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AND PROTECT AGAINST INFECTION

MLGYVLARIGQSAIVLLAVFSLVFWGVLSILPADPAIFVAKGEGYFNPDIVAQVKAFYGY
DRPLWQYFAQLNQVLHGHFGFSLSSGQAVTDRIIGGVIGETLKLAAATATGFAVLFAVSVT
ALATTCAPVRSVLRAIPPLEGAVPTF

Figure 1A

MKIEEGKLVINGDKGYNGLAIEVGKKFEKDTGIKVTVEHEDKLEEFQVAATGDGPDIIFWAHDFFGGYQOS
GLLAETPDKAFQDKLYPFTWDVRYNGKL IAYPIAVEALSILYNKDLNPPKTEWEEIPALDKELKAGKSAL
MENLQEPYFTWPLIADGGYAFKYENGKYD IKDVGVNDAGAKAGLTFLVDLIKKNHMNADTDYSIAEAAFNKGE
TAMTINGPWAKSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAA SPNKELEFLENYLLTDEGLEAVNK
DKPLGAVALKSYEEELAKDPRIATMENAKGKGEIMFIPOMSAFWYAVRTAVINAA SGRQTVDEALKDAQTNSS
SNNNNNNNNNLIEGRISFEFGSSRMLGYVLARIGQSAIVLLAVFSLVFWGVLSILPADPAIFVAKGEGYFNP
DIVAQVKAFYGYDRPLWQYFAQLNQVLHGHFGFSLSSGQAVTDRIIGGVIGETLKLAAATATGFAVLFAVSVTALA
ITCAPVRSVLRAIPPLEGAVPTF*

Figure 1B

(57) Abstract: Compositions of immunogenic proteins of Mycobacterium avium subspecies paratuberculosis (MAP) are effective for stimulating a protective immune response in animals against MAP. Combinations of MAP1087 with two or more of MAP1204, MAP1272c and MAP2077c are effective for stimulating a protective immune response in animals against MAP and can be administered as vaccines against paratuberculosis (Johne's disease). Induction of the immune response significantly reduces or eliminates colonization of the animal by MAP, and consequently reduces or eliminates the symptoms of clinical disease in animals infected with MAP and reduces or eliminates fecal shedding of MAP. Vaccination with the compositions provides protection against clinical disease and reduces transmission of MAP infection within a herd.



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RECOMBINANT MYCOBACTERIUM AVIUM SUBSP.
PARATUBERCULOSIS PROTEINS INDUCE IMMUNITY
AND PROTECT AGAINST INFECTION

Cross-Reference to Related Applications

[0001] This application claims the benefit under 35 U.S.C. 1.19(e) of United States provisional 61/717,723, filed October 24, 2012, the contents of which are incorporated by reference herein.

Background of the Invention

Field of the Invention

[0002] The invention relates to compositions of proteins effective as vaccines to induce immunity and protect animals against infection by *Mycobacterium avium* subspecies *paratuberculosis*.

Background of the Invention

[0003] Paratuberculosis vaccine studies have demonstrated the induction of both cellular and humoral immune responses, however, it is widely accepted that vaccination will not prevent infection. Some benefits of vaccination include reduced fecal shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and reduced clinical signs in infected animals, with evidence suggesting a reduction in the incidence of disease within herds or severity of disease for individual animals

(Kalis *et al.* Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. Am. J. Vet. Res. 2001. 62:270-4; Begg *et al.* Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. Vaccine. 2005. 23:4999-5008; and Juste *et al.* Significant reduction in bacterial shedding and improvement in milk production in dairy farms after the use of a new inactivated paratuberculosis vaccine in a field trial. BMC Res Notes. 2009. 2:233-9). The heat-killed whole cell vaccine that is approved for use in the US (Mycopar, Fort Dodge Animal Health) is not ideal because of potential adverse reactions, including severe inflammation and granuloma formation at the injection site. In addition, vaccination with whole cell vaccines has been shown to interfere with bovine tuberculosis skin testing and serologic detection of MAP infected animals (Köhler *et al.* Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections. J Vet Med B. 2001. 48:185-95; Muskens *et al.* Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. Vet. Microbiol. 2002. 86:269-78; and Nedrow *et al.* Antibody and skin-test responses of sheep vaccinated against Johne's disease. Vet Immunol Immunopathol. 116:109-12). Developing subunit or DNA vaccines would significantly reduce or eliminate some of the troubling aspects of the whole cell vaccine without sacrificing beneficial properties.

[0004] Several MAP proteins or protein complexes have demonstrated success for use as subunit vaccines, including a

70kDa heat shock protein, a 74F polyprotein, and a mixture of Ag85/SOD proteins. Immunization with these protein or protein complexes has provided protection against MAP challenge in mice, cattle and goat models, resulting in reduced colonization of tissues and decreased shedding in the feces (Koets *et al.* Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine*. 2006. 24:2550-9; Chen *et al.* Immune responses in mice to *Mycobacterium avium* subsp. *paratuberculosis* following vaccination with a novel 74F recombinant polyprotein. *Vaccine*. 2008. 26:1253-62; and Kathaperumal *et al.* Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine*. 2008. 26:1652-63). Each of these subunit vaccines has demonstrated that they are able to induce both cell-mediated and humoral immune responses in the respective hosts, suggesting strong protective measures. Further, it was recently demonstrated that the Hsp70 subunit vaccine does not cross-react with the comparative cervical skin test, a diagnostic tool commonly used for bovine tuberculosis in the field (Santema *et al.* Heat shock protein 70 subunit vaccination against bovine paratuberculosis does not interfere with current immunodiagnostic assays for bovine tuberculosis. *Vaccine*. 2009. 27:2312-19). Positive responses to AvPPD were noted in all vaccinated animals, however, responses to BoPPD were demonstrated only for cattle vaccinated with whole cell vaccine (Gudair) and not for those vaccinated with Hsp70 (Santema *et al. ibid*), demonstrating that a subunit vaccine can be more discriminative for identification of animals infected or vaccinated against MAP versus those animals infected with *M. bovis*.

[0005] However, despite these advances, the need remains for improved vaccines for protecting animals against paratuberculosis.

Summary of the Invention

[0006] We have now discovered compositions of immunogenic proteins of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) which are effective for stimulating a protective immune response in animals against MAP. These immunogenic proteins from MAP include MAP1087, MAP1204, MAP1272c and MAP2077c. Combinations of MAP1087 with two or more of MAP1204, MAP1272c and MAP2077c are effective for stimulating a protective immune response in animals against MAP and can be administered as vaccines against paratuberculosis (Johne's disease). Induction of the immune response significantly reduces or eliminates colonization of the animal by MAP, and consequently reduces or eliminates the symptoms of clinical disease in animals infected with MAP and reduces or eliminates fecal shedding of MAP. Vaccination with the compositions provides protection against clinical disease and reduces transmission of MAP infection within a herd.

[0007] In accordance with this discovery, it is an object of this invention to provide a novel protective vaccine against MAP in animals.

[0008] Another object of this invention is to provide a novel vaccine which significantly reduces or eliminates colonization of the animal by MAP.

[0009] A further object of this invention is to provide a novel vaccine which provides protection to animals against

clinical paratuberculosis and reduces transmission of MAP infection within a herd.

[00010] Other objects and advantages of this invention will become readily apparent from the ensuing description.

Brief Description of the Drawings

[0011] Figure 1A shows the amino acid sequence (SEQ ID NO: 1) of MAP1087, isolated from MAP and having a molecular weight of 15436 Daltons. Figure 1B shows the amino acid sequence of recombinant fusion protein (SEQ ID NO: 5) of MAP1087 and maltose-binding protein (MBP) as cloned into and produced by *E. coli* as described in Example 1. The amino acids of the MAP1087 are underlined (amino acids 396-541 of Figure 1B).

[0012] Figure 2A shows the amino acid sequence (SEQ ID NO: 2) of MAP1204, isolated from MAP and having a molecular weight of 25415 Daltons. Figure 2B shows the amino acid sequence of recombinant fusion protein (SEQ ID NO: 6) of MAP1204 and maltose-binding protein (MBP) as cloned into and produced by *E. coli* as described in Example 1. The amino acids of the MAP1204 are underlined (amino acids 396-639 of Figure 2B).

[0013] Figure 3A shows the amino acid sequence (SEQ ID NO: 3) of MAP1272c, isolated from MAP and having a molecular weight of 33404 Daltons. Figure 3B shows the amino acid sequence of recombinant fusion protein (SEQ ID NO: 7) of MAP1272c and maltose-binding protein (MBP) as cloned into and produced by *E. coli* as described in Example 1. The amino acids of the MAP1272c are underlined (amino acids 396-711 of Figure 3B).

[0014] Figure 4A shows the amino acid sequence (SEQ ID NO: 4) of MAP2077c, isolated from MAP and having a molecular weight of

11074 Daltons. Figure 4B shows the amino acid sequence of recombinant fusion protein (SEQ ID NO: 8) of MAP2077c and maltose-binding protein (MBP) as cloned into and produced by *E. coli* as described in Example 1. The amino acids of the MAP2077c are underlined (amino acids 396-504 of Figure 4B).

[0015] Figure 5 shows the MAP1087 nucleotide sequence (SEQ ID NO: 9) as cloned into the pMAL-c2 *E. coli* expression vector as described in Example 1. The underlined sequence (nucleotides 1186-1623) corresponds to the nucleotide sequence (Genome coordinates=1141173:1141613) of the cDNA from MAP encoding the isolated MAP1087 protein of Figure 1A. Nucleotides 1-1185 correspond to the nucleotide sequence encoding the MBP fusion protein.

[0016] Figure 6 shows the MAP1204 nucleotide sequence (SEQ ID NO: 10) as cloned into the pMAL-c2 *E. coli* expression vector as described in Example 1. The underlined sequence (nucleotides 1186-1917) corresponds to the nucleotide sequence (Genome coordinates=1265380:1266114) of the cDNA from MAP encoding the isolated MAP1204 protein of Figure 2A. Nucleotides 1-1185 correspond to the nucleotide sequence encoding the MBP fusion protein.

[0017] Figure 7 shows the MAP1272c nucleotide sequence (SEQ ID NO: 11) as cloned into the pMAL-c2 *E. coli* expression vector as described in Example 1. The underlined sequence (nucleotides 1186-2133) corresponds to the nucleotide sequence (Genome coordinates=1359174:1360124) of the cDNA from MAP encoding the isolated MAP1272c protein of Figure 3A. Nucleotides 1-1185 correspond to the nucleotide sequence encoding the MBP fusion protein.

[0018] Figure 8 shows the MAP2077c nucleotide sequence (SEQ ID NO: 12) as cloned into the pMAL-c2 *E. coli* expression vector as described in Example 1. The underlined sequence (nucleotides 1186-1512) corresponds to the nucleotide sequence (Genome coordinates=2285918:2286247) of the cDNA from MAP encoding the isolated MAP2077c protein of Figure 4A. Nucleotides 1-1185 correspond to the nucleotide sequence encoding the MBP fusion protein.

[0019] Figure 9 shows the secretion of interferon- γ (ng/ml; IFN- γ) in Example 2 by control non-infected cows and cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* upon incubation of whole blood with medium alone (NS); concanavalinA (ConA); pokeweed mitogen (PWM); a whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS); johnin purified protein derivative (JPPD); and MAP proteins (2077c, 1204, 1272c, and 1087). Data are expressed as means $\pm$ SEM. Significant differences between control and infection cows within in vitro treatment group are represented by asterisks (** $P < 0.01$; * $P < 0.05$).

[0020] Figure 10 shows the secretion of Th1-mediated cytokines, interferon- γ (**A**; IFN- γ , pg/ml) and interleukin-12 (**B**; IL-12, pg/ml), and Th2-mediated cytokines, interleukin-4 (**C**; IL-4, pg/ml), and interleukin-10 (**D**; IL-10; pg/ml) in Example 2 by splenocytes stimulated with medium alone (NS) or with a whole cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS). Splenocytes were isolated after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1-4. Data are expressed as means $\pm$ SEM. Significant differences between NS and MPS within treatment group are represented by asterisks (** $P < 0.01$; * $P < 0.05$).

[0021] Figure 11 shows the percentage of CD4+ (**A**), CD4CD25+ (**B**), CD8+ (**C**), and CD8CD25+ (**D**) T cells in Example 2 from splenocytes stimulated with medium alone (NS) or with a whole cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS). Splenocytes were isolated after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1-4. Data are expressed as means $\pm$ SEM. Significant differences between NS and MPS stimulants within a treatment group are represented by asterisks (** $P < 0.01$; * $P < 0.05$).

[0022] Figure 12 shows the serum IgG₁ and IgG_{2a} antibody responses in Example 2 against *Mycobacterium avium* subsp. *paratuberculosis* after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1-4. Data are expressed as means $\pm$ SEM. Significant differences between control uninfected, control infected and vaccinate groups are represented by asterisks (** $P < 0.01$; * $P < 0.05$).

