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INDUCTION OF A TH1-LIKE RESPONSE IN VITRO

Cross Reference to Related Applications

This application claims priority from U.S. Provisional Application No. 60/143,757, filed July 8, 1999. The content of this application is incorporated herein by reference in its entirety.

Field of the Invention

The invention relates to fusion proteins and methods of stimulating a Th1-like response *in vitro*.

Background

T lymphocytes can generally be divided into two classes based upon expression of the CD4 and CD8 antigens. The immune response mediated by CD4+ T cells is restricted by class II major histocompatibility complex (MHC) molecules. CD4+ T cells, also known as helper T lymphocytes, carry out their helper functions via the secretion of lymphokines. The immune response mediated by CD8+ T cells is restricted by class I MHC molecules. CD8+ T cells, also known as cytolytic T lymphocytes (CTLs), carry out cell mediated cytotoxicity and also secrete some lymphokines upon activation.

CD4+ T cells can be further divided into Th1 and Th2 subsets. Th1 cells participate in cell mediated immunity by producing lymphokines, such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-beta, that activate cell mediated immunity. Th2 cells provide help for humoral immunity by secreting lymphokines that stimulate B cells, such as IL-4 and IL-5. Antigenic stimuli that activate either the Th1 or Th2 pathway can inhibit the development of the other. For example, IFN-gamma produced by a stimulated Th1 cell can inhibit the formation of Th2 cells, and IL-4 produced by a stimulated Th2 cell can inhibit the formation of Th1 cells.

Certain disease conditions, such as cancer, allergy, and parasitic infections, are characterized by a predominantly Th2 response. Under certain circumstances, the induction of the Th1 response, typified by the production of IFN-gamma, may ameliorate these conditions.

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Summary of the Invention

The invention is based on the discovery that a cell sample containing naive lymphocytes can be stimulated *in vitro* to exhibit a Th1-like response.

Accordingly, the invention features a method of determining whether a fusion protein stimulates a Th1-like response by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a fusion protein containing (i) a heat shock protein (Hsp) or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length; (c) contacting the cell sample with the fusion protein; and (d) determining whether the fusion protein stimulates a Th1-like response in the cell sample.

"Naive lymphocytes" are lymphocytes that have not been exposed to the fusion protein (*in vivo* or *in vitro*) prior to their use in a method the invention. An "Hsp" is a polypeptide consisting of a sequence that is at least 40% identical to that of a protein whose expression is induced or enhanced in a cell exposed to stress, e.g., heat shock. A "fusion protein" is a non-naturally occurring polypeptide containing amino acid sequences derived from at least two different proteins.

The Hsp used in the method can be selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71. Additionally, the fusion protein can contain the full amino acid sequence of any of Hsp65, Hsp40, Hsp10, Hsp60, or Hsp71. In some embodiments, the fusion protein contains a fragment of an Hsp, e.g., amino acids 1-200 of Hsp65 of *Mycobacterium bovis*.

The heterologous polypeptide can contain a sequence identical to at least eight consecutive amino acids of (i) a protein of a human pathogen, e.g., a virus, or (ii) a tumor associated antigen. Examples of viruses include human papilloma virus (HPV), herpes simplex virus (HSV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza virus, measles virus, and human immunodeficiency virus (HIV). The heterologous polypeptide can contain an HPV E6 antigen, e.g., HPV16 E6, an HPV E7 antigen, e.g., HPV16 E7, or a fragment of any of these antigens that is at least eight amino acid residues in length.

In one example, the fusion protein contains *Mycobacterium bovis* BCG Hsp65 and HPV 16 E7.

The cell sample used in the methods of the invention can contain cells derived from a spleen, lymph node, peripheral blood, bone marrow, thymus, lung, respiratory

tract, or anogenital mucosa. In preferred embodiments, the cells are splenocytes or lymph node cells.

The stimulation of a Th1-like response can be determined by detecting the presence of a lymphokine produced by the cell sample, e.g. IFN-gamma or TNF-beta.

In one embodiment, the method also includes the steps of: (e) providing a second cell sample containing naive lymphocytes; (f) contacting the second cell sample with a second fusion protein; and (g) determining whether the second fusion protein stimulates a Th1-like response in the second cell sample. In this example, the first fusion protein contains the sequence of a full-length, naturally occurring Hsp, and the second fusion protein contains at least eight amino acids but less than all of the sequence of a naturally occurring Hsp.

In another aspect, the invention features a method of screening a compound by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a fusion protein containing (i) a Hsp or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length; (c) contacting the cell sample with the compound and the fusion protein; and (d) determining whether the cell sample exhibits a Th1-like response following the contacting step. In this method, a decrease in the Th1-like response in the presence of the compound compared to in the absence of the compound indicates that the compound inhibits a Th1-like response by the cell sample.

The invention also includes a method of screening a compound by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a fusion protein containing (i) a Hsp or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length; (c) contacting the cell sample with the compound and the fusion protein; and (d) determining whether the cell sample exhibits a Th1-like response following the contacting step. In this method, an increase in the Th1-like response in the presence of the compound compared to in the absence of the compound indicates that the compound promotes a Th1-like response by the cell sample.

In another aspect, the invention features a method of determining whether a hybrid compound stimulates a Th1-like response by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a hybrid compound that is non-naturally occurring and contains (i) a non-peptide compound having a molecular weight

of less than 1,500, covalently linked to (ii) a polypeptide of at least eight amino acids in length, wherein the hybrid compound is made by covalently linking the non-peptide compound to the polypeptide; (c) contacting the cell sample with the hybrid compound; and (d) determining whether the hybrid compound stimulates a Th1-like response in the cell sample. In one embodiment, the non-peptide compound has a molecular weight of at least 100.

In another aspect, the invention features a method of determining whether a hybrid compound stimulates a Th1-like response by: (a) producing a hybrid compound by covalently linking a non-peptide compound to a polypeptide of at least eight amino acids in length; (b) providing a cell sample containing naive lymphocytes *in vitro*; (c) contacting the cell sample with the hybrid compound; and (d) determining whether the hybrid compound stimulates a Th1-like response in the cell sample. In one embodiment, the non-peptide compound has a molecular weight between 100 and 1,500.

In another aspect, the invention features a method of determining whether a fusion protein stimulates a Th1-like response by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a fusion protein comprising (i) a first polypeptide at least eight amino acids in length, fused to (ii) a second polypeptide at least eight amino acids in length; (c) contacting the cell sample with the fusion protein; and (d) detecting a Th1-like response exhibited by the cell sample following the contacting step. In one embodiment, the detected Th1-like response is greater than a Th1-like response exhibited by a second cell sample containing naive lymphocytes when the second cell sample is contacted with either the first polypeptide, the second polypeptide, or a mixture of the first polypeptide and the second polypeptide. In one example, the detected Th1-like response is at least two times greater than the Th1-like response exhibited by the second cell sample. In another example, the detected Th1-like response is at least five times greater than the Th1-like response exhibited by the second cell sample.

In another aspect, the invention provides a fusion protein containing (i) a Hsp10 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length. The Hsp10 protein of the fusion protein can be a mycobacterial protein, e.g., *Mycobacterium tuberculosis* Hsp10 protein. The heterologous polypeptide can contain a sequence identical to at least eight

consecutive amino acids of a protein of a human virus, e.g., HPV. In one example, the heterologous polypeptide contains HPV16 E7.

In another aspect, the invention provides a fusion protein containing (i) a Hsp40 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length. The Hsp40 protein of the fusion protein can be a mycobacterial protein, e.g., *Mycobacterium tuberculosis* Hsp40 protein. The heterologous polypeptide can contain a sequence identical to at least eight consecutive amino acids of a protein of a human virus, e.g., HPV. In one example, the heterologous polypeptide contains HPV16 E7.

In another aspect, the invention provides a fusion protein containing (i) a Hsp71 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length. The Hsp71 protein of the fusion protein can be a mycobacterial protein, e.g., *Mycobacterium tuberculosis* Hsp71 protein. The heterologous polypeptide can contain a sequence identical to at least eight consecutive amino acids of a protein of a human virus, e.g., HPV. In one example, the heterologous polypeptide contains HPV16 E7.

In another aspect, the invention features a method of determining whether a compound stimulates a Th1-like response by: (a) providing a cell sample containing naive lymphocytes *in vitro*; (b) providing a compound; (c) contacting the cell sample with the compound; and (d) detecting a Th1-like response exhibited by the cell sample following the contacting step.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figures 1A-1B show the sequence of plasmid pET65 coding for expression of Hsp65.

Figure 2 shows the sequence of plasmid pET/E7 (NH) coding for expression of E7.

Figure 3 shows the sequence of plasmid pET/H/E7 coding for expression of (h)E7.

Figures 4A-4B show the sequence of plasmid pET65C/E7-1N coding for expression of HspE7.

Figures 5A-5B show the sequence of plasmid pETMT40E7 coding for expression of MT40-E7.

Figure 6 shows the sequence of plasmid pET/OVA coding for expression of ovalbumin (OVA).

Figures 7A-7C show the sequence of plasmid pET65H/OVA coding for expression of HspOVA.

Figure 8 shows the sequence of plasmid pGEX/K coding for expression of GST.

Figure 9 shows the sequence of plasmid pGEX/K/E7 coding for expression of GST-E7.

Figures 10A-10B show the sequence of plasmid pET/E7/5'65 coding for expression of E7-L-BCG65.

Figure 11 shows the sequence of plasmid pET65F1/E7 coding for expression of BCG65(F1)-E7.

Figure 12 shows the sequence of plasmid pETESE7 coding for expression of TB10-E7.

Figures 13A-13B show the sequence of plasmid pET/E7/71 coding for expression of E7-TB71.

Figures 14A-14B show the sequence of plasmid pET/E7/71' coding for expression of a fusion protein.

Figures 15A-15B show the sequence of plasmid pET/SP65c-E7 coding for expression of SP65(2)-E7.

Figures 16A-16B show the sequence of plasmid pETAF60E7 coding for expression of AF60-E7.

Figures 17A-17B show enhanced IFN-gamma release by splenocytes from C57BL/6 mice obtained from the Charles River Laboratory (Fig. 17A) and the Jackson Laboratory (Fig. 17B) upon exposure to HspE7.

Figures 18A-18C show enhanced IFN-gamma release by splenocytes from Balb/c (Fig. 18A), C57BL/6 (Fig. 18B), and C3HeB/FeJ (Fig. 18C) mice upon exposure to HspE7.

Figure 19 shows enhanced IFN-gamma release by splenocytes upon exposure to fusion proteins containing an antigen and a stress protein but not upon exposure to a fusion protein containing an antigen and a protein other than a stress protein.

Figures 20A-20B show enhanced IFN-gamma release by splenocytes upon exposure to fusion proteins containing stress proteins of different types, stress proteins from different organisms, or a fragment of a stress protein.

Figure 21 shows enhanced IFN-gamma release by lymph node cells and splenocytes upon exposure to fusion proteins containing an antigen and a stress protein.

Figures 22A-22B show a time course of tumor incidence (Fig. 22A) and tumor volume (Fig. 22B) in mice injected with TC-1 tumor cells followed by an injection with either saline, HspE7, SP65(2)-E7, or AF60-E7.

