

TITLE

Multivalent vaccines comprising recombinant viral vectors

FIELD OF THE INVENTION

5 The invention relates to the field of recombinant DNA and viral vector vaccines. Specifically, it relates to recombinant DNA and viral vectors harbouring nucleic acids encoding multiple antigens and/or adjuvants.

10 BACKGROUND OF THE INVENTION

 Tuberculosis (TB) has been a major worldwide threat to human health for several thousands of years. TB caused by *Mycobacterium tuberculosis* is an infectious disease of the lung caused by infection through exposure to air-borne *M.*
15 *tuberculosis* bacilli. These bacilli are extremely infectious and it has been estimated that currently approximately one-third of the world population (2 billion people) are infected. It has been further estimated that TB kills over 2 million people worldwide on an annual basis. Only 5 to 10%
20 of the immunocompetent humans are susceptible to TB, and over 85% of them will develop the disease exclusively in the lungs, while HIV-infected humans may also develop systemic diseases that will more easily lead to death.

 Approximately 90% of *M. tuberculosis*-infected humans
25 will not develop the disease. However, in these latently infected individuals the bacilli can survive for many years and become reactivated for instance in the case of a weakened immune system, such as after an HIV infection. Due to the latent nature, infected individuals generally have to
30 be treated by administration of several antibiotics for up to 12 months, which is not a very attractive treatment in general and due to costs and the possible occurrence of

multi-drug resistance, while it is also not a very effective treatment in most developing countries.

One relatively successful TB vaccine has been developed: the bacilli Calmette-Guerin (BCG) vaccine was generated in the early years of the twentieth century, and was first given to individuals in 1921. The BCG vaccine is an attenuated strain of bacteria based on a *Mycobacterium bovis* isolate obtained from a cow. It is a relatively safe vaccine, which is easy and rather cheaply produced. In the year 2000, BCG vaccination covered 86% of the world population. However, the vaccine appears to be not extremely effective for adult pulmonary TB and many regions in developing countries still have very high rates of TB, despite the BCG-vaccine programs. It has been estimated that BCG vaccine prevents only 5% of all vaccine-preventable deaths by TB (Kaufmann, 2000).

Due to the rather low protection rate of the BCG vaccine in general and due to the specific protection in respect to childhood and disseminated TB, more efforts were put in the development of new, more broadly applicable, vaccines against TB, based on other systems and knowledge acquired in other fields such as vaccination against other tropical infectious diseases and HIV (review by Wang and Xing, 2002).

Different approaches were taken to develop new TB vaccines, ranging from subunit vaccines and DNA vaccines to modified mycobacterium strains. Moreover, also recombinant viral-based vaccines were generated, enabling the transfer of *M. tuberculosis* antigens to antigen-presenting cells through gene delivery vehicles such as Modified Vaccinia Ankara (MVA) vectors and replication-defective adenovirus vectors.

Naked DNA vaccines against TB have been described in WO 96/15241 (see also EP 0792358), whereas many reports describe the use of numerous antigens from *Mycobacterium tuberculosis* in either recombinant or purified form for their application in vaccines: WO 95/01441, WO 95/14713, WO 96/37219, US 6,599,510, WO 98/31388, WO 98/44119, WO 99/04005, WO 99/24577, WO 00/21983, WO 01/04151, WO 01/79274, WO 2004/006952, US 2002/0150592. The use of fusion proteins comprising different TB antigens has also been suggested: See WO 98/44119, EP 0972045 and EP 1449922, disclosing the use of a fusion polypeptide between ESAT-6 and MPT59 (MPT59 is also referred to as Ag85B or the 85B antigen).

Despite all these and other efforts in generating a vaccine against tuberculosis that ensures both a strong cellular, a strong humoral response as well as a long-lasting high protection rate, no such vaccine is yet available.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1: Map of pAdApt35Bsu.myc
Figure 2: Map of pAdApt35Bsu.TB.LM
Figure 3: Map of pAdApt35Bsu.TB.SM
Figure 4: Map of pAdApt35Bsu.TB.FLM
Figure 5: Map of pAdApt35Bsu.TB.3M
Figure 6: Map of pAdApt35Bsu.TB.4M
Figure 7: Map of pAdApt35Bsu.TB.5M
Figure 8: Map of pAdApt35Bsu.TB.6M
Figure 9: Map of pAdApt35Bsu.TB.7M
Figure 10. Western blot with anti-TB antigen polyclonal on lysates from A549 cells infected with Ad35 viruses comprising nucleic acids encoding different sets of TB

antigens with the myc-tag (A) and without the myc-tag (B).
(C) similar to (B), with molecular weight markers. See for
notation Table I.

Figure 11. Experimental design of immunization protocol

5 using 7 different adenoviral vectors (DNA) harbouring
different sets of nucleic acids encoding tuberculosis
antigens.

Figure 12. Percentages of antigen-specific splenocytes that
stain positive for interferon-gamma production (IFN γ +) upon
10 stimulation with no peptide (A: CD4+ cells, B: CD8+ cells).

Figure 13. Percentages of antigen-specific splenocytes that
stain positive for interferon-gamma production (IFN γ +) upon
stimulation with a pool of peptides relevant for the Ag85A
antigen (A: CD4+ cells, B: CD8+ cells).

15 Figure 14. Percentages of antigen-specific splenocytes that
stain positive for interferon-gamma production (IFN γ +) upon
stimulation with a pool of peptides relevant for the Ag85B
antigen (A: CD4+ cells, B: CD8+ cells).

Figure 15. Percentages of antigen-specific splenocytes that
20 stain positive for interferon-gamma production (IFN γ +) upon
stimulation with a pool of peptides relevant for the TB10.4
antigen (A: CD4+ cells, B: CD8+ cells).

Figure 16. Overview of percentages of CD4+ and CD8+
splenocytes that stain positive in ICN, in sera obtained
25 from mice injected with the triple inserts TB-L (A) and TB-
S(B).

Figure 17. Dose response effect using different doses of TB-
S comprising a nucleic acid encoding Ag85A, Ag85B and Tb10.4
antigens. CD4 response towards Ag85A (A), Ag85B (C) and
30 TB10.4 (E). CD8 response towards Ag85A (B), Ag85B (D) and
TB10.4 (F). (G): CD8 response towards Ag85B with the

adjusted peptide pool (see example 6): left graph, upon TB-L infection; right graph, upon TB-S infection.

Figure 18. CD4 and CD8 responses after priming with BCG and boosting with different Ad-TB vectors. CD4 response towards Ag85A (A), Ag85B (C) and TB10.4 (E). CD8 response towards Ag85A (B), Ag85B (D) and TB10.4 (F).

Figure 19. Nucleotide sequence of TB-LM

Figure 20. Nucleotide sequence of TB-SM

Figure 21. Nucleotide sequence of TB-FLM

10 Figure 22. Amino acid sequence of TB-LM

Figure 23. Amino acid sequence of TB-SM

Figure 24. Amino acid sequence of TB-FLM

Figure 25. Ag85A stimulation in a BCG prime/Ad35-TB boost experiment with a long-term read-out. Upper panel: CD4 response, lower panel: CD8 response. Ad35.E = empty Ad35 virus.

Figure 26. Ag85B stimulation in a BCG prime/Ad35-TB boost experiment with a long-term read-out. Upper panel: CD4 response, lower panel: CD8 response. Ad35.E = empty Ad35 virus.

Figure 27. TB10.4 stimulation in a BCG prime/Ad35-TB boost experiment with a long-term read-out. Upper panel: CD4 response, lower panel: CD8 response. Ad35.E = empty Ad35 virus.

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SUMMARY OF THE INVENTION

The present invention relates to recombinant viral vectors, preferably replication defective adenoviruses, more preferably recombinant human adenovirus serotypes Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50, wherein the viral vectors comprise a heterologous nucleic acid sequence encoding for (fusion) polypeptides of at least two antigens

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from one or more tuberculosis causing bacilli. The encoded antigens may be directly linked, i.e. forming one single polypeptide. In one preferred embodiment, the antigens are present in a precursor polypeptide, in the sense that they
5 are connected via a linker sequence recognized by a specific protease that is co-expressed. The heterologous nucleic acid may comprise the gene encoding the protease. The fusion proteins with the direct linkages elicit desired immune responses due to the antigens present in the fusion product,
10 whereas the proteins comprising the protease sites are cleaved into separate discrete antigen forms, each contributing to the desired immune response. The protease is preferably linked to the antigens by a protease recognition-site recognized by a cellular protease. Both set-ups provide
15 additional or even synergistic effects in comparison to vaccination or therapy in which viral vectors are used that comprise only a single transgene-encoding unit. More generally, the invention also relates to viral vectors comprising a heterologous nucleic acid sequence encoding
20 multiple antigens separated by protease specific cleavage sites. It is to be understood that such antigens may be from a wide variety of sources including, but not limited to, infectious agents such as viruses, bacteria and parasites, and are thus according to this aspect of the invention not
25 limited to antigens from tuberculosis-causing bacilli. The antigens from *Tuberculosis mycobacterium* serve as non-limiting examples of how such multivalent viral vector vaccines are generated and how, upon entry into the host cell, the antigens are separated, and they are able to
30 contribute to the immune response.

The invention also relates to the use of genetic adjuvants that are co-expressed from the viral vector. These

adjuvants are encoded by a nucleic acid, which is part of the heterologous nucleic acid sequence introduced into the viral vector genome. The adjuvant is expressed together with the specific antigen(s) and is thereby able to stimulate the immune response towards the antigen(s). Clearly, the sequence encoding the adjuvant may be linked directly to the sequence encoding the antigen(s), but is preferably separated from the sequence encoding the one or more antigen(s) by the linker sequence encoding the protease recognition site. In the latter case, the adjuvant is present in the host separately from the antigen(s) and is able to provide its immune-stimulatory effects along with the antigen(s).

DETAILED DESCRIPTION

The present invention relates to multivalent vaccines comprising a recombinant viral vector. A preferred viral vector is a recombinant Adenovirus (Ad) vector. The recombinant adenoviral vector according to the invention comprises a heterologous nucleic acid sequence encoding at least two different antigens. The antigens may be within a single polypeptide. These determinants may be either antigens from viral, bacterial and parasitic pathogens, or host antigens, such as, but not limited to, autoimmune antigens or tumor antigens. In a preferred embodiment, the antigens are from tuberculosis (TB)-causing bacilli, more preferable from *Mycobacterium tuberculosis*, *M. africanum* or *M. bovis* or from a combination thereof. The antigens may be the full-length native protein, chimeric fusions between the antigen and a host protein or mimetic, a fragment or fragments thereof of an antigen that originates from the pathogen, or other mutants that still elicit a desired immune response. Genes

encoding TB antigens that may typically be used in the viral vectors of the present invention include, but are not limited to: Ag85A (MPT44), Ag85B (MPT59), Ag85C (MPT45), TB10.4 (CFP7), ESAT-6, CFP7A, CFP7B, CFP8A, CFP8B, CFP9, CFP10, 5 CFP10A, CFP11, CFP16, CFP17, CFP19, CFP19A, CFP19B, CFP20, CFP21, CFP22, CFP22A, CFP23, CFP23A, CFP23B, CFP25, CFP25A, CFP26 (MPT51) CFP27, CFP28, CFP29, CFP30A, CFP30B, CWP32, CFP50, MPT63, MTC28, LHP, MPB59, MPB64, MPT64, TB15, TB18, TB21, TB33, TB38, TB54, TB12.5, TB20.6, TB40.8, TB10C, TB15A, 10 TB17, TB24, TB27B, TB13A, TB64, TB11B, TB16, TB16A, TB32, TB32A, TB51, TB14, TB27, HBHA, GroEL, GroES (WO 95/01441, WO 98/44119, US 6,596,281, US 6,641,814, WO 99/04005, WO 00/21983, WO 99/24577), and the antigens disclosed in WO 92/14823, WO 95/14713, WO 96/37219, US 5,955,077, US 15 6,599,510, WO 98/31388, US 2002/0150592, WO 01/04151, WO 01/70991, WO 01/79274, WO 2004/006952, WO 97/09428, WO 97/09429, WO 98/16645, WO 98/16646, WO 98/53075, WO 98/53076, WO 99/42076, WO 99/42118, WO 99/51748, WO 00/39301, WO 00/55194, WO 01/23421, WO 01/24820, WO 20 01/25401, WO 01/62893, WO 01/98460, WO 02/098360, WO 03/070187, US 6,290,969, US 6,338,852, US 6,350,456, US 6,458,366, US 6,465,633, US 6,544,522, US 6,555,653, US 6,592,877, US 6,613,881, US 6,627,198. Antigen fusions that may be of particular use are those disclosed for the first 25 time herein (such as Ag85A-Ag85B-TB10.4 and combinations thereof), but also known fusions such as ESAT-6-MPT59 and MPT59-ESAT-6 disclosed in WO 98/44119 and in the above-referenced documents.