[0023] Figure 13 shows the recovery of viable *Mycobacterium avium* subsp. *paratuberculosis* (cfu/g tissue) in Example 2 from (**A**) spleen, (**B**) liver, (**C**) mesenteric lymph node (MLN), and (**D**) ileum of control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1-4 after 3 months of infection. Data are expressed as means $\pm$ SEM. Significant differences between control infected and vaccinate groups are represented by asterisks (** $P < 0.01$; * $P < 0.05$).

Definitions

The following terms are employed herein:

[0024] Cloning. The selection and propagation of (a) genetic material from a single individual, (b) a vector containing one gene or gene fragment, or (c) a single organism containing one such gene or gene fragment.

[0025] Cloning Vector. A plasmid, virus, retrovirus, bacteriophage or nucleic acid sequence which is able to replicate in a host cell, characterized by one or a small number of restriction endonuclease recognition sites at which the sequence may be cut in a predetermined fashion, and which contains a marker suitable for use in the identification of transformed cells, e.g., uracil utilization, tetracycline resistance, ampicillin resistance. A cloning vector may or may not possess the features necessary for it to operate as an expression vector.

[0026] Codon. A DNA sequence of three nucleotides (a triplet) which codes (through mRNA) for an amino acid, a translational start signal, or a translational termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA, and CTG encode for the amino acid leucine, while TAG, TAA, and TGA are translational stop signals, and ATG is a translational start signal.

[0027] Complement or Complementary Sequence. The product of complementary base pairing in which purines bond with pyrimidines, as it occurs in the two polynucleotide chains of DNA (adenine with thymine, guanine with cytosine) and between DNA and messenger RNA nucleotides during transcription.

[0028] DNA Coding Sequence. A DNA sequence which is transcribed and translated 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. A coding sequence can include, but is not limited to, prokaryotic sequences and cDNA from eukaryotic mRNA. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0029] DNA Sequence. A linear series of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

[0030] Expression. The process undergone by a structural gene to produce a polypeptide. Expression requires both transcription of DNA and translation of RNA.

[0031] Expression Vector. A replicon such as a plasmid, virus, retrovirus, bacteriophage, or nucleic acid sequence which is able to replicate in a host cell, characterized by a restriction endonuclease recognition site at which the sequence may be cut in a predetermined fashion for the insertion of a heterologous DNA sequence. An expression vector has a promoter positioned upstream of the site at which the sequence is cut for the insertion of the heterologous DNA sequence, the recognition site being selected so that the promoter will be operatively associated with the heterologous DNA sequence. A heterologous DNA sequence is "operatively associated" with the promoter in a cell when RNA polymerase, which binds the promoter sequence

transcribes the coding sequence into mRNA which is then in turn translated into the protein encoded by the coding sequence.

[0032] Fusion Protein. A protein produced when two heterologous genes or fragments thereof coding for two different proteins not found fused together in nature are fused together in an expression vector. For the fusion protein to correspond to the separate proteins, the separate DNA sequences must be fused together in correct translational reading frame.

[0033] Gene. A segment of DNA which encodes a specific protein or polypeptide, or RNA.

[0034] Genome. The entire DNA of an organism. It includes, among other things, the structural genes encoding for the polypeptides of the substance, as well as operator, promoter and ribosome binding and interaction sequences.

[0035] Heterologous DNA. A DNA sequence inserted within or connected to another DNA sequence which codes for polypeptides not coded for in nature by the DNA sequence to which it is joined. Allelic variations or naturally occurring mutational events do not give rise to a heterologous DNA sequence as defined herein.

[0036] Hybridization. The pairing together or annealing of single stranded regions of nucleic acids to form double-stranded molecules.

[0037] Nucleotide. A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. The base

characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C, and uracil ("U").

[0038] Plasmid. A non-chromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. A cell transformed by a plasmid is called a "transformant".

[0039] Polypeptide. A linear series of amino acids connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids.

[0040] Promoter. A DNA sequence within a larger DNA sequence defining a site to which RNA polymerase may bind and initiate transcription.

[0041] Reading Frame. The grouping of codons during translation of mRNA into amino acid sequences. During translation the proper reading frame must be maintained. For example, the DNA sequence may be translated via mRNA into three reading frames, each of which affords a different amino acid sequence.

[0042] Recombinant DNA Molecule. A hybrid DNA sequence comprising at least two DNA sequences, the first sequence not normally being found together in nature with the second.

[0043] Ribosomal Binding Site. A nucleotide sequence of mRNA, coded for by a DNA sequence, to which ribosomes bind so that translation may be initiated. A ribosomal binding site is required for efficient translation to occur. The DNA sequence

coding for a ribosomal binding site is positioned on a larger DNA sequence downstream of a promoter and upstream from a translational start sequence.

[0044] Start Codon. Also called the initiation codon, is the first mRNA triplet to be translated during protein or peptide synthesis and immediately precedes the structural gene being translated. The start codon is usually AUG, but may sometimes also be GUG.

[0045] Structural Gene. A DNA sequence which encodes through its template or messenger RNA (mRNA) a sequence of amino acids characteristic of a specific polypeptide.

[0046] Substantially Pure. The condition of a compound, such as a protein or a nucleotide, being cell free or being separated from other components that would interfere with or have a substantial qualitative effect on the activity of the compound or on a substrate on which the compound acts.

[0047] Transform. To change in a heritable manner the characteristics of a host cell in response to DNA foreign to that cell. An exogenous DNA has been introduced inside the cell wall or protoplast. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In prokaryotes and some fungi, for example, the exogenous DNA may be maintained on an episomal element such as a plasmid. With respect to most eukaryotic cells, a stably transformed cell is one in which the exogenous DNA has been integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish

cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

[0048] Transcription. The process of producing mRNA from a structural gene.

[0049] Translation. The process of producing a polypeptide from mRNA.

[0050] Vaccine. Vaccine is defined herein in its broad sense to refer to any type of biological agent in an administrable form capable of stimulating a protective immune response in an animal inoculated with the vaccine. For purposes of this invention, the vaccine may comprise combinations of three or more of the immunogenic (antigenic) proteins MAP1087, MAP1204, MAP1272c and MAP2077c.

Detailed Description of the Invention

[0051] In the following description, the nomenclature used to define the proteins and peptides is that specified by Schroder and Lubke ["The Peptides," Academic Press (1965)] wherein, in accordance with conventional representation, the N-terminal appears to the left and the C-terminal to the right. Where the amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented herein unless otherwise expressly indicated.

[0052] We have discovered that compositions of immunogenic proteins MAP1087, MAP1204, MAP1272c and MAP2077c of MAP are effective for stimulating an immune response in animals against MAP. Moreover, combinations or cocktails of MAP1087 with any

two or all of MAP1204, MAP1272c and MAP2077c are particularly effective for stimulating a protective immune response in animals against MAP and can be administered as vaccines against paratuberculosis (Johne's disease). The proteins have been isolated, substantially free from other proteins or cell components which are normally present in the cells of MAP, such that the MAP1087, MAP1204, MAP1272c and MAP2077c proteins are the only significant protein(s) or peptide(s) in the sample and may be used effectively as a vaccine. Moreover, all of the proteins have been produced in recombinant form as described herein below. The term "isolated" encompasses not only proteins which have been recovered from naturally occurring cells, but also recombinant proteins and synthesized proteins. Compositions of the MAP1087, MAP1204, MAP1272c and MAP2077c proteins, including recombinants, are immunogenic, effective for eliciting a protective immune response against MAP, which response is mediated by humoral, *i.e.*, antibodies, and/or cellular processes. Each of MAP1087, MAP1204, MAP1272c and MAP2077c has been previously described, including their amino acid sequences are corresponding gene sequences. However, their use as a vaccine has not been described.

[0053] The isolated MAP1087, MAP1204, MAP1272c and MAP2077c have been sequenced, and their amino acid sequences are shown in Figures 1A, 2A, 3A and 4A, and correspond to SEQ ID NOs: 1-4, respectively. MAP1087 is an ABC transporter permease protein with a calculated molecular weight of 15.4 kDa, MAP1204 and MAP1272c are putative invasion proteins of the NipC/P60 family having calculated molecular weights of 25.4 and 33.4 kDa, respectively, and MAP2077c is an STAS domain containing protein having a calculated molecular weight of 11.1 kDa. Moreover,

when utilizing recombinant proteins, the proteins may be modified to assist cloning in the selected vector and/or their separation and recovery, and the expressed proteins may further include optional, additional terminal amino acid sequences (not of MAP origin). For example, Figures 1B, 2B, 3B and 4B show the amino acid sequences (SEQ ID NOs: 5-8) of the MAP1087, MAP1204, MAP1272c and MAP2077c fusion proteins as cloned into and produced by the *E. coli* expression vector as described in Example 1. The underlined sequence of Figures 1B, 2B, 3B and 4B correspond to the amino acid sequences of Figures 1A, 2A, 3A and 4A. The N-terminal amino acids which are not underlined (amino acids 1-395) correspond to the MBP fusion protein. It is also understood that invention encompasses substantial equivalents of the MAP1087, MAP1204, MAP1272c and MAP2077c proteins which retain the ability to elicit an immune response in an animal against MAP. The practitioner of ordinary skill in the art will recognize that slight deviations of the amino acid sequences may be made without significantly affecting the efficacy of the proteins. Substantial equivalents of the MAP1087, MAP1204, MAP1272c and MAP2077c proteins include conservative substitutions of amino acids with other amino acids, including either naturally occurring or non-conventional amino acids, which maintain substantially the same charge and hydrophobicity as the original amino acid. Conservative substitutions include for example, replacement of glycine for alanine, valine for isoleucine, leucine for isoleucine, aspartic acid for glutamic acid, lysine for arginine, asparagine for glutamine, phenylalanine for tryptophan, and tryptophan for tyrosine. Examples of conservative substitutions with non-conventional amino acids are described in Rosenberg *et al.* (U.S. patent no.

5,679,782) the contents of which are incorporated by reference herein.

[0054] The MAP1087, MAP1204, MAP1272c and MAP2077c proteins may be produced for use in accordance with this invention by a variety of techniques, including production of recombinant proteins, isolation from cells of MAP, and chemical synthesis. For example, it is envisioned that the MAP1087, MAP1204, MAP1272c and MAP2077c proteins may be synthesized by methods well known to those skilled in the art of peptide synthesis, such as exclusively solid-phase techniques, partial solid-phase techniques, fragment condensation, or classical solution addition. Without being limited thereto, suitable solution phase synthesis methods are described by Finn and Hoffman [In "Proteins," Vol. 2, 3rd Ed., H. Neurath and R. L. Hill (eds.), Academic Press, New York, pp. 105-253 (1976)], while solid phase synthesis methods are described by Barany and Merrifield [In "The Peptides," Vol. 2, E. Gross and J. Meienhofer (eds.), Academic Press, New York, pp. 3-284 (1979)], and stepwise solid phase synthesis methods are described by Merrifield [J. Am. Chem. Soc. 85: 2149-2154 (1963)], the contents of each of which are incorporated herein by reference. However, the proteins are preferably produced by recombinant DNA techniques, which are particularly suitable for large-scale use.

[0055] Recombinant MAP1087, MAP1204, MAP1272c and MAP2077c proteins may be obtained by transforming a microorganism using an expression vector including a promoter or operator, or both, together with one or more of the MAP1087, MAP1204, MAP1272c and MAP2077c encoding genes and causing such transformed microorganisms to express the proteins. The genes encoding each of the MAP1087, MAP1204, MAP1272c and MAP2077c proteins have

been previously described, and the nucleic acid sequences of the cDNA encoding these proteins are available in GenBank as GeneID nos. 2719512, 2720812, 2720835 and 2719950, respectively. The nucleic acid sequences of the cDNA encoding the MAP1087, MAP1204, MAP1272c and MAP2077c proteins are also provided in Figures 5-8 (SEQ ID NOs. 9-12), respectively, each indicated by underlining. Specifically, the nucleic acid sequence of the cDNA encoding MAP1087 corresponds to nucleotides 1186-1623 of SEQ ID NO: 9 (Figure 5); the nucleic acid sequence of the cDNA encoding MAP1204 corresponds to nucleotides 1186-1917 of SEQ ID NO: 10 (Figure 6); the nucleic acid sequence of the cDNA encoding MAP1272c corresponds to nucleotides 1186-2133 of SEQ ID NO: 11 (Figure 7); the nucleic acid sequence of the cDNA encoding MAP2077c corresponds to nucleotides 1186-1512 of SEQ ID NO: 12 (Figure 8). Each of these sequences also include the optional nucleotide sequence encoding the MBP fusion protein, which corresponds to nucleotides 1-1185 of each of SEQ ID NOs: 9-12.

[0056] The DNA sequences encoding the MAP1087, MAP1204, MAP1272c and MAP2077c proteins can be used to prepare recombinant DNA molecules by cloning into any suitable vector. A variety of vector-host cell expression systems may be employed in practicing the present invention. *E. coli*, such as described in Example 1 hereinbelow, is particularly preferred. However, the novel invention described here can be applied with numerous hosts that would be desirable. Host strains may be of bacterial, fungal, or yeast origin. Ascertaining the most appropriate host-vector system is within the skill of the person in the art.

