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(50) Title:  
M. TUBERCULOSIS VACCINES

(57) Abstract:  
The present invention is directed to fusion proteins, 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 canetti, Mycobacterium pinnipedi or Mycobacterium mungi. The fusion proteins or antigen cocktails are based on ESX secreted or associated proteins e.g. proteins secreted by the ESAT-6 secretion system 1 (ESX-1) which are among the most immunodominant M. tuberculosis (MTB) antigens.
M. TUBERCULOSIS VACCINES

Field of invention
The present invention discloses new immunogenic compositions based on Esx-1 associated and esx family 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-\(\gamma\)).

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. 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 vast amount of expression data and the significant improvement of in silico prediction tools it is still not possible to predict 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. 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. Mycobacterium tuberculosis has several different types of secretion systems of which the ESX secretion system (type VII) is relevant for this invention. Mycobacterium tuberculosis has five of these systems, termed ESX-1 to ESX-5. 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. canetti*, *M. pinnipedii*, *Mycobacterium mungi*) by the use of a fusion protein or antigen cocktail comprising *M. tuberculosis* antigens selected from ESX-1 associated and esx family polypeptides possibly including latency 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 sequence selected from
(a) SEQ ID NO.1 (ESAT6), SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3665), SEQ ID NO 5 (Rv3849) and SEQ ID NO 6 (Rv3872), or
(b) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849) and SEQ ID NO 6 (Rv3872) or
(c) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872) and SEQ ID NO 7 (Rv3616), or
(d) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872) SEQ ID NO 7 (Rv3616) and SEQ ID NO 8 (Rv3881c), or
(e) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872) and SEQ ID NO 8 (Rv3881c), or
(f) SEQ ID NO 9 (Rv3891c), SEQ ID NO 10 (Rv3890), SEQ ID NO 11 (Rv0287), SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c) and SEQ ID NO 14 (Rv3619), or
(g) SEQ ID NO 11 (Rv0287), SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c), SEQ ID NO 14 (Rv3619), NO 7 (Rv3616c) and SEQ ID NO 3 (Rv3615c) or
(h) SEQ ID NO 11 (Rv0287), SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c), SEQ ID NO 14 (Rv3619), NO 7 (Rv3616c), SEQ ID NO 3 (Rv3615c) and SEQ ID 9 (Rv3881c), or
(i) SEQ ID NO 1 (ESAT6), SEQ ID NO 15 (Ag85B) and SEQ ID NO 16 (Rv1284), or
(j) an amino acid sequence analogue having at least 80% sequence identity to any-one of the sequences in (a) -(i) and at the same time being immunogenic;

The cysteines in the fusion protein according to the invention have preferably been re-placed by another amino acid to avoid sulphur-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.

Preferred fusion proteins according to the invention are suggested as SEQ ID NO 18 (H64), SEQ ID NO 19 (H68), SEQ ID NO 20 (H69), SEQ ID NO 21 (H70), SEQ ID NO 22 (H71), SEQ ID NO 23 (H65), SEQ ID NO 24 (H72), SEQ ID NO 25 (H73) or SEQ ID NO 26 (H67).
Another embodiment of the invention is using an antigen cocktail according to the invention e.g. the above mentioned amino acid sequences (a) - (i) (comprising SEQ ID NO 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16) without fusing the polypeptides together.

A preferred antigen cocktail comprises SEQ ID NO 16 and H1 (SEQ ID NO 17) where H1 is a fusion between SEQ ID NO 1 and SEQ ID NO 15.

In a still further embodiment, the invention discloses an immunogenic composition or pharmaceutical 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 prothetic 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 amino 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 pep-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 $1 \times 10^5$ to $3 \times 10^5$ 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-a, 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 $4 \times 10^6$ cells /ml and incubated for 18-22 hrs in the presence 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 $2 \times 10^5$ 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 Thymididine 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 subclinical<sup>6</sup> 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 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 subdominant epitopes are however as relevant as are the dominant 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 between 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 l:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Trehalose Dimycolate (TDM), Trehalose Dibenenate (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 l: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 Fey 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 seventy 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.
H64, H68, H69, H70 and H71: 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. 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.

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.

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. 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 McelB, McelC, McelF and Rv0177.

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.
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 H64 backbone fusion protein based on six of the ESX-1 associated proteins and from this backbone the H68, H69, H70 and H71 fusion proteins were made.

The H64 fusion protein consist of three experimentally proven ESX-1 substrates (ESAT-6, EspR, EspC) plus three secreted proteins associated with ESX-1 (EspD, PE35 and EspF). The order of proteins in the H64 fusion is: ESAT-6, EspD, EspC, EspF, EspR, PE35 but any other order can be used. H64 consists of 716 amino acids, the theoretical molecular weight is 75698 g/mol and the isoelectric point 4.56. 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 H64:

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

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.

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

EspD (Rv3614c) gene expression is regulated by EspR. EspD is co-transcribed with espC. 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.

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.

