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(54) Title: MYCOBACTERIUM RESUSCITATION PROMOTING FACTOR FOR USE AS ADJUVANT

(57) Abstract: The present invention relates to a product combination or acomposition comprising (a) one or more immunogen (s) and (b) a polypeptide comprising at least a Mycobacterium resuscitation promoting factor (Rpf) or a nucleic acid molecule encoding said polypeptide. The present invention also relates to such a polypeptide or its encoding nucleic acid molecule for use as an adjuvant in combination with one or more immunogen(s). The present invention also relates to a method for recombinant production of said polypeptide, product combination or composition as well as methods for therapeutic or prophylactic use using such elements.

Mycobacterium resuscitation promoting factor for use as adjuvant

FIELD OF THE INVENTION

The present invention relates generally to a novel polypeptide comprising a Mycobacterium resuscitation promoting factor or a nucleotide sequence encoding such a Rpf-comprising polypeptide, and its use as an adjuvant in combination with an immunogen with the goal of stimulating an immune response to said immunogen in comparison to the immunogen alone.

BACKGROUND OF THE INVENTION

Adjuvants are particularly useful in vaccine for enhancing host's immunity to immunogens of interest. However, if a number of adjuvants are available in the art to enhance humoral immunity (e.g. alumn), a clear need exists for immunological adjuvants having potential to stimulate immune pathways and notably the cellular immune responses, in particular the CD4+ and/or CD8+ T cell responses.

The need of new adjuvants is viewed as being especially useful in both prophylaxis and therapy, especially to strengthen immunotherapy candidates in areas where limited therapeutic solutions exist as well as to combat diseases affecting emerging countries for which access to therapy is limited or difficult to implement. Therefore, the present invention is particularly useful in the area of cancers and infectious diseases whatever the infecting organism (e.g. bacteria, viruses, parasites, etc), and in particular for preventing or treating diseases associated with Mycobacterium (e.g. tuberculosis, leprosy, etc) papillomavirus (HPV) and hepatitis virus (e.g. hepatitis B and C) infections.

Tuberculosis (TB) is one of the numerous diseases that illustrate the need of more efficient prophylactic and therapeutic approaches. With an estimated one third of the world's population infected with *Mycobacterium tuberculosis* (Mtb) (i.e. more than two billion individuals), 9 to 10 million new cases and 2 million deaths every year, tuberculosis is a global and worldwide health problem. The Mtb-caused million deaths every year are particularly dramatic considering that both vaccine (Bacille-Calmette-Guerin (BCG)) and antibiotics exist and are widely used. However, if BCG appears to be effective at preventing disease in newborns and toddlers, it does not protect adults and fails to prevent *Mtb* reactivation in latently infected persons. On the other hand, antibiotic treatment of active TB appears efficacious but requires strong patient compliance with daily administrations of

different drugs over several months. The last years saw an alarming rate of appearance of "MultiDrug Resistant" (MDR) and "eXtensively Drug-Resistant" (XDR) strains, mostly because of improper observance of this lengthy and costly drug regimen treatment. Therefore, in spite of vaccine and drug availability, tuberculosis is thus far from being controlled.

HCV is a major cause of acute hepatitis and chronic liver diseases. The HCV viral infection is associated with a high rate of chronicity that, in 5 to 24% of cases, can evolve to cirrhosis and subsequently to hepatocellular carcinoma over a 20- to 30- year period. Currently, in Europe and United States, 20-30% of liver transplantations and 15-33% of liver cancers are attributable to HCV infection. The World Health Organization estimated in 1999 that about 170 million people are chronically infected by HCV worldwide and 3 to 4 million persons are newly infected each year.

Hepatitis B is a major public health problem with more than 350 million persons chronically infected worldwide, 20 to 40% of them being at risk of developing chronic liver disease, cirrhosis and hepatocellular carcinoma. Despite the existence of effective preventive vaccines, the hepatitis B virus (HBV) infection is still rampant in many countries, even developed ones, with an estimation of 4.5 million of new cases of infection per year worldwide.

HPV infection is one of the most frequent sexually transmitted infections and about 25% of sexually active adults are infected with HPV. HPV infection progresses to pre-malignant CIN lesions which, if not diagnosed, may lead to invasive cervix cancer in approximately 20% of the infected subjects.

Cancer is the second leading cause of death worldwide and an explosion of the number of cancers is expected in the coming years.

There are several lines of evidence suggesting the immunogenic potential of the Rpf family during the Mtb infection or after immunization. In this regard, IFNg-producing T cell responses to Rpf proteins were measured in *ex vivo* stimulated PBMC from actively or latently-infected patients from American (Bertholet et al., 2008, J Immunol 181: 7948-57), German (Commandeur et al., 2011, Clinical and Vaccine Immunology 18: 676-83), Colombian (Riano et al., 2012, Tuberculosis 92: 148-159), and South African (Chegou et al., 2012, BMC Infectious Diseases 12: 10) cohorts. Moreover DNA vaccination with plasmid encoding the RpfB protein induced humoral and cellular immunity and a moderate protection against intra-tracheal Mtb challenge (Romano et al., 2012, Microbes and Infection 14: 86-95). Vaccinations with adjuvanted Rpf proteins also lead to humoral and cellular

responses providing a significant level of protection against a subsequent challenge with virulent *Mycobacterium tuberculosis* H37Rv (Yeremeev et al., 2003, Infect Immun 71: 4789-94).

There remains a need for improved strategies for preventing and treating a number of diseases and development of a novel and effective adjuvant is therefore a priority in the worrying context of cancer and infectious diseases.

The present invention fulfils this and other needs by providing Mycobacterium Rpf proteins and especially a RpfB and RpfD hybrid protein for use as an adjuvant with the goal of improving efficacy of existing therapeutic and preventive approaches.

This technical problem is solved by the provision of the embodiments as defined in the claims.

Other and further aspects, features and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF SUMMARY OF THE INVENTION

The present invention relates generally to a product combination comprising (a) one or more immunogen and (b) a polypeptide comprising at least a Mycobacterium resuscitation promoting factor (Rpf) or its encoding nucleotide sequence (e.g. vector expressing said Rfp-comprising polypeptide). Each main component (a) and (b) can be individually administered to the subject one or several times and concurrently or separately (administration of (a) being before or after (b)). However, in a preferred embodiment a product combination comprises in the same composition these two main components in adequate proportions. Other components may also be introduced (e.g. one or more pharmaceutical acceptable vehicle, etc).

In another aspect, the invention also concerns such Rpf-containing polypeptide or its encoding nucleotide sequence as well as its use as an adjuvant in association with an immunogen for stimulating immunity against said immunogen.

Rpf B and RpfD proteins were selected after an extensive bibliographic study and a score mining data system. An hybrid protein comprising the N-terminal domain of RpfB fused to the "catalytic domain" of the RpfD protein was designed after sequence alignment, biochemical and bioinformatics prediction studies. The presence of this RpfB and RpfD

containing polypeptide in association with immunogens (e.g. vector expressing Mtb antigens) offers improved and unexpected immunogenic properties as compared to those obtained with the individual immunogen(s).

The technical objective pursued with the present invention is, precisely, the development of novel adjuvants capable of enhancing the immune response to antigens administered to a subject in need thereof via any administration route (e.g. mucosal or systemic route). The present invention is particularly useful as an adjuvant in the field of human or veterinary immunotherapy. It can also be used in association with standard therapy (e.g. antibiotic, antiviral therapy) or any other novel treatment that is currently developed.The present invention also fulfills the need of providing a fusion polypeptide comprising Mtb RpfB and RpfD antigens that offers unexpected immunogenic properties. Such a fusion protein is particularly useful in the field of immunotherapy for the purposes of the diagnosis, prevention or treatment of a Mycobacterium infection or a condition associated with a Mycobacterium infection.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention it is reported for the first time a polypeptide comprising at least a Mycobacterium resuscitation promoting factor (Rpf) or a nucleotide sequence encoding such a Rpf-containing polypeptide, especially for use as an adjuvant in combination with one or more immunogen(s) with the goal of stimulating an immune response to said immunogen(s) in comparison to the immunogen(s) alone. The present invention also concerns compositions comprising such product combinations. A further aspect of the invention includes a fusion polypeptide comprising Mtb RpfB and RpfD antigens, nucleic acid and vectors encoding such a fusion polypeptide as well as composition comprising such a fusion polypeptide or encoding nucleic acid molecule that can be used as a stand alone or in combination (e.g. as a BCG booster, in combination with other Mtb immunogens) for preventing or treating Mtb infection or a Mtb-associated disease (e.g. primaty TB, reactivation of latent TB, etc), optionally or eventually in association with standard treatment (e.g. antibiotic treatment).

Definitions

As used herein throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced compounds or steps, unless the context dictates otherwise.

The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

The term "about" or "approximately" as used herein means within 10%, preferably within 8%, and more preferably within 5% of a given value or range.

The terms “amino acids”, “residues” and “amino acid residues” are synonyms and encompass natural amino acids as well as amino acid analogs (e.g. non-natural, synthetic and modified amino acids, including D or L optical isomers).

The term “polypeptide” refers to a polymer of amino acid residues which comprises at least nine or more amino acids bonded via covalent peptide bonds. The polypeptide can be linear, branched or cyclic and may comprise naturally occurring and/or amino acid analogs. It may be chemically modified by being glycosylated, lipidated, acetylated, cleaved, cross-linked by disulfide bridges and/or phosphorylated, or still by containing additional amino acids such as tag (His, myc, Flag, etc) or a targeting peptide (signal peptide, trans-membrane domain, etc). It will be understood that the term “polypeptide” encompasses proteins (usually employed for polypeptides comprising 50 or more amino acid residues), oligopeptides, and peptides (usually employed for polypeptides comprising less than 50 amino acid residues). Each polypeptide may thus be characterized by specific amino acids and be encoded by specific nucleic acid sequences.

As used herein, when used to define products, compositions and methods, the term "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are open-ended and do not exclude additional, unrecited elements or method steps. Thus, a polypeptide "comprises" an amino acid sequence (e.g. Rpf) when the amino acid sequence might be part of the final amino acid sequence of the polypeptide. Such a polypeptide can have up to several hundred additional amino acids residues (e.g. tag, targeting peptides, additional polypeptide as described herein). "Consisting essentially of" means excluding other components or steps of any essential significance. Thus, a composition consisting essentially of the recited components would not exclude trace contaminants and pharmaceutically acceptable carriers. A polypeptide "consists essentially of’ an amino acid sequence when such an amino acid sequence is present with optionally or eventually only a few additional amino acid residues. "Consisting of’ means excluding more than trace elements of other components or steps. For example, a polypeptide "consists

of' an amino acid sequence when the polypeptide does not contain any amino acids but the recited amino acid sequence.

The term "identity" refers to an amino acid to amino acid or nucleotide to nucleotide correspondence between two polypeptide or nucleic acid sequences. The percentage of identity between two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps which need to be introduced for optimal alignment and the length of each gap. Various computer programs and mathematical algorithms are available in the art to determine the percentage of identity between amino acid sequences, such as for example the Blast program available at NCBI or ALIGN in Atlas of Protein Sequence and Structure (Dayhoffed, 1981, Suppl., 3: 482-9). Programs for determining identity between nucleotide sequences are also available in specialized data base (e.g. Genbank, the Wisconsin Sequence Analysis Package, BESTFIT, FASTA and GAP programs). For illustrative purposes, "at least 80% identity" means 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%.

As used herein, "operably linked" means that the elements being linked are arranged so that they function in concert for their intended purposes. For example a promoter is operably linked to a nucleic acid molecule if the promoter effects transcription from the transcription initiation to the terminator resulting in the expression of the coding sequence present in the nucleic acid molecule in a permissive host cell.

As used herein, the term "product combination" refers to any arrangement possible of one or more immunogen(s) (e.g. polypeptide/peptide composition, immunogen-expressing vector(s), etc) with a polypeptide comprising at least a Rpf polypeptide or with the nucleotide sequence encoding such a polypeptide. In context of the invention, each of the immunogen(s) and Rpf-containing polypeptide/nucleic acid molecule can be individually administered one or several times and concurrently or separately. Such combinations include mixture of immunogen(s) and Rpf-containing polypeptide (e.g. mixture of immunogen polypeptide(s) or peptide(s) with the Rpf-containing polypeptide and/or fusion of such immunogen(s) and Rpf-containing polypeptide) or mixture of nucleic acid molecules (e.g. carried by one or more vector) as well as mixture of polypeptide(s) and nucleic acid molecule(s). The present invention encompasses combinations comprising equal molar concentrations of each component as well as combinations with very different concentrations. It is appreciated that optimal concentration of each component can be determined by the artisan skilled in the art.

As used herein the term "adjuvant" refers to a component that amplifies the immunogenic nature of an immunogen in the sense that it enhances the immune response provided by said immunogen – whether specific or non-specific; humoral or cellular, i.e. when the immune response observed with the addition of the adjuvant is greater or intensified in any way (duration, magnitude, intensity, etc) when compared to the same immune response measured without its addition. The adjuvanting effect can be performed through enhancement of any cell, molecule, etc that affects immunity in a positive way and/or through diminishment of any cell, molecule, etc that affects immunity in a negative way.

The term “treating” (and any form of treating such as “treatment”, “treat”) as used herein encompasses prevention and/or therapy. Treatment requires administer externally or internally to a subject an active agent (e.g. the product combination, fusion polypeptide, nucleic acid molecule, vector and/or composition described herein), optionally or eventually in association with conventional therapeutic modalities (e.g. SOC treatment currently employed for the disease or condition to be prevented or treated).

The term “subject” generally refers to a vertebrate and particularly a mammalian selected from the group consisting of laboratory animals, domestic animals, farm animals, sport animals, and primates. Preferably, the subject is a human thus is susceptible of having or at risk of having a disease or condition caused or associated with a pathogen (e.g. a bacteria, viral and/or parasitic infection) or a dysfunction (e.g. cancer) for which inducing or stimulating immunity would play a role to prevent or control such disease or condition.

The term "obtained from", “originating” or “originate” is used to identify the original source of a component (e.g. polypeptide, nucleic acid molecule) but is not meant to limit the method by which the component is made which can be, for example, by chemical synthesis or recombinant means.

Mycobacterium Resuscitation promoting factor (Rpf)

“A Rpf” refer to a polypeptide mainly expressed or involved into the transition between the dormancy state and active growth and replication (active state of Mycobacterium infection), i.e. in the resuscitation growth of the bacteria. This term encompasses native Rpf as well as fragment and variant thereof.

The present invention may employ the Rpf-comprising polypeptide (e.g. for adjuvanting immunogen such as polypeptide, peptide or peptide/polypeptide mixture, etc) or

its encoding nucleotide sequence (e.g. for adjuvanting immunogen such as expression vectors, viral particles, etc).

The Rpf(s) for use in the invention can independently be obtained from any member of a Mycobacterium (M.) species identified at present time. As used herein, the terms "Mycobacterium", "Mycobacterium species" and "mycobacterial" are used interchangeably to refer to any member of the genus of *Actinobacteria* belonging to the *Mycobacteriaceae* family and encompass laboratory strains as well as clinical isolates.

A vast number of Mycobacterium species have been described in the art including, without limitation, M. phlei, M. smegmatis, M. africanum, M. canetti, M. fortuitum, M. marinum, M. ulcerans, M. tuberculosis (Mtb), M. paratuberculosis, M. bovis, M. microti, M. celatum M. avium, M. leprae, M. lepraemurium, M. intracellulare, M. scrofulaceum, M. xenopi, M. genavense, M. kansasii, M. simiae, M. szulgai, M. haemophilum, M. asiaticum, M. malmoense, M. vaccae, M. caprae, M. pinnipedii and M. shimoidei.