[0057] Vectors used in practicing the present invention are selected to be operable as cloning vectors or expression vectors in the selected host cell. Numerous vectors are known to practitioners skilled in the art, and selection of an appropriate vector and host cell is a matter of choice. The vectors may, for example, be bacteriophage, plasmids, viruses, or hybrids thereof, such as those described in Maniatis *et al.* [Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, 1989] or Ausubel *et al.* [Current Protocols in Molecular Biology, John Wiley & Sons, Inc, 1995], the contents of each of which are herein incorporated by reference. Further, the vectors may be non-fusion vectors (*i.e.*, those producing the protein of the invention not fused to any heterologous polypeptide), or alternatively, fusion vectors (*i.e.*, those producing the protein fused to a vector encoded polypeptide). The fusion proteins would of course vary with the particular vector chosen. In accordance with a preferred embodiment, the vector is the pMAL-c2 expression vector (New England Biolabs, Beverly, MA) as described in the Examples.

[0058] Regardless of the specific vector utilized, various sites may be selected for insertion of the isolated DNA sequences. These sites are usually designated by the restriction enzyme or endonuclease that cuts them. The particular site chosen for insertion of the selected DNA fragment into the vector to form a recombinant vector is determined by a variety of factors. These include size and structure of the polypeptide to be expressed, susceptibility of the desired polypeptide to enzymatic degradation by the host cell components and contamination by its proteins, expression characteristics such as the location of start and stop codons,

and other factors recognized by those of skill in the art. None of these factors alone absolutely controls the choice of insertion site for a particular polypeptide. Rather, the site chosen reflects a balance of these factors, and not all sites may be equally effective for a given protein.

[0059] Each nucleotide sequence comprising the MAP1087, MAP1204, MAP1272c and MAP2077c encoding gene may be inserted into the desired vector by known techniques. If, however, the vector is to serve as an expression vector, the vector should have a promoter, and the DNA sequences should be inserted in the vector downstream of the promoter and operationally associated therewith (that is, the promoter should be recognized by the RNA polymerase of the host cell). In addition, the vector should have a region which codes for a ribosome binding site positioned between the promoter and the site at which the DNA sequence is inserted so as to be operatively associated with the DNA sequence of the invention once inserted (in correct translational reading frame therewith). The vector should be selected to provide a region which codes for a ribosomal binding site recognized by the ribosomes of the host cell into which the vector is to be inserted. The vector should contain a terminator with necessary 3' untranslated sequences for RNA termination, stability, and/or poly(A) tail addition (if eucaryotic). Alternatively, any or all of the above control sequences may be ligated to the coding sequence prior to insertion into the vector.

[0060] DNA constructs may be introduced into the appropriate host by numerous methods described in the technical and scientific literature. Transformation of bacteria or yeast may be performed using standard techniques described in Maniatis et

al., supra. Techniques for transforming filamentous fungi may include those described by Goosen *et al.* [Handbook for Applied Mycology, Arora, Elander & Mukerji, eds. (1992) pp. 151-195] and May *et al.* [Applied Molecular Genetics of Filamentous Fungi, Kinghorn and Turner, eds. (1992) pp. 1-27]. Transformation with *E. coli* is described in the Examples.

[0061] In general, linear or circular DNA constructs may be introduced into the host by techniques utilizing protoplast fusion, polyethylene glycol, liposomes, lithium acetate, electroporation, physical damage, biolistic bombardment, or *Agrobacterium* mediated transformation.

[0062] Successful transformants may be isolated by using markers, contained on the expression vectors, which confer a selectable trait to the transformed host. These may include nutritional selection related to substrate utilization (such as, growth on acetamide containing medium) or prototrophy of a required growth product (such as, arginine, leucine, or uracil). Dominant selectable markers (such as, resistance to ampicillin, G418, hygromycin, and phleomycin) are also useful in selecting transformants that have taken up the introduced DNA construct.

[0063] The DNA construct may be replicated autonomously or integrated into the genome of the host. Integration typically occurs by homologous recombination (for example, arginine selectable marker integrating in the chromosomal arginine gene) or at a chromosomal site unrelated to any genes on the DNA construct. Integration may occur by either a single or double cross-over event. It is also possible to have any number of these integration and replication types occurring in the same transformant.

[0064] For use in animal vaccinations, the combinations or cocktails of the isolated MAP1087, MAP1204, MAP1272c and MAP2077c proteins will typically be formulated in conjunction with a suitable pharmaceutically acceptable carrier or diluent. Specifically, combinations of MAP1087 with two or more of MAP1204, MAP1272c and MAP2077c are effective for stimulating a protective immune response in animals against MAP and can be administered as vaccines against paratuberculosis (Johne's disease). As illustrated in the Examples, cocktails comprising the combinations: (1) MAP1087, MAP1204 and MAP1272c, (2) MAP1087, MAP1204 and MAP2077c, (3) MAP1087, MAP1272c and MAP2077c, as well as (4) all of MAP1087, MAP1204, MAP1272c and MAP2077c, are all effective. The proteins in the vaccine compositions are provided in an immunologically effective amount or dosage to the animal. As used herein, an "effective amount" is defined as that amount of the proteins which will elicit a protective immune response against subsequent challenge to virulent MAP, which may include either or both of antibody production against the proteins or a cell-mediated immune response against MAP, in a treated animal in comparison to an untreated control animal. In a preferred embodiment, induction of a protective immune response (including complete or partial immunity) may be demonstrated by one or more of: a significant reduction in MAP shedding, a significant reduction in the average MAP concentration in animal tissues (including but not limited to the ileum, liver and/or lymph nodes), a significant reduction in the duration of MAP colonization, or a significant reduction in the percentage of animals colonized with MAP, all in a population of vaccinated animals as compared to an unvaccinated control group (measured at a confidence level of at least 80%, preferably measured at a confidence level of 95%).

The actual effective amount will vary with the route of administration, the target animal to be treated and its age and size, and may be readily determined empirically by the practitioner skilled in the art by routine antigen dose-response testing. By way of example and without being limited thereto, when treating bovine by intramuscular, subcutaneous or IV injection, it is envisioned that typical doses of the vaccine per animal will contain between approximately 0.1 to 0.5 mg of each MAP protein in the cocktail. For smaller and/or younger animals, it is envisioned that effective doses may be significantly reduced, with amounts of total protein as low as 0.1 mg being suitable. Although greater amounts of proteins may be administered, use of such higher levels is generally considered impractical. Dosage volumes may also vary, but will typically range between approximately 0.1 mL to 1 mL.

[0065] A variety of carriers or diluents are suitable for formulation with the MAP1087, MAP1204, MAP1272c and MAP2077c proteins. Such carriers or diluents include, but are not limited to, physiological saline, mineral oil, vegetable oils, aqueous carboxymethyl cellulose or polyvinylpyrrolidone. The skilled practitioner will recognize that such carriers should of course be compatible with the proteins. Phosphate buffered saline (PBS) is preferred. The vaccine formulations may also contain optional adjuvants, antibacterial agents or other pharmaceutically active agents as are known in the art. Suitable adjuvants for use herein will be effective for enhancing the immune response of the treated animal to the MAP protein immunogens of the vaccine. Preferred adjuvants include: dimethyl dioctadecylammonium bromide (DDA) in PBS, and the Sigma adjuvant system [*i.e.*, containing 0.5 mg Monophosphoryl Lipid A (MPL; isolated from *Salmonella Minnesota*) and 0.5 mg synthetic

trehalose dicorynomycolate (an analogue of trehalose dimycolate from the cord factor of the tubercle bacillus) in 44 μ L of squalene oil, 0.2% TWEEN 80 and water]. Other suitable adjuvants include but are not limited to mineral oil, vegetable oils, alum, aluminum hydroxide gel and alum, alum plus MPL, water in oil emulsions (such as Suprimm available through Novartis), Freund's incomplete adjuvant, Freund's incomplete adjuvant, and microparticles or nanoparticles or beads of biocompatible matrix materials such as (although not limited to) agar or polyacrylate. The amount of adjuvant will vary considerably with the particular adjuvant selected. By way of example, suitable amounts of DDA may vary between approximately 0.25 to 20 mg per dose in bovine, with 0.5 mg being preferred in calves. Other known immunogenic agents used in conventional vaccines for the target animals may also be included in the formulation.

[0066] The vaccines may be used for the treatment of any animal susceptible to infection by MAP. However, without being limited thereto, the vaccine is preferably used for the treatment of ruminant animals in the families Bovidae and Cervidae, particularly in meat and dairy animals of the Bovidae family, especially bovine (cattle), bison, caprine (goats), ovine (sheep) and the like. It is also envisioned that the vaccines may be used for the treatment of susceptible species that are traditionally free-ranging, but are subject to wildlife management practices. Examples of such species are reindeer, deer, antelope, elk, etc., and also exotic animals that are maintained in zoos and wildlife parks.

[0067] The vaccines may be effectively administered to an animal at any age. The vaccine is preferably administered to

neonatal animals, but may also be administered to older animals after they attain immunocompetence. The vaccines may be administered to the subject animal by any convenient route which enables an immune response. However, parenteral injection (e.g., subcutaneous, intravenous, or intramuscular) is preferred, with subcutaneous injection being particularly preferred. The vaccine products could also be administered using a needle-less device. The vaccine may be administered in a single dose or in a plurality of doses. Dependent upon rearing conditions, the vaccine may be administered in multiple doses, the timing of which may be readily determined by the skilled artisan. By way of example, for treatment of bovine, boost vaccinations are preferably administered approximately 2-3 weeks after the initial vaccination.

[0068] The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention which is defined by the claims.

Example 1

[0069] Recombinant MAP10897, MAP1204, MAP1272c and MAP2077c proteins were produced using the general techniques described by Bannantine and Paustian (Identification of diagnostic proteins in *Mycobacterium avium* subspecies *paratuberculosis* by a whole genome analysis approach. Methods Mol Biol. 2006. 345:185-96), the contents of which are incorporated by reference herein. In brief, each of the cDNA sequences encoding MAP10897, MAP1204, MAP1272c and MAP2077c fusion proteins as shown in Figures 5-8 were cloned into pMAL-c2 *E. coli* expression vectors (New England Biolabs, USA). The MAP were expressed as fusion proteins with

maltose binding protein (MBP) vectors to aid expression and protein recovery. The cDNA sequences and vectors were digested with XbaI and HindIII restriction enzymes, and the digested DNA ligated into the vector. *E. coli* DH5- α cells were transformed with the ligated expression vectors. *E. coli* harboring the expression plasmids were cultured by rotary shaking at 37°C in 1 L of LB media supplemented with glucose (0.2%) and ampicillin (100 mg/ml) and induced with 300 μ L 1M IPTG when the O.D._{600nm} measured between 0.4-0.6. After a 2-hr induction at 37°C, cells were harvested by centrifugation. A lysate of the harvested cells was prepared by freeze-thaw cycles followed by brief sonication. The heterologously expressed MAP/MBP fusion proteins were then affinity purified from the *E. coli* lysates using amylose resin column chromatography. The MAP/MBP fusion proteins were eluted with column buffer containing 10 mM maltose and recovered.

Example 2

Materials and methods

Antigen Screening

[0070] Four MAP proteins were evaluated for reactivity with control noninfected, subclinically and clinically infected cattle in a whole blood interferon- γ (IFN- γ) assay. Infection status of cattle was confirmed by bacteriologic culture for the fecal shedding of MAP and serologic assays by standard methods (Stabel. An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. J Vet Diagn Invest. 1997. 9:375-80; and Stabel *et al.* Mediation of host immune responses after immunization of neonatal calves with a heat-killed

Mycobacterium avium subsp. *paratuberculosis* vaccine. Clin Vaccine Immunol . 2011. 2079-89). Whole blood was incubated with medium only (nonstimulated; NS); concanavalinA (ConA, Sigma); pokeweed mitogen (PWM; 10 µg/ml; Sigma); johnin purified protein derivative (JPPD; 10 µg/ml; National Veterinary Services Laboratory, Ames, IA), a whole-cell sonicate of MAP (MPS; 10 µg/ml; NADC), and one of the following MAP proteins, MAP1087, MAP1204, MAP1272c, MAP2077c, (10 µg/ml). After incubation at 39°C for 18 hr, plasma was assayed using a Bovigam IFN-γ assay (Prionics, La Vista, NE). The selection of the 4 MAP proteins, MAP1087, MAP1204, MAP1272c, and MAP2077c, used in the present study was based upon their immunogenic potential as defined by robust antigen-specific IFN-γ and antibody responses in infected cattle and negligible responses in noninfected control cows. These 4 proteins were arrayed in 4 cocktails containing 3 of the 4 proteins as follows: cocktail 1: MAP1087, MAP1204, MAP1272c; cocktail 2: MAP1087, MAP1204, MAP2077c; cocktail 3: MAP 1087, 1272c, 2077c; and cocktail 4: MAP1204, MAP1272c, 2077c.