EspF (Rv3865) is a secreted protein. The amino acid sequence of EspF is 36% identical to EspC. Inactivation of EspF protein leads to attenuation of mycobacteria confirming its importance for survival. The attenuation of M. tuberculosis EspF mutants is not caused by...
the lack of ESAT-6 secretion but, rather, by the interruption of another, yet-unknown function of the ESX-1 system.17

The H68 fusion protein comprises the same proteins as H64 except that ESAT-6 is omitted in this constructs due to its importance in the diagnosis of tuberculosis:

\[ \text{Rv3614c-Rv3615c-Rv3865-Rv3849-PE35} \]

The H69 fusion protein comprises the same proteins as H68 but with the addition of EspC (Rv3616c):

\[ \text{Rv3614c-Rv3615c-Rv3865-Rv3849-PE35} \]

Info on the additional protein in H69

EspC (Rv3616c) gene expression is regulated by EspR. EspC is secreted by ESX-1.15

The H70 fusion protein comprises the same proteins as H69 but with the addition of EspB (Rv3881c):

\[ \text{Rv3881c-Rv3614c-Rv3615c-Rv3865-Rv3849-PE35} \]

Info on the additional protein in H70

EspB (Rv3881c) is secreted by the ESX-1 secretion system. During the secretion process it is cleaved by the membrane anchored proteinase MycP1.16

The H71 fusion protein comprises the same proteins as H68 but with the addition of EspB (Rv3881c):

\[ \text{Rv3881c-Rv3614c-Rv3615c-Rv3865-Rv3849-PE35} \]

H65, H72 and H73: A fusion protein comprising ESAT-6 family polypeptides

Several secreted proteins from mycobacteria have been shown to induce strong cellular immune responses.18 Two of the most frequently recognized T cell antigens from M. tuberculosis are the small secreted proteins ESAT-6 (early secretory antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa), the prototypes of the Esx family.19 Genes encoding ESAT-6 and CFP-10 are located directly adjacent to each other and co-transcribed.20 Analysis of the M. tuberculosis H37Rv genome sequence revealed 11 pairs of tandem genes encoding paralogous ESAT-6 family proteins.1

The ESAT-6 (esx) family has 23 members (11 gene pairs and a singleton, Rv0287, Rv0288, Rv1037c, Rv1038c, Rv1197, Rv1198, Rv1792, Rv1793, Rv2346c, Rv2347c,
Rv3017c, Rv3019c, Rv3020c, Rv3444c, Rv3619c, Rv3620c, Rv3874, Rv3875, Rv3890c, Rv3891c, Rv3904c and Rv3905c. The sequence identity varies between the Esx proteins from 35% to 98% but all of them belong to the WXG100 family, characterized by a size of ~100 amino acids and the presence of a Trp-Xaa-Gly (W-X-G) motif. Thus, far the precise biological function of the ESAT-6 family members are unknown but they are virulence factors.

ESAT-6 and CFP10 interact to form a 1:1 heterodimer, that is essential for their secretion via the ESX-1 secretion system. Proteins encoded by two other paralogous gene pairs, EsxR-EsxS and EsxH-EsxG, also form 1:1 complexes, suggesting that this may be typical of all Esx protein couplets.

The EsxH-EsxG complex is secreted by ESX-3. They are essential for in vitro growth, involved in iron/zinc homeostasis and regulated by the iron-dependent transcriptional repressor IdeR and the zinc-uptake regulator Zur.

ESX-5 is known to be necessary for the secretion of PE and PPE proteins in Mycobacterium marinum and for macrophage subversion. Most likely it is also responsible for the secretion of the five ESAT-6 paralogues esxL (rv1037c), esxO (rv2346c), esxV (rv3619c) and the five CFP10 paralogues esxJ (rv1038c), esxK (rv1197), esxP (rv2347c), esxW, (rv3620c). In M. marinum the function of ESX-5 mediated protein secretion is to establish a moderate and persistent infection. ESX-5 deficient Mycobacterium marinum is hypervirulent, ESX-5 is also found in M. tuberculosis.

The functions of ESX-2 and ESX-4 remain unknown but based on functional and physical homology to the other ESX secretion systems they are likely to secrete the esxC - esxD and esxT - esxU complexes, respectively.

Because the ESAT-6 family proteins are highly expressed during an Mycobacterium tuberculosis infection, are highly immunogenic and experimental data support their protective efficacy following vaccination, we constructed the H65 fusion protein based on six ESAT-6 family proteins. To make the vaccine compatible with the current diagnostic test the most prominent family members - ESAT-6 and CFP10 - where not included.
The order of the ESAT-6 proteins in the H65 fusion is: Rv3891c-Rv3890c- Rv0287- Rv0288-Rv3620c-Rv3619c, but any order can be used. Between each of the 3 protein couples the 9 amino acid GLVPRGSTG linker sequence is inserted to allow for protein-folding and dimer-formation. Between Rv3890c and Rv0287 the 20 amino acid LIGAHPRALNWKFGGAAFL linker is inserted and between Rv0288 and Rv3630c the 20 amino acid LGFGAGRLRLGFTNPGSWRI linker. The sequences of both 20 amino acid linkers are from the Rv1886 *M. tuberculosis* HRv37 protein sequence and corresponds to amino acid positions 61-80 and 161-180 in this protein. The linkers were included because they have been shown to be human epitopes and because T cells responding to these secrete large amounts of cytokine IL-2. IL-2 is necessary for the growth, proliferation, and differentiation of T cells to become effector T cells. In addition to increase the diversity of the vaccine induced T cell pool these IL-2 secreting T cells may provide cytokine help to other T cells in their differentiation from naive to effector T cells.

The order of the ESAT-6 proteins in the H72 fusion is: Rv3616c-Rv3615c-Rv3620c- Rv3619c- Rv0287-Rv0288-, but any order can be used.

