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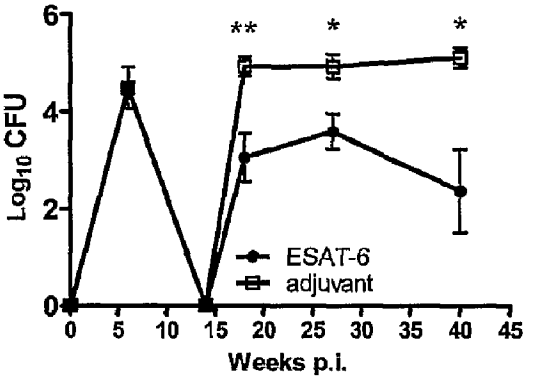
(54) **Titre : VACCIN CONTRE LA TUBERCULOSE TB POUR PREVENIR UNE REACTIVATION**
(54) **Title: A TUBERCULOSIS TB VACCINE TO PREVENT REACTIVATION**



(57) **Abrégé/Abstract:**
The present invention discloses a vaccine or immunogenic composition that can be administred to latently infected individuals to prevent reactivation of latent tuberculosis infection caused by species of the tuberculosis complex microorganisms (Mycobacterium tuberculosis., M.bovis, M.africanum), The invention is base on a number of M. tuberculosis derived proteins and protein fragments which are constitutively expressed in different stages of the infection. The invention is directed to the use of these polypeptides, immunologically active fragments thereof and the genes encoding them for immunological compositions such as vaccines.

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(54) **Title**: A TUBERCULOSIS TB VACCINE TO PREVENT REACTIVATION

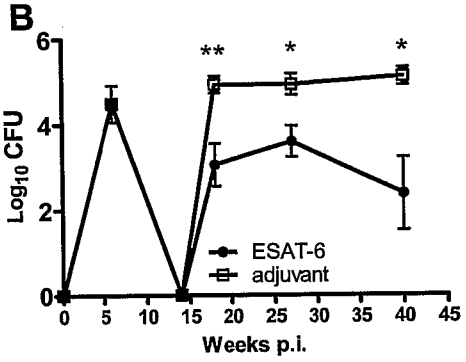

Figure 4

(57) **Abstract**: The present invention discloses a vaccine or immunogenic composition that can be administred to latently infected individuals to prevent reactivation of latent tuberculosis infection caused by species of the tuberculosis complex microorganisms (*Mycobacterium tuberculosis., M.bovis, M.africanum*), The invention is base on a number of *M. tuberculosis* derived proteins and protein fragments which are constitutively expressed in different stages of the infection. The invention is directed to the use of these polypeptides, immunologically active fragments thereof and the genes encoding them for immunological compositions such as vaccines.

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A tuberculosis TB vaccine to prevent reactivation

Field of invention

The present invention discloses a vaccine that can be administred to latently infected individuals to prevent reactivation of latent tuberculosis infection caused by species of the tuberculosis complex microorganisms (*Mycobacterium tuberculosis*, *M.bovis, M.africanum*), by targetting constitutively expressed antigens such as ESAT6, CFP10 and other antigens from the ESX-1 secretion system.

General Background

Human tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is a severe global health problem, responsible for approx. 3 million deaths annually, according to the WHO. The worldwide incidence of new tuberculosis (TB) cases had been falling during the 1960s and 1970s but during recent decades this trend has markedly changed in part due to the advent of AIDS and the appearance of multidrug resistant strains of *M. tuberculosis*.

Organisms of the tuberculosis complex can cause a variety of diseases, but the commonest route of invasion is by inhalation of bacteria. This initiates an infection in the lung, which can ultimately spread to other parts of the body. Normally, this infection is restricted in growth by the immune system, so that the majority of infected individuals show few signs apart from cough and fever, which eventually abates. Approximately 30% of individuals are unable to contain the infection and they will develop primary disease, which in many cases will eventually prove fatal. However, it is believed that even those individuals who apparently control the infection remain infected, probably for the rest of their life. Certainly, individuals who have been healthy for years or even decades can suddenly develop tuberculosis, which has proven to be caused by the same organism they were infected with many years previously. *M. tuberculosis* and other organisms of the TB complex are unique in that the mycobacteria can evade the immune response and survive for long periods in a refractory non-replicating or slowly-replicating stage. This is referred to as latent TB and is at present a very significant global health problem which is estimated to affect approximately 1/3 of the worlds population (Anon., 2001).

The only vaccine presently available for clinical use is BCG, a vaccine whose efficacy remains a matter of controversy. Although BCG consistently performs well in animal models of primary infection, it has clearly failed to control the TB epidemic. Consistent with that, BCG vaccination appears to provide protection against pediatric TB (which is due to primary infection), while offering little or no protection against adult disease (which is often reactivation of latent infection acquired in childhood). It has also been shown that vaccination of individuals who are currently sensitized to mycobacteria or latently infected is ineffective.

The course of a *M. tuberculosis* infection runs essentially through 3 phases, as illustrated in figure 1. During the acute phase, the bacteria proliferate in the organs, until the immune response increases to the point at which it can control the infection whereupon the bacterial load peaks and starts declining. After this, a latent phase is established where the bacterial load is kept stable at a low level. In this phase it has been the current thinking that *M. tuberculosis* goes from active multiplication to a state of dormancy, essentially becoming non-replicating and remaining inside the granuloma.

However, recently it has become clear that even in the stage of infection characterized by constant low bacterial numbers at least part of the bacterial population remain in a state of active metabolism (Talaat AM et al. 2007). These bacteria therefore survive, maintain an active metabolism and replicate in the face of a strong immune response. In the infected individual there is therefore a balance between non-replicating bacteria (that may be very difficult for the immune system to detect as they are located intracellularly) and slowly replicating bacteria that has an active but changed expression profile in an attempt to adapt to the hostile environement encountered in the immune host. Bacteria in this stage are typically not targeted by most of the preventive vaccines that are currently under development in the TB field as exemplified by the lack of activity when classical preventive vaccines are given to latently infected experimental animals (Turner 2000).

In some cases, the balance is tilted in favour of the pathogen and the infection goes into the reactivation phase, where the bacteria start replicating rapidly and bacterial numbers in the infected individual increases. Bacteria that replicates in latently infected individuals under very strong immune pressure is the target for the vaccination strategy in the present invention. Bacteria in this latent infective stage are typically not targeted by most of the preventive vaccines that are currently under development in the TB field as exemplified by

the lack of activity when preventive vaccines are given to latently infected experimental animals (Turner et al 2000). This is not surprising as it is now known that a strong host immune response results in the down regulation of many antigens such as the major preventive vaccine antigen Ag85 and PstS (Rogerson, BJ et al 2006). For Ag85B it has been shown that after infection there is an initial transient increase in Ag85B expression but already after two weeks infection the level of bacterial Ag85B expression had dropped from 0.3 transcripts per CFU of M.tb. during the peak period to 0.02 transcripts per CFU and this low level is maintained at least up to 100 days post infection. Thus at any time point after week 2 of infection less than 2 % of the bacteria actively express Ag85B (ibid.). The low expression of Ag85B is supported by a rapid drop in the number of T cells capable of making IFN-g in response to Ag85B in the lung 3 weeks post infection or later.

In contrast some antigens are more stably (constitutively) expressed throughout the different stages of infection and one example of this is ESAT6. After the initial infection phase the ESAT-6 expression level stabilizes at 0.8 transcrips per CFU *M. tuberculosis.* This is a transcription level much higher than for Ag85B and this level is maintained stabely up to at least 100 days post infection (Rogerson, BJ et al 2006). Again transcription data is supported by immune data that shows strong T cell recognition of ESAT-6 at the later stages of infection at the site of infection (ibid.). This constitutive expression pattern is an important feature that illustrates that these molecules fullfills essential functions of crucial importance for the pathogen, functions that depends upon genes that needs to be constitutively expressed for the pathogen to survive in the immune host. These molecules are the basis for the current invention and are particularly important antigens for vaccines administred to latently infected individuals as they targets all stages of the bacterial lifestyle and therefore has the broadest possible basis for activity. This is different from current thinking that has been focused on identifying the antigens upregulated by mycobacteria during non-replicating persistence (Andersen, P. 2007, WO02048391, WO04006952, Lin MYand Ottenhoff TH 2008; Leyten EM. et al. 2006). Although such antigens are upregulated during non-replicating persistence they may not always be available for immune recognition as the amounts available from non-replicating bacteria are below a reasonable threshold for detection or for the triggering of protective immune effector functions.

In contrast, several of the proteins from the ESX-1 secretion system have been shown to be highly immunogenic and expressed at high levels . ESX-1 is conserved in several

pathogenic mycobacteria and involved in virulence of tubercle bacilli. The contribution of the individual ESX-1 proteins in secretion of ESAT-6, CFP10 and EspA has been well documented (Pym AS et al 2003; Guinn KI et al, 2004;Stanley, SA et al. 2003; Brodin, P. et al. 2006; MacGurn JA et al. 2005; Raghavan, S. et al. 2008) and the function of the effector molecules has been shown to be membrane lysis, escape from the phagosome and bacterial spreading (Gao LY et al 2004; Smith J. et al. 2008).