Protein Expression and Purification

[0071] The four annotated coding sequences of the proteins were selected from a battery of recombinant proteins amplified from strain K-10 genomic DNA. The four proteins, MAP1087, MAP1204, MAP1272c, and MAP2077c, were expressed and purified as described in Example 1. 74F polyprotein, consisting of a combination of MAP1519 and MAP3527 peptides, was constructed as previously described (Chen *et al. ibid*). The 74F polyprotein had previously demonstrated efficacy in the mouse model as a vaccine for paratuberculosis (Chen *et al. ibid*).

Bacterial Strain

[0072] MAP used as the challenge strain was isolated from the ileum of clinical cow 167 (NADC) and was grown in Middlebrook 7H9 liquid medium (pH 5.9) supplemented with 0.5% Tween 80 (Sigma), 2 mg/ml mycobactin J (Allied Monitor Inc., Fayette, MO) and 10% oleic acid-albumin-dextrose complex (BD Biosciences, Franklin Lakes, NJ). The final concentration of the bacteria was adjusted to 10^9 cfu/ml and confirmed by serial dilution onto agar slants of Herrold's egg yolk medium (HEYM; BD) containing 2 mg/liter of mycobactin J (Allied Monitor) with a final read-out after 12 weeks of incubation. The presence of each of the 4 MAP proteins in the cocktails was confirmed in this strain by PCR and Western blot (data not shown). Clinical cow strain 167 was used as the challenge strain as it was expanded from a primary isolate from ileal tissue of a highly infected cow and had not been subjected to repeated passage in laboratory medium.

Mice

[0073] Six-week old, male Balb/c mice used in the study (Jackson Labs, Bar Harbor, Maine) were housed in biosecurity level-2 containment in disposable plastic cages with free access to water and standard mouse chow. All procedures were approved by the NADC Animal Care and Use Committee.

MAP vaccines

[0074] Mice were randomly assigned to 7 treatment groups containing 10 mice each as follows: control uninfected (no vaccine, no MAP), control infected (no vaccine, MAP infection), 74F protein only (74F vaccine, MAP infection), cocktail 1 (cocktail 1, MAP infection), cocktail 2 (cocktail 2, MAP infection), cocktail 3 (cocktail 3, MAP infection) and cocktail 4 (cocktail 4, MAP infection). The 74F group received 50 μ g

total protein as described in a previous study (Chen *et al. ibid*) and mice in cocktail 1-4 groups were given 100 µg total protein in 100 µl volume per mouse subcutaneously (SQ) in the dorsal region. The control uninfected and control infected groups received 100 µl PBS as sham injections. Mice within each treatment group were boosted with the identical vaccine three weeks after the initial immunization. Two weeks after boosting, mice were inoculated intraperitoneally with live, virulent MAP strain 167 (10^8 in 100 µl). Three months after infection, mice were anesthetized by inhalation of isoflurane and decapitated with a guillotine. The liver, spleen, ileum and mesenteric lymph node were removed from each mouse, weighed, and processed for tissue culture as previously described (Huntley *et al.* Expression library immunization confers protection against *Mycobacterium avium* subsp. *paratuberculosis* infection. Infect Immun. 2005. 73:6877-84). Splenocytes were isolated from a portion of the spleen and cultured at 2.0×10^6 cells/ml with medium only (NS); ConA, 10 µg/ml; PWM, 10 µg/ml; and MPS, 10 µg/ml. Quantitative cytokine analyses was performed on 24 hr supernatants for interleukins (IL)-2, IL-4, IL-10, IL-12, IL-23 and IFN-γ using commercial ELISA kits, according to accompanying protocol (R & D Systems, Minneapolis, MN). After 6 days of incubation, splenocytes were harvested for flow cytometric analyses of CD3, CD4, γδ T cells (BD Biosciences), CD8, B cells, monocytes, CD44, CD62L, and CD25 expression (BioLegend, San Diego, CA). Data analyses were performed using FlowJo software (TreeStar, Inc., San Carlos, CA).

[0075] Antigen-specific IgG₁ and IgG_{2a} responses were measured in mouse sera by ELISA. Briefly, plates (Nunc MaxiSorp module, Nunc, Roskilde, Denmark) were coated with 200 ng/well of a whole

cell sonicate preparation of MAP (clinical strain 167, NADC) and incubated at 4 °C overnight. After blocking (1% BSA in TBST), diluted sera were added to the wells and incubated at 25 °C for 1 hr, followed by incubation with either horseradish peroxidase-conjugated goat anti-mouse IgG₁ or IgG_{2a} (Kamiya Biochemical Co., Seattle, WA) for 20 min, and 3,3', 5,5'-tetramethylbenzidine substrate solution (TMB; Kamiya Biomedical Co.) for 10 min. Absorbance at 450 nm was measured in a Victor X₃ Microplate reader (Perkin-Elmer, Shelton, CT).

Statistical analysis

[0076] Data were analyzed using PROC MIXED procedure of the Statistical Analysis System (SAS Inst., Inc., Cary, NC). The model included the fixed effects of treatment (vaccination), stimulation (in vitro treatment), and treatment x stimulation interaction. When significant effects ($P < 0.05$) due to treatment, stimulation, or treatment x stimulation interactions were detected, means separation was conducted by the Student's *t* test option in SAS. Designation of statistical significance within figures is described in each figure legend. Broad measures of statistical significance due to treatment or treatment x stimulation interactions may only be denoted within the text of the manuscript.

Results

Immugenicity of MAP protein candidates

[0077] The MAP proteins (MAP1087, MAP1204, MAP1272c, and MAP2077c) used in the present study to formulate vaccine cocktails demonstrated strong immunogenic potential, producing antigen-specific IFN- γ responses similar to or greater than the whole-cell sonicate of MAP (MPS) in Johne's subclinical and clinical cows (Figure 9).

Cytokine results

[0078] Results for Th1 and Th2-mediated cytokine secretion from splenocytes stimulated with either medium alone (NS) or with MPS are presented in Figure 10. Stimulation of cells with MPS resulted in an upregulation ($P < 0.05$) of IFN- γ compared to NS cultures in all infected groups. Vaccination with MAP protein cocktails reduced IFN- γ responses to MPS overall when compared to the control infected and 74F treatment groups ($P < 0.05$). Secretion of IL-12 followed a similar trend with greater responses noted for the control infected and 74F groups compared to the protein cocktail vaccinates (Figure 10B). MPS-stimulated splenocytes had greater ($P < 0.05$) IL-4 responses compared to NS cultures only in control infected mice (Figure 10C).

Interestingly, immunization with MAP protein cocktails resulted in increased ($P < 0.05$) IL-4 secretion in NS cultures for mice immunized with cocktails 1 and 2 compared to control infected mice. There were no significant effects due to vaccination on the secretion of IL-10, regardless of in vitro stimulation, however, ($P < 0.05$) differences between NS and MPS-stimulated cultures were observed for control infected mice (Figure 10D). Secretion of IL-2 and IL-23 were not influenced by vaccination

but an upregulation of IL-23 was observed by stimulation of splenocytes with MPS in all infected mice (data not shown).

T cell populations

[0079] Vaccination with protein cocktails 1-3 and 74F resulted in higher ($P < 0.05$) CD4 T cells compared to control infected mice, regardless of in vitro treatment (Figure 11A). In addition, immunization of mice with MAP protein cocktails (1-3) or 74F resulted in dramatic ($P < 0.05$) upregulation of CD4CD25 T cells in MPS-stimulated cultures compared to NS cultures (Figure 11B). There were no major differences in CD8 T cells due to vaccination of mice (Figure 3C), however, the number of CD8CD25 T cells was significantly ($P < 0.05$) upregulated in MPS-stimulated splenocytes across treatment groups (Figure 3D). There were no differences in the percentage of $\gamma\delta$ T cells and $\gamma\delta$ CD25+ cells due to vaccination, yet there was a consistent trend towards reduced numbers of these cell types after MPS stimulation of splenocytes in all treatment groups (data not shown). There was a trend for increased total CD25 T cells in mice vaccinated with MAP proteins or 74F although only mice in cocktail 4 had significantly ($P < 0.05$) higher CD25 T cells after MPS stimulation of splenocytes than control infected mice (8.30 ± 1.76 vs 5.46 ± 0.70 , respectively; data not shown). Total percentages of CD44, CD62L, and monocytes in splenocyte cultures were unaffected by immunization of mice or in vitro treatment in the present study (data not shown).

B cells and MAP-specific serum IgG₁ and IgG_{2a} results

[0080] Interestingly, the percentages of B cells within MPS-stimulated splenocytes were lower ($P < 0.05$) in mice immunized

with cocktails 1, 2, and 4, compared to control infected mice (data not shown). Infection with MAP with or without immunization resulted in significant ($P < 0.05$) increases in serum IgG₁ and IgG_{2a} compared to control noninfected mice (Figure 12). Mice immunized with either 74F or the MAP protein cocktails demonstrated increased ($P < 0.05$) MAP-specific serum IgG_{2a} following challenge compared to control infected mice. In contrast, differences due to vaccination were not noted for MAP-specific serum IgG₁ antibodies with similar levels noted for all infected mice regardless of vaccination when compared to control infected mice.

Tissue culture

[0081] Immunization with cocktails 1-4 did not significantly impact tissue colonization in the spleen after challenge with live MAP, yet there was a trend towards reduced MAP colonization noted in the cocktail 1 group compared to the control infected group (Figure 13A). Similar reductions in MAP colonization were also noted in the MLN of mice receiving cocktails 1 and 2 prior to challenge, although these differences did not achieve statistical significance (Figure 13C). However, liver colonization was reduced ($P < 0.05$) in mice immunized with cocktails 1 and 3, as well as the 74F polyprotein that served as a positive control in the study (Figure 13B). Further, all protein cocktails significantly ($P < 0.05$) reduced MAP colonization in the ileum compared to infected controls (Figure 13D). Although immunization with the combination of proteins in cocktail 4 (MAP1204, MAP1272c, and MAP2077c) provided less protection against MAP colonization compared to the 3 other cocktails, it was still effective in reducing tissue burden in the ileum.

Discussion

[0082] The functions of the four MAP proteins include a peptide transport system permease protein, MAP1087; putative invasion proteins, MAP1204 and MAP1272c; and a STAS domain containing protein, MAP 2077c. Two of the 4 selected proteins (MAP1087 and MAP1204) had previously demonstrated strong reactivity with sera from naturally infected cattle in the subclinical stage of infection (Bannantine *et al.* Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. *Proteome Sci.* 2008. 6:5-16). In addition, MAP1087, MAP1204, and MAP2077c all reacted with sera from experimentally infected calves in the early stage of infection (Bannantine *et al. ibid*). The selection of these proteins was not based upon specificity to MAP and protein sequence analysis demonstrated significant alignment of all 4 proteins to both MAP and *M. avium*. This is not surprising given the high level of genetic homology (>98%) between MAP and *M. avium*, with few unique genes identified for MAP and even fewer immunogens (Bannantine and Paustian, *ibid*; Bannantine *et al.* Development and use of a partial *Mycobacterium avium* subspecies *paratuberculosis* protein array. *Proteomics.* 2008. 8:463-474). Although partial sequences of MAP1204 were also found in other mycobacterial species, including *M. bovis*, this would not be a major detractor from potential use as a vaccine candidate. Sero-diagnostic tests for the detection of *M. bovis* infection using antigens such as ESAT-6, CFP-10, and MPB83, have demonstrated a lack of cross-reactivity in calves vaccinated with the whole-cell vaccine for MAP (Stabel *et al.* 2011. *ibid*). In addition, IFN- γ responses to ESAT-6:CFP-10 antigens were observed to be highly specific for calves infected with *M.*

bovis, with negligible reactivity noted for MAP- and *M. avium*-infected calves (Waters et al. Use of recombinant ESAT-6:CFP-10 fusion protein for differentiation of infections of cattle by *Mycobacterium bovis* and by *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. Clin Diagn Lab Immunol. 2004. 11:729-735) or for calves vaccinated with a whole cell vaccine (Stabel et al. 2011. *ibid*). This would suggest that adequate tools are available to distinguish between MAP vaccinates and animals infected with *M. bovis*.

[0083] Although it is unknown how well immunogenic proteins translate into successful vaccines, it is understood that proteins can be highly antigenic, eliciting both humoral and cell-mediated immune responses. Many researchers utilize immune reactivity in the host as their primary method to screen protein candidates for subunit vaccines, with the hypothesis that an induced response is suggestive of protective immunity. Although proteomic and genomic screening approaches can be used to identify vaccine candidates, these tools are only useful if the candidates are recognized by the host immune system. Screening tools such as Western blotting have been effective for many bacterial pathogens, however, for intracellular pathogens such as MAP, antigen screening should include some measure of responsiveness in a cell-mediated assay since protection is aligned with Th1-mediated immunity in the host (Stabel. Immunology of paratuberculosis infection and disease. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, Disease, Control. Cambridge, MA. CAB International. 2010. p. 230-243). The proteins in the present study were selected upon observation of robust IFN- γ responses in naturally infected cattle, combined

with the ability to discriminate between infected and noninfected cattle with some measure of specificity.