Figures 23A-23B show a time course of tumor incidence (Fig. 23A) and tumor volume (Fig. 23B) in mice injected with TC-1 tumor cells followed by an injection with either saline, HspE7, MT40-E7, E7-MT71, or TB10-E7.

Detailed Description

The invention relates to methods of stimulating *in vitro* a Th1-like response in a cell sample containing naive lymphocytes. These methods are useful for assessing the ability of a protein, e.g., a fusion protein containing an Hsp linked to a heterologous polypeptide, to function as a stimulator of a Th1-like response. Additionally, the method can be used to identify compounds that can regulate a Th1-like response. Various materials and procedures suitable for use in the methods of the invention are discussed below.

The terms stress protein and heat shock protein (Hsp) are used synonymously herein. An Hsp is a polypeptide consisting of a sequence that is at least 40% identical to that of a protein whose expression is induced or enhanced in a cell exposed to stress. Turning to stress proteins generally, cells respond to a stressor (typically heat shock

treatment) by increasing the expression of a group of genes commonly referred to as stress, or heat shock, genes. Heat shock treatment involves exposure of cells or organisms to temperatures that are one to several degrees Celsius above the temperature to which the cells are adapted. In coordination with the induction of such genes, the levels of corresponding stress proteins increase in stressed cells. As used herein, a "stress protein," also known as a "heat shock protein" or "Hsp," is a protein that is encoded by a stress gene, and is therefore typically produced in significantly greater amounts upon the contact or exposure of the stressor to the organism. A "stress gene," also known as "heat shock gene" is used herein as a gene that is activated or otherwise detectably upregulated due to the contact or exposure of an organism (containing the gene) to a stressor, such as heat shock, hypoxia, glucose deprivation, heavy metal salts, inhibitors of energy metabolism and electron transport, and protein denaturants, or to certain benzoquinone ansamycins. Nover, L., *Heat Shock Response*, CRC Press, Inc., Boca Raton, FL (1991). "Stress gene" also includes homologous genes within known stress gene families, such as certain genes within the Hsp70 and Hsp90 stress gene families, even though such homologous genes are not themselves induced by a stressor. Each of the terms stress gene and stress protein as used in the present specification may be inclusive of the other, unless the context indicates otherwise.

An antigen can be any compound, peptide or protein to which an immune response is desired. Antigens of particular interest are tumor-associated antigens, allergens of any origin, and proteins from viruses, mycoplasma, bacteria, fungi, protozoa and other parasites.

Fusion Proteins

The invention provides Hsp fusion proteins. As used herein, a "fusion protein" is a non-naturally occurring polypeptide containing at least two amino acid sequences which generally are from two different proteins. The amino acid sequence of the full length fusion protein is not identical to the amino acid sequence of a naturally occurring protein or a fragment thereof. An Hsp fusion protein contains an Hsp or a fragment thereof at least eight amino acids in length linked to a heterologous polypeptide. An "Hsp polypeptide" refers to a polypeptide consisting of a sequence that is at least 40% identical to that of a protein whose expression is induced or enhanced in a cell exposed to stress, e.g., heat shock. A "heterologous polypeptide" refers to a polypeptide that is

fused to the Hsp protein or fragment thereof. The heterologous polypeptide is preferably at least eight amino acids in length. In some embodiments, the heterologous polypeptide is at least 10, 20, 50, 100, 150, 180, 200, or 300 amino acids in length. The heterologous polypeptide generally is not part or all of a naturally occurring Hsp. However, the fusion protein can also be a fusion between a first Hsp and a second, different, Hsp, or between all or portion of an Hsp fused to all or a portion of the same Hsp (as long as the resultant fusion is not identical to a naturally occurring protein). The Hsp polypeptide can be attached to the N-terminus or C-terminus of the heterologous polypeptide. Preferably the fusion protein is a purified protein.

The preferred Hsp fusion protein has one Hsp polypeptide linked to one heterologous polypeptide, but other conformations are within the invention. In one embodiment, the fusion protein comprises at least two copies of the heterologous polypeptide, e.g., HPV16 E7. In another embodiment, the fusion protein contains at least two copies of the Hsp polypeptide, e.g., Hsp65. Additionally, the fusion protein can contain at least two different heterologous polypeptides, e.g., two or more fragments of a single antigenic protein representing different epitopes or fragments of two or more different antigenic proteins derived from the same or different tumors or pathogens, and/or at least two different Hsp polypeptides.

The Hsp and heterologous polypeptide can be directly fused without a linker sequence. In preferred embodiments, the C-terminus of the Hsp can be directly fused to the N-terminus of the heterologous polypeptide or the C-terminus of the heterologous polypeptide can be directly fused to the N-terminus of the Hsp.

Alternatively, Hsp and heterologous polypeptides can be linked to each other via a peptide linker sequence. Preferred linker sequences (1) should adopt a flexible extended conformation, (2) should not exhibit a propensity for developing an ordered secondary structure which could interact with the functional Hsp and heterologous polypeptide domains, and (3) should have minimal hydrophobic or charged character, which could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Permutations of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other neutral or near-neutral amino acids, such as Thr and Ala, can also be used in the linker sequence. Any other amino acid can also be used in the linker. A linker sequence length of fewer than 20 amino acids can be used

to provide a suitable separation of functional protein domains, although longer linker sequences may also be used.

The Hsp fusion protein may be further fused to another amino acid sequence that facilitates the purification of the fusion protein. One useful fusion protein is a GST fusion protein in which the Hsp-heterologous polypeptide sequences are fused to the C-terminus or N-terminus of the GST sequence. Another useful fusion protein is a poly-histidine (His) fusion protein in which the Hsp-heterologous polypeptide sequences are fused to either the C-terminus or N-terminus of the poly-histidine sequence, e.g. His x 6. In another embodiment, the fusion protein contains the chitin-binding region of intein, thereby permitting the purification of the fusion protein by chitin beads (Hoang et al. (1999) *Gene* 1999 237:361-71). In another embodiment, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., mammalian host cells), expression and/or secretion of the Hsp fusion protein can be increased through use of a heterologous signal sequence. For example, the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). Prokaryotic signal sequences useful for increasing secretion by a prokaryotic host cell include the phoA secretory signal (Molecular Cloning, Sambrook et al., second edition, Cold Spring Harbor Laboratory Press, 1989) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

Fusion proteins of the invention, e.g., a fusion protein of Hsp65 and HPV16 E7, can be produced by standard recombinant techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together, in any order, in-frame in accordance with conventional techniques. Such techniques can include employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. Correct linkage of the two nucleic acids requires that the product of the linkage encode a chimeric protein consisting of a Hsp moiety and a heterologous polypeptide moiety. In another embodiment, the fusion gene can be synthesized by conventional techniques, including automated DNA synthesizers. Alternatively, PCR amplification of gene

fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments, which are subsequently annealed and reamplified to generate a chimeric gene sequence (see, e.g., Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons: 1992).

Expression vectors encoding fusion proteins containing a heterologous polypeptide and either an Hsp or a protein other than an Hsp can be prepared by the above procedures. Examples of Hsp fusion proteins can be found in international patent application WO 99/07860, incorporated herein by reference, that describes vector construction, expression and purification of *Mycobacterium bovis* BCG Hsp65 - HPV16 E7 (HspE7) fusion protein as well as of HPV16 E7 (E7), histidine tagged HPV16 E7 (hE7), and *M. bovis* BCG Hsp65 (Hsp65). Additional examples of nucleic acids encoding an Hsp optionally linked to a heterologous polypeptide, e.g., an HPV antigen, are described in WO 89/12455, WO 94/29459, WO 98/23735, and references cited therein, the contents of which are herein incorporated by reference.

A variety of heat shock proteins have been isolated, cloned, and characterized from a diverse array of organisms (Mizzen, Biotherapy 10:173-189, 1998). Any Hsp or fragment thereof may be suitable for use in the fusion polypeptides and conjugates of the invention. For example, Hsp70, Hsp60, Hsp20-30, and Hsp10 are among the major determinants recognized by host immune responses to infection by *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In addition, Hsp65 of Bacille Calmette Guerin (BCG), a strain of *Mycobacterium bovis*, was found to be an effective stimulatory agent, as described in the examples below.

Families of stress genes and proteins for use in the present invention are well known in the art and include, for example, Hsp100-200, Hsp100, Hsp90, Lon, Hsp70, Hsp60, TF55, Hsp40, FKBPs, cyclophilins, Hsp20-30, ClpP, GrpE, Hsp10, ubiquitin, calnexin, and protein disulfide isomerases. See, e.g., Macario, Cold Spring Harbor Laboratory Res. 25:59-70, 1995; Parsell et al., Rev. Genet. 27:437-496, 1993; and U.S. Patent No. 5,232,833. Preferred Hsps include Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

The Hsp portion of the fusion protein can include either a full length Hsp or a fragment of an Hsp at least eight amino acids in length. In some embodiments, the Hsp fragment is greater than 10 amino acids in length, and preferably is at least 20, 50, 100, 150, 180, 200, or 300 amino acids in length. In one embodiment, the Hsp portion of the

fusion protein consists of amino acids 1-200 of Hsp65 of *Mycobacterium bovis*. Other portions of Hsp65 and other Hsps can be used in a fusion protein to elicit a Th1-like response *in vitro*. Other preferred Hsps include Hsp40 of *M. tuberculosis*, Hsp10 of *M. tuberculosis*, Hsp65 of *Streptococcus pneumoniae*, and Hsp60 of *Aspergillus fumigatus*.

Heterologous polypeptides can contain any amino acid sequence useful for stimulating an immune response, *in vitro* and/or *in vivo*. Preferably, the heterologous polypeptide contains an MHC-binding epitope, e.g., an MHC class I or MHC class II binding epitope. The heterologous polypeptide can contain sequences found in a protein produced by a human pathogen, e.g., viruses, bacteria, mycoplasma, fungi, protozoa, and other parasites, or sequences found in the protein of a tumor associated antigen (TAA). Examples of viruses include human papilloma virus (HPV), herpes simplex virus (HSV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza virus, measles virus, and human immunodeficiency virus (HIV). Examples of tumor associated antigens include MAGE1, MAGE2, MAGE3, BAGE, GAGE, PRAME, SSX-2, Tyrosinase, MART-1, NY-ESO-1, gp100, TRP-1, TRP-2, A2 melanotope, BCR/ABL, Proeinase-3/Myeloblastin, HER2/neu, CEA, P1A, HK2, PAPA, PSA, PSCA, PSMA, pg75, MUM-1, MUC-1, E6, E7, GnT-V, Beta-catenin, CDK4 and P15.

HPV antigens from any strain of HPV are suitable for use in the fusion polypeptide. HPV expresses six or seven non-structural and two structural proteins. Viral capsid proteins L1 and L2 are the late structural proteins. L1 is the major capsid protein, the amino acid sequence of which is highly conserved among different HPV types. There are seven early non-structural proteins. Proteins E1, E2, and E4 play an important role in virus replication. Protein E4 also plays a role in virus maturation. The role of E5 is less well known. Proteins E6 and E7 are oncoproteins critical for viral replication, as well as for host cell immortalization and transformation. Fusion proteins of the invention can contain either the entire sequence of an HPV protein or a fragment thereof, e.g., a fragment of at least 8 amino acids. In one embodiment, the HPV antigenic sequence is derived from a "high risk" HPV, such as HPV16 or HPV18 E7 protein. The HPV antigenic sequence can include an MHC-binding epitope, e.g., an MHC class I and/or an MHC class II binding epitope.