The order of the ESAT-6 proteins in the H73 fusion is: Rv3881c-Rv3616c-Rv3615c- Rv3620c-Rv3619c- Rv0287-Rv0288-, but any order can be used.

*H67: A fusion protein comprising a latency polypeptide Ag85B-ESAT6-Rv1284*

The H1 vaccine, a protein fusion of Ag85B and ESAT-6 (Ag85B-ESAT6), is a very efficient vaccine against a primary infection but due to the expression profile of the two antigens it has its primary effect during the earlier stages of infection. To develop a vaccine that is effective in both early and late stage infection (persistence and reactivation from latency) the H1 fusion protein was enriched with Rv1284. Expression studies have shown that Rv1284 is strongly expressed under in vitro conditions simulating late stage infection. It should therefore be possible not only to increase the protective efficacy of H1 in the early infection stages but also extending the efficacy to the later stages of infection by the addition of Rv1284.

We designed the H67 fusion protein based on Ag85B, ESAT-6 and R1284 which reflects the order of the proteins, but any order of the polypeptides may be used. H67 consists of 549 amino acids, the theoretical molecular weight is 59548 g/mol and the isoelectric point 5.36. In the wild type sequence encoded from the *M. tuberculosis* chromosome there are
three cysteines in Ag85B, and three cysteines in Rv1284. 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 H67:

Ag85B (Rv1886c) is mycolyl transferase 85B, an extracellular protein, and is selected as the most immunogenic protein in the fusion protein characterized by an an initial transient increase in Ag85B expression but already after 10 days infection the level of bacterial Ag85B expression had dropped approx. 15 times per CFU and this low level is maintained at least up to 100 days post infection.

ESAT-6 (Rv3875) is, together with CFP10, secreted via ESX-1 as a heterologous dimer. The ESX-1 substrate ESAT-6 shows high expression at various time point during infection and display high immunogenicity.

Rv1284 is encoding a beta-carbonic anhydrase, and the gene has been shown to be essential for M. tuberculosis. The expression of the gene has previously been reported to be 14- to 40-fold increased in nutrient-starved cultures.

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Figure legends

Figure 1. Mycobacterial load in mice after preventive vaccination with single antigens. *M. tuberculosis* bacteria was enumerated in lungs of individual mice six weeks after aerosol challenge. Prior to challenge groups of mice were vaccinated with individual antigens or BCG and one group received saline injections. One way analysis of variance (ANOVA) in combination with Tukey’s multiple comparisons test was used to statistical test if the bacterial number in vaccinated groups were significantly lower than in lungs of animals from the saline control group (**p<0.01**) six weeks after challenge. The mean and standard error (SEM) are shown for each group.
Figure 2. Immune responses and mycobacteria load after H64 vaccination. After vaccination with the fusion proteins H64 or H56 (Ag85B-ESAT6-Rv2660c) in CAF01 adjuvant (DDA/TDB) the T cell response towards the individual proteins in the two fusions was tested (A). Readout was secreted IFN-γ after three days of in vitro stimulation with single antigens. Six weeks after being challenge with M.tb the bacterial burden in the lung was measured and compared among the groups using one-way-ANOVA combined with Tukey's multiple comparisons test (** p<0.01 * p<0.05. The mean and SEM are shown for each group.

Figure 3. Post-exposure vaccination with Rv3614 or Rv3615c resulted in significant vaccine specific immune responses. 1 week after vaccination lymphocytes obtained from the lung of the mice were used to evaluated vaccine-induced T cell immune response. Readout was frequency of cytokine positive CD4 T cells (expressing either IFN-γ, IL-2, TNF-α or any combination) in the cell culture after 5 hours of in vitro stimulation with the vaccine antigens i.e. (A) Rv3614 or (B) Rv3615c. TB10.4 was included as a control antigen for the infection driven immune response. Data shown as mean and represent a pool of 4 mice.

Figure 4. Post-exposure vaccination with H64. Vaccine responses and protective efficacy. After post-exposure vaccination with H64 the T cell response towards the individual protein and/or the whole fusion was tested (A,B and D). Readout was IFN-γ secretion after three days of in vitro stimulation with the single antigens or whole fusion-protein and was done for both vaccinated FvB (A) and CB6F1 mice(D) and control mice (B). (C) 37 weeks post M.tb. challenge (21 weeks post final vaccination) the bacterial burden in the lungs was measured and compared between the groups using one-way-ANOVA combined with Tukey's multiple comparisons test. The mean and SEM are shown for each group.

Figure 5. Mycobacterial tuberculosis load in mice after preventive vaccination with single antigens. M. tuberculosis bacteria was enumerated in lungs of individual mice six weeks after aerosol challenge. Prior to challenge groups of mice were vaccinated with individual antigens or BCG and one group received three saline injections. In A and B the B6C3F1 mouse strain was used and in C the CB6F1 strain. One way analysis of variance (ANOVA) in combination with Tukey's multiple comparisons test was used to statistical test if the bacterial number in vaccinated groups were significantly lower than in lungs of ani-
mals from the saline control group (** p<0.01) six weeks after challenge. The mean and standard error of mean (SEM) are shown for each group.