[0084] Numerous infection models have been developed for MAP, with emphasis on ruminant species such as cattle, sheep, goats, deer, and bison (Hines *et al.* Experimental challenge models for Johne's disease: a review and proposed international guidelines. Vet Microbiol. 2007. 122:197-222) since these are the target species for paratuberculosis. However, the protracted period of subclinical infection that occurs in naturally infected hosts is mimicked in experimentally infected animals, resulting in lengthy study periods (Hines *et al. ibid*; Begg and Whittington. Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. Vet J. 2008. 176:129-145). Mouse models provide a reasonable approach to efficiently evaluate vaccine candidates due to a shorter infection periods compared to ruminants, greater reproducibility due to more precise genetics amongst treatment animals, and reduced costs for care and housing, allowing for greater numbers of animals per treatment group. Mouse models for MAP infection have been adequately characterized and IP infection of Balb/c mice results in effective colonization of the major target tissues (Talaat. Experimental small animal models of paratuberculosis. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, Disease, Control. Cambridge, MA CAB International. 2010. p. 223-227).

[0085] Th1-mediated immune responses may be indicative of exposure to mycobacterial pathogens, including MAP, but also appear to be essential to keep infection from progressing from subclinical to clinical disease. In a neonatal calf infection model, we were able to demonstrate the upregulation of immune

markers including, robust antigen-specific IFN- γ responses as well as induction of antigen-specific CD25, CD26, and CD45RO expression less than 3 months after infection (Stabel and Robbe-Austerman. Early immune markers associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in a neonatal calf model. Clin Vaccine Immunol. 2011. 18:393-405). Although Th2 responses are not known to be protective in the host, both Th1- and Th2-mediated immunity has been induced after vaccination with a whole cell preparation of MAP (Gillan et al. Ovine immune parameters following immunization against *Mycobacterium avium* ssp. *paratuberculosis* using a lipid-based live-cell vaccine. Vet Immunol Immunopathol. 2010. 109-119; Platt et al. Evaluation of the cell-mediated immune response to reduced doses of *Mycobacterium avium* ssp. *paratuberculosis* vaccine in cattle. Vet Immunol Immunopathol. 2010. 122-126; and Kathaperumal et al. Evaluation of immune responses and protective efficacy in a goat model following immunization with a cocktail of recombinant antigens and a polyprotein of *Mycobacterium avium* subsp. *paratuberculosis*. Vaccine. 2009. 27:123-35). Recent studies have also demonstrated that MAP vaccines comprised of single proteins or protein complexes will also evoke strong Th1 responses (Koets et al. *ibid*; Chen et al. *ibid*; Kathaperumal et al. 2008. *ibid*; Kathaperumal et al. 2009. *ibid*; and Nguyen et al. Immune response of cattle immunized with a conjugate of the glycolipid glucose monomycolate and protein. Vet Immunol Immunopathol. 2011. 142:265-70). In the present study, immunization of mice with MAP protein cocktails prior to challenge with live MAP resulted in similar induction of antigen-specific IFN- γ when compared to control infected mice, although some attenuation of the IFN- γ response was noted. Differences in IFN- γ secretion between NS and MPS-stimulated

cultures were lower for mice vaccinated with protein cocktails, particularly cocktail 1. This would suggest that immunization with this triad of proteins may have resulted in greater constitutive secretion of IFN- γ but lower antigen-specific IFN- γ secretion after challenge, an effect that may be advantageous to the host. Pro-inflammatory effects of IFN- γ can be both beneficial and detrimental to the host and a finite balance must be achieved in order to maintain effective immunity (Schroder *et al.* Interferon-gamma: an overview of signals, mechanisms, and functions. *J Leuko Biol.* 2004. 75:163-9). Interleukin-23 was upregulated by MAP infection, with increased secretion noted after stimulation with MPS antigen in vitro for all treatment groups. This is the first study describing effects of MAP infection on the secretion of IL-23. IL-23 is a newly recognized cytokine that is involved in the inflammatory response to mycobacterial infections, although little is known about its role in the immunopathology of MAP infections. IL-23 is required for the generation of effector memory T cells and is also needed for generation of IL-17-producing T cells, which play an important role in the inflammatory response (Khader and Cooper. IL-23 and IL-17 in tuberculosis. *Cytokine.* 41:79-83).

[0086] Although Th2-mediated cytokine production was not markedly different due to vaccination, an interesting observation was the pattern of IL-4 secretion noted between NS and MPS-stimulated splenocytes in mice vaccinated with MAP protein cocktails, particularly for mice immunized with cocktail 1. Vaccination with MAP proteins 1087, 1204, and 1272c, in cocktail 1 resulted in higher constitutive secretion of IL-4 compared to the other treatment groups, something that was not apparent upon further exposure of splenocytes to MAP antigen in

vitro. These results would suggest that this cocktail of MAP proteins modulated Th2 responses of the host after infection, an important consideration for selection of vaccine candidates. This is further substantiated by the increase in MAP-specific serum IgG_{2a} noted for mice immunized with either 74F or MAP protein cocktails. Secretion of IgG_{2a} antibodies is most closely associated with Th1-mediated immunity and cytokines such as IFN- γ , IL-4, and IL-10 can influence the isotype switch to IgG_{2a} (Stevens *et al.* Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature*. 1998. 334:255-258).

[0087] Immune responses to vaccination are critical for the control of infection in the host. Immunization with the MAP protein cocktails effectively reduced MAP colonization of the liver and ileum. In particular, cocktail 1 also demonstrated efficacy for reduced colonization of the spleen and mesenteric lymph node, providing the most consistent effect on retardation of infection in the host. Reduced colonization of tissues is a beneficial characteristic for paratuberculosis vaccines and has been reported for commercial vaccines such as Mycopar and Gudair, as well as for more recently developed subunit vaccines (Koets *et al. ibid*; Kathaperumal *et al.* 2008. *ibid*; Patton. *Vet Clin North Am Food Anim Pract.* 2011. 27:573-580; Reddacliff *et al.* Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Vet Micro.* 2006. 115:77-90). Decreased tissue burdens result in reduced shedding of MAP in the feces thereby allaying spread of infection within a herd (Kalis *et al.* Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. *Am J Vet Res.* 2001. 62:270-

274). Similar reductions in tissue burden have been reported for mice immunized with the 74F polyprotein, with reduced recoveries of viable MAP from spleen, liver and mesenteric lymph nodes at 12 and 16 weeks post-challenge (Chen *et al. ibid*). The highly positive results achieved with the 74F polyprotein prompted us to incorporate it into our study as a positive control. The Mycopar vaccine was an undesirable choice as a positive control vaccine in the current study as effects have not been previously evaluated in a mouse model. In addition, the Mycopar vaccine typically causes large granulomatous nodules at the injection site in ruminants, an effect that would not be handled well in a young mouse (Patton. *ibid*). In ruminants, this local inflammatory effect is managed somewhat by injecting the vaccine in the fatty area of the brisket. Much of the benefit previously noted for this immunogen was repeated in the present study, with concurrent reductions in tissue colonization and activation of T cells as previously described. Despite this, cocktail 1 seemed to invoke the most consistent responses in protection against tissue colonization. Further, this triad of MAP proteins, 1087, 1204, and 1272c, appeared to more tightly regulate the immune response post-immunization both before and after challenge with live MAP. Host responses after immunization with either cocktail 2 or 3 more closely aligned themselves to results observed for cocktail 1. The common protein between these 3 cocktails was MAP1087, whose known function is a peptide transport system permease protein.

[0088] In summary, the present study evaluated cocktails of MAP proteins as potential subunit vaccines for paratuberculosis. Cocktails of MAP proteins proved effective in protection against

tissue colonization and invoked cell-mediated and humoral immunity in the host.

[0089] It is understood that the foregoing detailed description is given merely by way of illustration and that modifications and variations may be made therein without departing from the spirit and scope of the invention.

We claim:

1. A composition comprising immunogenic proteins of *Mycobacterium avium* subspecies *paratuberculosis* and a pharmaceutically acceptable carrier, wherein said proteins are provided in an amount effective to stimulate an immune response in an animal against said *Mycobacterium avium* subspecies *paratuberculosis*, and said proteins comprise MAP1087 which comprises SEQ ID NO: 1 and at least two of MAP1204 which comprises SEQ ID NO: 2, MAP1272c which comprises SEQ ID NO: 3 and MAP2077c which comprises SEQ ID NO: 4.

2. The composition of claim 1 further comprising an adjuvant.

3. The composition of claim 1 wherein said proteins comprise MAP1087, MAP1204 and MAP1272c.

4. The composition of claim 1 wherein said proteins comprise MAP1087, MAP1204 and MAP2077c.

5. The composition of claim 1 wherein said proteins comprise MAP1087, MAP1272c and MAP2077c.

6. The composition of claim 1 wherein said proteins comprise MAP1087, MAP1204, MAP1272c and MAP2077c.

7. The composition of claim 1 wherein said MAP1087 comprises SEQ ID NO: 5, said MAP1204 comprises SEQ ID NO: 6, said MAP1272c comprises SEQ ID NO: 7 and said MAP2077c comprises SEQ ID NO: 8.

8. A method for protecting an animal against infection with *Mycobacterium avium* subspecies *paratuberculosis* comprising administering the composition of claim 1 to said animal.

9. The method of claim 8 wherein said animal is a ruminant.

10. The method of claim 9 wherein said animal is selected from the group consisting of bovine, ovine and caprine.

11. The method of claim 8 wherein said composition further comprises an adjuvant.

12. The method of claim 8 wherein said proteins of said composition comprise MAP1087, MAP1204 and MAP1272c.

13. The method of claim 8 wherein said proteins of said composition comprise MAP1087, MAP1204 and MAP2077c.

14. The method of claim 8 wherein said proteins of said composition comprise MAP1087 and MAP1272c and MAP2077c.

15. The method of claim 8 wherein said proteins of said composition comprise MAP1087, MAP1204, MAP1272c and MAP2077c.

16. The method of claim 8 wherein said MAP1087 comprises SEQ ID NO: 5, said MAP1204 comprises SEQ ID NO: 6, said MAP1272c comprises SEQ ID NO: 7 and said MAP2077c comprises SEQ ID NO: 8.

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MLGYVLARIGQSAIVLLAVFSLVFWGVSILPADPAAIFVAKGEGYFNPDIVAQVKAFYGY
DRPLWVQYFAQLNQVLHGFGFSLSSGQAVTDRIGGVIGETLKLAATATGFAVLFAVSVT
ALATTCAPVRSVLRAIPPLFGAVPTF

Figure 1A

MKIEEGKLVIWINGDKGYNGLAIEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQS
GLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSAL
MFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVNAGAKAGLTFLVDLIKKNHNMADTDYSIAEAAFNKGE
TAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAA SPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNI PQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSS
SNNNNNNNNNNLGI EGRISEFGSSRMLGYVLARIGQSAIVLLAVFSLVFWGVSILPADPAAIFVAKGEGYFNP
IVAQVKAFYGYDRPLWVQYFAQLNOVLHGFGFSLSSGQAVTDRIGGVIGETLKLAATATGFAVLFAVSVTALA
TTCAPVRSVLRAIPPLFGAVPTF*

Figure 1B

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MRRNRFRLLIVFAWITAMVTGLMFSVAPTPAALADPGEWDPTLPAQISAGAPGDPLAVANA
SLQATAQATQTTLNLGKQFLGGLGINLGGNDAPAAAAATPSNPGGKIPRVYGRQAIEYVIK
RMGSQMGVPYSWGGSGLDGPSKGVGDGANITGFDCSGLMRYGFAGVGVLIPRFSGDQYNA
GRHIPQDQARRGDLIFYGPGGSQHVTMYLNGQMLEASGSAGKVTVSPVRKPGMTPFLTRIIEY

Figure 2A

MKIEEGKLVIWINGDKGYNGLAIEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQS
GLLAIEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSIIYNKDLLPNPPKTWEEIPALDKELKAKGKSAL
MFNLQEPYFTWPLIAADGGYAFKYENKDYDIKDVGVNDAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGE
TAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKSYYYELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSS
SNNNNNNNNNNNLGIEGRISEFGSSRMRRNRFRLLIVFAWITAMVTGLMFSVAPTPAALADPGEWDPTLPAQISAG
APGDPLAVANASLOATAQATQTTLNLGKQFLGGLGINLGGNDAPAAAAATPSNPGGKIPRVYGRQAIEYVIKRMG
SOMGVPIYSWGGSGLDGPSKGVGDGANITGFDCSGLMRYGFAGVGVLIPRFSGDQYNAGRHIPQDQARRGDLIFY
GPGGSQHVTMYLNGQMLEASGSAGKVTVSPVRKPGMTPFLTRIIEY*

Figure 2B

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VRSQRGGPRPVHEPGRTREVTAPRPDECRRGQERPGMKMKRIYAF AIGLALLGAPAAPMVV
PPVATADPGVRAMDYQQATDVV IARGLSQRGV PFSWAGGGINGPTRGTGTGANTVGF DAS
GLMQYAYAGAGIKLPRSSGAMYRVGQKILPQQARKGDLIFYGPEGTQSVAMYLGNNO MLE
VGDDVVQVSPVRTAGMAPYMRVLGTTAPTQQVPQQAPLQQTTPAQQAPLQQTTPGQQAPLQQ
TPGQQLPQTQQAPLQQVPGQQVPGQQLPQTQQAPQQAPLQLAPTQQAPLQQLPQTQQSPLQQ L
PVQQSPLQPAGAGLTR