Preferably, the Rpf(s) for use in this invention is/are obtained from a Mycobacterium species of the tuberculosis complex which includes those species traditionally considered as causing the disease tuberculosis, as well as Mycobacterium environmental and opportunistic species that cause tuberculosis and pulmonary disease in immune compromised subjects (e.g. HIV-infected patients). Exemplary species of the tuberculosis complex for use herein include without limitation *M. tuberculosis* (Mtb), M. bovis, M. bovis BCG, M. africanum, M. canetti, M. caprae, and M. microti.

A preferred embodiment is directed to Rpf(s) obtained from the *M. tuberculosis* and especially from Mtb laboratory strains such as H37Rv and H37Ra.

M. tuberculosis (Mtb) genome encodes five different Rpf, namely Rv0867c (RpfA), Rv1009 (RpfB), Rv1884c (RpfC), Rv2389c (RpfD) and Rv2450c (RpfE) that are all involved in the reactivation phase of the bacteria (transition from dormancy to active growth and replication) (e.g. Mukamolova et al., 2002, Mol Microbiol 46: 623-35; Yeremeev et al., 2003, Infection and Immunity 71: 4789-94; Mukamolova et al., 2006, Mol Microbiol 59: 84-98; Tufariello et al., 2006, Infect Immun 74: 2985-95; Biketov et al., 2007, MMC Infect Dis 7: 146; Kana et al., 2008, Mol Microbiol 67: 672-84; Kana et al., 2009, FEMS Immunol Med Microbiol 58: 39-50; Russel-Goldman et al., 2008, Infect Immun 76: 4269-81; Gupta et al., 2010, Microbiol 156: 2714-22 and Commandeur et al., 2011, Clin Vaccine Immunol. 18: 676-83). There is no significant similarity among these five proteins except that they all contain a conserved catalytic domain (lysozyme like domain). A preferred Rpf-containing

polypeptide for use in the product combination of the invention comprises at least RpfB and RpfD (or fragment thereof).

Amino acid sequences of a variety of Rpfs and the encoding nucleotide sequences are readily available in the literature and in specialized data banks. More particularly, the Mtb Rpf sequences can be found in Cole et al. (1998, Nature 393: 537) or at websites such as those maintained by the Wellcome Trust Sanger Institute, Institut Pasteur and others (e.g. TB database (@tbdb.org) and tuberculist (@tuberculist.epfl.ch)). However, the present invention is not limited to these exemplary Rpf. Indeed the nucleotide and amino acid sequences can vary between different isolates and strains and this natural genetic variation is included within the scope of the invention as well as non-natural modification(s) such as those described below.

As mentioned above the Rpf(s) contained in the polypeptide comprised /encoded by the product combination of the present invention may be native Rpf or modified with respect to the native counterpart (e.g. fragment(s) and/or variant(s) thereof).

A "native" Rpf comprises the amino acid sequence of a Rpf obtained (e.g. found or isolated) from a source of Mycobacterium in nature. Such sources include biological samples (e.g. blood, plasma, sera, saliva, sputum, tissue sections, biopsy specimen etc.) collected from a subject infected or that has been exposed to a Mycobacterium, cultured cells as well as recombinant materials available in depositary institutions (e.g. ATCC or TB institutions), libraries or described in the literature (e.g. Mycobacterium isolates, Mycobacterium genomes, genomic fragments, genomic RNA or cDNA as well as any plasmid and vector known in the art to include such elements).

A "modified" Rpf typically differs from its native counterpart in one or more position. Any modification (s) can be envisaged, including substitution, insertion, addition and/or deletion of one or more amino acid residue(s), non-natural arrangements and any combination of these possibilities. Amino acid substitution can be conservative or not. When several modifications are contemplated, they can concern consecutive residues and/or non-consecutive residues. Modification(s) can be generated by a number of ways known to those skilled in the art, such as site-directed mutagenesis (e.g. using the Sculptor™ *in vitro* mutagenesis system of Amersham, Les Ullis, France), PCR mutagenesis, DNA shuffling and by synthetic techniques (e.g. resulting in a synthetic nucleic acid molecule encoding the desired polypeptide variant).

The present invention encompasses any modification that might be beneficial to the cloning, expression, stability, and/or adjuvanting properties of the Rpf(s) in use in the invention, including any Rpf fragment and Rfp variant resulting from N and/or C truncation, internal deletion of one or more amino acid residue(s) and/or amino acid substitution(s).

For example, a particularly appropriate RpfB is modified with respect to the native counterpart by (i) deletion of all or part of the signal peptide (from the first residue following the initiator Met to approximately residue in position 29), and/or (ii) deletion of all or part of its catalytic domain. Another particularly appropriate RpfD is modified with respect to the native counterpart by (i) deleting all RpfD except its catalytic domain and/or (b) mutating. In one or more positions the residues involved in the catalytic site so as to abolish or significantly reduce the associated enzymatic activity. Suitable mutations are described in the art (e.g. Mukamolova *et al.* 2006, Mol Microbiol 59: 84-98; Cohen-Gonsaud, *et al.* 2005, Nat Struct Mol Biol 12, 270-3).

In one embodiment, the Mycobacterium Rpf comprised in the polypeptide for use in the present invention is fused to one or more fusion partner(s) which can originate from a Mycobacterium (homologous fusion partner) and/or from a non Mycobacterium source (heterologous fusion partner). Typically, the fusion is performed by genetic means by fusing in frame the nucleotide sequences encoding the Rpf-containing polypeptide and its fusion partner(s) resulting in a single polypeptide chain without any translational terminator between each of the fused polypeptides. The fusion can be direct (i.e. without any additional amino acid residues in between) or indirect (e.g. through a linker between the fused polypeptides). The presence of a linker may facilitate correct folding and/or functioning of the fusion polypeptide. The present invention is not limited by the form, size or number of linker sequences employed. For illustrative purposes, typical linkers are 3 to 30 amino acids long and composed of repeats of amino acid residues such as glycine, serine, threonine, asparagine, alanine and/or proline. Additionally, each of the fusion partner(s) can be fused at the N terminus, at the C-terminus and/or in internal position of Rpf.

According to an advantageous aspect of this embodiment, at least one of the fusion partner is a homologous fusion partner and desirably a second Rpf originating from a Mycobacterium species as defined above which can be as the first one a native Rpf, a fragment or a variant thereof. Preferably, the homologous fusion partner is a RpfD polypeptide originating from a Mycobacterium of the tuberculosis complex and especially

from Mtb with a specific preference for a Mtb laboratory strain (e.g. H37Rv). The present invention also concerns a fusion polypeptide comprising Mtb antigens RpfB and RfpD fused each other either directly or indirectly. Preferably, the polypeptide for use in the context of the present invention and the fusion polypeptide of this invention comprise a Mtb RpfB modified by deletion of all or part of its catalytic domain which is fused to all or part of the catalytic domain of a Mtb RpfD. In other words, all or part of the RpfD catalytic domain is inserted in replacement of all or part of the deleted RpfB catalytic domain. As described above, other modification(s) can be envisaged such as the deletion of all or part of the RpfB signal sequence, the substitution/deletion of one or more amino acid residues of the catalytic site to significantly reduce or abolish the associated enzymatic activity (e.g. E292K, T315A and Q347A by reference to amino acid positions of the native Rpf). A particularly preferred Rpf-containing polypeptide is illustrated by the so-called RpfB-Dhyb polypeptide described in the appended example section. In a more preferred embodiment, the Rpf-containing polypeptide comprises an amino acid sequence at least 80% (e.g. 80%, 85%, 90%, 95%, 98% or 100%) identical to the amino acid sequence shown in SEQ ID NO: 1 optionally or eventually with an initiator Met depending of the context.

Alternatively or in addition, at least one of the fusion partner is a heterologous fusion partner. Appropriate heterologous fusion partners include without any limitation tag peptide(s) and/or targeting peptide(s) and/or any immunogenic polypeptide /peptide from a non Mycobacterium source .

Targeting peptides are well known in the art and comprises signal and transmembrane peptides (see for example WO99/03885). Briefly, signal peptides (SS) are generally present at the N-terminus of membrane-presented or secreted polypeptides and initiate their passage into the endoplasmic reticulum (ER). They comprise 15 or more essentially hydrophobic amino acids which are then removed by a specific ER-located endopeptidase to give the mature polypeptide. Trans-membrane peptides (TM) are usually highly hydrophobic in nature and serve to anchor the polypeptides in the cell membrane. The choice of the trans-membrane and/or signal peptides which can be used in the context of the present invention is vast. They may be obtained from any membrane-anchored and/or secreted polypeptide (e.g. cellular or viral polypeptides) such as those of immunoglobulins, tissue plasminogen activator (tPA), insulin, rabies glycoprotein, the HIV virus envelope glycoprotein or the measles virus F protein or may be synthetic (WO2008/138649). The preferred site of insertion of the signal peptide is the N-terminus downstream of the codon

for initiation of translation and that of the trans-membrane peptide is the C-terminus, for example immediately upstream of the stop codon.

Tag peptides are useful to facilitate polypeptide isolation and detection or to facilitate identification of host cells expressing such polypeptide. A vast variety of tag peptides can be used in the context of the invention including without limitation PK, FLAG, MYC and polyhistidine tag. Tag peptides can be detected by immunodetection assays using anti-tag antibodies as described in the appended examples. The tag peptide(s) may be independently positioned at the N-terminus of the Rpf-containing polypeptide (tag-polypeptide) or alternatively at its C-terminus (polypeptide-tag) or alternatively internally or at any of these positions when several tags are employed.

In a more preferred embodiment, the Rpf-containing polypeptide for use in the invention comprises an amino acid sequence at least 80% (e.g. 80%, 85%, 90%, 95%, 98% or 100%) identical to the amino acid sequence shown in SEQ ID NO: 2 or SEQ ID NO: 3. More specifically, SEQ ID NO: 2 represents the so-called RpfB-Dhyb polypeptide comprising RpfB and RpfD with targeting SS and TM peptides and and SEQ ID NO: 3 without such targeting peptides (as illustrated respectively by fusions n°3 and 12 in the appended examples).

Immunogen

The Rpf-containing polypeptide is for use as an adjuvant in combination with one or more immunogen(s). As used herein, the term "immunogen" refers to any component capable of inducing or stimulating a measurable specific and/or non-specific T and/or B cell-mediated immune response in a subject into which it has been introduced. Typically, such an immunogen contains one or more B and/or T epitope(s), in particular CTL or T_H epitope(s) or both, involved in recognition by a particular antibody or T-cell receptor in the context of the Major Histocompatibility Complex (MHC).

Suitably, immunogens can be whole organisms (pathogenic virus, bacterium, fungus or parasite) which can be killed, live or attenuated, structural components thereof (e.g. cell walls, envelopes, capsids) or any component obtained from or expressed by such a pathogen or not and directly or indirectly involved in a disease condition (e.g. polypeptides, polysaccharides, lipids, toxins, etc.). In the context of the invention, this term encompasses native antigens, fragments thereof (e.g. epitopes, immunogenic domains, etc), modified versions thereof (i.e. variants) and recombinant material (polyepitope strings, subunit vaccine, etc) as well as vectors for expression of such components. For information, an

epitope is a. minimal peptide motif (8-11 amino acid long that can be consecutive (linear epitope) or not (conformational epitope that includes residues that are not immediately adjacent to one another)) whereas an immunogenic domain usually comprises 11 to 50 amino acid residues. Preferred immunogens in the context of this invention are polypeptides, fragments thereof (e.g. comprising one or more epitopes, immunogenic domains, etc) as well as expressing vectors. The immunogenic nature of the component qualified as immunogen can be tested using a vast variety of *in vitro* or *in vivo* direct or indirect biological assays well known in the art. For a general description of techniques available to evaluate the onset and activation of an immune response, see for example Coligan et al. (1992 and 1994, Current Protocols in Immunology; ed J Wiley & Sons Inc, National Institute of Health; incorporated herein by reference).

Advantageously, the one or more immunogen(s) for use in this invention independently originate (e.g. isolated, cloned, recombinantly produced, etc) from a pathogenic organism that is associated with a disease condition (e.g. bacteria, virus, parasite, fungus) or from a subject's component which in certain context (e.g. lack of expression or surexpression) are directly or indirectly involved in a disease condition (e.g. a tumor-associated antigens).

Representative examples of suitable immunogens for use in this invention include without limitation:

- immunogen(s) of the Hepatitis B virus (HBV) such as the surface antigen PreS1, PreS2, S antigens, core, polymerase or any combination thereof (see e.g. WO2011/015656);
- Immunogen(s) of the Hepatitis C virus (HCV) including structural immunogens such as the Core (C) and the envelop glycoproteins E1 and E2 as well as non-structural immunogen NS2, NS3, NS4 (NS4a and/or NS4b), NS5 (NS5a and/or NS5b) or any combination thereof (see e.g. WO2004/111082);
- Immunogen(s) of the HIV-1 virus, especially gp120 and gp160 (as described WO 87/06260);
- Immunogen(s) of Human Papilloma Virus (HPV), especially HPV associated with cervical cancer (HPV16, HPV18, HPV 31, HPV-33 and others). Appropriate HPV immunogens include E1, E2, E6, E7, L1, and L2 and any combination thereof (see e.g. WO99/03885 and WO2008/092854).

- Immunogen(s) from Mycobacteria and especially from Mtb. (see e.g Thaissa et al., 2010, Yale J. of Biol. and Medicine 83: 209-15; Andersen, 2007, Nature 5: 484 and Kaufman, 2012, Trend in Immunology 241: 1-7). Appropriate Mtb immunogen(s) can be active antigens and/or latent antigens.
- Tumour-associated antigens such as those associated with prostrate, breast, colorectal, lung, pancreatic, renal, liver, bladder, sarcoma or melanoma cancers. Exemplary immunogens include those associated with breast cancer such as BRCA-1, BRCA-2 and lung cancer such as MUC-1 (see for example WO 92/07000).

Preferred immunogens in the context of the present invention comprise or encode one or more TB antigen(s), HPV antigen(s), HCV antigen(s), and/or HBV antigen(s). A preferred product combination comprises or encodes one or more Mtb antigens selected from the group consisting of ESAT-6 (Rv3875), CFP-10 (Rv3874), TB10.4 (Rv0288), Ag85A (Rv3804), Ag85B (Rv1886), Rv3619, Rv3620, RpfA, RpfB, RpfC, RpfD, RpfE, Rv0081, Rv0111, Rv0198, Rv0569, Rv1733c, Rv1735, Rv1737, Rv1806, Rv1807, Rv1813, Rv2005c, Rv2029c, Rv2032, Rv2626, Rv2627, Rv2628, Rv2660c, Rv3407 Rv3478, and Rv3812.

The immunogen can be used in the product combination of the invention in the form of polypeptide (alone or in fusion) or in the form of immunogen-encoding nucleotide sequence. Preferably, the immunogen(s) is/are expressed from a suitable vector, which can be the one used for expression of the adjuvant Rpf-containing polypeptide or a separate one. It/They is/are placed under the control of appropriate regulatory elements to permit expression in the selected host cell or subject in either a constitutive or inducible fashion. The choice of such regulatory elements is within the reach of the skilled artisan with a specific promoter as described in connection with the expression of the adjuvant polypeptide.

