



US 20170340751A1

(19) **United States**(12) **Patent Application Publication**
JIANG et al.(10) **Pub. No.: US 2017/0340751 A1**(43) **Pub. Date: Nov. 30, 2017**(54) **ARTIFICIAL MULTI-ANTIGEN FUSION
PROTEIN AND PREPARATION AND USE
THEREOF**(71) Applicants: **Shisong JIANG**, Changzhou, Jiangsu
(CN); **OXFORD VACMEDIX UK
LTD.**, Oxfordshire (GB)(72) Inventors: **Shisong JIANG**, Changzhou, Jiangsu
(CN); **Xiaobing XIA**, Oxfordshire
(GB); **Hanghai DING**, Oxfordshire
(GB)(73) Assignees: **OXFORD VACMEDIX UK LTD.**,
Oxfordshire (GB); **Shisong JIANG**,
Changzhou, Jiangsu (CN)(21) Appl. No.: **15/536,428**(22) PCT Filed: **Dec. 15, 2015**(86) PCT No.: **PCT/CN2015/097470**

§ 371 (c)(1),

(2) Date: **Jun. 15, 2017**(30) **Foreign Application Priority Data**

Dec. 15, 2014 (CN) 201410777861.0

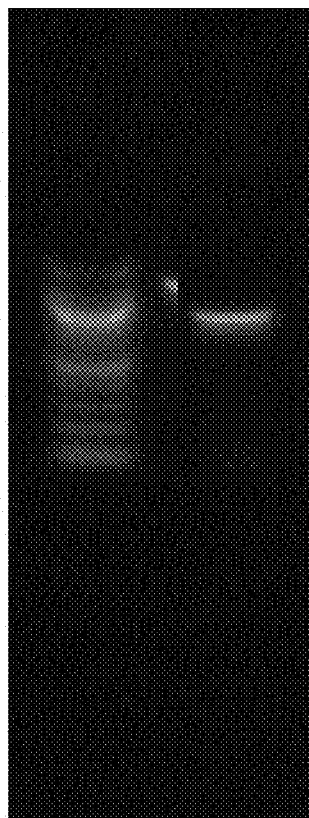
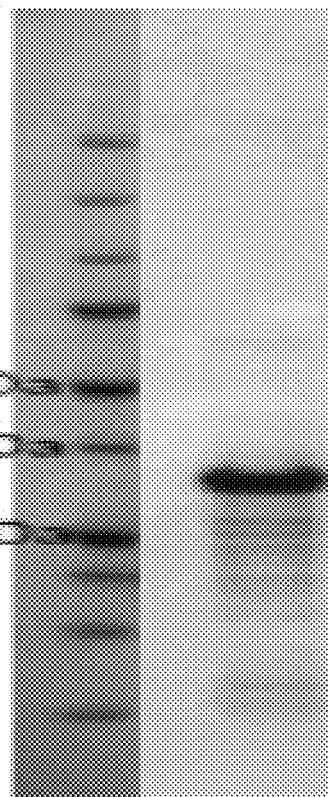
Publication Classification(51) **Int. Cl.****A61K 47/68** (2006.01)**C12N 5/0783** (2010.01)**C12Q 1/68** (2006.01)**C12N 15/70** (2006.01)**C07K 2/00** (2006.01)**C07K 16/00** (2006.01)**A61K 39/39** (2006.01)(52) **U.S. Cl.**CPC **A61K 47/6849** (2017.08); **C12Q 1/686**
(2013.01); **C12N 5/0636** (2013.01); **A61K**
47/6855 (2017.08); **C12N 15/70** (2013.01);
C07K 16/00 (2013.01); **A61K 39/39** (2013.01);
C07K 2/00 (2013.01); **C12N 2501/505**
(2013.01)

(57)

ABSTRACT

Provided are an artificial multi-antigen fusion protein and a preparation method thereof. The fusion protein can effectively stimulate CD8+T and CD4+ T cell immunities, and can be applied to immunodiagnostics or serve as a prophylactic or therapeutic vaccine.

1kb

50kDa
37kDa
25kDa

```

          T7-forward          pLIC-forward
          ----->          ----->
                                lac operator
                                -----
7220 CTGATCCCG CGAATTAAAT ACACATCACT ATAGGGGAAT TGTGACCGGA TAACAATTCC
     GAGTAGGGC GCTTTAAATA TGTGAGTGA TATCCCTTA ACATCGCCT ATTGTTAAGG

                                NdeI
                                -----
7282 CCTCTAGAAA TAATTTGTT TAACITTAAG AAGGAGATAT ACATATGCAC CAICATCAIC
     GGAGATCTTT ATTAAAACAA ATTGAATTC TTCCCTAATA TGTATACGTG GTAGTAGTAG

                                Upper-LIC          BsaI
                                -----
     . H S S G V D L G T E N L Y F Q S
58  ATCAITCTTC TGTSTTAGAT CTGGGTACCG ASAACCTGTA CTTCCAATCC ATGAGAGCCG
     TASTAAGAG ACCACATCTA GACCCATGGC TCTTGGACAT GAAGTTTAGG TACCTCTGGC

118  ACGTCCACAT ..... (SacB fragment) .....
     TCCAGGTGTA

                                BsaI          Lower-LIC          BamHI          EcoRI          SacI
                                -----
3910 GATATCTAT TGGCATTAGC GGTCTCCAGT AAAGGTGGAT ACGATCCGA ATTGAGCTC
     CTATAGGATA ACGTAACTG CCAGAGGTGA TTTCACCTA TCCCTAGGCT TAAGCTGGAG

     SalI
     HindIII
     *****
2070 CGTCGACAG CTTCGGGCG CACTCGAGCA CCACCACAC CACCACTGAG ATCGGCTGC
     SCAGCTCTTC GACGCGCGC GTGAGCTCGT GTTGGTGGTG GTTGTGACTC TAGGCGGAGC

                                T7-reverse
                                -----
2130 TAACAAAGCC CGAAGGAAG CTGAGTTGGC TGTGCCACC GCTGAGCAAT AACTAGCATA
     ATTGTTTCG GCTTTCTTC GACTCAACCG ACGAGGTGG CCACTCGTA TGTATCGAT

                                pLIC-rev
                                -----

```

Fig. 1

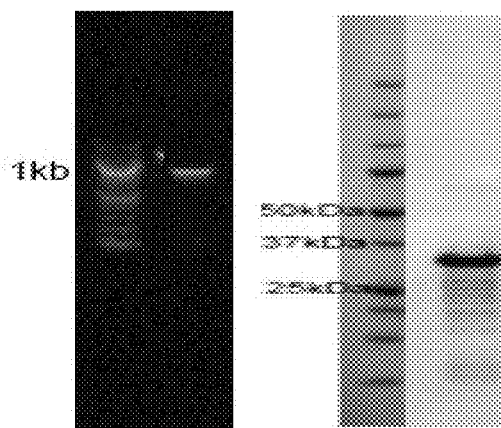


Fig. 2

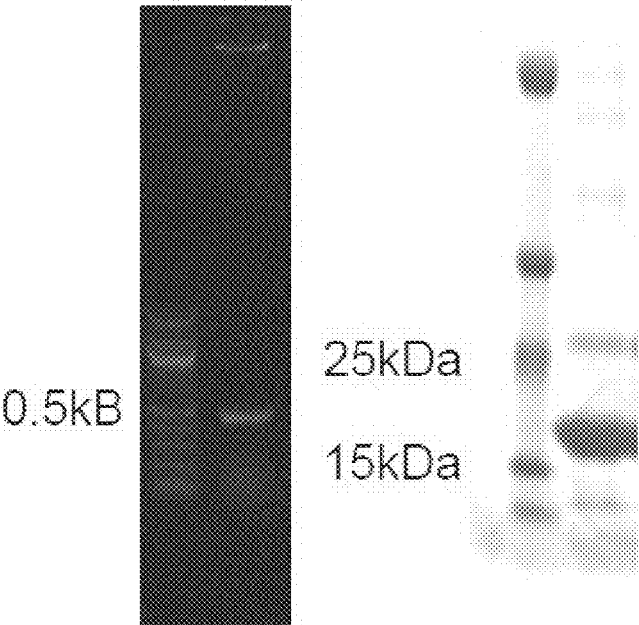


Fig. 3

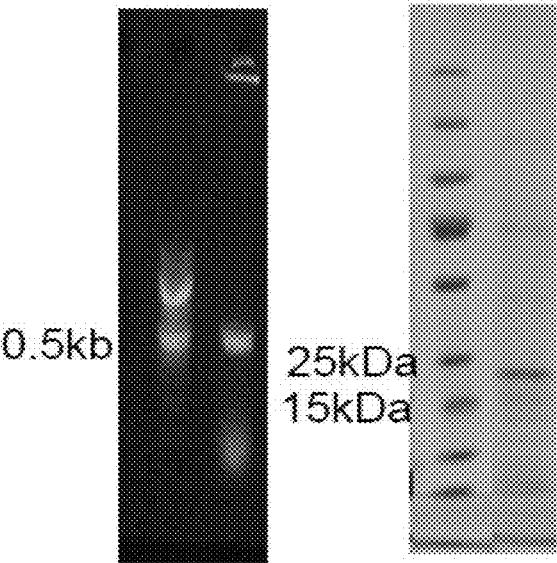


Fig. 4

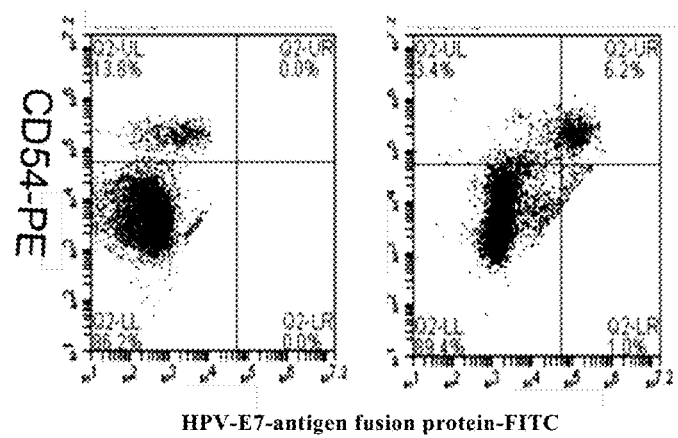


Fig. 5

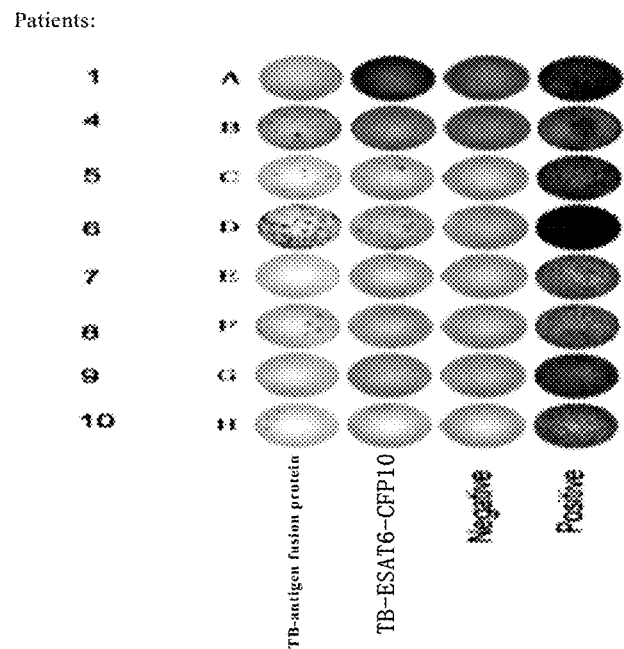
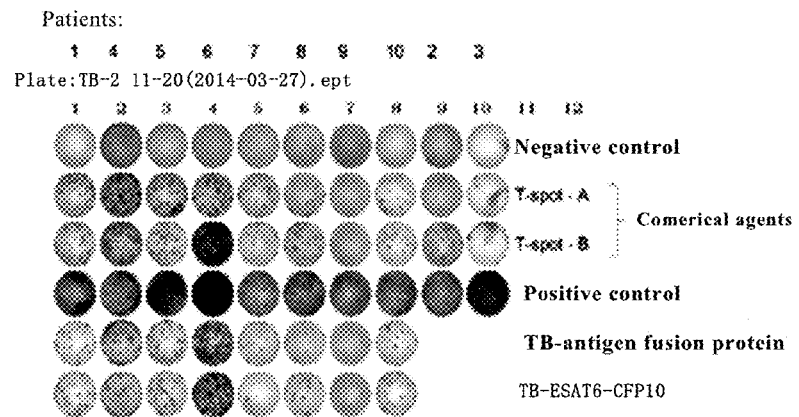