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The full nature of the immune response that controls latent infection and the factors that lead to reactivation are largely unknown. However, there is some evidence for a shift in the dominant cell types responsible. While CD4 T cells are essential and sufficient for control of infection during the acute phase, studies suggest that CD8 T cell responses are more important in the latent phase (van Pinxteren LA et al . 2000).

As one skilled in the art will readily appreciate, expression of a gene is not sufficient to make it a good vaccine candidate. The only way to determine if a protein is recognized by the immune system during latent infection with *M. tuberculosis* is to produce the given protein and test it in an appropriate assay as described herein. In this regard, our group has demonstrated that antigens strongly expressed by mycobacteria, such as ESAT-6 (Early Secretory Antigen Target-6) are recognized in individuals in all stages of infection and in fact in particular in latently infected individuals (Boesen , Ravn, Doherty 2002). However the ESAT-6 specific T cells primed during the natural infection are although they may be present in large numbers, almost exclusively of the socalled effector phenotype that are terminally differentiated T cells with a very limited lifespan and of low activity as protective T cells against infectious diseases (Seder R, et al. 2008). This is markedly different from the high quality, socallled polyfunctional T cells that are promoted by the vaccine demonstrated in the present study to protect against reactivation of TB.

It is far from all highly expressed and immunogenic proteins that are useful as post exposure vaccines because many will provoke hypersensitivity reactions and thereby worsen the situation instead. This was clearly demonstrated in the clinical trial of Kock´s original tuberculin vaccine. The vaccine was given as a post exposure vaccine to patients suffering from different forms of the disease including skin and pulmonary TB. The trial was a complete failure and several of the enrolled patients died because of severe hypersensitive reactions (Guttstadt A. 1891). Of the several hundred antigens known to be expressed during primary infection, and tested as vaccines, less than a half dozen have

demonstrated significant potential. So far only one antigen has been shown to have any potential as a postexposure vaccine (Lowrie, 1999). However this vaccine only worked if given as a DNA vaccine, an experimental technique so far not approved for use in humans. Moreover, the technique has proved controversial, with other groups claiming that vaccination using this protocol induces either non-specific protection or even worsens disease (Turner, 2000).

Therefore, an effective postexposure vaccination strategy to protect infected individuals against reactivation of the disease is highly desireable.

Summary of the invention

The invention is related to treating infections caused by species of the tuberculosis complex (*Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*) by a vaccine that can be administred to latently infected individuals to prevent reactivation of latent tuberculosis infection caused by species of the tuberculosis complex microorganisms (*Mycobacterium tuberculosis.*, *M.bovis, M.africanum*), by targetting constitutively expressed antigens such as ESAT6, CFP10 and EspA. ESAT6, CFP10 and EspA are all interdependently required for secretion and all belong to the ESX-1 secretion system known to be essential for virulence. These secreted antigens are crucial for bacterial dessimination and lysis of cellular membranes. ESAT6, CFP10 and EspA are also antigens that are constitutively expressed in the different stages of disease - whereas eg the expression of Ag85 is downregulated shortly after infection. Surprisingly immunogenic constitutively expressed antigens are preventing reactivation of latent tuberculosis infection when administered as a post exposure vaccine thereby keeping the infection latent.

Detailed disclosure of the invention

The invention discloses a vaccine or immunogenic composition that is administred postexpoure to latently infected individuals that prevents reactivation of tuberculosis comprising an antigen which is constitutively expressed during infection with M.tuberculosis or a nucleic acid encoding said antigen.

Preferably the composition comprises constitutively expressed antigens belonging to the ESX-1 secretion system, ESAT6 (SEQ ID NO. 1) , CFP10 (SEQ ID NO. 2), EspA (SEQ ID NO.3), Rv3614c (SEQ ID NO. 4), Rv3615c (SEQ ID NO. 5), EspR (SEQ ID NO. 6), Rv3868 (SEQ ID NO. 7) Rv3869 (SEQ ID NO. 8), Rv3870 (SEQ ID NO. 9), Rv3871 (SEQ ID NO. 10), Rv3872 (SEQ ID NO. 11), Rv3873 (SEQ ID NO. 12), Rv3876 (SEQ ID NO. 13), Rv3877 (SEQ ID NO. 14), Rv3878 (SEQ ID NO. 15), Rv3879c (SEQ ID NO. 16), Rv3880c (SEQ ID NO. 17), Rv3881c (SEQ ID NO. 18), Rv3882c (SEQ ID NO 32), Rv3883c (SEQ ID NO 33), Rv3865c (SEQ ID NO 34) or an immunogenic portion, e.g. a T-cell epitope, of any one of these sequences or an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in and at the same time being immunogenic.

Alternatively the composition comprises a mix of immunogenic portions preferably selected from the group consisting of SEQ ID NO. 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 and 31.

Another embodiment of the invention is a composition where said polypeptides are fused to an antigen expressed by bacteria within the mycobacteria family preferably where the fusion partner is a antigen which is constitutively expressed. A preferred fusion protein comprises ESAT6 fused to CFP10.

The composition according to the invention preferably comprises an additional delivery system selected among, live recombinant vaccines, that is gene-modified organisms such as bacteria or viruses expressing mycobacterial genes, or immunogenic delivery systems such as, DNA vaccines, that is plasmids expressing genes or gene fragments for the proteins described above, or protein vaccines, that is the proteins themselves or synthetic peptides derived from the proteins themselves delivered in a delivery system such as an adjuvant. The adjuvant is preferably selected from the group consisting of dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Treholose Dimycolate (TDM), Trehalose Dibehenate and muramyl dipeptide (MDP) most preferably an adjuvant promoting a polyfuctional T-cell response such as DDA/TDB and IC31.
The most preferred adjuvant comprises DDA/TDB and/or poly I:C. Alternatively the amino acid sequence is lipidated so as to allow a self-adjuvanting effect of the polypeptide.

The invention also discloses antigens described above for use in treatment of latent tuberculosis and preventing reactivation of the infection.

A method for treating an animal, including a human being, against reactivation of the tuberculosis infection caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, comprising administering to the animal the vaccine or immunogenic composition described above, wherein said vaccine or immunogenic composition is administered post infection, such as during or after acute stage infection and/or during latent stage infection.

The method can comprise a step of identifying a subject latently infected with a virulent mycobacteria e.g. by a diagnostic procedure such as the Mantoux tuberculin skin test (TST), the Quantiferon test, in vitro detection of responses to HBHA or the detection of IP10 after stimulation with a constitutively expressed antigen.

The invention also discloses the use of an antigen described above for the manufacture of a postexposure vaccine or immunogenic composition against reactivation of latent infections caused by species of the tuberculosis complex e.g. Mycobacterium tuberculosis, M.bovis and M.africanum, wherein said vaccine or immunogenic composition is for administration post infection, such as during or after acute stage infection and/or during latent stage comprising one or more immunogenic portions described above.

Mycobacteriums success as a pathogen is due to the complex and delicated way it interacts with ist host - a process controlled in part by the specialized ESX-1 bacterial protein-secretion system. The ESX-1 system delivers bacterial proteins (e.c. ESAT-6, CFP10 and EspA) into host cells and it critical for virulence. After being secreted from the bacilli the ESAT-6 proteins forms pores in the phagosomal membrane, allowing the bacilli to escape into the cytosol from its containment in the phagosome and thereby it facilitates cell-to-cell spread.

The constitutive expression pattern is an important feature that illustrates that these molecules fullfills essential functions of crucial importance for the pathogen, functions that depends upon genes that needs to be constitutively expressed for the pathogen to survive in the immune host. These molecules are the basis for the current invention and are particu-

larly important antigens for vaccines administred to latently infected individuals as they targets all stages of the bacterial lifestyle and therefore has the broadest possible basis for activity.

ESAT6, CFP10 and EspA are all interdependently required for secretion and all belong to the ESX-1 secretion system known to be essential for virulence. These secreted antigens are crucial for bacterial dessimination and lysis of cellular membranes. ESAT6, CFP10 and EspA are also antigens that are constitutively expressed in the different stages of disease - whereas eg the expression of Ag85 is downregulated shortly after infection. Immunogenic constitutively expressed antigens prevent reactivation of latent tuberculosis infection when administered as a therapeutic vaccine thereby keeping the infection latent.

Definitions

Polyfunctional T cells

By the term Polyfunctional T cells is understood T cells that simultaneously express all the cytokines IFN-γ, IL-2, and TNF-α, or IL-2 plus at least one of the two other cytokines IFN-γ and TNF-α.

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 his-tag or a signal peptide.

Each polypeptide may thus be characterised by specific amino acids and be encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide and still be immunogenic

in any of the biological assays described herein. Substitutions are preferably "conservative". These are defined according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. The amino acids in the third column are indicated in one-letter code.

ALIPHATIC	Non-polar	GAP
		ILV
	Polar-uncharged	CSTM
		NQ
	Polar-charged	DE
		KR
AROMATIC		HFWY

A preferred polypeptide within the present invention is an immunogenic antigen from *M. tuberculosis* produced when the organism is subjected to the stresses associated with latent infection. Such antigen can for example also be derived from the *M. tuberculosis* cell and/or *M. tuberculosis* culture filtrate. Thus, a polypeptide comprising an immunogenic portion of one of the above antigens may consist entirely of the immunogenic portion, or may contain additional sequences. The additional sequences may be derived from the native *M. tuberculosis* antigen or be heterologous and such sequences may, but need not, be immunogenic.

