(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 October 2015 (29.10.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/161853 A1

(51) International Patent Classification:
A61K 39/04 (2006.01) *C07K 14/35* (2006.01)

(21) International Application Number:
PCT/DK2015/050086

(22) International Filing Date:
9 April 2015 (09.04.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2014 00226 24 April 2014 (24.04.2014) DK

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

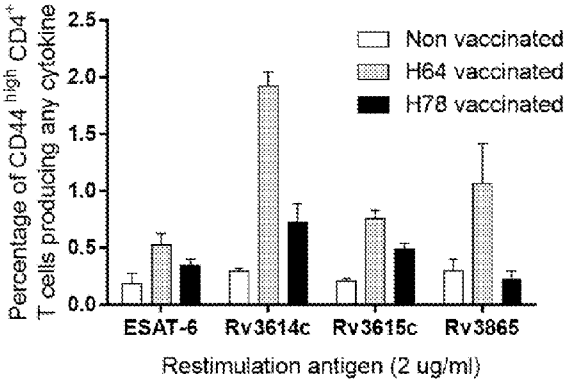
Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: NEW M.TUBERCULOSIS VACCINES

Figure 6



(57) Abstract: The present invention is directed to a fusion protein, antigen cocktails and immunological compositions such as vaccines against infections caused by virulent mycobacteria, e.g. by Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium microti, Mycobacterium canettii, Mycobacterium pinnipedii or Mycobacterium mungi. The fusion protein, antigen cocktails and immunological compositions are based on proteins secreted by the ESAT-6 secretion system 1 (ESX-1) and are among the most immunodominant M. tuberculosis (MTB) antigens.

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New M.tuberculosis vaccines

Field of invention

The present invention discloses new immunogenic compositions based on Esx-1 associated polypeptides derived from *M. tuberculosis*

General Background

Immunity to *M. tuberculosis* is characterized by some basic features; specifically sensitized T lymphocytes mediates protection, and the most important mediator molecule seems to be interferon gamma (IFN-γ).

M. tuberculosis holds, as well as secretes, several proteins of potential relevance for the generation of a new TB vaccine. In 1998 Cole et al. published the complete genome sequence of *M. tuberculosis* and predicted the presence of approximately 4000 open reading frames [1]. However importantly, this sequence information cannot be used to predict if the DNA is translated and expressed as proteins *in vivo*. The genome sequence has been used extensively to design DNA arrays for RNA expression analysis and in proteome studies to identify expressed proteins. Even with the vaste amount of expression data and the significant improvement of in silico prediction tools it is still not possible to predictic with certainty that a given sequence will encode an immunogenic molecule. The only way to determine if a molecule is recognized by the immune system during or after an infection with *M. tuberculosis* is to produce the given molecule and test it in an appropriate assay as described herein.

Currently there are several new TB vaccines in clinical trials. However, they are primarily classical preventive vaccines based on a limited number of antigens expressed in the early stage of infection. As a direct consequence of the expression dynamic the epitope pattern that is presented to T cells changes radically over time – implicating how new vaccines should be designed. E.g. for the transiently expressed early antigen, Ag85B, two independent T cell transfer studies have shown that 3-4 weeks after infection, Ag85B is no longer being presented to T cells and as a result there is no Ag85B specific production of cytokine's , chemokine's etc. at this or later time points of the infection [2,3]. Thus, it is of limited value for a chronic disease that establish long-term co-existence with the host to

vaccinate and induce memory T cells specific for epitopes in proteins that are only expressed during a brief period of the infection.

For vaccine development it is therefore vital to identify antigens that are highly expressed in the later stage of infection and among these select those that are immunogenic and can contribute to protection and include this special subset of proteins in TB vaccines. By doing so it is not only possible to improve vaccine potency and epitope coverage but also target latent infections.

Mycobacteria secretion systems are responsible for the export of proteins into the extracellular environment. The 6-kDa early secretory antigenic target of Mycobacterium tuberculosis (ESAT-6) and the 10-kDa culture filtrate antigen (CFP-10), are proteins secreted by the ESAT-6 secretion system 1 (ESX-1) and are among the most immunodominant *M. tuberculosis* (MTB) antigens. These attributes makes them important for tuberculosis (TB) vaccine development. Based upon this knowledge we tested other ESX-1 associated proteins as potential TB vaccine antigens.

Summary of the invention

The invention is related to preventing and treating infections caused by species of the tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum, M. microti, M. canettii, M. pinnipedii, Mycobacterium mungi*) by the use of a fusion protein or antigen cocktail comprising *M. tuberculosis* antigens selected from ESX-1 associated polypeptides. The fusion proteins or antigen cocktails are used in vaccines preferably together with an adjuvant and/or an immunemodulator.

Detailed disclosure of the invention

The invention discloses a fusion protein or antigen cocktail, which comprises the amino acid sequences selected from:

a) H74 = SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 3 (Rv3614c), SEQ ID NO 4 (Rv3615c), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or
b) H164 = SEQ ID NO. 2 (ESAT6), SEQ ID NO. 3 (Rv3614c), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or

c) H78 = SEQ ID NO. 2 (ESAT6), SEQ ID NO. 3 (Rv3614c), SEQ ID NO. 15 (part of Rv3615), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or
d) H174 = SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 3 (Rv3614c), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or
e) H264 = SEQ ID NO. 2 (ESAT6), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or
f) H274 = SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or
g) H374 = SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6) and SEQ ID NO 6 (Rv3849), or

an amino acid sequence analogue having at least 80% sequence identity hereto and at the same time being immunogenic.

The cysteines in the fusion protein according to the invention have preferably been replaced by another amino acid to avoid sulhur-bridge formation and protein aggregation. A preferred replacement amino acid is serine.

The fusion partners of the fusion protein according to the invention is preferably linked with a linker molecule to allow for protein folding and dimer formation.

A preferred embodiment is the fusion protein comprising SEQ ID NO 7 (H74), SEQ ID NO.9 (H164), SEQ ID NO 10 (H174), SEQ ID NO 11 (H264), SEQ ID NO 12 (H274), SEQ ID NO 13 (H374) or SEQ ID NO 14 (H78).

Another embodiment of the invention is using an antigen cocktail according to the invention e.g. the above mentioned amino acid sequences SEQ ID NOS. 1-6, SEQ ID NOS 2, 3, 6 and 8, SEQ ID NOS. 2, 3, 15, 6 and 8, SEQ ID NOS. 1, 2, 3, 5 and 6, SEQ ID NOS 2, 6 and 8, SEQ ID NOS 1,2 5 and 6 or SEQ ID NOS 1, 2 and 6 without fusing the polypeptides together.

In a still further embodiment, the invention discloses an immunogenic composition comprising a fusion protein or antigen cocktail as defined above, preferably in the form of a vaccine.

In another embodiment, the invention discloses a method for immunising an animal, including a human being, against tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum,Mycobacterium bovis M. microti, M. canettii, M. pinnipedii or Mycobacterium mungi*, comprising administering to the animal the polypeptide as defined above, the immunogenic composition according to the invention, or the vaccine according to the invention.