In a preferred embodiment, the Rpf-containing adjuvant polypeptide for use in this invention is fused to one or more immunogen(s) with a specific preference for a fusion comprising the Rpf-containing polypeptide described herein together with one or more Mtb antigens, especially Mtb antigens selected from the group consisting of ESAT-6 (Rv3875), CFP-10 (Rv3874), TB10.4 (Rv0288), Ag85A (Rv3804), Ag85B (Rv1886), Rv3619, Rv3620, RpfA, RpfB, RpfC, RpfD, RpfE, Rv0081, Rv0111, Rv0198, Rv0569, Rv1733c, Rv1735, Rv1737, Rv1806, Rv1807, Rv1813, Rv2005c, Rv2029c, Rv2032, Rv2626, Rv2627,

Rv2628, Rv2660c, Rv3407 Rv3478, and Rv3812; and preferably from the group consisting of ESAT-6 (Rv3875), TB10.4 (Rv0288), Ag85B (Rv1886), RpfB, RpfD, Rv0111, Rv0569, Rv1733c, Rv1807, Rv1813, Rv2029c, Rv2626, Rv3407 and Rv3478.

A preferred product combination comprises or encodes a fusion of RpfB, RpfD (e.g. RpfB-Dhyb), Ag85B, TB10.4 and ESAT6; or a fusion of Ag85B, Rv2626, RpfB, RpfD (e.g. RpfB-Dhyb) and Rv1733. In the context of the invention the Mtb antigens can be in any order from the N to the C terminus and not necessary in the recited order. Moreover, such fusions can additionally comprise additional fusion partner(s) such as tag and/or targeting peptide(s) as described above.

An even more preferred product combination comprises, or alternatively consists essentially of, or alternatively consist of an amino acid sequence which exhibits at least 80% of identity, advantageously at least 85% of identity, desirably at least 90% of identity, preferably at least 95% of identity, and more preferably 98% identity and even more preferably 100% identity with any of the amino acid sequence shown in SEQ ID NO: 4-6. More specifically, SEQ ID NO: 4 and 5 represent the fusion polypeptide comprising RpfB-Dhyb, Ag85B, TB10.4 and ESAT6, with and without targeting peptides respectively (as illustrated by fusions n°4 and 11 in the appended examples) and SEQ ID NO: 6 represents the fusion polypeptide comprising Ag85B, Rv2626, RpfB-Dhyb, and Rv1733, with a signal peptide (as illustrated by fusion n°6 in the appended examples).

The present invention also encompasses a Rpf-containing polypeptide as described herein, and especially a polypeptide comprising at least Mtb RfpB and RpfD antigens or variant thereof or part thereof, with a specific preference for a polypeptide comprising a Mtb RpfB modified by deletion of all or part of its catalytic domain which is fused to the catalytic domain of a Mtb RpfD, and even more preferably a polypeptide, comprising or consisting of an amino acid sequence which exhibits at least 80% of identity, advantageously at least 85% of identity, desirably at least 90% of identity, preferably at least 95% of identity, and more preferably 98% identity and even more preferably 100% identity with any of the amino acid sequence shown in SEQ ID NO: 1-3.

Nucleic acid molecules

The present invention also provides isolated nucleic acid molecules encoding the Rpf-containing polypeptide and the product combination (e.g. adjuvant-immunogen fusion) of the present invention as well as compositions comprising such nucleic acid molecule(s).

Within the context of the present invention, the terms "nucleic acid", "nucleic acid molecule", "polynucleotide" and "nucleotide sequence" are used interchangeably and define a polymer of any length of either polydeoxyribonucleotides (DNA) (e.g., cDNA, genomic DNA, plasmids, vectors, viral genomes, isolated DNA, probes, primers and any mixture thereof) or polyribonucleotides (RNA) (e.g., mRNA, antisense RNA) or mixed polyribo-polydeoxyribonucleotides. They can be single or double-stranded, linear or circular, natural or synthetic nucleic acids.

The present invention encompasses any nucleotide modifications aimed to improve cloning, expression, stability (e.g. introduction of appropriate restriction sites, degeneration and/or optimisation of nucleotide sequence to optimize translation in a given host cell and/or suppression of potentially negative elements that may destabilize the nucleic acid molecule or its transcript). The modification(s) contemplated by the present invention can be silent (i.e. they do not change the amino acid sequence of the encoded polypeptide) and/or not silent (i.e. they are translated into the encoded polypeptide).

Of particular interest is a nucleic acid molecule which encodes a Rpf-containing polypeptide exhibiting at least 80% of identity (e.g. 80%, 85%, 90%, 95%, 98%, 100%) with any of the amino acid sequence shown in SEQ ID NO: 1-3 or a product combination exhibiting at least 80% of identity with any of the amino acid sequence shown in SEQ ID NO: 4-6.

A particularly preferred embodiment of the present invention is directed to a nucleic acid molecule comprising, alternatively essentially consisting of or alternatively consisting of a nucleotide sequence which exhibits at least 80% of identity (i.e. a nucleic acid molecule that hybridizes to the recited nucleic acid molecule under stringent conditions), advantageously at least 85% of identity, preferably at least 90% of identity, more preferably at least 95% of identity, and even more preferably 100% identity with the nucleotide sequence shown in any of SEQ ID NO: 7-11.

Typically, the nucleic acid molecules of the present invention can be generated using sequence data accessible in the art and the sequence information provided herein. For example, they may be isolated using routine techniques well known in the art, e.g. by PCR isolation and/or cloning by conventional molecular biology from a Mycobacterium genome of a particular species or genomic fragment thereof, cDNA and genomic libraries or any prior art vector known to include it. Alternatively, the nucleic acid molecules of the

invention can also be generated by chemical synthesis in automatised process (e.g. assembled from overlapping synthetic oligonucleotides).

Vectors

The present invention also concerns vectors comprising one or more nucleic acid molecule(s) of the present invention, a product combination comprising such one or more vector(s) as well as compositions comprising such vector(s).

In one embodiment, the nucleic acid molecules encoding the Rpf-containing polypeptide for use as an adjuvant and the immunogen(s) are carried out by a single vector. In an alternative embodiment, they are carried out by two or more vectors (depending of the number of immunogens to be used), which can be administered to the subject substantially simultaneously, or sequentially. The present invention also relates to a vector for expression a Rpf-containing polypeptide as described herein.

The term "vector" as used herein refers to a vehicle, preferably a nucleic acid molecule or a viral particle that contains the elements necessary to allow delivery, propagation and/or expression of any of the nucleic acid molecule(s) described herein within a host cell or subject. This term encompasses vectors for maintenance (cloning vectors) or vectors for expression in various host cells or subjects (expression vectors), extrachromosomal vectors (e.g. multicopy plasmids) or integration vectors (e.g. designed to integrate into the host cell genome and produce additional copies of the nucleic acid molecules when the host cell replicates) as well as shuttle vectors (e.g. functioning in both prokaryotic and/or eukaryotic hosts) and transfer vectors (e.g. for transferring nucleic acid molecule(s) in a viral genome). For the purpose of the invention, the vectors may be of naturally occurring genetic sources, synthetic or artificial, or some combination of natural and artificial genetic elements.

In the context of the invention, the term "vector" has to be understood broadly as including plasmid and viral vectors. A "plasmid vector" as used herein refers to a replicable DNA construct. Usually plasmid vectors contain selectable marker genes that allow host cells carrying the plasmid vector to be selected for or against in the presence of a corresponding selective drug. A variety of positive and negative selectable marker genes are known in the art. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be selected in the presence of the corresponding antibiotic.

The term "viral vector" as used herein refers to a nucleic acid vector that includes at least one element of a virus genome and may be packaged into a viral particle or to a viral particle. The terms “virus”, “virions”, “viral particles” and “viral vector particle” are used interchangeably to refer to viral particles that are formed when the nucleic acid vector is transduced into an appropriate cell or cell line according to suitable conditions allowing the generation of viral particles. In the context of the present invention, the term “viral vector” has to be understood broadly as including nucleic acid vector (e.g. DNA viral vector) as well as viral particles generated thereof. The term “infectious” refers to the ability of a viral vector to infect and enter into a host cell or subject. Viral vectors can be replication-competent or -selective (e.g. engineered to replicate better or selectively in specific host cells), or can be genetically disabled or impaired for replication and/or propagation (e.g. replication and propagation-defective; replication-competent and propagation-defective; etc.).

Vectors which are appropriate in the context of the present invention, include, without limitation, bacteriophage, plasmid or cosmid vectors for expression in prokaryotic host cells such as bacteria (e.g. *E. coli, BCG or Listeria*); vectors for expression in yeast (e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*); baculovirus vectors for expression in insect cell systems (e.g. Sf 9 cells); viral and plasmid vectors for expression in plant cell systems (e.g. Ti plasmid, cauliflower mosaic virus CaMV; tobacco mosaic virus TMV); as well as plasmid and viral vectors for expression in higher eukaryotic cells or subjects. Typically, such vectors are commercially available (e.g. in Invitrogen, Stratagene, Amersham Biosciences, Promega, etc.) or available from depositary institutions such as the American Type Culture Collection (ATCC, Rockville, Md.) or have been the subject of numerous publications describing their sequence, organization and methods of producing, allowing the artisan to apply them.

Representative examples of suitable plasmid vectors include, without limitation, pREP4, pCEP4 (Invitrogen), pCI (Promega), pVAX (Invitrogen) and pGWiz (Gene Therapy System Inc).

Representative examples of suitable viral vectors are generated from a variety of different viruses including but not limited to viruses selected from the group consisting from the group consisting of retrovirus, adenovirus, adenovirus-associated virus (AAV), poxvirus, herpes virus, measles virus, foamy virus, alphavirus and vesicular stomatis virus. As described above, the term "viral vector" encompasses vector DNA, genomic DNA as well as viral particles generated thereof, and especially infectious viral particles. In one embodiment,

the viral vector employed in this invention is defective or impaired for at least one function involved in the viral replication or propagation which means that it cannot replicate or propagate to any significant extent in normal cells (eg. normal human cells) or in the subject to whom it is administered (the impairment or defectiveness of such function(s) can be evaluated by conventional means - eg. via measuring DNA synthesis and/ or viral titre in non-permissive cells). Such defective or impaired vectors typically require for propagation, permissive cell lines which bring up or complement the missing/impaired functions.

Examples of viral vectors that are useful in the context of the invention include adenoviral vectors which have a number of well-documented advantages for gene transfer or for recombinant production (for a review, see "Adenoviral vectors for gene therapy", 2002, Ed D. Curiel and J. Douglas, Academic Press). The adenoviral vectors of the present invention can be derived from a variety of human or animal sources (e.g. canine, ovine, simian adenovirus, etc). Any serotype can be employed with a special preference for human adenoviruses and a specific preference for subgenus C such as Ad2, Ad5, Ad6, and subgenus B such as Ad11, Ad34 and Ad35. It may also be advantageous to use animal Ad with a special preference for chimp Ad, such as chimp Ad3 and Ad63. The cited adenovirus are available from ATCC or have been the subject of numerous publications describing their sequence, organization and methods of producing, allowing the artisan to apply them (see for example US 6,136,594; US 6,133,028; WO00/50573; WO00/70071; WO2004/083418; WO2004/097016 and WO2005/071093).

Preferred replication-defective adenoviral vectors are E1-defective with an E1 deletion extending from approximately positions 459 to 3328 or from approximately positions 459 to 3510 (by reference to the sequence of Ad5 disclosed in the GeneBank under the accession number M 73260). The cloning capacity can further be improved by deleting additional portion(s) of the adenoviral genome (all or part of the non-essential E3 region (e.g. deletion from approximately positions 27867 to 30743) or of other essential E2 and/or E4 regions as described in WO94/28152 and Lusky et al., 1998, J. Virol 72: 2022).

The nucleic acid molecules of the present invention can be independently inserted in any location of the adenoviral genome, with a specific preference for insertion in replacement of the E1 and/or E3 region. They may be positioned in sense or antisense orientation relative to the natural transcriptional direction of the region in question.

Other examples of viral vectors particularly appropriate in the context of the invention include poxvirus vectors such as fowlpox vectors (e.g. FP9), canarypox vectors (e.g. ALVAC) and vaccinia virus vectors, the latter being preferred. Suitable vaccinia viruses include without limitation the Copenhagen strain, the Wyeth strain, NYVAC (US 5,494,807) and the modified Ankara (MVA) strain (Antoine et al., 1998, Virol. 244: 365; WO02/42480). The general conditions for constructing and producing recombinant poxvirus are well known in the art (see for example WO2010/130753; WO03/008533; US 6,998,252; US 5,972,597 and US 6,440,422). The nucleic acid molecules of the present invention are preferably inserted within the poxviral genome in a non-essential locus. Thymidine kinase gene is particularly appropriate for insertion in Copenhagen vaccinia vectors and deletion II or III for insertion in MVA vector (WO97/02355).

Other viral vectors suitable in the context of the invention are morbillivirus which can be obtained from the paramyxoviridae family, with a specific preference for measles virus. Various attenuated strains are available in the art (Brandler et al, 2008, CIMID, 31: 271; Singh et al., 1999, J. virol. 73(6): 4823), such as and without limitation, the Edmonston A and B strains (Griffin et al., 2001, Field's in Virology, 1401-1441), the Schwarz strain (Schwarz A, 1962, Am J Dis Child, 103: 216), the S-191 or C-47 strains (Zhang et al., 2009, J Med Virol. 81 (8): 1477). Insertion between P and M genes or between H and L genes is particularly appropriate.

Suitable vector for use in the present invention also include bacterium cell which can be wild-type or mutant (e.g. avirulent). Well-known examples of such bacterium cells include without limitation avirulent Mycobacterium (e.g. Mycobacterium bovis BCG), Lactobacillus (e.g. *Lactococcus lactis*), Listeria (e.g. *Listeria monocytogenes*) and other microorganisms such as Salmonella and Pseudomona. A preferred embodiment is directed to a BCG vector into the genome of which has been incorporated nucleic acid molecule(s) encoding one or more mycobacterial antigen(s) or fusion polypeptide (s) as defined above in a manner allowing the BCG vector to express such element(s).

The present invention also encompasses vectors (e.g. plasmid DNA) complexed to lipids or polymers to form particulate structures such as liposomes, lipoplexes or nanoparticles.

In accordance with the present invention, the nucleic acid molecule(s) comprised in the vector of the invention are in a form suitable for expression in a host cell or subject, which means that each of the nucleic acid molecules set forth herein is operably linked to appropriate regulatory sequences. As used herein, the term "regulatory elements" or "regulatory sequence" refers to any element that allows, contributes or modulates the expression of nucleic acid molecule(s) in a given host cell or subject, including replication, duplication, transcription, splicing, translation, stability and/or transport of the nucleic acid(s) or its derivative (i.e. mRNA).

It will be appreciated by those skilled in the art that the choice of the regulatory sequences can depend on such factors as the vector itself, the host cell or subject, the level of expression desired, etc. The promoter is of special importance. In the context of the invention, it can be constitutive directing expression of the nucleic acid molecule in many types of host cells or specific to certain host cells (e.g. lung-specific regulatory sequences) or regulated in response to specific events or exogenous factors (e.g. by temperature, nutrient additive, hormone, etc) or according to the phase of a viral cycle (e.g. late or early). One may also use promoters that are repressed during the production step in response to specific events or exogenous factors, in order to optimize vector production and circumvent potential toxicity of the expressed polypeptide(s).