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.

In the present context the term "substantially pure polypeptide fragment" means a polypeptide preparation which contains at most 5% by weight of other polypeptide material with which it is natively associated (lower percentages of other polypeptide material are preferred, e.g. at most 4%, at most 3%, at most 2%, at most 1%, and at most ½%). It is preferred that the substantially pure polypeptide is at least 96% pure, *i.e.* that the polypep-

tide constitutes at least 96% by weight of total polypeptide material present in the preparation, and higher percentages are preferred, such as at least 97%, at least 98%, at least 99%, at least 99,25%, at least 99,5%, and at least 99,75%. It is especially preferred that the polypeptide fragment is in "essentially pure form", *i.e.* that the polypeptide fragment is essentially free of any other antigen with which it is natively associated, *i.e.* free of any other antigen from bacteria belonging to the tuberculosis complex or a virulent mycobacterium. This can be accomplished by preparing the polypeptide fragment by means of recombinant methods in a non-mycobacterial host cell as will be described in detail below, or by synthesizing the polypeptide fragment by the well-known methods of solid or liquid phase peptide synthesis, e.g. by the method described by Merrifield or variations thereof. For the purpose of the present invention it will be understood that the above definition of "substantially pure polypeptide or polypeptide fragment" does not exclude such polypeptides or polypeptide fragments when present in combination with other purified or synthetic antigens of mycobacterial or non-mycobacterial origin.

By the term "virulent mycobacterium" is understood a bacterium capable of causing the tuberculosis disease in an animal or in a human being. Examples of virulent mycobacteria include but are not limited to *M. tuberculosis, M. africanum,* and *M. bovis*. Examples of relevant animals are cattle, possums, badgers and kangaroos.

By "an infected individual" is understood an individual with culture or microscopically proven infection with virulent mycobacteria, and/or an individual clinically diagnosed with TB and who is responsive to anti-TB chemotherapy. Culture, microscopy and clinical diagnosis of TB are well known by any person skilled in the art.

By the term "PPD-positive individual" is understood an individual with a positive Mantoux test or an individual where PPD (purified protein derivative) induces a positive *in vitro* recall response determined by release of IFN-γ.

By "a latently infected individual" is understood an individual, who has been infected by a virulent mycobacterium, e.g. *M. tuberculosis* , but shows no sign of active tuberculosis. It is likely that individuals who have been vaccinated, e.g. by BCG, or treated for TB may still retain the mycobacteria within their bodies, although this is currently impossible to prove since such individuals would be expected to be postive if tested for PPD reactivity. Nonetheless, in its most accurate sense, "latently-infected" may be used to describe any

individual who has *M. tuberculosis* residing in their tissues but who is not clinically ill. A latently infected individual can be identified by a number of methods in clinical use today such as the Mantoux tuberculin skin test (TST), the Quantiferon test and in the future there may be even more sensitive means of diagnosing this particular stage of the infection such as the recently suggested in vitro detection of responses to HBHA (Hougardy 2007) or the detection of IP10 after stimulation in vitro with ESAT6 (Ruhwald 2008)

By the term "reactivation" is understood the situation where the balance between non-replicating bacteria (that may be very difficult for the immune system to detect as they are located intracellularly) and slowly replicating bacteria that has an active but changed expression profile in an attempt to adapt to the hostile environement encountered in the immune host is tilted in favour of the pathogen and the infection goes into the phase, where the bacteria start replicating rapidly again and bacterial numbers in the infected individual increases. These bacteria that replicates in latently infected individuals under very strong immune pressure is the target for the vaccination strategy in the present invention.

By the term "IFN-γ" is understood interferon-gamma. The measurement of IFN-γ is used as an indication of an immunological response.

By the terms "nucleic acid fragment" and "nucleic acid sequence" are understood any nucleic acid molecule including DNA, RNA , LNA (locked nucleic acids), PNA, RNA, dsRNA and RNA-DNA-hybrids. Also included are nucleic acid molecules comprising non-naturally occurring nucleosides. The term includes nucleic acid molecules of any length e.g. from 10 to 10000 nucleotides, depending on the use. When the nucleic acid molecule is for use as a pharmaceutical, e.g. in DNA therapy, or for use in a method for producing a polypeptide according to the invention, a molecule encoding at least one epitope is preferably used, having a length from about 18 to about 1000 nucleotides, the molecule being optionally inserted into a vector.

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.

Constitutively expressed genes are defined as genes that after a detailed analysis of mRNA at a population level are equally well expressed in vivo in the lung at time points later than three weeks post infection after being correlated for M. tb CFU numbers in the lung. From this definition it follows that a constitutive gene may be differentially expressed at a single bacteria level. The method to quantitate gene expression is quantitative PCR. "Equaly well" is defined as being within +/- 5 fold the level from the previous measurement The comparison is always to the time point immediately preceeding the current. Time between measurements cannot be longer than the time between infection and the previous measurement. E.c if expression of a gene is measured the fist time at week 3 post infection the second measurement can not be done later than 6 weeks post infection and the third 12 weeks post infection etc.

Constitutively expressed antigens are polypeptides or part of these polypeptides which are products of constitutively expressed genes.

Sequence identity

The term "sequence identity" indicates a quantitative measure of the degree of homology between two amino acid sequences of equal length or between two nucleotide sequences of equal length. The two sequences to be compared must be aligned to best possible fit allowing the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as $\frac{(N_{ref}-N_{dif})100}{N_{ref}}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N_{dif}=2 and N_{ref}=8). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC (N_{dif}=2 and N_{ref}=8). Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson, 1988, or www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one aspect of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., *et al* 1994, available at http://www2.ebi.ac.uk/clustalw/.

A preferred minimum percentage of sequence identity is at least 80%, such as at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and at least 99.5%.

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 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 ESAT-6 and CFP10 such studies have shown that every part of the antigen contains T-cell epitopes (Mustafa et al. 2000, Arend SM et al. 2000). 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. Consistent with this definition, an immunogenic portion of a polypeptide as described herein can be identified as a portion which elicits an immune response, c.f. the definition of "immune response" herein below.

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. Hence, it is preferred that the polypeptide fragment of the invention has a length of at least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, and at least 30 amino acid residues. Hence, in important embodiments of the inventive method, it is preferred that

the polypeptide fragment has a length of at most 50 amino acid residues, such as at most 40, 35, 30, 25, and 20 amino acid residues. It is expected that the peptides having a length of between 10 and 20 amino acid residues will prove to be most efficient as MHC class II epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 18, such as 15, 14, 13, 12 and even 11 amino acid residues. It is expected that the peptides having a length of between 7 and 12 amino acid residues will prove to be most efficient as MHC class I epitopes and therefore especially preferred lengths of the polypeptide fragment used in the inventive method are 11, such as 10, 9, 8 and even 7 amino acid residues.

Immunogenic portions of polypeptides may be recognised by a broad part (high frequency) or by a minor part (low frequency) of the genetically heterogeneous 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 (Sinigaglia, 1988, Kilgus, 1991).

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 shown (Olsen, 2000) that such epitopes can induce protection regardless of the fact that they are not as strongly or broadly recognised.

Variants

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 may also be immunogenic as determined by any of the assays described herein.

Immune individual

An immune individual is defined as a person or an animal, which has cleared or controlled an infection with virulent mycobacteria or has received a prophylactic vaccination, such as vaccination with *M.bovis* BCG.

Immune response

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

- An in vitro cellular response is determined by induction of the release of a relevant cytokine such as IFN-γ from, or the induction of proliferation in lymphocytes withdrawn from an animal or human being currently or previously infected with virulent mycobacteria or immunized with the relevant polypeptide.The induction being performed by the addition of the polypeptide or the immunogenic portion of the polypeptide to a suspension comprising from $2x10^5$ cells to $4x10^5$ 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 is defined as 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 detecting the immune response is the ELISpot method, in which the frequency of IFN-γ producing cells is determined. In an ELIspot plate (MAHA, Millipore) precoated with anti-murine IFN-γ antibodies (PharMingen) graded numbers of cells isolated from either blood, spleen, or lung (typically between 1 to 4 x 10^5 cells /well) are incubated for 24-32 hrs in the presence of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μg per ml. The plates are subsequently incubated with biotinylated anti-IFN-γ antibodies followed by a streptavidin-alkaline phosphatase incubation. The IFN-γ producing cells are identified by adding BCIP/NBT (Sigma), the relevant substrate giving rise to spots. These spots 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 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 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.

Preparation methods

In general, *M. tuberculosis* antigens, and DNA sequences encoding such antigens, may be prepared using any one of a variety of procedures.
They may be purified as native proteins from the *M. tuberculosis* cell or culture filtrate by procedures such as those described above. Immunogenic antigens may also be produced recombinantly using a DNA sequence encoding the antigen, which has been inserted into an expression vector and expressed in an appropriate host. Examples of host cells are *E. coli*. The polypeptides or immunogenic portion hereof can also be produced synthetically having fewer than about 100 amino acids, and generally fewer than 50 amino acids and may be generated using techniques well known to those ordinarily skilled in the art, such as commercially available solid-phase techniques where amino acids are sequentially added to a growing amino acid chain.