Figure 3A

MKIEEGKLVIWINGDKGYNGLA EVGKKFEKDTGIKVTVEHPDKLEEF PQVAATGDGPD IIFWAHDRFGGYAQS
GLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALS LIYNKDLLPNPPKTWEEIPALDKELKAKGKSAL
MFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGV DNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGE
TAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAA SPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKS YEEELAKDPRI AATMENAQKGEIMPNI PQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSS
SNNNNNNNNNNL GIEGRISEFGSSRVRSQRGGPRPVHEPGRTREVTAPRPDECRRGQERPGMKMKRIYAF AIGLA
LLGAPAAPMVVPPVATADPGVRAMDYQQATDVV IARGLSQRGV PFSWAGGGINGPTRGTGTGANTVGF DASGLM
QYAYAGAGIKLPRSSGAMYRVGQKILPQQARKGDLIFYGPEGTQSVAMYLGNNO MLEVGDVVQVSPVRTAGMAP
YMRVLGTTAPTQQVPQQAPLQQTTPAQQAPLQQTTPGQQAPLQQTTPGQQLPQTQQAPLQQVPGQQVPGQQLPQTQA
PQQAPLQLAPTQQAPLQQLPQTQQSPLQQLPVQOSPLQPAGAGLTR*

Figure 3B

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MVTPLTLDTGRGSDGNPVLVAVGEIDLSNIDAFHRLATATAEVTGSDGAVLVDLSAVEY
VDSAAINALAAHADHIALVAHPVLMFVFRVSGLTELTVEAAPPPAPR

Figure 4A

MKIEEGKLVIWINGDKGYNGLAEVGGKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQS
GLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSAL
MFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVNAGAKAGLTFLVDLIKKNHNMNADTDYSIAEAAFNKGE
TAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAA SPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKSYYYEELAKDPRIAATMENAQKGEIMPNI PQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSS
SNNNNNNNNNNLGI EGRISEFGSSRMVTPLTLDTGRGSDGNPVLVAVGEIDLSNIDAFHRLATATAEVTGSDG
AVLVDLSAVEYVDSAAINALAAHADHIALVAHPVLMFVFRVSGLTELTVEAAPPPAPR*

Figure 4B

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ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGG
TAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATCCCAC
AGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGGTACGCTCAATCT
GGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACG
TTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGC
CGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAAGCTGAAAGCGAAAAGGTAAGAGCGCGCTG
ATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGA
AAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTG
ACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAA
ACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAAACGGT
ACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTC
CGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACCTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAATAAA
GACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCAC
TATGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTA
CTGCGGTGATCAACGCCGCCAGCGGTGCTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGC
TCGAACAACAACAATAACAATAACAACCTCGGGATCGAGGGAAGGATTTCAGAATTCGGATCCTCTAG
AATGCTCGGCTACGTTTCTCGCCCGGATCGGCCAGTCGGCCATCGTGCTGCTGGCGGTCTTCAGCCTGGTGTCT
GGGGCGTCAGCATCCTGCCGGCCGATCCGGCGGCGATCTTCGTGGCCAAGGGGGAGGGCTACTTCAACCCCGAC
ATCGTCGCGCAGGTCAAGGCGTTCTACGGCTACGACCGGCCGCTGTGGGTGCAGTACTTCGCGCAGCTGAACCA
GGTGCTGCACGGGCATTTTCGGCTTCTCGCTGTCCAGCGGTTCAGGCCGTCACCGACCGGATCGGCGGGGTGATCG
GCGAGACCCTGAAACTGGCGGCCACCGCCACCGGGTTCGCGGTGCTGTTTCGCGGTGTCGGTCAACGCGCTGGCG
ACCACCTGCGCGCCGGTGCGGTGCTGCGCGCGATCCCGCCGCTGTTTCGGCGCGGTCCCCACGTTTTGA

Figure 5

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ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGG
TAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCAC
AGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCT
GGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACG
TTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGC
CGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAAGCTGAAAGCGAAAAGGTAAGAGCGCGCTG
ATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGA
AAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTG
ACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAA
ACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAAACGGT
ACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTC
CGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACCTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAATAAA
GACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCAC
TATGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTCTGTTATGCCGTGCGTA
CTGCGGTGATCAACGCCGCCAGCGGTGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGC
TCGAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAAGGATTTCAGAATTCGGATCCTCTAG
AATGCGCCGCAACCGGTTTTCGTCTCATCGTCTTCGCTGCGCTGGATCACCGCGATGGTGACCGGGGTGATGTTTTCCG
TGGCACCGACGCCGCGCGCTGGCCGACCCCGGCGAGTGGGATCCCACCTGCCGGCCAGATCAGTGCCGGC
GCCCCGGGCGATCCGCTCGCCGTCGCCAACGCCTCGCTGCAGGCCACCGCGCAGGCCACCCAGACCACGCTGAA
CCTGGGCAAGCAATTCTCGGCGGGCTCGGCATCAACCTGGGCGGCAACGACGCGCCCGCGGCCGCGGCCACGC
CGTCCAACCCGGGCGGCAAGATCCCGCGGGTCTACGGCCGGCAGGCCATCGAGTACGTGATCAAGCGGATGGGG
TCGCAGATGGGGGTGCCGTACTCGTGGGGCGGCGGCTCGCTGGACGGTCCCAGCAAGGGTGTCGGCGACGGCGC
CAACATCACCGGGTTCGACTGCTCGGGGCTGATGCGCTACGGCTTCGCCGGGGTCGGCGTGCTGATCCCGCGGT
TCTCCGGCGACCAAGTACAACGCCGGGCGTCACATCCCGCAGGATCAGGCCCGCCGCGGCGACCTCATCTTCTAC
GGCCCGGGCGGGTCCCAGCACGTCACCATGTACCTGGGCAACGGGCAGATGCTCGAGGCGTCCGGCAGCGCCGG
CAAGGTCACCGTCAGCCCGGTGCGCAAGCCCGGCATGACACCGTTCTGACTAGGATCATCGAGTACTGA

Figure 6

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ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGG
TAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATCCCAC
AGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCT
GGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACG
TTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGC
CGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAAGCTGAAAGCGAAAGGTAAGAGCGCGCTG
ATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTATGCGTTCAAGTATGA
AAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCTTGTTG
ACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAA
ACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGT
ACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTC
CGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACCTATCTGCTGACTGATGAAGGTCTGGAAGCGGTAAATAAA
GACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCAC
TATGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTCTGTTATGCCGTGCGTA
CTGCGGTGATCAACGCCGCCAGCGGTGTCGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGC
TCGAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAAGGATTTCAGAATTCGGATCCTCTAG
AGTGCGATCCCGAGCGTGGGGGTCTCTCGCCCGGTCCATGAACCGGGGCGGACGCGCGAGGTACCCGCGCCGAGGC
CCGACGAGTGCCGGAGAGGCCAGGAAAGGCCAGGAAAAATGAAACGCATCTACGCCTTCGCGATCGGTTTGGCC
CTACTCGGGGCGCCGGCGCGCGATGGTGGTTCCCCCGTTCGCGACCGCCGACCGGGCGTCAGGGCGATGGA
CTATCAGCAGGCCACCGACGTGGTGATCGCGCGCGGTCTGTGCGCAGCGCGGTGTGCCGTTCTCCTGGGCCGGCG
GCGGCATCAACGGCCCCACCCGCGGCACCGGGACCGGCGCCAACACCGTCCGTTTCGACCGGTCCGGGCTGATG
CAGTACGCGTACGCCGGCGCCGGCATCAAGCTGCCGCGCTCGTCCGGCGCGATGTACCGCGTCGGCCAGAAGAT
CCTGCCGAGCAGGCCCGCAAGGGTGACCTGATCTTCTACGGCCCCGAGGGCACCCAGAGCGTCGCAATGTACC
TGGGCAACAACCAGATGCTCGAGGTGCGCGACGTGGTGCAGGTGTGCGCCGTGCGTACCGCCGGCATGGCGCCC
TACATGGTCCGGGTGTTGGGAACACGGCGCCCACCCAGCAGGTTCCGCAGCAGGCGCCGCTGCAGCAGACCCC
GGCGCAGCAGGCGCCCTTGCAACAGACCCCGGGCCAGCAGGCGCCCTTGAGCAGACCCCGGGCCAGCAACTGC
CCACCCAGCAGGCCCCGCTGCAACAGGTTCCGGGGCAGCAGGTTCCGGGGCAGCAGCTGCCACCCAGCAAGCG
CCCCAGCAGGCACCCCTGCAGCTGGCGCCGACCCAGCAGGCGCCGCTGCAACAGCTGCCGACCCAGCAGTCACC
GCTGCAGCAGCTGCCGGTCCAGCAGTCGCCACTGCAGCCGGCGGGCGCGGACTCACCCGGTAG

Figure 7

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ATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGG
TAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCAC
AGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCT
GGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACG
TTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGC
CGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAAGCTGAAAGCGAAAAGGTAAGAGCGCGCTG
ATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGTTATGCGTTCAAGTATGA
AAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTG
ACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAA
ACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAAACGGT
ACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTC
CGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACCTATCTGCTGACTGATGAAGGTCTGGAAGCGGTAAATAAA
GACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCAC
TATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTCTGTTATGCCGTGCGTA
CTGCGGTGATCAACGCCGCCAGCGGTGTCGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGC
TCGAACAACAACAATAACAATAACAACAACCTCGGGATCGAGGGAAGGATTTCAGAATTCGGATCCTCTAG
AATGGTCACCCCGCTGACGCTGGACACCGGCCGTGGTAGCGACGGCAACCCGGTGCTGGTGGCAGTGGGCGAAA
TCGACCTGAGCAACATCGACGCATTCCACCGGGCGCTGGCCACCGCCACCGCGGAGGTCACCGGGAGTGACGGC
GCGGTGCTCGTCGACCTCAGCGCCGTGGAGTATGTGGACAGCGCCGCCATCAATGCGTTGGCCGCGCACGCCGA
CCACATCGCGCTCGTCGCGCACCCGGTCCTGATGCCCGTCTTCAGGGTCAGCGGTTTGACCGAGCTGACCACCG
TCGAAGCCGCACCCCCGCCGCCGGCGCCTCGTTGA

Figure 8

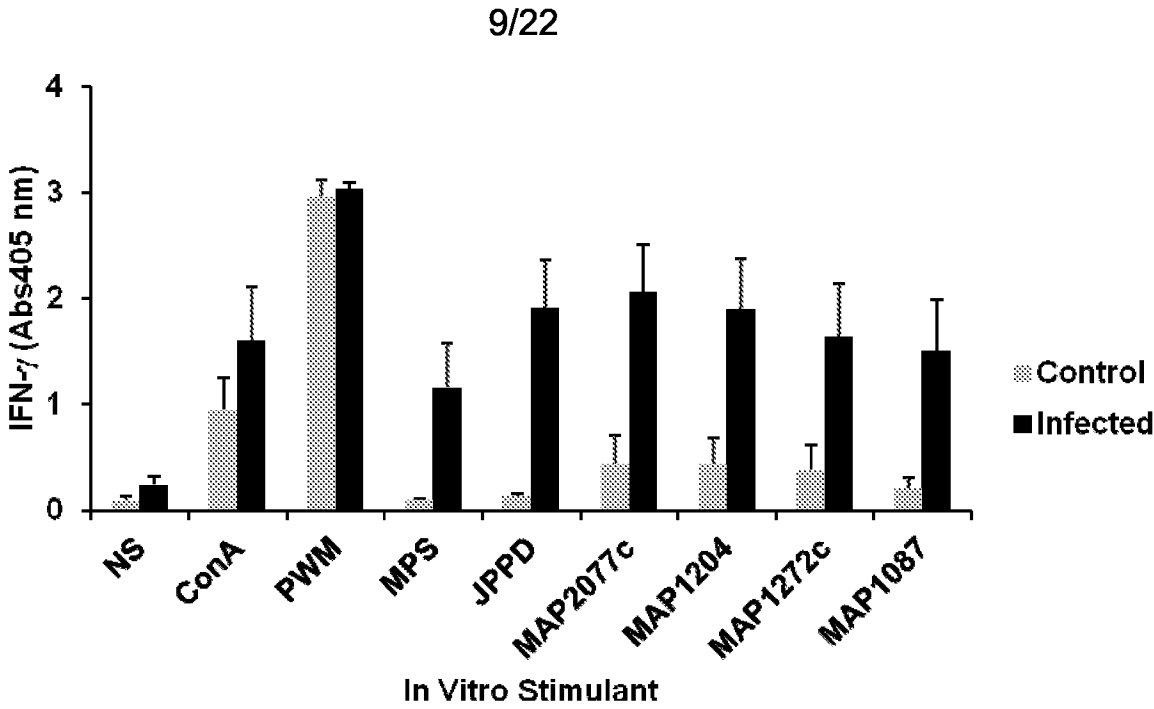
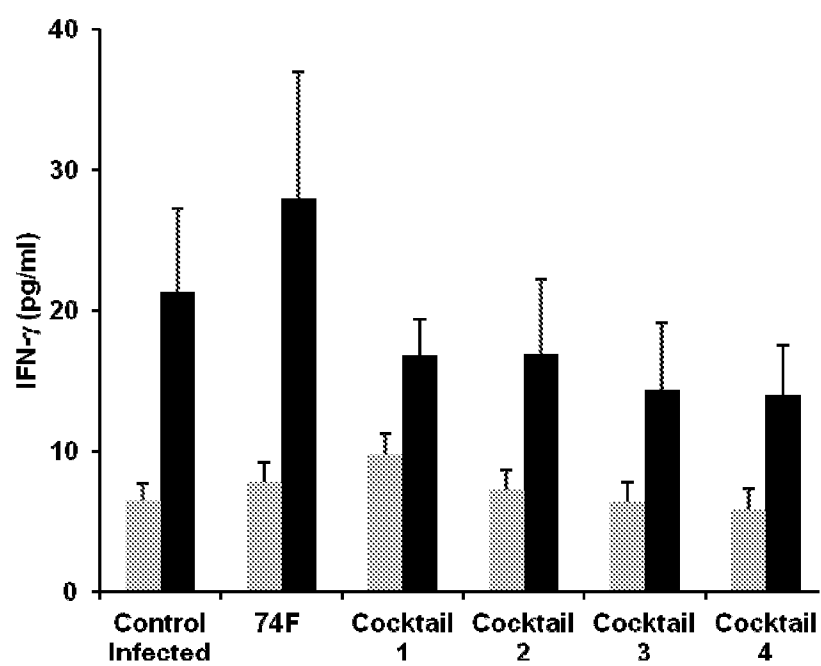


