG2.Hav in 50μl sterile endotoxin free TE buffer i.m. into each thigh. Control Group 3 mice received 50μl of sterile endotoxin free TE buffer i.m. into each thigh. This priming procedure was repeated after 1 week. Ten days later control Group 1 mice were given 5×10^6 pfu Modified Vaccinia Ankara.GFP (MVA) vector alone i.v. into the tail vein in100 μl sterile endotoxin free TE buffer. Experimental Group 2 mice were boost vaccinated with the same dose i.v. of recombinant MVA.Hav expressing the Havilah construct. Control Group 3 mice were given 100 μl sterile endotoxin free TE buffer i.v. into the tail vein. Eight days later all mice were infected with 4×10^7 MAP bovine strain K10 i.p. in 200 μl sterile saline. The study was terminated 24½ weeks later and the infective load of MAP organisms was measured in the spleens and livers of all the mice using quantitative real-time IS900 PCR as before.

Results.

No adverse effects of the vaccine were seen in any of the Havilah vaccinated or control mice throughout the study or at autopsy. In the therapeutic vaccination study the numbers of MAP organisms in the spleens of the mice in control Groups 1 and 3 were generally in the range 10 to 10,000 per gram of tissue. In the vaccine treated Group 2 no MAP organisms could be detected at all in the spleens of 6 of the 8 mice (Figure 5B). In the other 2 animals in this group the number of MAP was around the lower limit of detection of the qRT-PCR in the range 1-10 per gram of tissue $p=0.003$ for Group 2 versus controls. The infective load of MAP in the liver of Havilah vaccine treated mice in Group 2 was also significantly reduced $p=0.019$. In the study of protective vaccination the efficacy of the vaccine was not as dramatic as therapeutic vaccination but the infective load of MAP in the liver was significantly lower than control mice $p=0.0074$.

Conclusions.

Therapeutic vaccination against MAP using plasmid and MVA vectors expressing the Havilah construct results in a highly significant attenuation of the MAP infection. Vaccination using these vectors expressing the Havilah construct has a smaller but significant protective effect against subsequent MAP infection. The preexistence of MAP infection appears to confer advantage by priming the response to the vaccine used in its therapeutic role. Vaccination in the presence of MAP infection using vectors containing Havilah is safe and is not associated with any adverse effect.

4. Safety and immunogenicity of vaccination using Ad5.HAV then MVA.HAV

A further study was carried out in 3 groups of six naïve 4-6 week old female C57/BL6 mice following the format previously described. Group 1 received 10^8 pfu of Ad5 vector in 50µl buffer intradermally (i.d) into the pinna of the ear; group 2 received the same dose of Ad5.HAV i.d and group 3 received 50µl buffered saline only. Two weeks later the groups 1-3 were vaccinated with 10^8 pfu MVA vector only, 10^8 pfu MVA.HAV or buffer i.d respectively. The animals were killed at 4 weeks noting clinical and autopsy conditions and body weights. Spleen cells and serum were obtained to quantify immunological responses to vaccine antigens.

Results.

No adverse effects due to the vaccination were seen in any of the animals. Significant recognition of rec.AhpC, rec.gsd and rec.mpa in ELISPOT assays as well as of pooled peptide antigens especially peptide 9.1 GFAEINPIA and pool J compared with controls, again occurred. There was also significant recognition of the recombinant antigens AhpC and mpa by antibody in the vaccinated group compared with control groups.

To test the reproducibility of these findings the study was repeated using 3 similar groups of eight mice and a dose of 2 x 10^7 pfu of either viral vector alone or 2 x 10^7 pfu Ad5.HAV followed by 2 x 10^7 pfu MVA.HAV i.d in 50µl into the pinna of the ear. The animals were killed after 4 weeks.

There was no evidence of adverse effects in any of these animals. Immune responses shown in Figure 6 A and B were the same as seen previously. There was again strong ELISPOT recognition of peptide 9.1 and significant responses to recombinant antigens and peptide pool J compared with vector only controls. By contrast there was no recognition of peptide 9.1 or any of the peptide pools by antibody and substantial antibody recognition of recombinant antigens AhpC and mpa.

Conclusions.

Vaccination of uninfected female C57/BL6 mice using Ad5.HAV followed by MVA.HAV expressing Havilah is highly immunogenic resulting in significant populations of antigen specific spleen cells recognizing both recombinant antigens and synthetic peptide epitope pools. Of particular note is the repeated demonstration of the strong specific T cell epitope GFAEINPIA comprising the 5th extracellular loop of mpa recognized in vaccinated mice. Antigen specific immunity to Havilah polyprotein following vaccination is reproducibly not associated with any adverse effect.

5. Safety and efficacy of therapeutic and protective vaccination against a virulent recent disease isolate of MAP using Ad5.HAV then MVA.HAV.

Two further studies were carried out each using 2 or 3 groups of eight 4-6 week old female C57/BL6 mice to determine the safety and efficacy of vaccination against a fast growing virulent recent disease isolate of bovine MAP using 2×10^7 pfu Ad5.HAV then 2×10^7 pfu MVA.HAV compared with control groups receiving vector-only or buffered saline alone, following the format previously described. In a high dose study the animals received 10^7 MAP i.p and in a low dose study 10^5 MAP i.p. The study was terminated after 8 weeks of MAP infection and the infective load of MAP organisms was measured in the spleens and livers of all the mice using a sensitive and specific quantitative real-time IS*900* PCR.

Results.

None of the animals demonstrated any adverse effects of vaccination. The numbers of MAP organisms in the spleen tissues of the mice vaccinated therapeutically or prophylactically was significantly reduced compared to control groups both in the low dose and high dose studies (Figure 7). With the exception of the high dose study the numbers of MAP organisms in the liver tissues of the mice vaccinated therapeutically or prophylactically was also significantly reduced compared to control groups (Figure 8). Figure 9 shows that the strong T cell epitope GFAEINPIA in peptide 9.1 is again prominently recognised despitethe presence of virulent MAP infection. Peptide pool J is significantly recognised in both prophylactic and therapeutic vaccination. Peptide pool L is again significantly recognised when vaccination is given to animals which are already MAP infected consistent with the prior infection 'priming' the response to the vaccine used therapeutically.

Conclusions.

Vaccination using Havilah expressed in plasmid, Adenovirus and MVA pox virus vectors given by different immunisation routes in different doses demonstrates a reproducible and highly significant antigen-specific T cell immunogenicity in mice without adverse effect. Vaccination induced significant T cell recognition both of recombinant Havilah proteins and of synthetic peptide epitopes within the Havilah sequence, especially the strong T cell epitope GRAEINPIA corresponding to the 5^{th} extracellular loop of the mpa moiety. Significant antibody responses to recombinant AhpC and mpa also occurred. Using different vector combinations in different doses and different routes of administration against different strains of MAP, vaccination using Havilah constructs repeatedly results in significant therapeutic attenuation of pre-existing MAP infection and protection against subsequent MAP infection. Furthermore, responses to therapeutic vaccination may be enhanced by the 'priming' effect of pre-existing MAP infection. Havilah may be used as a vaccine to confer protection against MAP infections and to treat MAP infections of animals and humans such as Johne's disease and Crohn's disease and Irritable Bowel syndrome as well as to potentiate the clinical response to treatment with anti-MAP drugs.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS

1. A polypeptide comprising an ahpC polypeptide sequence, a gsd polypeptide sequence, a p12 polypeptide sequence and an mpa polypeptide sequence, wherein said ahpC polypeptide comprises

(ai) the sequence of SEQ ID NO: 2,

(aii) a variant of SEQ ID NO: 2 having more than 70% amino acid sequence identity to SEQ ID NO: 2 across the full length of SEQ ID NO: 2 which comprises at least one amino acid sequence selected from the group consisting of amino acids 48 to 56 of SEQ ID NO: 4, amino acids 90 to 101 of SEQ ID NO: 4 and amino acids 161 to 169 of SEQ ID NO: 4, wherein said variant of SEQ ID NO: 2 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 2, or