In the construction and preparation of plasmid DNA encoding the polypeptide as defined for DNA vaccination a host strain such as *E. coli* can be used. Plasmid DNA can then be prepared from cultures of the host strain carrying the plasmid of interest, and purified using e.g. the Qiagen Giga -Plasmid column kit (Qiagen, Santa Clarita, CA, USA) including an endotoxin removal step. It is preferred that plasmid DNA used for DNA vaccination is endotoxin free.

Fusion proteins

The immunogenic polypeptides may also be produced as fusion proteins, by which methods superior characteristics of the polypeptide of the invention can be achieved. For instance, fusion partners that facilitate export of the polypeptide when produced recombinantly, fusion partners that facilitate purification of the polypeptide, and fusion partners which enhance the immunogenicity of the polypeptide fragment of the invention are all interesting possibilities. Therefore, the invention also pertains to a fusion polypeptide

comprising at least one polypeptide or immunogenic portion defined above and at least one fusion partner. The fusion partner can, in order to enhance immunogenicity, be another polypeptide derived from *M. tuberculosis*, such as of a polypeptide fragment derived from a bacterium belonging to the tuberculosis complex, such as ESAT-6, CFP10, EspA, TB10.4, RD1-ORF5, RD1-ORF2, Rv1036, MPB64, MPT64, Ag85A, Ag85B (MPT59), MPB59, Ag85C, 19kDa lipoprotein, MPT32 and alpha-crystallin, or at least one T-cell epitope of any of the above mentioned antigens (Skjøt et al 2000; WO0179274; WO0104151; US patent application 09/0505,739; Rosenkrands *et al* 1998; Nagai et al 1991). The invention also pertains to a fusion polypeptide comprising mutual fusions of two or more of the polypeptides (or immunogenic portions thereof) of the invention. Other fusion partners, which could enhance the immunogenicity of the product, are lymphokines such as IFN-γ, IL-2 and IL-12. In order to facilitate expression and/or purification, the fusion partner can e.g. be a bacterial fimbrial protein, e.g. the pilus components pilin and papA; protein A; the ZZ-peptide (ZZ-fusions are marketed by Pharmacia in Sweden); the maltose binding protein; gluthatione S-transferase; β-galactosidase; or poly-histidine. Fusion proteins can be produced recombinantly in a host cell, which could be *E. coli*, and it is a possibility to induce a linker region between the different fusion partners.

Other interesting fusion partners are polypeptides, which are lipidated so that the immunogenic polypeptide is presented in a suitable manner to the immune system. This effect is e.g. known from vaccines based on the Borrelia burgdorferi OspA polypeptide as described in e.g. WO 96/40718 A or vaccines based on the Pseudomonas aeruginosa OprI lipoprotein (Cote-Sierra J 1998). Another possibility is N-terminal fusion of a known signal sequence and an N-terminal cystein to the immunogenic polypeptide. Such a fusion results in lipidation of the immunogenic polypeptide at the N-terminal cystein, when produced in a suitable production host.

Uses

Vaccine

A vaccine is a biological preparation that establishes or improves immunity to a particular disease. Vaccines can be prophylactic (e.g. to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), postexposure (e.g. to prevent reactivation in latently infected individuals without clinical symptoms) or therapeutic (e.g. vaccines used

to treat active disease either alone or combined with antibiotic treatment to shorten treatment)

An animal model for latent TB

To induce a low grade latent infection with M.tb, animals are first given an aerosol infection using a normal dose of *M.tb* (approximately 150 bacteria in the lungs). After 6 weeks of infection, the animals are then given a suboptimal chemotherapy treatment of 6 weeks during which most – but not all - of the bacteria are eradicated. The remaining bacteria will establish a latent infection. Following the chemotherapy treatment some animals will be vaccinated to examine the ability of the vaccine to prevent re-activation of the latent infection, which will occur spontaneously 5-15 weeks after the chemotherapy treatment. See figure 2.

Protein Vaccine

Another part of the invention pertains to a vaccine composition comprising a polypeptide (or at least one immunogenic portion thereof) or fusion polypeptide according to the invention. 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.

An effective vaccine, wherein a polypeptide of 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

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 dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), Treholose Dimycolate (TDM), Trehalose Dibehenate and muramyl dipeptide (MDP).

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231 and 4,599,230.

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. 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 immunogenic and effective in preventing reactivation. 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 intra pulmonary, e.g by aerosol or inhalation, 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. In instances where the individual has already become infected or is suspected to have become infected, the previous vaccination may have provided sufficient immunity to prevent primary disease, but as discussed previously, boosting this immune response will not help against the latent infection. In such a situation, the vaccine will necessarily have to be a post exposure vaccine designed for efficacy against the latent stage of infection or re-emerging active tuberculosis infection.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response. The vaccine may comprise two or more polypeptides or immunogenic portions, where all of the polypeptides are as defined above, or some but not all of the peptides may be derived from virulent mycobacteria. In the latter example, the polypeptides not necessarily fulfilling the criteria set forth above for polypeptides may either act due to their own immunogenicity or merely act as adjuvants.

The vaccine may comprise 1-20, such as 2-20 or even 3-20 different polypeptides or fusion polypeptides, such as 3-10 different polypeptides or fusion polypeptides.

DNA Vaccine.

The nucleic acid fragments of the invention may be used for effecting *in vivo* expression of antigens, *i.e.* the nucleic acid fragments may be used in so-called DNA vaccines as reviewed in Ulmer et al 1993, which is included by reference.

Hence, the invention also relates to a post exposure vaccine comprising a nucleic acid fragment according to the invention, the vaccine effecting *in vivo* expression of antigen by an animal, including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer treatment of the infections caused by virulent mycobacteria in an animal, including a human being.

The efficacy of such a DNA vaccine can possibly be enhanced by administering the gene encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response.

Live recombinant vaccines

One possibility for effectively activating a cellular immune response for a post exposure vaccine can be achieved by expressing the relevant antigen in a vaccine in a non-pathogenic microorganism or virus. Well-known examples of such microorganisms are *Mycobacterium bovis* BCG, *Salmonella* and *Pseudomona* and examples of viruses are Vaccinia Virus and Adenovirus.

Therefore, another important aspect of the present invention is an improvement of the living BCG vaccine presently available, wherein one or more copies of a DNA sequence encoding one or more polypeptide as defined above has been incorporated into the genome of the micro-organism in a manner allowing the micro-organism to express and secrete the polypeptide. The incorporation of more than one copy of a nucleotide sequence of the invention is contemplated to enhance the immune response.

Another possibility is to integrate the DNA encoding the polypeptide according to the invention in an attenuated virus such as the vaccinia virus or Adenovirus (Rolph et al 1997). The recombinant vaccinia virus is able to replicate within the cytoplasma of the infected host cell and the polypeptide of interest can therefore induce an immune response, which is envisioned to induce protection against TB.

The invention will now be described in further details in the following non-limiting examples.

Figure legends

Figure 1: The course of a *M. tuberculosis* infection runs essentially through 3 phases

Figure 2: Model for postexposure vaccination to prevent reactivation

Figure 3: TB vaccination model.
A schematic overview of the model used at the SSI for the testing of postexposure vaccines. Mice are infected with virulent *M.tb* by the aerosol route. From weeks 6 to week 12 post infection mice are treated with antibiotics to establish a state of latent TB. The mice are vaccinated 2 to three times with 3 weeks interval initiated at week 10 post infection with the postexposure vaccine candidates. The mice are allowed time to reactivate the disease and approximately 20 weeks later the lungs are assessed for bacterial numbers to assess the protective efficacy of the vaccine.

Figure 4: Post-exposure vaccine induced protection by ESAT6 but not Ag85.
Mice were infected, treated and vaccinated according to the schematic overview in example 1. Mice were killed between week 30-40 post infection and at this timepoint lungs were assessed for bacterial load (Figure A, C-E) or as displayed in figure 4B where the bacterial load was determined at several timepoints throughout infection for ESAT6. (A and B) Bacterial load of ESAT6 vaccinated compared to control animals. (C) Bacterial load of Ag85B vaccinated compared to control animals. (D) Bacterial of ESAT-6 pepmix vaccinated (pool of overlapping peptides covering the entire ESAT6 sequence) compared to both Ag85B vaccinated and control animals. (E) Protection against reactivation following postexposure vaccination with Ag85B-ESAT-6 (H1) vaccinated compared to non-vaccinated control mice. All data in figure 4A, C-E are displayed as dot plots representing each individual animal with the mean depicted whereas each timepoint in figure 4B is representative of 6 individual animals and displayed as mean ± standard error of the mean (SEM) (B). All statistical analyses were performed using either an unpaired t-test (Figure

A-C and E) or Tukey's multiple comparison test (figure D) where $p<0.05$ was considered significant.