30 One approach for applying multiple antigens may be by having two or more separate expression cassettes present in a single vector, each cassette comprising a separate gene of

interest. This approach clearly has disadvantages, for instance related to space availability in the vector: separate cassettes generally comprise separate promoters and/or inducers and separate polyadenylation signal
5 sequences. Such cassettes typically require separate positions in the viral vector, resulting in more laborious cloning procedures, whereas a phenomenon known as 'promoter interference' or 'squenching' (limited availability of cellular factors required by the promoters to act) may
10 restrict the expression levels from the different promoters.

As exemplified by the recombinant viral vectors disclosed herein relating to fusions between multiple TB antigens, one is now able to make recombinant adenoviral vectors comprising several nucleic acids encoding more than
15 one antigen, which viral vector elicits a strong immune response, whereas the use of single inserts elicit limited effects. Clearly, these vectors encode recombinant genetic chimeras, which express the two or more antigens in a single cistronic mRNA, for example in the form of a fusion protein.
20 This approach can be effective when DNA vaccines or the viral vectors are being used to invoke T cell immunity to the passenger antigens. However, such fusion proteins may have additional drawbacks that cannot always be envisioned beforehand, as it was found that such fusions might skew
25 immunodominant patterns and do not always invoke immunity to all target antigens with equal potency, whereas a second and perhaps more significant drawback to expression of genetic fusions is that the individual components may not fold to a native conformation, due to the close presence of their
30 fusion partner or other reasons. As a result of this, genetic fusions may invoke antibody responses to nonsense

epitopes and such antibodies do not recognize native epitopes displayed by the founder pathogens and may be poor at combating infection.

The inventors of the present invention have now
5 developed a system wherein multiple antigens are encoded by a single heterologous nucleic acid sequence, wherein the expressed polyprotein is processed into the discrete antigenic polypeptides. Thus, in one embodiment, the present invention relates to viral vectors that enable the
10 expression of multiple antigens that are subsequently processed into the discrete antigens thus avoiding the possible limitations associated with genetic fusions, while also excluding the need for separate expression cassettes. Heretofore, no compositions or methods have been described
15 that enable precise processing of viral vector-expressed genetic fusions into discrete antigens. The expression of multiple antigens encoded by nucleic acids comprised in a DNA or viral vector, which antigens are subsequently processed into discrete antigens is demonstrated by the use
20 of a protease (PR), such as the viral protease encoded by Avian Leucosis Virus (ALV; referred to as PR-ALV herein). In ALV, ALV-PR forms the C-terminal domain of the gag protein, which is known to catalyse the processing of gag and gag-pol precursors, a critical step during ALV replication (reviewed
25 by Skalka. 1989).

A unique ALV-PR directed-processing system was created. A polyprotein containing ALV-PR and given antigens is expressed by DNA or viral vectors, in which ALV-PR preferably forms the N-terminus of a polyprotein followed by
30 antigen sequences that are linked with ALV-PR digestion sites. Two different cleavage sites are preferably used in

the system. One cleavage site (GSSGPWPAPEPPAVSLAMTMEHRDRPLV;
SEQ ID NO:22) is to release ALV-PR and the other cleavage
site (PPSKSKKGGAAAMSSAIQPLVMAVVRERDGTG; SEQ ID NO:21) is
recognized by ALV-PR and used to separate the other encoded
5 antigens in discrete polypeptides.

Alternatively, the PR and its cleavage sites may be
encoded by or based on other retroviruses such as Human
Immunodeficiency Virus (HIV), murine leukaemia virus, Simian
Immunodeficiency Virus (SIV) and Rous Sarcoma Virus.

10 According to a preferred embodiment, the invention
discloses recombinant viral vectors comprising nucleic acid
sequences encoding multiple antigens from *Mycobacterium*
tuberculosis, wherein the different nucleic acid sequences
are separated from each other by sequences encoding the ALV
15 protease recognition site. In this the discrete TB antigens
are produced as a polyprotein and subsequently processed
such that they are cleaved into discrete antigenic
polypeptides, each contributing to the immune response. It
is to be understood that the ALV protease system is not to
20 be limited to the use of TB-specific antigens. A person
skilled in the art will appreciate the possibility that the
system has for applying other antigens, different from or in
combination with TB antigens, and its applicability in other
therapeutic settings such as gene therapy and tumor
25 vaccination.

Preferably, the viral vector comprising the multiple
antigen-encoding sequences separated by protease sites is an
adenoviral vector. The viral vector may be the viral
particle itself, whereas the term viral vector also refers
30 to the nucleic acid encoding the viral particle. The
adenoviral vector is preferably a recombinant vector based

on, or derived from, an adenovirus species or serotype that encounters neutralizing activity in a low percentage of the target population. Such adenoviruses are also sometimes referred to as 'rare' adenoviruses as they generally do not
5 regularly circulate within the human population. Preferred serotypes are therefore Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50.

As used herein, "antigen" means a protein or fragment thereof, which when expressed in an animal or human cell or
10 tissue is capable of triggering an immune response. Examples include but are not limited to, viral proteins, bacterial proteins, parasite proteins, cytokines, chemokines, immunoregulatory agents, and therapeutic agents. The antigen may be a wild type protein, a truncated form of that protein,
15 a mutated form of that protein or any other variant of that protein, in each case capable of contributing to immune responses upon expression in the animal or human host to be vaccinated. It is to be understood that when antigens are directly fused, this fusion is the result of recombinant
20 molecular biology; thus, a direct fusion of two antigens as used herein does not refer to two antigenic parts of a single wild type protein as it occurs in nature. For the sake of clarity, when two antigenic parts of a single wild type protein (which two parts are normally directly linked within
25 the protein) are linked via linkers as disclosed herein (such as through the ALV protease site, as discussed below), such fusion is part of the present invention. In preferred embodiments, the present invention relates to different proteins (having antigenic activity) that are either directly
30 linked or that are linked through one or more protease sites. In a more preferred embodiment, the gene encoding the

protease is linked to the protein(s) of interest, even more preferably through yet another protease site.

The different antigens are not necessarily from one pathogenic species. Combinations of different antigens from multiple species, wherein the different antigens are encoded by nucleic acid sequences within a single vector are also encompassed by the present invention.

A "host antigen" means a protein or part thereof that is present in the recipient animal cell or tissue, such as, but not limited to, a cellular protein, an immunoregulatory agent, or a therapeutic agent.

The antigen may be encoded by a codon-optimized, synthetic gene and may be constructed using conventional recombinant DNA methods.

As mentioned, the antigen that is expressed by the recombinant viral vector comprising the ALV protease system can be any molecule that is expressed by any viral, bacterial, or parasitic pathogen prior to or during entry into, colonization of, or replication in their animal host. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

The viral pathogens, from which the viral antigens are derived, include, but are not limited to: Orthomyxoviruses, such as influenza virus; Retroviruses, such as RSV, HTLV-1, and HTLV-II, Herpesviruses such as EBV; CMV or herpes simplex virus; Lentiviruses, such as HIV-1 and HIV-2; Rhabdoviruses, such as rabies virus; Picornaviruses, such as Poliovirus; Poxviruses, such as vaccinia virus; Rotavirus; and Parvoviruses, such as Adeno-Associated Viruses (AAV).

Examples of viral antigens can be found in the group including but not limited to the Human Immunodeficiency Virus (HIV) antigens Rev, Pol, Nef, Gag, Env, Tat, mutant derivatives of Tat, such as Tat-Δ31-45, T- and B-cell epitopes of gp120, chimeric derivatives of HIV-1 Env and gp120, such as a fusion between gp120 and CD4, a truncated or modified HIV-1 Env, such as gp140 or derivatives of HIV-1 Env and/or gp140. Other examples are the hepatitis B surface antigen, rotavirus antigens, such as VP4 and VP7, influenza virus antigens such as hemagglutinin, neuraminidase, or nucleoprotein, and herpes simplex virus antigens such as thymidine kinase.

Examples of bacterial pathogens, from which the bacterial antigens may be derived, include but are not limited to, *Mycobacterium spp.*, *Helicobacter pylori*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Rickettsia spp.*, *Listeria spp.*, *Legionella pneumoniae*, *Fansicella spp.*, *Pseudomonas spp.*, *Vibrio spp.*, and *Borellia burgdorferi*.

Examples of protective antigens of bacterial pathogens include the somatic antigens of enterotoxigenic *E. coli*, such as the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin; pertactin of *Bordetella pertussis*, adenylate cyclase-hemolysin of *B. pertussis*, fragment C of tetanus toxin of *Clostridium tetani*, OspA of *Borellia burgdorferi*, protective paracrystalline-surface-layer proteins of *Rickettsia prowazekii* and *Rickettsia typhi*, the listeriolysin (also known as "Llo" and "Hly") and/or the superoxide dismutase (also know as "SOD" and "p60") of *Listeria monocytogenes*, urease of *Helicobacter pylori*, and the receptor-binding domain of lethal toxin and/or the protective antigen of *Bacillus anthrax*.

The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to: *Plasmodium* spp. such as *Plasmodium falciparum*, *Trypanosome* spp. such as *Trypanosoma cruzi*, *Giardia* spp. such as *Giardia intestinalis*, *Boophilus* spp., *Babesia* spp. such as *Babesia microti*, *Entamoeba* spp. such as *Entamoeba histolytica*, *Eimeria* spp. such as *Eimeria maxima*, *Leishmania* spp., *Schistosoma* spp., *Brugia* spp., *Fasciola* spp., *Dirofilaria* spp., *Wuchereria* spp., and *Onchocerca* spp.

Examples of protective antigens of parasitic pathogens include the circumsporozoite (CS) or Liver Stage Specific (LSA) antigens LSA-1 and LSA-3 of *Plasmodium* spp. such as those of *P. bergerii* or *P. falciparum*, or immunogenic mutants thereof; the merozoite surface antigen of *Plasmodium* spp., the galactose specific lectin of *Entamoeba histolytica*, gp63 of *Leishmania* spp., gp46 of *Leishmania major*, paramyosin of *Brugia malayi*, the triose-phosphate isomerase of *Schistosoma mansoni*, the secreted globin-like protein of *Trichostrongylus colubriformis*, the glutathione-S-transferase of *Fasciola hepatica*, *Schistosoma bovis* and *S. japonicum*, and KLH of *Schistosoma bovis* and *S. japonicum*.

As mentioned earlier, the recombinant viral vectors comprising nucleic acids encoding the ALV or ALV-like protease may encode host antigens, which may be any cellular protein, immunoregulatory agent, or therapeutic agent, or parts thereof, that may be expressed in the recipient cell, including but not limited to tumor, transplantation, and autoimmune antigens, or fragments and derivatives of tumor, transplantation, and autoimmune antigens thereof. Thus, in the present invention, viral vectors may encode tumor, transplant, or autoimmune antigens, or parts or derivatives

thereof. Alternatively, the viral vectors may encode synthetic genes (made as described above), which encode tumor-specific, transplant, or autoimmune antigens or parts thereof. Examples of such antigens include, but are not
5 limited to, prostate specific antigen, MUC1, gp100, HER2, TAG-72, CEA, MAGE-1, tyrosinase, CD3, and IAS beta chain.

Clearly, the ALV protease site technology as disclosed herein is also applicable for gene therapy applications by introducing multiple polypeptides in a single polyprotein and
10 having the polyprotein processed into discrete polypeptides in hosts in need of these multiple (discrete) polypeptides.