Figure 9

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**Figure 10A**

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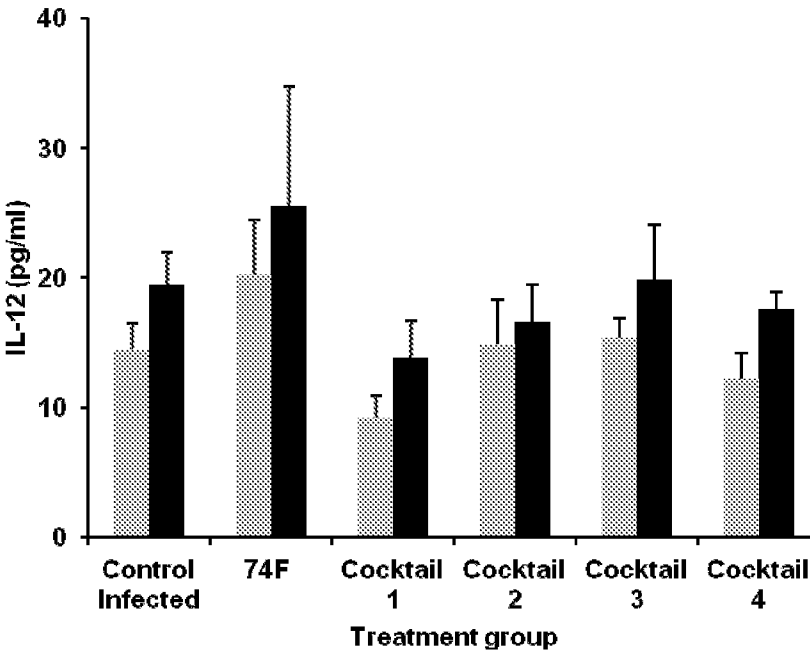
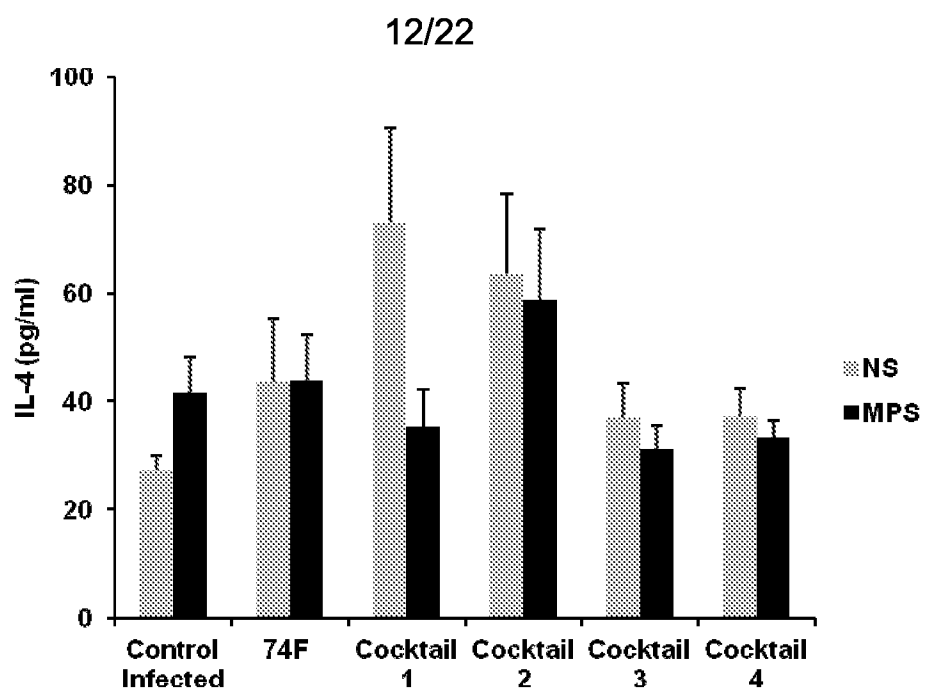
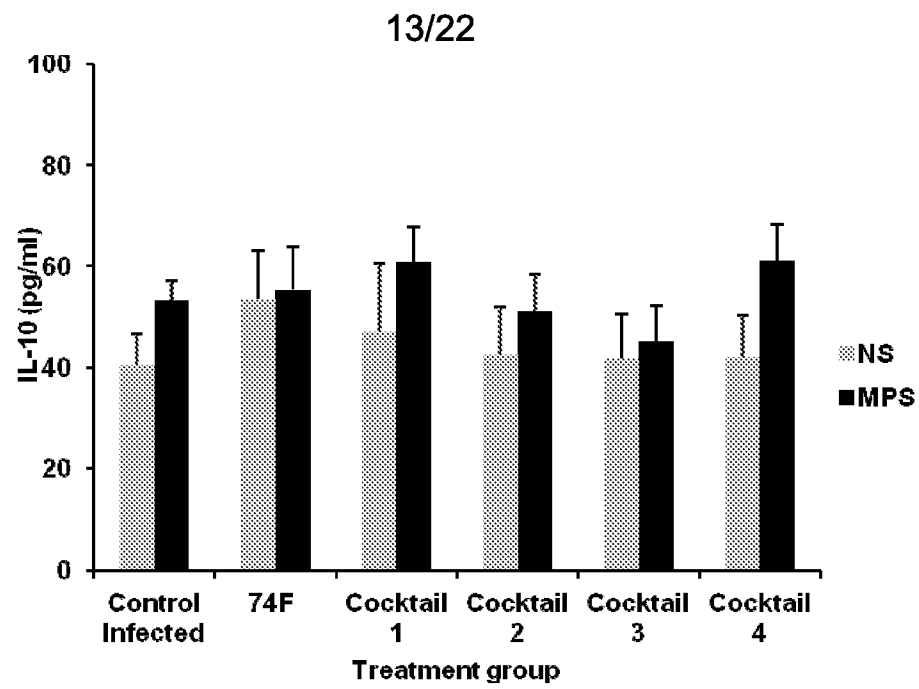


Figure 10B

**Figure 10C**

**Figure 10D**

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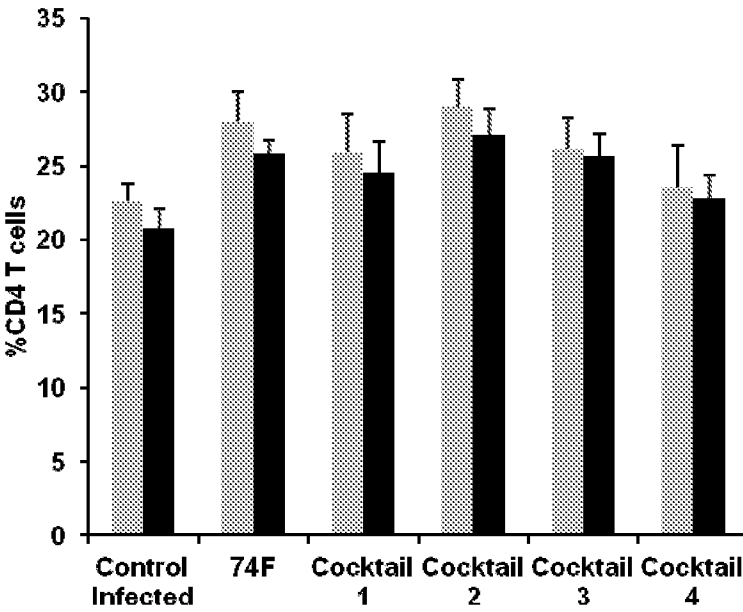


Figure 11A

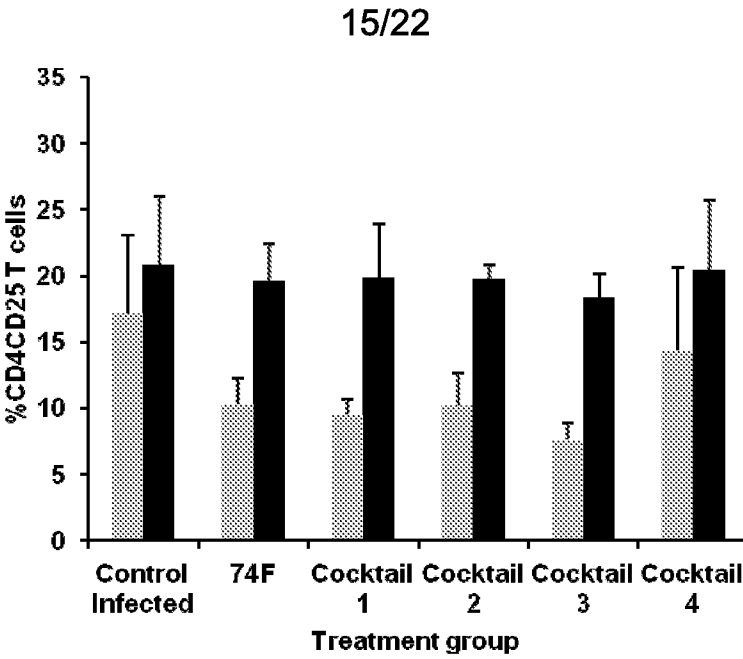


Figure 11B

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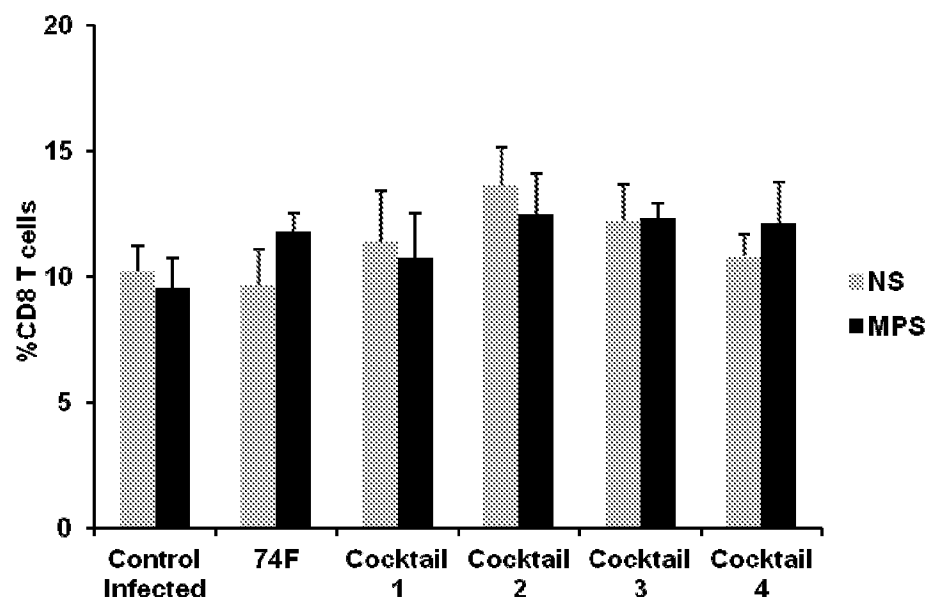