Promoters suitable for constitutive expression in mammalian cells include but are not limited to the cytomegalovirus (CMV) immediate early promoter (US 5,168,062), the RSV promoter, the adenovirus major late promoter, the phosphoglycero kinase (PGK) promoter, the thymidine kinase (TK) promoter of herpes simplex virus (HSV)-1 and the T7 polymerase promoter. Promoters such as the trp, lac, phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic hosts. Useful yeast promoters include the promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization. Vaccinia virus promoters are particularly adapted for expression in poxviral vectors. Representative example include without limitation the vaccinia 7.5K, H5R, 11K7.5 (Erbs et al., 2008, Cancer Gene Ther. 15: 18), TK, p28, p11 and K1L promoter, as well as synthetic promoters such as those described in Chakrabarti et al. (1997, Biotechniques 23: 1094-7; Hammond et al., 1997, J. Virol Methods 66: 135-8; and Kumar and Boyle, 1990, Virology 179: 151-8) as well as early/late chimeric promoters. Promoters suitable for measles-mediated expression include without limitation

any promoter directing expression of measles transcription units (Brandler and Tangy, 2008, CIMID 31: 271).

Those skilled in the art will appreciate that the regulatory elements controlling the expression of the nucleic acid molecule(s) of the invention may further comprise additional elements for proper initiation, regulation and/or termination of transcription (e.g. polyA transcription termination sequences), mRNA transport (e.g. nuclear localization signal sequences), processing (e.g. splicing signals), and stability (e.g. introns and non-coding 5' and 3' sequences), translation (e.g. an initiator Met, tripartite leader sequences, IRES ribosome binding sites, Shine-Dalgarno sequences, etc.) into the host cell or subject and purification steps (e.g. a tag as described herein).

According to a preferred embodiment, the vector(s) of the invention is/are in the form of infectious viral particles. Typically, such viral particles are produced by a process comprising the steps of (i) introducing the viral vector into a suitable cell line, (ii) culturing said cell line under suitable conditions so as to allow the production of said infectious viral particle, (iii) recovering the produced viral particle from the culture of said cell line, and (iv) optionally purifying said recovered viral particle. When the viral vector is replication-defective or replication-impaired, the particles are usually produced in a permissive cell line or via the use of a helper virus, which supplies *in trans* the missing/impaired functions. For example, suitable cell lines for complementing E1-deleted adenoviral vectors include the 293 cells (Graham et al., 1997, J. Gen. Virol. 36: 59-72) as well as the HER-96 and PER-C6 cells (e.g. Fallaux et al., 1998, Human Gene Ther. 9: 1909-17; WO97/00326) or any derivative of these cell lines. Avian cells are particularly suitable for propagating poxvirus vectors including without limitation primary chicken embryo fibroblasts (CEF) prepared from chicken embryos obtained from fertilized eggs, and duck cell lines (e.g. as described in WO03/076601, WO2009/004016, WO2010/130756 and US2011-008872). The infectious viral particles may be recovered from the culture supernatant and/or from the cells after lysis. They can be further purified according to standard techniques (chromatography, ultracentrifugation techniques, etc).

Host cells and production methods

In another aspect, the invention also relates to host cells which comprise the product combinations, the nucleic acid molecules or vectors (e.g. viral particles) of the invention as well as compositions comprising such a host cell.

As used herein, the term "host cell" should be understood broadly without any limitation concerning particular organization in tissue, organ, or isolated cells. Such cells may be of a unique type of cells or a group of different types of cells such as cultured cell lines, primary cells and proliferative cells. In the context of the invention, the term "host cells" include prokaryotic cells, lower eukaryotic cells such as yeast, and other eukaryotic cells such as insect cells, plant and mammalian (e.g. human or non-human) cells as well as cells capable of producing the vector of the invention (e.g. 293, HER96, PERC.6 cells, CEF, duck cell lines, etc). This term also includes cells which can be or has been the recipient of the vector described herein as well as progeny of such cells.

According to a specific embodiment of the invention, the host cell can be further encapsulated. Cell encapsulation technology is known in the art.

Still a further aspect of the present invention is a method for recombinant production of the Rpf-containing polypeptide or product combination of the invention employing the vectors (or infectious viral particles) and/or host cells of the invention. Typically, the method comprises the steps of (i) introducing a vector into a suitable host cell to produce a transfected or infected host cell, (ii) culturing *in-vitro* said transfected or infected host cell under conditions suitable for growth of the host cell, (iii) recovering the cell culture, and (iv) optionally, purifying the produced Rpf-containing polypeptide or product combination from the recovered cell and/or culture supernatant.

It is expected that those skilled in the art are knowledgeable in the numerous expression systems available in the art for expressing polypeptides and of the methods for introducing a vector into a host cell (see e.g. Sambrook et al., 2001, Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory). Suitable methods include DNA transfection (e.g. $CaPO_4$--mediated), microinjection, electroporation, lipofection/liposome fusion, gene guns, virus transduction, viral infection as well as systemic or mucosal administration into a subject. The method may also be used in association with conventional transfection reagents that facilitate introduction of nucleic acids in host cells, such as

polycationic polymers (e.g. chitosan, polymethacrylate, PEI, etc) and cationic lipids (e.g.DC-Chol/DOPE, transfectam, lipofectin, etc).

Host cells can be cultured in conventional fermentation bioreactors, flasks, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a given host cell. No attempts will be made here to describe in detail the various prokaryotic and eukaryotic expression systems available in the art for such purposes.

In a preferred embodiment, the method employs an *E coli* host cell and in particular a *E. coli* strain carrying the D13 prophage in its genome for allowing inducible expression of T7 polymerase by lactose or analogue of lactose (e.g. IPTG: IsoPropyl b-D-1-Thio Galactopyranoside). Such strains are available for various manufacturers (e.g. Lucigen, Merck, etc). After plasmid introduction, the transformed *E. coli* cell can be cultured at a temperature comprised between approximately 18°C to approximately 39°C (specific preference for approximately 30°C or approximately 37°C) for a time period varying from 6 to 48 hours (specific preference from approximately 8 to approximately 24h) in conventional medium adapted to the vector selection marker (e.g. presence of antibiotic) and to the host strain (e.g. in the presence of an inducer such as IPTG). The cell culture is recovered and can be lysed (e.g. chemical lysis with a detergent, sonication, etc). After centrifugation of the cell lysate, both the supernatant and the pellet can be collected for further analysis (e.g. by SDS PAGE) to evaluate the level of expression as well as the solubility of the expressed material (e.g. soluble material can be found in the cell lysate supernatant and insoluble material can be trapped in inclusion bodies).

The recovered Rpf-containing polypeptide or product combination (e.g. fusion adjuvant-immunogen) of the invention can optionally be purified by well-known purification methods including ammonium sulfate precipitation, acid extraction, gel electrophoresis; filtration and chromatographic methods (e.g. reverse phase, size exclusion, ion exchange, affinity, hydrophobic-interaction, hydroxyapatite, high performance liquid chromatography, etc). The conditions and techniques to be used depend on factors such as net charge, molecular weight, hydrophobicity, hydrophilicity as well as the presence of additional peptides such as His tag that permits IMAC-based purification (Immobilized Metal ion Affinity Chromatography). Moreover, the level of purification will depend on the intended use.

Compositions

In another aspect, this invention provides a composition comprising at least one of the Rpf-containing polypeptide, the product combination, nucleic acid molecule, vector (e.g. infectious viral particle), or host cell of the invention (also referred herein to "active agent") or any combination thereof (e.g. combination of different polypeptides or vectors/viral particles). Preferably, the composition is a pharmaceutical composition which comprises further to a therapeutically effective amount of the active agent(s), one or more pharmaceutically acceptable vehicle(s).

As used herein, a "pharmaceutically acceptable vehicle" is intended to include any and all carriers, solvents, diluents, excipients, adjuvants, dispersion media, coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like, compatible with administration in a subject and in particular in a human.

As used herein a “therapeutically effective amount” is a dose sufficient for the intended use. When prophylactic use is concerned, this term means a dose sufficient to prevent or to delay the onset and/or establishment of a disease condition (e.g. Mtb infection). For therapeutic use, this term means a dose sufficient to treat a disease condition (e.g. cure), optionally or eventually in combination with one or more conventional therapeutic modalities (e.g. SOC). In particular, a therapeutically effective amount of the composition of the invention could be that amount necessary to cause stimulation of the immune response in the administered subject (e.g. innate and/or specific response).

The composition of the invention is suitably buffered in order to be appropriate for human or animal use at a physiological or slightly basic pH (e.g. from approximately pH 7 to approximately pH 9). Suitable buffers include without limitation phosphate buffer (e.g. PBS), bicarbonate buffer and/or Tris buffer.

The composition of the invention can further comprise a diluent appropriate for human or animal use. It is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength. Representative examples include sterile water, physiological saline (e.g. sodium chloride), Ringer’s solution, glucose, trehalose or saccharose solutions, Hank’s solution, and other aqueous physiologically balanced salt solutions (see for example the most current edition of Remington : The Science and Practice of Pharmacy, A. Gennaro, Lippincott, Williams&Wilkins).

Additional pharmaceutically acceptable excipients may be used for providing desirable pharmaceutical or pharmacodynamic properties, including for example modifying or maintaining the pH, osmolarity, viscosity, clarity, colour, sterility, stability, rate of

dissolution, modifying or maintaining release or absorption into a human or animal organism, promoting transport across the blood barrier or penetration in a particular organ.

The pharmaceutically acceptable vehicles included in the composition of the invention must also permit to preserve its stability under the conditions of manufacture and long-term storage (i.e. at least one month with a preference for at least one year) at freezing (e.g. -70°C, -20°C), refrigerated (e.g. 4°C), ambient temperatures. Such "long term" formulations are known in the art (e.g. WO98/02522; WO03/053463). One may cite (a) 1M saccharose, 150 mM NaCl, 1mM $MgCl_2$, 54 mg/l Tween 80, 10 mM Tris pH 8.5, (b) 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl and (c) physiological saline which are particularly adapted to the composition of the invention.

The composition of the invention can be in various forms, e.g. solid, liquid or frozen. Solid (e.g. dry powdered or lyophilized) compositions can be obtained by a process involving vacuum drying and freeze-drying. In a specific embodiment, the composition of the invention is formulated for delivery in the respiratory tract (e.g. by inhalation, intranasal or intrapulmonary route) in a spray-dried (see e.g. WO2010/135495) or droplet form (with a specific preference for droplets having an average diameter of 100-5000 μm).

The Rpf-containing polypeptide, product combination, nucleic acid molecule, vector, host cell or composition of the present invention is suitable for a variety of modes of administration. Any of the conventional administration routes are applicable in the context of the invention including systemic, topical, oral or mucosal routes.

Systemic administration includes for example subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal, intravascular, intraarterial injection as well as scarification. Injections can be made with conventional syringes and needles, or any other appropriate devices available in the art (e.g. electroporation). Mucosal administration includes without limitation oral/alimentary, intranasal, intratracheal, intrapulmonary, intravaginal or intra-rectal route. Administration in the respiratory tract can be performed through nebulisation or aerosolization of droplet, spray, or dry powdered compositions using appropriate dispenser. Topical administration can also be performed using transdermal means (e.g. patch and the like). Intramuscular, intradermal and subcutaneous routes are particularly preferred in the context of the invention.

The appropriate dosage can be adapted as a function of various parameters, in particular the active agent(s) comprised in the composition, the mode of administration; the age, health, and weight of the subject; the nature and extent of disease condition; kind of concurrent treatment; the frequency of treatment; and/or the need for prevention or therapy.

Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by a practitioner, in the light of the relevant circumstances.

For general guidance, suitable dosage for a viral vector-comprising composition varies from about 10^4 to about 10^{13} vp (viral particles), iu (infectious unit) or pfu (plaque-forming units) depending on the vector and the quantitative technique used. Techniques available to evaluate the quantity of vp, iu and pfu present in a sample are conventional in the art. For example, the number of adenoviral particles (vp) is usually determined by measuring the A260 absorbance or HPLC, iu titers by quantitative DBP immunofluorescence and pfu by counting the number of plaques following infection of permissive cells. Preferably, the vp/iu ratio is below 100 in accordance with FDA guidelines. A preferred dose contains from about 10^5 to about 10^{12} vp of an adenoviral vector (e.g. about $5x10^8$, about 10^9, about $5x10^9$, about 10^{10}, about $5x10^{10}$ vp or about 10^{11} vp). A dose from about $5x10^5$ to about 10^9 pfu are preferred for vaccinia (e.g. MVA)-based composition with a specific preference for about $5x10^6$, about 10^7, about $5x10^7$, about 10^8 or about $5x10^8$ pfu. A dose from about $5x10^4$ to about 10^7 pfu are preferred for measles-based composition, with a specific preference for about 10^5, $5x10^5$, 10^6 or $5x10^6$ pfu. A composition based on plasmid vector may be administered in doses of between 10 µg and 20 mg, advantageously between 100 µg and 2 mg. A polypeptide composition may be administered in doses of between 10 µg and 20 mg, with a special preference for about 0.1 mg to about 2 mg per kg body weight for each of the polypeptide comprised in the composition.

The present invention also concerns a Rpf-containing polypeptide, a nucleotide sequence, a vector or a composition as described herein for use as an immunogen or as an adjuvant, optionally or eventually in combination with one or more other immunogen(s).

In a specific embodiment, the Rpf-comprising polypeptide/nucleotide sequence may be administered once or several times. Moreover, when used in combination, the Rpf-containing polypeptide (e.g. RpfB-Dhyb fusion polypeptide) and the immunogen(s) can be administered together or separately. For example, they can be co-administered in the form of a mixture (polypeptide combination) or as a fusion or they can be provided together via one or more expressing vector (a single vector co-expressing said Rpf-containing polypeptide and immunogen(s) or separate vectors that are administered concurrently or as a vector mixture). In case of separate administrations, administration(s) of the Rpf-containing polypeptide can take place before or preferably after immunogen administration or they can be interspersed. The various administrations of the Rpf-containing polypeptide/nucleotide

sequence and/or the immunogen(s) can be separated from each other by an appropriate period of time and carried out by the same route or by different routes of administration, either at the same site or at different sites and using the same or different dosage. Moreover, each administration can use the same active agent(s) or different ones. Optimum regimens can be determined by one skilled in the art and can vary with, for example, the immunogen, the patient and the specific effect sought.

Prophylactic and therapeutic use

In one embodiment, the Rpf-containing polypeptide, product combination, nucleic acid molecule, vector, host cell or composition of the invention is for use in methods for preventing, attenuating or delaying the risk of a disease condition in connection with the selected immunogen(s), the method comprising the step of administering to a subject in need thereof a therapeutically effective amount of at least one of the product combination, nucleic acid molecule, vector, host cell or composition described herein, so as to enhance the anti-immunogen immune response, thereby delaying or reducing the risk of development of said disease condition.

In another embodiment, the Rpf-containing polypeptide, product combination, nucleic acid molecule, vector, host cell or composition of the invention is for use in methods for treating a disease condition in connection with the selected immunogen(s) used, the method comprising the step of administering to the subject having developed such a disease, a therapeutically effective amount of at least one of the product combination, nucleic acid molecule, vector, host cell or composition described herein, thereby reducing clinical signs and/or symptoms associated with such a disease (stabilized disease condition, reduction of the number or severity of clinical symptoms, cure, improved conversion rate, etc).

For illustrative purposes, the Rpf-containing polypeptide described herein or the product combination comprising or encoding Mtb immunogens may be used to treat tuberculosis; a product combination comprising or encoding NS3, NS4 and NS5B antigens may be used to treat chronic HCV hepatitis and a product combination comprising or encoding HbS, core and/or polymerase antigens may be used to treat chronic HBV hepatitis.