As a means to further enhance the immunogenicity of the viral vectors, expression cassettes are constructed that encode at least one antigen and an adjuvant, and can be used
15 to increase host responses to the antigen expressed by said viral vectors. Such adjuvants are herein also referred to as 'genetic adjuvants' as genes encode the proteins that act as adjuvant. A preferred use is made of the protease and the linking protease sites as described above to have the
20 antigen cleaved from the adjuvant after translation, although in certain embodiments the adjuvant may also be directly linked to the antigen.

The particular adjuvant encoded by the viral vectors may be selected from a wide variety of genetic adjuvants. In a
25 preferred embodiment, the adjuvant is the A subunit of cholera toxin (CtxA; examples: GenBank accession no. X00171, AF175708, D30053, D30052), or functional parts and/or functional mutant derivatives thereof, such as the A1 domain of the A subunit of Ctx (CtxA1; GenBank accession no.
30 K02679). Alternatively, any bacterial toxin that is a member of the family of bacterial adenosine diphosphate-

ribosylating exotoxins may be used. Non-limiting examples are the A subunit of heat-labile toxin (EltA) of enterotoxigenic *E. coli*, and the pertussis toxin S1 subunit. Other examples are the adenylate cyclase-hemolysins such as
5 the *cyaA* genes of *Bordetella pertussis*, *B. bronchiseptica* or *B. parapertussis*. Alternatively, the particular ADP-ribosyltransferase toxin may be any derivative of the A subunit of cholera toxin (i.e. CtxA), or parts thereof (i.e. the A1 domain of the A subunit of Ctx (i.e. CtxA1), from any
10 classical *Vibrio cholerae* strain (e.g. strain 395) or El Tor *V. cholerae* (e.g. strain 2125) that display reduced ADP-ribosyltransferase catalytic activity but retain the structural integrity, including but not restricted to replacement of arginine-7 with lysine (R7K), serine-41 with
15 phenylalanine (S41F) serine-61 with lysine (S61K), serine-63 with lysine (S63K), valine-53 with aspartic acid (V53D), valine-97 with lysine (V97K) or tyrosine-104 with lysine (Y104K), or combinations thereof. Alternatively, the particular ADP-ribosyltransferase toxin may be any derivative
20 of cholera toxin that fully assemble, but are nontoxic proteins due to mutations in the catalytic-site, or adjacent to the catalytic site, respectively. Such mutants are made by conventional site-directed mutagenesis procedures, as described below.

25 In another embodiment, the ADP-ribosyltransferase toxin is any derivative of the A subunit of heat-labile toxin (LtxA) of enterotoxigenic *Escherichia coli* isolated from any enterotoxigenic *E. coli*, including but not restricted to *E. coli* strain H10407 that display reduced ADP-
30 ribosyltransferase catalytic activity but retain the structural integrity, including but not restricted to R7K,

S41F, S61K, S63K, V53D, V97K or Y104K, or combinations thereof. Alternatively, the particular ADP-ribosyltransferase toxin may be any fully assembled derivative of cholera toxin that is nontoxic due to
5 mutations in, or adjacent to, the catalytic site. Such mutants are made by conventional site-directed mutagenesis procedures, as described below.

Although ADP-ribosylating toxins are potent adjuvants, the adjuvant encoded by the viral vectors in the present
10 invention may also be any bioactive protein from viral, bacterial or protozoan organisms, immunoregulatory DNA, double stranded RNA or small inhibitory RNA (herein referred to as siRNA). The particular bioactive protein can be selected from but not restricted to the following classes:

15 Class 1. This class of adjuvants induce apoptosis by inhibiting Rho, a host small GTPase. Inhibition of Rho has been clearly associated with the induction of apoptosis. Induction of apoptosis is useful method to drive bystander T cell responses and a potent method for the induction of
20 CTLs. This strategy has not been evaluated in any experimental system thus far. The active domain of Sope is contained in amino acids 78-240 and it only requires a 486 bp gene for expression. The catalytic domain of *E. coli* CNF-1 is likely to possess similar properties.

25 Class 2. Bacterial porins have been shown to possess immune-modulating activity. These hydrophobic homotrimeric proteins form pores that allow passage of molecules of Mr < 600 Da through membranes. Examples of porins include the OmpF, OmpC and OmpD proteins of the *Enterobacteriaceae*.

30 Class 3. Double stranded RNA (dsRNA) activates host cells, including dendritic cells. Expression of an mRNA that

encodes an inverted repeat spaced by an intro or ribozyme will result in the expression of dsRNA.

Class 4. The peptide motive [WYF]xx[QD]xx[WYF] is known to induce CD1d-restricted NK T cell responses (Kronenberg and Gapin, 2002). Expression of a peptide with this motif fused to a T4 fibritin coiled-coiled motif will produce trimeric peptide that will cross-link TCRs on CD1d-restricted T cells, thereby activated innate host responses.

Class 5. siRNA can be used to target host mRNA molecules that suppress immune responses (e.g. kir), regulate immune responses (e.g. B7.2) or prevent cross presentation (e.g. Rho).

Class 6. siRNA's can be used for vaccines that target costimulatory molecules, such as CD80 and CD86. Inhibition of these molecules will prevent co-stimulation thereby resulting in T cell anergy.

Surprisingly, as disclosed herein, it has been found that antigens from TB-causing bacilli, such as the TB10.4 protein may not only act as an antigen in itself, but even act as an adjuvant towards other TB antigens, for instance Ag85A: In the case where a triple insert was present (Ag85A-Ag85B-TB10.4) it was surprisingly found that the presence of TB10.4 in this construct stimulated the immune response towards Ag85A, whereas the absence of TB10.4 revealed a minor effect towards CD8+ splenocytes (see example 4 and figure 13B). The adjuvant effect of TB10.4 was further investigated and it was found that TB10.4 indeed stimulated the activation of CD8 cells directed against Ag85A when present in a triple construct, whereas an infection with separate vectors each encoding the separate antigens did not result in such stimulation, strongly suggesting that the

TB10.4 antigen should be present, either in the same vector, or present within the same translated product.

The present invention also relates to viral vectors
5 that encode at least one antigen and a cytokine fused by a protease recognition site. Such vectors are used to increase host responses to the passenger antigen(s) expressed by said viral vectors. Examples of cytokines encoded by the viral vectors are interleukin-4 (IL-4), IL-5, IL-6, IL-10, IL-
10 12_{p40}, IL-12_{p70}, TGF β , and TNF α .

Recombinant DNA and RNA procedures for the introduction of functional expression cassettes to generate viral vectors capable of expressing an immunoregulatory agent in eukaryotic cells or tissues are known in the art.

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Herein, compositions and methods are described for the construction of viral vectors that express more than one antigen from a TB-causing bacillus, preferably *Mycobacterium tuberculosis*, *M. africanum* and/or *M. bovis*. Preferably, the
20 viral vector is a replication-defective recombinant adenoviral vector. One extensively studied and generally applied adenovirus serotype is adenovirus 5 (Ad5). The existence of anti-Ad5 immunity has been shown to suppress substantially the immunogenicity of Ad5-based vaccines in
25 studies in mice and rhesus monkeys. Early data from phase-1 clinical trials show that this problem may also occur in humans.

One promising strategy to circumvent the existence of pre-existing immunity in individuals previously infected
30 with the most common human adenoviruses (such as Ad5), involves the development of recombinant vectors from

adenovirus serotypes that do not encounter such pre-existing immunities. Human adenoviral vectors that were identified to be particularly useful are based on serotypes 11, 26, 34, 35, 48, 49, and 50 as was shown in WO 00/70071, WO 02/40665 and WO 2004/037294 (see also Vogels et al. 2003). Others have found that also adenovirus 24 (Ad24) is of particular interest as it is shown to be a rare serotype (WO 2004/083418). In a preferred embodiment said viral vector is thus an adenovirus derived from a serotype selected from the group consisting of: Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50. The advantage of this selection of human adenoviruses as vaccine vectors lies clearly in the fact that humans are not regularly infected with these wild type adenoviruses. As a consequence, neutralizing antibodies against these serotypes is less prevalent in the human population at large. This is in contrast to serotype 5, because humans are quite regularly infected with this wild type serotype. The immune responses raised during an infection with a parental wild-type serotype can negatively impact the efficacy of the recombinant adenovirus serotype when used as a subsequent recombinant vaccine vector, such as a vaccine against malaria in which adenoviruses are applied. The spread of the different adenovirus serotypes in the human worldwide population differs from one geographic area to the other. Generally, the preferred serotypes encounter a low neutralizing activity in hosts in most parts of the world, as outlined in WO 00/70071. In another preferred embodiment, the adenovirus is a simian, canine or a bovine adenovirus, since these viruses also do not encounter pre-existing immunity in the (human) host to which the recombinant virus is to be administered. The applicability of simian adenoviruses for use in human gene

therapy or vaccines is well appreciated by those of ordinary skill in the art. Besides this, canine and bovine adenoviruses were found to infect human cells in vitro and are therefore also applicable for human use. Particularly
5 preferred simian adenoviruses are those isolated from chimpanzee. Examples that are suitable include C68 (also known as Pan 9; US 6,083,716) and Pan 5, 6 and 7 (WO 03/046124); See also WO 03/000851.

Thus, choice of the recombinant vector is influenced by
10 those that encounter neutralizing activity in a low percentage of the human population in need of the vaccination. The advantages of the present invention are multi-fold. Recombinant viruses, such as recombinant adenoviruses, can be produced to very high titers using
15 cells that are considered safe, and that can grow in suspension to very high volumes, using medium that does not contain any animal- or human derived components. Also, it is known that recombinant adenoviruses elicit a dramatic immune response against the protein encoded by the heterologous
20 nucleic acid sequence in the adenoviral genome.

The inventors of the present invention realized that a vaccine comprising multiple antigens would provide a stronger and broader immune response towards the TB-causing bacillus. Moreover, despite the fact that a single antigen
25 could by itself induce protection in inbred strains of mice, a cocktail comprising several antigens is conceivably a better vaccine for applications in humans as it is less likely to suffer from MHC related unresponsiveness in a heterogeneous population.

30 However, from a practical standpoint of vaccine development, a vaccine consisting of multiple constructs would be very expensive to manufacture and formulate. In

addition to simplifying the manufacturing process, a single construct may ensure equivalent uptake of the components by antigen presenting cells and in turn, generate an immune response that is broadly specific.

5 In one particular aspect of the invention the replication-defective recombinant viral vector comprises a nucleic acid sequence coding for an antigenic determinant wherein said heterologous nucleic acid sequence is codon-optimized for elevated expression in a mammal, preferably a
10 human. Codon-optimization is based on the required amino acid content, the general optimal codon usage in the mammal of interest and a number of aspects that should be avoided to ensure proper expression. Such aspects may be splice donor or -acceptor sites, stop codons, Chi-sites, poly(A)
15 stretches, GC- and AT-rich sequences, internal TATA boxes, etcetera. Methods of codon optimization for mammalian hosts are well known to the skilled person and can be found in several places in molecular biology literature.

 In a preferred embodiment, the invention relates to a
20 replication-defective recombinant adenoviral vector according to the invention, wherein the adenine plus thymine content in said heterologous nucleic acid, as compared to the cytosine plus guanine content, is less than 87%, preferably less than 80%, more preferably less than 59% and
25 most preferably equal to approximately 45%.

 The production of recombinant adenoviral vectors harboring heterologous genes is well-known in the art and typically involves the use of a packaging cell line, adapter constructs and cosmids and deletion of at least a functional
30 part of the E1 region from the adenoviral genome (see also below for packaging systems and preferred cell lines).

The vaccines of the present invention are typically held in pharmaceutically acceptable carriers or excipients. Pharmaceutically acceptable carriers or excipients are well known in the art and used extensively in a wide range of therapeutic products. Preferably, carriers are applied that work well in vaccines. More preferably, the vaccines further comprise an adjuvant. Adjuvants are known in the art to further increase the immune response to an applied antigenic determinant.

10 The invention also relates to the use of a kit according to the invention in the therapeutic, prophylactic or diagnostic treatment of TB.