Figure 6. Groups of CB6F1 mice were either vaccinated three times with H64 fusion protein using protein doses from 0.01 to 25 µg per animal per vaccination round or 5 µg of the H56 fusion protein. Three weeks after third vaccination the vaccine specific T cell response was measured in spleen (A)(n=3) and blood (B) by restimulation with vaccine antigens. Six weeks after vaccination all animals were aerosolly challenged with virulent M.tb strain Erdman. Six weeks after infection M. tb bacteria was enumerated in lungs of individual mice (C). ANOVA and Tukey's multiple comparisons test was used to statistical test if the bacterial number in vaccinated groups were significantly lower than in lungs of animals from the saline control group (** p<0,01; * p<0,05)

Figure 7. Mycobacterial load in mice after preventive vaccination with single antigens. M. tuberculosis bacteria were enumerated in lungs of individual mice six weeks after aerosol challenge. Prior to challenge groups of mice were vaccinated with Rv3891, Rv3619, Rv3620 or BCG or received saline injections. One way analysis of variance (ANOVA) in combination with Tukey's multiple comparisons test was used to compare the bacterial burden among the groups (** p<0.01; * p<0.05 relative to the saline group).

Figure 8. Dimer fusions of ESAT-6 family proteins. Schematic representation of the six fusions (A). CFP10 paralogue is either Rv3891c, Rv0287, Rv3445c, Rv3620c, Rv3905c or CFP10. The ESAT-6 paralogue is either Rv3890c, Rv0288, Rv3444c, Rv3619c, Rv3904c or ESAT-6. The amino acid sequence for the linker is GLVPRGSTG in all six constructs. Protective efficacy was measured in lungs of individual mice six weeks after aerosol challenge in CB6F1 mice (B) and B6C3F1 mice (C). Vaccine induced responses were determined in B6C3F1 mice by stimulating PBMC's isolated 3 weeks after third vaccination (D). Each panel illustrate results from one vaccination group. PBMC's from each groups were either stimulated with the vaccine antigen or a control protein (BSA).

Figure 9. Vaccination of B6C3F1 mice with H65. Schematic representation of the H65 fusion consisting of six ESAT-6 family proteins separated by either a 9 aa linker (L) or 20 aa linkers (LL1 and LL2) (A). The 9 aa linker is identical to the linker used for the dimer fusions (GLVPRGSTG). The sequence of the LL1 linker is LIGAHPRALNWKFGGAAFL
and for the LL2 linker LGFGAGRLRGLFTNPGSWRI. Cytokine responses measured by flowcytometry against each of the ESX-2 (Rv3891c-Rv3890c), ESX-3 (Rv0287-Rv0288) and ESX-5 (Rv3620c-Rv3619c) dimer substrates in splenocytes isolated three weeks after third H65 vaccinated (B). Mycobacterial load in B6C3F1 mice after preventive vaccination with H65 or BCG (C). *M. tuberculosis* bacteria were enumerated in lungs of individual mice six weeks after aerosol challenge. One-way-ANOVA in combination with Tukey's multiple comparisons test was used to compare the bacterial burden among the groups (*p<0.1 relative to the saline group).

Figure 10. H65 vaccination in CB6F1 mice - immune response and protective efficacy. Antigen specific secretion of the cytokine IFN-γ by splenocytes isolated three weeks after third vaccination with H65/CAF01 (A) or third injection with saline (B). CFU in four groups of CB6F1 mice six (C) and twenty-four (D) weeks after challenge.

Figure 11. Vaccine-induced immune response after post-exposure vaccination with H65. After post-exposure vaccination with H65 the T cell response towards the individual protein and/or the whole fusion was tested (A-C). Readout was IFN-γ secretion after three days of in vitro stimulation with the single antigens for the CB6F1 mouse strain (C) and both single antigens and whole fusion-protein in the FVB mouse strain (A: vaccinated and B: control).

Figure 12. Rv1284 (canA) vaccination in two mice strains. Secreted cytokine IFN-γ was measured in cell medium after incubating PBMC's with either 2 μg of Rv1284 or Rv0287 or with buffer control for 72 hours. The PBMC's were isolated from CB6F1 mice (A) or B6C3F1 mice (B) two weeks after the third Rv1284/CAF01 vaccination. Six weeks after Mtb challenge the number of mycobacteria was measured in CB6F1 mice (C) or B6C3F1 mice (D).

Figure 13. H1+Rv1284 vaccination. Bacteria load (CFU) in groups of FVB mice six weeks after an aerosol Mtb challenge. The mice were either vaccinated three times with 5 μg H1(Ag85B-ESAT6)/CAF01, 5 μg (total) H1(Ag85B-ESAT6)+Rv1284 formulated in CAF01 or injected three times with saline (control group).

Figure 14. Immune response following post-exposure vaccination with the antigens Ag85B, ESAT-6 or Rv1284. 1 week after vaccination lymphocytes obtained from the lung
of the mice were used to evaluate vaccine-induced T cell immune response to either Ag85B, ESAT6 or Rv1284, all components of H67. Readout was frequency of cytokine positive CD4 T cells (expressing either IFN-γ, IL-2, TNF-α or any combination) in the cell culture after 5 hours of in vitro stimulation with the vaccine antigens and shown for (A) Ag85B, (B) ESAT6 and (C) Rv1284. TB10.4 was included as a control antigen for the infection driven immune response. Data shown as mean and represent a pool of 4 mice.

Figure 15. 2D DIGE image of culture filtrate proteins from log phase and starvation conditions. The numbered protein spots were excised from 2D DIGE gels post-stained with silver and subjected to MS identification. Spots # 1666 and 1669 were identified by PMF and MS/MS, respectively, as Rv1284.