Fig. 6

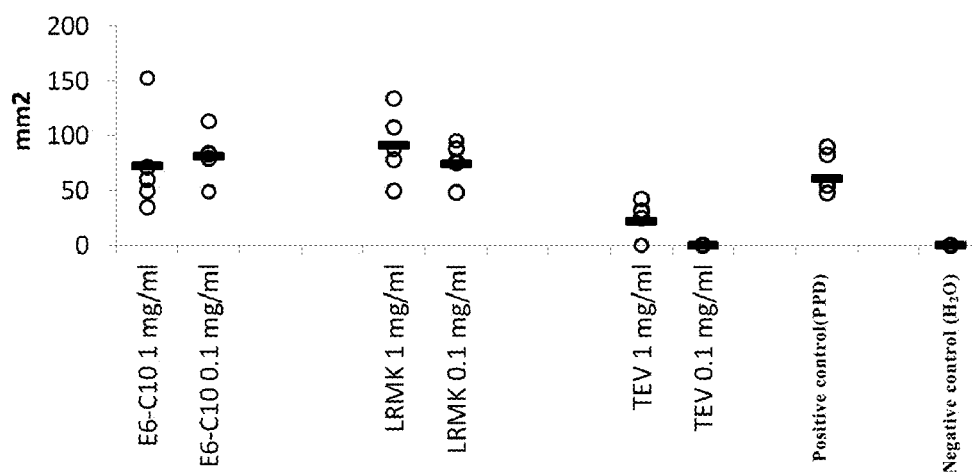


Fig. 7

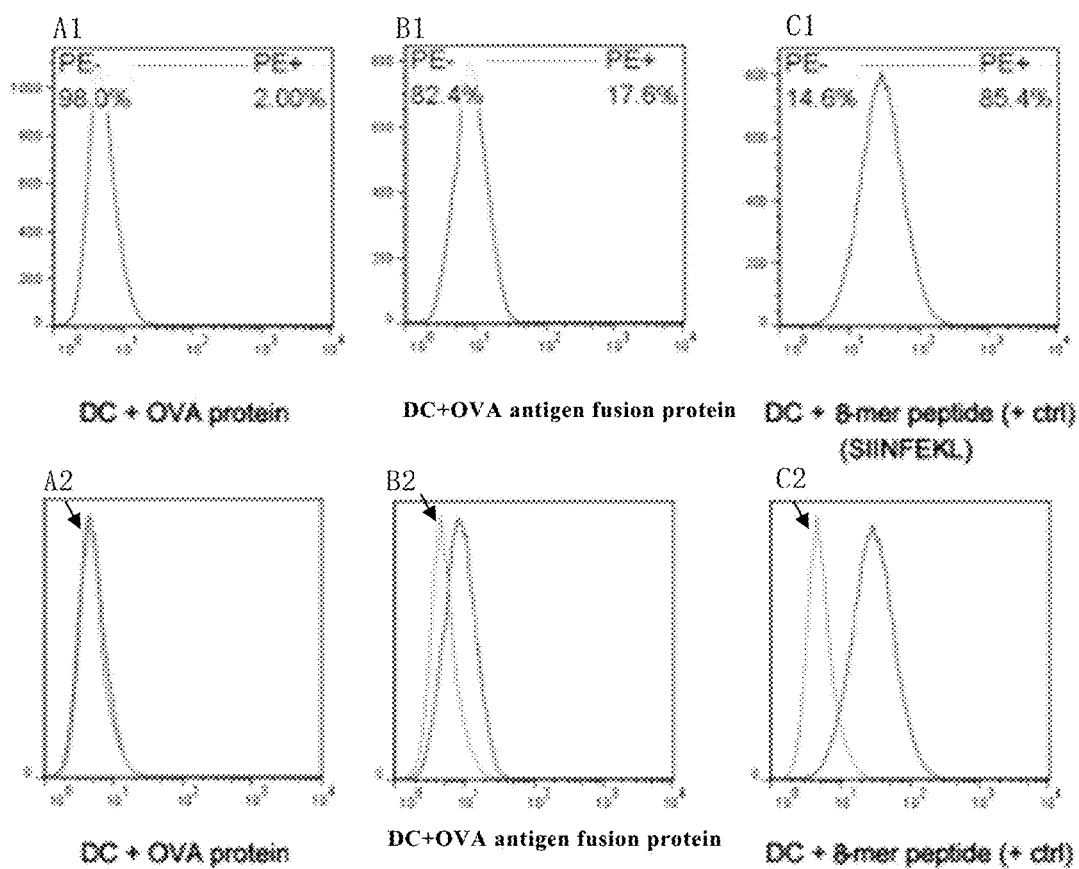


Fig. 8

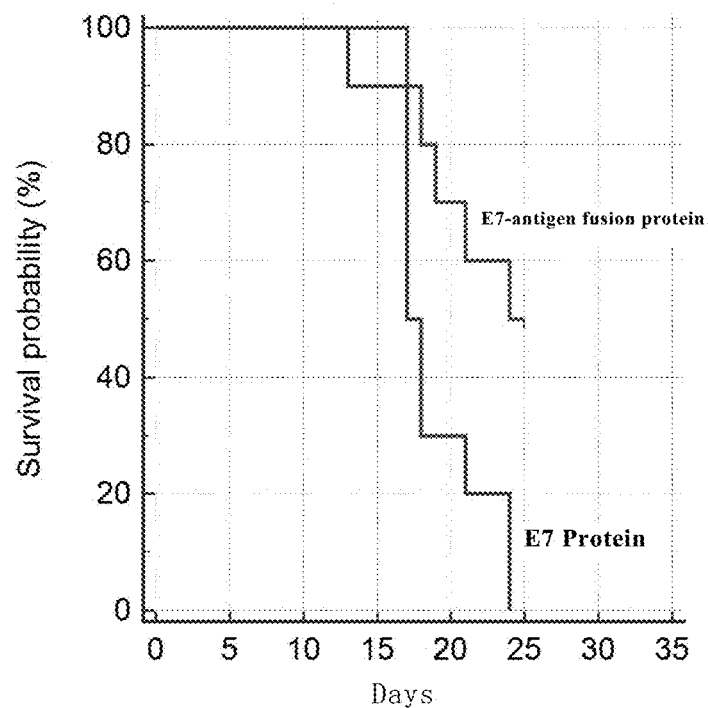


Fig. 9

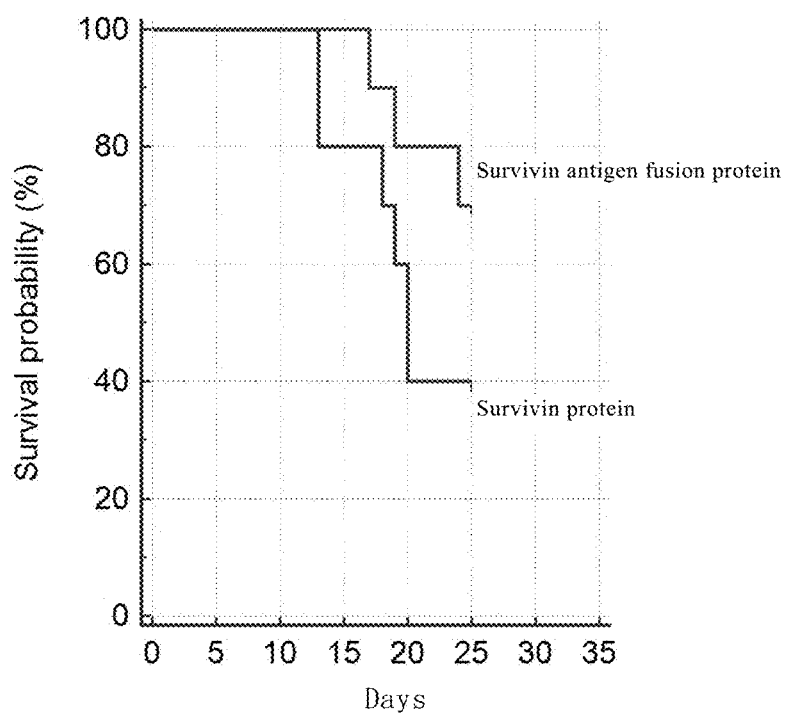


Fig. 10

ARTIFICIAL MULTI-ANTIGEN FUSION PROTEIN AND PREPARATION AND USE THEREOF

TECHNICAL FIELD

[0001] The present invention relates to the field of medicine and, more particularly, to an artificial multi-antigen fusion protein capable of simultaneously stimulating CD4⁺ and CD8⁺ T cell immune responses and methods as well as uses thereof. After expressed and purified, the artificial multi-antigen fusion protein of the present invention can be directly used for immuno-diagnosis or as a prophylactic or therapeutic vaccine.

BACKGROUND

[0002] A series of changes in humor immunity and cellular immune will occur in human body due to infection with bacteria, viruses or stimulation by cancer cells. Cellular immunity is important for tumor cells and infectious bacteria and viruses that have infected and entered into cells. After the human body encounters for the first time the above exogenous stimulus, T lymphocytes will be sensitized, and converted into memory T lymphocytes. When the human body comes into contact with the same antigen again, a quickly specific immune response will be produced, including the effect of T lymphocyte response, thereby producing a variety of cytokines to exert immune effects (cellular immunity). Because cellular immunity plays an important role in the control and removal of bacteria, viruses and tumors, cellular immune vaccines play a role in the prevention of infection by bacterial and viral and cancer therapy.

[0003] Currently, there is no clinically effective drug for some intracellular infectious bacteria and viruses. Therefore, therapeutic vaccines that stimulate cell immunity play a very important role in controlling tumor and infection by bacterial and virus. At present, US FDA has approved the therapeutic vaccine for the treatment of prostate cancer.