(aiii) a fragment of SEQ ID NO: 2 which comprises at least one amino acid sequence selected from the group consisting of amino acids 48 to 56 of SEQ ID NO: 4, amino acids 90 to 101 of SEQ ID NO: 4 and amino acids 161 to 169 of SEQ ID NO: 4, wherein said fragment of SEQ ID NO: 2 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 2;

said gsd polypeptide comprises

(bi) the sequence of SEQ ID NO: 6,

(bii) a variant of SEQ ID NO: 6 having more than 70% amino acid sequence identity to SEQ ID NO: 6 across the full length of SEQ ID NO: 6, which comprises at least one amino acid sequence selected from the group consisting of amino acids 1 to 32 of SEQ ID NO: 8, amino acids 58 to 68 of SEQ ID NO: 8, amino acids 99 to 119 of SEQ ID NO: 8, amino acids 123 to 147 of SEQ ID NO: 8, amino acids 159 to 169 of SEQ ID NO: 8, amino acids 180 to 194 of SEQ ID NO: 8, amino acids 200 to 231 of SEQ ID NO: 8, amino acids 64 to 76 of SEQ ID NO: 8, amino acids 95 to 110 of SEQ ID NO: 8, amino acids 192 to 206 of SEQ ID NO: 8 and amino acids 223 to 240 of SEQ ID NO: 8, wherein said variant of SEQ ID NO: 6 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 6, or

(biii) or a fragment of SEQ ID NO: 6 which comprises at least one amino acid sequence selected from the group consisting of amino acids 1 to 32 of SEQ ID NO: 8, amino acids 58 to 68 of SEQ ID NO: 8, amino acids 99 to 119 of SEQ ID NO: 8, amino acids 123 to 147 of SEQ ID NO: 8, amino acids 159 to 169 of SEQ ID NO: 8, amino acids 180 to 194 of SEQ ID NO: 8, amino acids 200 to 231 of SEQ ID NO: 8, amino acids 64 to 76 of SEQ ID NO: 8, amino acids 95 to 110 of SEQ ID NO: 8, amino acids 192 to 206 of SEQ ID NO: 8 and amino acids 223 to 240 of SEQ ID NO: 8, wherein said fragment of SEQ ID NO: 6 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 6;

said p12 polypeptide comprises

(ci) the sequence of SEQ ID NO: 10,

(cii) a variant of SEQ ID NO: 10 having more than 70% amino acid sequence identity to SEQ ID NO: 10 across the full length of SEQ ID NO: 10 which comprises at least one amino acid sequence selected from the group consisting of amino acids 33 to 56 of SEQ ID NO: 12, amino acids 98 to 117 of SEQ ID NO: 12 and amino acids 3 to 10 of SEQ ID NO: 12, wherein said variant of SEQ ID NO: 10 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 10, or

(ciii) a fragment of SEQ ID NO: 10 which comprises at least one amino acid sequence selected from the group consisting of amino acids 33 to 56 of SEQ ID NO: 12, amino acids 98 to 117 of SEQ ID NO: 12 and amino acids 3 to 10 of SEQ ID NO: 12, wherein said fragment of SEQ ID NO: 10 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 10; and

said mpa polypeptide comprises

(di) the sequence of SEQ ID NO: 14,

(dii) a variant of SEQ ID NO: 14 having more than 70% amino acid sequence identity to SEQ ID NO: 14 across the full length of SEQ ID NO: 14 which comprises at least one amino acid sequence selected from the group consisting of amino acids 130 to 160 of SEQ ID NO: 16, amino acids 56 to 64 of SEQ ID NO: 16, amino acids 150 to 160 of SEQ ID NO: 16 and amino acids 177 to 185 of SEQ ID NO: 16, wherein said variant of SEQ ID NO: 14 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 14, or

(diii) a fragment of SEQ ID NO: 14 which comprises at least one amino acid sequence selected from the group consisting of amino acids 130 to 160 of SEQ ID NO: 16, amino acids 56 to 64 of SEQ ID NO: 16, amino acids 150 to 160 of SEQ ID NO: 16 and amino acids 177 to 185 of SEQ ID NO: 16, wherein said fragment of of SEQ ID NO: 14 retains the ability to stimulate an immune response against a polypeptide of SEQ ID NO: 14.

2. The polypeptide according to claim 1 wherein said ahpC polypeptide has the amino acid sequence given in SEQ ID NO: 4.

3. The polypeptide according to claim 1 or 2 wherein said gsd polypeptide has the amino acid sequence given in SEQ ID NO: 8.

4. The polypeptide according to any one of claims 1 to 3 wherein said p12 polypeptide has the amino acid sequence given in SEQ ID NO: 12.

5. The polypeptide according to any one of claims 1 to 4 wherein said mpa polypeptide has the amino acid sequence given in SEQ ID NO: 16.

6. The polypeptide according to any one of claims 1 to 4 wherein said mpa polypeptide comprises the amino acid sequence GFAEINPIA.

7. The polypeptide according to claim 1 which comprises the amino acid sequences of SEQ ID Nos: 4, 8, 12 and 16.

8. The polypeptide according to claim 1 which comprises the amino acid sequence given in SEQ ID NO: 24.

9. A polynucleotide which encodes a polypeptide according to any one of claims 1 to 8.

10. The polynucleotide according to claim 9 which comprises

(a) the ahpC polynucleotide of SEQ ID NO: 1 or a variant thereof having at least 70% identity with SEQ ID NO: 1 across the full length of SEQ ID NO: 1 or a fragment of SEQ ID NO: 1 which encodes at least one amino acid sequence selected from the group consisting of amino acids 48 to 56 of SEQ ID NO: 4, amino acids 90 to 101 of SEQ ID NO: 4

and amino acids 161 to 169 of SEQ ID NO: 4, wherein said ahpC polynucleotide, variant or fragment of SEQ ID NO: 1 encodes a polypeptide that is capable of stimulating an immune response against a polypeptide of SEQ ID NO: 2;

(b) the gsd polynucleotide of SEQ ID NO: 5 or a variant thereof having at least 70% identity with SEQ ID NO: 5 across the full length of SEQ ID NO: 5 or a fragment of SEQ ID NO: 5 which encodes at least one amino acid sequence selected from the group consisting of amino acids 1 to 32 of SEQ ID NO: 8, amino acids 58 to 68 of SEQ ID NO: 8, amino acids 99 to 119 of SEQ ID NO: 8, amino acids 123 to 147 of SEQ ID NO: 8, amino acids 159 to 169 of SEQ ID NO: 8, amino acids 180 to 194 of SEQ ID NO: 8, amino acids 200 to 231 of SEQ ID NO: 8, amino acids 64 to 76 of SEQ ID NO: 8, amino acids 95 to 110 of SEQ ID NO: 8, amino acids 192 to 206 of SEQ ID NO: 8 and amino acids 223 to 240 of SEQ ID NO: 8, wherein said gsd polynucleotide, variant or fragment of SEQ ID NO: 5 encodes a polypeptide that is capable of stimulating an immune response against a polypeptide of SEQ ID NO: 6;

(c) the p12 polynucleotide of SEQ ID NO: 9 or a variant thereof having at least 70% identity with SEQ ID NO: 9 across the full length of SEQ ID NO: 9 or a fragment of at least 24 nucleotides of SEQ ID NO: 9 which encodes at least one amino acid sequence selected from the group consisting of amino acids 33 to 56 of SEQ ID NO: 12, amino acids 98 to 117 of SEQ ID NO: 12 and amino acids 3 to 10 of SEQ ID NO: 12, wherein said p12 polynucleotide, variant or fragment of SEQ ID NO: 9 encodes a polypeptide that is capable of stimulating an immune response against a polypeptide of SEQ ID NO: 10; and

(d) the mpa polynucleotide of SEQ ID NO: 13 or a variant thereof having at least 70% identity with SEQ ID NO: 13 across the full length of SEQ ID NO: 13 or a fragment of SEQ ID NO: 13 which encodes at least one amino acid sequence selected from the group consisting of amino acids 130 to 160 of SEQ ID NO: 16, amino acids 56 to 64 of SEQ ID NO: 16, amino acids 150 to 160 of SEQ ID NO: 16 and amino acids 177 to 185 of SEQ ID NO: 16, wherein said mpa polynucleotide, variant or fragment of SEQ ID NO: 13 encodes a polypeptide that is capable of stimulating an immune response against a polypeptide of SEQ ID NO: 14.

11. The polynucleotide according to claim 10 wherein said ahpC polynucleotide has the sequence given in SEQ ID NO: 3.

12. The polynucleotide according to claim 10 or 11 wherein said gsd polynucleotide has the sequence given in SEQ ID NO: 7.