In a preferred embodiment, the product combination, nucleic acid molecule, vector, host cell and/or composition of the invention is for use for enhancing an immune response in the administered subject as compared to administration of the immunogen alone. Accordingly, the present invention also encompasses a method for stimulating an anti-

immunogen response upon administration of the product combination, nucleic acid molecule, vector, host cell and/or composition of the invention.

The stimulated immune response can be specific (i.e. directed to the immunogen or epitope thereof) and/or non-specific (innate), humoral and/or cellular, Th1 and/or Th2. In the context of the invention, the immune response is preferably a T cell response CD4+ or CD8+-mediated or both and the enhancement effect can be at various levels (duration, magnitude, intensity, etc).

The stimulation of immunity provided by the active agents(s) described herein can be evaluated either *in vitro* (e.g. in a biological sample), *ex vivo* (e.g. in cells collected from a human or animal subject) or *in vivo* (e.g. animal or human being) by a vast variety of direct or indirect biological assays that permit to measure, e.g. the activation, differentiation, maturation and/or proliferation of one or more immune effector cells (e.g. cytotoxic T cells, B, T lymphocytes, antigen presenting cells, etc) and/or to the production of appropriate immune mediators (e.g. cytokines), and/or to the improvement of antigen presentation, and/or to the onset of a clinical benefit (e.g. prevention of a disease, amelioration of a disease condition, reduction of symptom severity, tumor regression, etc). Testing and validation are also illustrated in the appended Example section.

For example, non-specific immunity can be evaluated by measurement of the NK/NKT-cells (e.g. representativity and level of activation), as well as IFN-related cytokine and/or chemokine producing cascades, activation of TLRs and other markers of innate immunity (e.g. Riano et al., 2012, Tuberculosis 92: 148-59).

The ability to stimulate a humoral response can be determined by an increase in antibody titer that is specific for at least one of the antigens comprised in or encoded by the immunogenic combination and fusion polypeptides described herein. Exemplary techniques include without limitation antibody binding, binding competition as well as ELISA and Western blot.

Evaluation of cellular immunity can be estimated for example by an increased frequency in immune cells such as immunogen-specific T lymphocytes. One may also monitor cell proliferation upon radioactive labelling (e.g. T cell proliferation assays by [^{3}H] thymidine incorporation assay). Another and sensitive method for detecting the immune response is ELISpot in which the frequency of IFNg-producing cells is determined. Cytotoxic capacity for antigen-specific T lymphocytes can also be evaluated in a sensitized subject or by immunization of appropriate animal models. It is also possible to proceed by quantification of the release of relevant Th1 and/or Th2 cytokine(s) produced by activated T

cells using routine bioassays (e.g. by multiparameters flow cytometry (ICS), by cytokine profile analysis using multiplex technologies or ELISA, etc.). PCR techniques can also be used to determine the presence of mRNA coding for the relevant cytokines. It will be appreciated by a skilled person that a significant increase or decrease in the amount of such relevant cytokines can be used to assess the immunogenic activity of one or more of the active agent(s) described herein. The immune response can also be evaluated *ex vivo*, e.g. using peripheral blood cells collected from a subject and submitted to *in vitro* stimulation with adjuvanted immunogen(s) described herein versus the same immunogen(s) without the adjuvant.

Finally, the immune response can be evaluated *in vivo* in appropriate experimental animal, e.g. a mouse, a rat or a guinea pig or by implementing any read out that correlates with a clinical benefit or protective state. The comparison between the group treated with the active agent(s) described herein (adjuvant + immunogen) and the group treated with only the immunogen (control group) can be assessed on animal survival (an increased survival in the treated group will correlate with a protective immune response) and/or on animal protection (e.g. reduced pathogen load in an appropriate tissue homogenate (e.g. splenocytes) collected from challenged animals immunized according to the invention as compared to the challenged group which has only received the immunogen), etc

Such measurements can be performed before the administration of the active agent(s) described herein (baseline) and at various time points during treatment and at least for some (e.g. 12) weeks after cessation of the treatment.

As the immune responses may vary considerably from a subject to another, it is not required that the immune stimulation be observed in each subject treated but in a significant number of subjects (e.g. statistically significant differences between two groups can be determined by any statistical test known in the art, such as a Tukey parametric test, the Kruskal-Wallis test the U test according to Mann and Whitney, the Student's t-test, the Wilcoxon test, etc).

Association with chemotherapy

The Rpf-containing polypeptide, product combination, nucleic acid molecule, vector, host cell or composition of the invention may be employed in association with one or more conventional therapy. The chemotherapy is typically determined by the physician using current practice and depending on the disease condition to be treated, e.g. antibiotics for treating bacterial infection (e.g. Mtb infection), antiviral compounds for treating viral

infection (e.g. IFNa, ribavirin and/or protease inhibitor for treating chronic HCV infection; nucleoside analogs for treating chronic HBV infection, chemotherapy for treating cancers, etc).

In accordance with the present invention, the product combination, nucleic acid molecule, vector, host cell or composition of the invention can be administered before, concurrently with, or after administration of the one or more conventional therapy. In one embodiment, the active agent described herein is administered at least 2 weeks after starting administration of the chemotherapy.

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication or entry were specifically and individually indicated to be incorporated by reference.

DESCRIPTION OF THE DRAWINGS

Figure 1 represents IFNg-producing cells produced following immunization of animals with fusion-encoding plasmids pTG18270 (SS-Ag85B-Rv2626-RpfB-Dhyb-Rv1733; Figure 1A) and pTG18272 (SS-Ag85B-Rv2626- Rv1733; Figure 1B). Cellular immune response was evaluated 2 weeks following the last DNA injection by Elispot IFNγ assays after *ex vivo* re-stimulation with specific peptide pools covering either Ag85B, Rv2626, Rv1733 or RpfB-Dhyb antigens.

Figure 2 represents IL-2 production capacity of T cells following immunization of animals with plasmids pTG18310, pTG18305, pTG18309 and pTG18307 encoding Ag85B, Rv2626, Rv1733 and RpfB-Dhyb antigens, respectively. Immune response was evaluated 2 weeks following the last DNA injection by Elisa IL-2 assays after *ex vivo* re-stimulation with specific peptide pools covering MtB antigens. Full bars represent measures for individual mice, and hatched bars represent median measure of the group. Dotted line represents the cut-off value equal to 9.8 pg/mL. * $p \leq 0.05$.

Figure 3 represents SDS-PAGE analysis of the purified RpfB-Dhyb protein. Lanes 1 to 8 represent intermediate purification fractions and lanes 9 to 11 increasing volumes (5, 10 and 15µL) of purified pool)

EXEMPLE 1: Selection of the Rpf proteins

Existing data on Rpf antigens were investigated from the available literature and data bases with the goal of identifying among the five existing Rpf gene products the one(s) that may be useful in an immunotherapeutic vaccine capable of raising immunity.

A data-mining scoring system was developed to transcribe and compare data from different sources. An overall final "score" was generated reflecting the value of each antigen in terms of immunogenic potential as well as its capacity to protect against an infectious challenge in animal models and in humans (for example protection data in humans will be better scored than inducing immunogenicity in animal models). Once all data for a particular antigen were collected, a grade from 0 to 5 was attributed to each category, 0 being the worse possible grade while 5 being the best. The choice of the grade was also based on the quality of the data (e.g. right controls used in the experiments, rigorous interpretation) but also on the robustness of the data (e.g. number of times experiments were run, number of publications confirming/supporting the findings).

Among the 5 resuscitation gene products, three Rpfs stood out (RpfB, D and E) with very similar and high score after the running data mining scoring process but only RpfB and D were selected for 2 main reasons. Firstly, the reported cross-reactivity in term of cellular and humoral responses between 4 out of 5 Rpfs (Yeremeev et al., 2003, Infect Immun 71: 4789-94), except for RpfB justified in our view the selection of the latter. Secondly, RpfD was chosen instead of RpfE after sequence analysis based on a lower sequence homology in the lysozyme domain (LD) between RpfB and D than between RpfB and E. It is thus assumed that keeping Rpfs B and D would be sufficient to generate a broad immune response.

EXEMPLE 2: Design of RpfB-Dhyb

An hybrid protein gathering RpfB and RpfD proteins was constructed. The fusion design was carefully studied permitting to anticipate potential expression problems. Indeed, it is well known in the art that biochemical and biological data are key data for optimizing expression. For example, the biological functions of a protein may lead to a potential toxicity

resulting in genetic unstability and/or safety profile especially in view of human use. Moreover, protein unfolding may impact stability and expression levels due to a higher cellular degradation rate.

2.1 Search analyses

A bibliographic search was first carried out in order to better understand and characterize the Rfp structure and function. Additionally, extensive biochemical and bioinformatics prediction analyses were performed to better characterize Rpf antigens and identify the relevant structural regions to retain in the hybrid polypeptide. Search for protein homologs with a known structure was undertaken in protein data bank (program BLASTP with the default parameters and UNIPROT-SWISSPROT database). Search for known protein motif associated with protein domains, families and functional sites was made using PROSITE SCAN. Prediction of signal peptides was searched in the UNIPROT-SWISSPROT database or using the hidden Markov model of signal v3.0 algorithm. Prediction of potential transmembrane domains (TM) was made using three different programs (e.g. dense Alignment surface (DAS) method, Algorithm TMHMM and TopPred0.01). Prediction of secondary structures was carried out using several prediction methods (namely: SOPM, MLRC, HNN, DSC, PHD, PREDATOR). Hydrophobic clusters along the protein sequence were identified by HCA plots (these clusters are characteristic of folded proteins with a hydrophobic buried core). Natively unfolded regions were predicted using MetaPrDOS predictions program that complements HCA plot (all areas above the threshold 0.5 were considered as unfolded parts of the protein). Coiled coil domains were predicted using the COILS program. Bioinformatic prediction tools (Nielsen et al., 2007 PLos One 2: e796; Nielsen et al., 2008, PLoS Comput Biol 4: e1000107) were also used to look at predicted epitopes for class I and II HLA molecules that need to be retained in the hybrid polypeptide.

2.2 Structure prediction

The Rpf proteins all contain a catalytic domain (lysozyme like domain) that is highly conserved between the 5 members of this family. Apart from this domain, there is no significant similarity among the Rpf proteins. RpfB structure has been obtained for about half of the molecule (residues 194-362) and a signal peptide was predicted (residues 1-29; Ruggiero *et al.* 2009, J Mol Biol 385: 153-62). The full length protein (without its signal peptide) behaves as a monomer when expressed in *E. coli.*

In silico predictions and analyses were performed on RpfB to analyse the part of the protein (30-193) for which no structure was available. Except for the signal peptide, no transmembrane domain was predicted. HCA plots, secondary structure prediction and natively disordered regions predictions are in agreement with a well-defined fold of the 30-193 region. Coiled coils predictions and search for known motifs using PROSCAN did not yield any significant result.

Activity of the catalytic domain has been shown to depend on a conserved residue essential in the resuscitation activity of *Micrococcus luteus* Rpf in a *Mycobacterium smegmatis* resuscitation assay (E292; Mukamolova *et al.* 2006, Mol Microbiol 59: 84-98). Furthermore, the two residues T315 and Q347 are involved in substrate binding in lysozyme, and conserved in RpfB (Cohen-Gonsaud, *et al.* 2005, Nat Struct Mol Biol 12, 270-3).

Based on these results, the inventors designed a RpfB-Dhyb polypeptide that comprises a signal peptide-deleted RpfB molecule with its catalytic domain replaced by the most divergent catalytic domain among Rpfs (*i.e.* RpfD catalytic domain) with a neutralized catalytic activity by three mutations (E292K, T315A and Q347A) and with deletion of the 7 last residues. The recommended primary structure for this RPFB-D hybrid protein used in fusions corresponds to the amino acid sequence shown in SEQ ID NO: 1, optionally or eventually with a initiator Met.

EXEMPLE 3: Construction of vectors for expression of RpfB-Dhyb and in association with Mtb antigens.

RpfB-Dhyb polypeptide was expressed with and without signal sequence (SS) and TM domain so as to study the influence of cell location (membrane presentation in the presence of SS and TM peptides versus cytoplasmic location in the absence of such peptides) on expression level and immunogenic activity.

The immunogenic and adjuvanting properties of such RpfB-Dhyb protein were studied in association with immunogens originating from *M. tuberculosis*. For these purposes, various fusions were designed. The first series comprises the RpfB-Dhyb polypeptide fused to 3 active Mtb antigens (Ag85B*-TB10.4-ESAT6), resulting in pTG18268 and pTG18297 vectors encoding the anchored fusion (with a SS at its N-terminus and a TM-at its C terminus) and the cytoplasmic version, respectively. The second series comprises the RpfB-Dhyb polypeptide fused to active Ag85B* and latent Rv2626 and Rv1733* antigens, resulting in pTG18270. In this case, RpfB-Dhyb is positioned internally in the fusion (in between the latent Rv2626 and Rv1733* antigens) and the encoded fusion is

membrane-anchored with a signal peptide (SS) at its N-terminus (instead of at the RpfB-Dhyb N terminus). Addition of a TM domain was not necessary for fusions ending with Rv1733*, as this protein already contains such domains).

Whether expressed alone or in fusion, a series of Tag peptides were added to facilitate detection of the encoded gene products, namely a N-terminal Flag (DYKDDDDK; SEQ ID NO: 12) and C-terminal c-myc (EQKLISEEDL; SEQ ID NO: 13) and His (HHHHHH; SEQ ID NO: 14) Tag peptides.

3.1 Vector constructions

All together 5 Rpf-containing constructions were generated

- pTG18267: Fusion n°3 (anchored RpfB-Dhyb*);
- pTG18307: Fusion n°12 (cytoplasmic RpfB-Dhyb*),
- pTG18268: fusion n°4 (anchored RpfB-Dhyb*-Ag85B*-TB10.4-ESAT6),
- pTG18297: fusion n° 11 (cytoplasmic RpfB-Dhyb*-Ag85B*-TB10.4-ESAT6),
- pTG18270: Fusion n°6 (anchored Ag85B*-Rv2626-RpfB-Dhyb*-Rv1733*);

Other plasmids encoding Mtb antigens were also generated for comparative purposes:

- pTG18266: Fusion n°2 (anchored Ag85B*-TB10.4-ESAT6),
- pTG18296: Fusion n°10 (cytoplasmic Ag85B*-TB10.4-ESAT6),
- pTG18272: Fusion n°8 (cytoplasmic Ag85B*-Rv2626-Rv1733*)
- pTG18310: cytoplasmic Ag85B*,
- pTG18305: cytoplasmic Rv2626,
- pTG18309: anchored Rv1733*,

For each construction, synthetic genes coding for RpfB-Dhyb and Mtb fusions were synthesized by Geneart (Regensburg, Germany). The sequences were optimized for human codon usage and a Kozak sequence (ACC) was added before the ATG starting codon. Moreover some motives were excluded: TTTTTNT, GGGGG, CCCCC which are deleterious for expression in poxvirus vector and AAAGGG, AAAAGG, GGGAAA, GGGGAA, (and complementary sequences TTCCCC, TTTCCC, CCTTTT, CCCCTT) which can be deleterious for expression in some others vectors.

The synthetic genes were then cloned in pGWIZ plasmid (Gelantis) digested by *Not*I and *BamH*. This plasmid contains a modified CMV promoter, followed by intron A from the CMV immediate early gene, and a high-efficiency artificial transcription terminator.