[0004] In addition, monitoring of cellular immunity is critical to the treatment, prognosis of diseases and effectiveness of vaccines.

[0005] A commonly used method for detecting cellular immunity is enzyme linked immunospot assay (ELISPOT), which can detect T cells secreting cytokines on single cell level. For example, cellular immune function can be assessed by detecting the number of T cells secreting cytokines after stimulation by tuberculosis (TB) specific antigen. For tuberculosis, the detection of cellular immunity is one way to diagnose TB infection. In 2008, US FDA has approved a ELISPOT Commercialization Kit T-SPOT for the diagnosis of tuberculosis infection. In these diagnostic kits, a mixture of polypeptides of 18-20 amino acids is basically used as a stimulating source. These polypeptides are phagocytosed by the antigen presenting cells in peripheral blood, processed and present together with their own HLA antigens to the cell surface, and then recognized by T cell receptors of memory T lymphocytes. After being stimulated, T cells produce cytokines for example gamma interferon and therefore, can be detected.

[0006] There are many different clones of T cells in human body, wherein each clone needs to be stimulated by different short peptide with HLA; there are different T cell clones in different human bodies; and there are different HLA antigens in different human bodies. To select T cells from some HLA

population, it is necessary to identify, select T cell epitope antigens from the population. Identification of HLA phenotypes and T cell epitope sequences are time-consuming and laborious work. To cover all T cell recognition epitopes of all populations and certain stimulating sources (proteins), the most common way is to synthesize a polypeptide of 18-20 amino acids or even more in length. However, for being clinically used, it is necessary to establish a strict production process and quality testing standards for each polypeptide. For example, for ESAT6 (96 amino acids) and CFP10 (100 amino acids) to be used in TB detection, a mixture of more than 20 polypeptides is typically required to cover all epitopes of the two protein antigens, resulting in high cost and expensive kits, and therefore limiting, to a certain extent, its wide application. An alternative strategy is to select a limited number of polypeptides as a source of stimulation, however, due to HLA polymorphism among different populations and difference in antigen epitopes recognized by T cell, epitope coverage is reduced by this strategy despite the reduced cost saving.

[0007] Prophylactic and therapeutic vaccines are delivered to antigen-presenting cells, especially dendritic cells (DC), and then stimulate and elicit antigen-specific cytotoxic CD8⁺ (CTL) and helper CD4⁺ T lymphocytes. Helper CD4⁺ T cells can effectively stimulate and amplify cytotoxic CD8⁺ T cells and help B cells to produce antibodies. CD8⁺ T cells can specifically recognize and dissolve target cells containing target antigens. Activation of specific CD8⁺ T cells depends on the antigen that can be efficiently presented to MHC class I molecule (HLA-I antigen in humans). CD8⁺ cytotoxic T lymphocytes are the main active ingredient in prophylactic and therapeutic cellular immune vaccines because they can directly recognize and destroy tumor cells or cells infected by viruses. Therefore, most of therapeutic vaccines are designed according to how to stimulate the body to produce CD8⁺ T cells specifically recognizing a tumor or viral antigen.

[0008] At present, it is commonly believed that only the antigen in cytoplasm of antigen-presenting cells (APC) can be degraded into small molecule peptides by proteasome and transported into endoplasmic reticulum by TAP protein, wherein CTL epitope polypeptide binds to MHC-I (HLA-I), is presented to the surface of antigen presenting cells, and binds to specific receptor of CD8⁺ T cells, thereby specifically activating the latter. At the same time, it is also a traditional point of view that only the protein antigen transcribed and translated in cytoplasm can enter MHC-I (HLA-I) presentation pathway. Currently, it is also the core strategy for designing therapeutic vaccines, that is, development of vaccines that can be replicated, transcribed and translated in cytoplasm. If a protein antigen is phagocytosed by a cell, the antigen will not enter cytoplasm, but into endosome and lysosomes; therefore, the protein antigen can not effectively stimulate CD8⁺ T cells.

[0009] Currently, vaccines which can effectively stimulating CD8⁺ T cells include DNA vaccines, epitope polypeptide vaccines and bacterial or viral vector vaccines. These vaccines have some obvious drawbacks: the efficiency of DNA vaccines to enter cells is low and there is a risk of integration into genome; a single epitope polypeptide vaccine covers a very small population, thus reducing its use; bacterial/viral vector vaccine has side effects, and immunity against the vector itself is prone to be elicited in the body, thus reducing the immunity against the target antigen. Addi-

tionally, many populations may possess immunity against these bacteria/viruses before they are immunized, and therefore the vaccine is destroyed before it takes effect, thereby significantly reducing its uses.

[0010] Protein vaccine is a safe vaccine since a protein can not be self-replicated. In general, proteins are phagocytosed into endosomes and lysosomes by antigen presentation cells (APC), so that they won't enter cytoplasm and can not be degraded by cytoplasmic proteasomes and presented to MHC-Class I molecules, and the protein antigen vaccine does not stimulate CTL response. A protein is phagocytosed into APC cells, then enters lysosome, and is degraded into small molecule peptides, and then some polypeptides (CD4 epitopes) bind to MHC-Class II molecules on lysosomal membrane and are presented to cell surface to stimulate CD4⁺ T cells. CD4⁺ can help B cells produce antibodies. Additionally, a protein vaccine can stimulate B cells. Therefore protein antigens have been mainly used to stimulate body to produce antibodies. Studies have found that APC can also intake antigens from outside, and present them to MHC-class I molecules, thus stimulating CTL. This process is called antigen cross presentation. However, the efficiency of this process is very low, so that no one can design a protein vaccine which can stimulate CD8⁺ T cell immune.

[0011] Similarly, the best way for preparing a wide range of peptide-based vaccines is to prepare a series of polypeptides due to the limitation on human HLA, so that their sequences can cover all CD4 and CD8 epitopes of a protein, however, it is necessary to establish a strict production process and quality testing standards for each polypeptide, resulting in high cost and difficulties in industrialization.

[0012] Summing up, currently there is no satisfactory vaccines that can stimulate CD8⁺ T cell immunity. Therefore, there is an urgent need in the art for developing new vaccines that are effective in stimulating CD8⁺ T cell immunity, easily quality-controlled and of high safety.

SUMMARY OF THE INVENTION

[0013] It is an object of the present invention to provide a protein vaccine with advantages of effectively stimulate CD8⁺ T cell immunity, easily quality-controlled and of high safety.

[0014] In the first aspect of the present invention, an artificial multi-antigen fusion protein is provided, wherein, when administered to a mammalian subject, the artificial multi-antigen fusion protein simultaneously stimulates CD4⁺ and CD8⁺ T cell immune responses in said subject.

[0015] In another preferred embodiment, the CD4⁺ T cell immune response and CD8⁺ T cell immune response result in the recognition of target cells carrying the antigen on the cell surface by the T cells.

[0016] In another preferred embodiment, the antigen in the artificial multi-antigen fusion protein is derived from a virus, a bacterium, a parasite, a *chlamydia*, a tumor cell, or a combination thereof.

[0017] In another preferred embodiment, the artificial multi-antigen fusion protein comprises ≥ 3 , preferably ≥ 5 , more preferably ≥ 10 antigen segments.

[0018] In another preferred embodiment, the upper limit of the number of antigen segments is ≤ 200 , preferably ≤ 100 , more preferably ≤ 50 .

[0019] In another preferred embodiment, each antigen segment may be different, partially identical, or completely identical.

[0020] In another preferred embodiment, each antigen segment is from the same or different pathogens, or from the same or different species, or each antigen segment is an artificial sequence (i.e., artificially designed sequence, which is not present in nature).

[0021] In another preferred embodiment, each antigen segment is from a protein or a plurality of different proteins.

[0022] In another preferred embodiment, each antigen segment is from a viral antigen, a bacterial antigen, a parasitic antigen, a chlamydial antigen, a tumor antigen, or a combination thereof.

[0023] In another preferred embodiment, each antigen segment comprises at least one (preferably at least 2) CD8⁺ epitope or a motif sequence capable of stimulating CD8⁺ T cell immune response; and at least one (preferably at least 2) CD4⁺ epitope or a motif sequence capable of stimulating CD4⁺ T cell immune response.

[0024] In another preferred embodiment, each antigen segment comprises at least one (preferably at least 2) amino acid sequence simultaneously serving as CD8⁺ epitope and CD4⁺ epitope.

[0025] In another preferred embodiment, each antigen segment is of 8-50 amino acids, preferably 10-40 amino acids, more preferably 15-35 amino acids in length.

[0026] In another preferred embodiment, the artificial multi-antigen fusion protein further comprises sequences of cleavage site located between antigen segments.

[0027] In another preferred embodiment, the sequence of cleavage site comprises a cleavage site of cathepsin.

[0028] In another preferred embodiment, the cleavage site of cathepsin is selected from a group consisting of a cleavage site of cathepsin S (e.g., Leu-Arg-Met-Lys or a similar cleavage site), a cleavage site of cathepsin B (e.g., Met-Lys-Arg-Leu or a similar cleavage site), a cleavage site of cathepsin K (e.g., His-Pro-Gly-Gly or a similar restriction site), or combinations thereof.

[0029] In another preferred embodiment, the cleavage site of cathepsin S is selected from a group consisting of Arg-Cys-Gly ↓-Leu, Thr-Val-Gly ↓-Leu, Thr-Val-Gln ↓-Leu, X-Asn-Leu-Arg ↓, X-Pro-Leu-Arg ↓, X-Ile-Val-Gln ↓ and X-Arg-Met-Lys ↓; wherein each X is independently any natural amino acid, and ↓ represents cleavage position.

[0030] In another preferred embodiment, the cleavage site of cathepsin S is X-Arg-Met-Lys (e.g., Leu-Arg-Met-Lys), X-Ile-Val-Gln, or a combination thereof.

[0031] In another preferred embodiment, each antigen segment is directly connected in the artificial multi-antigen fusion protein via said sequence of cleavage site.

[0032] In another preferred embodiment, the sequence of cleavage site used to connect each antigen segment is the same or different.

[0033] In another preferred embodiment, the sequence of cleavage site used to connect each antigen segment is the same.

[0034] In another preferred embodiment, the sequence of cleavage site is not contained in each antigen segment; or the sequence of cleavage site is contained in the antigen segment, while at least one cleavage product (or some or all of the cleavage products) formed after the antigen segment is digested can still be used as CD8⁺ epitope or CD4⁺ epitope.

[0035] In another preferred embodiment, the artificial multi-antigen fusion protein further comprises a sequence of one or more optional elements selected from a group consisting of:

[0036] (a) a label sequence (e.g., 6His for purification);

[0037] (b) a signal peptide sequence;

[0038] (c) a membrane-penetrating sequence (e.g., CPP)

[0039] (d) an adjuvant element sequence (e.g., LRMK);

[0040] (e) a cell necrosis inductive factor sequence.

[0041] In another preferred embodiment, the artificial multi-antigen fusion protein is of 100-2000 amino acids, preferably 150-1500 amino acids, more preferably 200-1000 amino acids or 300-800 amino acids in length.