Figure 5: ESAT-6 postexposure vaccination induce polyfunctional T cells.
Cells from infected lungs from non-vaccinated or ESAT-6 vaccinated animals were stimulated in vitro with ESAT-6 prior to staining with anti-CD4, -CD8, -IFN-γ, -TNF-α and –IL-2. (A and B) Cytokine profiles were determined by first dividing the CD4 T cells into IFN-γ positive (+) or IFN-γ negative (-) cells. Both the IFN-γ+ and IFN-γ- cells were analyzed with respect to the production of TNF-α and IL-2. The pie charts (A and B) are colour coded according to the cytokine production profile and summarizes the fractions of the $CD4^+$ T cell response (out of the ESAT-6 specific CD4 T cells) that are positive for a given cytokine production profile. (C) Every possible combination of cytokines is shown on the x-axis of the bar chart and the percentage of ESAT-6 specific $CD4^+$ T cells in non vaccinated mice (grey bars) or ESAT-6 vaccinated mice (Black bars) expressing any combination of cytokines is given for each immunization group. D. Latently infected mice were vaccinated twice with ESAT-6, and 20 weeks after the last vaccination, lungs were assessed for bacterial number to determine protective efficacy. (**$p<0.01$, One way ANOVA Tukey's multiple comparisons test).

Figure 6: Pooled analysis of all postexposure experiments
For an individual experiment where either ESAT6, Rv3871, Ag85B, Rv3905, Rv3445, Rv0569 or Rv2031c (Figure A), Ag85B-ESAT6 (H1) or Ag85B-ESAT6-Rv2660 (H56) (Figure B) was used for post-exposure vaccination the median of the bacterial load of the adjuvant control group was compared to the bacterial load of each individual mouse in a vaccinated group vaccinated with either one of the antigens mentioned above. In figure A and B each dot corresponds to the level of protection i.e. ΔLog10 CFU conferred by the vaccination compared to the adjuvant control group and consists of several independent experiments. (A) Log10 protection for the single antigens ESAT6, Rv3871, Ag85B, Rv3905, Rv3445, Rv0569 or Rv2031c (B) or for the hybrid antigens H1 and H56 compared to ESAT6 alone. A statistical analysis was applied for comparisons of medians between the different groups either using the Kruskall Wallis multiple comparison test. $p<0.05$ was considered significant.

Figure 7: Effect of postexposure vaccination with Rv3871 compared to ESAT6 and control animals.

Mice were infected, treated and vaccinated at week 10, 13 and 18 post infection. At week 36 post infection the mice were terminated and lung lymphocytes from both vaccinated and non-vaccinated saline control mice were restimulated in vitro with Rv3871 (Fig.7A) or ESAT6 (Fig.7B). IFN-γ releases assessed by ELISA and samples were performed in triplicated. Data are depicted as mean ± SEM. The protective efficacy conferred by the vaccines was determined by enumeration of bacteria in the lung cultured from full lung homogenate (n=16-18). Data displated as a dot plot where each dot represents an individual animal and depicted with the median (red line).

Examples

EXAMPLE 1: Murine TB model for vaccination

The Cornell model has widely been used as a murine model for the study of latent TB. This model has been adapted in our laboratory for the testing of the ability of vaccine candidates to prevent reactivation. Mice are initially aerosolly infected with virulent *M.tb.* and at week 6 post infection antibiotic treatment is initiated to reduce the bacterial load. This is to mimic the latent stage of a human infection which does not occur spontaneously in mice. During this latent stage (a stage with continous low bacterial numbers) the mice are being immunized twice and the ability to prevent reactivation by the vaccine is determined by culturing the spleen and lungs for live *M.tb.* 20 weeks after the last immunization. The long timespan of the experiments is necessary to allow sufficient time for reactivation of the disease which is a prerequisite for readout of vaccine efficacy (Figure 3).

EXAMPLE 2 : Postexposure vaccine induced protection by ESAT6 but not Ag85.

ESAT-6 and Ag85B have proven to be protective in prophylactic vaccination both as single components and also as the fusion molecule Ag85B-ESAT6 (H1). However, when these antigens were tested in the postexposure model (as described above in example 1) only ESAT6 has a protective effect and control bacteria growth during the reactivation phase (Figure 4). Furthermore, as seen in figure 4B ESAT6 protection against reactivation manifests itself as early as W18 post infection and this protection was maintained throughout the course of the experiment (up until week 40 post infection). This is in contrast to what is observed when Ag85B is used as a post exposure vaccine (Figure 4C and

D), where there is no significant decrease in bacterial load compared to the control. In addition, we evaluated the H1 fusion protein which is composed of the TB antigens Ag85B and ESAT-6 which has shown promising efficacy in a prophylactic setting. When this molecule was used as a post exposure vaccine in the SSI postexposure model it was able to significantly reduce the bacterial numbers (Figure 4E).

EXAMPLE 3: Post exposure vaccine induced protection by ESAT6 peptide mix

As shown in the examples above, the ESAT-6 molecule is very active when given postexposure resulting in a decrease in bacterial load compared to the control group and also compared to Ag85B. Furthermore we have shown that ESAT-6 given as a pool of overlapping peptides instead of a recombinant protein also lead to a better protection against reactivation compared to both the control group and Ag85B demonstrating the strong activity of ESAT6, and ability to function as a post exposure vaccine (Figure 4D).

Overlapping ESAT-6 peptides (P1-P13) used for protection experiment :

P1 MTEQQWNFAGIEAAA (SEQ ID NO. 19)
P2 NFAGIEAAASAIQGN (SEQ ID NO. 20)
P3 ASAIQGNVTSIHSLL (SEQ ID NO. 21)
P4 NVTSIHSLLDEGKQS (SEQ ID NO. 22)
P5 SLLDEGKQSLTKLAA (SEQ ID NO. 23)
P6 KQSLTKLAAAWGGSG (SEQ ID NO. 24)
P7 AAWGGSGSEAYQGVQ (SEQ ID NO. 25)
P8 GSEAYQGVQQKWDAT (SEQ ID NO. 26)
P9 QQKWDATATELNNAL (SEQ ID NO. 27)
P10 TATELNNALQNLART (SEQ ID NO. 28)
P11 ALQNLARTISEAGQA (SEQ ID NO. 29)
P12 TISEAGQAMASTEGN (SEQ ID NO. 30)
P13 QAMASTEGNVTGMFA (SEQ ID NO. 31)

EXAMPLE 5: Post exposure vaccination with ESAT-6 induce polyfunctional T cells

To examine the effect of a post exposure vaccination with ESAT-6 on the cytokine expression profile of the ESAT-6 specific cells, mice were first aerosolly infected with virulent *M.tb.* and at week 6 post infection antibiotic treatment was initiated to reduce the bacterial

load and establish a latent infection. During the latent stage the mice were vaccinated (as shown in figure 3) three times with 3 weeks interval and the ability of the ESAT-6 vaccine influence the number of polyfunctional T cells and to prevent reactivation of M.tb was determined 20 weeks after the last vaccination. The results showed that there was a substantial ESAT-6 response in the non-vaccinated group, but the cytokine expression profile was markedly different compared to the ESAT-6 vaccinated group (Fig. 5), in particularly in terms of polyfunctional T cells (IFN-γ+TNF-α+IL-2+ CD4 T cells). Thus, compared to the non vaccinated group, we observed decreased numbers of IFN-γ/TNF-α CD4 T cells, and increased numbers of triple positive polyfunctional CD4 T cells co-expressing IFN-γ/TNF-α/IL-2. The increased presence of polyfunctional T cells correlated with decreased bacterial numbers in the lungs of ESAT-6 vaccinated animals (Fig. 5 D).

EXAMPLE 6: Post-exposure vaccination with ESAT6 more consistently protects against reactivation compared to other antigens associated with both early and late stage infection.

To determine which antigens most consistently protect against reactivation we made a pooled analysis of normalized data based on all post-exposure experiments conducted. Data sets from individual experiments was normalized by comparing the bacterial load of each individual mice within a group to the median of the control group i.e. each data point represents the difference (Log10 CFU control median-Log10CFU vaccine group) between the control median CFU and the CFU of each individual animal (Figure 6). In figure 6A comparison of the pooled data set for protection for the antigens latency associated antigens Rv0569, Rv2031c and the early antigens Ag85B, ESAT6, Rv3871, Rv3905 and Rv3445 of which the two latter are ESAT6 family proteins show that ESAT6 vaccinated animals are significantly better protected against reactivation compared to other antigens evaluated. Furthermore, protective levels attained following post-exposure vaccination with Rv3871, an ESX-1 protein also seem to be elevated compared to the other antigens (Figure 6A). To further demonstrate the activity of ESAT6 in particular we compared the protection conferred by ESAT6 to the two fusion constructs H1 (Ag85B-ESAT6) and H56 (Ag85B-ESAT6-Rv2660) both of which contain ESAT6 (figure 6B). The analysis show that ESAT6 activity still result in protection against reactivation when included in the two above mentioned fusion constructs.

EXAMPLE 7: Postexposure vaccination with another member of the ESX-1 family, Rv3871 seems to have an inhibitory effect on the reactivation process.