Construction of pTG18267 (fusion n°3 encoding membrane-anchored RpfB-Dhyb)

The amino acid sequence of the fusion n°3 is shown in SEQ ID NO: 2. Amino acids 1 to 23 correspond to the signal peptide present at the N-terminus of the glycoprotein precursor of rabies virus PG strain (described in Genbank n° ay009097 and in WO2008/138649), amino acids 25 to 31 correspond to the Flag TAG, amino acids 32 to 380 correspond to RpfB-Dhyb*, amino acids 381 to 390 correspond to the c-myc TAG, amino acids 392 to 457 correspond to the membrane-anchoring peptide derived from the rabies glycoprotein of PG strain (SEQ ID NO: 3 in WO2008/138649) and amino acids 458 to 463 correspond to the His TAG. The fusion n°3-encoding nucleotide sequence shown in SEQ ID NO: 7 was generated by synthetic way and the synthetic gene was cloned in pGWIZ restricted by *Not*I and *BamH*1 to give pTG18267.

Construction of pTG18307 (fusion n°12 encoding cytoplasmic RpfB-Dhyb)

The targeting sequences were deleted from plasmids pTG18267 by directed mutagenesis (Quick Change Site-Directed mutagenesis kit, Stratagene) using appropriate pairs of primers, OTG20188 (CGCGGCCGCACCATGGATTACAAGGATGACGACG; SEQ ID NO: 15) and OTG20189 (CGTCGTCATCCTTGTAATCCATGGTGCGGCCGCG; SEQ ID NO: 16) for deleting signal peptide sequence and OTG20190 (CATCTCAGAAGAGGATCTGCATCATCATCATCATTG; SEQ ID NO: 17) and OTG20191 (CAATGATGATGATGATGATGCAGATCCTCTTCTGAGATG; SEQ ID NO: 18) for deleting TM sequence. The resulting plasmid was pTG18307 with the nucleotide sequences SEQ ID NO: 8 encoding the amino acid sequence SEQ ID NO: 3 (fusion n° 12).

Construction of pTG18268 (fusion n°4 encoding membrane-anchored fusion of RpfB-Dhyb with Ag85B, TB10.4 and ESAT6 antigens)

The amino acid sequence of the fusion n°4 is shown in SEQ ID NO: 4. Amino acids 1 to 23 correspond to the signal peptide present at the N-terminus of the glycoprotein precursor of rabies virus PG strain (described in Genbank n° ay009097), amino acids 25 to 31 correspond to the Flag TAG, amino acids 32 to 380 correspond to RpfB-Dhyb*, amino acids 381 to 666 correspond to Ag85B*, amino acids 667 to 761 correspond to TB10.4, amino acids 762 to 855 correspond to ESAT6, amino acids 856 to 865 correspond to the c-myc TAG, amino acids 867 to 932 correspond to the membrane-anchoring peptide derived

from the rabies glycoprotein of PG strain and amino acids 933 to 938 correspond to the His TAG. The fusion n°4-encoding nucleotide sequence shown in SEQ ID NO: 9 was generated by synthetic way and the synthetic gene was cloned in pGWIZ restricted by *Not*I and *BamH*1 to give pTG18268.

Construction of pTG18297 (fusion n°11 encoding cytoplasmic fusion of RpfB-Dhyb with Ag85B, TB10.4 and ESAT6 antigens)

The targeting sequences were deleted from plasmids pTG18268 by directed mutagenesis (Quick Change Site-Directed mutagenesis kit, Stratagene) using pairs of primers, OTG20188 (SEQ ID NO: 15) and OTG20189 (SEQ ID NO: 16) for deleting signal peptide sequence and OTG20190 (SEQ ID NO: 17) and OTG20191 (SEQ ID NO: 18) for deleting TM sequence. The resulting plasmid was pTG18297 with the nucleotide sequences SEQ ID NO: 10 encoding the amino acid sequence SEQ ID NO: 5 (fusion n° 11).

Construction of pTG18270 (fusion n°6 encoding membrane-anchored fusion of RpfB-Dhyb with Ag85B, Rv2626 and Rv1733* antigens)*

The amino acid sequence of the fusion n°6 is shown in SEQ ID NO: 6. Amino acids 1 to 23 correspond to the signal peptide present at the N-terminus of the glycoprotein precursor of rabies virus ERA strain (described in Genbank n° M38452), amino acids 25 to 31 correspond to the Flag TAG, amino acids 32 to 317 correspond to Ag85B*, amino acids 318 to 459 correspond to Rv2626, amino acids 460 to 808 correspond to RpfB-Dhyb*, amino acids 809 to 956 correspond to Rv1733*, amino acids 957 to 966 correspond to the c-myc TAG and amino acids 968 to 973 correspond to the His TAG. The fusion n°6-encoding nucleotide sequence shown in SEQ ID NO: 11 was generated by synthetic way and the synthetic gene was cloned in pGWIZ restricted by *Not*I and *BamH*1 to give pTG18270.

3.2 Construction of Mtb gene fusions without RpfB-Dhyb

For comparative purposes, plasmids encoding the Mtb immunogens in the same configuration as with RpfB-Dhyb-comprising fusions were constructed and cloned in pGWIZ downstream the CMV promoter and fused to Flag tag in 5'and c-myc-and His tags in 3' and optionally SS and TM peptides. The generated plasmids were named respectively pTG18266 (SS-Ag85B*-TB10.4-ESAT6-TM), pTG18296 (cytoplasmic Ag85B*-TB10.4-ESAT6), and pTG18272 (Ag85B*-Rv2626-Rv1733*).

3.3 Construction of individual Mtb gene expression plasmids

pGWiz-based plasmids were generated for expression of individual Mtb antigens Rv2626, Rv1733* and Ag85B*. Each Mtb-encoding gene was placed under the same configuration as the above-described fusion polypeptides under the transcriptional control of the CMV promoter and with Flag and c-myc-His sequences. More specifically, a synthetic DNA fragment containing the end of CMV promoter, Flag and c-myc-His sequences was synthetized by Geneart and inserted in the FLAG-TAG1 plasmid before being digested by PvuII and BglII and inserted in pGWiz restricted by the same enzyme, giving rise to pTG18282. The individual Rv2626 gene sequence was amplified by PCR from pTG18272 using appropriate primer pairs. The resulting amplicon was cloned by "In fusion Advantage" PCR cloning method (Clontech) in the linearized pTG18282 so as to allow the fusion of the *Mtb* gene with Tag sequences. The generated plasmid was named pTG18305.

(Rv2626).

The expression cassettes for Rv1733* and Ag85B* fused to Flag in 5'and c-myc-His sequences in 3' were synthesized by Geneart and inserted in pGWiz. They were named respectively pTG18309 (Rv1733*) and pTG18310 (Ag85B*). As Rv1733* proteins contain a TM domain, the signal peptide presents at the N-terminus of the glycoprotein precursor of rabies virus ERA strain was fused upstream to the Flag sequence to avoid expression issues.

Whether encoding individual or fused *Mtb* genes, plasmids used for immunization were produced in endotoxin-free conditions.

EXAMPLE 4: Analysis of expression of Mtb antigens and Mtb antigen fusions

Expression of RpfB-Dhyb polypeptide alone or in association with the Mtb immunogens was analyzed by Western Blot from cell lysates obtained from transfected HEK293 cells.

4.1 Western blot protocols

More specifically, $2x10^6$ HEK293 cells were transfected with 5µg of the plasmids encoding RpfB-Dhyb and RpfB-Dhyb-immunogen fusions using Lipofectamine 2000 (Invitrogen; #11668-019) in presence of proteasome inhibitor MG132 (10µM) added to growth medium 18h after transfection. pGWIZ plasmid was used as negative control. After 48 hours medium was discarded and cells were lysed with 450 µL/dish of Tris-Glycin-SDS 2 X buffer (ref: LC2676; Novex) supplemented with β-mercaptoethanol (5 % v:v). The lysate

was then sonicated and boiled for 5 min at 95 °C. Thirty microliters of cell lysates were submitted to electrophoresis onto precasted 10% Criterion gel using the Criterion Precast gel system (Biorad). Following electrophoresis, proteins were transferred onto a PVDF membrane (Macherey Nagel, 741260). Immunodetection of the RpfB-Dhyb alone or in association with Mtb antigens was performed with either antibodies directed to the tag peptides included in the expression cassettes (Sigma's anti-Flag M2 peroxydase (HRP) antibody #A8592 diluted 1/500; Invitrogen's monoclonal anti-His peroxydase antibody #R931-25 diluted 1/5000 and anti-c-myc peroxidase antibody) or with specific antibodies. Specifically, the sera of immunized rabbits (following 4 injections of two 15 or 16 mers specific peptides; sera diluted 1/1000) were used for detection of Rv1733* and RpfB-D whereas commercial antibodies were used for the detection of ESAT6, Ag85B*, TB10.4 and Rv2626 (mouse monoclonal antibody HYB076-08 (Santa-Cruz; #sc-57730, diluted 1/500) for ESAT6, rabbit polyclonal anti-serum NR-13800 (BEI, diluted 1/5000) for Ag85B*, mouse monoclonal antibody 26A11 (Lifespan-Biosciences;#LS-C91052 diluted 1/1000) for Rv2626 and polyclonal rabbit antibody ABIN361292 (Antibodies–online, diluted 1/1000) for TB10.4). Immune-complexes were revealed using the ImmunStar WesternC kit (Biorad, ref 170.5070).

4.2 Western blot analysis of cell lysate.

The expected sizes of the various constructs are indicated below:

- membrane-anchored RpfB-Dhyb (pTG18267) : 49.0 kDa
- Cytoplasmic RpfB-Dhyb (pTG18307) : 39.3 kDa
- membrane-anchored fusion RpfB-Dhyb-Ag85B*-TB10.4-ESAT6 (pTG18268): 99.7 kDa
- cytoplasmic fusion RpfB-Dhyb-Ag85B*-TB10.4-ESAT6 (pTG18297) : 90.0 kDa
- membrane-anchored fusion Ag85B*-Rv2626-RpfB-Dhyb-Rv1733 (pTG18270) : 103.5 kDa

Whatever the immunodetection system, a band corresponding to the expected size was highlighted for RpfB-Dhyb polypeptide and Mtb fusions expressed from the above constructs with the anti-Flag and anti-His monoclonal antibodies as well as with the anti-c myc monoclonal antibody except for pTG18267 and pTG18268. The c-myc epitope might be inaccessible in these fusions due to adjacent TM domains since the cytoplasmic counterparts (pTG18307 and pTG18297) are well detected with the anti-myc antibody. In

some cases, additional products were also observed. In particular, dimers were detected for pTG18270. This fusion contains Rv2626 which has the ability to form dimers resistant to reducing conditions. Moreover, additional products higher than the expected size were detected for pTG18268 and pTG18270 with anti-Flag and anti-His antibodies. These bands correspond to N-glycosylated products as it was demonstrated by *in vitro* treatment with N-Glycosidase F (i.e. expression products at the expected size were obtained after N-glycosidase treatment of cellular extracts). Two weak bands ≈40 kDa were also observed in pTG18307-transfected cell lysate only with anti-RpfB-Dhyb rabbit sera.

Similar and high levels of expression were obtained for all fusions and higher amounts of products were detected in the presence of MG132. The expression levels of membrane-anchored fusions were comparable to those detected with their cytoplasmic counterparts.

Expression of the individual genes and the fusion polypeptides containing or not RpfB-Dhyb was confirmed by Western blot and found in conformity to what is expected.

EXAMPLE 5: DNA immunization evaluation

Immunogenic activity provided by the above-described constructs was evaluated in a mouse model following DNA immunization.

5.1. DNA Immunization protocols

Balb/c mice were immunized three times at 3-week interval either with the RpfB-Dhyb and fusion encoding plasmids. 100µg of DNA in 100 µl of sterile PBS were injected via intramuscular route in the tibialis anterior muscle. Cellular immune response was evaluated 2 weeks following the last DNA injection by Elispot IFNγ assays.

5.2 Peptide libraries

A peptide library was used to restimulate *ex-vivo* the splenocytes from immunized mice. More precisely, 15mers peptides overlapping by 11 amino acids covering all Mtb antigens described above were synthetized (ProImmune). Pools of peptides were prepared in DMSO with a final concentration of 1µmol/L. One to 4 pools were needed so as to cover the full length of each Mtb antigen.

RpfB-Dhyb was covered by 3 pools of 22 peptides (covering RpfB residues 30 to 127; residues 117 to 215 and residues 205 to 284, respectively) and one pool of 19 peptides (covering RpfD residues 61 to 146).

Ag85B was covered by 3 pools of 23 peptides (covering Ag85B residues 39 to 141; residues 131 to 233; and residues 223 to 325, respectively).

ESAT-6 was covered by 1 pool of 21 peptides covering ESAT-6 from residues 1 to 95.

TB10.4 was covered by 1 pool of 21 peptides covering TB10.4 from residues 1 to 95.

Rv1733 was covered by 2 pools of 18 and 17 peptides (covering Rv1733 residues 62 to 144 and residues 134 to 210, respectively).

Rv2626 was covered by 2 pools of 17 and 16 peptides (covering Rv2626 residues 1 to 79 and residues 69 to 143, respectively).

5.3 IFNγ Elispot assays

Splenocytes from immunized mice were collected and red blood cells were lysed (Sigma, R7757). $2x10^5$ cells per well were cultured in triplicate for 40 h in Multiscreen plates (Millipore, MSHA S4510) coated with an anti-mouse IFNγ monoclonal antibody (BD Biosciences; 10 µg/ml, 551216) in αMEM culture medium (Gibco, 22571) supplemented with 10 % FCS (JRH, 12003-100M), 80 U/mL penicillin / 80 µg/mL streptomycin (PAN, P06-07-100), 2 mM L-glutamine (Gibco, 25030), 1x non-essential amino acids (Gibco, 11140), 10 mM Hepes (Gibco, 15630), 1 mM sodium pyruvate (Gibco, 31350) and 50 µM β-mercaptoethanol (Gibco, 31350) and in presence of 10 units/ml of recombinant murine IL2 (Peprotech, 212-12), alone as negative control, or with:

- The above-described pool of peptides at a final concentration of 1µmol/L
- 5 µg/ml of Concanavalin A (Sigma, C5275) for positive control.
- Irrelevant peptide

IFNγ-producing T cells were quantified by Elispot (cytokine-specific enzyme linked immunospot) assay as previously described (Himoudi et al., 2002, J. Virol. 76: 12735-46). Results are shown as the mean value obtained for triplicate wells. An experimental threshold of positivity for observed responses (or cut-off) was determined by calculating a threshold value which corresponds to the mean value of spots observed with medium alone + 2 standard deviations, reported to 10^6 cells. A technical cut-off linked to the CTL Elispot reader was also defined as being 50 spots/10^6 cells (which is the value above which the CV (coefficient of variation) of the reader was systematically less than 20%). Statistical analyses of Elispot responses were conducted by using a Kruskal-Wallis test followed, when a

significant difference was obtained, by a Mann-Whitney test. P value equal or inferior to 0.05 will be considered as significant.