The vaccine, immunogenic composition and pharmaceutical composition according to the invention can be used prophylactically in a subject not infected with a virulent mycobacterium or therapeutically in a subject already infected with a virulent mycobacterium.

Definitions

Polypeptides

The word "polypeptide" in the present invention should have its usual meaning. That is an amino acid chain of any length, including a full-length protein, oligopeptides, short peptides and fragments thereof, wherein the amino acid residues are linked by covalent peptide bonds.

The polypeptide may be chemically modified by being glycosylated, by being lipidated (e.g. by chemical lipidation with palmitoyloxy succinimide as described by Mowat et al. 1991 or with dodecanoyl chloride as described by Lustig et al. 1976), by comprising prosthetic groups, or by containing additional amino acids such as e.g. a purification tag (e.g. his-tag) or a signal peptide. Purification tag's are used to obtain highly pure protein preparations and for e.g the His-tag comprises a methionine as the first animo acid followed by 6-8 histidines if used N-terminal, and 6-8 histidines followed by a STOP-codon if used C-terminal. When used N-terminal the methionine start codon in the gene coding for the polypeptide fusion can be deleted to avoid false translational start sites. The same is true if the gene contains one of the alternative start codons GUG or UUG which normally codes for valine and leucine, respectively, but, as a start codon, they are translated as methionine or formylmethionine.

Each polypeptide is encoded by a specific nucleic acid sequence. It will be understood that such sequences include analogues and variants hereof wherein such nucleic acid sequences have been modified by substitution, insertion, addition or deletion of one or more

nucleic acid. Substitutions are preferably silent substitutions in the codon usage which will not lead to any change in the amino acid sequence, but may be introduced to enhance the expression of the protein.

Secretion systems

Type VII secretion system (T7SS) is a recent discovery in bacterial secretion systems that was first identified in *Mycobacterium tuberculosis*. The corresponding gene clusters were referred to as the ESX (ESAT-6 Secretion System) regions [4-6]. The genome of *M. tuberculosis* H37Rv contains five gene clusters that have evolved through gene duplication events and include components of the T7SS secretion machinery. These clusters are called ESAT-6 secretion system (ESX) 1 through 5. The ESX systems have been shown to secrete proteins lacking classical signal peptides. Furthermore, most of the proteins secreted by ESX1-5 follow a pairwise dependency for secretion [7].

Esx-family

Except for Rv3017c (esxR) the genes encoding the ESAT-6 family proteins are arranged in tandem pairs at 11 loci on the *M. tuberculosis* H37Rv chromosome and are often preceded by a *pe-ppe* gene pair. They encode proteins that are approximately 100 amino acids in length and are secreted by the ESX1-5 systems

Throughout this specification, unless the context requires otherwise, the word “comprise”, or variations thereof such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

An immunogenic polypeptide is defined as a polypeptide that induces an immune response in a biological sample or an individual currently or previously infected with a virulent mycobacterium.

The immune response may be monitored by one of the following methods:

- An in vitro cellular response is determined by release of a relevant cytokine such as IFN-γ, from lymphocytes withdrawn from an animal or human being currently or previously infected with virulent mycobacteria, or by detection of proliferation of these T cells. The induction being performed by the addition of the polypeptide or the immunogenic portion to a suspension comprising from $1x10^5$ cells to $3x10^5$ cells per

well. The cells being isolated from either the blood, the spleen, the liver or the lung and the addition of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml suspension and the stimulation being performed from two to five days. For monitoring cell proliferation the cells are pulsed with radioactive labeled Thymidine and after 16-22 hours of incubation detecting the proliferation by liquid scintillation counting. A positive response being a response more than background plus two standard deviations. The release of IFN-γ can be determined by the ELISA method, which is well known to a person skilled in the art. A positive response being a response more than background plus two standard deviations. Other cytokines than IFN-γ could be relevant when monitoring the immunological response to the polypeptide, such as IL-12, TNF-α, IL-4, IL-5, IL-10, IL-6, TGF-β. Another and more sensitive method for determining the presence of a cytokine (e.g. IFN-γ) is the ELISPOT method where the cells isolated from either the blood, the spleen, the liver or the lung are diluted to a concentration of preferable of 1 to 4 x 10^6 cells /ml and incubated for 18-22 hrs in the presence of of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml. The cell suspensions are hereafter diluted to 1 to 2 x 10^6/ ml and transferred to Maxisorp plates coated with anti–IFN-γ and incubated for preferably 4 to 16 hours. The IFN-γ producing cells are determined by the use of labelled secondary anti-IFN-γ antibody and a relevant substrate giving rise to spots, which can be enumerated using a dissection microscope. It is also a possibility to determine the presence of mRNA coding for the relevant cytokine by the use of the PCR technique. Usually one or more cytokines will be measured utilizing for example the PCR, ELISPOT or ELISA. It will be appreciated by a person skilled in the art that a significant increase or decrease in the amount of any of these cytokines induced by a specific polypeptide can be used in evaluation of the immunological activity of the polypeptide.

- An *in vitro* cellular response may also be determined by the use of T cell lines derived from an immune individual or an *M. tuberculosis* infected person where the T cell lines have been driven with either live mycobacteria, extracts from the bacterial cell or culture filtrate for 10 to 20 days with the addition of IL-2. The induction being performed by addition of not more than 20 μg polypeptide per ml suspension to the T cell lines containing from 1x10^5 cells to 3x10^5 cells per well and incubation being performed from two to six days. The induction of IFN-γ or release of another relevant cytokine is detected by ELISA. The stimulation of T cells can also be monitored by detecting cell proliferation using radioactively labeled Thymidine as described

above. For both assays a positive response being a response more than background plus two standard deviations.

- An *in vivo* cellular response which may be determined as a positive DTH response after intradermal injection or local application patch of at most 100 μg of the polypeptide or the immunogenic portion to an individual who is clinically or subclinically infected with a virulent Mycobacterium, a positive response having a diameter of at least 5 mm 72-96 hours after the injection or application.

- An *in vitro* humoral response is determined by a specific antibody response in an immune or infected individual. The presence of antibodies may be determined by an ELISA technique or a Western blot where the polypeptide or the immunogenic portion is absorbed to either a nitrocellulose membrane or a polystyrene surface. The serum is preferably diluted in PBS from 1:10 to 1:100 and added to the absorbed polypeptide and the incubation being performed from 1 to 12 hours. By the use of labeled secondary antibodies the presence of specific antibodies can be determined by measuring the OD e.g. by ELISA where a positive response is a response of more than background plus two standard deviations or alternatively a visual response in a Western blot.

- Another relevant parameter is measurement of the protection in animal models induced after vaccination with the polypeptide in an adjuvant or after DNA vaccination. Suitable animal models include primates, guinea pigs or mice, which are challenged with an infection of a virulent Mycobacterium. Readout for induced protection could be decrease of the bacterial load in target organs compared to non-vaccinated animals, prolonged survival times compared to non-vaccinated animals and diminished weight loss compared to non-vaccinated animals.