We evaluated other members of the ESX-1 family in parallel with ESAT6 and found that Rv3871 postexposure vaccination led to an induction of Rv3871 specific immune response (Fig. 7B) although not to the extent of the ESAT6 induced immune response (Fig. 7A). Nevertheless both ESAT6 and Rv3871 induced immune response were greater compared to saline control animals. The induction of vaccine specific immune response was associated with a lowered (median) bacterial load in both vaccine groups compared to the saline group. This indicated that Rv3871 may have a similar effect in protection against reactivation compared to ESAT6 demonstrated by the similar levels of bacterial numbers in these two groups compared to the somewhat elevated level in the control group (Figure 7C)

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CLAIMS:

1. A vaccine for use in the prevention of reactivation of tuberculosis in an individual latently infected with *Mycobacterium*, comprising an antigen belonging to the ESX-1 secretion system which is constitutively expressed during infection with *Mycobacterium tuberculosis* or a nucleic acid encoding said antigen,

wherein the antigen belonging to the ESX-1 secretion system is selected from the group consisting of:

i) ESAT6 (SEQ ID NO. 1), CFP10 (SEQ ID NO. 2), Rv3614c (SEQ ID NO. 4), Rv3615c (SEQ ID NO. 5), EspR (SEQ ID NO. 6), Rv3868 (SEQ ID NO. 7), Rv3869 (SEQ ID NO. 8), Rv3870 (SEQ ID NO. 9), Rv3871 (SEQ ID NO. 10), Rv3872 (SEQ ID NO. 11), Rv3873 (SEQ ID NO. 12), Rv3876 (SEQ ID NO. 13), Rv3877 (SEQ ID NO. 14), Rv3878 (SEQ ID NO. 15), Rv3879c (SEQ ID NO. 16), Rv3880c (SEQ ID NO. 17), Rv3881c (SEQ ID NO. 18), Rv3882c (SEQ ID NO. 32), Rv3883c (SEQ ID NO. 33) and Rv3865 (SEQ ID NO. 34);

ii) at least one immunogenic portion comprising an epitope for a B-cell or T-cell of any one of the sequences in (i); and

iii) an analogue having an amino acid sequence having at least 80% sequence identity to any one of the sequences in (i) or (ii) and at the same time being immunogenic.

2. The vaccine for use according to claim 1, wherein the at least one immunogenic portion is a mix of immunogenic portions.

3. The vaccine for use according to claim 2, wherein the mix of immunogenic portions is a mix of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 immunogenic portions as defined in ii) of claim 1.

4. The vaccine for use according to claim 1, wherein said antigen belonging to the ESX-1 secretion system is fused to an antigen expressed by bacteria within the mycobacteria family.

5. The vaccine for use according to claim 4, wherein the antigen belonging to the ESX-1 secretion system expressed by bacteria within the mycobacterium family is constitutively expressed.

6. The vaccine for use according to claim 5, comprising ESAT6 fused to CFP10.

7. The vaccine for use according to any one of claims 1 to 6, further comprising a delivery system selected from the group consisting of live recombinant vaccines, immunogenic delivery systems and protein vaccines.

8. The vaccine for use according to claim 7, further comprising an adjuvant, wherein the adjuvant comprises DDA/TDB and/or poly I:C.

9. The vaccine for use according to any one of claims 1 to 8, in which the amino acid sequence is lipidated so as to allow a self-adjuvanting effect of the polypeptide.

10. An antigen belonging to the ESX-1 secretion system which is constitutively expressed during infection with *Mycobacterium tuberculosis* or a nucleic acid encoding said antigen for use in treatment of latent tuberculosis, wherein the antigen belonging to the ESX-1 secretion system is selected from the group consisting of

(i) ESAT6 (SEQ ID NO. 1), CFP10 (SEQ ID NO. 2), Rv3614c (SEQ ID NO. 4), Rv3615c (SEQ ID NO. 5), EspR (SEQ ID NO. 6), Rv3868 (SEQ ID NO. 7), Rv3869 (SEQ ID NO. 8), Rv3870 (SEQ ID NO. 9), Rv3871 (SEQ ID NO. 10), Rv3872 (SEQ ID NO. 11), Rv3873 (SEQ ID NO. 12), Rv3876 (SEQ ID NO. 13), Rv3877 (SEQ ID NO. 14), Rv3878 (SEQ ID NO. 15), Rv3879c (SEQ ID NO. 16), Rv3880c (SEQ ID NO. 17), Rv3881c (SEQ ID NO. 18), Rv3882c (SEQ ID NO. 32), Rv3883c (SEQ ID NO. 33) and Rv3865 (SEQ ID NO. 34);

(ii) at least one immunogenic portion comprising an epitope for a B-cell or T-cell of any one of the sequences in (i); and

(iii) an analogue having an amino acid sequence having at least 80% sequence identity to any one of the sequences in (i) or (ii) and at the same time being immunogenic.

11. The vaccine according to any one of claims 1 to 9, wherein the latent *Mycobacterium* infection is caused by a species of the tuberculosis complex selected from the group consisting of *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*.

12. The vaccine according to claim 11, wherein the individual is an animal.

13. The vaccine according to claim 11, wherein the individual is a human being.

14. The vaccine according to any one of claims 11 to 13, wherein said vaccine is for administration after acute stage tuberculosis infection and/or during latent stage tuberculosis infection.

15. The vaccine according to any one of claims 11 to 13, wherein the use of vaccine comprises a step of identifying a subject latently infected with a virulent mycobacteria.

16. The vaccine according to claim 15, wherein said subject latently infected with a virulent mycobacterium is identified in a diagnostic procedure selected from the group consisting of the Mantoux tuberculin skin test (TST), the Quantiferon test, in vitro detection of responses to HBHA and the detection of IP10 after stimulation with a constitutively expressed antigen.

17. An antigen belonging to the ESX-1 secretion system which is constitutively expressed during infection with *Mycobacterium tuberculosis* or a nucleic acid encoding said antigen for use in the manufacture of a vaccine against reactivation of latent infections caused by species of the tuberculosis complex selected from the group consisting of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum,* wherein the antigen belonging to the ESX-1 secretion system is selected from the group consisting of:

i) ESAT6 (SEQ ID NO. 1), CFP10 (SEQ ID NO. 2), Rv3614c (SEQ ID NO. 4), Rv3615c (SEQ ID NO. 5), EspR (SEQ ID NO. 6), Rv3868 (SEQ ID NO. 7), Rv3869 (SEQ ID NO. 8), Rv3870 (SEQ ID NO. 9), Rv3871 (SEQ ID NO. 10), Rv3872 (SEQ ID NO. 11), Rv3873 (SEQ ID NO. 12), Rv3876 (SEQ ID NO. 13), Rv3877 (SEQ ID NO. 14), Rv3878 (SEQ ID NO. 15), Rv3879c (SEQ ID NO. 16), Rv3880c (SEQ ID NO. 17), Rv3881c (SEQ ID

NO. 18), Rv3882c (SEQ ID NO. 32), Rv3883c (SEQ ID NO. 33) and Rv3865 (SEQ ID NO. 34);

ii) at least one immunogenic portion comprising an epitope for a B-cell or T-cell of any one of the sequences in (i); and

iii) an analogue having an amino acid sequence having at least 80% sequence identity to any one of the sequences in (i) or (ii) and at the same time being immunogenic.

18. The antigen for use according to claim 17, wherein said vaccine is for administration after acute stage tuberculosis infection and/or during latent stage tuberculosis infection.

19. The antigen according to any one of claims 10, 17 and 18, wherein the at least one immunogenic portion is a mix of immunogenic portions.

20. The antigen according to claim 19, wherein the mix of immunogenic portions is a mix of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 immunogenic portions as defined in ii) of claim 10 or 17.

21. The antigen according to any one of claims 17 to 20, wherein said antigen belonging to the ESX-1 secretion system is fused to an antigen expressed by bacteria within the mycobacteria family.

22. The antigen according to claim 21, wherein the antigen belonging to the ESX-1 secretion system expressed by bacteria within the mycobacterium family is constitutively expressed.

23. The antigen according to claim 22, comprising ESAT6 fused to CFP10.

24. The vaccine according to any one of claims 1 to 9, wherein the analogue in (iii) has at least 90% sequence identity to any one of the sequences in (i) or (ii) and is at the same time immunogenic.

25. The antigen according to claim 10, wherein the analogue in (iii) has at least 90% sequence identity to any one of the sequences in (i) or (ii) and is at the same time immunogenic.

26. The antigen according to claim 17, wherein the analogue in (iii) has at least 90% sequence identity to any one of the sequences in (i) or (ii) and is at the same time immunogenic.