5.4 IL-2 ELISA assays

Splenocytes from immunized mice were collected and red blood cells were lysed (Sigma, R7757). $2x10^5$ cells per well were cultured in triplicate for 72 h in Tissue Culture plate (Falcon, 353072) in αMEM culture medium (Gibco, 22571) supplemented with 10 % FCS (JRH, 12003-100M), 80 U/mL penicillin / 80 µg/mL streptomycin (PAN, P06-07-100), 2 mM L-glutamine (Gibco, 25030), 1x non-essential amino acids (Gibco, 11140), 10 mM Hepes (Gibco, 15630), 1 mM sodium pyruvate (Gibco, 31350) and 50 µM β-mercaptoethanol (Gibco, 31350), alone as negative control, or with:

- The above-described pool of peptides at a final concentration of 1 µmol/L
- 5 µg/ml of Concanavalin A (Sigma, C5275) for positive control.
- Irrelevant peptide

IL-2 concentrations in culture supernatant were quantified by ELISA assay, using commercial kit (Ebioscience 88-7024-22, associated with Corning Costar plates 9018). An experimental threshold of positivity for observed responses (or cut-off) was determined by calculating a threshold value which corresponds to the mean value observed with medium alone + 2 standard deviations. Statistical analyses were conducted by using a Mann-Whitney test. P value equal or inferior to 0.05 will be considered as significant.

5.5. Evaluation of the immunogenicity induced by RpfB-Dhyb polypeptide.

BALB/c mice were immunized with pTG18267 (membrane anchored RpfB-Dhyb) or pTG18307 (cytoplasmic RpfB-Dhyb) or with pGWIZ as negative control. Cellular immune response was evaluated 2 weeks following the last DNA injection by Elispot IFNγ assays after *ex vivo* re-stimulation with the RpfB-Dhyb pools described above or with irrelevant peptides (negative control).

Comparable and very strong immune responses (no significant difference) were induced against RpfB-Dhyb although responses seem more homogeneous with the membrane-anchored version. T cells responses as high as 1500 spots per 10^6 cells were measured in both groups of mice with a median of 750 spots/10^6 cells in the group of animals immunized with pTG18267 versus 550 spots/10^6 cells in the group immunized with pTG18307. These results illustrate the unexpected immunogenic properties provided by the RpfB-RpfD hybrid protein

5.6. Evaluation of the adjuvanting effect of RpfB-Dhyb polypeptide on immunogenic response provided by Mtb antigens

BALB/c mice were immunized three times at 3-week interval via intramuscular route with the plasmid expressing the *"Ag85B – Rv2626 – RpfB-Dhyb –Rv1733*"* fusion (pTG18270*)* or the same fusion without RpfB-Dhyb (pTG18272) and with empty pGWIZ as negative control. Cellular immune response was evaluated 2 weeks following the last DNA injection by Elispot IFNγ assays after *ex vivo* re-stimulation with the various peptide pools described above.

As illustrated in Figure 1, high levels of IFNγ producing cells were observed in pTG18270-vaccinated mice against Ag85B and Rv2626 and RpfB-Dhyb, indicating that these mice mounted a strong specific cellular response against these three Mtb immunogens. Immunization with pTG18272 (expressing the Ag85B-Rv2626-Rv1733* fusion), also resulted in activation of IFNγ producing cells against pools of peptides specific of Ag85B and Rv2626 but to a lesser extent. As expected, immunization with the empty plasmid and stimulation with irrelevant peptides did not rise any specific immune response.

The adjuvanting effect provided by RpfB-Dhyb protein can also be evaluated by comparing the cellular immune response obtained following immunization of BALB/c mice with the plasmid expressing the *"RpfB-Dhyb – Ag85B – TB10.4 – ESAT6"* fusion (pTG18268*)* or with the plasmid encoding the same fusion devoid of RpfB-Dhyb (pTG18266). The same experimental conditions as those described above in connection with plasmids pTG18270 and pTG18272 can be applied except that *ex vivo* re-stimulation will be performed with the peptide pools adapted to the Mtb antigens present in the fusion. Induction of a higher number of IFNγ producing cells against one or more of the Mtb antigens present in the fusion in the group of mice immunized with pTG18268 as compared to the group of mice injected with pTG18266 would be indicative of RpfB-Dhyb-mediated adjuvanting properties.

5.7. Evaluation of the IL-2 secretion capacity by RpfB-Dhyb-specific T cells.

BALB/c mice were immunized with individual plasmids coding for Ag85B (pTG18310), Rv2626 (pTG18305), Rv1733 (pTG18309) and RpfB-Dhyb (pTG18307). Cellular immune response was evaluated 2 weeks following the last DNA injection by

ELISA IL-2 assays after *ex vivo* re-stimulation with the specific Mtb antigens peptide pools, as described above.

As illustrated in Figure 2, re-stimulation with peptide pool specific of Ag85B, Rv2626 or Rv1733 induced significant production of IL-2 when compared to unstimulated cells (Medium). In addition, significant higher levels of IL-2 were measured in culture supernatant of splenocytes re-stimulated with RpfB-Dhyb specific peptide pool, as compared to re-stimulations with Ag85B, Rv2626 and Rv1733 peptide. So, DNA immunization induced RpfB-Dhyb specific T lymphocytes with high capacity of IL-2 production.

IL-2 plays pivotal roles in the immune response. Discovered as a T cell growth factor, IL-2 additionally promotes CD8 T cell and natural killer cell cytolytic activity and modulates T cell differentiation programs in response to antigen, promoting naïve CD4 T cell differentiation into T helper 1 and T helper 2 cells. As regard to these functions, the capacity of RpfB-Dhyb specific T lymphocytes to highly express IL-2 could contribute at least partially to the adjuvanting properties of RpfB-Dhyb.

EXEMPLE 6: Recombinant production of RpfB-Dhyb

The RpfB-Dhyb polypeptide was produced in E. coli by recombinant means and purified. The recombinant product may be used for adjuvanting therapeutic or vaccinal protein preparation.

6.1 Production assays

Four *E. coli* strains have been tested for the expression of the RpfB-Dhyb protein. All the strains carry the DE3 prophage in their genome that allows the induction of expression of T7 polymerase by lactose or analogue of lactose (*i.e.* IPTG). The four strains were Bl21(DE3) (Lucigen) as a classic strain for protein expression, C41(DE3) (Lucigen) for the expression of toxic protein, Bl21(DE3) Rosetta (Merck Chemical) for expression of protein with a codon usage that is different of the *E. coli* one, and C43(DE3) (Lucigen) for the expression of protein with trans-membrane peptides. Moreover, three different temperatures and production time were tested for optimizing antigen production.

Each *E. coli* strain was transformed with the RpfB-Dhyb-encoding plasmid. Five colonies were isolated from a freshly transformed plate, inoculated in 50 ml of LB (Luria Broth) medium in the presence of ampicillin and allowed to grow overnight at 37°C under shaking. A flask of autoinducible medium (AI medium containing glucose/lactose and

antibiotic; Studier, 2005, Protein Expr Purif. 41: 207-34) was inoculated with preculture specimen and was then cultured at either 18°C, 30°C and 37°C for 24, 8 and 8 hours, respectively. At the end of incubation, the absorbance at 600nm was measured and the cells were harvested by centrifugation. The cell pellet was resuspended in PBS and the OD 600 nm adjusted around 50 for each culture condition tested before lysing the cells by sonication. The cell lysate was then centrifuged at 10,000 g for 10 minutes at 4°C and a specimen (typically 10 µL) of the supernatant and the pellet were then loaded on a SDS-PAGE to estimate optimal conditions.

These assays highlight that RpfB-Dhyb is easily produced in the various *E. coli* strains tested and whatever the culture conditions (expression clearly visible as the major protein of the bacterial lysate). In each assays, the produced RpfB-Dhyb is present in insoluble material collected from the pellet after cell lysis.

6.2. Purification of RpfB-Dhyb protein

Purification was undertaken from 500mL culture grown in 2L flasks applying the optimal conditions determined previously. The cells were harvested by centrifugation and pellets corresponding to 250 mL of culture were kept at -20°C until use. The harvested bacteria were resuspended in guanidine due to the insolubility of the protein and submitted to sonication for cell lysis. Due to the presence of an His tag, the RpfB-Dhyb was purified by IMAC affinity chromatography on Ni sepharose 6 fast Flow resin (GE Healthcare; reference 17-5318) in denaturing conditions according to the provider's recommendations. The protein was eluted by applying increasing concentrations of Imidazole (50 mM, 100 mM and 250mM). Fractions containing the pure protein were pooled and dialysed against Urea.

A variety of tests can be performed to estimate the quantity and quality of the purified RpfB-Dhyb polypeptide present in the eluted fractions. For example, purity of the recovered material can be evaluated by electrophoresis on SDS-PAGE (4-12% Invitrogen); protein concentrations can be determined by Bradford assay (Bioroad); endotoxin levels can be measured using Portable Test System (e.g. using cartridges with a range of detection of 0.005 to 0.5 EU/mL; Charles River Laboratories). Mass of the purified polypeptide can be measured using MALDI (Matrix-Assisted Laser Desorption/ionization) or electrospray methods. Measured and calculated masses were compared in order to determine if the protein is intact or not. Identity of the protein either in solution or in a band of gel was

checked by mass measurement of peptides generated after trypsin digestion. Masses of peptides were determined by MALDI and/or liquid chromatography coupled to tandem mass Spectrometry (LC/MS/MS).

When visualized on SDS-PAGE, the RpfB-Dhyb purified pool did not show any visible contaminant (see Figure 3). Endotoxin levels were measured in the purified pools and showed to be at a maximum level of 10 EU/mg protein.

Therefore, the RpfB-Dhyb polypeptide has been purified with acceptable amount, purity and endotoxin level.

CLAIMS

1. A product combination comprising (a) one or more immunogen(s) and (b) a polypeptide comprising at least a Mycobacterium resuscitation promoting factor (Rpf) or a nucleic acid molecule encoding said polypeptide.

2. The product combination according to claim 1, wherein said polypeptide comprises at least a Mycobacterium RpfB and RpfD

3. The product combination according to claim 1 or 2, wherein said Rpf(s) comprised in said polypeptide or encoded by said nucleotide sequence is/are obtained from a Mycobacterium species of the tuberculosis complex selected from the group consisting of *M. tuberculosis* (Mtb), M. bovis, M. bovis BCG, M. africanum, M. canetti, M. caprae, and M. microti

4. The product combination according to anyone of claims 1 to 3, wherein said Mycobacterium Rpf is fused to one or more fusion partner(s).

5. The product combination according to claim 4, wherein said one or more fusion partner originate from a Mycobacterium and desirably is a second Rpf originating from a Mycobacterium species as defined in claim 2.

6. The product combination according to claim 5, wherein said polypeptide comprises a Mtb RpfB modified by deletion of all or part of its catalytic domain which is fused to the catalytic domain of a Mtb RpfD.

7. The product combination according to claim 4, wherein said one or more fusion partner originate from a non-Mycobacterium source.

8. The product combination according to claim 7, wherein said fusion partners comprise tag peptide(s) and/or targeting peptide(s).

9. The product combination according to anyone of claims 2 to 8, wherein said product combination comprises or encodes a polypeptide comprises an amino acid sequence at least 80% identical to any of the amino acid sequence shown in SEQ ID NO: 1-3.

10. The product combination according to anyone of claims 1 to 9, wherein said one or more immunogen(s) comprise or encode one or more TB antigen(s), HPV antigen(s), HCV antigen(s) and/or HBV antigen(s).

11. The product combination according to claim 10, wherein said one or more Mtb antigens, are selected from the group consisting of ESAT-6 (Rv3875), CFP-10 (Rv3874), TB10.4 (Rv0288), Ag85A (Rv3804), Ag85B (Rv1886), Rv3619, Rv3620, RpfA, RpfB, RpfC, RpfD, RpfE, Rv0081, Rv0111, Rv0198, Rv0569, Rv1733c, Rv1735, Rv1737, Rv1806, Rv1807, Rv1813, Rv2005c, Rv2029c, Rv2032, Rv2626, Rv2627, Rv2628, Rv2660c, Rv3407 Rv3478, and Rv3812.

12. The product combination according to anyone of claims 1 to 11, wherein said polypeptide is fused to said one or more immunogen(s).

13. The product combination according to claim 12, wherein said product combination comprises or encodes a fusion of RpfB, RpfD Ag85B, TB10.4 and ESAT6; or a fusion of Ag85B, Rv2626, RpfB, RpfD and Rv1733.

14. The product combination according to claim 13, wherein said product combination comprises or encodes an amino acid sequence which exhibits at least 80% of identity with any of the amino acid sequence shown in SEQ ID NO: 4-6.

15. The product combination according to claim 14, wherein said product combination comprises a nucleic acid molecule comprising a nucleotide sequence which exhibits at least 80% of identity with the nucleotide sequence shown in any of SEQ ID NO: 7-11.

16. The product combination according to anyone of claims 1 to 15, wherein the nucleic acid molecules encoding said polypeptide and said immunogen(s) are carried out by a single vector.

17. The product combination according to anyone of claims 1 to 15, wherein the nucleic acid molecules encoding said polypeptide and said immunogen(s) are carried out by two or more vectors.

18. The product combination according to claim 16 to 17, wherein said vector is a plasmid or a viral vector obtained viruses selected from the group consisting from the group consisting of retrovirus, adenovirus, adenovirus-associated virus (AAV), poxvirus, herpes virus, measles virus, foamy virus, alphavirus and vesicular stomatis virus.

19. A method for recombinant production of said polypeptide comprised or encoded by the product combination according to anyone of claims 16 to 17 comprising the steps of (i) introducing a vector as defined in anyone of claims 16 to 18 into a suitable host cell to produce a transfected or infected host cell, (ii) culturing *in-vitro* said transfected or infected host cell under conditions suitable for growth of the host cell, (iii) recovering the cell culture, and (iv) optionally, purifying the produced polypeptide or product combination from the recovered cell and/or culture supernatant.

20. The method according to claim 19, wherein said host cell is an *E coli* host cell and in particular a *E. coli* strain carrying the D13 prophage in its genome for allowing inducible expression of T7 polymerase by lactose or analogue of lactose.

21. A vector for expression of a Rpf-containing polypeptide as defined in anyone of claims 2 to 14.

22. A composition comprising the product combination according to anyone of claims 1 to 18, or produced according to claim 19 or 20 or or the polypeptide as defined in anyone of claims 2 to 14, the nucleic acid molecule as defined in claim 15, the vector as defined in anyone of claims 16 to 18 and 21 or any combination thereof.

23. The composition according to claim 22, wherein said composition further comprises one or more pharmaceutically acceptable vehicle(s).

24. The composition according to claim 22 or 23, wherein said composition is suitable for intramuscular, intradermal or subcutaneous route.

25. The polypeptide as defined in anyone of claims 2 to 14 or produced according to claim 19 or 20, the nucleic acid molecule as defined in claim 15, the vector as defined in anyone of claims 16 to 18 and 21 or the composition according to anyone of claims 22 to 23 for use as an adjuvant in combination with one or more immunogen(s).

26. The polypeptide, nucleotide sequence, vector or composition for use as an adjuvant according to claim 25, wherein said polypeptide, nucleotide sequence, vector or composition and said immunogen(s) are administered together or separately.

27. A method for preventing or delaying the risk of a disease, wherein said method comprises the step of administering to a subject in need thereof a therapeutically effective amount of the product combination according to anyone of claims 1 to 18, the composition according to anyone of claims 22 to 24 or the polypeptide, nucleotide sequence, vector or composition for use according to claim 25 or 26, so as to enhance the anti-immunogen immune response, thereby delaying or reducing the risk of development of said disease condition.

28. A method for treating a disease condition, wherein said method comprises the step of administering to the subject having developed such a disease, a therapeutically effective amount of the product combination according to anyone of claims 1 to 18, the composition according to anyone of claims 22 to 24 or the polypeptide, nucleotide sequence, vector or composition for use according to claim 25 or 26, thereby reducing clinical signs and/or symptoms associated with such a disease.