Immunogenic portion

In a preferred embodiment of the invention, the polypeptide comprises an immunogenic portion of the polypeptide, such as an epitope for a B-cell or T-cell. The immunogenic portion of a polypeptide is a part of the polypeptide, which elicits an immune response in an animal or a human being, and/or in a biological sample determined by any of the biological assays described herein. The immunogenic portion of a polypeptide may be a T-cell epitope or a B-cell epitope. Immunogenic portions can be related to one or a few relatively small parts of the polypeptide, they can be scattered throughout the polypeptide sequence or be situated in specific parts of the polypeptide. For a few polypeptides epitopes have even

been demonstrated to be scattered throughout the polypeptide covering the full sequence (Ravn et al 1999).

In order to identify relevant T-cell epitopes which are recognised during an immune response, it is possible to use a "brute force" method: Since T-cell epitopes are linear, deletion mutants of the polypeptide will, if constructed systematically, reveal what regions of the polypeptide are essential in immune recognition, e.g. by subjecting these deletion mutants e.g. to the IFN-γ assay described herein. Another method utilises overlapping oligopeptides for the detection of MHC class II epitopes, preferably synthetic, having a length of e.g. 20 amino acid residues derived from the polypeptide. These peptides can be tested in biological assays (e.g. the IFN-γ assay as described herein) and some of these will give a positive response (and thereby be immunogenic) as evidence for the presence of a T cell epitope in the peptide. For the detection of MHC class I epitopes it is possible to predict peptides that will bind (Stryhn et al. 1996) and hereafter produce these peptides synthetic and test them in relevant biological assays e.g. the IFN-γ assay as described herein. The peptides preferably having a length of e.g. 8 to 11 amino acid residues derived from the polypeptide. B-cell epitopes can be determined by analysing the B cell recognition to overlapping peptides covering the polypeptide of interest as e.g. described in Harboe et al 1998.

Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids.

Immunogenic portions of polypeptides may be recognised by a broad part (high frequency) or by a minor part (low frequency) of the genetically heterogenic human population. In addition some immunogenic portions induce high immunological responses (dominant), whereas others induce lower, but still significant, responses (subdominant). High frequency><low frequency can be related to the immunogenic portion binding to widely distributed MHC molecules (HLA type) or even by multiple MHC molecules (Kilgus et al. 1991, Sinigaglia et al 1988).

In the context of providing candidate molecules for a new vaccine against tuberculosis, the subdominat epitopes are however as relevant as are the dominat epitopes since it has been show (WO2008000261) that such epitopes can induce protection regardless of being subdominant.

A common feature of the polypeptides of the invention is their capability to induce an immunological response as illustrated in the examples. It is understood that a variant of a polypeptide of the invention produced by substitution, insertion, addition or deletion is also immunogenic determined by any of the assays described herein.

Fusion proteins

By the term "fusion protein" is understood a random order of two or more immunogenic polypeptides from M. tuberculosis or analogues thereof fused together with or without an amino acid linker/spacer(s) of arbitrary length and sequence. To avoid protein aggregation in the down-stream production all cysteines in the fusion protein can be replaced with any amino acid but serine is the preferred substitute because of its high structural similarity with cysteine

Linkers

Linkers or spacers are short peptide sequences that occur between polypeptide partners in a fusion protein. Linkers are often composed of flexible residues like glycine and serine so that the adjacent protein domains are free to move relative to one another and for independent proper folding during secretion/manufacturing. Longer linkers are used when it is necessary to ensure that two adjacent domains do not sterically interfere with one another.

Paralogue, ortologue and homologue

By the term "paralogue" is understood proteins or genes that share some degree of homology because of shared ancestry followed by one or more duplication event(s). Paralogues are genes related by duplication within a genome while orthologs, which are homologous genes in different species that evolved from a common ancestral gene by speciation, The term, homologue apply to the relationship between genes separated by the event of speciation (ortholog) or to the relationship betwen genes separated by the event of genetic duplication (paralog).

Analogue

By the term sequence analogue is meant polypeptides which are structurally and immunogenically similar to each other but differs in amino acid composition

Vaccine

Another part of the invention pertains to a vaccine composition comprising a fusion protein according to the invention. An effective vaccine, wherein a fusion protein the invention is recognized by the animal, will in an animal model be able to decrease bacterial load in target organs, prolong survival times and/or diminish weight loss after challenge with a virulent Mycobacterium, compared to non-vaccinated animals

In order to ensure optimum performance of such a vaccine composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.

Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of cationic liposomes (e.g.dimethyldioctadecylammonium bromide (DDA)), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Trehalose Dimycolate (TDM), Trehalose Dibehenate (TDB), Muramyl Dipeptide (MDP) and monomycolyl glycerol (MMG) or combinations hereof.

Other methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), synthetic polymers of sugars (Carbopol), aggregation of the protein in the vaccine by heat treatment, aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Other possibilities involve the use of immune modulating substances such as cytokines or synthetic IFN-γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

Another interesting possibility for achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (which is hereby incorporated by reference herein). In brief, a relevant antigen such as an antigen of the present invention can be conjugated to an

antibody (or antigen binding antibody fragment) against the Fcγ receptors on monocytes/macrophages.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μg to 1000 μg, such as in the range from about 1 μg to 300 μg, and especially in the range from about 10 μg to 50 μg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and advantageously contain 10-95% of active ingredient, preferably 25-70%.

In many instances, it will be necessary to have multiple administrations of the vaccine. Especially, vaccines can be administered to prevent an infection with virulent mycobacteria

and/or to treat established mycobacterial infection. When administered to prevent an infection, the vaccine is given prophylactically, before definitive clinical signs or symptoms of an infection are present.

The invention also pertains to a method for immunising an animal, including a human being, against TB caused by virulent mycobacteria, comprising administering to the animal the polypeptide of the invention, or a vaccine composition of the invention as described above, or a living vaccine described above.

Therapeutic vaccine.

The invention also relates to the use of a fusion protein of the invention for use as therapeutic vaccines based on their ability to diminish the severity of *M. tuberculosis* infection in experimental animals or prevent reactivation of previous infection, when administered as a vaccine. The composition used for therapeutic vaccines can be prepared as described above for vaccines.

Fusion proteins comprising ESX-1 associated polypeptides

Mycobacteria secretion systems are responsible for the export of virulence factors either to extracellular environment or directly into the host cell and thus, play a vital role in the virulence and survival of the bacteria. Part of the ESX-1 secretion system was identified during the comparative genomic analysis of attenuated M. bovis BCG and pathogenic mycobacterial species [8]. One of the main genome differences was a major deletion in the M. bovis genome that included the region encoding the secreted antigens CFP10 and ESAT-6. This region was observed to be especially responsible for virulence and restoration of the region not only enabled the secretion of ESAT-6, but also led to increased virulence in M. bovis BCG [4].

The ESX-1 secretion system is conserved among slow growing mycobacteria including all pathogenic mycobacteria within the M. tuberculosis complex and is required for survival of mycobacteria in vivo. The functions of the secreted effector molecules are required for initiation of granuloma formation and phagosome maturation, essential for escape from phagosomes, cell lysis and cell-to-cell spreading, apoptosis through caspase activation and immune modulation by interfering with TLR2 signaling [6,9].