[0042] In another preferred embodiment, the mammal includes human, domestic animal (e.g., cattle, sheep, pig), pet (e.g., dog, cat), rodent, rabbit, monkey and the like.

[0043] In another preferred embodiment, the antigen segment in the antigen fusion protein covers $\geq 10\%$, $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$, $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, more preferably 100% of the amino acid sequence of one or two, or more target proteins.

[0044] In another preferred embodiment, the fusion protein is shown in the structure of formula I:



[0045] Wherein,

[0046] A is an antigen segment;

[0047] C is a sequence of cleavage site of cathepsin;

[0048] n is a positive integer ≥ 3 ;

[0049] Y is absent or is a sequence represented by "Y0-B", wherein Y0 is a signal peptide sequence, a tag sequence, a membrane-penetrating element sequence, an adjuvant element sequence, a cell necrosis-inductive element sequence, or any combination of the above sequences, and B is absent or a sequence of cleavage site;

[0050] Z is absent, or a tag sequence, a membrane-penetrating element sequence, an adjuvant element sequence, a cell necrosis-inductive element sequence, or any combination of the above sequences;

[0051] provided that when Z is absent, C in the last "A-C" can be absent.

[0052] In another preferred embodiment, the cleavage site sequence is different from C (i.e., $B \neq C$).

[0053] In another preferred embodiment, the cleavage site sequence is identical to C (i.e., $B = C$).

[0054] In another preferred embodiment, n is any integer from 5 to 100, preferably from 6 to 50, more preferably from 7 to 30.

[0055] In the second aspect of the present invention, a composition is provided, comprising the artificial multi-antigen fusion protein according to the first aspect of the invention and a pharmaceutically acceptable carrier.

[0056] In another preferred embodiment, the composition includes a pharmaceutical composition and a vaccine composition.

[0057] In another preferred embodiment, the dosage form of the composition includes: an injection, a lyophilized powder, a liquid formulation, an oral formulation, or a transdermal formulation.

[0058] In the third aspect of the present invention, use of the artificial multi-antigen fusion protein according to the first aspect of the present invention is provided, which is used for the preparation of a prophylactic and/or therapeutic vaccine composition or pharmaceutical composition.

[0059] In another preferred embodiment, the vaccine composition includes an anti-pathogen (e.g., a virus) vaccine composition, and an anti-tumor vaccine composition.

[0060] In another preferred embodiment, the pharmaceutical composition is used for the treatment and/or prevention of a disease selected from the group consisting of a bacterial associated disease, a virus associated disease, an autoimmune disease, a tumor associated disease, or a combination thereof.

[0061] In the fourth aspect of the present invention, use of the artificial multi-antigen fusion protein according to the first aspect of the present invention is provided, which is used for preparing an agent or a kit for detecting specific T cell immunity.

[0062] In another preferred embodiment, the specific T cell immunity includes $CD4^+$ T cell immunity and $CD8^+$ T cell immunity.

[0063] In another preferred embodiment, such agent or kit is used in skin tests.

[0064] In the fifth aspect of the present invention, a method (e.g., a therapeutic or prophylactic method) is provided, comprising steps of: administering the artificial multi-antigen fusion protein according to the first aspect of the present invention or the composition according to the second aspect of the present invention to a subject in need thereof.

[0065] In another preferred embodiment, the method is used for the treatment and/or prevention of an infection of pathogen or a tumor.

[0066] In another preferred embodiment, the subject includes both human and non-human mammals.

[0067] In another preferred embodiment, the manner of administration includes intravenous, subcutaneous, oral, transdermal administration, and the like.

[0068] It is to be understood that, within the scope of the present invention, the above-described technical features of the present invention and the technical features specifically described in the following (e.g., examples) may be combined with each other to form a new or preferred technical solution, which is not necessary to be listed one by one due to the limited contents.

DESCRIPTION OF DRAWINGS

[0069] FIG. 1 shows cloning sites of pNIC28a-Bsa4 expression vector.

[0070] FIG. 2 shows electrophoresis identification of PCR products of *Escherichia coli* containing ESAT6/CFP10 gene (left) and SDS-PAGE electrophoresis analysis of purified ESAT6/CFP10 multi-antigen fusion protein (right).

[0071] FIG. 3 shows electrophoresis identification of PCR products of *Escherichia coli* containing OVA: 242-352 gene (left) and SDS-PAGE electrophoresis analysis of purified OVA: 242-352 multi-antigen fusion protein (right).

[0072] FIG. 4 shows electrophoresis identification of PCR products of *Escherichia coli* containing HPV16-E7 gene (left) and SDS-PAGE electrophoresis analysis of purified HPV16-E7 multi-antigen fusion protein (right).

[0073] FIG. 5 shows the flow cytometry analysis of normal human PBMC which is treated by HPV16-E7 multi-antigen fusion protein, and the two-parameter scatter plot shows distribution of CD54 and antigen in PBMC. CD54 is labeled with PE and the antigen is labeled with FITC.

[0074] FIG. 6 shows the spots formed after stimulated by ESAT6/CFP10 multi-antigen fusion protein, ESAT6/CFP10 mature protein, and antigens in a commercially-available T-SPOT.TB kit, respectively. The results shows that for stimulation of total T lymphocytes, effects of ESAT6/CFP10 multi-antigen fusion protein and antigens in the commer-

cially-available T-SPOT.TB kit are equivalent (above panel), while for the stimulation of CD8⁺ lymphocytes, effects of ESAT6/CFP10 multi-antigen fusion protein are significantly better than those of ESAT6/CFP10 mature protein (below panel).

[0075] FIG. 7 shows results of skin test for ESAT6/CFP10 multi-antigen fusion protein. In the figure, results of skin test for four groups of guinea pigs sensitized by proteins/polypeptides derived from inactivated *Mycobacterium tuberculosis* H37Rv are shown. 0.1 ml of positive control (PPD), negative control, recombinant ESAT6/CFP10 multi-antigen fusion protein (marked as “LRMK”), recombinant ESAT6/CFP10 mature protein (marked as “E6-C10”) were intradermally injected by alternative skin test, and the area of red and swollen skin at each injection site of guinea pigs was measured 24 hours after injection.

[0076] FIG. 8 shows flow cytometry analysis of distribution of SIINFEKL-MHC-I on DC2.4 cells. Histograms show that SIINFEKL peptide in OVA (242-252) multi-antigen fusion protein can be efficiently presented by DC cells at a concentration of 100 µg/ml (delivery efficiency is 17.6%). Whereas original protein fragments containing SIINFEKL peptide can not be presented to MHC class I molecule.

[0077] FIG. 9 shows survival-time curve for B16-HPV-E7 tumor-bearing mice (the date of inoculation of tumor cell is deemed as day 0).

[0078] FIG. 10 shows survival-time curve for B16-survivin mice (the date of inoculation of tumor cell is deemed as day 0).

MODES FOR CARRYING OUT THE INVENTION

[0079] After extensive and in-depth research as well as continuous and repeated exploration, the present inventors have developed a protein vaccine capable of stimulating CD8⁺ T cell immunity for the first time, and the protein vaccine possesses advantages, such as low production cost and convenient control. Additionally, compared with existing vaccines, the vaccine of the present invention can also simultaneously stimulate CD4⁺ T cell immunity. Experiments demonstrate that the protein vaccine of the present invention is effective in stimulating CD8⁺ cells via MHC-I antigen presenting pathway. The present invention has been completed based on the above findings.

[0080] In particular, the present inventors have unexpectedly found that a multi-antigen fusion protein vaccine connected through cleavage site of cathepsin can be effectively presented by antigen-presenting cells via MHC-I pathway. In vitro and in vivo experiments have shown that the protein vaccine can effectively stimulate CD8⁺ T cells, and also have the ability to stimulate CD4⁺ T cells.

Terms

[0081] As used herein, the terms “protein of the present invention”, “fusion protein of the present invention”, “artificial multi-antigen fusion protein”, “antigen fusion protein”, “fusion protein of the present invention stimulating CD4⁺ and CD8⁺ T cell immune responses” can be interchangeably used, and refer to an artificial recombinant protein having a structure of Formula I and capable of effectively and simultaneously activating CD4⁺ and CD8⁺ T cell immune responses.

[0082] Lysosome and MHC-I Pathway

[0083] Polypeptide antigens of short fragment (such as commercially-available T-SPOT.TB kit used to stimulate antigens of human antigen presenting cells) can not only enter lysosome and can be presented to MHC-II molecules, but also can be absorbed into cytoplasm by presenting cells, processed and presented to MHC-I class molecules. Protein antigens are firstly endocytosed by antigen-presenting cells, and then pass through early phagosome, late phagosome, and finally enter into lysosome. In general, proteins that enter lysosomes are completely degraded to amino acids. However, under certain situation, a protein will gradually degrade, and the resulting polypeptide can bind to MHC-II molecules located at lysosomal to form a relatively stable complex, presented to the cell surface, and stimulate CD4⁺ T cell immunity. In general, protein antigens do not leak from lysosomes into cytoplasm and therefore do not enter MHC-I pathway to stimulate CD8⁺ cells. The present inventors have unexpectedly found that the antigen fusion protein specially designed in the present invention can not only stimulate CD8⁺ T cells, but also retain the original function of stimulating CD4⁺ T cell.

[0084] The major protease in lysosomes of antigen-presenting cells is cathepsin S. Leu-Arg-Met-Lys is a preference sequence for cathepsin S. In the present invention, Leu-Arg-Met-Lys is preferably used to connect a group of antigen segments to form a new artificial multi-antigen fusion protein and stimulate CD4⁺ and CD8⁺ T cells.

[0085] Common antigen polypeptides of short fragment (generally ≤70aa, ≤60aa or shorter) can be directly absorbed into cytoplasm by antigen-presenting cells, and thus presented to MHC-class I molecules. Common protein antigens can only be endocytosed into lysosomes by antigen-presenting cells, and the common mature protein in lysosome only binds to MHC-II, and only stimulates CD4⁺ T cells.

[0086] In contrast, the artificial multi-antigen fusion protein of the present invention is endocytosed into lysosomes by antigen presenting cells, and then cleavage occurs firstly in the ligation sequence in the presence of cleavage sites of cathepsin, such as cathepsin S, thereby rapidly generating a group (plurality) of polypeptides as well as achieving and facilitating cross-presentation, and simultaneously stimulating CD8⁺ T cells and CD4⁺ T cells.

[0087] Experiments of the present invention show that after the fusion protein of the present invention enters lysosomes, the fusion protein still disappears rapidly even lysosomal function is inhibited, indicating that the protein of the present invention or cleavage produces thereof in lysosomes can leak into cytoplasm.

[0088] Fusion Protein that Stimulates CD4⁺ and CD8⁺ T Cell Immune Response

[0089] In the present invention, a single protein, i.e., a multi-antigen fusion protein, is provided, which is formed by connecting a plurality of antigen epitopes (or antigen segments) and used for specific cellular immunoassays and as a prophylactic and therapeutic vaccine.

[0090] In the multi-antigen fusion protein of the present invention, there are at least one, more preferably 2, 3, 4, 5 or more (e.g., any positive integer of 6-20) CD8⁺ T cell epitopes.