 The recombinant viral vectors comprising TB antigens of the present invention may be used in vaccination settings in which they are applied in combination with BCG. They may also be applied as a priming agent or a boosting agent, respectively preceding or following a BCG vaccination to increase the desired immune responses. It can also be envisioned that different viral vectors as disclosed herein are used in prime-boost setups, wherein one vector is followed by another. Moreover, vectors comprising directly linked antigens may be combined as such with vectors comprising the protease-site linked antigens. Prime-boost settings using one adenovirus serotype as a prime and another serotype as a boost (selected from the preferred human, simian, canine or bovine adenoviruses) are also envisioned. The viral vectors according to the invention may also be used in combination with vaccines comprising purified (recombinantly produced) antigens and/or with vaccines comprising naked DNA or RNA encoding similar or the same antigens.

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Thus, the invention relates to a recombinant replication-defective adenovirus comprising a nucleic acid sequence encoding two or more antigens from at least one tuberculosis (TB)-causing bacillus. It is to be understood
5 that a polypeptide may comprise several antigenic parts or antigenic fragments (= antigens). Also, a protein itself may be considered as being an 'antigen'. Preferably, said recombinant adenovirus is a human or a simian adenovirus. More preferably, the adenovirus used as a recombinant vector
10 in the present invention is selected from the group consisting of human adenovirus serotypes Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50. The TB-causing bacillus used for providing the preferred antigen(s) is preferably *Mycobacterium tuberculosis*, *Mycobacterium africanum* and/or
15 *Mycobacterium bovis*, and said two or more antigens are preferably selected from the group consisting of antigens encoded by the Ag85A, Ag85B, ESAT-6, f72 and TB10.4 open reading frames of *M. tuberculosis*. In a highly preferred embodiment, said nucleic acid sequence encodes at least two
20 antigens selected from the group consisting of antigens encoded by the Ag85A, Ag85B, and TB10.4 open reading frames of *M. tuberculosis*. In an even more preferred embodiment, the adenovirus according to the invention comprises a nucleic acid sequence encoding the full length proteins
25 Ag85A, Ag85B and TB10.4, wherein it is even more preferred that these three proteins are encoded by a nucleic acid comprising a sequence in which the genes encoding the respective proteins are cloned in that 5' to 3' order (Ag85A-Ag85B-TB10.4).

30 The invention relates to a recombinant adenovirus according to the invention, wherein at least two of said antigens are expressed from one polyprotein. In one

preferred embodiment at least two of said antigens are linked so as to form a fusion protein. The linkage may be direct or via a connecting linker of at least one amino acid. Where a linker is used to connect two separate
5 antigens and thus to provide a fusion protein of two or more antigens according to the invention, preferably one or more linkers according to SEQ ID NO:23 is used.

The invention also relates to a multivalent TB vaccine comprising a recombinant adenovirus according to the
10 invention or a recombinant polynucleotide vector according to the invention, further comprising a pharmaceutically acceptable excipient, and optionally an adjuvant. Many pharmaceutically acceptable recipients and adjuvants are known in the art.

15 The invention furthermore relates to a method of vaccinating a mammal for the prevention or treatment of TB, comprising administering to said mammal a recombinant adenovirus, a multivalent TB vaccine or a recombinant polynucleotide vector according to the invention. In one
20 aspect, the invention relates to a method of vaccinating a mammal for the prevention or treatment of TB, comprising the steps of administering to said mammal a recombinant adenovirus, a multivalent TB vaccine, or a recombinant polynucleotide vector according to the invention as a
25 priming vaccination; and administering to said mammal a recombinant adenovirus, a multivalent TB vaccine, or a recombinant polynucleotide vector according to the invention as a boosting vaccination. The invention also relates to a recombinant adenovirus, a multivalent TB vaccine, or a
30 recombinant polynucleotide vector according to the invention, either one for use as a medicament, preferably in the prophylactic-, therapeutic- or diagnostic treatment of

tuberculosis. The invention also relates to the use of a recombinant adenovirus, a multivalent TB vaccine, or a recombinant polynucleotide vector according to the invention in the preparation of a medicament for the prophylactic-, or
5 therapeutic treatment of tuberculosis.

In one particular aspect, the invention relates to a recombinant polynucleotide vector comprising a nucleic acid sequence encoding two or more antigens and a protease-recognition site, wherein said antigens are expressed as a
10 polyprotein, said polyprotein comprising the protease recognition site separating at least two of the two or more antigens. Preferably, said polynucleotide vector is a naked DNA-, a naked RNA-, a plasmid-, or a viral vector. In a preferred embodiment, said viral vector is packaged into a
15 replication-defective human or simian adenovirus. It is to be understood that a viral vector may be seen as two kinds of entities: the viral DNA encoding the virus may be used as a nucleic acid vector, while the virus (comprising the viral vector DNA) may also be used to transfer the nucleic acid of
20 interest to a host cell through infection of said host cell. Thus, a 'vector' as used herein refers to a means for transferring a gene or multiple genes of interest to a host. This may be achieved by direct injections of the DNA, RNA, plasmid, or the viral nucleic acid vector, but may also be
25 achieved by infecting host cells with a recombinant virus (which then acts as the vector). As exemplified herein, viruses may be used to immunize mammals (for example mice), whereas the DNA (for instance in the form of the adapter plasmid carrying the gene(s) of interest and a part of the
30 viral DNA) may also be directly injected in the mammal for immunizing said mammal. Vaccines based on naked DNA, or RNA, or plasmids are known in the art, whereas vaccines based on

recombinant viruses are also known. For clarity issues, all entities that deliver a gene or more genes of interest to a host cell are regarded as a 'vector'.

In one preferred embodiment the nucleic acid present in
5 said vector comprises a sequence encoding a protease,
wherein it is preferred that said protease upon expression
is expressed as part of the polyprotein and is linked to at
least one of said antigens by a protease-recognition site.
Particularly preferred protease-recognition sites comprise a
10 sequence according to SEQ ID NO:21 or 22. More preferred is
a recombinant polynucleotide vector according to the
invention, wherein said protease is from an Avian Leukosis
Virus (ALV). In a preferred aspect, the antigens that are
linked through a protease recognition site are from at least
15 one tuberculosis (TB)-causing bacillus, wherein said TB-
causing bacillus is preferably *Mycobacterium tuberculosis*,
Mycobacterium africanum and/or *Mycobacterium bovis*. The two
or more antigens are preferably selected from the group
consisting of antigens encoded by the Ag85A, Ag85B, ESAT-6,
20 and TB10.4 open reading frames of *M. tuberculosis*, wherein
said heterologous nucleic acid sequence encodes most
preferably at least two antigens selected from the group
consisting of antigens encoded by the Ag85A, Ag85B, and
TB10.4 open reading frames of *M. tuberculosis*. Even more
25 preferred are polynucleotides according to the invention
wherein the antigens are the full length Ag85A, Ag85B and
TB10.4 polypeptides, of which the encoding genes are cloned
in that 5' to 3' order. Fusion proteins based on these and
other tuberculosis antigens were described in US 5,916,558,
30 WO 01/24820, WO 03/070187 and WO 2005/061534. However, the
use of the nucleic acids according to the present invention,
encoding the fusion proteins disclosed herein, for

incorporation into recombinant adenoviral vectors was not disclosed.

In yet another aspect, the invention relates to a recombinant polynucleotide vector comprising a heterologous nucleic acid sequence encoding an antigen and a genetic adjuvant. The term 'genetic adjuvant' refers to a proteinaceous molecule that is encoded by a nucleic acid sequence. Said antigen and said genetic adjuvant may be linked directly or in another embodiment linked indirectly, for instance by a connection comprising a first protease-recognition site. In another preferred aspect, said polynucleotide vector is a naked DNA-, a naked RNA-, a plasmid-, or a viral vector. The viral vector is preferably packaged into a replication-defective human or simian adenovirus, wherein said adenovirus is even more preferably selected from the group consisting of human adenovirus serotypes Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50. Also preferred are nucleic acids comprising a sequence encoding a protease, wherein said protease is preferably linked to said antigen and/or to said genetic adjuvant by a second protease-recognition site. The preferred second protease-recognition site comprises a sequence according to SEQ ID NO:22, whereas the preferred first protease-recognition site comprises a sequence according to SEQ ID NO:21. A preferred protease is a protease from an Avian Leukosis Virus (ALV), while the antigens are preferably from at least a tuberculosis (TB)-causing bacillus, more preferably *Mycobacterium tuberculosis*, *Mycobacterium africanum* and/or *Mycobacterium bovis*. Preferred antigens are selected from the group consisting of: Ag85A, Ag85B, ESAT-6 and TB10.4. A most preferred embodiment is a vector wherein said heterologous nucleic acid sequence encodes at least two

antigens selected from the group consisting of *M. tuberculosis* antigens Ag85A, Ag85B, and TB10.4, wherein it is further preferred to have a fusion polypeptide comprising the full length Ag85A, Ag85B and TB10.4 proteins, in that
5 order from N- to C-terminus.

As disclosed herein, the TB10.4 has unexpected adjuvant activity, as it was found that it stimulates the immune response towards the other (especially Ag85A) antigen present in the polyprotein. The TB10.4 adjuvant is a
10 preferred genetic adjuvant. Thus, the invention also provides a recombinant vector comprising a nucleic acid encoding the TB10.4 antigen with at least one other antigen, which antigen is preferably a tuberculosis antigen, more preferably the Ag85A antigen. In an even more preferred
15 embodiment, the vector comprises a nucleic acid encoding the TB10.4 antigen and at least the Ag85A and Ag85B antigens. As outlined below, the TB10.4 is suggested to increase the processing of the multiple-antigen translation product towards the proteosome, resulting in a highly significant
20 increase in CD8 response. It is very likely that the effect is not limited to Ag85A and TB10.4 alone, with a wider applicability of the TB10.4 antigen than limited to tuberculosis vaccines alone. Thus, the invention, in yet another embodiment, also relates to a recombinant vector
25 comprising a nucleic acid encoding TB10.4 and at least one other antigen, wherein the other antigen is not a *Mycobacterium* antigen. The invention discloses the use of the *Mycobacterium* TB10.4 antigen as a genetic adjuvant. Moreover, the invention discloses the use of the TB10.4
30 antigen in the manufacture of a medicament for the treatment, diagnosis and/or prophylaxis of a disease other than tuberculosis, and at least in a disease in which the

immune response towards the antigen of interest needs to be stimulated by the action of an adjuvant. So, it is disclosed that an antigen within TB10.4 of *Mycobacterium tuberculosis* can act as an adjuvant towards other antigens, such as

5 Ag85A. Thus, the invention also relates to the use of the *Mycobacterium* antigen TB10.4 as a genetic adjuvant. Furthermore, in another embodiment, the invention relates to the use of the *Mycobacterium* antigen TB10.4 in the preparation of a medicament for the treatment or prophylaxis
10 of a disease in which the immune response of a host towards a certain antigen or therapeutic component of interest needs to be stimulated. The skilled person would be able to determine the level of immune response towards a given antigen of interest and whether an extra stimulation by the
15 use of an adjuvant, such as a genetic adjuvant, herein exemplified by TB10.4, might be beneficial in a treated subject.

The invention further relates to a recombinant polynucleotide vector according to the invention, wherein
20 said genetic adjuvant comprises a cholera toxin (CtxA1) or a mutant derivative thereof, said mutant derivative comprising a serine to lysine substitution at amino acid position 63 (Al_{K63}). The invention also relates to a multivalent TB vaccine comprising a recombinant polynucleotide vector
25 according to the invention, further comprising a pharmaceutically acceptable excipient, and optionally an adjuvant.

EXAMPLES

Example 1. Construction of Ad35-based adapter plasmids carrying *M. tuberculosis* antigens.

5 Here, the construction of adapter plasmids suitable to generate E1-deleted Ad35-based vectors capable of expressing single or multiple TB antigens is described. The examples relate to TB antigens Ag85A (Swissprot #P17944), Ag85B (Swissprot #P31952) and TB10.4 (Swissprot #053693) as non-
10 limiting examples of means and methods to generate single- and multi-antigen vaccine preparations using Adenovirus-based replication-defective vectors. As already stated above, the principles applied here can also be applied to any combination of prophylactic or therapeutic polypeptide.