Examples

**EXAMPLE 1: Single protein and protection in a preventive TB vaccination model**

Groups of CB6F1 mice were either vaccinated three times with 5 μg of one of the recombinant proteins formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 μL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and six weeks after third vaccination all animals were aerosolly challenge with virulent M. tuberculosis Erdman. Six 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 1). Vaccination with the individual ESX-1 related proteins - Rv3615c, Rv3614c and Rv3849 - induced comparable and significant protection against tuberculosis thus not at the level of BCG.

**EXAMPLE 2: The H64 fusion protein - immune responses and protection in a preventive TB vaccination model.** Groups of CB6F1 mice were either vaccinated three times with 5 μg of one of the fusion proteins H56 or H64 formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 μL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and. Three weeks after 3rd vaccination animals were bleed, PBMC's isolated and the vaccine induced T cell responses were measured. 5 x 106 PMBC's were incubated with 2 μg of the individual pro-
teins present in the two fusion proteins for three days and secreted IFN-g was measured in the media by ELISA (Figure 2A). In the H56 vaccinated animals there is strong recognition of Ag85B and ESAT-6 and a weak recognition of the third protein, Rv2660c. H64 vaccinated animals have a strong response specific for ESAT-6 and Rv3614 and a moderate response towards PE35 (Rv3872) and Rv3849. In this inbred mice strain there is no response towards Rv3865 and Rv3615c. There is no response in the saline injected animals confirming the responses are vaccine specific.

Six weeks after third vaccination all animals were aerosolly challenge with virulent M. tuberculosis H37Rv (Figure 2B) or M. tuberculosis Erdman (Figure 2C). Six 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 2B and 2C).

In both experiments vaccination with either H64 or H56 induced significant protection compared to the saline control group. Furthermore, statistical comparison of the CFU's in H56 and H64 vaccinated animals revealed that H64 reduced the bacteria numbers significantly more than H56 did after H37Rv challenge (Figure 2B).

**EXAMPLE 3: Rv3614c and Rv3615c immune responses after vaccination in a post-exposure TB vaccination model**

In the post-exposure TB vaccination model mice are initially challenged with *M. tb* via the aerosol route. To mimic the latent stage of infection mice are given antibiotics *ad libitum* in the drinking water from 6 to 12 weeks post-infection (p.i.). Groups of mice were vaccinated at week 10, 13 and 16 p.i. with either 5 µg of the recombinant proteins formulated in the liposome based adjuvant CAF01 or an equal volume of saltwater (200 µl). 1 week after the final vaccination (week 17 p.i.) vaccine-induced immune responses was assessed in the lung and determined by intracellular staining for cytokines following 6 hours of re-stimulation of lung lymphocytes with the vaccine-antigen or TB10.4 (Figure 3). Vaccination with Rv3614c or Rv3615c induced a significant vaccine-specific immune response not measurable at comparable levels in the lungs of control mice.
EXAMPLE 4: The H64 fusion protein - immune responses and protection in a post-exposure TB vaccination model.

The post-exposure TB vaccination model was generated as described in example 3 and was used to evaluate the effect of post-exposure immunization with H64 fusion-protein. Groups of mice were either vaccinated three times with 5 μg H64 formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 μL) or vaccinated once with BCG. H64 immunogenicity was evaluated in both CB6F1 (C57BL/6xBALB/c) (Figure A-C) and FvB mice (Figure 4D). Following the three immunizations, lymphocytes were obtained from the lungs of the mice, and stimulated in vitro with the individual components and/or the fusion protein. At week 17 p.i. where vaccine-induced response was measured the infection-driven response was negligible as measured in the saline-injected control group (Figure 4B). This is in contrast to the IFN-γ responses measured in the vaccinated group (Figure 4A and D). As expected the pattern of recognition did differ between the two distinct mouse strains FvB (Figure 4A) and CB6F1 (Figure 4D). H64 vaccination of FvB mice primarily led to an induction of a IFN-γ response directed towards Rv3615c whereas a response was induced to ESAT6, Rv3614c, Rv3849 and to a lesser extent Rv3872 in the CB6F1 mice. Thus, H64 is a highly immunogenic vaccine resulting in substantial amounts of IFN-γ production. At 37 weeks p.i. all mice were euthanized and the number of bacteria in the lungs of individual mice were determined by serial plating dilutions of lung homogenate and counting the number of colonies after 2-3 weeks of incubation at 37 °C. At this timepoint the control animals had a mean bacterial load of 4.021 log10 CFU comparable to the mean bacterial load following BCG vaccination (3.807 log10 CFU), whereas the bacterial load of the H64 vaccinated was slightly lower (2.917 log10 CFU) (Figure 4C).
EXAMPLE 5: Single protein protection of seven ESX-1 antigens against aerosol TB challenge in two preventive TB vaccination models

Groups of CB6F1 or B6C3F1 mice were either vaccinated three times with 5 μg of one of the recombinant proteins formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 μL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and six weeks after third vaccination all animals were aerosolly challenge with virulent *M. tuberculosis* Erdman. Six 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 5A-C).

In the B6C3F1 strain (Figure 5A and B) vaccination with seven individual ESX-1 related proteins - Rv3616c, Rv3615c, Rv3614c Rv3865, ESAT-6, Rv3849, og Rv3872 - induced variable degrees of protection ranging from 0.30 to 1.31 log<sub>10</sub> reduction of the bacteria load in the lung of individual animals relative to the saline control group. None of the single proteins reached the 1.53 log<sub>10</sub> reduction obtained after *M. bovis* BCG vaccination. In the CB6F1 mouse strain (Figure 5C) vaccination with four of the seven proteins, Rv3615c, Rv3614c, ESAT-6 and Rv3849, induced a protective immune response resulting in a CFU reduction between 0.24 and 0.52 log<sub>10</sub>.