[0091] In the multi-antigen fusion protein of the present invention, at least one, more preferably two, three, four, five or more (e.g., any positive integer of 6-20) CD4⁺ T cell epitopes are included.

[0092] In the present invention, the sequence of antigen segment may be from any polypeptide capable of stimulating immune response, preferably a sequence capable of stimulating CD4⁺ or CD8⁺ T cell responses (i.e., T cell epitopes).

[0093] Cathepsin is a group of cysteine proteases, the main role of which is to degrade lysosomal proteins. The known primary function of cathepsin-S is to participate in antigen presentation of MHC-2.

[0094] In the multi-antigen fusion protein of the present invention, these polypeptides containing CD8⁺ T cell epitope and CD4⁺ T cell epitope may be joined by recognition sites of cathepsin in any antigen presenting cell, and preferred Leu-Arg-Met-Lys sequence for cathepsin S is preferably used.

[0095] In another preferred embodiment, several other cleavage sites of cathepsin B and K, which are relatively more expressed in presenting cells, may be used; for example, Met-Lys-Arg ↓-Leu is the cleavage site of cathepsin B, and His-Pro-Gly ↓-Gly is the cleavage site of cathepsin K. However, the effect of cleavage site of cathepsin S is particularly excellent.

[0096] In another preferred embodiment, different cleavage sites of cathepsin S can be used in the artificial multi-antigen fusion protein, including, but not limited to, Arg-Cys-Gly ↓-Leu, Thr-Val-Gly ↓-Leu, Thr-Val-Gln ↓-Leu, X-Asn-Leu-Arg ↓, X-Pro-Leu-Arg ↓, X-Ile-Val-Gln ↓ and X-Arg-Met-Lys ↓; wherein X-Arg-Met-Lys ↓ and X-Asn-Leu-Arg ↓ are preferred linking cleavage sites, where X is any amino acid and ↓ is the cleavage site.

[0097] Any protein may be used into the present invention. In general, the sequence covered by the multi-antigen fusion protein accounts for more than 40% of the protein, and in a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, preferably 100% of the sequence of protein antigen is covered by the antigen fusion protein.

[0098] As a preferred example of the present invention, the coverage of HPV16-E7 multi-antigen fusion protein is 100%. Of course, even if the coverage is reduced, it is sometimes feasible, especially when the MHC-I antigen epitope in a target protein is less and distinct. For example, in a preferred embodiment of the present invention, the coverage of OVA is 40% because OVA has only a single MHC-I antigen epitope and the selected fragment includes the epitope.

[0099] The multi-antigen fusion protein may cover more than one antigenic protein. In another preferred embodiment, the antigen fusion protein of *tubercule bacillus* of the present invention covers both ESAT6 and CFP10 protein sequences, which may reduce the cost of production and facilitate quality control.

[0100] The artificial multi-antigen fusion protein of the present invention is different from natural proteins (such as proteins or tumor antigens from viruses, bacteria, parasites). One of the main differences is the loss of spatial structure of the natural protein and therefore does not possess the function of a natural protein, thus avoiding the potential hazards of, for example, pathogen proteins (such as viral capsid proteins).

[0101] The artificial multi-antigen fusion protein of the present invention is also different from the antigen polypeptide of short fragment (e.g., antigen peptide and epitope polypeptide of ≤70 or ≤60 amino acids). One of the main differences is that the protein of the invention has a large

molecular weight and belongs to a macromolecule protein (rather than a small molecule polypeptide), and thus the pathway into a cell is not the same, resulting in that the pathway through which the antigen is presented, and the mode and mechanism for stimulating T cells are different from those of the antigen polypeptides of short fragment.

[0102] Preparation Method and Engineered Cell

[0103] Once the antigen protein sequence is determined, the protein of the present invention can be obtained by mass production using recombinant method. Generally, encoding DNAs artificially synthesized are cloned into an expression vector, transferred into a cell and then isolated from a host cell or a fermentation product by conventional methods.

[0104] A typical method for preparing an antigen fusion protein comprises steps of:

[0105] Constructing an artificial multi-antigen fusion protein according to the amino acid sequence of the protein antigen, and the multi-antigen fusion protein comprises or consists of a series of antigen segments of a specific length, wherein each antigen segment is connected by the same or different cleavage sites of cathepsin (e.g., leu-Arg-Met-Lys).

[0106] In addition, the cells used to express the fusion protein of the present invention are also included in the present invention. "Host cells" include prokaryotic cells and eukaryotic cells. Commonly used prokaryotic cells are *E. coli*. Commonly used eukaryotic cells include, but are not limited to, yeast cells, insect cells, and mammalian cells. As a preferred embodiment of the present invention, the used host cells are *Escherichia coli*, such as BL21 (DE3) and yeast, and CHO cells.

[0107] Vaccine Composition and Pharmaceutical Composition

[0108] In the present invention, the use of the fusion proteins of the invention is provided, for example, for immuno-diagnosis and preparation of vaccines.

[0109] Immunological diagnosis includes skin test and T cell dot enzyme immunoassay. The skin test is to immunize a subject with the multi-antigen fusion protein of the present invention, and then the skin reaction of the individual is observed to determine whether the individual has been exposed or infected with similar antigens. As a preferred embodiment of the present invention, the immuno-diagnosis is to use the fusion protein of the present invention as a source of stimulation to detect T cell immune response of an individual. For example, generation of gamma interferon after stimulating T cells of an individual with ESAT6-CFP10 multi-antigen fusion protein of the present invention can be observed to determine whether a patient is infected with tuberculosis or infection stage or prognosis.

[0110] Vaccines include prophylactic vaccines and therapeutic vaccines. The latter is mainly related to the activation of T cells, especially CD8⁺ killer T cells (CTL). The activation of CTL cells is limited by MHC-I molecules. As a preferred embodiment of the present invention, it has been demonstrated in vitro that HPV16-E7 multi-antigen fusion protein of the present invention can be endocytosed by human antigen presenting cells and activate antigen presenting cells. In another preferred embodiment, the OVA multi-antigen fusion protein of the present invention is used as a model to demonstrate that it can be processed by antigen presenting cells and presented to MHC class I molecules.

[0111] Uses

[0112] In the fusion protein of the present invention, recognition sequences of cathepsin in the endosome and

lysosome of antigen presenting cells are used to connect a plurality of antigen epitope peptides containing CD4⁺ and CD8⁺ epitopes into a single protein, which, after expression and purification, can be directly used in immune diagnosis or as a prophylactic and therapeutic vaccine.

[0113] When used as a prophylactic and therapeutic vaccine, the protein or composition of the present invention can be injected into an animal directly or with an adjuvant or used in vitro with a cell therapy. For example, it is added into DC/NK cell culture to stimulate the production of specific antigen presenting cells and then to stimulate the production of specific CD8⁺ T cells (CTL) in vivo/in vitro with the antigen presenting cells.

[0114] In the present invention, these externally sensitized specific antigen presenting cells and/or in vitro produced CTL antigens can be returned to the body of a corresponding subject for antiviral, antitumor and other different uses.

[0115] In the present invention, the protein or in vitro sensitized cells (or corresponding formulation) of the invention are preferably administered to a subject by the following administration mode: intravenous injection, perfusion, subcutaneous injection, transdermal administration, and the like.

[0116] Main advantages of the present invention include:

[0117] (1) It has been found for the first time that the artificial multi-antigen fusion protein of the present invention can be efficiently presented onto MHC-I molecules.

[0118] (2) It has been found for the first time that a single multi-antigen fusion protein instead of several antigen short peptides, can specifically stimulate human T cells to release gamma interferon.

[0119] (3) It has been demonstrated for the first time that the antigen fusion protein of the present invention can be phagocytosed by antigen-presenting cells and activate CD8⁺ cells.

[0120] (4) The fusion protein of the present invention can be used in the development of reagents and techniques for immuno-diagnosis. For example, specific cellular immunity is detected by skin tests, or specific cellular immunity is detected by ELISPOT assays.

[0121] (5) The fusion protein of the present invention can be used in the development of therapeutic or prophylactic drugs or vaccines, including DC cells sensitized by the fusion protein of the present invention and/or CTL cell preparation, and the industrial application value is immeasurable.

[0122] (6) Compared with a conventional solution using a group of short fragment polypeptides, the cost of the method

and product of the present invention is significantly reduced (typically at least 80% or more); and quality control is easy and huge amount of manpower, material and time can be saved.

[0123] The invention will be further illustrated with reference to the following specific examples. It is to be understood that these examples are only intended to illustrate the invention, but not to limit the scope of the invention. For the experimental methods in the following examples without particular conditions, they are performed under routine conditions, such as conditions described in Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989, or as instructed by the manufacturer. Unless otherwise stated, the percentages and parts are percentages by weight and parts by weight.

Example 1

[0124] Clone of Multi-Antigen Fusion Protein

[0125] The antigen fusion protein was designed according to the amino acid sequence of the protein. The antigen fusion protein was formed by connecting a series of polypeptide fragments (short fragment antigen peptide) of 25-35 amino acids in length, and the antigen peptides are ligated by the same preferred sequence of cathepsin S (Leu-Arg-Met-Lys).

[0126] Codons in the DNA encoding the antigen fusion protein are optimized into *E. coli* preferred codons. DNA coding sequences were prepared by whole artificial synthesis and TACTCCCATATATAT (SEQ ID NO.: 7) was added at 5'-end and TATCCACCTTTACTGTTA (SEQ ID NO.: 8) was added at 3'-end. Synthesized DNA molecules were treated with T4 DNA polymerase and dCTP for 30 minutes.

[0127] A conventional pNIC28-Bsa4 vector (obtained from Oxford University; U.S. Pat. No. 8,148,100 B2; GenBank ID: EF198106) was digested with BsaI for 1 hour. The important components involved in the expression of the clone in this vector are shown in FIG. 1. The linearized vector was isolated by 1% agarose gel electrophoresis and treated with T4 DNA polymerase and dGTP for 30 minutes. After two products from T4 DNA polymerase treatment were mixed, regular competent *E. coli* DN5α was transformed, and inoculated into a plate, and mono colony was picked up for culture. Positive colonies were identified by PCR performed on the culture.

[0128] Results are shown in FIGS. 2, 3 and 4, indicating that the size of the coding sequence of the obtained fusion protein and molecular weight of the protein are consistent with the design or predicted value.

[0129] The amino acid and nucleotide sequences of each multi-antigen fusion protein are shown as below.