15

Construction of pAdApt35Bsu.myc.

 Adapter plasmid pAdApt35Bsu is described in applicant's application WO 2004/001032. This plasmid contains the left part of the Ad35 genome (including the left Inverted
20 Terminal Repeat (ITR)), further lacking a functional E1 region, and an expression cassette comprising a CMV promoter inserted into the E1 region. The adapter also comprises a functional pIX promoter and a region of Ad35 downstream of the E1 region, which region is sufficient for a homologous
25 recombination event with a cosmid comprising the remaining part of the Ad35 genome, leading to the generation of a recombinant replication-defective adenovirus in a packaging cell, which packaging cell provides all necessary elements and functions for a functional replication and packaging of
30 the virus to be produced. The generation of recombinant adenoviruses using such adapter plasmids is a process well known to the skilled person.

pAdApt35Bsu was digested with NheI and XbaI and the 5kb vector-containing fragment was isolated from agarose gel using the Qiaquick gel extraction kit (Qiagen) according to manufacturer's instructions. A double-stranded (ds) linker was prepared from the following single stranded (ss) oligos (synthesized by Sigma): Myc-oligo 1: 5'- CTA GCA AGA AAA CCG AGC AGA AGC TGA TCT CCG AGG AGG ACC TGT GAT AAT -3' (SEQ ID NO:1) and Myc-oligo 2: 5'- CTA GAT TAT CAC AGG TCC TCC TCG GAG ATC AGC TTC TGC TCG GTT TTC TTG -3' (SEQ ID NO:2). The two oligonucleotides were mixed using 2 µl of 0.5 µg/µl stocks in a total volume of 20 µl annealing buffer (10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 1 mM Dithiotreitol), incubated at 98°C for 2 min and subsequently cooled down to 4°C at a rate of 0.6°C per min using a PCR machine. The resulting ds linker was then ligated with the above prepared pAdApt35Bsu vector in 3x, 6x or 9x molar excess of the linker. Colonies were tested for insertion of the linker sequence in correct orientation by digestion with NheI or XbaI, sites that are restored only in correct orientation. Sequencing confirmed that the linker consisted of the expected sequence. The resulting adapter plasmid is named pAdApt35Bsu.myc (Figure 1).

Below, methods for cloning a large set of different constructs is provided. An overview of all constructs and their respective inserts is found in Table I.

Table I. Construct names and their respective inserts comprising nucleic acids encoding TB antigens. ALV = Avian Leukosis Virus protease; dig* = a protease recognition site recognized by a cellular protease; dig = protease

5 recognition site recognized by the ALV protease; X = flexible linker, not being a protease recognition site; myc = myc-tag.

Construct	Insert
TB-3	ALV-dig*-Ag85A-dig-Ag85B
TB-3M	ALV-dig*-Ag85A-dig-Ag85B-myc
TB-4	Ag85A-Ag85B
TB-4M	Ag85A-Ag85B-myc
TB-5	Ag85A
TB-5M	Ag85A-myc
TB-6	Ag85B
TB-6M	Ag85B-myc
TB-7	TB10.4
TB-7M	TB10.4-myc
TB-S	Ag85A-Ag85B-TB10.4
TB-SM	Ag85A-Ag85B-TB10.4-myc
TB-L	ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4
TB-LM	ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4-myc
TB-FL	Ag85A-X-Ag85B-X-TB10.4
TB-FLM	Ag85A-X-Ag85B-X-TB10.4-myc

10

Construction of pAdApt35Bsu-based adapter plasmids containing three TB antigens

The heterologous nucleic acids of the present invention

encode the three *M. tuberculosis* antigens Ag85A, Ag85B and

15 TB10.4 as a poly-protein from one mRNA. All fusion sequences

indicated with an 'M' contain a myc epitope (myc-tag:

SKKTEQKLISEEDL; SEQ ID NO:9) attached to the 3' end of the

sequence to allow analysis of expression using myc-specific antibodies in case the antibodies specific for the separate TB antigens do not recognize the fusions properly. Thus, the 'M' in the names of all constructs described below relates to the myc-tag, whereas also all constructs were made without a myc-tag.

In a first embodiment (TB-SM) the three antigens are expressed as a direct fusion polyprotein: Ag85A-Ag85B-TB10.4-myc (TB-S = Ag85A-Ag85B-TB10.4).

10 In a second embodiment (TB-LM) the polyprotein precursor contains a protease, which cleaves the three antigens intracellularly on incorporated digestion sites that separate them: linker/digestion site sequence: PPSKSKKGGAAAMSSAIQPL VMAVVNRERDGQTG (SEQ ID NO:21). This digestion occurs through a sequence-specific protease fused to the N-terminus of the fusion protein. This protease, derived from the gag gene of the Avian Leukosis Virus (ALV) is also cleaved resulting in four separate proteins after protease digestion. The polyprotein may be as follows: ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4-myc (in which 'dig*' relates to the digestion site separating the protease from the antigens [GSSGPWPAPPEPPAV SLAMTMEHRDRPLV; SEQ ID NO:22] of the protease and 'dig' relates to the digestion site between the antigens, see above; TB-L = ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4). Both protease-cleavable linkers as well as self-cleavage linkers may be used in the vectors of the present invention and are encompassed herein. The use of self-processing cleavage sites has been described in WO 2005/017149.

25 In a third embodiment the poly-protein (TB-FLM) comprises the mentioned *M. tuberculosis* antigens separated by a linker sequence that is not cleaved (as in the second embodiment described above) but allows proper and independent folding

of each of the three antigens: Ag85A-X-Ag85B-X-TB10.4-myc
(in which 'X' relates to a flexible linker:
GTGGSGGTGSGTGGSV; SEQ ID NO:23). All these fusion proteins
were also made without the myc-tag (referred to as TB-S, TB-
5 L and TB-FL respectively) using similar construction methods
(see below).

The desired protein sequences were assembled using the
above indicated published protein sequences for the *M.*
tuberculosis antigens, the ALV protease PR p15 sequence as
10 published in Genbank (Acc. No.CAA86524) and the protease
digestion site as in Genbank Acc. No. AAK13202 amino acids
476-500. The three protein sequences were then back
translated to DNA coding sequences optimised for expression
in humans and subsequently synthesized, assembled and cloned
15 in pCR-Script vectors by Geneart (Germany).

The codon-optimized DNA sequences for TB-LM are
provided (Fig. 19; SEQ ID NO:3), TB-SM (Fig. 20; SEQ ID
NO:4) and TB-FLM (Fig. 21; SEQ ID NO:5), as well as the
protein sequences for TB-LM (Fig. 22; SEQ ID NO:6), TB-SM
20 (Fig. 23; SEQ ID NO:7) and TB-FLM (Fig. 24; SEQ ID NO:8).
The myc epitope is contained within the C-terminal sequence
SKKTEQKLISEEDL (SEQ ID NO:9) in each of the fusion proteins,
which sequence is not present in the case of TB-L, TB-S and
TB-FL, respectively.

25 The cloned fusion genes were then digested with
HindIII, XbaI and ApaLI after which the 2.7 kb (TB-L), 2.2
kb (TB-FL) and 2.1 kb (TB-S) fragments were isolated from
agarose gel as described above. ApaLI digestion was done to
digest the plasmid vector in fragments that were better
30 separable from the inserts. Plasmid pAdApt35Bsu was also
digested with HindIII and XbaI and the vector-containing
fragment was isolated from gel as above. The isolated

pAdApt35Bsu vector was ligated in separate reactions to each of the isolated fragments containing the TB sequences and transformed into DH5 α competent bacteria (Invitrogen). Resulting colonies were analysed by digestion with HindIII and XbaI and plasmid clones containing the expected insert were selected. This resulted in pAdApt35Bsu.TB.LM (Figure 2), pAdApt35Bsu.TB.SM (Figure 3) and pAdApt35Bsu.TB.FLM (Figure 4), all containing a myc-tag. To generate adapter plasmids expressing the fusion genes without myc-tag the inserts are first PCR-amplified using the following primers and templates:

Fragment TB-L

ALVprot.FW: 5'- GCC CAA GCT TGC CAC CAT GCT GGC CAT GAC CAT GG -3' (SEQ ID NO:10) and 10.4.RE.stop: 5'- GCT AGT CTA GAT TAT CAG CCG CCC CAC TTG GC -3' (SEQ ID NO:11) with TB-LM as template.

Fragment TB-FL and TB-S

85A.FW: 5'- GCC CAA GCT TGC CAC CAT GTT CAG C -3' (SEQ ID NO:12) and 10.4.RE.stop with TB-FLM or TB-SM as template.

The amplifications were done with Phusion DNA polymerase (Bioke) according to manufacturer's instructions. The following program was used: 2 min at 98°C followed by 30 cycles of (20 sec at 98°C, 30 sec at 58°C and 2 min + 30 sec at 72°C) and ended by 10 min at 72°C. The resulting fragments were purified using Qiaquick PCR purification kit (Qiagen) and digested with HindIII and XbaI. The digested fragments were then again purified over a Qiaquick PCR purification column as above and ligated with pAdApt35Bsu digested with the same enzymes and purified over a Qiaquick PCR purification column. Transformation into competent DH5 α bacteria (Invitrogen) and selection of the clones containing

the correct inserts using HindIII and XbaI as diagnostic enzymes results in pAdApt35Bsu.TB-L, pAdApt35Bsu.TB-S and pAdApt35Bsu.TB-FL. These constructs differ from the constructs presented in Figures 2, 3 and 4 in that they do not contain the myc epitope at the C-terminal end.

Construction of pAdApt35Bsu-based adapter plasmids containing two TB antigens

Here, the construction of adapter plasmids containing two TB antigens using Ag85A and Ag85B as an example is also described. Obvious to the person with general skill in the art, other combinations and a different order of *M. tuberculosis* antigens can be made using the general strategy outlined herein. The fusions that are described here are TB-3M: ALV-dig*-Ag85A-dig-Ag85B-myc and TB-4M: Ag85A-Ag85B-myc, and the same constructs without the myc-tag. Specific primers were designed to amplify the Ag85A and Ag85B sequences from the above described TB-LM and TB-SM fusion proteins (see below). Fusions are generated with and without (TB-3 and TB-4 respectively) myc-tag as above. Hereto, different primer sets and templates are used.

Fragment TB.3M:

ALVprot.FW and 85B.RE myc: 5'- GCC TAG CTA GCG CCG GCT CCC AGG CTG C -3' (SEQ ID NO:13) with TB-LM as template.

Fragment TB.4M:

85A.FW.TB.L: 5'- GCC CAA GCT TGC CAC CAT GTT CAG CAG ACC CGG CCT G -3' (SEQ ID NO:14) and 85B.RE myc (see above) with TB-SM as template.

All reactions were done using Phusion (Bioke) DNA polymerase with the conditions as described above. PCR fragments were purified using a Qiaquick PCR purification kit, digested with HindIII and NheI and again purified using

the PCR purification kit. The amplified fragments were subsequently ligated into plasmid pAdApt35Bsu.myc that was digested with the same enzymes. After transformation into competent DH5 α bacteria (Invitrogen) clones were selected
5 that contained an insert of the correct length. This resulted in constructs pAdApt35Bsu.TB.3M (Figure 5) and pAdApt35Bsu.TB.4M (Figure 6).

Fragments TB.3 and TB.4 were generated using the same forward primers and templates indicated above for fragments
10 TB.3M and TB.4M but using a different reverse primer named 85B.RE.stop: 5'- GCT AGT CTA GAT TAT CAG CCG GCT CCC AGG CTG C -3' (SEQ ID NO:15). Amplified fragments were purified as above, digested with HindIII and XbaI, and again purified as described intra and cloned into pAdApt35Bsu using HindIII
15 and XbaI as cloning sites. This gave pAdApt35Bsu.TB.3 and pAdApt35Bsu.TB.4 that only differ from the constructs in Figure 5 and 6 in that they have no myc-epitope at the C-terminus.