Today we know that the ESX-1 secretion system is encoded by three different loci, the ESX-1 locus, the espA operon and the locus for the transcriptional regulator EspR [10,11].

The exact number of components that are involved in ESX-1 secretion is still debated and seems to vary between different mycobacterial species. Currently the following Mycobacterium tuberculosis genes have shown relation to the ESX-1 system: espR, espA; espB; espC, espD, espF esxA; esxB; mycP1; PE35; Rv3862 (WhiB6), Rv3866, Rv3868; Rv3869; Rv3870; Rv3871; Rv3876; Rv3877; Rv3879c; Rv3881c Rv3882c and the MCE1 proteins Mce1B, Mce1C MCe1F and Rv0177 [12,13].

The six experimentally verified ESX-1 substrates, Rv3616c (EspA), Rv3615c (EspC), Rv3849 (EspR), ESAT-6, CFP-10 and Rv3881c (EspB) are mutually dependent on each other for secretion[7].

All known ESX-1 secreted substrates are strong antigens that are highly expressed in different stages of infection – in contrast to eg. Ag85 and other metabolic related antigens that are downregulated shortly after infection.

Given the high expression at various time point during infection and the high immunogenicity of many ESX-1 associated proteins we made the H74 backbone fusion protein based on six of the ESX-1 associated.

The H74 fusion protein consist of three experimentally proven ESX-1 substrates (ESAT-6, EspR, EspC) plus three secreted proteins associated with ESX-1 (EspD, EspA, EspB). The order of proteins in the H74 fusion is: EspB, ESAT-6, Rv3614, EspC, EspA, EspR. H74 consists of 1319 amino acids, the theoretical molecular weight is 137.917 g/mol and the isoelectric point 4,77. In the wild type sequence encoded from the *Mycobacterium tuberculosis* chromosome there is one cysteine in EspD, EspC and EspR. To avoid problems with sulphur-bridge formation and protein aggregation during refolding all three cysteines have been replaced with the amino acid serine.

Info on the individual proteins in H74:

ESAT-6 (Rv3875) is, together with CFP10, secreted via ESX-1 as a heterologous dimer [14].

EspR (Rv3849) is a transcriptional activator of its own expression and an operon that includes espA (Rv3616c), C and D. The EspR protein is secreted via ESX-1 [15].

EspC (Rv3615c) gene expression is regulated by EspR. EspC is secreted by ESX-1 [11].

EspD (Rv3614c) gene expression is regulated by EspR. EspD is co-transcribed with espC and espA. EspD expression but not secretion is required for EsxA secretion. EspD stabilizes the EspA- EspC complex. EspD secretion does not exclusively require the ESX-1 system [16].

EspA (Rv3616) gene expression is regulated by EspR. EspA is secreted by ESX-1 [7].
EspB (Rv3881) is secreted by ESX-1 {Xu, 2007 #912; McLaughlin, 2007 #591}.

Info on the individual proteins in H78:
ESAT-6 (Rv3875) is, together with CFP10, secreted via ESX-1 as a heterologous dimer [14].

EspD (Rv3614c) gene expression is regulated by EspR. EspD is co-transcribed with espC and espA. EspD expression but not secretion is required for EsxA secretion. EspD stabilizes the EspA- EspC complex. EspD secretion does not exclusively require the ESX-1 system [16].

Amino acid 1-56 of the EspC (Rv3615c) protein sequence. The espC gene expression is regulated by EspR. EspC protein is secreted by ESX-1 [11].
EspR (Rv3849) is a transcriptional activator of its own expression and an operon that includes espA (Rv3616c), C and D. The EspR protein is secreted via ESX-1 [15].
PE35 (Rv3872) is a secreted PE protein. Inactivation of gene pe35 (Rv3872) impaired expression of CFP-10 and ESAT-6, suggesting a role in regulation.

Figure legends

Figure 1. Antigen specific immune responses after vaccination.
After vaccination of animals with either the fusion proteins H74 or H56 formulated in CAF01 adjuvant the strength and specificity of T cell responses were measured. Responses towards the combined six proteins in H74 or three proteins in H56 was quantitated in PBMC´s isolated from blood (A and C) or in spelocytes (B and D) by stimulating the cells with either 2 ug H74 or H56 antigen pools The antigen specificity was measured by restimulaing with 2 ug of each of the single antigens in the two vaccines (E and F).

Readout was secreted IFN-γ that was measured in culture supernatants by ELISA after three days of in vitro stimulation.

Figure 2. Vaccine induced T cell polyfunctionality and protective efficacy.

Using flow cytometry the frequency of vaccine specific CD4 T cells capable of producing at least one of the cytokines IFN-γ, TNF-α or IL-2 was measured after stimulation with vaccine antigens (A). PBS stimulation controls were substtracted. The cytokine expression profile of the three cytokines was measured for individual CD4 T cells and the frequency of tridple, double and single cytokine producing T cells tabulated for four individual animals per group. In total there is seven combinations of the three cytokines The pie charts illustrate the distribution of cytokine producing subtypes for each vaccination group (B). The same color coding is used for all six pie´s. After being aerosolly challenged with Mycobacterium tuberculosis the bacterial burden in the lung of individual animals was measured and compared among the groups (C). Statistical comparison of the groups was done by one-way-ANOVA and Tukey's multiple comparison post test (*** p<0,001; ** p<0,01; * p<0,05). The mean and SEM are shown for each group.

Figure 3. A broad range of H74 vaccine doses can improve BCG protection.

(A) The number of Mycobacterial tuberculosis bacteria was determined in groups of mice after twelve weeks infection with M.tb strain Erdman. The animals were vaccinated with either BCG alone or BCG followed by H74 formulated in CAF01 adjuvant three months later. To identify a relevant H74 vaccine dose five different doses of H74 were tested (from 0.01 ug to 25 ug). Based upon one way analysis of variance (ANOVA) and Tukey's multiple comparisons test showed that all vaccinated groups had significant lower CFU numbers relative to the saline control (black circles, p<0.001). Even though the 0.1; 1 and 5 ug H74 vaccination groups had lower CFU´s than the BCG only vaccinated group (red circles) the difference was not significant. (B) Repeat of the experiment using 0.1 or 5 ug H74 showed that H74 significantly reduced the CFU load in protected animals with a prior BCG vaccination (*, p<0.05 compared to the BCG only vaccinated groups).

Figure 4. H64 and H74 can supplement the BCG vaccine and induce long-term protection against clinical isolates of Mtb.

The number of Mycobacterial tuberculosis bacteria was determined in groups of mice after twenty-four weeks infection with either M.tb strain Beijing (A) or Kazakhstan (B). In

both experiments animals were vaccinated with either BCG alone or BCG followed by either 2ug of H64 or H74 formulated in CAF01 adjuvant two months later. Only in animals from vaccination groups where BCG was supplemented with either H64 or H74 was the reduction in bacteria load statistical significant ($p<0.05$) based upon one way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

Figure 5. H74 protects against M.tb challenge and can supplement BCG for improved protection when injected at the same time as BCG.
Groups of mice were either vaccinated once with BCG, three times with H74/CAF01 or once with BCG and H74/CAF01 (injected at two different sites) followed by two H74/CAF01 vaccinations. After six weeks infection with Mtb strain Erdman the bacterial load was determined. All vaccines gave significant protection ($p<0.001$) but the injection of both BCG and H74 in the first vaccination round induced significantly better protection than either of the vaccines on their own ($p<0.001$).