In addition to Hsp fusion proteins, other fusion proteins can be used in the *in vitro* assay described herein. These non-Hsp fusion proteins contain a first polypeptide at least eight amino acids in length, fused to a second polypeptide at least eight amino acids in length, wherein the first and second polypeptides are derived from different proteins (preferably naturally occurring proteins). The fusion protein itself does not have the sequence of a naturally occurring protein.

In the fusion protein of the invention, neither the first nor second polypeptide is an amino acid sequence that is commonly used for protein purification or detection, e.g., GST or poly-histidine.

In order to produce the fusion protein, a nucleic acid encoding the fusion protein can be introduced into a host cell, e.g., a bacterium, a primary cell, or an immortalized cell line using an expression vector. The recombinant cells are then used to produce the fusion protein. The transfection can be transient or stable, the later sometimes accomplished by homologous recombination.

The nucleotide sequence encoding a fusion protein will usually be operably linked to one or more regulatory sequences, selected on the basis of the host cells to be used for expression. The term "regulatory sequence" refers to promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA, the content of which is incorporated herein by reference. Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cells, those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences), and those that direct expression in a regulatable manner (*e.g.*, only in the presence of an inducing agent). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of fusion protein desired, and the like.

Recombinant expression vectors can be designed for expression of fusion proteins in prokaryotic or eukaryotic cells. For example, fusion proteins can be expressed in bacterial cells such as *E. coli*, insect cells (e.g., in the baculovirus expression system), yeast cells or mammalian cells. Some suitable host cells are discussed further in Goeddel (1990) *Gene Expression Technology: Methods in*

Enzymology 185, Academic Press, San Diego, CA. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.* (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.* (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of fusion proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When intended for use in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory control sequences discussed above, the recombinant expression vector can contain additional nucleotide sequences. For example, the recombinant expression vector may encode a selectable marker gene to identify host cells that have incorporated the vector. Moreover, to facilitate secretion of the fusion protein from a host cell, in particular mammalian host cells, the recombinant expression vector can encode a signal sequence linked to the amino-terminus of the fusion protein, such that upon expression, the fusion protein is synthesized with the signal sequence fused to its amino terminus. This signal sequence directs the fusion protein into the secretory pathway of the cell and is then usually cleaved, allowing for release of the mature fusion protein (*i.e.*, the fusion protein without the signal sequence) from the host cell. Use of a signal sequence to facilitate secretion of proteins or peptides from mammalian host cells is known in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press (1989)), and other laboratory manuals.

Often only a small fraction of mammalian cells integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) can be introduced into the host cells along with the gene encoding the fusion protein. Preferred selectable markers include those that confer resistance to drugs such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding the fusion protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

Alternatively, a recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

In addition to the recombinant techniques described above, a fusion protein of the invention can be formed by linking two polypeptides, e.g., a Hsp and a heterologous polypeptide, to form a conjugate. Methods of forming Hsp conjugates are described in WO 89/12455, WO 94/29459, WO 98/23735, and WO 99/07860, the contents of which are herein incorporated by reference. As used herein, an Hsp "conjugate" comprises an Hsp that has been covalently linked to a heterologous polypeptide via the action of a coupling agent. A conjugate thus comprises two separate molecules that have been coupled one to the other. The term "coupling agent," as used herein, refers to a reagent capable of coupling one polypeptide to another polypeptide, e.g., a Hsp to a heterologous polypeptide. Any bond which is capable of linking the components such that the linkage is stable under physiological conditions for the time needed for the assay (e.g., at least 12 hours, preferably at least 72 hours) is suitable. The link between two components may be direct, e.g., where a Hsp is linked directly to a heterologous polypeptide, or indirect, e.g., where a Hsp is linked to an intermediate, e.g., a backbone, and that intermediate is also linked to the heterologous polypeptide. A coupling agent should function under conditions of temperature, pH, salt, solvent system, and other reactants that substantially retain the chemical stability of the Hsp, the backbone (if present), and the heterologous polypeptide.

A coupling agent can link components, e.g., a Hsp and a heterologous polypeptide, without the addition of the coupling agent to the resulting fusion protein. Other coupling agents result in the addition of the coupling agent to the resulting fusion

protein. For example, coupling agents can be cross-linking agents that are homo- or hetero-bifunctional, and wherein one or more atomic components of the agent is retained in the composition. A coupling agent that is not a cross-linking agent can be removed entirely following the coupling reaction, so that the molecular product is composed entirely of the Hsp, the heterologous polypeptide, and a backbone moiety (if present).

Many coupling agents react with an amine and a carboxylate, to form an amide, or an alcohol and a carboxylate to form an ester. Coupling agents are known in the art, see, e.g., M. Bodansky, "Principles of Peptide Synthesis", 2nd ed., referenced herein, and T. Greene and P. Wuts, "Protective Groups in Organic Synthesis," 2nd Ed, 1991, John Wiley, NY. Coupling agents should link component moieties stably, but such that there is minimal or no denaturation or deactivation of the Hsp or the heterologous polypeptide.

The conjugates of the invention can be prepared by coupling a Hsp to a heterologous polypeptide using methods known in the art. A variety of coupling agents, including cross-linking agents, can be used for covalent conjugation. Examples of cross-linking agents include N,N'-dicyclohexylcarbodiimide (DCC; Pierce), N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), ortho-phenylenedimaleimide (*o*-PDM), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). See, e.g., Karpovsky *et al.* (1984) *J. Exp. Med.* 160:1686 and Liu *et al.* (1985) *Proc. Natl. Acad. Sci. USA 82*:8648. Other methods include those described by Paulus (1985) *Behring Ins. Mitt.* 78:118-132; Brennan *et al.* (1985) *Science* 229:81-83; and Glennie *et al.* (1987) *J. Immunol.* 139: 2367-2375. A large number of coupling agents for peptides and proteins, along with buffers, solvents, and methods of use, are described in the Pierce Chemical Co. catalog, pages T-155 –T-200, 1994 (3747 N. Meridian Rd., Rockford IL, 61105, U.S.A.; Pierce Europe B.V., P.O. Box 1512, 3260 BA Oud Beijerland, The Netherlands), which catalog is hereby incorporated by reference.

DCC is a useful coupling agent (Pierce #20320; Rockford, IL). It promotes coupling of the alcohol NHS in DMSO (Pierce #20684), forming an activated ester which can be cross-linked to polylysine. DCC (N,N'-dicyclohexylcarbodiimide) is a carboxy-reactive cross-linker commonly used as a coupling agent in peptide synthesis,

and has a molecular weight of 206.32. Another useful cross-linking agent is SPDP (Pierce #21557), a heterobifunctional cross-linker for use with primary amines and sulfhydryl groups. SPDP has a molecular weight of 312.4 and a spacer arm length of 6.8 angstroms, is reactive to NHS-esters and pyridyldithio groups, and produces cleavable cross-linking such that upon further reaction, the agent is eliminated so the Hsp can be linked directly to a backbone or heterologous polypeptide. Other useful conjugating agents are SATA (Pierce #26102) for introduction of blocked SH groups for two-step cross-linking, which are deblocked with hydroxylamine-HCl (Pierce #26103), and sulfo-SMCC (Pierce #22322), reactive towards amines and sulfhydryls. Other cross-linking and coupling agents are also available from Pierce Chemical Co. (Rockford, IL). Additional compounds and processes, particularly those involving a Schiff base as an intermediate, for conjugation of proteins to other proteins or to other compositions, for example to reporter groups or to chelators for metal ion labeling of a protein, are disclosed in EP 243,929 A2 (published Nov. 4, 1987).

Polypeptides that contain carboxyl groups can be joined to lysine ε-amino groups in the heterologous polypeptide either by preformed reactive esters (such as N-hydroxy succinimide ester) or esters conjugated *in situ* by a carbodiimide-mediated reaction. The same applies to Hsps containing sulfonic acid groups, which can be transformed to sulfonyl chlorides that react with amino groups. Hsps that have carboxyl groups can be joined to amino groups on the polypeptide by an *in situ* carbodiimide method. Hsps can also be attached to hydroxyl groups of serine or threonine residues, or to sulfhydryl groups of cysteine residues.

In addition to conjugates of two polypeptides, e.g., a Hsp and a heterologous polypeptide, hybrid compounds can be constructed containing a non-peptide compound covalently linked to a polypeptide at least eight amino acids in length. The polypeptide component of this hybrid compound can be any of the heterologous polypeptides described herein as a component of a Hsp fusion protein or conjugate. Examples of the non-peptide component of this hybrid compound include polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, preferably between about 1,500 and 100 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such non-peptide compounds.

In Vitro Assays for Th1-Like Activity

Cell samples containing naive lymphocytes are prepared from any mammal, e.g., a mouse, rat, rabbit, goat, or human, and are plated at an appropriate density in one or more tissue culture plates. A naive lymphocyte is a lymphocyte that has not been exposed (either *in vivo* or *in vitro*) to the fusion protein (or to either of the polypeptides that are joined to make the fusion protein) prior to the cell's use in the *in vitro* assay. The cell sample can be derived from any of various primary or secondary lymphoid organs or tissues of an animal, e.g., spleen, lymph node, peripheral blood, bone marrow, or thymus. The sample may also be derived from any tissue in the body containing lymphoid cells, such as the lung, respiratory tract (including pharynx, larynx, trachea, bronchi, etc), and anogenital mucosa. The cell sample can include naive lymphocytes selected from NK cells, NK T cells, αβT cells and γδT cells. The cell sample can be either unfractionated or enriched for a particular cell type or cell types. In addition to naive lymphocytes, the cell sample can optionally include naive antigen presenting cells such as macrophages, dendtritic cells, and/or B cells. The cell sample can optionally include cell lines, e.g., a transformed T cell line or a T cell clone.

The cell sample is exposed *in vitro* to a fusion protein or a conjugate described herein. Following a period of incubation between the cell sample and the fusion protein or conjugate, e.g., 6, 12, 24, 36, 48, 60, 72, or 96 hours, a determination is made as to whether a Th1-like response has been elicited in the cell sample. A Th1-like response can be detected, for example, by measuring the production of particular lymphokines, e.g., IFN-gamma or TNF-beta, by the cell sample. Alternatively, a Th1-like response can be detected by assaying for cell surface marker expression, such as SLAM (signaling lymphocytic activation molecule), or for cytokine expression, using a variety of techniques (for example, flow cytometry).

In one example, pooled, unfractionated splenocyte cultures containing naive lymphocytes are prepared from a mouse and are plated in tissue culture plates. Methods of isolating and culturing splenocytes are described in Current Protocols in Immunology, Coligan et al., eds., John Wiley & Sons, 2000. Cultures of splenocytes are then exposed to different concentrations of a test protein, e.g., a recombinant Hsp fusion protein, Hsp, the antigen alone, or another antigen-containing fusion protein, for a time that is sufficient to elicit a measurable IFN-gamma response against a standard antigen-stress protein fusion protein such as, for example, HspE7, described in patent

application WO 99/07860 and employed in the Examples below. Following exposure of the cell sample to the test protein, the IFN-gamma level in the extracellular medium is determined using a suitable assay such as an IFN-gamma ELISA.