29. A method for stimulating an anti-immunogen response upon administration of the product combination according to anyone of claims 1 to 18, the composition according to anyone of claims 22 to 24 or the polypeptide, nucleotide sequence, vector or composition for use according to claim 25 or 26.

30. The method according to claim 29, wherein said stimulated immunity is humoral and/or cellular.

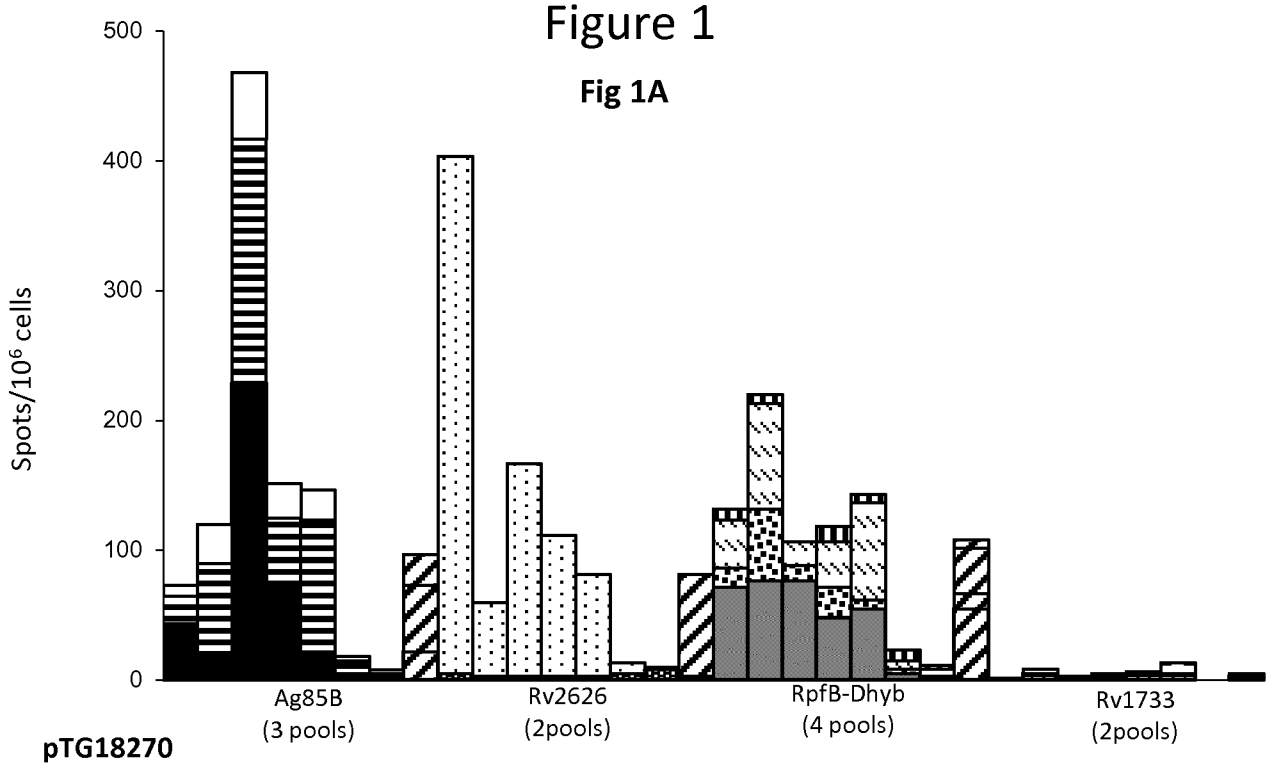
31. The method according to claim 30, wherein said stimulated immunity is specific or non specific, Th1 and/or Th2, CD4+ and/or CD8+ T cell mediated.

32. The method according to anyone of claims 27 to 31, wherein said polypeptide or or encoding nucleic acid molecule and said immunogen are administered separately.

33. The method according to claim 32, wherein said administration is by intramuscular, intradermal or subcutaneous route.

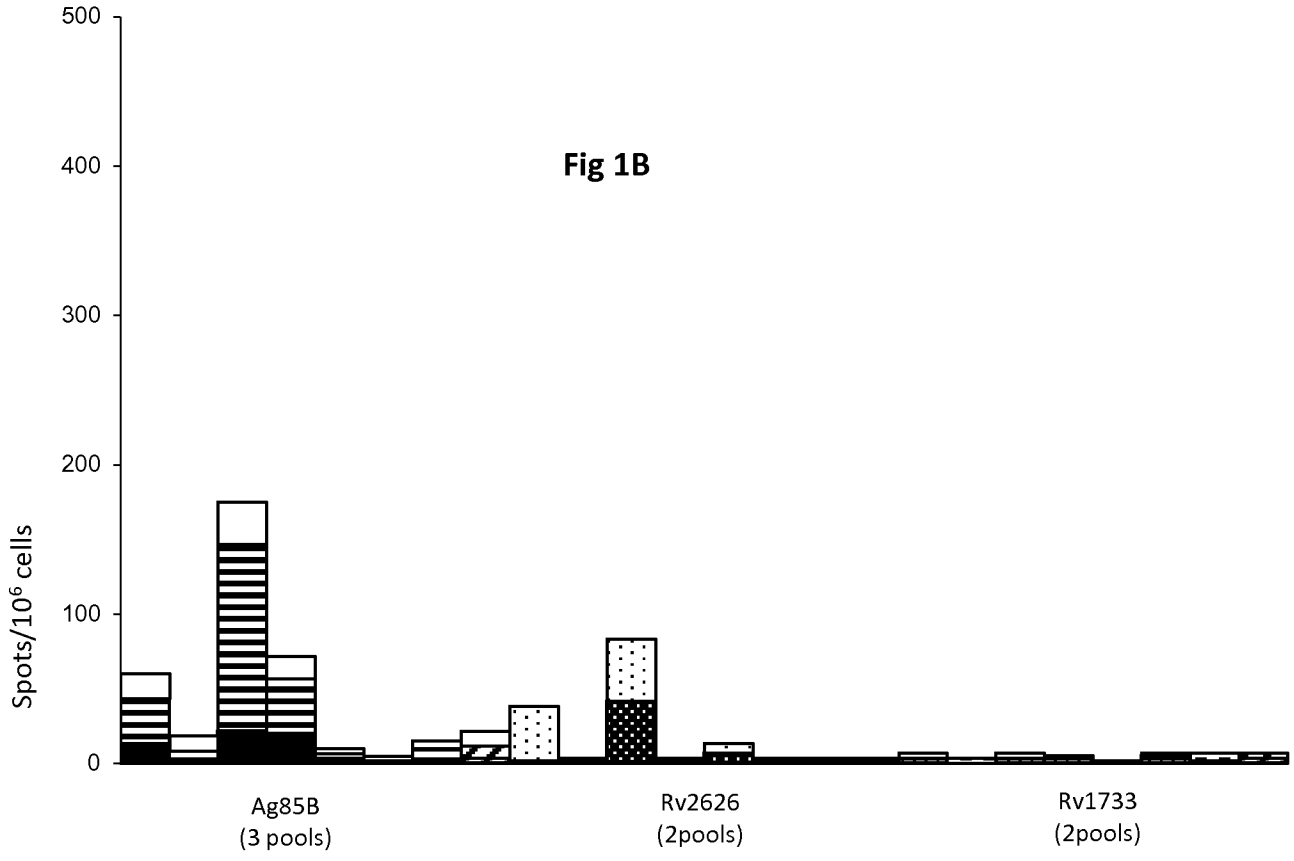
Figure 1

Fig 1A



pTG18270

Fig 1B



pTG18272

Figure 2

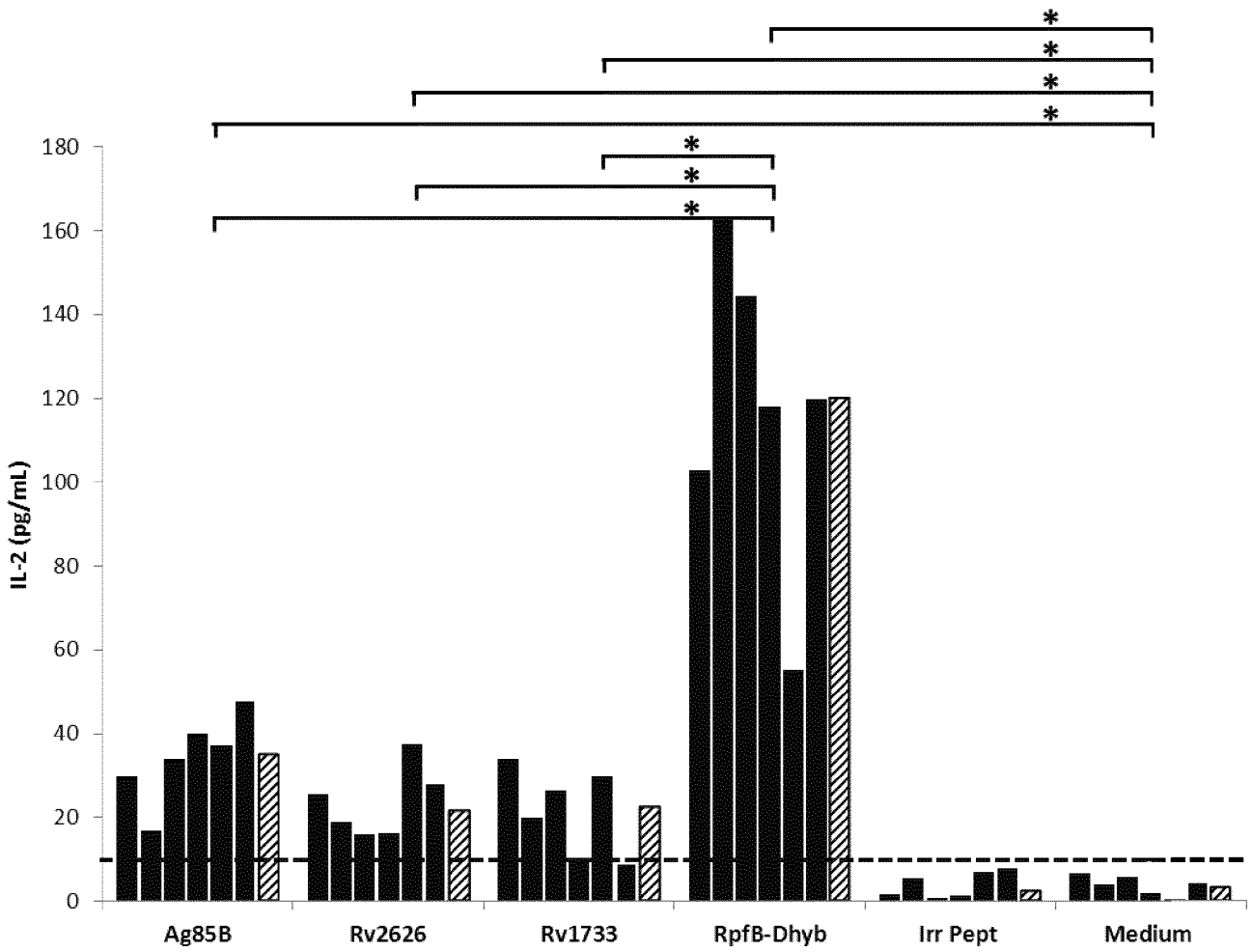
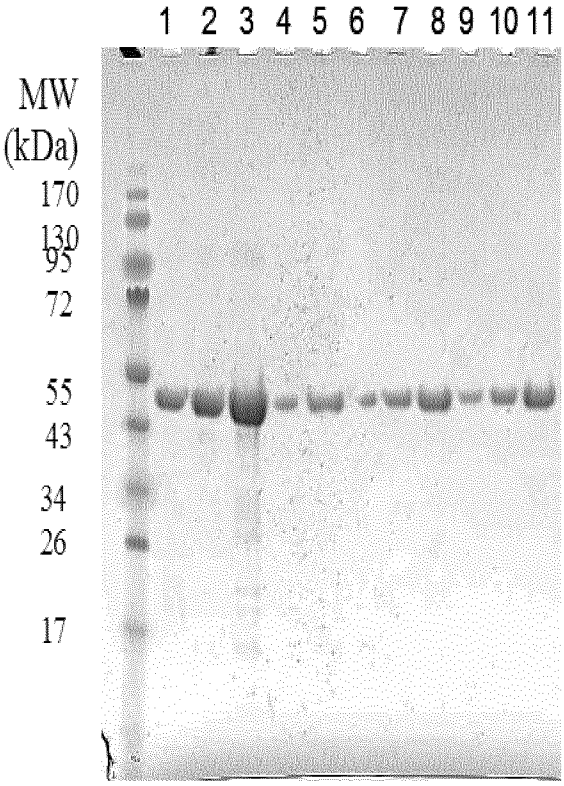

IL-2 (pg/mL)
0
20
40
60
80
100
120
140
160
180
Ag85B
Rv2626
Rv1733
RpfB-Dhyb
Irr Pept
Medium
*
*
*
*
*
*
*

Figure 3



Lanes 1 to 8: intermediate fractions of purification
Lanes 9 to 11: final pool 5, 10 and 15 µL

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/064617

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/16 A61K39/04 A61K39/39
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 201172 Thomson Scientific, London, GB; AN 2011-N36995 XP002713794, -& CN 102 190 733 A (UNIV LANZHOU) 21 September 2011 (2011-09-21)	1,3,4, 10-12, 16,19, 21-24, 27,29-31
Y	abstract	1-5, 7-12, 16-33

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

[X] See patent family annex.

* Special categories of cited documents :

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2013	23/10/2013

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

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/144951 A1 (HEALTH PROT AGENCY [GB]; CARROLL MILES [GB]; HALL YPER [GB]; WILLIAMS) 24 November 2011 (2011-11-24)	1-5, 8-12,16, 17,19, 21-24, 27-33
Y	page 156 - page 157; claims; examples page 75, paragraph 6 - page 76, paragraph 1	1-5, 7-12, 16-33

A	ROMANO M ET AL: "Potential of Mycobacterium tuberculosis resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines", MICROBES AND INFECTION, vol. 14, no. 1, January 2012 (2012-01), pages 86-95, XP055080935, ELSEVIER MASSON SAS FRA ISSN: 1286-4579 cited in the application abstract; figures	1-33

A	YEREMEEV VLADIMIR V ET AL: "Proteins of the Rpf family: Immune cell reactivity and vaccination efficacy against tuberculosis in mice.", INFECTION AND IMMUNITY, vol. 71, no. 8, August 2003 (2003-08), pages 4789-4794, XP055080939, ISSN: 0019-9567 cited in the application abstract; figures	1-33

A	BAVESH D. KANA ET AL: "The resuscitation-promoting factors of Mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro", MOLECULAR MICROBIOLOGY, vol. 67, no. 3, 1 February 2008 (2008-02-01), pages 672-684, XP055081375, ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2007.06078.x the whole document	1-33

T	XIN QI ET AL: "Subunit Vaccine Consisting of Multi-Stage Antigens Has High Protective Efficacy against Mycobacterium tuberculosis Infection in Mice.", PLOS ONE, vol. 8, no. 8, E72745, 2013, pages 1-12, XP002713795, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0072745	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/064617

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 102190733 A	21-09-2011	NONE	
WO 2011144951 A1	24-11-2011	CA 2800139 A1	24-11-2011
		CN 102905724 A	30-01-2013
		EP 2571518 A1	27-03-2013
		US 2013142800 A1	06-06-2013
		WO 2011144951 A1	24-11-2011