13. The polynucleotide according to any one of claims 10 to 12 wherein said p12 polynucleotide has the sequence given in SEQ ID NO: 11.

14. The polynucleotide according to any one of claims 10 to 13 wherein said mpa polynucleotide has the sequence given in SEQ ID NO: 15.

15. The polynucleotide according to claim 10 which comprises the nucleic acid sequences of SEQ ID Nos: 3, 7, 11 and 15.

16. The polynucleotide according to claim 10 which comprises the nucleic acid sequence given in SEQ ID NO: 23.

17. A vector comprising a polynucleotide according to any one of claims 9 to 16.

18. A vector expressing an ahpC polypeptide, a gsd polypeptide, a p12 polypeptide and an mpa polypeptide as defined in any one of claims 1 to 8.

19. The vector according to claim 17 or 18 which is a poxvirus vector, an adenovirus vector or a plasmid.

20. A host cell comprising a polypeptide according to any one of claims 1 to 8, a polynucleotide according to any one of claims 9 to 16 or a vector according to any one of claims 17 to 19.

21. A host cell which expresses a polypeptide according to any one of claims 1 to 8.

22. Use of a polypeptide according to any one of claims 1 to 8, a polynucleotide according to any one of claims 9 to 16, a vector according to any one of claims 17 to 19 or a host cell according to claim 20 or 21 in the manufacture of a medicament for treating or preventing *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection.

23. The use according to claim 22 wherein said medicament is for treating or preventing chronic inflammation of the intestine, inflammatory bowel disease, Irritable Bowel Syndrome, chronic enteritis, Johne's disease or Crohn's disease.

24. The use according to claim 22 or 23 wherein said medicament is for use concurrently with another therapeutic agent which has activity against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection or a further therapeutic agent used in the treatment of a condition which is associated with MAP infection.

25. Use of a polypeptide according to any one of claims 1 to 8, a polynucleotide according to any one of claims 9 to 16, a vector according to any one of claims 17 to 19 or a host cell according to claim 20 or 21 for treating or preventing *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection.

26. The use according to claim 25 for treating or preventing chronic inflammation of the intestine, inflammatory bowel disease, Irritable Bowel Syndrome, chronic enteritis, Johne's disease or Crohn's disease.

27. The use according to claim 25 or 26 concurrently with another therapeutic agent which has activity against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection or a further therapeutic agent used in the treatment of a condition which is associated with MAP infection.

28. A polypeptide according to any one of claims 1 to 8, a polynucleotide according to any one of claims 9 to 16, a vector according to any one of claims 17 to 19 or a host cell

according to claim 20 or 21 for treating or preventing *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection.

29. The polypeptide, polynucleotide, vector or host cell according to claim 28 for treating or preventing chronic inflammation of the intestine, inflammatory bowel disease, Irritable Bowel Syndrome, chronic enteritis, Johne's disease or Crohn's disease.

30. The polypeptide, polynucleotide, vector or host cell according to claim 28 or 29 for use concurrently with another therapeutic agent which has activity against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection or a further therapeutic agent used in the treatment of a condition which is associated with MAP infection.

```
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                                     M  Q  I  F  V
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 K  L  P  L  L  T  I  G  D  Q  F  P  A  Y  E  L
CAGCTCTTATCGCTGGAGATCTGAGTAAGGTTGACGCCAAACAGCCCGGC   150
T  A  L  I  A  G  D  L  S  K  V  D  A  K  Q  P  G
GATTATTTCACTACCGTTACCAGTGAGGATCACGCAGGTAAATGGAGAGT   200
 D  Y  F  T  T  V  T  S  E  D  H  A  G  K  W  R  V
CGTCTTCTTCTGGCCTAAAGACTTCACCTTTGTGTGCCCTACTGAGATCG   250
 V  F  F  W  P  K  D  F  T  F  V  C  P  T  E  I
CAACATTCGGGAAGCTGAACGATGAGTTCGAAGATCGAGACGCACAGGTT   300
A  T  F  G  K  L  N  D  E  F  E  D  R  D  A  Q  V
TTGGGCGTGTCTATCGATTCCGAGTTCGTGCACTTCAACTGGAGAGCACA   350
 L  G  V  S  I  D  S  E  F  V  H  F  N  W  R  A  Q
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 H  E  D  L  K  N  L  P  F  P  M  L  S  D  I  K
GAGAACTGAGCTTGGCAACAGGTGTTCTGAATGCTGATGGCGTTGCTGAC   450
R  E  L  S  L  A  T  G  V  L  N  A  D  G  V  A  D
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 R  A  T  F  I  V  D  P  N  N  E  I  Q  F  V  S  V
TACTGCTGGTTCTGTCGGTAGAAACGTTGAAGAGGTCCTGAGAGTTCTCG   550
 T  A  G  S  V  G  R  N  V  E  E  V  L  R  V  L
ACGCACTTCAGAGTGATGAACTGTGTGCCTGCAATTGGCGGAAAGGAGAT   600
D  A  L  Q  S  D  E  L  C  A  C  N  W  R  K  G  D
CCTACTCTCAATGCCACAGAGCTGCTTAAAGCAAGTGCTCTCGGATCCAT   650
 P  T  L  N  A  T  E  L  L  K  A  S  A  L  G  S  I
TGTCGGACAGACCTATAGAGAGGTGGAAGTTGTCCTGGTCGATGGTGGAT   700
 V  G  Q  T  Y  R  E  V  E  V  V  L  V  D  G  G
CTACAGATAGGACTCTCGACATTGCCAACTCCTTTAGACCAGAGCTCGGT   750
S  T  D  R  T  L  D  I  A  N  S  F  R  P  E  L  G
TCAAGGCTCGTTGTTCATTCTGGACCAGATGATGGACCATACGACGCCAT   800
 S  R  L  V  V  H  S  G  P  D  D  G  P  Y  D  A  M
GAACAGAGGTGTTGGAGTTGCTACAGGAGAATGGGTCTTGTTCCTTGGAG   850
 N  R  G  V  G  V  A  T  G  E  W  V  L  F  L  G
CTGATGACACTCTGTACGAACCGACTACATTGGCTCAGGTTGCAGCATTT   900
A  D  D  T  L  Y  E  P  T  T  L  A  Q  V  A  A  F
TTGGGAGATCATGCAGCTTCTCACCTTGTGTACGGAGATGTGGTCATGAG   950
 L  G  D  H  A  A  S  H  L  V  Y  G  D  V  V  M  R
ATCCACCAAGTCCAGACATGCTGGACCATTCGATCTTGACAGACTCCTGT   1000
 S  T  K  S  R  H  A  G  P  F  D  L  D  R  L  L
TCGAGACCAACCTCTGTCATCAGAGCATCTTCTACAGACGGGAACTCTTC   1050
F  E  T  N  L  C  H  Q  S  I  F  Y  R  R  E  L  F
GACGGAATTGGACCTTACAACCTCAGGTACAGGGTTTGGGCAGACTGGGA   1100
 D  G  I  G  P  Y  N  L  R  Y  R  V  W  A  D  W  D
TTTCAACATCAGGTGCTTCTCGAACCCAGCTTTGATCACACGGTACATGG   1150
 F  N  I  R  C  F  S  N  P  A  L  I  T  R  Y  M
ATGTTGTGATCTCCGAGTACAACGATATGACCGGCTTCTCCATGAGACAG   1200
D  V  V  I  S  E  Y  N  D  M  T  G  F  S  M  R  Q
GGAACCGACAAAGAGTTCAGGAAGCGCTTGCCAATGTACTTCTGGGTTGC   1250
 G  T  D  K  E  F  R  K  R  L  P  M  Y  F  W  V  A
TGGATGGGAAACATGTCGGAGAATGCTTGCTTTCCTGAAGGACAAGGAGA   1300
 G  W  E  T  C  R  R  M  L  A  F  L  K  D  K  E
ACAGGAGACTTGCTCTCAGGACTAGACTCATCAGGGTCAAAGCAGTGTCC   1350
N  R  R  L  A  L  R  T  R  L  I  R  V  K  A  V  S
AAGGAAAGGAGTGCTGAACCTAGAATTCGGAGACATAGACATGCAGAGAT   1400
 K  E  R  S  A  E  P  R  I  R  R  H  R  H  A  E  I
CATCCTGAGCATGCCTGGATTTGGCGTTATCCTCGGAGCTGAATTTCTTG   1450
 I  L  S  M  P  G  F  G  V  I  L  G  A  E  F  L
CAGCAACAGGAGGTGATATGGCAGCTTTCGCATCAGCTGACAGATTGGCT   1500
A  A  T  G  G  D  M  A  A  F  A  S  A  D  R  L  A
GGAGTTGCAGGTTTGGCTCCAGTTCCAAGAGATTCAGGGAGAATCAGCGG   1550
 G  V  A  G  L  A  P  V  P  R  D  S  G  R  I  S  G
TAACCTCAAGAGACCTAGACGCTACGACAGAAGACTGCTTAGAGCCTGCT   1600
 N  L  K  R  P  R  R  Y  D  R  R  L  L  R  A  C
ATCTGAGTGCTTTGGTTAGCATTAGAACCGACCCCTCTAGTCGAACCTAC   1650
Y  L  S  A  L  V  S  I  R  T  D  P  S  S  R  T  Y
TACGATAGGAAGCGGACTGAAGGTAAGAGACATACCCAGGCAGTGTTGGC   1700
 Y  D  R  K  R  T  E  G  K  R  H  T  Q  A  V  L  A
ACTTGCTAGAAGACGGCTTAATGTTCTGTGGGCTATGCTGAGAGATCATG   1750
 L  A  R  R  R  L  N  V  L  W  A  M  L  R  D  H
CCGTGTACCATCCTGCTACCACAACAGCTGCTGCTAGACTTAAGCTTCGC   1800
A  V  Y  H  P  A  T  T  T  A  A  A  R  L  K  L  R
AGAGGTGAGAGACCTATGAGTCTTGGCCAGGTCTTTGATCCTAGAGCTAA   1850
 R  G  E  R  P  M  S  L  G  Q  V  F  D  P  R  A  N
TGCACTGCACTCTTTCCCTCTTACAGGACGCATGCCTTGGGCTCCATTTA   1900
 A  L  H  S  F  P  L  T  G  R  M  P  W  A  P  F
TCGTTAGTTCCTGGCTCAGAAACCCTCATCCAGCTCAGTACTTCACAGCC   1950
I  V  S  S  W  L  R  N  P  H  P  A  Q  Y  F  T  A
AGATGTCTCAGAATCCTTCCTGGTCTTTGGATTGGAGCACAGGGTGGTTC   2000
 R  C  L  R  I  L  P  G  L  W  I  G  A  Q  G  G  S
CGCAGCTAAGCTGTTGATGAGTGGTGCACCAATCGAATACGTCCTGAAAG   2050
 A  A  K  L  L  M  S  G  A  P  I  E  Y  V  L  K
ACTCAGCAGTGTGGATGTTCAAGTTCGACATTGGAGGAACACCAAGGGAT   2100
D  S  A  V  W  M  F  K  F  D  I  G  G  T  P  R  D
ATTCCTGTCGCTGGTATCTGGAATGGAAGTTTGTGGACCCCAGCATGGGG   2150
 I  P  V  A  G  I  W  N  G  S  L  W  T  P  A  W  G
AGGTATTCATGCTATCGCTTCCAACGCTTACCAGTTCCGAAATGTGATCC   2200
 G  I  H  A  I  A  S  N  A  Y  Q  F  R  N  V  I
CTGCAAGATGGTCTGTGAGTTCAGCCGTGTTGCCAAACTATAGACTTGTT   2250
P  A  R  W  S  V  S  S  A  V  L  P  N  Y  R  L  V
GCTGCTCTCCCCATGGCCTACCATAATCAGCGAATGAGGTTTCGGACAGA   2300
 A  A  L  P  M  A  Y  H  N  Q  R  M  R  F  R  T  D
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 L  S  Y  G  V  Y  G  F  A  E  I  N  P  I  A  L
TTGAGAAACCTGCCCTGTCTTGGAAATCCAGACTGAGACGGAAGAACTCT   2400
V  E  K  P  A  L  S  W  K  S  R  L  R  R  K  N  S
TCCATCGCTCTCGCAAACATGGAAGATGGTGGTAGTGTTGGAAGGAGTAA   2450
 S  I  A  L  A  N  M  E  D  G  G  S  V  G  R  S  N
CGACATCCCTGGGAGGAGGGCTAGATTTATTGGTGAGAAAGCCGAAGATC   2500
 D  I  P  G  R  R  A  R  F  I  G  E  K  A  E  D
CTCCTGCTCCATCTCCTAGACCCGCCTTGAGGATTCCAAACCCTCTTCTC   2550
P  P  A  P  S  P  R  P  A  L  R  I  P  N  P  L  L
GGTCTTGATTGAATATCTAGACAGTGACCCGGGATCGACTAGATCGATCA   2600
 G  L  D
```