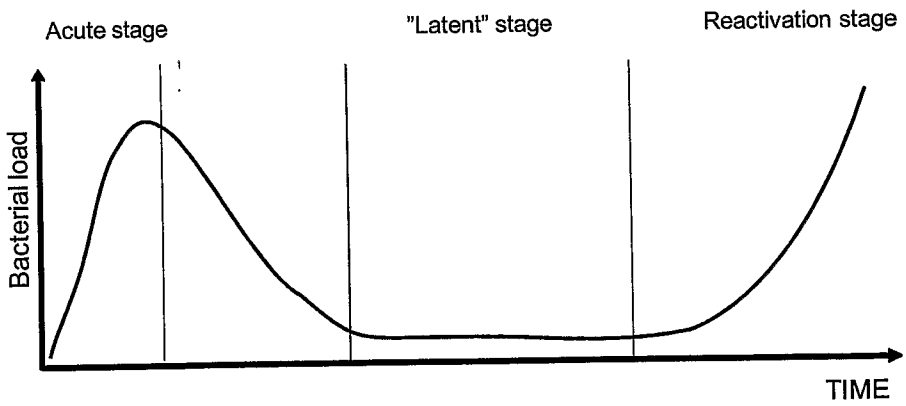

Acute stage
"Latent" stage
Reactivation stage
Bacterial load
TIME

Figure 1

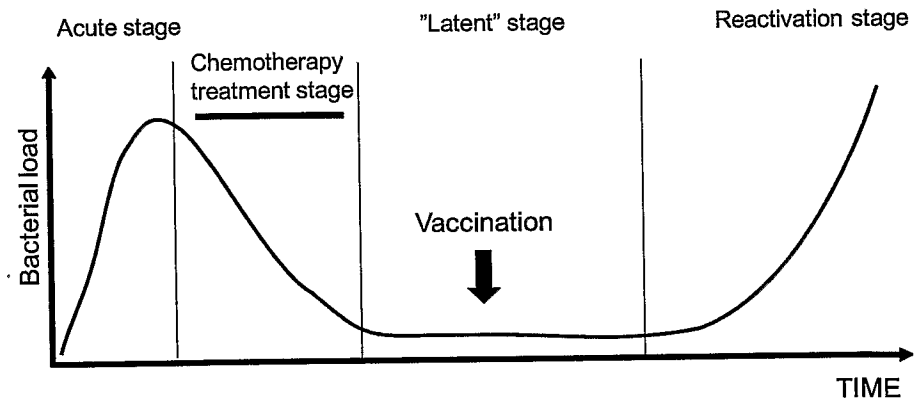

Acute stage
"Latent" stage
Reactivation stage
Chemotherapy
treatment stage
Bacterial load
Vaccination
TIME

Figure 2

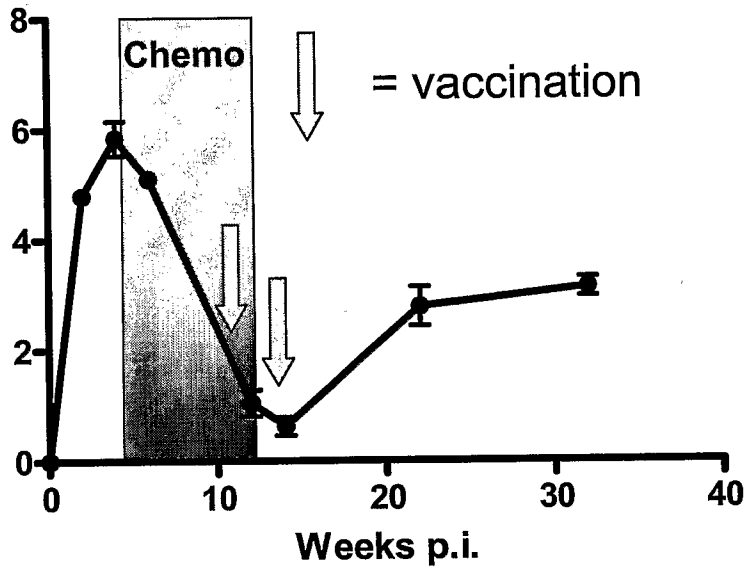


Figure 3

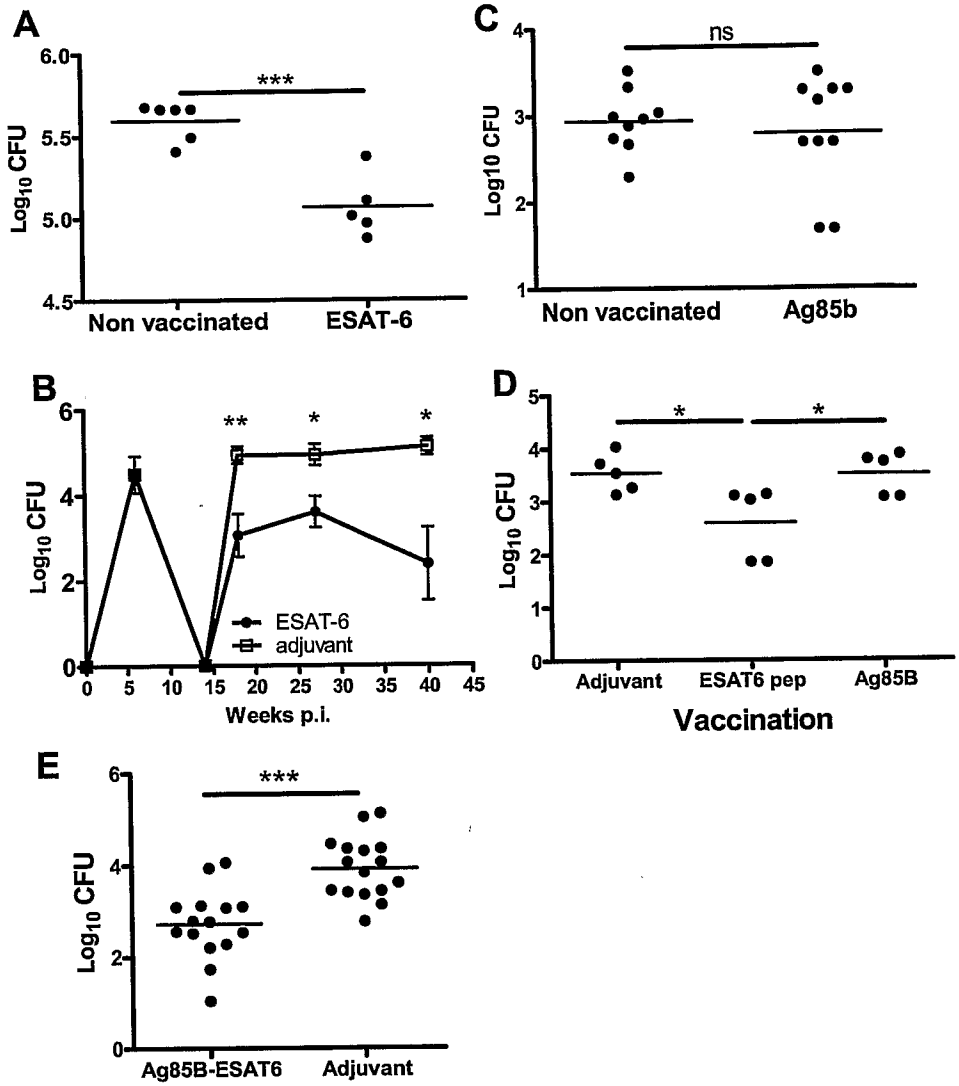

A
6.0
5.5
5.0
4.5
Log10 CFU
***
Non vaccinated
ESAT-6
C
4
3
2
1
Log10 CFU
ns
Non vaccinated
Ag85b
B
6
4
2
0
Log10 CFU
**
*
*
ESAT-6
adjuvant
0 5 10 15 20 25 30 35 40 45
Weeks p.i.
D
5
4
3
2
1
0
Log10 CFU
*
*
Adjuvant
ESAT6 pep
Ag85B
Vaccination
E
6
4
2
0
Log10 CFU
***
Ag85B-ESAT6
Adjuvant

Figure 4

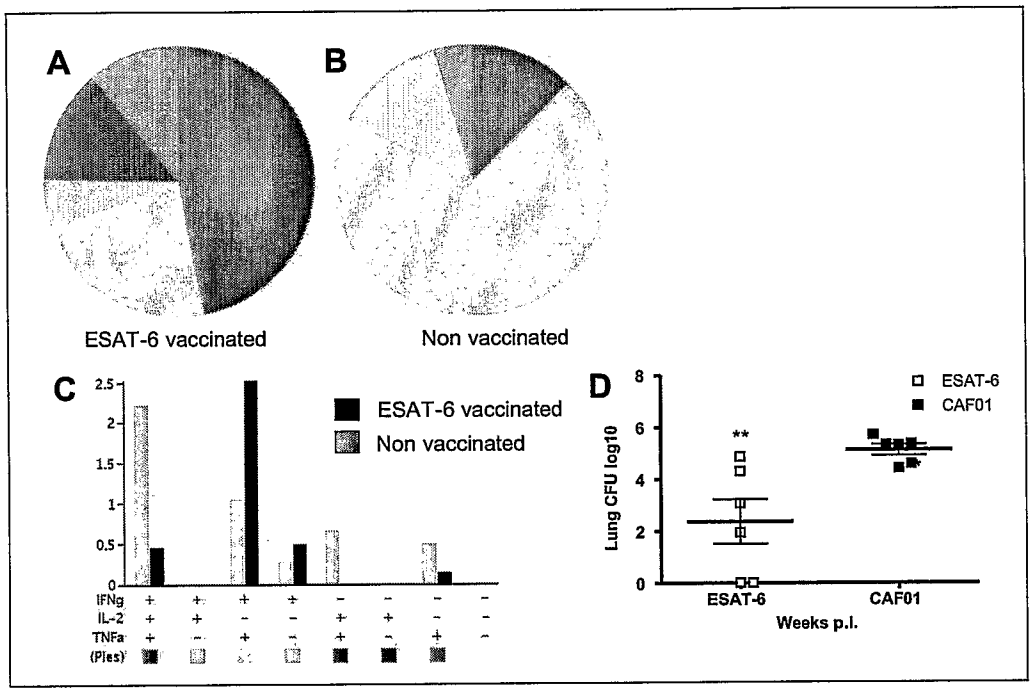

A
B
ESAT-6 vaccinated
Non vaccinated
C
ESAT-6 vaccinated
Non vaccinated
IFNg
IL-2
TNFa
(Pies)
D
ESAT-6
CAF01
Lung CFU log10
Weeks p.l.