Results of the assays described below reveal that IFN-gamma release elicited by exposure of splenocytes or lymph node cells to an Hsp fusion protein is much more substantial than that induced by exposure to the antigen itself, the Hsp itself, an admixture of antigen and Hsp, or a fusion between antigen and a protein other than a Hsp.

The assay of the invention can be used to evaluate a preparation of an Hsp fusion protein (e.g., as a quality control assay) or compare different preparations of Hsp fusion proteins. The measurements taken in the assay constitute a method for identifying a particularly active batch or to eliminate substandard batches of fusion protein preparations. The assay may also be used to optimize production procedures, storage regimes, etc. In cases in which a maximal Th1-like response to a particular antigen is desired, the assays can be used to test different fusions between the antigen and different types of Hsps or Hsps of different origins. Furthermore, the assay can be used to test a series of different candidate antigens, to identify the antigen that gives rise to the most pronounced Th1-like response when fused to a Hsp.

The assay can also be used to identify regions in an antigen sequence or an Hsp sequence that are primarily responsible for eliciting a Th1-like response and thus have therapeutic potential. To identify such active regions in an antigen, fusions containing individual subregions of the antigen fused to an Hsp can be prepared and tested in the assay of the invention. To identify active regions in an Hsp, fusions containing individual subregions of the Hsp fused to the antigen can be prepared and tested. These determinations will provide the basis for the construction of shortened fusion proteins comprising subregions of antigen and/or Hsp that are sufficient to elicit a Th1-like response. Fusions containing subregions of a Hsp and/or subregions of an antigen can be tested by comparing the elicited Th1-like response to that induced by a full length fusion protein with known activity, e.g., HspE7.

The fusion proteins described herein are useful in assays for screening compounds for their effectiveness in stimulating a Th1-like response. For example, the Hsp fusion proteins that were found to stimulate IFN-gamma secretion in the *in vitro*

assay can be used as controls to test candidate compounds for their ability to produce the same effect.

The system described herein for stimulating a Th1-like response *in vitro* can be used to generate activated Th1 cells *ex vivo* for reimplantation into an individual. This may be useful for treating conditions characterized by a dominant Th2 immune response and an insufficient Th1 response.

The assay can also be used to identify compounds that can regulate a Th1-like response. Compounds can be screened for their ability to inhibit an Hsp-fusion protein-induced Th1-like response, or to promote a Th1-like response in a manner similar to a Hsp fusion protein, or to enhance the Th1-like response induced by a Hsp fusion protein (or any other protein found to act in a manner comparable to a Hsp fusion protein). Inhibitory compounds may be useful to treat conditions characterized by an inappropriate Th1 response, e.g., inflammatory and autoimmune diseases. Potential inhibitors (e.g., of binding of antigen-stress protein fusion proteins to antigen-presenting cells or of stress protein fusion-enhanced antigen processing) can be screened as follows. A cell sample comprising naive lymphocytes is mixed with a fusion protein or conjugate that is known to induce a Th1-like response, e.g., IFN-gamma secretion. Compounds to be screened as potential inhibitors are added to the cell culture either before, after, or simultaneous to the addition of the fusion protein or conjugate. The effect of the compound on the induction of a Th1-like response, e.g., as measured by IFN-gamma release, can be determined by comparing the response to that obtained when the fusion protein or conjugate alone is added to the cell sample.

In a similar manner, compounds can be screened for their ability to promote a Th1-like response. Any compound can be screened for its ability to regulate a Th1-like response, including both peptides and non-peptide chemicals. These compounds include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. In this case, a cell sample comprising naive lymphocytes is contacted with a test compound. The effect of the test compound on the induction of a Th1-like response, e.g., as measured by IFN-gamma release, is then measured and compared to a control (no test sample) or compared to an Hsp fusion known to stimulate a Th1-like

response. This assay can be used to identify novel compounds that can be used to stimulate a Th1-like response. Preferably the Th1-like response stimulated by the compound is at least 25%, e.g., at least 40%, 50%, 60%, 70%, or 80%, the level of the maximum response induced by an HspE7 fusion protein. In one embodiment, the compound is preferably not a naturally occurring compound. In another embodiment, the compound is a peptide, wherein the peptide does not correspond to the fragment of a naturally occurring protein.

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

Examples

Example 1: Bacterial Growth and Cell Lysis for Production of Recombinant Proteins

E. coli strains BL21(DE3) or BLR(DE3) (Novagen) were used as the host for all recombinant protein production, with the exception of pET65, which was transformed into BL21(DE3) pLysS (Novagen). BL21(DE3) pLysS cells harboring pET65 were grown in 2xYT media (20 g/L tryptone; 10 g/L yeast extract, 20 g/L NaCl; Milli-Q™ quality water) containing 30 µg/ml kanamycin and 34 µg/ml chloramphenicol, while all other transformants were grown in 2xYT media containing 30 µg/ml kanamycin. All bacterial cultures were grown in 2 L shaker flasks at 200-400 rpm to OD_{600}=0.5 and then induced with 0.5 mM IPTG for 3 hours at 37°C. Cells were then harvested by centrifugation at 4°C and 4,000 – 8,000 *g* for 5 minutes, then suspended in 300 ml of Lysis Buffer (10 mM TRIS·HCl, 10 mM 2-mercaptoethanol, pH 7.5), lysozyme was added to 200 µg/mL, and the suspension mixed and frozen at -70°C.

To purify the recombinant protein, the cells were thawed using a 37°C waterbath and proteinase inhibitors were added (2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin and 2 mM PMSF). The cell suspension was split into 50 mL samples, stored on ice, and sonicated 3–4 times for 30 seconds at Power-Level 5 - 8 (Sonicator 450, Branson, Corp.). The supernatant was separated from the pellet by centrifugation at 35,000 – 60,000 *g* for 10 –20 minutes at 4°C. For soluble proteins, the supernatant was kept and processed as the Soluble Fraction. For proteins found in inclusion bodies,

the supernatant was discarded and the pellet was washed with Lysis Buffer (optionally containing 1 M urea, 1 %(v/v) Triton X-100). The resulting mixture was then centrifugation at 35,000 – 60,000 *g* for 10 –20 minutes at 4°C and the supernatant discarded. The pellet was dissolved in Lysis Buffer containing 8 M urea. This mixture was then centrifuged at 4°C for 10 - 20 minutes at 35,000 – 60,000 *g* and the pellet was discarded and the supernatant stored at -70°C as the Inclusion Body fraction.

Example 2: Production of Recombinant *M. bovis* BCG Hsp65 (Hsp65)

A plasmid encoding Hsp65 was constructed as follows. The *M. bovis* BCG Hsp65 coding sequence was PCR amplified from pRIB1300 (van Eden *et al.* (1988) Nature 331:171-173) using the following primers. The forward primer (w046: 5' TTC GCC ATG GCC AAG ACA ATT GCG 3'; SEQ ID NO:1) contains an ATG start codon at an NcoI site. The reverse primer (w078: 5' TTC TCG GCT AGC TCA GAA ATC CAT GCC 3'; SEQ ID NO:2) contains an Nhe I site downstream of a TGA stop codon. The PCR product was digested with NcoI and NheI, purified and ligated to pET28a (Novagen) which had been cut with NcoI and NheI. Plasmid pET65 encodes the *M. bovis* BCG Hsp65 protein, abbreviated Hsp65. The nucleotide sequence (SEQ ID NO:3) coding for expression of Hsp65 (SEQ ID NO:4) is shown in Figs. 1A-1B.

The Hsp65 protein was purified as follows. The Soluble Fraction was prepared as described above from *E. coli* BL21(DE3) pLysS cells transformed with plasmid pET65. The *M. bovis* BCG Hsp65 protein (Hsp65) present in the Soluble Fraction was purified by the following chromatographic steps: SP-Sepharose (200 ml column, Amersham Pharmacia), Q-Sepharose (200 ml column, Amersham Pharmacia), Sephacryl S-300 (500 ml column, Amersham Pharmacia) and ceramic hydroxyapatite (HAP; 100 ml column, Biorad). Purified Hsp65 was exchanged into Dulbecco's modified phosphate buffered saline (DPBS)/15% (v/v) glycerol and stored at -70°C.

Example 3: Production of Recombinant HPV16 E7 (E7)

A plasmid encoding HPV16 E7 was constructed as follows. The HPV16 E7 coding sequence was PCR-amplified from pSK/HPV16 (ATCC) using primers w280 and w134 (w280: CCA GCT GTA ACC ATG GAT GGA GAT (SEQ ID NO:5) and w134: AGC CAT GAA TTC TTA TGG TTT CTG (SEQ ID NO:6)). The PCR product was digested with restriction enzyme Nco I and EcoR I and purified from an agarose

gel. The purified PCR product was ligated to pET28a that had been previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the HPV16 E7 gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of entire gene, promoter and termination regions. DNA of the confirmed construct, named pET/E7 (NH), was then introduced by electroporation into *E. coli* strain BL21(DE3). The nucleotide sequence (SEQ ID NO:7) coding for expression of E7 (SEQ ID NO:8) is shown in Fig. 2.

The HPV16 E7 protein was purified as follows. The Soluble Fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pET/E7 (NH). The HPV 16 E7 protein was purified by the following chromatographic steps: Q-Sepharose (100 ml column, Amersham Pharmacia); Superdex 200 (26/60 column, Amersham Pharmacia); and Ni-chelating Sepharose (100 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100, and the pooled fractions containing HPV E7 protein were then dialyzed overnight against 30 mM TRIS·HCl, 1 M NaCl, 1 mM 2-mercaptoethanol, pH 7.5. The dialyzed protein was further purified by Ni-chelating Sepharose (75 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v)Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS/10 %(v/v) glycerol.

Example 4: Production of Recombinant Histidine-tagged HPV 16 E7 ((h)E7)

A plasmid encoding (h)E7 was constructed as follows. The HPV16 E7 coding sequence was PCR amplified from HPV16 genomic DNA (pSK/HPV16) using the following primers. The forward primer (w133: 5' AAC CCA GCT GCT AGC ATG CAT GGA GAT 3'; SEQ ID NO:9) contains an NheI site upstream of an ATG start codon. The reverse primer (w134: 5' AGC CAT GAA TTC TTA TGG TTT CTG 3'; SEQ ID NO:10) contains an EcoRI site downstream of a TAA stop codon. The PCR product was digested with NheI and EcoRI, purified and ligated to pET28a which had been cut with NheI and EcoRI. pET/H/E7 which encodes the HPV16 E7 protein containing an N-terminal histidine tag, abbreviated (h)E7, was used to transform *E. coli*

BL21(DE3) cells. The nucleotide sequence (SEQ ID NO:11) coding for expression of (h)E7 (SEQ ID NO:12) is shown in Fig. 3.

The (h)E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pET/H/E7. The N-terminal histidine-tagged HPV16 E7 protein ((h)E7) present in the Inclusion Body fraction was purified using the following chromatographic steps: Ni-chelating Sepharose (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. Bound (h)E7 was refolded on the resin and eluted by a 50-500 mM imidazole gradient. Purified (h)E7 was dialyzed against DPBS/25% (v/v) glycerol.