Figure 1

A

PLLTIGDQFPAYELTALIAGDLSKVDAKQPGDYFTTVTSEDHAGKWR
VVFFWPKDFTFVCPTEIATFGKLNDEFEDRDAQVLGVSIDSE***FVHFN***
WRAQHEDLKNLPFPMLSDIKRELSLATGVLNADGVADRATFIVDPNN
EIQFVSVTAGSVGRNVEEV***LRVLDALQS***DELCACNWRKGDPTLNAT
ELLKASAL

B

GSIVGQTYREVEVVLVDGGSTDRTLDIANSFRPELGSRLVVHSGPDD
GPYDAMNRGVGVATGE***WVLFLGADDTLYE***PTTLAQVAAFLGDHAA
SH***LVYGDVVMRSTKSRHA***GPFDLDRLLFETNLCHQSIFYRRELFDGI
GPYNLRYRVWADWDFNIRCFSNPALITRYMDVVISEYNDMTGFSMR
QGTDKE***FRKRLPMYFWVAGWE***TCRRMLAFLKDKENRR***LALRTRLI***
RVKAVSKERSAEP

C

RI***RRHRHAEI***ILSMPGFGVILGAEFLAATGGDMAAFASADRLAGVAG
LAPVPRDSGRISGNLKRPRRYDRRLLRACYLSALVSIRTDPSSRTYY
DRKRTEGKRHTQAVLALARRRLNVLWAMLRDHAVYHPATTTAAARL