Name of Protein	Amino acid sequence	Nucleotide sequence	
ESAT6-CFP10 antigen fusion protein (also named as "TB-antigen fusion protein")	SEQ ID NO.: 1	SEQ ID NO.: 2	
OVA antigen fusion protein	SEQ ID NO.: 3	SEQ ID NO.: 4	
HPV16-E7 antigen fusion protein	SEQ ID NO.: 5	SEQ ID NO.: 6	
SEQ ID NO.: 1			
MAEMKTDAA	LAQEAGNFER	ISGDLTKTQID QVESTLRMKT QIDQVESTAG SLQGQWRGAA	60
GTAAQAQAVVR	PQELRMKAQA	AVVRFQEAAN KQKQELDEIS TNIRQAGVQY SRLRMKIRQA	120
GVQYSRADEE	QQQALSSQMG	FLRMKMTEQQ WNFAGIEAAA SAIQGNVTSI HSLLDEGKQS	180
LRMKHSLLE	GKQSLTKLAA	AWGGSSEAY QGVQKWDAL RMKYQGVQKQ WDATATELNN	240
ALONLARTIS	EAGQAMASLR	MKISEAGOAM ASTEGNVTGM FA	282

-continued

Name of Protein	Amino acid sequence	Nucleotide sequence
SEQ ID NO.: 2		
caaatcgatc	aagtggaaag	taccgcaggt
ggcaccgcag	cacaggcagc	agttgttcgt
gccgtggtgc	gcttccaaga	agccgcaa
accaaattc	gtcaggcagg	cgttcagtat
gggtgtcagt	attcacgtgc	agatgaagaa
tttttaagaa	tgaaaatgac	cgagcagcag
agcgcaattc	agggtaatgt	taccagcatt
ctgcggatga	agcatagtct	gttagatgaa
gcattgggtg	gtagcggtag	cgaagcatat
cgtatgaagt	atcagggcgt	gcaacaaaag
gcactgcaga	atctggcagc	taccattagt
atgaagattt	ctgaagcagg	ccaagctatg
tttgcataa		
SEQ ID NO.: 3		
MLVLLPDEVS	GLEQLESIIN	FEKLTEWTSS
YLPKMKMEEK	YNLTSVLMAM	GITDVFSSSA
RMKISQAVHA	AHAEINEAGR	EVVGSAAEAGV
SEQ ID NO.: 4		
atgctggttc	tgctgccgga	tgaagttagc
tttgaaaaac	tgaccgaatg	gaccagcagc
ttcgagaaac	tgacagagtg	gacaagcagc
tacctgcctc	gcattgaaat	ggaagagaaa
gggtattaccg	atgttttttag	cagcagcgca
ctgaaaatta	gccaggcagc	tcattgcagc
cggatgaaaa	tttcacaggc	cgtgcattgt
gaagtgtgtg	gtagtgcgca	agccggtgtt
SEQ ID NO.: 5		
MHGDTPTLHE	YMLDLQPETT	DLYCYEQLND
PDRAHYNIIVT	FCCKLRMKHY	NIVTFCKKCD
LEDLLMGTGLG	IVCPICSQKP	
SEQ ID NO.: 6		
atgcatggtg	ataccccgac	cctgcattgaa
gatctgtatt	gttatgagca	gctgaatgat
cagctgaacg	attcaagcga	agaagaggac
ccggatcgtg	cacattacaa	cattgtttacc
aatatcgtga	ccttcctgctg	taaatgtgat
catgttgata	ttcgtacatt	agaggacctg
ctggaagacc	tggttaatggg	caccctgggt
taa		

[0130] In addition, LC-MS analysis also showed that the measures molecular weight of purified TB-antigen fusion protein (30974 Da), OVA antigen fusion protein (16589 Da) and HPV16-E7 antigen fusion protein (16231 Da) were consistent with the predicted values.

Example 2

[0131] Expression of Antigen Fusion Protein

[0132] Plasmid DNA of positive colonies containing encoding sequence of the artificial multi-antigen fusion protein was extracted and transformed into *Escherichia coli* BL21 (DE3).

[0133] Transformed single colonies were inoculated into low salt LB broth and incubated overnight at 37° C. The culture was diluted at 1:100 in low salt LB broth, incubated at 37° C. with shaking until OD₆₀₀=1.0 and cooled to 18° C. 0.2 mM IPTG was added for inducing expression of protein. Cells were incubated at 18° C. for another 16 hours

and then centrifuged at 4000 rpm. The cells were collected and re-suspended in phosphate buffer.

Example 3

[0134] Purification of Antigen Fusion Protein

[0135] (a) Purification of ESAT6-CFP10 and OVA Antigen Fusion Protein

[0136] Cells were lysed by ultrasonic method and inclusion bodies were collected by centrifugation. The inclusion bodies were dissolved in denaturation buffer (8 M urea in HEPES buffer at pH 7.4) and passed through a Ni-NTA column, and the antigen fusion protein containing histidine label bound to the column. After impurity proteins were removed by sufficient washing, the antigen fusion protein was eluted with urea elution buffer. The eluted antigen fusion protein was firstly diluted 8-fold with PBS containing 0.5 M arginine (pH 9.5) and the urea was removed through dialysis against PBS (right panel in FIG. 2 and right panel in FIG. 3).

[0137] (b) Purification of HPV16-E7 Antigen Fusion Protein

[0138] The cells were lysed by ultrasonic method. The supernatant containing target proteins was collected by centrifugation and passed through a Ni-NTA column. The histidine-labeled antigen fusion protein was bound to the column. After impurity proteins were removed by sufficient washing, HPV16-E7 antigen fusion protein was eluted with an elution buffer containing imidazole (right panel in FIG. 4).

Example 4

[0139] Intake of Antigen Fusion Protein by Antigen Presenting Cells

[0140] Human peripheral blood lymphocytes PBMCs were isolated from blood of voluntary blood donors by Ficoll and washed. The cell concentration was adjusted to 2×10^6 /ml in cell culture medium and 5 ml of cell suspension was placed in a cell culture flask. HPV16-E7 antigen fusion protein (50 μ g/ml) was added or FITC-HPV16-E7 (5 μ g/ml) was added at the same time, and cultured hours at 37° C. and 5% CO₂ for 24 hours and for another 12 hours. Cells were stained with anti-human CD54 antibody at 4° C. for 30 minutes and analyzed by flow cytometry. After treated for 13 hours, the cells were treated by EDTA, harvested and washed with 1 ml of ice-cold FACS buffer (2% FCS in PBS) for two times. And then a commercially available PE-tagged monoclonal antibody 25.D1-16 was added to a concentration of 0.6 μ g/ml, and incubate at 4° C. for 30 min. Cells were washed for three times with FACS buffer and the cells were re-suspended in 0.2 ml of FACS fixation buffer (BD) and analyzed by flow cytometry.

[0141] Results showed that the major cells which phagocytose multi-antigen fusion protein were CD54 positive cells (FIG. 5).

Example 5

[0142] Detection of γ -Interferon Secreting T Cells in Patients with Antigen Fusion Protein

[0143] In this example, the ability of TB-antigen fusion protein to stimulate CD8⁺ T cells was tested by γ -interferon release assay. The method is described as below:

[0144] After peripheral blood mononuclear cells were isolated from tuberculosis sputum smear positive patients, the cell concentration was adjusted to 5×10^6 live cells/ml. 50 μ l of the above mentioned cell suspension was added into negative wells, positive wells (with CONA as stimulating source), detection wells (With TB-antigen fusion protein as stimulating source), and control wells (with antigen T-spot A and T-spot B in the commercially available T-SPOT.TB kit from Oxford Immunology Technology Ltd., as stimulating source; or with mature CFP10-ESAT6 fusion natural protein (marked as TB-ESAT6-CFP10) as control stimulating source)) respectively.

[0145] Cells were incubated at 37° C., 5% CO₂ for 16-20 hours, plates were washed, and 50 μ l of labeled antibody working solution was added, and incubated at 2-8° C. for 60 minutes. Afterwards, the plates were washed, and 50 μ l of BCIP INBT substrate solution was added, incubated at room temperature in darkness, dried, and counted. Results were judged according to the instruction of T-SPOT.TB kit: when the number of spots in a blank control well is 0-5, the number of spots in an antigen-containing well—the number

of spots in a blank control well ≥ 6 ; or when the number of spots in a blank control well is 6-10, the number of spots in an antigen-containing well ≥ 2 times of the number of spots in a blank control well.

[0146] The results showed that effects obtained from stimulation with ESAT6/CFP10 antigen fusion protein was consistent with those obtained from stimulation with antigens in T-SPOT.TB kit (FIG. 6).

[0147] In addition, after CD4⁺ T cells of the patient were removed by using specific anti-CD4 magnetic beads, remaining T cells were mainly CD8⁺ T cells, and multi-antigen fusion protein and CFP10 mature protein were compared on this basis.

[0148] Results showed that TB-antigen fusion protein of the present invention exerted a significant stimulating effect on CD8⁺ T cells. In contrast, the wild-type sequence of ESAT6-CFP10 mature protein exerted little effect on CD8⁺ T cells (FIG. 6).

Example 6

[0149] In Vitro Induction of Specific CD8⁺ T Cells by Antigen Fusion Protein-Loaded DCs

[0150] Preparation of antigen-loaded DC cells: After mononuclear peripheral cells (PBMC) were isolated, part of the cells were frozen and the remaining cells were adjusted to a cell concentration of 1×10^7 cells/mL and incubated in conventional AIM-V culture for 2 hours. Suspended cells were removed (cryo-preserved) and AIM-V medium containing GM-CSF (1000 IU/ml) and IL-4 (50 IU/ml) was added and incubated at 37° C., 5% CO₂. HPV16-E7 antigen fusion protein (50 μ g/ml) or recombinant HPV16-E7 protein (50 μ g/ml) was added at day 3.

[0151] CD8⁺ T cells were sensitized in vitro by antigen-loaded DCs: At day 5, adherent DC cells were collected, cryo-preserved PBMCs were thawed, in which CD8-positive cells were separated with CD8 beads, and CD8-negative cells were frozen. CD8 positive cells: DC cells were co-cultured in a 5:1 ratio and IL-2 100 IU/ml, IL-7 25 IU/ml were added. In addition, IL-2 (100 IU/ml) and IL-7 (25 IU/ml) were added every 2 days or half-volume of fluid was changed. On day 7, the frozen CD8-negative cells were thawed and treated with mitomycin. Afterwards, CD8-negative cells were added according to $\frac{1}{10}$ of the number of CD8⁺ T cells for secondary stimulation. 50 μ g/ml of corresponding protein was supplemented, and IL-2 100 IU/ml, IL-7 25 IU/ml were supplemented. IL-2 100 IU/ml and IL-7 25 IU/ml were supplemented every two days.

[0152] Preparation of antigen presenting cells: the suspended cells frozen at day 1 were thawed, and incubated in a plate coated with CD19 monoclonal antibody for 2 h. Non-adherent cells were discarded, CD19 positive cells were suspended in the culture medium. The cell concentration was adjusted to 5×10^5 /ml, 2 ml of the cell suspension was cultured at 37° C., 5% CO₂ for 48 h, and IL-4 was added to a concentration of 100 μ g/ml.

[0153] Effects of in vitro induction of specific CD8⁺ T by multi-antigen fusion protein-loaded DCs evaluated through γ -interferon release assay: 5×10^4 cells/50 μ l of CD19 positive cells were added to a 96-well plate, and corresponding proteins (HPV16-E7 antigen fusion Protein (SEQ ID NO.: 5) or conventional recombinant HPV16-E7 protein) were added to 50 μ g/ml, and incubated at 37° C., 5% CO₂ for 2 h. After centrifugation, supernatant was discarded, and cells were re-suspended in 50 μ l of fresh medium. 5×10^4 cells/50

μl of CD8 positive cells were added and incubated at 37° C. for 18 hr. The concentration of IFN-γ in the supernatant was measured by ELISA.