Example 5: Production of Recombinant HPV 16 E7 - *M. bovis* BCG 65 Fusion Protein (HspE7)

A plasmid encoding HspE7 was constructed as follows. The *M. bovis* BCG Hsp65 coding sequence was PCR amplified from pRIB1300 using the same forward primer (w046) as for pET65. The reverse primer (w076: 5' CGC TCG GAC GCT AGC TCA CAT ATG GAA ATC CAT GCC 3'; SEQ ID NO:13) contains an NdeI site upstream and an NheI site downstream of a TGA stop codon. The PCR product was digested with NcoI and NheI, purified and ligated to pET28a which had been cut with NcoI and NheI.

The HPV16 E7 coding sequence was PCR amplified from HPV16 genomic DNA (pSK/HPV16) using the following primers. The forward primer (w151: 5' CCA GCT GTA CAT ATG CAT GGA GAT 3'; SEQ ID NO:14) contains an ATG start codon at an NdeI site. The reverse primer (w134: 5' AGC CAT GAA TTC TTA TGG TTT CTG 3'; SEQ ID NO:15) contains an EcoRI site downstream of a TAA stop codon. The PCR product was digested with NdeI and EcoRI, purified and ligated to pET65C which had been cut with Nde I and EcoRI and the resulting plasmid (pET65C/E7-1N) was transformed into *E. coli* BL21(DE3) cells. pET65C/E7-1N encodes a fusion protein consisting of Hsp65 linked via its C-terminus to HPV16 E7, abbreviated HspE7. The nucleotide sequence (SEQ ID NO:16) coding for expression of HspE7 (SEQ ID NO:17) is shown in Figs. 4A-4B.

The HspE7 protein was purified as follows. The Soluble Fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pET65C/E7-1N. Hsp65-HPV16 E7 fusion protein (HspE7) present in the Soluble Fraction was purified by the following chromatographic steps: 0-15% ammonium sulfate precipitation, Ni-chelating Sepharose (100 ml column, Amersham Pharmacia) and Q-Sepharose (100 ml column, Amersham Pharmacia). Endotoxin was removed by extensive washing with 1% (v/v) Triton X-100 on a Ni-chelating Sepharose column in the presence of 6M guanidine-HCl (Gu-HCl). Purified HspE7 was exchanged into DPBS/15% (v/v) glycerol and stored at -70°C.

Example 6: Production of Recombinant *M. tuberculosis* Hsp40 – HPV 16 E7 Fusion Protein (MT40-E7)

pETMT40E7 is a plasmid encoding chimeric recombinant protein MT40E7 composed of *Mycobacterium tuberculosis* (strain H37RV - ATCC 27294) hsp40 protein with hu HPV16 (ATCC 45113) E7 protein attached at the C-terminus of Hsp40. The plasmid was transformed into *E. coli* BL21(DE3) cells for protein production and purification. The nucleotide sequence (SEQ ID NO:18) coding for expression of MT40-E7 (SEQ ID NO:19) is shown in Figs. 5A-5B.

The MT40-E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pETMT40E7. MT40-E7 protein was purified using the following chromatographic steps: Q-Sepharose (100 ml column, Amersham Pharmacia), Ni-chelating Sepharose (70 ml, Amersham Pharmacia) under native conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS/25% (v/v) glycerol.

Example 7: Ovalbumin (OVA)

Ovalbumin (Lot # 37H7010) was purchased from Sigma Chemicals and purified by chromatography using 20 mL of Con A Sepharose (Amersham-Pharmacia). Fractions containing the purified product were pooled and dialyzed overnight against DPBS.

Example 8: Production of Recombinant *M. bovis* BCG Hsp65-Ovalbumin Fusion Protein (HspOva)

A plasmid encoding HspOva was constructed as follows. The full length chicken ovalbumin-coding sequence was excised from pET/OVA with Nhe I and EcoR I digestion and purified from an agarose gel. The sequence (SEQ ID NO:54) coding for expression of OVA (SEQ ID NO:55) is shown in Fig. 6. The purified product was ligated to pET65H previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the chicken ovalbumin gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of the entire fusion gene, promoter and termination regions. DNA of the confirmed construct, named pET65H/OVA, was used to transform *E. coli* BL21(DE3). The nucleotide sequence (SEQ ID NO:20) coding for expression of HspOVA (SEQ ID NO:21) is shown in Figs. 7A-7C.

The HspOva protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pET65H/OVA. The HspOva fusion protein present in the Inclusion Body fraction was purified using the following chromatographic steps: Q-Sepharose (100 ml column, Amersham Pharmacia) and Ni-chelating Sepharose (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS/15% (v/v) glycerol, followed by a dialysis against DPBS/2.5 %(w/v) sucrose.

Example 9: Production of Recombinant Glutathione-S-Transferase (GST)

A plasmid encoding Gst was constructed as follows. The kanamycin resistance-coding sequence was excised from pET28a DNA with AlwN I and Xho I digestion and purified from an agarose gel. The purified product was ligated to pGEX-4T-2 that had been previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the kanamycin resistance gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of the entire insert

coding sequence, promoter and termination regions. DNA of the confirmed construct, named pGEX/K, was used to transform *E. coli* strain BL21(DE3). The nucleotide sequence (SEQ ID NO:22) coding for expression of GST (SEQ ID NO:23) is shown in Fig. 8.

The GST protein was purified as follows. The Soluble fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pGEX/K. The GST protein present in the Soluble Fraction was purified by Glutathione-Agarose Chromatography as follows. Approximately 20 mL of Glutathione-Agarose (Sigma-Aldrich; Cat. #: G4510) was equilibrated with DPBS, and mixed and incubated overnight with the sample at room temperature on a shaker. The next morning, the resin was packed into a column and serially washed with DPBS. Endotoxin was removed by washing with 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. Finally, the protein was eluted using 10 mM glutathione (reduced form), 50 mM TRIS·HCl, pH 8.0.

Example 10: Production of Recombinant Glutathione-S-Transferase – HPV 16 E7 Fusion Protein (GST-E7)

A plasmid encoding GST-E7 was constructed as follows. The HPV16 E7 coding sequence was excised from pETOVA/E7 with BamH I and EcoR I digestion and purified from an agarose gel. The purified product was ligated to pGEX/K that had been previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the HPV16-E7 gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of entire fusion gene, promoter and termination regions. DNA of the confirmed construct, named pGEX/K/E7, was used to transform *E. coli* strain BL21(DE3). The nucleotide sequence (SEQ ID NO:24) coding for expression of GST-E7 (SEQ ID NO:25) is shown in Fig. 9.

The GST-E7 protein was purified as follows. Bacteria containing the expression vector pGEX/K/E7 were grown and the protein purified using the affinity chromatography procedure essentially as described above for GST.

Example 11: Production of Recombinant HPV 16 E7 – Linker – *M. bovis* BCG Hsp65 Fusion Protein (E7-L-BCG65)

A plasmid encoding E7-L-BCG65 was constructed as follows. The HPV16 E7-coding sequence was PCR-amplified from pSK/HPV16 (ATCC) using primers w280 and w396 (w280: CCA GCT GTA ACC ATG GAT GGA GAT (SEQ ID NO:26) and w396: GCC ATG GTA CTA GTT GGT TTC TGA GAA(SEQ ID NO27:)). The PCR product was digested with restriction enzyme Nco I and Spe I and purified from an agarose gel. The purified PCR product was ligated to pET5'65 (pET5'65 is pET65 with a polyglycine linker sequence inserted at the 5' end of the *M. bovis* BCG hsp65 sequence) that had been previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the HPV16 E7 gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of entire fusion gene, promoter and termination regions. DNA of confirmed construct, named pET/E7/5'65, was used to transform *E. coli* strain BLR(DE3). The nucleotide sequence (SEQ ID NO:28) coding for expression of E7-L-BCG65 (SEQ ID NO:29) is shown in Figs. 10A-10B.

The E7-L-BCG65 protein was purified as follows. The Soluble Fraction was prepared as described above from *E. coli* BLR(DE3) cells transformed with plasmid pET/E7/5'65. The E7-L-BCG65 fusion protein present in the Soluble Fraction was purified using the following chromatographic steps: Butyl Sepharose (100 ml, Amersham-Pharmacia), Q-Sepharose (100 ml column, Amersham Pharmacia), Superdex 200 Gel Filtration (26/60 column, Amersham Pharmacia), and Ni-chelating Sepharose Fast Flow Chromotography (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS. In order to reduce the amount of endotoxin contained in the sample, it was further purified using a pre-packed 1 ml column of DetoxiGel™ (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Example 12: Production of Recombinant HPV 16 E7 – *M. bovis* BCG Hsp65 Fragment Fusion Protein (BCG65(F1)-E7)

A plasmid encoding BCG65(F1)-E7 was constructed as follows. The first 600 amino terminal base pairs of *M. bovis* BCG hsp65 gene were PCR-amplified from pET65C/E7-1N using primers w046 and w293 (w046: TTC GCC ATG GCC AAG ACA ATT GCG (SEQ ID NO:30) and w293: GTA CCC CGA CAT ATG GCC CTT GTC GAA CCG CAT AC(SEQ ID NO:31)). The PCR product was digested with the restriction enzymes Nco I and Nde I and purified from an agarose gel. The purified PCR product was ligated to pET65C/E7-1N that had been previously digested with the same enzymes. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the truncated BCG65 gene were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of the entire fusion gene, promoter and termination regions. The confirmed plasmid construct, named pET65F1/E7, was used to transform *E. coli* strain BLR(DE3). The nucleotide sequence (SEQ ID NO:32) coding for expression of BCG65(F1)-E7 (SEQ ID NO:33) is shown in Fig. 11.

The BCG65(F1)-E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BLR(DE3) cells transformed with plasmid pET65F1/E7. The BCG65(F1)-E7 fusion protein present in the Inclusion Body fraction was purified using the following chromatographic steps: Source 15Q Sepharose (Amersham-Pharmacia) and Ni-chelating Sepharose (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS.

Example 13: Production of Recombinant *M. tuberculosis* Hsp10 – HPV 16 E7 Fusion Protein (TB10-E7)

Expression plasmid pETESE7 contains a chimeric gene composed of the *Mycobacterium tuberculosis* strain H37RV (ATCC 27294) groES (hsp10) coding sequence fused, at its 3' end, to the HPV16 (ATCC 45113) E7 coding. The chimeric gene was cloned into expression vector pET28a and transformed into *E. coli* BL21(DE3) cells for protein production and purification. The nucleotide sequence

(SEQ ID NO:34) coding for expression of TB10-E7 (SEQ ID NO:35) is shown in Fig. 12.

The TB10-E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pETESE7. The TB10-E7 fusion protein present in the Inclusion Body fraction was purified using the following chromatographic steps: DEAE Sepharose (100 ml column, Amersham Pharmacia), Source 15Q Sepharose (100 ml column, Amersham Pharmacia) and Ni-chelating Sepharose (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS/10 %(v/v) glycerol.