D

KLRRGERPMSLGQVFDPRANALHSFPLTGRMPWAPFIVSSWLRNP
HPAQYFTARC***LRILPGLWI***GAQGGSAAKLLMSGAPIEYVLKDSAVW
MFKFDIGGTPRDIPVAGIWNGSLWTPAWGGIHAIASNAYQFRNVIPA
RWSVSSAVLPN***YRLVAALPMAY***HNQRMRFRTDLSYGVYGFAEINPI
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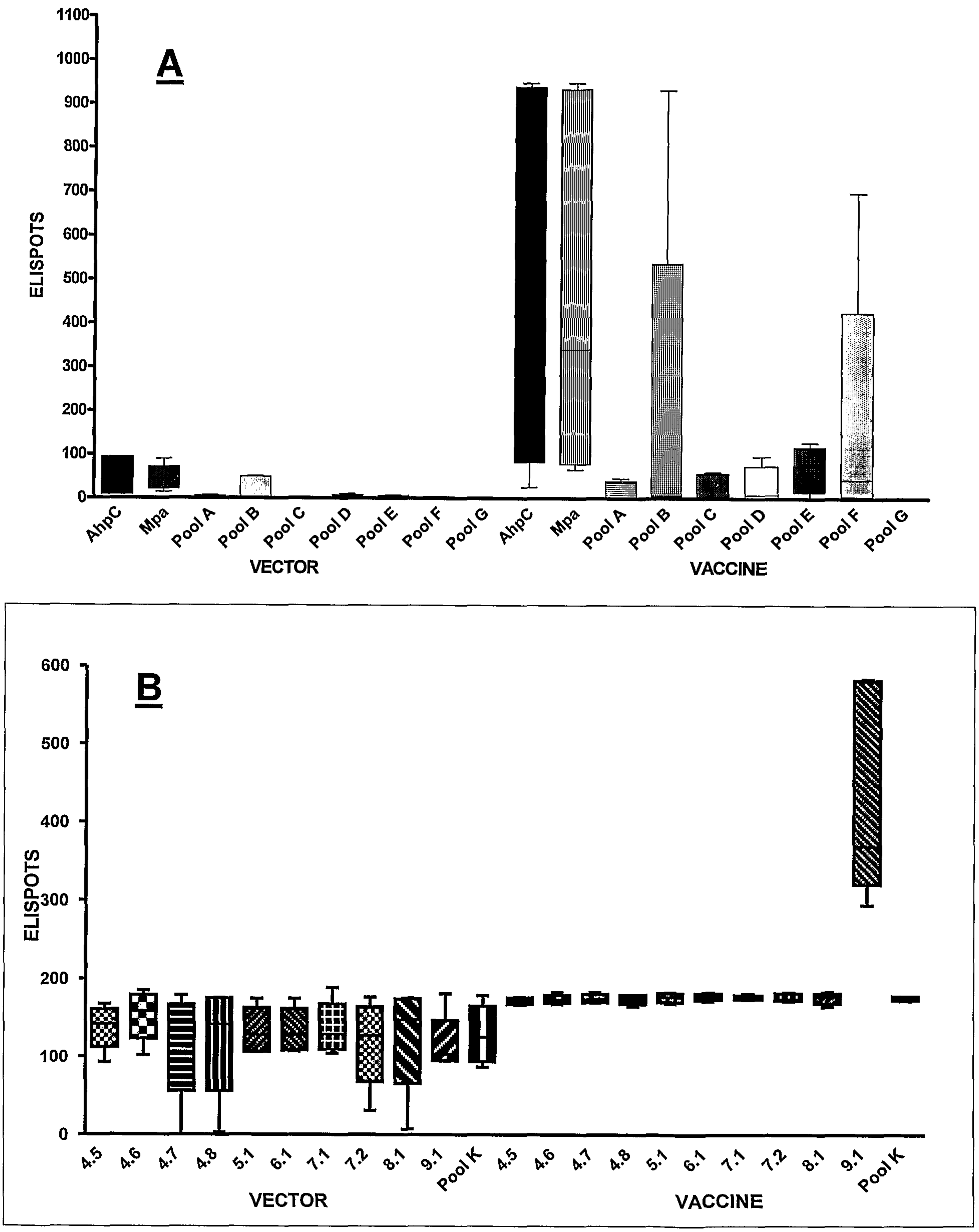
Figure 2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ELISPOTS
0
100
200
300
400
500
600
700
800
900
1000
1100
AhpC
Mpa
Pool A
Pool B
Pool C
Pool D
Pool E
Pool F
Pool G
AhpC
Mpa
Pool A
Pool B
Pool C
Pool D
Pool E
Pool F
Pool G
VECTOR
VACCINE
B
ELISPOTS
0
100
200
300
400
500
600
4.5
4.6
4.7
4.8
5.1
6.1
7.1
7.2
8.1
9.1
Pool K
4.5
4.6
4.7
4.8
5.1
6.1
7.1
7.2
8.1
9.1
Pool K
VECTOR
VACCINE

Figure 3

a

LPLLTIGDQFPAYEL
IGDQFPAYELTALIA
PAYELTALIAGDLSK
TALIAGDLSKVDAKQ
GDLSKVDAKQPGDYF
VDAKQPGDYFTTVTS
PGDYFTTVTSEDHAG
TTVTSEDHAGKWRVV
EDHAGKWRVVFFWPK
KWRVVFFWPKDFTFV

b

FFWPKDFTFVCPTEI
DFTFVCPTEIATFGK
CPTEIATFGKLNDEF
ATFGKLNDEFEDRDA
LNDEFEDRDAQVLGV
EDRDAQVLGVSIDSE
QVLGVSIDSEFVHFN
SIDSEFVHFNWRAQH
FVHFNWRAQHEDLKN
WRAQHEDLKNLPFPM

c

EDLKNLPFPMLSDIK
LPFPMLSDIKRELSL
LSDIKRELSLATGVL
RELSLATGVLNADGV
ATGVLNADGVADRAT
NADGVADRATFIVDP
ADRATFIVDPNNEIQ
FIVDPNNEIQFVSVT
NNEIQFVSVTAGSVG
FVSVTAGSVGRNVEE

d

AGSVGRNVEEVLRVL
RNVEEVLRVLDALQS
VLRVLDALQSDELCA
DALQSDELCACNWRK
DELCACNWRKGDPTL
CNWRKGDPTLNATEL
GDPTLNATELLKASA
NATELLKASALGSRI

e

RRGERPMSLGQVFDP
PMSLGQVFDPRANAL
HSFPLTGRMPWAPF
IVSSWLRNPHPAQYF
LRNPHPAQYFTARCL
PAQYFTARCLRILPGLWI
GAQGGSAAKLLMSGA
SAAKLLMSGAPIEYV
LMSGAPIEYVLKDSA
PIEYVLKDSAVWMFK

f

LKDSAVWMFKFDIGG
VWMFKFDIGGTPRDI
FDIGGTPRDIPVAGI
TPRDIPVAGIWNGSLWT
PAWGGIHAIASNA
YQFRNVIPARWS
VSSAVLPNYRLVAAL
LPNYRLVAALPMAY
HNQRMRFRTDLSYGVY
GFAEINPIA

g

LVEKPALSWKSRLRR
ALSWKSRLRRKNSSI
SRLRRKNSSIALANM
KNSSIALANMEDGGS
ALANMEDGGSVGRSN
EDGGSVGRSNDIPGR
VGRSNDIPGRRARFI
DIPGRRARFIGEKAE
RARFIGEKAEDPPAP
GEKAEDPPAPSPRPAL

h

RIRRHRHAEIILSMP
RHAEIILSMPGFGVI
ILSMPGFGVILGAEF
GFGVILGAEFLAATG
LGAEFLAATGGDMAA
LAATGGDMAAFASAD
GDMAAFASADRLAGV
FASADRLAGVAGLAP
RLAGVAGLAPVPRDS
AGLAPVPRDSGRISG

i

VPRDSGRISGNLKRP
GRISGNLKRPRRYDR
NLKRPRRYDRRLLRA
RRYDRRLLRACYLSA
RLLRACYLSALVSIR
CYLSALVSIRTDPSS
LVSIRTDPSSRTYYD
TDPSSRTYYDRKRTE
RTYYDRKRTEGKRHT
RKRTEGKRHTQAVLA

j

GKRHTQAVLALARRR
QAVLALARRRLNVLW
LARRRLNVLWAMLRD
LNVLWAMLRDHAVYH
AMLRDHAVYHPATTT
HAVYHPATTTAAARL
IVGQTYREVEVVLVD
TYREVEVVLVDGGST
EVVLVDGGSTDRTLD
DGGSTDRTLDIANSF

k

DRTLDIANSFRPELG
IANSFRPELGSRLVV
RPELGSRLVVHSGPD
SRLVVHSGPDDGPYD
HSGPDDGPYDAMNRG
DGPYDAMNRGVGVAT
AMNRGVGVATGEWVL
VGVATGEWVLFLGAD
GEWVLFLGADDTLYE
FLGADDTLYEPTTLA

l

DTLYEPTTLAQVAAF
PTTLAQVAAFLGDHA
QVAAFLGDHAASHLV
LGDHAASHLVYGDVV
ASHLVYGDVVMRSTK
YGDVVMRSTKSRHAG
MRSTKSRHAGPFDLD
SRHAGPFDLDRLLFEPF
DLDRLLFETNLCHRLL
FETNLCHQSIFY

m

TNLCHQSIFYRRELF
QSIFYRRELFDGIGP
RRELFDGIGPYNLRY
DGIGPYNLRYRVWAD
YNLRYRVWADWDFNI
RVWADWDFNIRCFSN
WDFNIRCFSNPALIT
RCFSNPALITRYMDV
PALITRYMDVVISEY
RYMDVVISEYNDMTG
VISEYNDMTGFSMRQ