20 Other combinations that may be useful but not described in detailed cloning procedures herein are:

ALV-dig*-Ag85B-dig-Ag85A-myc

ALV-dig*-Ag85A-dig-TB10.4-dig-Ag85B-myc

ALV-dig*-TB10.4-dig-Ag85A-dig-Ag85B-myc

25 ALV-dig*-TB10.4-dig-Ag85B-dig-Ag85A-myc

ALV-dig*-Ag85B-dig-Ag85A-dig-TB10.4-myc

ALV-dig*-Ag85B-dig-TB10.4-dig-Ag85A-myc

ALV-dig*-Ag85A-dig-TB10.4-myc

ALV-dig*-Ag85B-dig-TB10.4-myc

30 ALV-dig*-TB10.4-dig-Ag85A-myc

ALV-dig*-TB10.4-dig-Ag85B-myc

Ag85B-Ag84A-myc

- Ag85A-TB10.4-myc
Ag85B-TB10.4-myc
TB10.4-Ag85A-myc
TB10.4-Ag85B-myc
5 Ag85A-X-Ag85B-myc
Ag85B-X-Ag85A-myc
Ag85A-X-TB10.4-myc
Ag85B-X-TB10.4-myc
TB10.4-X-Ag85A-myc
10 TB10.4-X-Ag85B-myc
Ag85A-X-TB10.4-X-Ag85B-myc
Ag85B-X-Ag85A-X-TB10.4-myc
Ag85B-X-TB10.4-X-Ag85A-myc
TB10.4-X-Ag85A-X-Ag85B-myc
15 TB10.4-X-Ag85B-X-Ag85A-myc
Dig*, dig, myc and X all relate to the same features as outlined above. It is to be understood that these constructs may also be produced without the myc-tag.

20 Construction of pAdApt35Bsu-based adapter plasmids containing single TB antigens

- Here, the Ad35 adapter plasmids containing single TB antigens are also described. As above, proteins are expressed with or without myc-tag. Hereto, the appropriate
25 coding regions were amplified from the TB-L and TB-S templates using specific primers sets:

Fragment TB.5M:

85A.FW.TB.L and 85A.RE myc: 5'- GCC TAG CTA GCG CCC TGG GGG
G -3' (SEQ ID NO:16) using TB-LM as template.

30 Fragment TB.6M:

85B.FW: 5'- GCC CAA GCT TGC CAC CAT GTT CAG CCG GCC TGG CCT
G -3' (SEQ ID NO:17) and 85B.RE myc using TB-LM as template.

Fragment TB.7M:

10.4.FW: 5'- GCC CAA GCT TGC CAC CAT GAG CCA GAT CAT GTA CAA
CTA CCC -3' (SEQ ID NO:18) and 10.4.RE myc: 5'- GCT AGT CTA
GAT TAT CAC AGG TCC TCC TCG -3' (SEQ ID NO:19) using TB-LM
5 as template.

The fragments without myc-tag were generated with the
same forward primers but with different reverse primers:
85A.RE.stop (for TB.5): 5'- GCT AGT CTA GAT TAT CAG CCC TGG
GGG GCA G -3' (SEQ ID NO:20), 85B.RE.stop (for TB.6), and
10 10.4.RE.stop (for TB.7). All reactions were done with
Phusion (Bioke) as described above. All amplified fragments
were purified using the Qiaquick PCR purification kit. The
fragments TB-5M and TB-6M were then digested with HindIII
and NheI and after purification as described above, cloned
15 into pAdApt35Bsu.myc digested with the same enzymes. The
fragments TB-7M, TB-5, TB-6 and TB-7 were digested with
HindIII and XbaI. After purification as above fragments were
ligated into pAdApt35Bsu digested with the indicated
restriction enzymes. This resulted in pAdApt35Bsu.TB.5M
20 (Figure 7), pAdApt35Bsu.TB.6M (Figure 8), pAdApt35Bsu.TB.7M
(Figure 9), pAdApt35Bsu.TB.5, pAdApt35Bsu.TB.6 and
pAdApt35Bsu.TB.7. The latter three differ only from the ones
in Figure 7, 8 and 9 in that no myc-tag is present at the C-
terminus.

25

**Example 2. Generation of replication-deficient Ad35 viruses
carrying nucleic acids encoding TB antigens**

Methods to generate stable replication-defective
recombinant Ad35-based adenoviral vectors carrying
30 heterologous expression cassettes are well known to the
person of skill in the art and were previously described in
published patent applications WO 00/70071, WO 02/40665, WO

03/104467 and WO 2004/001032. This example describes the generation of Ad35-based TB vectors using PER.C6[®] cells and Ad35 viruses comprising the Ad5-derived E4-Orf6 and E4-Orf6/7 genes replacing the homologous E4-Orf6 and 6/7 sequences in the Ad35 backbone (generally as described in WO 03/104467 and WO 2004/001032). For the purposes of the invention, PER.C6[®] cells refer to cells as deposited on February 29, 1996 as patent deposit under no. 96022940 at the European Collection of Cell Cultures (ECACC) at the Centre of Applied Microbiology & Research (CAMR), Porton Down, Salisbury, Wiltshire, SP4 0JG United Kingdom.

The generated adapter plasmids described herein, containing the different TB antigens were digested with PspI to liberate the Ad35 sequences and transgene cassette (adapter fragment) from the plasmid backbone. Construct pWE.Ad35.pIX-EcoRV (see WO 03/104467 and WO 2004/001032) was digested with NotI and EcoRV (fragment 2) and construct pBr.Ad35.ΔE3.PR5Orf6 (see WO 03/104467 and WO 2004/001032) was digested with PacI and NotI (fragment 3). The digested DNA mixes were incubated at 65°C to inactivate the enzymes. For each transfection, digested adapter fragment (360 ng), fragment 2 (1.4 µg) and fragment 3 (1 µg) were mixed to a (maximum) volume of 15 µl and adjusted to 25 µl with DMEM (culture medium, Invitrogen). A second mixture was prepared by mixing 14,4 µl Lipofectamine (Invitrogen) with 10.6 µl DMEM, after which the two mixes were added together and mixed by tapping the tube. The resulting DNA-Lipofectamine mixture was then incubated 30-40 min at room temperature after which 4.5 ml DMEM was added to the tube. During incubation, PER.C6 cells that were seeded the day before in 6-well plates at 1.5×10^6 cells/well in DMEM containing 10%

FBS (Invitrogen/GIBCO) and 10 mM MgCl₂, were washed with DMEM. Then, to the first two wells 0.5 ml DMEM was added and 0.5 ml of the incubated transfection mixture. To the second two wells 0.25 ml medium and 0.75 ml of the transfection mixture was added. The last two wells received 1 ml of the transfection mixture. The 6-well plate was then incubated at 37°C and 10% CO₂ for 4 h after which an agar overlay was placed as follows. 30 min before the end of the 4 h incubation period, a mixture containing 9 ml 2xMEM (Invitrogen), 0.36 ml FBS, 0.18 ml 1M MgCl₂ and 1.3 ml PBS was prepared and placed at 37°C. A sterile pre-made solution of 2.5% agarose (Seaplaque; Cambrex) in H₂O was melted and also kept at 37°C (at least 15 min prior to use). The transfection medium was then removed from the cells and cells were washed with PBS once. Then 7.5 ml of the agar solution was added to the MEM medium mixture, mixed and 3 ml was quickly added to each well. The overlay was allowed to coagulate in the flow after which the plates were incubated at 37°C/10% CO₂ for at least 7 days. When large enough, single plaques were picked from the wells with the lowest number of plaques using pipettes with sterile filter tips (20 µl). The picked plaques were mixed in 200 µl culture medium each and 100 µl of this was used to inoculate PER.C6 cells in 6-well plates. Upon CPE and after one more amplification of the viruses on PER.C6 cells in T25 flasks cells and medium were harvested and freeze/thawed once and stored as crude lysates. These virus stocks were used to confirm the presence of the correct transgene by PCR on isolated virus DNA and to test expression. One of the amplified plaques was then chosen to generate virus seed stocks and to produce batches of purified virus according to

procedures known in the art using a two-step CsCl purification method. The concentration of purified viruses was typically determined by HPLC as described by Shabram et al. (1997).

5

Example 3. Analysis of expression of TB antigens upon infection with Ad35 viral vectors

The expression of the fused TB antigens was determined by western blotting. Hereto, A549 cells were infected with
10 the different Ad35 viruses containing the genes encoding the TB antigens. 48 h post infection, cells were washed twice with PBS (NPBI), lysed and scraped in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% DOC, 1% Tween-20 in dH₂O supplemented with 1% SDS and Protease inhibitor added as a
15 pill (Roche)). After 5-10 min in lysis buffer on ice, lysates were collected and cleared by centrifugation. Equal amounts of whole-cell extract were fractionated by using 4-12% Bis-Tris NuPAGE® Pre-Cast Gels (Invitrogen). Proteins were transferred onto Immobilon-P membranes (Millipore) and
20 incubated with a polyclonal antibody directed to the Culture Filtrate Protein of *M. tuberculosis*. This polyclonal serum was raised in rabbits against an *M. tuberculosis* culture comprising secreted proteins. In principle the polyclonal serum contains antibodies against Ag85A, Ag85B and TB10.4,
25 which are all secreted proteins. The secondary antibody was a horseradish-peroxidase-conjugated goat-anti-rabbit antibody (Biorad). The western blotting procedure and incubations were performed according to general methods known in the art. The complexes were visualized with the ECL
30 detection system (Amersham) according to the manufacturer's protocol.

Figure 10A shows the results using Ad35 viruses carrying the TB encoding nucleic acids including the myc epitope as described herein. The different lanes in figure 10A show the different viral vectors used and Table I indicates which name refers to what insert. In the same way, expression of the TB antigens from the Ad35 viruses that do not contain a myc epitope was measured (figure 10B). Figure 10C shows a similar result, with the molecular weight indicated on the right hand side. Specific TB (fusion) proteins expressed from Ad35 viruses are detected by this method and, in addition, certain cleavage products of TB-3 and TB-L. From figure 10A it can be concluded that the polyprotein including all three TB antigens is expressed, since a higher band in lane TB-LM is present as compared to TB-3M (and the band in lane TB-S is higher than the specific band in TB-4M). Since the TB10.4 is the most C-terminal polypeptide in the TB-LM and TB-SM polyproteins, this indicates that the entire polyproteins are translated. It is also noted that cleavage is not complete, although cleavage products can be seen in lanes TB-3M and TB-LM. The Ag85A and Ag85B antigens (lanes TB-5(M) and TB-6(M) respectively) are expressed. No specific staining is found in lanes TB-7(M) related to the TB10.4 antigen. It may be that the antigen is not recognized in a western blot setting by the CFP polyclonal, whereas it may also be that the protein has run from the gel or that is poorly expressed in A549 cells when present in a single expression construct (TB-7M), while present in a triple construct (as TB-LM, TB-L and TB-S). In figure 10A, lane TB-LM a slightly shorter band is visible under the highest (probably non-cleaved) band. This suggests cleavage of the TB10.4 antigen from the remaining portion of the polyprotein.

Further experiments should reveal the physical presence of the protein, although it is clear that the TB10.4 antigen contributes to the immune response (see below), strongly indicating that the antigen is present and actively involved in the immune response.

Example 4. Immunogenicity of vectors encoding *M tuberculosis* antigens in mice

First, the immunogenicity of the adapter plasmids as described in example 1 (DNA constructs) was studied in mice. The constructs encoded one, two or three TB antigens: Ag85A, Ag85B and TB10.4. The DNA constructs encoding for the multiple TB antigens were designed in two ways as described above, i.e. expressing a polyprotein comprising direct fusions not containing the myc tag and expressing a polyprotein comprising a sequence encoding a protease and the protease recognition sites resulting in the cleavage of the polyprotein (also not containing the myc tag) into discrete polypeptides. The following DNA constructs were used (see example 1):

Single antigen constructs

TB-5 (Ag85A), TB-6 (Ag85B) and TB-7 (TB10.4)

Double antigen constructs

TB-3 (ALV-dig*-Ag85A-dig-Ag85B) and TB-4 (Ag85A-Ag85B direct fusion)

Triple antigen constructs

TB-L (ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4) and TB-S (Ag85A-Ag85B-TB10.4 direct fusion).