Example 14: Production of Recombinant HPV 16 E7 – *M. tuberculosis* Hsp71 Fusion Protein (E7-TB71)

A plasmid encoding E7-TB71 was constructed as follows. The *M. tuberculosis* hsp71 gene was PCR-amplified from clone pY3111/8 (Mehlert and Young (1989) Mol.Microbiol. 3:125-130) using primers w048 and w079 (w048: 5'-TTC ACC ATG GCT CGT GCG GTC GGG (SEQ ID NO:36) and w079: ACC TCC GCG TCC ACA GCT AGC TCA GCC(SEQ ID NO:37)). The PCR product was digested with Nco I and Nhe I, gel-purified and ligated to pET28a digested with the same enzymes to generate pET/71.

The HPV16 E7-coding sequence was PCR-amplified from pSK/HPV16 (ATCC) using primers w280 and w344 (w280: CCA GCT GTA ACC ATG GAT GGA GAT (SEQ ID NO:38) and w344: GGA TCA GAC ATG GCC ATG GCT GGT TTC TG (SEQ ID NO:39)). The PCR product was digested with restriction enzyme Nco I and purified from an agarose gel. The purified PCR product was ligated to pET/71 DNA that had been previously digested with Nco I and CIAP to remove 5' phosphate. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the HPV16 E7 gene insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of entire fusion gene, promoter and termination regions. The confirmed construct, named pET/E7/71, was used to transform *E. coli* strain BL21(DE3). The nucleotide sequence

(SEQ ID NO:40) coding for expression of E7-TB71 (SEQ ID NO:41) is shown in Figs. 13A-13B. The resulting construct, pET/E7/71, was further modified (to complete sequences at the 3' end of the hsp71 gene) by replacement of a Kpn I to Nhe I fragment containing sequences from the 3' end of the hsp71 gene by a Kpn I- and Nhe I-digested PCR fragment amplified from pY3111/8 using primers w391 and w392 (w391: GAG GGT GGT TCG AAG GTA CC (SEQ ID NO:42) and w392: TTT GAT TTC GCT AGC TCA CTT GGC CTC(SEQ ID NO:43)). The resulting final plasmid, pET/E7/71', expresses HPV16 E7 fused to the amino-terminus of full-length Hsp71 protein and was used to transform *E. coli* strain BL21(DE3). The nucleotide sequence (SEQ ID NO:44) coding for expression of the fusion protein (SEQ ID NO:45) of pET/E7/71' is shown in Figs. 14A-14B.

The E7-TB71 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pET/E7/71'. The E7-TB71 fusion protein present in the Inclusion Body fraction was purified using the following chromatographic steps: Q-Sepharose (100 ml column, Amersham Pharmacia) and Ni-chelating Sepharose (80 ml, Amersham Pharmacia) under native conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS/10 %(v/v) glycerol.

Example 15: Production of Recombinant *Streptococcus pneumoniae* HSP65(2) – HPV 16 E7 Fusion Protein (SP65(2)-E7)

A plasmid encoding SP65(2)-E7 was constructed as follows. The *Streptococcus pneumoniae* hsp65 gene was PCR-amplified from plasmid pETP60-2 (PCT patent application WO 99/35720) using primers w384 and w385 (w384: GCA GCC CCA TGG CAA AAG AAA (SEQ ID NO:46) and w385: GCT CGA ATT CGG TCA GCT AGC TCC GCC CAT (SEQ ID NO:47)). The PCR product was digested with Nco I and EcoR I, gel-purified and ligated to pET28a digested with the same enzymes to generate pET/SP65-2C.

The HPV16 E7-coding sequence was PCR-amplified from pSK/HPV16 (ATCC) using primers w133 and w134 (w133: AAC CCA GCT GCT AGC ATG CAT GGA GAT (SEQ ID NO:48) and w134: AGC CAT GAA TTC TTA TGG TTT CTG

(SEQ ID NO:49)). The PCR product was digested with restriction enzymes Nhe I and EcoR I and purified from an agarose gel. The purified PCR product was then ligated to pET/SP65-2C that had been previously digested with Nhe I and EcoR I. The ligation reaction was used to transform *E. coli* DH5alpha and putative clones containing the HPV16 E7 insert were selected based on diagnostic restriction digestion. This initial restriction analysis was confirmed by DNA sequence analysis of entire fusion gene, promoter and termination regions. DNA of the confirmed construct, named pET/SP65c-E7, was used to transform *E. coli* strain BLR(DE3). The nucleotide sequence (SEQ ID NO:50) coding for expression of SP65(2)-E7 (SEQ ID NO:51) is shown in Figs. 15A-15B.

The SP65(2)-E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BLR(DE3) cells transformed with plasmid pET/SP65c-E7. The SP65(2)-E7 fusion protein present in the Inclusion Body fraction was purified using the following chromatographic steps: Q-Sepharose (100 ml column, Amersham Pharmacia) and Ni-chelating (60 ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS.

Example 16: Recombinant Production of *Aspergillus fumigatus* Hsp60- HPV 16 E7 Fusion Protein (AF60-E7)

pETAF60E7 is a plasmid encoding a recombinant protein, AF60-E7, composed of the *Aspergillus fumigatus* (ATCC 26933) Hsp60 protein (without leader) (obtained as described in PCT/CA99/01152) fused at its C-terminus to the HPV16 (ATCC 45113) E7 protein sequence. Plasmid pETAF60E7 was used to transform *E. coli* BL21(DE3) cells for protein production and purification. The nucleotide sequence (SEQ ID NO:52) coding for expression of AF60-E7 (SEQ ID NO:53) is shown in Figs. 16A-16B.

The AF60-E7 protein was purified as follows. The Inclusion Body fraction was prepared as described above from *E. coli* BL21(DE3) cells transformed with plasmid pETAF60E7. AF60-E7 protein was purified using the following chromatographic steps: Source 15Q Sepharose (Amersham-Pharmacia) and Ni-chelating Sepharose (60

ml, Amersham Pharmacia) under denaturing conditions with serial washings containing 2% (v/v) Triton X-100 followed by serial washing to remove residual Triton X-100. The purity of the protein was checked by SDS-PAGE, the appropriate fractions pooled and dialyzed overnight at 4°C against DPBS.

Example 17: Stimulation of IFN-Gamma Release by a Hsp65-HPVE7 (HspE7) Fusion Protein

Pooled, unfractionated splenocytes were prepared from untreated naive C57BL/6 mice obtained from two different sources (Charles River Laboratory and Jackson Laboratory) and were plated in complete medium (complete RPMI) at 6 x 10^5 cells/well in flat bottom 96-well tissue culture plates. Replicate cultures (5) were incubated for 72 hours with 0.05 to 1.4 nmol/mL concentrations of recombinant *M. bovis* BCG Hsp65 (Hsp65), HPV16 E7 (E7) or histidine-tagged E7 ((h)E7), an admixture of *M. bovis* BCG Hsp65 and HPV16 E7 (Hsp65 + E7), or *M. bovis* BCG Hsp65 - HPV16 E7 fusion protein (HspE7). Subsequent to incubation, cells were pelleted, and supernatants were transferred to IFN-gamma capture ELISA plates.

After incubation, the replicate samples were harvested, pooled in eppendorf tubes and pelleted at 1200 rpm for 7 minutes in Beckman GS-6R centrifuge (300 x g). The supernatants were removed into cryovials and frozen at -70°C until time of analysis.

Maxisorp ELISA plates (Nunc cat# 442404A) were coated overnight at 4°C with 1 µg/mL purified rat anti-mouse IFN-gamma (PharMingen cat. no 18181D) in 0.1 M $NaHCO_3$ buffer, pH 8.2. The plates were washed with 0.05% Tween 20 in PBS then blocked with 3% BSA (albumin fraction V: Amersham cat. no 10857) in DPBS (blocking buffer) for 2 hours. After the plates were washed, recombinant mouse IFN-gamma (8000, 4000, 2000, 1000, 500, 250, 125, 62.5 pg/mL in complete RMPI) was placed in triplicate onto each ELISA plate. Sample supernatants were removed from -70°C, thawed quickly at 37°C, and placed undiluted onto the ELISA plates in duplicate. The samples were then serially diluted by seven, 3-fold dilutions in complete RPMI followed by incubation at 4°C overnight. Background ELISA values were established by measuring eight wells containing all reagents except the target antigen.

Detection of bound murine IFN-gamma was accomplished using 1 µg/mL of a rat anti-mouse IFN-gamma biotin conjugate (PharMingen cat. no 18112D) in blocking

buffer. Following washing, bound biotin-conjugated antibody was detected using a 1:1000 dilution of a streptavidin-alkaline phosphatase conjugate (Caltag cat. no SA1008). The plates were washed as before followed by the addition of a chromogenic substrate, p-nitrophenyl phosphate (pNPP; Sigma cat# N-2765) at 1 mg/mL in diethanolamine buffer, pH 9.5. After 30 minutes incubation, the color reaction was stopped using 50 µL of 100 mM EDTA, pH 8.0. The absorbance was measured at 410 nm using a Dynatech MR5000 ELISA plate reader equipped with Biolinx 2.0 software. The levels of IFN-gamma detected in test samples were extrapolated from the standard curves generated on each of the respective ELISA plates. Data is expressed as IFN-gamma released (pg/mL ± SD).

Results of assays are shown in Figs. 17A-17B. The averages from five replicates are shown along with the standard deviation. Substantial secretion of IFN-gamma was elicited by exposure of splenocytes to 0.05, 0.15, 0.46 and 1.4 nmol/mL HspE7. Hsp65 alone, E7 alone, hE7 alone, and an admixture of Hsp65 and E7 were virtually incapable of stimulating IFN-gamma release. Similar results were obtained with splenocytes prepared from mice obtained from the Charles River Laboratory (Fig. 17A) and from the Jackson Laboratory (Fig. 17B).

Example 18: Stimulation of IFN-Gamma Release by a HspE7 Fusion Protein in Splenocyte Cultures from Mice Having Different Genetic Backgrounds

Experiments similar to those presented in Example 17 were carried out using splenocytes from mice (from Jackson Laboratory) of three different haplotypes: C57BL/6 (H-2^b); Balb/c (H-2^d); and C3HeB/FeJ (H-2^k). The relative effects of the fusion protein on the different splenocyte preparations were similar, although there were differences in the absolute amounts of IFN-gamma released: the observed order being Balb/c (highest; Fig. 18A), C57BL/6 (intermediate; Fig. 18B), and C3HeB/FeJ (lowest; Fig. 18C). As in Example 17, substantially increased IFN-gamma release was induced by HspE7, but not by E7 alone, Hsp65 alone, or an admixture of E7 and Hsp65.

Example 19: Stimulation of IFN-Gamma Release by Fusion Proteins is Independent of the Nature of the Linked Antigen but Requires a Linked Stress Protein Moiety

Experiments were performed as discussed under the previous examples. It was observed that stimulation of naive splenocytes by (h)E7 or Hsp65 (*M. bovis* BCG) produced negligible IFN-gamma release, but that fusion proteins containing E7 and Hsp65 (*M. bovis* BCG) or Hsp40 (*M. tuberculosis*) substantially enhanced IFN-gamma release (Fig. 19). Virtually no induction of IFN-gamma release was mediated by a fusion protein containing E7 and glutathione-S-transferase (GST). When a fusion protein including an ovalbumin fragment and an Hsp (*M. bovis* BCG Hsp65) was tested, high levels of IFN-gamma release were detected. The IFN-gamma release mediated by the HspOVA fusion protein exceeded that resulting from addition of OVA alone to the cell culture. These results demonstrate that the induced release of IFN-gamma is not dependent on the presence of the E7 antigen in the fusion protein, but that other antigens fused to an Hsp can similarly enhance IFN-gamma production.