n

NDMTGFSMRQGTDKE
FSMRQGTDKEFRKRL
GTDKEFRKRLPMYFW
FRKRLPMYFWVAGWE
PMYFWVAGWETCRRM
VAGWETCRRMLAFLK
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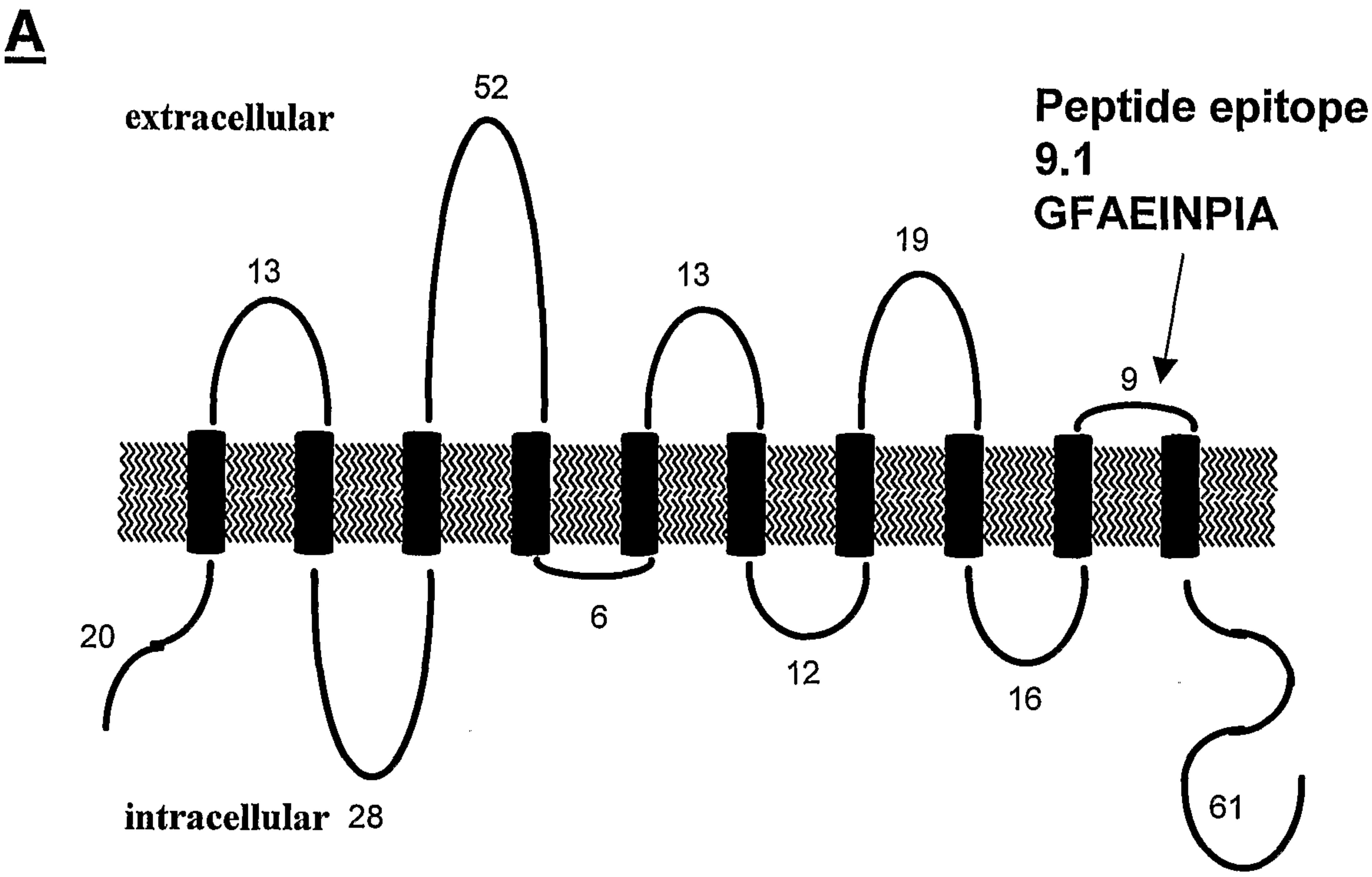
Figure 4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extracellular
52
Peptide epitope
9.1
GFAEINPIA
13
13
19
9
20
6
12
16
intracellular 28
61

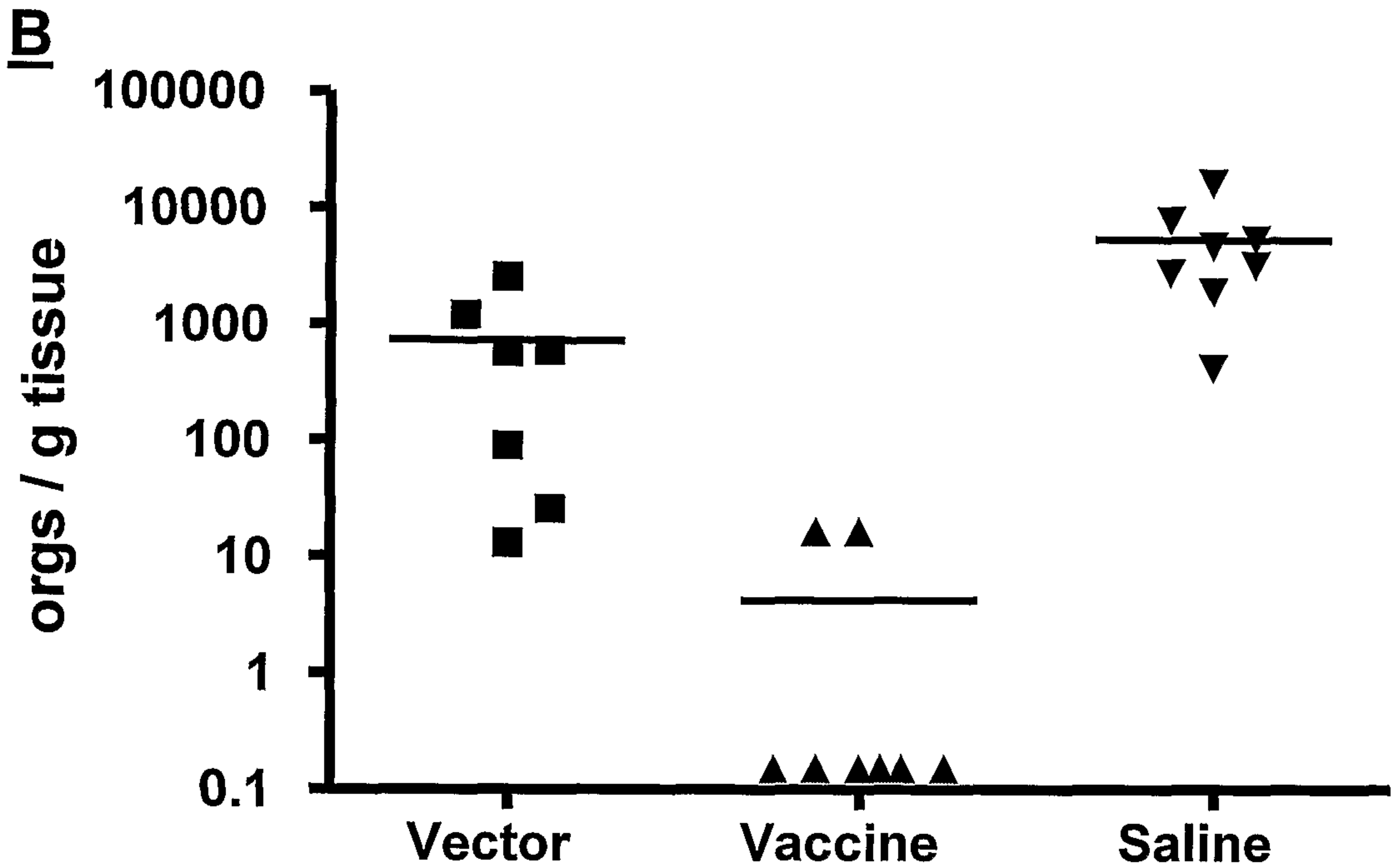
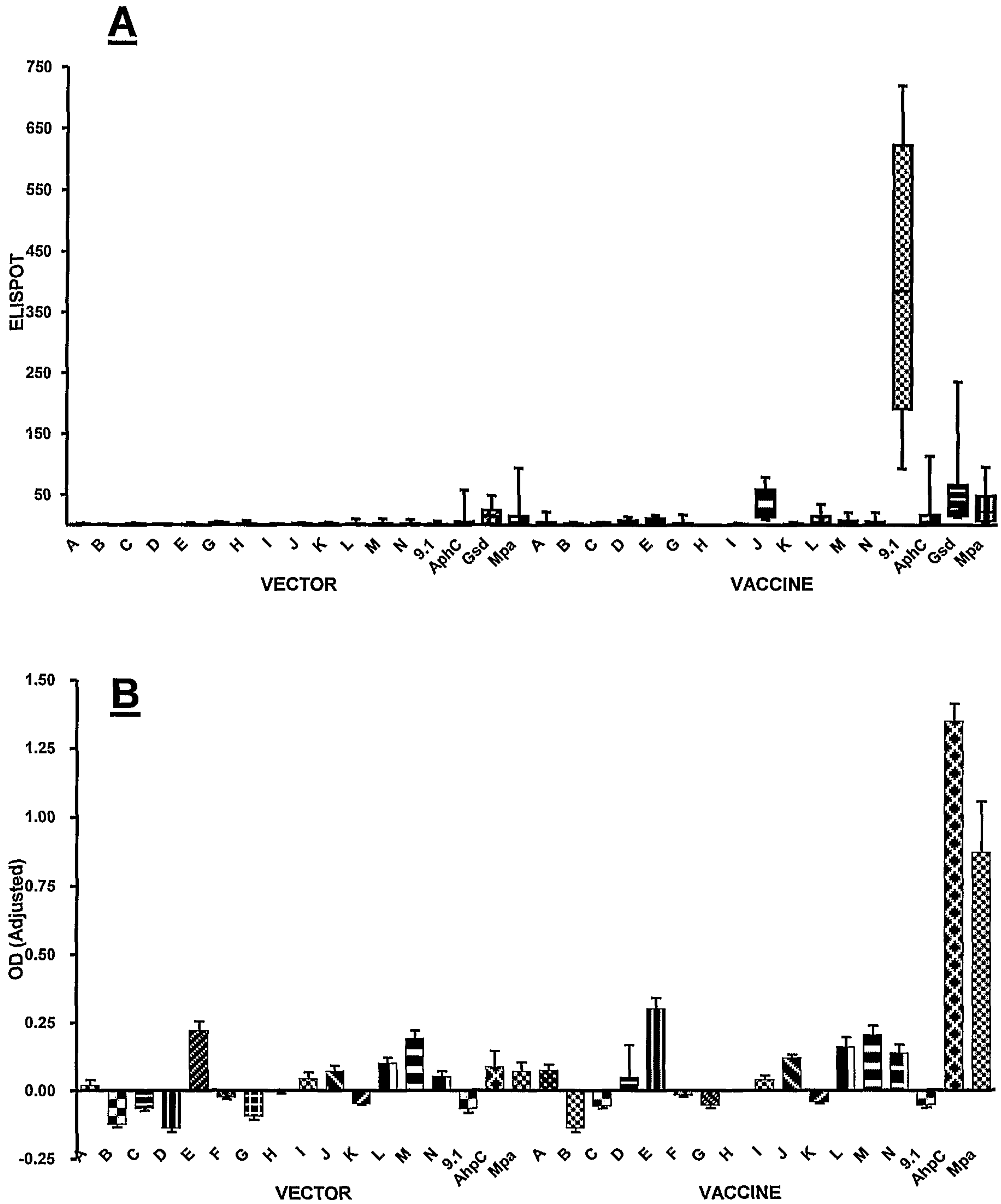