Figure 5

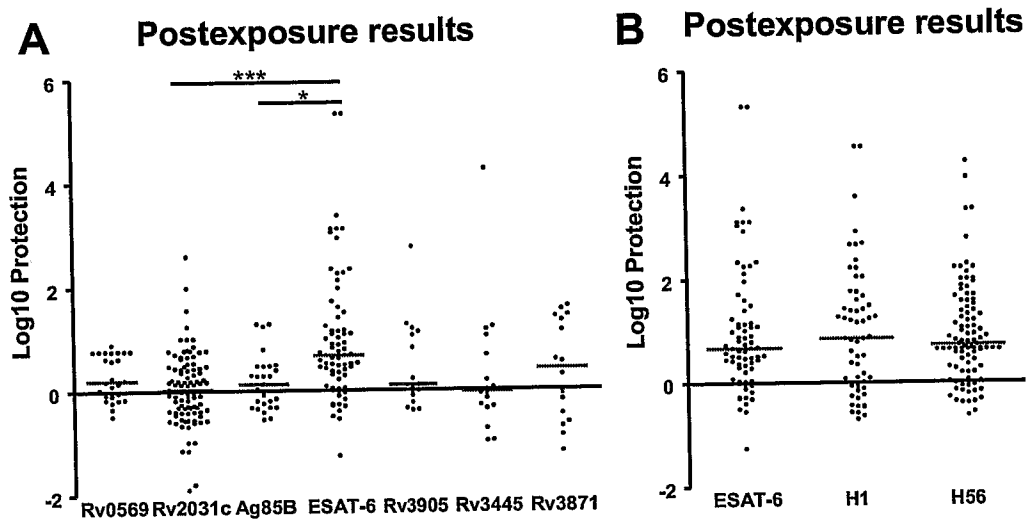

A
Postexposure results
***
*
Log10 Protection
6
4
2
0
-2
Rv0569
Rv2031c
Ag85B
ESAT-6
Rv3905
Rv3445
Rv3871
B
Postexposure results
Log10 Protection
6
4
2
0
-2
ESAT-6
H1
H56

Figure 6

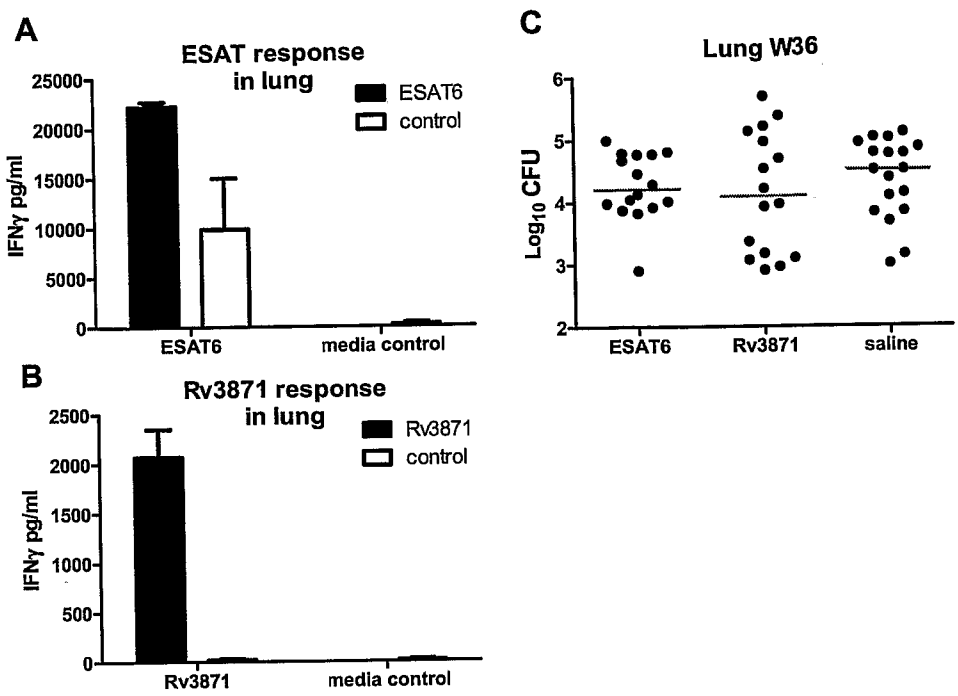
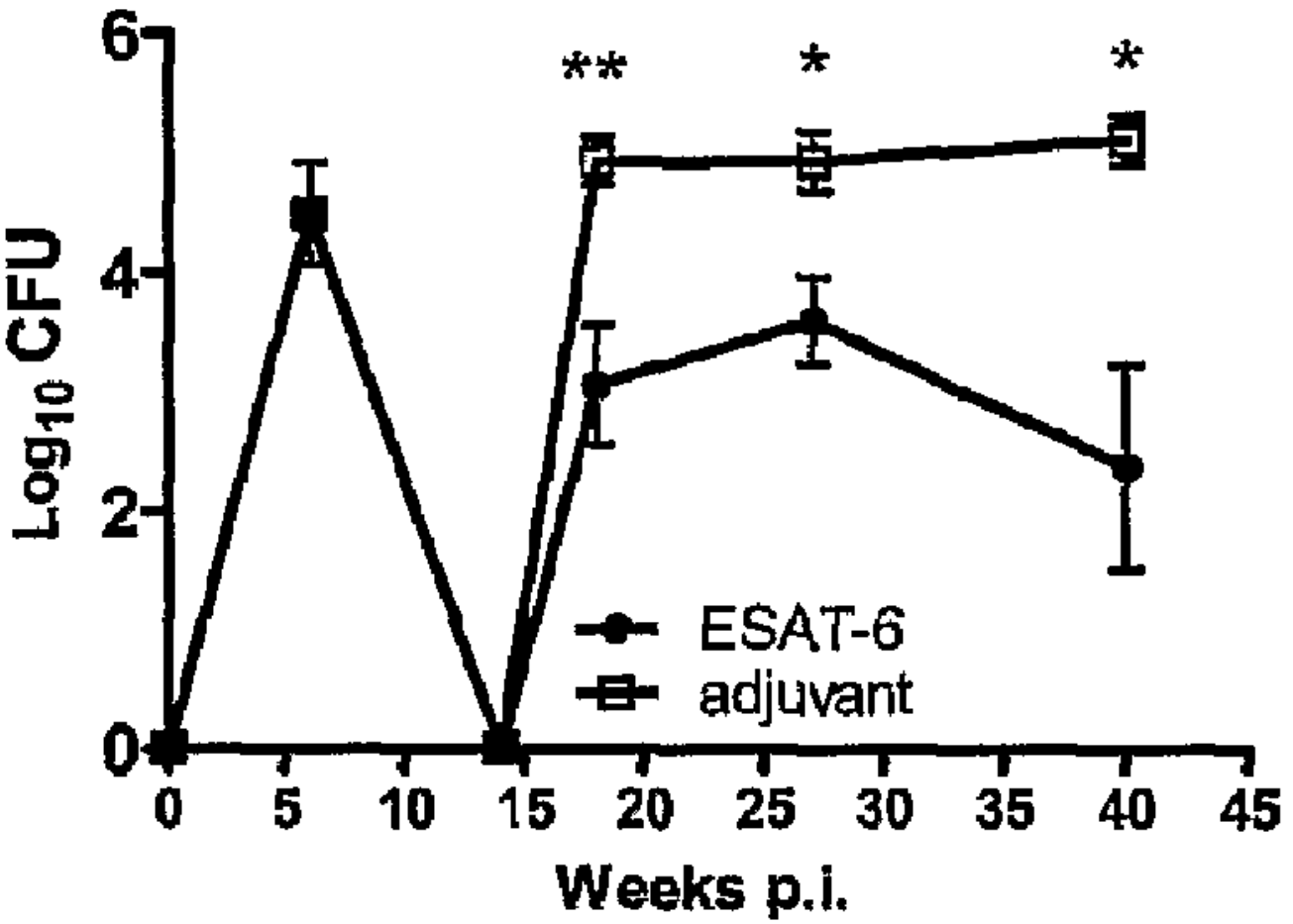

A
ESAT response in lung
ESAT6
control
IFNγ pg/ml
25000
20000
15000
10000
5000
0
ESAT6
media control
B
Rv3871 response in lung
Rv3871
control
IFNγ pg/ml
2500
2000
1500
1000
500
0
Rv3871
media control
C
Lung W36
Log10 CFU
6
5
4
3
2
ESAT6
Rv3871
saline

Figure 7


6
4
2
0
Log10 CFU
**
*
*
ESAT-6
adjuvant
0
5
10
15
20
25
30
35
40
45
Weeks p.i.