Example 20: Stimulation of IFN-Gamma Release by E7 Fusion Proteins Having Different Stress Protein Moieties

Experiments were performed as discussed under the previous examples. HPV16 E7 was fused to different Hsps, i.e., *M. tuberculosis* Hsp10 (TB10-E7), *M. bovis* BCG Hsp65 (HspE7), *Streptococcus pneumoniae* Hsp65 (2) (SP65(2)-E7), and *Aspergillus fumigatus* Hsp60 (AF60-E7). Furthermore, in two cases (E7-L-BCG65 and E7-TB71) the Hsp (*M. bovis* BCG Hsp65 and *M. tuberculosis* Hsp71, respectively) was added to the carboxy terminus of the E7 antigen instead of to the amino terminus as in the other fusions.

Additionally, one construct was tested, in which the E7 antigen was linked to the amino terminal one third (residues 1-200) of the *M. bovis* BCG Hsp65 sequence (BCG65(F1)-E7), rather than an intact Hsp. It was observed (Figs. 20A-20B) that stimulation of IFN-gamma release occurred upon exposure of splenocytes to all the different fusion proteins, although differences in the magnitude of the responses were noted. Thus, fusions containing different Hsps, including Hsp65 from different organisms as well as different types of Hsps, were capable of eliciting enhanced IFN-gamma release. Furthermore, fusions containing a stress protein at either the amino terminal end or at the carboxy terminal end of the E7 antigen were active. Finally,

BCG65(F1)-E7, containing amino acids 1-200 of *M. bovis* BCG Hsp65, induced IFN-gamma secretion in a manner similar to the full-length Hsp65 sequence (HspE7).

Example 21: Stimulation of IFN-Gamma Release by HspE7 Fusion Protein in Lymph Node Cell Cultures

To test for their ability to induce IFN-gamma release, various concentrations of the HspE7 proteins (diluted to the desired starting concentration in complete medium, defined as RPMI 1640 with 10% fetal calf serum) were added as replicate samples (3 to 5 replicates) to flat bottom 96-well tissue culture plates. For the cellular component of the assay, three inguinal lymph nodes were aseptically removed from untreated C57BL/6 mice and placed in 5 ml of Hank's balanced salt solution supplemented with 5% fetal calf serum (medium). Following their transfer to a sterile 0.22 micron nylon mesh, a sterile syringe plunger was used to disperse the cells through the mesh. Medium was used to rinse the cells, yielding a pooled, unfractionated single cell suspension. Cells were washed once, resuspended in complete medium and added to wells at 6×10^5 cells/well, to a final volume of 0.2 ml. Cultures were exposed to the HspE7 protein in medium or to medium alone for 72 hours at 37^0C in a 5% CO_2 atmosphere. Following incubation, replicate cultures were pooled, cells pelleted by centrifugation and supernatants either measured for IFN-gamma content by ELISA according to the procedure described in Example 17, or frozen immediately at -70^0C for later analysis.

Fig. 21 shows the results of the above experiment, comparing induction of IFN-gamma release by lymph node cells and by splenocytes. The fusion protein was found to elicit a release of IFN-gamma in both cell types. The IFN-gamma release elicited by the fusion protein greatly exceeded that induced by Hsp65 alone.

Example 22: Regression of Pre-Established Tumors *in vivo* Induced by Administration of Hsp Fusion Proteins

Human papilloma virus type 16 (HPV16) is an infectious agent associated with the induction of cervical cancer and its premalignant precursor, cervical intraepithelial neoplasia. The following experiments use Hsp - HPV16 E7 fusion proteins of the invention to target immune recognition as part of a strategy to eliminate HPV16 E7-expressing host cells.

The H-2^b murine epithelial cell-derived tumor line, TC-1 (co-transformed with HPV16 E6 and E7 and activated human Ha-*ras*), was obtained from T.C. Wu of Johns Hopkins University (Baltimore, MD). The use of TC-1 cells in assays similar to those used herein is described in PCT patent application WO 99/07860. TC-1 was maintained in complete medium, consisting of: RPMI 1640 (ICN, cat no. 1260354) supplemented with 10% FBS (Hyclone, cat no. SH30071); 2 mM L-Glutamine (ICN, cat no. 16-801-49); 10 mM HEPES (ICN, cat no. 16-884-49); 0.1mM MEM Non Essential Amino Acid Solution (Gibco BRL, cat no. 11140-050); 1 mM MEM Sodium Pyruvate (Gibco BRL, cat no. 11360-070); 50 µM 2-Mercaptoethanol (Sigma, cat no. M-7522); and 50 mcg/mL Gentamycin Sulfate (Gibco BRL, cat no. 15750-011). The medium was also supplemented with G418 (0.4 mg/mL active, Gibco BRL, cat no. 11811-023) and Hygromycin B (0.2 mg/mL active, Calbiochem, cat no. 400051).

Since the TC-1 cell line was derived from a C57BL/6 mouse, this mouse strain was used as the host in these experiments. Female C57BL/6 mice of approximately 8 to 10 weeks of age were purchased from Charles River Canada (St-Constant, Quebec, Canada) and housed using filter top cages (four animals per cage).

TC-1 cells were prepared for implantation as follows. TC-1 cells were seeded at a density of 2 - 5x10^4 cells /mL and incubated for two to four days until 70 to 90% confluent. Cells were trypsinized using a 30 second exposure to 0.25% Trypsin (10x stock, Gibco cat. no. 1505-065, diluted to 1x with DPBS), then diluted four-fold with supplemented complete medium. Following trypsinization, TC-1 cells were pelleted at 4°C at 1000 rpm (250x g) for 4 minutes, the supernatant removed by aspiration and 30 mL of cold DPBS added. The cells were then pelleted at 4°C at 700 rpm (100x g) for 4 minutes, the supernatant removed by aspiration, and a minimal amount (approx. 5 mL) of cold DPBS added. The final cell density for injection was adjusted to 6.5 x 10^5 viable cells per mL, as measured by the trypan blue dye exclusion method. At least 90% of the cells used for TC-1 inoculations were viable. The cells were stored on ice for immediate injection into mice.

TC-1 cells were implanted as follows. Between 24 to 72 hours prior to implantation, the hind flank of each mouse was shaved. TC-1 cells were prepared as described above and held on ice until injected. All injections were performed within two hours of cell trypsinization. The cells were swirled gently in the centrifuge tube and drawn into a 1 mL syringe (Becton-Dickinson, cat. no. 309602) without a needle.

A 25 gauge needle (Becton-Dickinson, cat. no. 305122) was then attached and any air bubbles were expelled. The shaved skin was raised gently and the needle was inserted bevel side up just beneath the skin surface. Cells (1.3×10^5) were injected in a 0.2 mL volume for all studies. A fresh syringe and needle was used for every fifth injection.

Fusion proteins were injected as follows. On treatment days, the fusion proteins HspE7, SP65(2)-E7, AF60-E7, E7-TB71 (shown if Figs. 23A and 23B as E7-MT71), MT40-E7 and TB10-E7 (prepared as described above) were removed from –70°C storage and thawed in a 37°C water bath. Dulbecco's phosphate buffered saline (DPBS) (4°C) was added to obtain the protein concentration desired for injection. The diluted fusion protein was held on ice until drawn into a 1 mL syringe (Becton-Dickinson, cat no. 309602) with a 30 gauge needle (Becton-Dickinson, cat no. 3095106). The same syringe was used to inject 0.2 mL of fusion protein into each mouse within a dose group; the syringe was refitted with a fresh needle for every fifth injection. Mice were injected subcutaneously in the scruff of the neck, as high on the neck as possible.

Tumor incidence (TI) was measured as follows. TI was generally recorded three times per week, beginning eight days after tumor implantation and continuing for eight weeks. Mice were assessed for the presence or absence of subcutaneous tumor by palpation and visual observation of the tumor injection site.

Tumor volume was measured as follows. Volumes of palpable subcutaneous tumor nodules were measured beginning on approximately Day 8 post implantation. The two longest orthogonal dimensions were measured using a Fowler Sylvac Ultra-Cal Mark III digital caliper with computerized data collection. Data points were tabulated in a Microsoft Excel spreadsheet. Tumor nodule measurements were extrapolated to mm^3 using the formula $V = W^2 \times L \times 0.5$ (where V represents volume, W represents width and L represents length) and are presented as average tumor volume ± standard error of the mean. The Student's t test function of Excel (two-tailed, unpaired samples, equal variances) was used to test the significance ($p < 0.05$) of the difference of the means of tumor volumes in each group.

Seven different HPV16 E7 fusion proteins linked to various hsps were tested for their ability to regress a tumor *in vivo*.

In the first experiment, C57BL/6 mice (18 per group) were inoculated subcutaneously with 1.3×10^5 TC-1 cells in the right hind flank (Day 0). After 7 days,

groups of mice were treated with 0.2 mL of either DPBS (saline), 115 ug HspE7, 100 ug SP65(2)-E7, or 100 ug AF60-E7. The doses of the two latter proteins were chosen based on the same molar equivalent of E7 contained in HspE7. The mice were monitored for the presence or absence of tumor in addition to tumor volume. The data are represented as percent tumor incidence (TI) per group (Fig. 22A) and tumor volume, expressed as average tumor volume ± standard error of the mean (Fig. 22B).

As indicated in Fig. 22A, the majority of animals had detectable tumor by Day 8 post implantation and by Day 13 tumor was evident in 94 to 100% of the mice. After this timepoint, TI in all of the mice declined until day 25 when the incidence for the DPBS-treated animals stabilized to approximately 50% for the remainder of the observation period. In contrast, the animals treated with fusion proteins showed a comparatively sharp decline in TI until day 28, when none of the animals had detectable tumor. This complete absence of tumor was observed for the remainder of the observation period for most of these animals. The complete regression of tumor in the animals treated with the fusion proteins was also clearly seen when measured by tumor volume. Figure 22B shows that by day 28, the average tumor volume of the animals treated with the fusion proteins was not detectable. By comparison, the average tumor volume of those animals treated with DPBS rose steadily from day 25 onwards.

In the second experiment, C57BL/6 mice (18 per group) were inoculated subcutaneously with 1.3×10^5 TC-1 cells in the right hind flank (Day 0). After 7 days, groups of mice were treated with 0.2 mL of either DPBS (saline), 100 ug HspE7, 100 ug MT40-E7, 100 ug E7-TB71 (shown if Figs. 23A and 23B as E7-MT71), or 100 ug TB10-E7. The mice were monitored for the presence or absence of tumor in addition to tumor volume. The data are represented as percent tumor incidence (TI) per group (Fig. 23A) and tumor volume, expressed as average tumor volume ± standard error of the mean (Fig. 23 B).