EXAMPLE 6: The optimal prophylactic vaccination dose of H64 for the CB6F1 mice strain is 5 μg of protein in CAF01. Groups of CB6F1 or mice were either vaccinated three times with various doses of the recombinant H64 fusion protein formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 μL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and six weeks after third vaccination all animals were aerosolly challenge with virulent *M. tuberculosis* Erdman. Three weeks after immunization the potential vaccine specific T cell present in spleen (Figure 6A) and blood (Figure 6B) was measured by *in vitro* stimulation of isolated PBMC-s and splenocytes. The general tendency being that the higher the vaccine dose the stronger T cell responses. Six 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 6C). Immunization with 5 μg H64 resulted in the lowest number of bacteria and though the best protection.
EXAMPLE 7: Single protein and protection in a preventive TB vaccination model
Groups of CB6F1 mice were either vaccinated three times with 5 ug of one of the recombinant proteins formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 uL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and six weeks after third vaccination all animals were aerosoil challenge with virulent M. tuberculosis Erdman. Six 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 7). Vaccination with the individual ESX-2 and ESX-5 secreted proteins - Rv3891, Rv3619 and Rv3620 - induced comparable and significant protection against tuberculosis though not at the level of BCG.

EXAMPLE 8: CFP10-ESAT6 family fusions - immune responses and protection in a preventive TB vaccination model.
Twelve of the paralogous ESAT-6 family proteins were fused as six protein dimers: CFP10-ESAT6, Rv3891c-3890c, Rv0287-0288, Rv3445c-3444c, Rv3620c-3619c and Rv3905c-3904c (Figure 8A). In all construes the two proteins were separated by a nine amino acid long spacer sequence (GLVPRGSTG). Groups of CB6F1 or B6C3F1 mice were either vaccinated three times with 5 ug of each of the recombinant dimer proteins formulated in the liposome based adjuvant CAF01, injected 3 times with an equal volume of saltwater (200 uL) or vaccinated once with BCG. Spacing between vaccination were 2 weeks and six weeks after third vaccination all animals were aerosoil challenged with virulent M. tuberculosis Erdman. Six 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 8B and C). In CB6F1 mice, vaccination with CFP10-ESAT6, Rv3891c-3891c or Rv0287-0288 resulted in a log10 reduction of the bacterial number in the lung between 0.25-0.45 (Figure 8B). The same three dimer fusion plus Rv3620c-3619c protected B6C3F1 mice (Figure 8C). In none of the two strains was the protective efficacy of the tested dimer fusions on level with the live vaccine BCG that induced a log10 reduction of 1.4 and 0.85. In B6C3F1 mice vaccine specific responses was found in blood 3 weeks post third vaccination in the CFP10-ESAT6, Rv0287-0288, Rv3445c-3444c, Rv3620c-3619c and Rv3905c-3904c vaccinated groups (Figure 8D).
EXAMPLE 9: The H65 fusion protein - immune responses and protection in a preventive TB vaccination model.

Groups of B6C3F1 mice were vaccinated three times with 5 ug of H65 (Figure 9A) formulated in CAF01 adjuvant or once with the live vaccine M. bovis BCG. To confirm vaccine induced responses against each of the three ESX secreted dimers, splenocytes were isolated 3 weeks post third vaccination. 5 x 10⁶ splenocytes were stimulated for 6 hours with 2 ug of either Rv3891c-Rv3890c, Rv0287-Rv0288 or Rv3620c-Rv3619c dimer fusion-protein. The CD4 T cell expression of IL-2, TNF-a and IFN-γ cytokines in response to antigen stimulation was measured by polychromatic flow cytometry (Figure 9B). The ranking of response was Rv3891c-Rv3890c > Rv0287-Rv0288 > Rv3620c-Rv3619c however for all three dimer proteins we observed vaccine specific polyfunctional T cells including IL-2⁺, TNF-a⁺, IFN-γ⁺ and IL-2⁺, TNF-oc⁺ CD4 T cells. Six weeks after third vaccination all animals were aerosolly challenge with virulent M. tuberculosis Erdman and euthanized six weeks later. The number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 9C). H65 and BCG both induced significant protection (log₁₀ reduction ~ 0.7).

Groups of CB6F1 mice were vaccinated three times with 5 ug of H65 (Figure 9A) or H56 (fusion of Ag85B-ESAT6 and Rv2660c) formulated in CAF01 adjuvant or once with the live vaccine M. bovis BCG. To establish which of the 6 antigens in H65 that are immunogenic in the CB6F1 inbred mice strain, splenocytes were isolated 3 weeks post third vaccination. 5 x 10⁶ splenocytes were stimulated for 72 hours with 2 ug of either Rv3891c, Rv3890c, Rv0287, Rv0288, Rv3620c or Rv3619c single protein (Figure 10A). Significant amounts of IFN-γ was released to the medium from cells isolated from H65 vaccinated animals and stimulated with Rv0287, Rv0288, Rv3620c or Rv3619c whereas there was no response towards Rv3891c or Rv3890c (Figure 10A). In saline injected animals there was no response to any of the 6 antigens after stimulation (Figure 10B).