[0154] Results are shown in Table 1: CD8⁺ T cells can be sensitized in vitro and re-activated by antigen fusion protein HPV16-E7-loaded DC, effects of the antigen fusion protein effect is much higher than that of the control protein HPV-E7, i.e., compared with the recombinant HPV16-E7 protein as the control, the release of gamma-interferon caused by the fusion protein of the present invention was increased by 372.5%. $(152.7-58.2)/(2.5-(-17.5))-1 \times 100\% = 372.5\%$.

TABLE 1

Effects of HPV16-E7 antigen fusion protein and recombinant HPV16-E7 protein on in vitro stimulating DC to induce specific CD8 ⁺ T cells detected through γ-interferon release assay Concentration of IFN-γ (ng/l)			
	CD8 ⁺ CD19 + antigen	CD8 ⁺ CD19 (negative control)	Net release of IFN-γ
HPV16-E7-antigen fusion protein	152.7	58.2	94.5
HPV16-E7-natural protein	2.5	-17.5	20.0
Non-antigen	-29.0	-27.3	-1.7

Example 7

[0155] Skin Sensitive Test for Tuberculosis

[0156] A guinea pig weighing about 250 g was intradermally injected with 0.1 ml of PPD and the skin reaction was observed at 24 hours. Skin reaction-negative guinea pigs were randomly divided into 5 groups, subcutaneously injected with 0.2 ml of 40 mg/ml of *Mycobacterium tuberculosis* H37Rv suspension at the inguinal inguinal, and immunized once every week for five times. One week after the fifth immunization, the guinea pigs were randomly divided into five groups. Furs on the back of guinea pig were plucked, 0.1 ml of positive control (PPD), negative control (tuberculosis-irrelevant recombinant protein), recombinant ESAT6-CFP10 protein, a mixture liquid of recombinant ESAT6-CFP10-antigen fusion protein (1 mg/ml, 0.1 mg/ml) were intradermally injected by alternative skin test respectively. The vertical diameter and horizontal diameter (mm) of skin redness at each injection spot on the back of a guinea pig were measured 24 hours after the injection, and average value was recorded.

[0157] Results of 24-hour skin reaction are shown in FIG. 7. In TB-antigen fusion protein group (SEQ ID NO: 1), the average diameter of skin induration is greater than that of PPD group and ESAT6-CFP10 natural protein group. In the control group, no swelling, or induration reaction was observed on local skin of a guinea pig. This suggests that TB-antigen fusion proteins can be used in a skin test to effectively detect tuberculosis infections.

[0158] In the figure, E6-C10 represents ESAT6-CFP10-natural protein; LRMK represents TB-antigen fusion protein, in which cleavage site of cathepsin S is used to connect each antigen segment, and sequence of which is shown in SEQ ID NO: 1; and TEV represents a control protein formed by replacing all cleavage sites of cathepsin S in SEQ ID NO: 1 with TEV cleavage site (cleavage site of non-cathepsin).

Example 8

[0159] Treatment and Delivery Mechanism of Antigen Presenting Cells on Antigen Fusion Proteins and Common Antigens

[0160] In order to determine after antigen presenting cells intake antigen fusion proteins and are activated by antigen fusion proteins, whether T cell epitopes can be efficiently presented to MHC-I molecule (CD8⁺ T cells can be only activated by presenting on MHC-I molecule, thereby producing cytotoxic reaction, and killing bacteria/virus-infected cells or cancerous cells). In the present example, OVA antigen fusion protein was designed, expressed and purified according to positions 242-352 of amino acid sequence of chicken ovalbumin (OVA), which contains MHC-I epitope SIINFEKL. And the OVA protein fragment OVA 255-340 containing the epitope was used as a control. The mouse dendritic cell line (DC2.4) was used as antigen presenting cells to study the presentation of SIINFEKL in OVA antigen fusion protein. Finally, T cell receptor-like antibodies (identifying SIINFEKL/MHC-I complex) were detected.

[0161] 100 μg/ml of OVA protein fragment (or 30 μg/ml of OVA antigen fusion protein (SEQ ID NO: 3)) was mixed with DC2.4 cells, cultured in RPMI 1640(Sigma) containing 10% heat-inactivated fetal bovine serum (sigma), 2 mM L-glutamine (Sigma) for 13 hours, then stained with a commercially available 25.D1-16 antibody labeled with PE (the monoclonal antibody 25. D1-16 specifically recognizes MHC-1 molecule binding to SIINFEKL), and washed by 1 ml of ice-cold FACS buffer (2% FCS in PBS) for two times. Afterwards, PE-labeled monoclonal antibody 25.D1-16 was added to a concentration of 0.6 μg/ml, and incubate at 4° C. for 30 min. Cells were washed for three times with FACS buffer, re-suspended in 0.2 ml of FACS fixation buffer (BD) and analyzed by flow cytometry.

[0162] Results are shown in FIG. 8. In the figure, the blue peak (indicated by the arrow) is DC+PBS.

[0163] As a positive control, 8 peptide (SIINFEKL) can directly bind to MHC-1 molecule on cell surface, so that more than 80% of the cells were positively stained (C1 and C2 in FIG. 8).

[0164] SIINFEKL peptide in OVA natural protein can not be processed by DC cells and presented on MHC-1 molecule (A1 and A2 in FIG. 8). (Note: The blue and red peaks in FIG. 8A2 almost overlap).

[0165] Surprisingly, as a protein with large molecular weight, SIINFEKL peptide in OVA-antigen fusion protein (SEQ ID NO: 3) can be efficiently processed by mouse dendritic cell line DC2.4 and presented to the cell surface, resulting in up to about 17.6% of cells being 25.D1-16 antibody positively-stained (B1 and B2 in FIG. 8).

Example 9

[0166] Anti-Tumor Effects of C57/BL6 Mice Immunized with Antigen Fusion Protein on Murine Melanoma Cell B16 Overexpressing Survivin or HPV-E7

[0167] 2 mg/ml of antigen solution (control group: recombinant HPV-E7 protein, recombinant Survivin protein; experimental group: HPV-E7 antigen fusion protein (SEQ ID NO: 5), Survivin antigen fusion protein) was mixed with 1 mg/ml of MPL at equal volume to prepare an immunization suspension. Afterwards, 10 randomly assigned C57/B6 female mice aged 7-8 weeks and weighing 25 grams were

subcutaneously injected at neck with 100 μ l of immunization suspension on the 0 day, 21 day and 42 day.

[0168] For each immunized mouse, 100 μ l of homogeneously mixed mice B16 cells stably transfected with human HPV-E7 or Survivin were subcutaneously inoculated at right flank of the mice, and the amount of injected cells was 7×10^5 cells. The survival of mice was recorded and evaluated. Results are shown in FIG. 9 and FIG. 10.

[0169] The results showed that compared with the recombinant HPV-E7 protein control group, HPV-E7 antigen fusion protein of the present invention can significantly prolong the survival rate of mice (FIG. 9) and 50% of mice still survived on day 25; while all mice in recombinant protein control group were dead.

[0170] Compared with the recombinant Survivin protein, the Survivin antigen fusion protein of the present invention can significantly prolong the survival of mice (FIG. 10), and 70% of mice still survived on day 25; while only 40% of mice in the recombinant protein control group survived.

DISCUSSION

[0171] Prior to the present invention, clinically protein antigens were not successfully used as vaccines to stimulate CD8⁺ cell immunity.

[0172] Production and quality control of protein-like vaccine are mature technology and many clinical drugs and vaccines (stimulating antibodies) are proteins. It would be ideal if vaccines that could stimulate CD8⁺ T cell immunity can be produced by the proven method of producing protein. However, how to convert protein antigen presentation from endosome-lysosome-MHC-II pathway to cytoplasm-MHC-I pathway is a very challenging subject. Many immunologists and vaccine scientists are making efforts but there is no breakthrough yet.

[0173] Main obstacles include:

[0174] 1. There are a lot of enzymes in Lysosomes, their function is generally to completely degrade a protein, and a protein will be degraded into amino acids instead of pep-

tides. Therefore, a general recombinant protein antigen will be completely degraded and can not stimulate T cells.

[0175] 2. Since lysosomal membrane is to isolate acid substances and cytoplasm in lysosome, lysosome is not an organelle which is easy to leak. Even a conventionally expressed protein or recombinant protein is not completely degraded, whether it can escape from or leak out of lysosome is unknown.

[0176] 3. For protein antigens that can stimulate CD4⁺ T cells, if a fusion protein, after CD8⁺ antigen is added, is degraded and leaks into cytoplasm (stimulating CD8⁺ T cells), whether the function of stimulating CD4⁺ T cell will decrease or disappear, is also unknown.

[0177] In the present invention, the present inventors have designed a variety of antigen fusion proteins based on enzymatic principles and biological recombination techniques, and identified a novel structure of antigen fusion protein, after a huge amount of screening: a series of long peptides containing antigen segment are ligated by cleavage sites of cathepsin. When the antigen fusion protein is phagocytosed in a cell and enters into lysosome, cathepsin in lysosome will degrade the antigen fusion protein into polypeptides containing different antigen epitopes.

[0178] The experiments of the present invention have surprisingly confirmed that after the fusion protein with particular structure of the present invention is degraded, degraded polypeptides are capable of leaking out of lysosomes and entering into cytoplasm and being processed to MHC class I molecule, thereby initiating MHC-I antigen presenting pathway and in turn stimulating CD8⁺ cells; and the original function of stimulating CD4⁺ T cell can be retained or improved.

[0179] All documents mentioned in the present invention are incorporated herein by reference, as if each document were individually recited for reference. It is to be understood that those skilled in the art will be able to make various changes or modifications to the present invention after reading the teachings of the present invention, which also fall within the scope of the claims appended hereto.