Figure 6. T cell responses after H64 or H78 vaccination.
Spleen cells from H64 or H78 vaccinated mice were isolated from individual animals and stimulated with 2 ug/ml of either ESAT-6, Rv3614c, Rv3615c or Rv3865 at 37ºC for 6 hours. After washing the cells were stained with fluorescent labelled antibodies against the surface markers CD4 and CD44 and the cytokines IFN-g, TNF-a, IL-2 and IL17. The expression of the labelled markers and cytokines was measured by flowcytometri. After gating for high expression of CD4 and CD44 the frequency of cytokine producing cells in this subgroup of activated T cells was plotted.

Examples

EXAMPLE 1:

The H74 fusion protein – immune responses and protection in a preventive TB vaccination model. Groups of CB6F1 mice were vaccinated three times with either 0.01; 0.1, 1, 5 or 25 ug of the fusion protein H74 or 5 ug of the fusion protein H56. Both proteins were formulated in the liposome based adjuvant CAF01 prior to injection. Control groups were injected 3 times with an equal volume of saltwater (200 uL) or vaccinated once with

200 uL BCG (5 x10^7 CFU/mL). Spacing between vaccination were 2 weeks and. Three weeks after 3rd vaccination PBMC´s and splenocytes were isolated and the vaccine induced T cell responses were measured. Isolated cells (5 x 10^6/well) were incubated with 2 ug of the individual proteins present in the two fusion proteins or 2 ug of the fusion proteins for three days and sected IFN-g measured in the media by ELISA (Figure 1A-F). In the H56 vaccinated control animals there is strong recognition of the H56 protein and Ag85B and a moderate ESAT-6 recognition. H74 vaccinated animals have a strong response specific for Rv3881c and a moderate reposnse against Rv3849c and Rv3616c. In this inbred mice strain there is no response towards Rv3615c or Rv3614c. There is no response in the saline injected animals confirming the responses are vaccine specific. The strength of the H74 response was strongest in the animals receiving either 1 or 5 ug of the H74 protein depending upon the organ (Figure 1A and B). However, even the low 0.1 ug dose of H74 gave higher IFN-γ release than vaccinating with 5 ug of H56 protein. Looking at additional cytokines produced by spleen T cells the maximum frequency of responding T cells was found in the 1 ug H74 group but again 0.01 ug H74 gave higher response than 5 ug of H56 (Figure 2A). In therms of vaccine dose and polyfunctionality of the T cells there was an inverse correlation between the relative fraction of IL-2 producing T cells and H74 vaccination dose (Figure 2B).

Six weeks after third vaccination all animals were aerosolly challenge with virulent M. tuberculosis strain Erdman. Twelve weeks after challenge all mice were euthanized and the number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 2C). Three of the H74 vacine doses and BCG all induced significant protection compared to the saline control group. Importantly the control vaccine, H56, vaccine did not induce a statistically level of protection at this timepoint.

EXAMPLE 2:

The H74 and H64 fusion proteins as a supplement vaccine to BCG. Except for control animals, all CB6F1 mice were BCG vaccinated.

To determine the optimal H74 dose for vaccination on top of a BCG vaccine groups of BCG vaccinated animals where vaccinated three times with different doses of the fusion proteins H74 formulated in the liposome based adjuvant CAF01. The first H74 vaccination was injected three month after the BCG vaccination. Control animals received four injections with volume of saltwater (no BCG, no H74) or where vaccinated once with BCG.

Spacing between the H74 vaccinations were 2 weeks. Six weeks after the 3rd H74 vaccination animals were aerosolly challenge with virulent M. tuberculosis Erdman. Twelve weeks later all mice were euthanized and the number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 3A and B).

H74 doses from 0.1 ug to 5 ug induces a similar level of protection with CFU numbers slightly lower than in the BCG only vaccinated animals. All vaccinated groups induced significant protection comared to the saline control ($p<0.001$). In a repeat experiment (Figure 3B) only including the H74 vaccination doses 0.1 ug and 5 ug H74 the results were similar. All vaccinated animals had significantly lower bacteria load ($p<0.001$) but this time the H74 supplement groups also had significantly lower CFU´s that the BCG only vaccinated group. To compare the H74 and H64 as BCG supplement vaccines animals were BCG vaccinated and two months later vaccinated with either 0.1 ug H64/CAF01 or H74/CAF01 as above. In this experiment the animals were challenge with either the clinical Mtb isolate Beijing (Figure 4A) or clinical M.tb isolate Kazakhstan (Figure 4B) six weeks after the third H64 or H74 vaccination. After twenty-four weeks infection the bacterial load was enumerated in individual lungs. At this late time point, the protective efficacy of BCG against any of the isolates was not significant. However, supplementing BCG with either H64 or H74 gave significant protection against both clinical isolates of M.tb ($p<0.001$).

EXAMPLE 3:

H74 can supplement BCG even If the first vaccination is at the same time point as BCG vaccination.

There is four vaccination groups and one saline control group in this experiment. One vaccination group received only BCG and one group received three times 5 ug H74/CAF01. The remaining two groups received a BCG vaccination with one syringe and either a H74/CAF01 or CAF01 vaccination with another syringe (side-by-side vaccination) as their first vaccination. In the second and third vaccination, they received H74/CAF01 or CAF01 respectively. Six weeks after third vaccination all animals were challenge with Mtb strain Erdman and six weeks later the CFU numbers were determined in lungs as above (Figure 5). All vaccines induces significant protection ($p<0.001$) and the level for the BCG, BCG:CAF01 and H74 were similar. However, the animals in the group receiving BCG:H74 and two times H74 boost had significantly lower bacteria in the lungs than the other vaccinations groups - BCG, BCG:CAF01 and H74 ($p<0.001$).

Example 4:

H78 and H64 vaccination induces comparable T cells responses against the major antigens in the fusion proteins.

The H64 fusion protein is comprised of six proteins from Mycobacterium tuberculosis. Two of these, Rv3865 and Rv3615, are recognized with high frequency and specificity in TB patients and therefore have important value as protein antigens in coming TB diagnostic kits. Due to the antigen overlap, a worldwide use of a H64 vaccine would compromise the diagnostic kits. To avoid this problem full-length Rv3865 and half of the Rv3615 protein were removed from H64 to create a slightly shorter fusion protein named H78. To compare the ability of H64 and H78 to induce immune responses groups of animals were vaccinated three times with either 2 ug H64 or H78 formulated in CAF01 adjuvant or injected with saline three times. Three weeks after third vaccination three animals from each group were sacrificed and the T cell responses against the most important antigens was measured in spleen cells by flow cytometry (Figure 6). Both H64 and H78 induces the expected antigen pattern. H64 induced T cell responses against all four antigens investigated (ESAT-6, Rv3615c, Rv3614c and Rv3865). As expected, there was no response against Rv3865 in H78 vaccinated animals but, quite importantly, there was still a specific response against Rv3615c with a T cell frequency similar to that found in H64 vaccinated animals despite H78 is lacking half of the Rv3615c protein.