The experimental set up is given in Figure 11. Seven groups of mice were immunized with individual TB DNA constructs (two experiments, see below). For each

immunization, DNA was injected intramuscularly three times (3 x 50 µg) with intervals of 2.5 weeks. As a negative control, one group of mice received three injections of PBS. Additional control group received single dose of 6×10^5 cfu BCG (strain SSI1331) subcutaneously.

One week after the last DNA immunization, and six weeks after the BCG immunization the mice were sacrificed. Spleens were isolated to serve as a source of cells for cellular immunological assays. Sera, required for humoral response analysis, were collected by heart puncture and pooled per group.

The level of specific cellular immune response was determined using intracellular IFN γ staining (ICS) FACS assay, by measuring the frequency of IFN γ + CD4+ and IFN γ + CD8+ splenocytes after in vitro re-stimulation with peptide pools of corresponding antigens. The immune sera were tested using immunofluorescence of A549 cells transduced with adenovirus encoding for corresponding antigen.

Two independent immunization experiments were performed. For the first experiment, 3 mice per group were used and the immune response was analyzed for each mouse individually. For the second experiment, 8 mice per group were used for DNA immunizations and 4 mice per group for control immunizations. After in vitro stimulation with peptides, samples of two-by-two mice from the same group were pooled and stained for FACS analysis. Similar results were obtained in both experiments and the data were brought together for statistical analysis.

The intracellular IFN γ staining (ICS) was performed as follows. Splenocytes (10^6 per well of 96-well plate) were

stimulated in duplicate with appropriate peptide pool as indicated (final concentration 2 µg/ml per peptide), in the presence of co-stimulatory antibodies: anti-mouse-CD49d and anti-mouse-CD28 (Pharmingen) in a final dilution of 1:1000.

- 5 Peptide pools consisted of 15-mer peptides spanning whole antigens, with 10-mer (Ag84B) or 11-mer (Ag85A, TB10.4) overlapping sequences, or adjusted for Ag85B with peptide p1 and p2 from Ag85A, as outlined below in example 6 and 7. Samples from BCG and PBS immunized mice were stimulated
- 10 additionally with CFP (Culture Filtrate Protein; final concentration 10 µg/ml) and PPD (Purified Protein Derivative; final concentration 10 µg/ml), which are antigens commonly used for in vitro stimulation upon BCG immunization. As a positive control, samples were stimulated
- 15 with PMA/ionomycin (final concentrations: 50 ng/ml and 2 µg/ml, respectively) whereas the incubation with medium served as a negative control (no stimulation). After 1 h stimulation at 37°C, secretion blocker GolgiPlug was added (Pharmingen; final dilution 1:200) and the incubation was
- 20 continued for an additional time period of 5 h. The corresponding duplicate samples were pooled and processed for FACS analysis. Briefly, cells were washed with PBS containing 0.5% BSA and incubated with FcR Blocker (Pharmingen; dilution 1:50) for 10 min on ice. After a
- 25 washing step, the cells were incubated with CD4-FITC (Pharmingen; dilution 1:250) and CD8-APC (Pharmingen; dilution 1:50) for 30 min on ice. Upon washing cells were fixed and permabilized with Cytofix/Cytoperm (Pharmingen) for 20 min on ice, followed by a washing step with Perm/Wash
- 30 buffer (Pharmingen). Intracellular IFNγ was stained using anti-IFNγ-PE (Pharmingen; dilution 1:100) for 30 min on ice.

After final washing steps cells were resuspended in CellFix (BD) and analyzed using flow cytometer. At least 10,000 CD8+ cells were measured for each individual sample. Results are expressed as a percentage of CD4+ or CD8+ cells that express

5 IFN γ .

An overview of the in vitro re-stimulation samples is given in Table II. The results of the ICS are presented in Figure 12-16.

10

Table II. Overview of the in vitro re-stimulation samples.

Immunization	In vitro antigen stimulation						Medium
	Ad85A	Ad85B	TB10.4	CFP	PPD	PMA	
Ad85A (TB-5)	X	X				X	X
Ad85B (TB-6)	X	X				X	X
TB10.4 (TB-7)			X			X	X
Ad85A.Ad85B (TB-3)	X	X				X	X
Ad85A.Ad85B (TB-4)	X	X				X	X
Ad85A.Ad85B.TB10.4 (TB-L)	X	X	X			X	X
Ad85A.Ad85B.TB10.4 (TB-S)	X	X	X			X	X
BCG	X	X	X	X	X	X	X
PBS	X	X	X	X	X	X	X

Figures 12A and B show that background levels were very low when the cells were not stimulated. Figure 13A shows a high frequency of IFN γ + CD4+ splenocytes after stimulation with peptides of the Ag85A pool. There is a clear cross-reactivity with CD4+ cells obtained from mice injected with the construct harboring the Ag85B encoding gene, which is not unexpected due to the high structural homology between Ag85A and Ag85B. In contrast to what was found for CD4+ cells, no stimulation of CD8+ splenocytes (see figure 13B) was detected of cells from mice injected with constructs encoding either Ag85A alone or in the context of Ag85B (lanes Ag85A, Ag85B, TB-3L and TB-4S). However, there was a striking increase in IFN γ + CD8+ splenocytes in mice injected with the triple constructs TB-L and TB-S, clearly indicating an important role of the additional antigen (TB10.4) present in these constructs. Apparently, in this setting, the TB10.4 antigen is able to strongly increase the frequency of CD8+ splenocytes reactive towards the Ag85A peptides, where Ag85A alone (or in combination with Ag85B) provides no responses. Figure 14A shows that Ag85B in all settings in which it was present is able to increase the frequency of IFN γ + CD4+ splenocytes, whereas the effect on IFN γ + CD8+ splenocytes is minimal (see figure 14B). Also here, cross-reactivity is found between Ag85B and Ag85A (figure 14A) as discussed above. Figure 15A shows that the frequency of IFN γ + CD4+ splenocytes responding to the TB10.4 related peptide pool is present, where no real difference can be found between mice injected with either a construct with TB10.4 alone or a construct comprising the triple inserts. However, as shown in figure 15B, the frequency of IFN γ + CD8+ splenocytes from mice that were injected with constructs comprising the gene

encoding the TB10.4 antigen, is dramatically increased upon stimulation with TB10.4 related peptides, especially in the context of the triple inserts (Note the y-axis, indicating that an average of 1.5% of the splenocytes was reactive).

5 The results are summarized in figure 16A (triple insert in TB-L: with protease and protease digestion sites) and figure 16B (TB-S: direct linked antigens). Clearly, the different antigens contribute in different manners to the immune response: Ag85A induces both CD4 and CD8 responses; Ag85B
10 only induces a strong CD4 response and hardly any CD8 response. In contrast to Ag85B, the TB10.4 antigen invokes a strong CD8 response and a minor CD4 response. This indicates the clear beneficial subsidiary effect of the different antigens encoded by the sequences present in the triple
15 inserts.

The BCG immunization did not result in significant ICS response. However, splenocytes of BCG immunized mice did produce high levels of IFN γ after 72 h stimulation with CFP or PPD, as determine using an IFN γ ELISA kit, which
20 indicates that mice were immunized efficiently (data not shown).

To determine whether any antigen-specific antibodies were actually raised in the mice injected with the different
25 DNA constructs, A549 cells were transduced with Ad35 recombinant adenoviruses encoding the TB antigens in 96 well plates. The adenoviruses were produced as described in example 2. For this, 1×10^4 cells were seeded per well and viruses were infected with a multiplicity of infection of
30 5000. Two days after infection cells were fixed with Cytofix/Cytoperm (20 min at 4°C), followed by a washing step

with Perm/Wash buffer. Cells were incubated with immunized mice sera, diluted 1:2 in Perm/Wash buffer, for 1 h at 37°C. Upon washing, goat anti-mouse-FITC, diluted 1:5 in Perm/Wash buffer, was added and incubated for 30 min at 37°C. After a
5 final wash, cells were analyzed using a fluorescence microscope.

The immunofluorescence analysis revealed strong antigen specific staining of cells with sera obtained from mice immunized with TB-6 (Ag85B alone), TB-3 (ALV-dig*-Ag85A-dig-
10 Ag85B), TB-4 (Ag85A-Ag85B direct fusion) and TB-L (ALV-dig*-Ag85A-dig-Ag85B-dig-TB10.4). Weak staining was observed with sera from mice immunized with TB-S (Ag85A-Ag85B-TB10.4 direct fusion), while sera obtained upon immunization with TB-5 (Ag85A alone) and TB-7 (TB10.4 alone) did not exhibit
15 any staining. This indicates that at least some of the antigens are able to elicit an antibody response. Full cleavage of the protease from the remaining part of the polyprotein and expression levels of the separate antigens was not determined in this experiment.

20

Example 5. Construction of rAd vectors encoding an antigen and an adjuvant.

Here, a novel recombinant replication-defective adenoviral vector is constructed, herein designated Ad35-X-
25 Al_{K63}, which co-expresses an antigen (referred to as X) and a mutant derivative of CtxA1 that harbors a lysine substitution at amino acid no. 63 (i.e. herein referred to as Al_{K63}) in place of the serine that is present in the parental CtxA1.

The construction of adapter plasmids suitable to
30 generate E1-deleted Ad35-based vectors capable of expressing X and Al_{K63} is achieved by introducing PCR-amplified X using

standard PCR procedures known to persons skilled in the art, and introducing appropriate cloning restriction sites. The resultant PCR-generated DNA fragment is digested with the respective restriction endonuclease(s) and annealed to an adapter plasmid generally as described above for Ag85A, Ag85 and TB10.4. Additional analysis by restriction endonuclease digestion, PCR and sequencing of the cloned PCR fragment are conducted to verify that the DNA was not altered during construction.

10 DNA encoding Al_{K63} is amplified from plasmid pOGL1-A comprising a copy of CtxA1. The nucleotide sequence of ctxA1 is available in GenBank (Accession # A16422) and modified by replacing the serine-63 TCA codon (nt 187-189) with a lysine codon AAA. The mutant derivative is generated using the

15 QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). The site-directed mutagenesis process entails whole-plasmid PCR using pOGL1-A1 DNA as template, forward primer 5'-TGT TTC CCA CCA AAA TTA GTT TGA GAA GTG C-3' (SEQ ID NO:24) and reverse primer 5'- CAA ACT AAT TTT GGT GGA AAC ATA TCC ATC-

20 3' (SEQ ID NO:25). This procedure modifies nucleotides 187-189 by replacing the TCA codon with an AAA codon (see underlined sequences). The resultant PCR-generated plasmid is digested with DpnI to remove the template DNA and the digested DNA is introduced into *E. coli* by chemical

25 transformation and grown on agar at 30°C for 16 hr. Isolated colonies are selected and DNA was extracted from overnight liquid cultures. Plasmid PCR using primers specific for Al_{K63}, and agarose gel electrophoresis are conducted to screen for an appropriate derivative. The mutant insert is

30 cloned thereafter into the same adapter plasmid that

contains X, either upstream or downstream of X and adenoviruses are produced as described above.

Example 6. Dose response of Ad35.TB vectors in mice

5 Ad35 recombinant viruses expressing the different
single and fused TB antigens were used to test antigenicity
in mice. Methods to quantify T-cells that produce
interferon-gamma (IFN- γ) after stimulation with proteins or
peptide pools are typically performed using methods known in
10 the art and for example described in WO 2004/037294, Sander
et al. (1991) and Jung et al. (1993). Details are described
below.

The triple insert vector TB-S was used in a dose-
response immunogenicity test. Four different doses of viral
15 particles (10^7 , 10^8 , 10^9 and 10^{10} vp) were injected
intramuscularly in different groups of C57BL/6 mice (5 mice
per group), whereas 3 mice served as a negative control and
were injected with 10^{10} vp of the empty viral vector. Two
weeks after immunization, the mice were sacrificed and
20 splenocytes were isolated to serve as the source of cells
for cellular immunological studies. The level of antigen-
specific cellular immune responses was determined using the
intracellular IFN γ staining (ICS) FACS assay, by measuring
the frequency of IFN γ CD4+ and CD8+ splenocytes as described
25 above. The results are shown in figure 17. Clearly, the Ad35
based TB-S vector induces an antigen specific immune
response in a dose dependent manner, especially in relation
to the increase in response with Ag85B specific CD4 cells
(figure 17C). No response was found related to TB10.4
30 specific CD4 cells (figure 17E), and no response was found
related to Ag85B specific CD8 cells (figure 17D). While
hardly any responses were detected with the 10^7 and 10^8

doses in respect to Ag85A and TB10.4 specific CD8 cells (figure 17B and 17F respectively), a marked increase was found using the 10^9 and 10^{10} doses. The 10^7 dose did not give any significant effects in any of the settings, while the
5 10^8 dose also resulted in an increase in Ag85A and Ag85B specific CD4 cells (figure 17A and 17C respectively). Similar data were found using the TB-L construct (data not shown).