Figure 11C

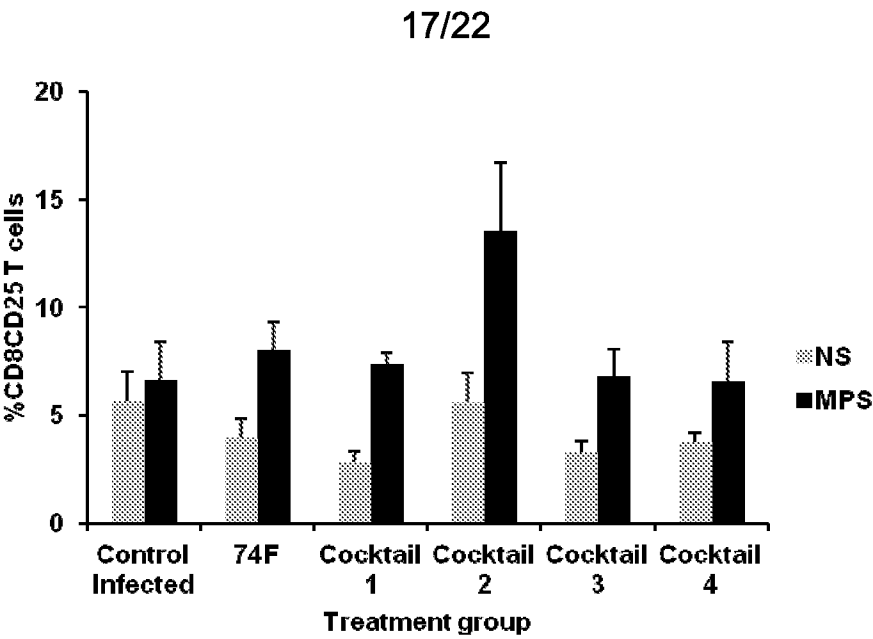
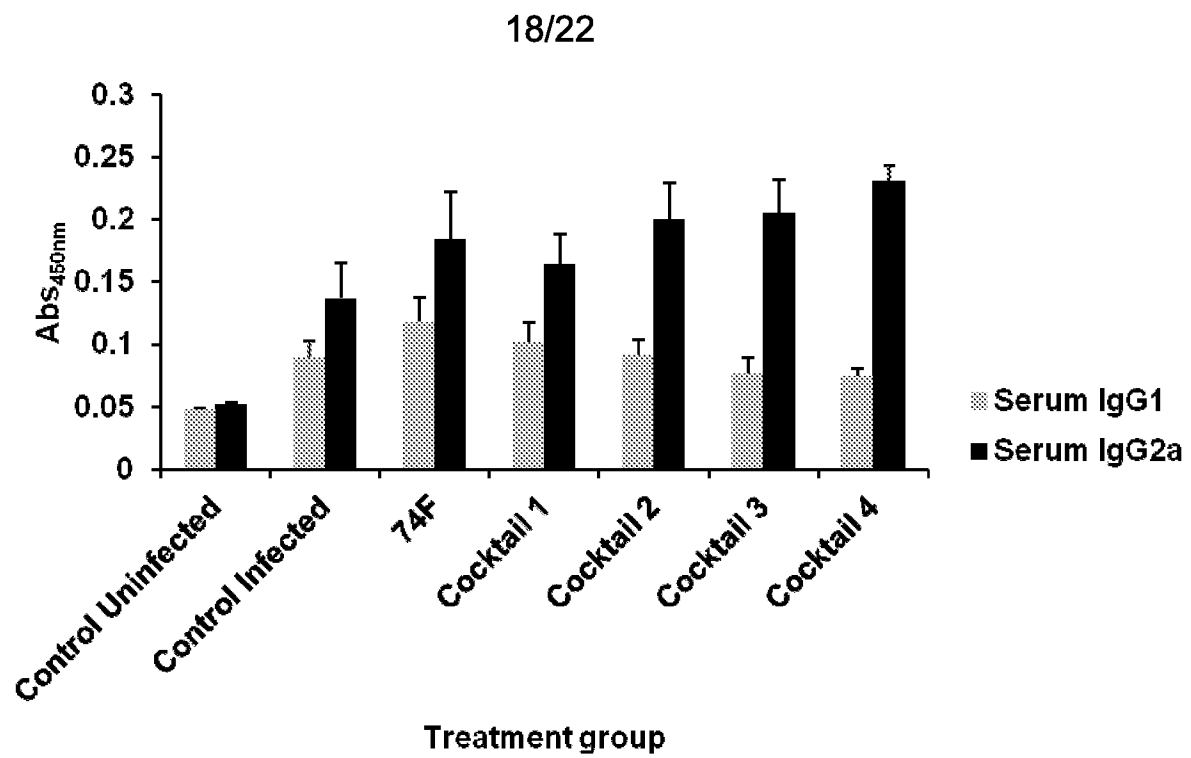
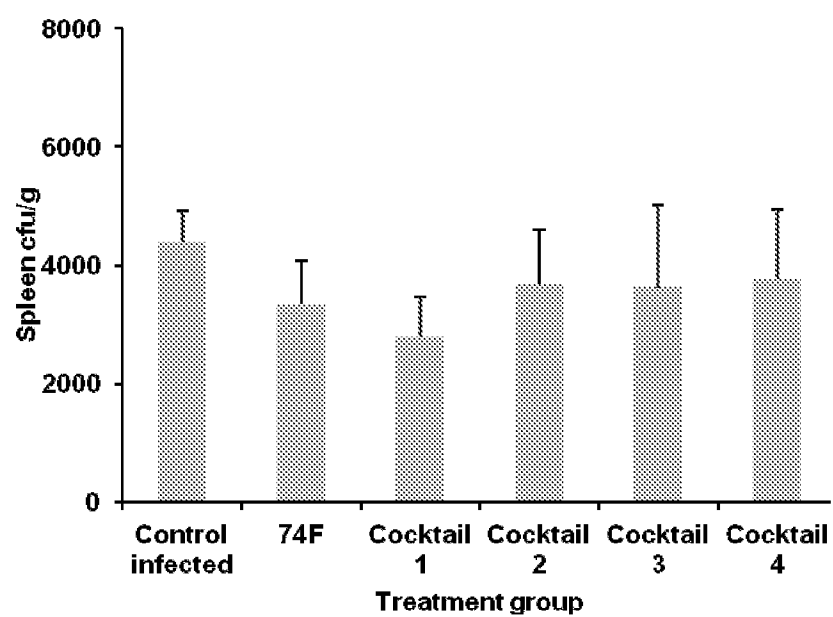


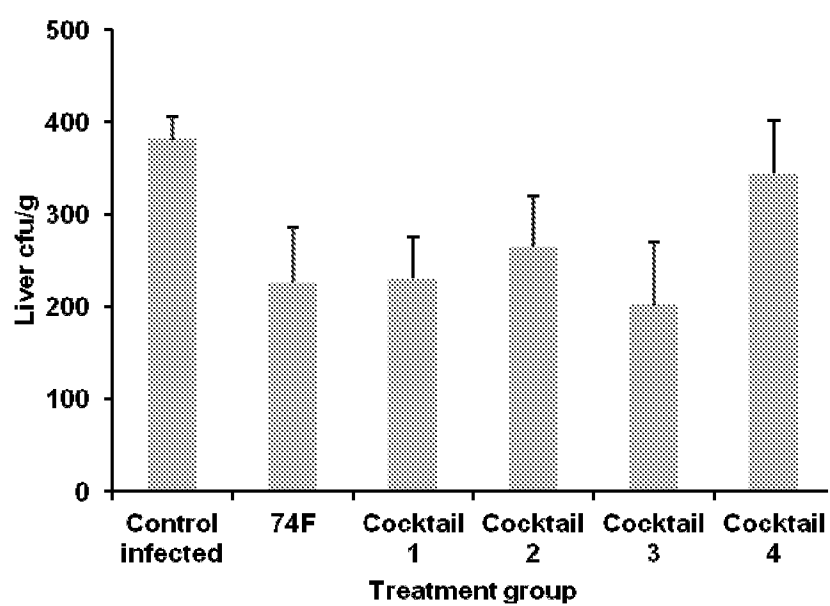
Figure 11D

**Figure 12**

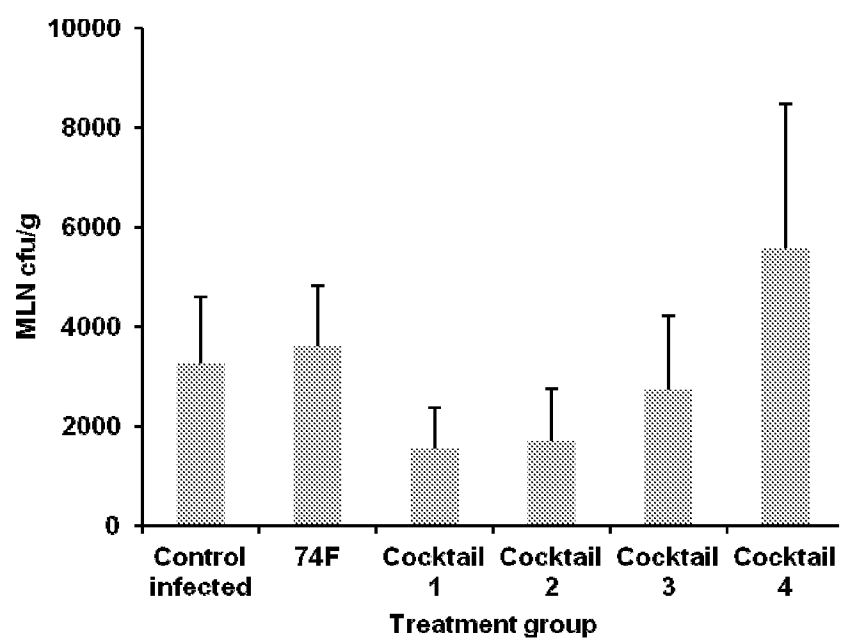
19/22

**Figure 13A**

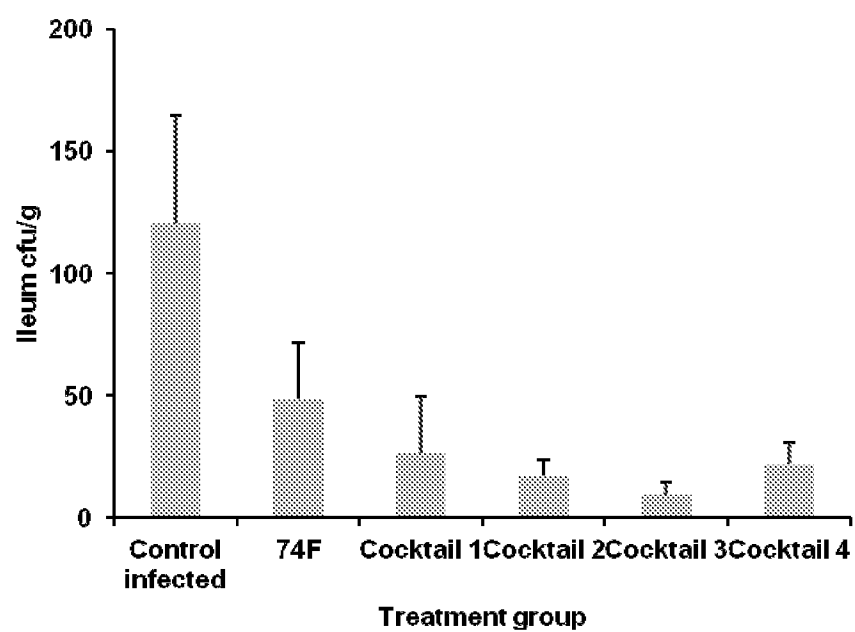
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**Figure 13B**

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**Figure 13C**

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**Figure 13D**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/065651**A. CLASSIFICATION OF SUBJECT MATTER****A61K 38/17(2006.01)i, A61K 38/16(2006.01)i, A61P 37/00(2006.01)i, A61P 31/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/17; A61K 39/02; C12N 1/36; A61K 39/00; C12N 1/12; A61K 48/00; A61P 37/04; A61K 38/16; C12Q 1/68; A61K 39/12; A61P 37/00; A61P 31/00

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: Mycobacterium avium, paratuberculosis, animal, immune response

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009-0099083 A1 (CHANG) 16 April 2009 See abstract, claims 1, 6, 9-10, 12-14 and paragraph [0011].	1-16
A	US 7867704 B2 (KAPUR et al.) 11 January 2011 See abstract, claim 1 and SEQ ID NOs: 4065, 3944, 3872, 3041.	1-16
A	US 2011-0274712 A1 (ALDWELL et al.) 10 November 2011 See claims 1, 13-17 and paragraphs [0072]-[0073], [0075].	1-16
A	WO 2010-051210 A1 (BOEHRINGER INGELHEIM VETMEDICA, INC.) 6 May 2010 See abstract and claims 1, 6-8, 10.	1-16
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A	WO 2010-124154 A1 (BOEHRINGER INGELHEIM VETMEDICA, INC.) 28 October 2010 See abstract and claims 11, 16-18.	1-16



Further documents are listed in the continuation of Box C.



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

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Date of the actual completion of the international search

19 December 2013 (19.12.2013)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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