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Claims

1. A fusion protein or antigen cocktail, which comprises the amino acid sequences selected from:

a) SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 3 (Rv3614c), SEQ ID NO 4 (Rv3615c), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or

b) SEQ ID NO. 2 (ESAT6), SEQ ID NO. 3 (Rv3614c), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or

c) SEQ ID NO. 2 (ESAT6), SEQ ID NO. 3 (Rv3614c), SEQ ID NO. 9 (part of Rv3615), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or

d) SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 3 (Rv3614c), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or

e) SEQ ID NO. 2 (ESAT6), SEQ ID NO. 6 (Rv3849) and SEQ ID NO. 8 (Rv3872), or

f) SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6), SEQ ID NO 5 (Rv3616c) and SEQ ID NO 6 (Rv3849), or

g) SEQ ID NO.1 (Rv3881), SEQ ID NO 2 (ESAT-6) and SEQ ID NO 6 (Rv3849), or

an amino acid sequence analogue having at least 80% sequence identity hereto and at the same time being immunogenic

2. A fusion protein or antigen cocktail according to claim 1, wherein the amino acid sequence analogue has at least 90% or more preferred 95 % sequence identity to said sequences.

3. A fusion protein according to claim 1 or 2 wherin the cysteines have been replaced by another amino acid to avoid sulphur-bridge formation and protein aggregation.

4. A fusion protein according to claim 3, wherin the cysteine have been replaced with serine.

5. A fusion protein according to claim 1-4, wherin the fusion partners are linked with a linker molecule.

6. A fusion protein according to claim 5 with the amino acid sequence is SEQ ID NO 7 (H74), SEQ ID NO. 9 (H164), SEQ ID NO. 10 (H174), SEQ ID NO 11 (H264), SEQ ID NO 12 (H274), SEQ ID NO 13 (H374) or SEQ ID NO. 14 (H78).

7. Use of a fusion protein or a antigen cocktail according to any of the preceding claims for the preparation of a pharmaceutical composition for the vaccination against infections caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

8. A vaccine comprising a fusion protein or a antigen cocktail according to claim 1-6.

9. A vaccine according to claim 8 additionally comprising an adjuvant.

10. A vaccine according to claim 9 where the adjuvant is selected from the group consisting of cationic liposomes (e.g.dimethyldioctadecylammonium bromide (DDA)), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Trehalose Dimycolate (TDM), Trehalose Dibehenate (TDB), Muramyl Dipeptide (MDP) and monomycolyl glycerol (MMG) or combinations hereof.

11. A method for immunising an animal, including a human being, against tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, comprising administering to the animal the vaccine according to any of claims 8-10.

Figure 1

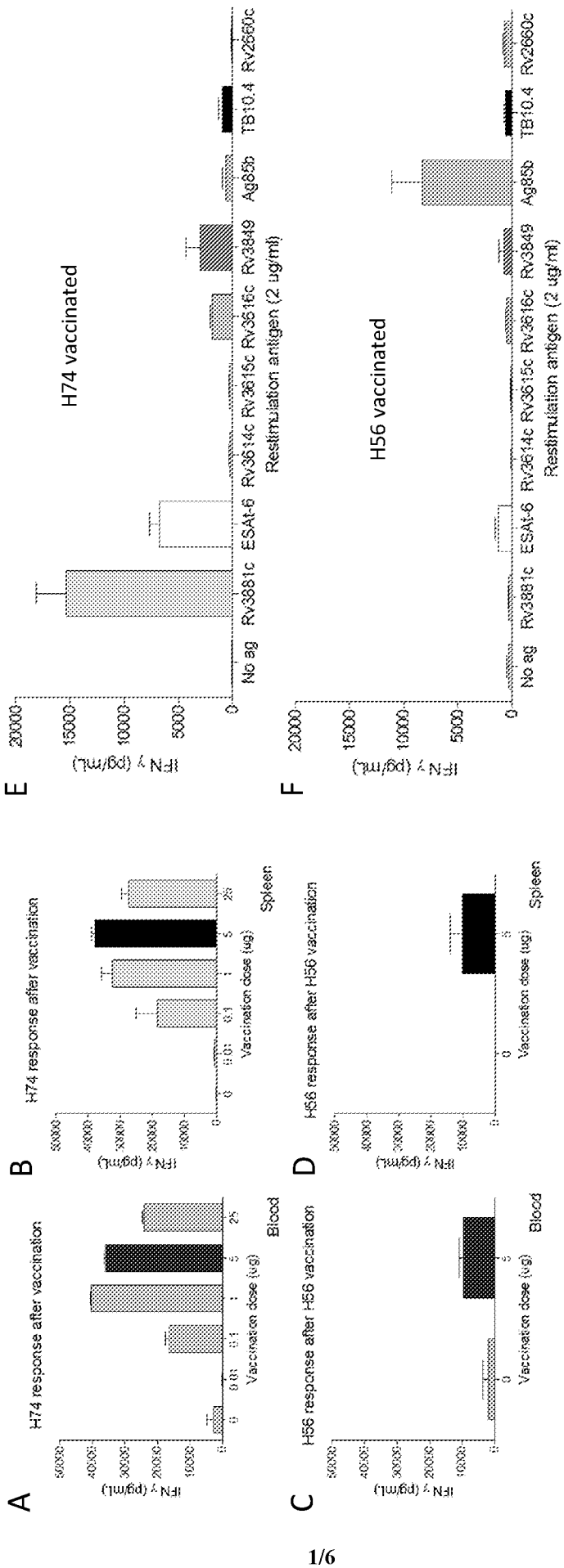

A
H74 response after vaccination
IFN γ (pg/mL)
Vaccination dose (ug)
Blood
B
H74 response after vaccination
IFN γ (pg/mL)
Vaccination dose (ug)
Spleen
C
H56 response after H56 vaccination
IFN γ (pg/mL)
Vaccination dose (ug)
Blood
D
H56 response after H56 vaccination
IFN γ (pg/mL)
Vaccination dose (ug)
Spleen
E
H74 vaccinated
IFN γ (pg/mL)
No ag
Rv3881c
ESAt-6
Rv3614c
Rv3615c
Rv3616c
Rv3849
Ag85b
TB10.4
Rv2660c
Restimulation antigen (2 ug/ml)
F
H56 vaccinated
IFN γ (pg/mL)
No ag
Rv3881c
ESAt-6
Rv3614c
Rv3615c
Rv3616c
Rv3849
Ag85b
TB10.4
Rv2660c
Restimulation antigen (2 ug/ml)