B
100000
10000
1000
100
10
1
0.1
orgs / g tissue
Vector
Vaccine
Saline

Figure 5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750
650
550
450
350
250
150
50
ELISPOT
A B C D E G H I J K L M N 9.1 AphC Gsd Mpa A B C D E G H I J K L M N 9.1 AphC Gsd Mpa
VECTOR
VACCINE
B
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
OD (Adjusted)
A B C D E F G H I J K L M N 9.1 AhpC Mpa A B C D E F G H I J K L M N 9.1 AhpC Mpa
VECTOR
VACCINE

Figure 6

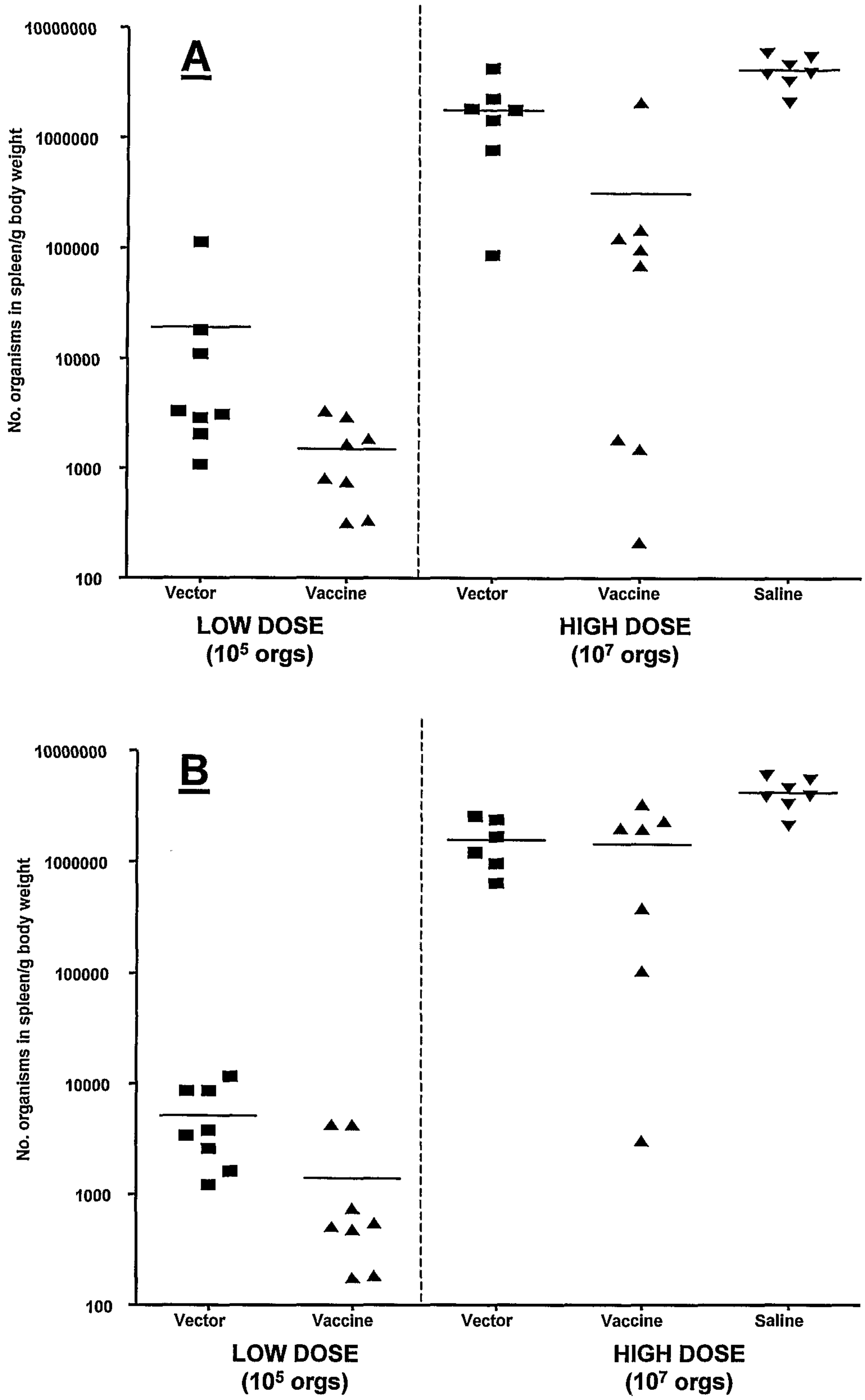

A
No. organisms in spleen/g body weight
10000000
1000000
100000
10000
1000
100
Vector
Vaccine
Vector
Vaccine
Saline
LOW DOSE
(10^5 orgs)
HIGH DOSE
(10^7 orgs)
B
No. organisms in spleen/g body weight
10000000
1000000
100000
10000
1000
100
Vector
Vaccine
Vector
Vaccine
Saline
LOW DOSE
(10^5 orgs)
HIGH DOSE
(10^7 orgs)