Six weeks after third vaccination all animals were aerosolly challenged with virulent M. tuberculosis Erdman and euthanized six or twenty-four weeks later. The number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 10C and D). At week six after challenge (Figure 9C) H65 and H56 both resulted in similar and significant protection (log₁₀ reduction ~ 0.8). At week 24 post challenge (Figure 10D) vaccination with H65 or H56 still in-
duced a comparable reduction of bacteria number in the lung but compared to the control group (CAF01) the difference was no longer statistical significant (log_{10} reduction = 0.37 for H65 and 0.34 for H56).

**EXAMPLE 10: The H65 fusion protein - immune responses after post-exposure vaccination in two mice strains.**

In the post-exposure TB vaccination model mice are initially challenged with M. tb. via the aerosol route. To mimic the latent stage of infection mice are given antibiotics *ad libitum* in the drinking water from 6 to 12 weeks post-infection (p.i.). Groups of FvB or CB6F1 mice were either vaccinated three times with 5 μg H65 formulated in the liposome based adjuvant CAF01 or injected 3 times with an equal volume of saltwater (200 uL).

Infection-driven immune responses specific for the components of H65 (Rv3891c, Rv3890c, Rv0287, Rv0288, Rv3620c, Rv3619c) at week 17 p.i. were measured in the lung of infected FvB mice and the responses were barely detectable (Figure 11B). Following vaccination, we measured a pronounced response in the lungs directed towards Rv3619c in the FvB mouse strain (Figure 11A). In the CB6F1 mouse strain there was in addition a response raised towards Rv0287 and Rv0288 (Figure 11C). Hence, the H65 fusion-protein is highly immunogenic resulting in a robust induction of vaccine-specific IFN-y release. After 37 weeks infection the number of bacteria was enumerated in individual mice (Figure 10D). Comparison between H65 vaccinated and the negative control group shows a reduction of CFU's in H65 vaccinated animals. The protection level was comparable to the positive control (H56) and more pronounced than for the BCG vaccine.

**EXAMPLE 11: The abundance of the Rv1284 protein is increased under nutrient starvation**

Rv1284 has previously been identified by proteomics in *M. tuberculosis* lysates. To investigate the abundance of the protein under nutrient starvation, two-dimensional difference gel electrophoresis (2D DIGE) was applied to investigate the culture filtrate (CF) and lysate proteome of *M. tuberculosis* H37Rv bacteria in normal log phase growth and after six weeks of nutrient starvation.

Erlenmeyer flasks containing 200 ml of modified Sauton medium were inoculated with \(2 \times 10^6\) bacteria per ml and placed in a standard shaking incubator at 37°C. After 7 days of
growth to log phase, cultures were pelleted, washed twice with PBS, and resuspended in 200 ml PBS followed by incubation for six weeks without shaking. Control log phase cultures were obtained after 7 days of culturing in 200 ml modified Sauton medium in 500 ml flasks at 37°C under shaking conditions. After harvesting of cultures, the bacterial pellet was washed twice in PBS, resuspended in 10 mM Tris, 250 mM sucrose buffer, pH 7.0, and broken with glass beads using a Mini-Beadbeater. The lysates were sterile filtered and protein concentration determined by the 2-D Quant kit (GE Healthcare). In addition, the culture medium was collected, sterile filtered and concentrated approx. 160 times in Centrprep-3 ultrafiltration units.

Lysate and CF samples were analysed in two separate experiments. Each 2D DIGE experiment included triplicate log phase and starvation samples. 50 µg of each sample was prepared for 2D DIGE by the 2D Clean-up kit (GE Healthcare) and resolubilized in 30 mM Tris, 7 M Urea, 2 M Thio-Urea, 4% CHAPS, pH 8.5. Cy2, Cy3 and Cy5 minimal labeling was performed with 125 pmol of each CyDye, followed by isoelectric focusing with pH 4-7 IPG strips. Cy2, Cy3 and Cy5 labeled samples were applied during the rehydration step in 8 M urea, 2% CHAPS, 0.5% IPG buffer, 18 mM DTT. The second dimension separation was performed in 10 to 20% Tris-glycine SDS-PAGE gradient gels. After electrophoresis, the gels were scanned by a Typhoon 9410 gel imager, and spot images were analyzed with the Image Master Platinum 2.0 software. Spots which displayed more than 1.5 fold difference in volume ratio, p<0.05 (student's t-test), were selected for identification. The 2D DIGE gels were silver stained and spots were excised for MALDI-TOF MS or MALDI-TOF MS/MS analysis. Two spots (#1666 and #1669 on Figure 12) displayed increased abundance in CF from six week nutrient starved cultures compared to log phase cultures. These spots were identified by MALDI-TOF MS and MALDI-TOF MS/MS as Rv1284. In parallel, these spots were also selected as increased in lysates from nutrient starved cultures, and confirmed as Rv1284 by MS.

EXAMPLE 12: Rv1284 immune responses and protection in a preventive TB vaccination model.

Groups of CB6F1 and B5C3F1 mice were vaccinated three times with 5 µg of Rv1284 formulated in CAF01 adjuvant or once with the live vaccine M. bovis BCG. To measure vaccine induced responses blood was drawn from individual aniamsl and PBMC's isolated 2 weeks after third vaccination. 5 x 10⁶ PBMC's were stimulated for 72 hours with 2
ug of vaccine antigen (Rv1284) or a control antigen (Rv0287) and released IFN-γ was measured in the cell medium by ELISA (Figure 13A and B). In both mice strain vaccination with Rv1284 induced a significant antigen specific immune response.