Figure 2

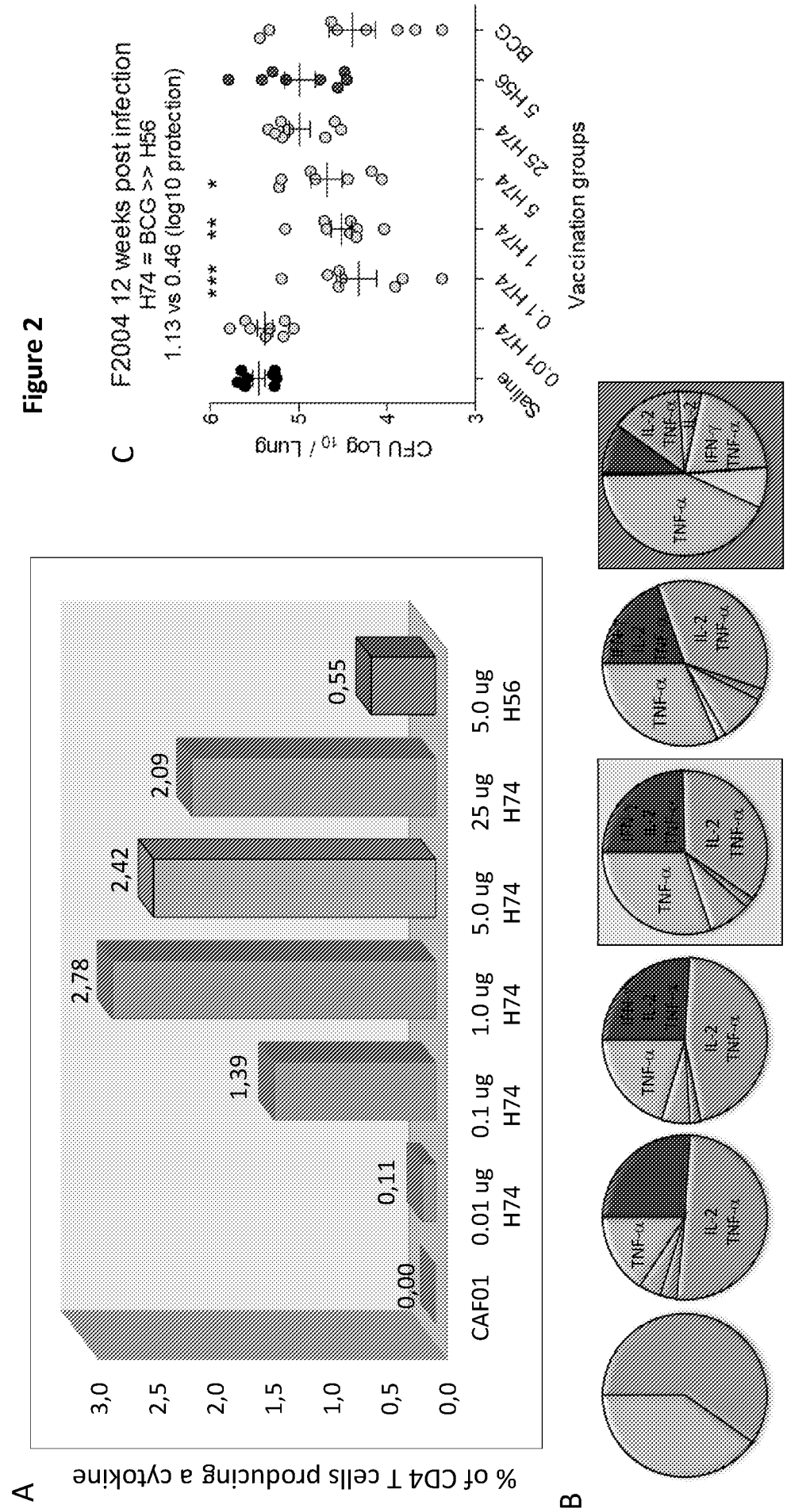

A
% of CD4 T cells producing a cytokine
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,00
0,11
1,39
2,78
2,42
2,09
0,55
CAF01
0.01 ug H74
0.1 ug H74
1.0 ug H74
5.0 ug H74
25 ug H74
5.0 ug H56
B
TNF-α
IL-2
IFN-γ
Vaccination groups
C
F2004 12 weeks post infection
H74 = BCG >> H56
1.13 vs 0.46 (log10 protection)
*** ** *
CFU Log 10 / Lung
6
5
4
3
Saline
0.01 H74
0.1 H74
1 H74
5 H74
25 H74
5 H56
BCG

Figure 3

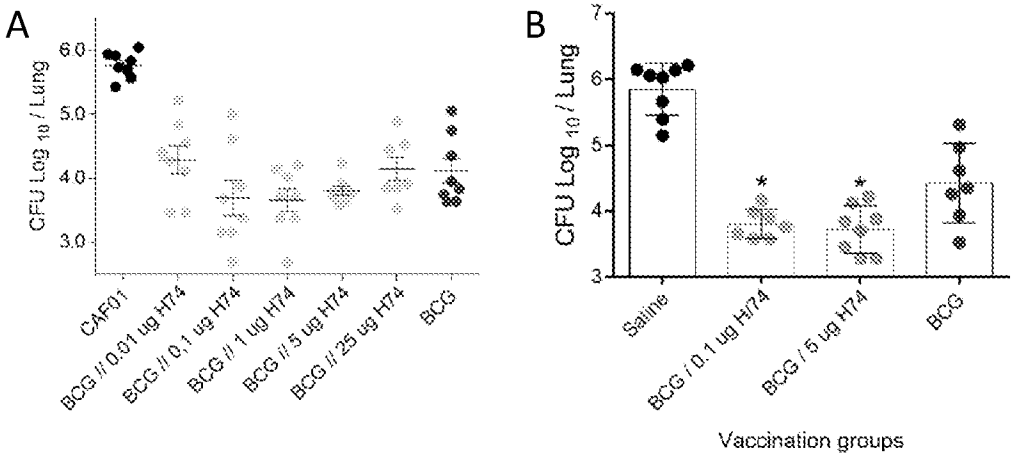

A
CFU Log 10 / Lung
6.0
5.0
4.0
3.0
CAF01
BCG // 0.01 ug H74
BCG // 0.1 ug H74
BCG // 1 ug H74
BCG // 5 ug H74
BCG // 25 ug H74
BCG
B
CFU Log 10 / Lung
7
6
5
4
3
Saline
BCG / 0.1 ug H74
BCG / 5 ug H74
BCG
Vaccination groups