SEQUENCE LISTING

```
<160> NUMBER OF SEQ ID NOS: 8

<210> SEQ ID NO 1
<211> LENGTH: 282
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: ESAT6-CFP10 antigen fusion protein

<400> SEQUENCE: 1

Met Ala Glu Met Lys Thr Asp Ala Ala Thr Leu Ala Gln Glu Ala Gly
1          5          10          15

Asn Phe Glu Arg Ile Ser Gly Asp Leu Lys Thr Gln Ile Asp Gln Val
20          25          30

Glu Ser Thr Leu Arg Met Lys Thr Gln Ile Asp Gln Val Glu Ser Thr
35          40          45

Ala Gly Ser Leu Gln Gly Gln Trp Arg Gly Ala Ala Gly Thr Ala Ala
50          55          60

Gln Ala Ala Val Val Arg Phe Gln Glu Leu Arg Met Lys Ala Gln Ala
65          70          75          80
```


-continued

Ala Val Val Arg Phe Gln Glu Ala Ala Asn Lys Gln Lys Gln Glu Leu
85 90 95

Asp Glu Ile Ser Thr Asn Ile Arg Gln Ala Gly Val Gln Tyr Ser Arg
100 105 110

Leu Arg Met Lys Ile Arg Gln Ala Gly Val Gln Tyr Ser Arg Ala Asp
115 120 125

Glu Glu Gln Gln Gln Ala Leu Ser Ser Gln Met Gly Phe Leu Arg Met
130 135 140

Lys Met Thr Glu Gln Gln Trp Asn Phe Ala Gly Ile Glu Ala Ala Ala
145 150 155 160

Ser Ala Ile Gln Gly Asn Val Thr Ser Ile His Ser Leu Leu Asp Glu
165 170 175

Gly Lys Gln Ser Leu Arg Met Lys His Ser Leu Leu Asp Glu Gly Lys
180 185 190

Gln Ser Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly Ser Glu
195 200 205

Ala Tyr Gln Gly Val Gln Gln Lys Trp Asp Ala Leu Arg Met Lys Tyr
210 215 220

Gln Gly Val Gln Gln Lys Trp Asp Ala Thr Ala Thr Glu Leu Asn Asn
225 230 235 240

Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser Glu Ala Gly Gln Ala Met
245 250 255

Ala Ser Leu Arg Met Lys Ile Ser Glu Ala Gly Gln Ala Met Ala Ser
260 265 270

Thr Glu Gly Asn Val Thr Gly Met Phe Ala
275 280

<210> SEQ ID NO 2
 <211> LENGTH: 849
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <223> OTHER INFORMATION: encoding sequence of ESAT6-CFP10 antigen
 fusion protein

<400> SEQUENCE: 2

atggccgaaa tgaaaaccga tgcagcaacc ctggcacaag aagcaggtaa ttttgaacgt	60
attagcgggtg atctgaaaac ccagattgat caggttgaaa gcacctgctg tatgaaaaca	120
caaatcgatc aagtggaaag taccgcaggt agcctgcagg gtcagtggcg tgggtgcagca	180
ggcaccgcag cacaggcagc agttgttcgt tttcaagaac tgcgcatgaa agcccaggca	240
gccgtggtgc gcttccaaga agccgcaaat aaacagaaac aagagctgga tgaaatcagc	300
accaatatcc gtcaggcagg cggttcagtat agccgtttac ggatgaaaaa tcgtcaagcc	360
ggtgtgcagt attcacgtgc agatgaagaa cagcagcaag cactgagcag ccagatgggt	420
tttttaagaa tgaatatgac cgagcagcag tggaattttg caggtattga agcagccgca	480
agcgcaattc agggtaattg taccagcatt catagcctgc tggacgaagg taaacagagc	540
ctgcggatga agcatagtct gttagatgaa ggcaaacagt cactgaccaa actggcagca	600
gcatgggggtg gtacgggtag cgaagcatat caggggtgttc agcagaaatg ggatgcatta	660
cgtatgaagt atcagggcgt gcaacaaaag tgggacgcaa ccgcaaccga actgaataat	720

-continued

```
gcactgcaga atctggcacg taccattagt gaagccggtc aggcaatggc cagcttacgc 780
atgaagattt ctgaagcagg ccaagctatg gcaagcaccg aaggcaatgt gaccggtatg 840
tttgcataa 849
```

```
<210> SEQ ID NO 3
<211> LENGTH: 152
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: OVA antigen fusion protein
```

```
<400> SEQUENCE: 3
```

```
Met Leu Val Leu Leu Pro Asp Glu Val Ser Gly Leu Glu Gln Leu Glu
1      5      10      15
Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr Ser Ser Leu Arg
20     25     30
Met Lys Leu Glu Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr
35     40     45
Ser Ser Asn Val Met Glu Glu Arg Lys Ile Lys Val Tyr Leu Pro Arg
50     55     60
Met Lys Met Glu Glu Lys Tyr Asn Leu Thr Ser Val Leu Met Ala Met
65     70     75     80
Gly Ile Thr Asp Val Phe Ser Ser Ser Ala Asn Leu Ser Gly Ile Ser
85     90     95
Ser Ala Glu Ser Leu Lys Ile Ser Gln Ala Val His Ala Ala His Ala
100    105    110
Glu Ile Asn Glu Ala Gly Arg Leu Arg Met Lys Ile Ser Gln Ala Val
115    120    125
His Ala Ala His Ala Glu Ile Asn Glu Ala Gly Arg Glu Val Val Gly
130    135    140
Ser Ala Glu Ala Gly Val Asp Ala
145    150
```

```
<210> SEQ ID NO 4
<211> LENGTH: 459
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: encoding sequence of OVA antigen fusion
protein
```

```
<400> SEQUENCE: 4
```

```
atgctggttc tgctgccgga tgaagttagc ggtctggaac agctggaaaag cattatcaat 60
tttgaanaaa tgaccgaatg gaccagcagc ctgcgtatga aactggaatc catcattaac 120
ttcgagaaaac tgacagagtg gacaagcagc aatgttatgg aagaacgtaa aatcaaagtg 180
tacctgcctc gcatgaaaat ggaagagaaa tataacctga ccagcgttct gatggcaatg 240
ggattaccg atgttttttag cagcagcgca aatctgagcg gtattagcag cgagaaaagc 300
ctgaaaatta gccaggcagt tcattgcagca catgccgaaa ttaatgaagc aggtcgctctg 360
cggatgaaaa ttccacaggc cgtgcattgt gcccatgcag aaatcaacga agctggccgt 420
gaagttgttg gtagtgcga agccggtgtt gatgcataa 459
```

-continued

<210> SEQ ID NO 5
 <211> LENGTH: 140
 <212> TYPE: PRT
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <223> OTHER INFORMATION: HPV16-E7 antigen fusion protein

<400> SEQUENCE: 5

Met	His	Gly	Asp	Thr	Pro	Thr	Leu	His	Glu	Tyr	Met	Leu	Asp	Leu	Gln
1				5					10					15	
Pro	Glu	Thr	Thr	Asp	Leu	Tyr	Cys	Tyr	Glu	Gln	Leu	Asn	Asp	Ser	Ser
			20					25					30		
Glu	Glu	Glu	Leu	Arg	Met	Lys	Glu	Gln	Leu	Asn	Asp	Ser	Ser	Glu	Glu
			35				40					45			
Glu	Asp	Glu	Ile	Asp	Gly	Pro	Ala	Gly	Gln	Ala	Glu	Pro	Asp	Arg	Ala
	50					55					60				
His	Tyr	Asn	Ile	Val	Thr	Phe	Cys	Cys	Lys	Leu	Arg	Met	Lys	His	Tyr
65					70					75				80	
Asn	Ile	Val	Thr	Phe	Cys	Cys	Lys	Cys	Asp	Ser	Thr	Leu	Arg	Leu	Cys
			85						90					95	
Val	Gln	Ser	Thr	His	Val	Asp	Ile	Arg	Thr	Leu	Glu	Asp	Leu	Leu	Met
			100					105					110		
Gly	Leu	Arg	Met	Lys	Ile	Arg	Thr	Leu	Glu	Asp	Leu	Leu	Met	Gly	Thr
			115				120					125			
Leu	Gly	Ile	Val	Cys	Pro	Ile	Cys	Ser	Gln	Lys	Pro				
	130					135				140					

<210> SEQ ID NO 6
 <211> LENGTH: 423
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <223> OTHER INFORMATION: encoding sequence of HPV16-E7 antigen fusion protein

<400> SEQUENCE: 6

atgcatggtg	ataccgcgac	cctgcatgaa	tatatgctgg	atctgcaacc	ggaaccacc	60
gatctgtatt	gttatgagca	gctgaatgat	agcagcgaag	aggaattacg	catgaaggaa	120
cagctgaacg	attcaagcga	agaagaggac	gaaattgacg	gtccggcagg	tcaggcagaa	180
ccggatcgtg	cacattacaa	cattgttacc	ttttgttgca	aactgagaat	gaaacactac	240
aatatcgtga	ccttctgctg	taaattgtgat	agcaccctgc	gtctgtgtgt	tcagagcacc	300
catgttgata	ttcgtaacatt	agaggacctg	ctgatgggcc	tgcggatgaa	aattcgtaac	360
ctggaagacc	tgtaaatggg	caccctgggt	attgtttgtc	cgattttag	ccagaaaccg	420
taa						423

<210> SEQ ID NO 7
 <211> LENGTH: 15
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <223> OTHER INFORMATION: Polynucleotide

<400> SEQUENCE: 7

-continued

tacttccaat ccatg

15

<210> SEQ ID NO 8
 <211> LENGTH: 18
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <221> NAME/KEY: misc feature
 <223> OTHER INFORMATION: Polynucleotide

<400> SEQUENCE: 8

tatccacatt tactgtta

18

1. An artificial multi-antigen fusion protein, wherein, when administered to a mammalian subject, the artificial multi-antigen fusion protein simultaneously stimulates CD4⁺ and CD8⁺ T cell immune responses in said subject.

2. The artificial multi-antigen fusion protein of claim 1, wherein the artificial multi-antigen fusion protein comprises ≥ 3 , preferably ≥ 5 , more preferably ≥ 10 antigen segments.

3. The artificial multi-antigen fusion protein of claim 2, wherein each antigen segment is from a viral antigen, a bacterial antigen, a parasite antigen, a chlamydial antigen, a tumor antigen, or a combination thereof.

4. The artificial multi-antigen fusion protein of claim 1, wherein the artificial multi-antigen fusion protein further comprises sequences of cleavage site located between antigen segments.

5. The artificial multi-antigen fusion protein of claim 4, wherein the sequence of cleavage site comprises a cleavage site of cathepsin; and

Preferably, the cleavage site of cathepsin is selected from a group consisting of a cleavage site of cathepsin S (e.g., Leu-Arg-Met-Lys or a similar cleavage site), a cleavage site of cathepsin B (e.g., Met-Lys-Arg-Leu or a similar cleavage site), a cleavage site of cathepsin K (e.g., His-Pro-Gly-Gly or a similar restriction site), and combinations thereof.

6. The artificial multi-antigen fusion protein of claim 1, wherein the fusion protein is shown in the structure of formula I:

Y-(A-C)_n-Z

(I)

wherein,

A is an antigen segment;

C is a sequence of cleavage site of cathepsin;

n is a positive integer ≥ 3 ;

Y is absent or is a sequence represented by "Y0-B", wherein Y0 is a signal peptide sequence, a tag sequence, a membrane-penetrating element sequence, an adjuvant element sequence, a cell necrosis-inductive element sequence, or any combination of the above sequences, and B is absent or a sequence of cleavage site;

Z is absent, or a tag sequence, a membrane-penetrating element sequence, an adjuvant element sequence, a cell necrosis-inductive element sequence, or any combination of the above sequences;

provided that when Z is absent, C in the last "A-C" can be absent.

7. A composition, wherein the composition comprises the artificial multi-antigen fusion protein of claim 1 and a pharmaceutically acceptable carrier.

8. Use of the artificial multi-antigen fusion protein of claim 1 used for the preparation of a prophylactic and/or therapeutic vaccine composition or pharmaceutical composition.

9. Use of the artificial multi-antigen fusion protein of claim 1 used for preparing an agent or a kit for detecting specific T cell immunity.

10. A method, comprising administering the artificial multi-antigen fusion protein of claim 1.

11. A method, comprising administering the composition of claim 7 to a subject in need thereof.

* * * * *