Six weeks after third vaccination all animals were aerosolly challenge with virulent M. tuberculosis Erdman and euthanized six weeks later. The number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 13C and D). Rv1284 vaccination reduced the number of bacteria significantly in both strains compared to the saline control group (log₁₀ reduction = 0.43 in CB6F1 and 0.54 in B6C3F1).

EXAMPLE 13: The protective efficacy of H1 + Rv1284 in a preventive TB vaccination model.

Groups of FVB (H2a)q mice were vaccinated three times with 5 ug of H1 fusion protein or H1 + Rv1284 formulated in CAF01 adjuvant. The control group received three saline injections. Six weeks after third vaccination/injection all animals were aerosolly challenge with virulent M. tuberculosis Erdman and euthanized six weeks later. The number of bacteria in lungs of individual animals was determined by plating dilutions of lung homogenate and counting the number of colonies (Figure 14). Vaccination of FVB mice with the H1 fusion protein only reduced the number of bacteria in half (log₁₀ reduction = 0.32) whereas the mixture of H1+Rv1284 reduced the bacteria number 6.6 times (log₁₀ reduction = 0.82). Statistically the H1+Rv1284 vaccinated animals had significantly lower bacteria load than both the saline injected and H1 vaccinated group.

EXAMPLE 14: Immune response of Ag85B, ESAT-6 and Rv1284 after post exposure vaccination with single proteins.

Mice were infected, treated with antibiotics and vaccinated according to the protocol described in example 3. Here, groups of mice were vaccinated three times with either Ag85B, ESAT6 or Rv1284, all formulated in the liposome based adjuvant CAF01. The control mice were vaccinated in a similar way with saltwater. One week following the final vaccination lymphocytes were obtained from the lungs and used for 6 hour in vitro culture with the respective vaccine antigen i.e. Ag85B, ESAT6 or Rv1284 and TB10.4 as a measurement for infection-driven responses. Vaccine-or infection-driven responses were measured by staining for intracellular cytokines and cumulative frequency of CD4 T cell
responders, expression either IFN-γ, IL-2, TNF-a or any combination of the three, were determined. Vaccination with Ag85B, ESAT-6 or Rv1284 all induced significant vaccine-specific CD4 T cell responses (Figure 15) that could not be measured at comparable levels in the control animals. The TB10.4 infection-driven CD4 T cell response in comparison was low but this is expected given that at this particular timepoint the bacterial load is still relatively low.
References

Claims

1. A fusion protein or antigen cocktail, which comprises the amino acid sequence selected from
   (a) SEQ ID NO.1 (ESAT6), SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849) and SEQ ID NO 6 (Rv3872), or
   (b) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849) and SEQ ID NO 6 (Rv3872), or
   (c) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872) and SEQ ID NO 7 (Rv3616c) or
   (d) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872), SEQ ID NO 7 (Rv3616c) and
      SEQ ID NO 8 (Rv3881c) or
   (e) SEQ ID NO 2 (Rv3614c), SEQ ID NO 3 (Rv3615c), SEQ ID NO 4 (Rv3865), SEQ ID NO 5 (Rv3849), SEQ ID NO 6 (Rv3872) and SEQ ID NO 8 (Rv3881c) or
   (f) SEQ ID NO 9 (Rv3891c), SEQ ID NO 10 (Rv3890), SEQ ID NO 11 (Rv0287),
      SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c) and SEQ ID NO 14
      (Rv3619), or
   (g) SEQ ID NO 11 (Rv0287), SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c),
      SEQ ID NO 14 (Rv3619), NO 7 (Rv3616c) and SEQ ID NO 3 (Rv3615c) or
   (h) SEQ ID NO 11 (Rv0287), SEQ ID NO 12 (Rv0288), SEQ ID NO 13 (Rv3620c),
      SEQ ID NO 14 (Rv3619), NO 7 (Rv3616c), SEQ ID NO 3 (Rv3615c) and SEQ
      ID 9 (Rv3881c), or
   (i) SEQ ID NO 1 (ESAT6), SEQ ID NO 15 (Ag85B) and SEQ ID NO 16 (Rv1284), or
   (j) an amino acid sequence analogue having at least 80% sequence identity to any-
      one of the sequences in (a) - (i) and at the same time being immunogenic;

2. A fusion protein or antigen cocktail according to claim 1, wherein the amino acid se-
   quence analogue has at least 90% or more preferred 95 % sequence identity to the se-
   quences in (a), (b), (c), (d), (e), (f), (g), (h) or (i).

3. A fusion protein according to claim 1 or 2 wherein 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, wherein the cysteine have been replaced with serine.

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

6. A fusion protein according to claim 5 with the amino acid sequence selected from SEQ ID NO 18 (H64), SEQ ID NO 19 (H68), SEQ ID NO 20 (H69), SEQ ID NO 21 (H70), SEQ ID NO 22 (H71), SEQ ID NO 23 (H65), SEQ ID NO 24 (H72), SEQ ID NO 25 (H73) or SEQ ID NO 26 (H67).

7. An antigen cocktail according to claim 1-2, wherein the cocktail comprises SEQ ID NO 16 and SEQ ID NO 17.

8. Use of a fusion protein or an 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.

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

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

11. 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 thereof.

12. 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 2

A

B

Log_{10} CFU/lung

Saline  H64  H56  BCG

Vaccination groups

**  ***  **  ***
Figure 12
Figure 13