Figure 4

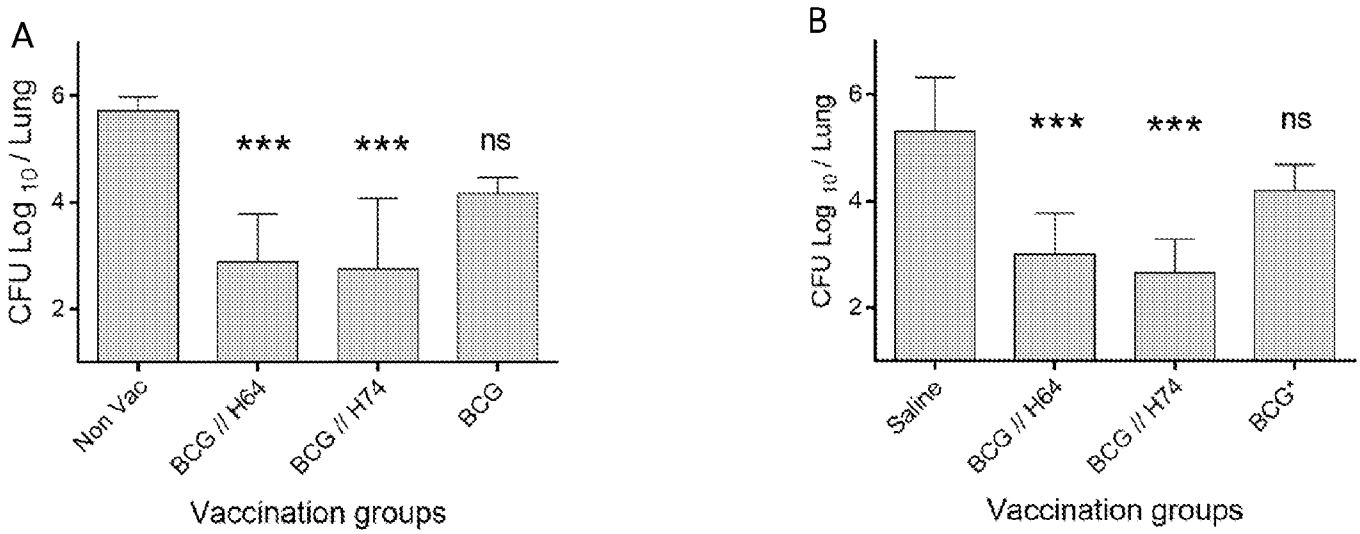

A
CFU Log 10 / Lung
6
4
2
***
***
ns
Non Vac
BCG // H64
BCG // H74
BCG
Vaccination groups
B
CFU Log 10 / Lung
6
4
2
***
***
ns
Saline
BCG // H64
BCG // H74
BCG'
Vaccination groups

Figure 5

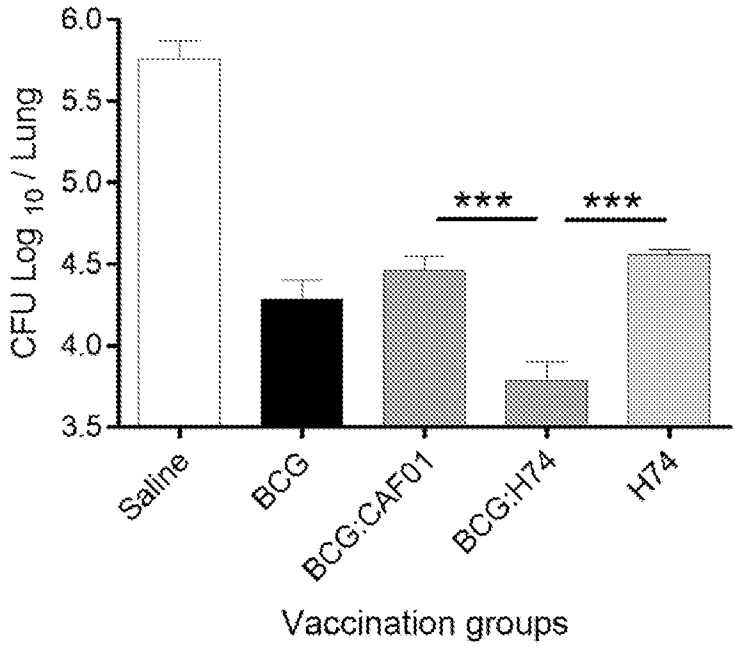
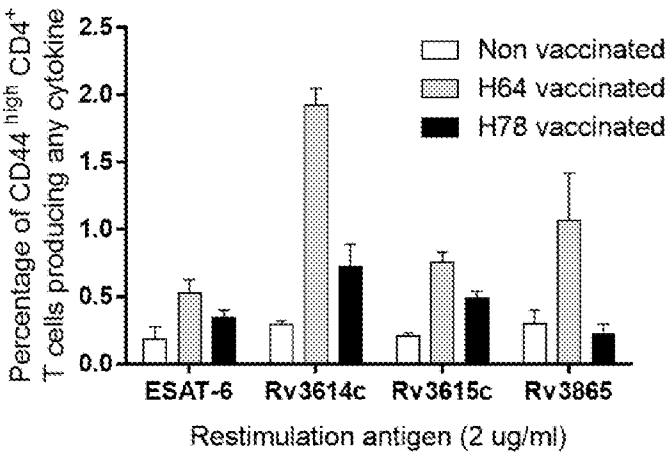

6.0
5.5
5.0
4.5
4.0
3.5
CFU Log 10 / Lung
***
***
Saline
BCG
BCG:CAF01
BCG:H74
H74
Vaccination groups

Figure 6


Percentage of CD44 high CD4+ T cells producing any cytokine
2.5
2.0
1.5
1.0
0.5
0.0
Non vaccinated
H64 vaccinated
H78 vaccinated
ESAT-6
Rv3614c
Rv3615c
Rv3865
Restimulation antigen (2 ug/ml)

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050086

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/04 C07K14/35
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/121618 A1 (STATENS SERUMINSTITUT [DK]; DIETRICH JES [DK]; ANDERSEN PETER [DK]; LU) 28 October 2010 (2010-10-28) page 6, paragraph 1; claim 1	1-11
X	WO 2012/057904 A1 (INFECTIOUS DISEASE RES INST [US]; IRETON GREGORY C [US]; REED STEVEN G) 3 May 2012 (2012-05-03) claim 1	1-11

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2015	23/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Griesinger, Irina

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050086

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLAUS AAGAARD ET AL: "A multistage tuberculosis vaccine that confers efficient protection before and after exposure", NATURE MEDICINE, vol. 17, no. 2, 23 January 2011 (2011-01-23), pages 189-194, XP055098420, ISSN: 1078-8956, DOI: 10.1038/nm.2285 abstract ----	1-11
A	CLAUS AAGAARD ET AL: "TB vaccines: current status and future perspectives", IMMUNOLOGY AND CELL BIOLOGY, vol. 87, no. 4, 7 April 2009 (2009-04-07), pages 279-286, XP055098495, ISSN: 0818-9641, DOI: 10.1038/icb.2009.14 the whole document ----	1-11
X,P	WO 2014/063704 A2 (STATENS SERUMINSTITUT [DK]) 1 May 2014 (2014-05-01) claim 1 ----	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2015/050086

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-11

relate to a fusion protein or antigen cocktail comprising at least three of the antigens shown in SEQ ID NOs: 1, 2, 3, 4, 5, 6, 8 and 9 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.1. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 1, 2, 3, 4, 5 and 6 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.2. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 2, 3, 6 and 8 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.3. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 2, 3, 6, 8 and 9 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.4. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 1, 2, 3, 5 and 6 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.5. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 2, 6 and 8 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.6. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 1, 2, 5 and 6 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

1.7. claims: 1-11(partially)

relate to a fusion protein or antigen cocktail comprising SEQ ID NOs: 1, 2 and 6 or a sequences with at least 80% identity thereto; the corresponding vaccines and uses.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/DK2015/050086

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