Figure 7

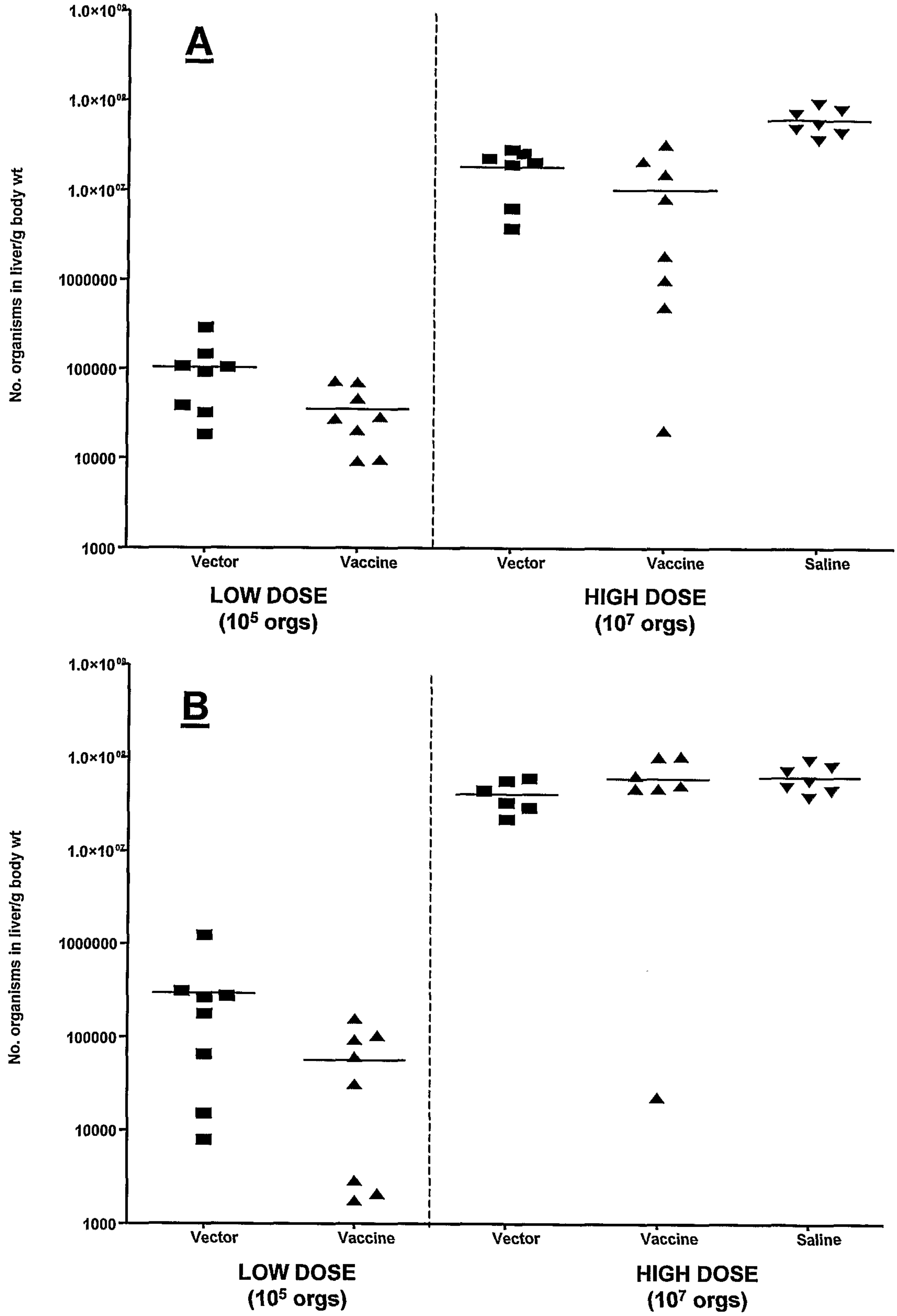
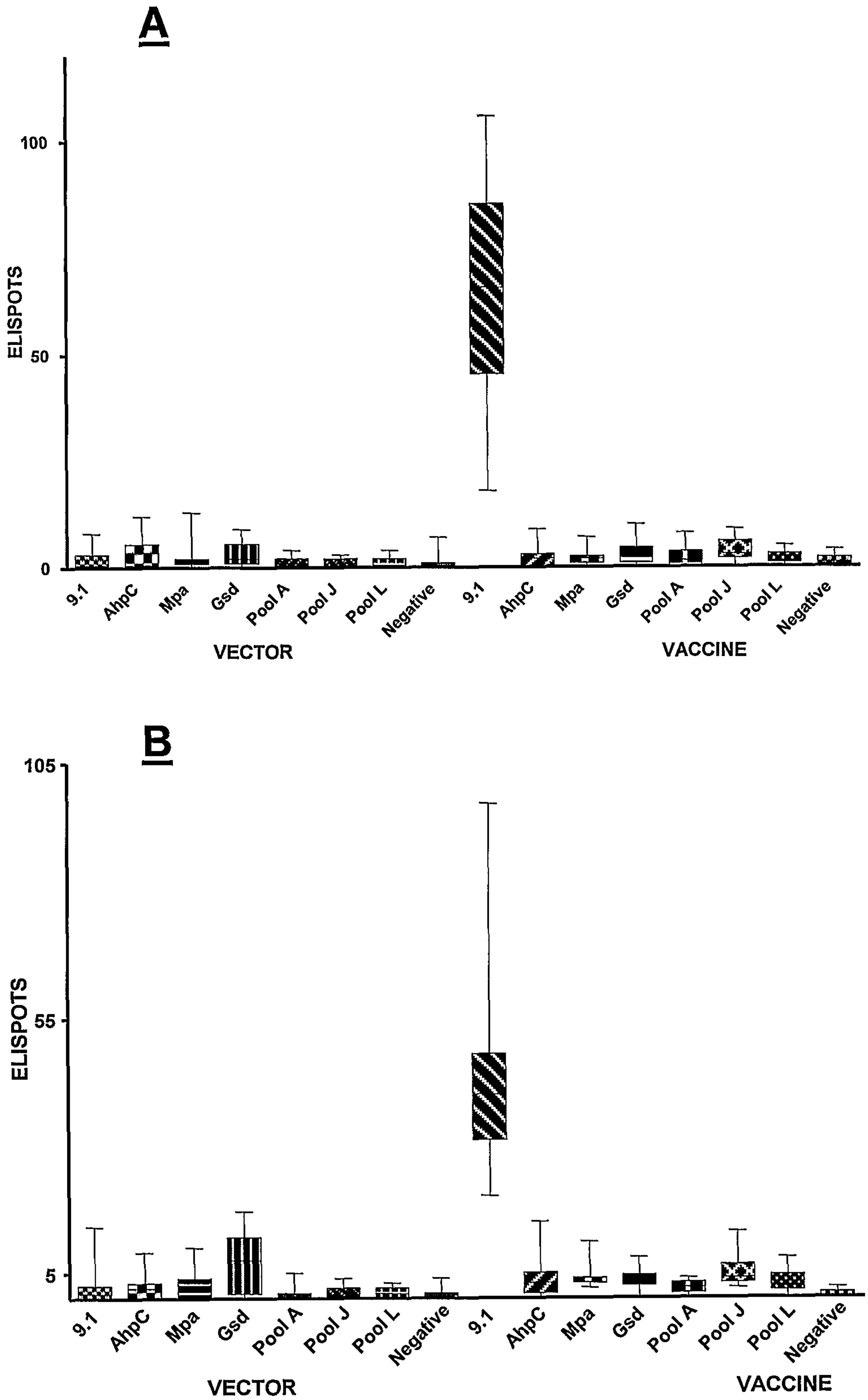

A
No. organisms in liver/g body wt
1000
10000
100000
1000000
Vector
Vaccine
Vector
Vaccine
Saline
LOW DOSE
(10⁵ orgs)
HIGH DOSE
(10⁷ orgs)
B
No. organisms in liver/g body wt
1000
10000
100000
1000000
Vector
Vaccine
Vector
Vaccine
Saline
LOW DOSE
(10⁵ orgs)
HIGH DOSE
(10⁷ orgs)

Figure 8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ELISPOTS
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0
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AhpC
Mpa
Gsd
Pool A
Pool J
Pool L
Negative
9.1
AhpC
Mpa
Gsd
Pool A
Pool J
Pool L
Negative
VECTOR
VACCINE
B
ELISPOTS
105
55
5
9.1
AhpC
Mpa
Gsd
Pool A
Pool J
Pool L
Negative
9.1
AhpC
Mpa
Gsd
Pool A
Pool J
Pool L
Negative
VECTOR
VACCINE

Figure 9