During an assessment for the CD8 immunodominant
10 sequence epitope mapping of *M. tuberculosis* antigens in mice, it was found that the peptides referred to as p1 (FSRPGLPVEYLQVPS; SEQ ID NO:26) and p2 (GLPVEYLQVPSPSMG; SEQ ID NO:27) of Ag85A were the only CD8 immunodominant epitopes for C57BL/6 mice. The underlined stretch should
15 theoretically fit in the MHC molecules of C57BL/6 mice. The sequence of the Ag85A antigen in this region of the protein (amino acids 1-19: FSRPGLPVEYLQVPSPSMG; SEQ ID NO:28) is identical to the sequence of Ag85B in the same region. However, the peptides p1 and p2 from the Ag85B pool,
20 although comprised of the same sequence as peptides from Ag85A, did not give any CD8 response (see Figure 17D). This suggests that the peptides p1 and p2 from Ag85B were not in order, perhaps due to production effects or contaminations. Therefore, an additional dose response experiment was
25 performed in which the in vitro stimulation peptide pool of Ag85B was reconstituted with p1 and p2 from the Ag85A pool. The experiment was performed with both TB-S and TB-L vectors, using doses of 10^7 , 10^8 , 10^9 , and 10^{10} vp. The T cell response was determined two weeks after immunization,
30 generally as described above. As negative controls, one group of mice was injected with PBS, while one group was injected with an empty Ad35 virus (10^{10} vp). The results

with respect to CD8 cells are presented in Figure 17G (TB-L, left graph, TB-S, right graph). Clearly, CD8 positive cells were measured upon in vitro stimulation with the adjusted Ag85B pool, although the peptides from the Ag85A antigen
5 were identical to the peptides of the Ag85B antigen, which were originally used and did not provide any positive results. These observations nevertheless show that also the Ag85B protein as encoded by the Ad35-based adenoviruses can induce a CD8 positive T cell response after infection of
10 said viruses.

Example 7. Ad35 based TB vectors used as a boost upon priming with BCG

In another experiment Ad35 vectors expressing TB
15 antigens were tested as a boosting agent for BCG immunization. Hereto, groups of mice were injected subcutaneous with BCG vaccine (Bacilli Calmette-Guerin; reference standard FDA and generally known in the art of tuberculosis vaccination) according to protocols delivered
20 by the FDA (standards and testing section CBER).

Four groups of mice (8 mice per group) were primed with BCG (6×10^5 cfu/mouse) subcutaneously ten weeks prior to infection with the adenoviral vectors based on Ad35 carrying the three directly linked TB antigens (TB-S) or with the
25 adenoviral Ad35 vectors carrying the following combinations of antigens:

- TB-4 alone (comprising the Ag85A and Ag85B direct fusion)
- TB-4 + TB-7 (comprising TB10.4 alone)
- TB-5 (comprising Ag85A alone) + TB-6 (comprising Ag85B
30 alone) + TB-7.

Two control groups (4 mice per group) were primed with PBS or with BCG, whereafter the PBS group received PBS as mock-

immunization, and the BCG primed control group received 10^9 vp of the empty Ad35 vector. Injections with the Ad35 based vectors were performed in all cases with 10^9 vp, intramuscularly. Four weeks post-infection (14 weeks after prime), mice were sacrificed and splenocytes were isolated and used as described above. The results are shown in figure 18. The presence of the Ag85A antigen resulted in a significant effect towards Ag85A specific CD4 cells (figure 18A). As expected (see also figure 13B), the triple construct TB-S induced an Ag85A specific CD8 response, while the TB-4 vector did not induce such a response (figure 18B). Similar results were found earlier (figure 13B), indicating that the presence of Ag85A alone or in combination with Ag85B does not give a CD8 response, whereas such a response is found when TB10.4 is present. Interestingly, no effect was determined when the separate vectors were injected but in a single shot (TB-4/TB-7 or TB-5/TB-6/TB-7 in figure 18B), indicating that the TB10.4 antigen can not induce an Ag85A specific CD8 response when co-injected, but rather that the antigen should be present in the same construct or at least in the same cell. The mechanism for the adjuvant effect of TB10.4 is yet unclear.

The effects seen with the Ag85B antigen are in concert with what was found earlier (figure 18C and D). It must be noted that the presence of the TB10.4 antigen in the triple construct TB-S does not give rise to a Ag85B-specific CD8 response, in contrast to what is found with Ag85A. Both antigens are well expressed from the constructs, as was shown in figure 10B. The negative effect may be due to a corrupted peptide pool used to measure any CD8 response towards Ag85B (see example 6 and below).

The induction of CD4+ cells using TB10.4 is very low (figure 18E). The induction of CD8+ cells using TB10.4 in a separate vector (TB-5/TB-6/TB-7) is significant (note the scale on the y-axis; see also figure 15B). The induction of
5 TB10.4 specific CD8 cells using TB-S is very high (figure 18F), with an average of around 12% IFN γ positive CD8 cells.

It can be concluded that the TB10.4 antigen is capable of inducing a CD8 response towards an antigen, which as a single construct does not give rise to a CD8 response
10 (Ag85A). It is known that activation of CD8 cells requires a somewhat higher antigenic threshold than the activation of CD4 cells, which is at least partly due to complex machinery involved in antigen processing and presentation by MHC class I molecules (Storin and Bachmann. 2004). Here, it was found
15 that when TB10.4 was coupled to antigens Ag85A and Ag85B in a triple-antigen construct, strong CD8 responses were triggered, not only against TB10.4 itself but also against Ag85A. It is likely that the physical presence of TB10.4 in the construct increases the efficiency of transport of the
20 fusion protein to the proteosome, which is necessary for the efficient presentation to and activation of CD8 cells. The reason for the higher TB10.4-specific CD8 cell response is most likely due to an increased expression level of the triple construct in comparison to the vector carrying the
25 TB10.4 antigen alone. Although the CD8 response towards TB10.4 alone was also significant, no expression levels of TB10.4 could be determined due to lack of TB10.4 specific antisera for western blotting.

The increased targeting to the proteosome might be the
30 result of the presence of specific sites in TB10.4 molecule, such as sequences involved in binding of ubiquitin (or other molecules responsible for labeling the proteins destined for

processing), or transporter proteins, or sequences that otherwise increase processing and presentation in the context of MHC class I molecules (Wang et al. 2004). Alternatively, the presence of TB10.4 protein in the
5 construct might physically destabilize the fusion protein, leading to increased degradation rate of the molecule. Increased level of antigen processing leads in general to increased CD8 cell activation. Furthermore, if much protein ends up in the proteosome for class I presentation, less
10 will be present in cytosol and extracellularly and, thus, not be available for activation of B cells. It has been reported that an inverse correlation exists between antigen processing (i.e. CD8 activation) and antigen specific antibody titer (Delogu et al. 2000). It is interesting to
15 mention that a much stronger antigen-immunofluorescence was observed in sera from mice immunized with double-antigen constructs rather than from the triple-antigen construct immunized mice. This finding suggests that our triple-antigen molecules, containing TB10.4, are highly susceptible
20 to proteosome degradation and CD8 cell activation and, thus, less available for antibody induction. As a strong T cell response is a preferable response against tuberculosis, it is concluded that an Ad35-based triple-antigen vector, which comprises a nucleic acid encoding the TB10.4 antigen and at
25 least one other TB antigen, preferably Ag85A and more preferably, both Ag85A and Ag85B, is very suited to be used in a vaccine against tuberculosis. The found effects may be even further increased by using BCG as a priming agent, as indicated by the results shown in figure 18.

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Using the new peptide pool for Ag85B with the peptides p1 and p2 of Ag85A added (as described in example 6), also

the prime/boost study with BCG prime, Ad35-TB boost was repeated, although now the splenocytes were removed from mice that were sacrificed 26 weeks after prime (16 weeks after immunization). Mice (8 per group) were immunized with
5 PBS, Ad35.Empty, Ad35.TB-S, or Ad35.TB-L with either 10^9 or 10^{10} vp of the respective viral vectors. Results are shown in Figure 25 (Ag85A stimulation), 26 (Ag85B stimulation) and 27 (TB10.4 stimulation). The results clearly indicate that significant CD4 and CD8 responses can still be measured
10 after prolonged period of time.

Example 8. Prime-boost-challenge experiment in Guinea pigs

In a subsequent experiment, it was investigated whether priming with BCG, followed by a boost with Ad35-based TB
15 vectors, would protect against a *Mycobacterium tuberculosis* infection in a challenging set-up.

Guinea pigs were initially primed with BCG typically as indicated above (6×10^5 cfu per animal). After 14 weeks, the animals were either immunized with 10^{10} vp Ad35.TB-S (Ag85A-
20 Ag85B-TB10.4) or Ad35.TB-4 (Ag85A-Ag85B) recombinant viruses, or injected with PBS (control group). Eight weeks later, the animals were challenged with ~100 cfu *M. tuberculosis* per animal. The animals are monitored up to approximately 78 weeks post prime for survival. Intermediate
25 observations suggest that the BCG prime followed by an Ad35-TB boost ensures a higher survival rate than BCG alone.

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The invention claimed is:

1. A recombinant replication-defective adenovirus comprising a nucleic acid sequence encoding antigens from the Ag85A, the Ag85B and the TB10.4 open reading frames of *Mycobacterium tuberculosis*, wherein the nucleic acid sequence comprises, in the 5' to 3' direction, an expression control sequence, the Ag85A coding sequence, the Ag85B coding sequence, and the TB10.4 coding sequence.
2. The recombinant replication-defective adenovirus of claim 1, wherein the three antigens are expressed as one polypeptide.
3. The recombinant replication-defective adenovirus of claim 1, wherein the three antigens are linked so as to form a fusion protein.
4. The recombinant replication-defective adenovirus of claim 1, wherein the antigens comprise amino acids 1-676 of SEQ ID NO:7.
5. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 4, and
a pharmaceutically acceptable excipient.
6. The recombinant replication-defective adenovirus of claim 1, wherein the nucleic acid encoding the antigens comprises nucleotides 13-2043 of SEQ ID NO:4.
7. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 6, and
a pharmaceutically acceptable excipient.
8. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 1, and
a pharmaceutically acceptable excipient.
9. The recombinant replication-defective adenovirus of claim 1, wherein the adenovirus is selected from the group consisting of human adenovirus serotypes Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49, and Ad50.
10. The recombinant replication-defective adenovirus of claim 1, wherein the adenovirus is serotype 35.

11. The recombinant replication-defective adenovirus of claim 10, wherein the antigens comprise amino acids 1-676 of SEQ ID NO:7.
12. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 11, and
a pharmaceutically acceptable excipient.
13. The recombinant replication-defective adenovirus of claim 10, wherein the nucleic acid encoding the antigens comprises nucleotides 13-2043 of SEQ ID NO:4.
14. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 13, and
a pharmaceutically acceptable excipient.
15. The recombinant replication-defective adenovirus of claim 1, wherein the adenovirus is serotype 26.
16. The recombinant replication-defective adenovirus of claim 15, wherein the antigens comprise amino acids 1-676 of SEQ ID NO:7.
17. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 16, and
a pharmaceutically acceptable excipient.
18. The recombinant replication-defective adenovirus of claim 15, wherein the nucleic acid encoding the antigens comprises nucleotides 13-2043 of SEQ ID NO:4.
19. A multivalent tuberculosis vaccine comprising:
the recombinant adenovirus of claim 18, and
a pharmaceutically acceptable excipient.