As in Figure 22A, a majority (approximately 95%) of the animals had visible and palpable tumors on day 8 post tumor implantation (Fig. 23A). By day 19, a decrease in TI was apparent. Following this, a sharp decrease in TI for all of the fusion protein-treated animals was observed such that by day 33, practically all of the animals were tumor-free. In contrast, the TI of the mice treated with DPBS had stabilized to approximately 75%. Fig. 23B shows the average tumor volumes of the mice treated

with the respective fusion proteins. The decrease in TI was reflected by the marked decrease in tumor volumes. Average tumor volumes for the animals treated with any of the fusion proteins was essentially not measurable by day 30.

Claims

1. A method of determining whether a fusion protein stimulates a Th1-like response, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a fusion protein comprising (i) a heat shock protein (Hsp) or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length;

(c) contacting the cell sample with the fusion protein; and

(d) determining whether the fusion protein stimulates a Th1-like response in the cell sample.

2. The method of claim 1, wherein the Hsp is selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

3. The method of claim 2, wherein the fusion protein comprises a polypeptide selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

4. The method of claim 1, wherein the fusion protein comprises amino acids 1-200 of Hsp65 of *Mycobacterium bovis*.

5. The method of claim 1, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of (i) a protein of a human pathogen or (ii) a tumor associated antigen.

6. The method of claim 1, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a human virus.

7. The method of claim 6, wherein the virus is selected from the group consisting of human papilloma virus (HPV), herpes simplex virus (HSV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza virus, measles virus, and human immunodeficiency virus (HIV).

8. The method of claim 7, wherein the heterologous polypeptide comprises HPV E6.

9. The method of claim 7, wherein the heterologous polypeptide comprises HPV E7.

10. The method of claim 1, wherein the heterologous polypeptide comprises HPV 16 E7 or a fragment thereof at least eight amino acid residues in length.

11. The method of claim 1, wherein the heterologous polypeptide comprises HPV 16 E6 or a fragment thereof at least eight amino acid residues in length.

12. The method of claim 10, wherein the fusion protein comprises *Mycobacterium bovis* Hsp65 and HPV 16 E7.

13. The method of claim 1, wherein the cell sample comprises cells derived from a spleen, lymph node, peripheral blood, bone marrow, thymus, lung, respiratory tract, or anogenital mucosa

14. The method of claim 1, wherein the cell sample comprises splenocytes or lymph node cells.

15. The method of claim 1, wherein the detecting step comprises detecting IFN-gamma produced by the cell sample.

16. The method of claim 1, comprising the further steps of

(e) providing a second cell sample comprising naive lymphocytes;

(f) contacting the second cell sample with a second fusion protein; and

(g) determining whether the second fusion protein stimulates a Th1-like response in the second cell sample,

wherein the first fusion protein comprises the sequence of a full-length, naturally occurring Hsp, and the second fusion protein comprises at least eight amino acids but less than all of the sequence of a naturally occurring Hsp.

17. A method of screening a compound, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a fusion protein comprising (i) a Hsp or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length;

(c) contacting the cell sample with the compound and the fusion protein; and

(d) determining whether the cell sample exhibits a Th1-like response following the contacting step,

wherein a decrease in the Th1-like response in the presence of the compound compared to in the absence of the compound indicates that the compound inhibits a Th1-like response by the cell sample.

18. The method of claim 17, wherein the determining step comprises detecting IFN-gamma produced by the cell sample.

19. The method of claim 17, wherein the cell sample comprises cells derived from a spleen, lymph node, peripheral blood, bone marrow, thymus, lung, respiratory tract, or anogenital mucosa

20. The method of claim 17, wherein the cell sample comprises splenocytes or lymph node cells.

21. The method of claim 17, wherein the Hsp is selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

22. The method of claim 21, wherein the fusion protein comprises a polypeptide selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

23. The method of claim 17, wherein the heterologous polypeptide comprises HPV E6.

24. The method of claim 17, wherein the heterologous polypeptide comprises HPV E7.

25. The method of claim 17, wherein the fusion protein comprises *Mycobacterium bovis* Hsp65 and HPV 16 E7.

26. A method of screening a compound, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a fusion protein comprising (i) a Hsp or a fragment thereof at least eight amino acid residues in length, fused to (ii) a heterologous polypeptide at least eight amino acid residues in length;

(c) contacting the cell sample with the compound and the fusion protein; and

(d) determining whether the cell sample exhibits a Th1-like response following the contacting step,

wherein an increase in the Th1-like response in the presence of the compound compared to in the absence of the compound indicates that the compound promotes a Th1-like response by the cell sample.

27. The method of claim 26, wherein the determining step comprises detecting IFN-gamma produced by the cell sample.

28. The method of claim 26, wherein the cell sample comprises cells derived from a spleen, lymph node, peripheral blood, bone marrow, thymus, lung, respiratory tract, or anogenital mucosa

29. The method of claim 26, wherein the cell sample comprises splenocytes or lymph node cells.

30. The method of claim 26, wherein the Hsp is selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

31. The method of claim 30, wherein the fusion protein comprises a polypeptide selected from the group consisting of Hsp65, Hsp40, Hsp10, Hsp60, and Hsp71.

32. The method of claim 26, wherein the heterologous polypeptide comprises HPV E6.

33. The method of claim 26, wherein the heterologous polypeptide comprises HPV E7.

34. The method of claim 26, wherein the fusion protein comprises *Mycobacterium bovis* BCG Hsp65 and HPV 16 E7.

35. A method of determining whether a hybrid compound stimulates a Th1-like response, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a hybrid compound that is non-naturally occurring and comprises (i) a non-peptide compound having a molecular weight of less than 1,500, covalently linked to (ii) a polypeptide of at least eight amino acids in length, wherein the hybrid compound is made by covalently linking the non-peptide compound to the polypeptide;

(c) contacting the cell sample with the hybrid compound; and

(d) determining whether the hybrid compound stimulates a Th1-like response in the cell sample.

36. The method of claim 35, wherein the non-peptide compound has a molecular weight of at least 100.

37. A method of determining whether a hybrid compound stimulates a Th1-like response, the method comprising:

(a) producing a hybrid compound by covalently linking a non-peptide compound to a polypeptide of at least eight amino acids in length;

(b) providing a cell sample comprising naive lymphocytes *in vitro*;

(c) contacting the cell sample with the hybrid compound; and

(d) determining whether the hybrid compound stimulates a Th1-like response in the cell sample.

38. The method of claim 37, wherein the non-peptide compound has a molecular weight between 100 and 1,500.

39. A method of determining whether a fusion protein stimulates a Th1-like response, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a fusion protein comprising (i) a first polypeptide at least eight amino acids in length, fused to (ii) a second polypeptide at least eight amino acids in length;

(c) contacting the cell sample with the fusion protein; and

(d) detecting a Th1-like response exhibited by the cell sample following the contacting step.

40. The method of claim 39, wherein the detected Th1-like response is greater than a Th1-like response exhibited by a second cell sample comprising naive lymphocytes when the second cell sample is contacted with either the first polypeptide, the second polypeptide, or a mixture of the first polypeptide and the second polypeptide.

41. The method of claim 40, wherein the detected Th1-like response is at least two times greater than the Th1-like response exhibited by the second cell sample.

42. The method of claim 40, wherein the detected Th1-like response is at least five times greater than the Th1-like response exhibited by the second cell sample.

43. A fusion protein comprising (i) a Hsp10 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

44. The fusion protein of claim 43, comprising a Hsp10 protein.

45. The fusion protein of claim 44, wherein the Hsp10 protein is a mycobacterial protein.

46. The fusion protein of claim 45, comprising the *Mycobacterium tuberculosis* Hsp10 protein.

47. The fusion protein of claim 43, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a human virus.

48. The fusion protein of claim 47, wherein the human virus is HPV.

49. The fusion protein of claim 48, wherein the heterologous polypeptide comprises HPV16 E7.

50. A fusion protein comprising (i) a Hsp40 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

51. The fusion protein of claim 50, comprising a Hsp40 protein.

52. The fusion protein of claim 51, wherein the Hsp40 protein is a mycobacterial protein.

53. The fusion protein of claim 52, comprising the *Mycobacterium tuberculosis* Hsp40 protein.

54. The fusion protein of claim 50, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a human virus.

55. The fusion protein of claim 54, wherein the human virus is HPV.

56. The fusion protein of claim 55, wherein the heterologous polypeptide comprises HPV16 E7.

57. A fusion protein comprising (i) a Hsp71 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

58. The fusion protein of claim 57, comprising a Hsp71 protein.

59. The fusion protein of claim 58, wherein the Hsp71 protein is a mycobacterial protein.

60. The fusion protein of claim 59, comprising the *Mycobacterium tuberculosis* Hsp71 protein.

61. The fusion protein of claim 57, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a human virus.

62. The fusion protein of claim 61, wherein the human virus is HPV.

63. The fusion protein of claim 62, wherein the heterologous polypeptide comprises HPV16 E7.

64. A method of determining whether a compound stimulates a Th1-like response, the method comprising:

(a) providing a cell sample comprising naive lymphocytes *in vitro*;

(b) providing a compound;

(c) contacting the cell sample with the compound; and

(d) detecting a Th1-like response exhibited by the cell sample following the contacting step.

65. A nucleic acid encoding a fusion protein comprising (i) an Hsp10 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

66. The nucleic acid of claim 65, wherein the fusion protein comprises an Hsp10 protein.

67. The nucleic acid of claim 66, wherein the Hsp10 protein is a mycobacterial protein.

68. The nucleic acid of claim 67, wherein the Hsp10 protein is a *Mycobacterium tuberculosis* Hsp10 protein.

69. The nucleic acid of claim 65, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a virus.

70. The nucleic acid of claim 69, wherein the virus is a human papilloma virus (HPV).

71. The nucleic acid of claim 70, wherein the heterologous polypeptide comprises HPV16 E7.

72. A nucleic acid encoding a fusion protein comprising (i) an Hsp40 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

73. The nucleic acid of claim 72, wherein the fusion protein comprises an Hsp40 protein.

74. The nucleic acid of claim 73, wherein the Hsp40 protein is a mycobacterial protein.

75. The nucleic acid of claim 74, wherein the Hsp40 protein is a *Mycobacterium tuberculosis* Hsp40 protein.

76. The nucleic acid of claim 72, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a virus.

77. The nucleic acid of claim 76, wherein the virus is an HPV.

78. The nucleic acid of claim 77, wherein the heterologous polypeptide comprises HPV16 E7.

79. A nucleic acid encoding a fusion protein comprising (i) an Hsp71 protein or a fragment thereof at least eight amino acid residues in length, and (ii) a heterologous polypeptide at least eight amino acids in length.

80. The nucleic acid of claim 79, wherein the fusion protein comprises an Hsp71 protein.

81. The nucleic acid of claim 80, wherein the Hsp71 protein is a mycobacterial protein.

82. The nucleic acid of claim 81, wherein the Hsp71 protein is a *Mycobacterium tuberculosis* Hsp71 protein.

83. The nucleic acid of claim 79, wherein the heterologous polypeptide comprises a sequence identical to at least eight consecutive amino acids of a protein of a virus.

84. The nucleic acid of claim 83, wherein the virus is an HPV.

85. The nucleic acid of claim 84, wherein the heterologous polypeptide comprises HPV16 E7.

86. The nucleic acid of claim 69, wherein the virus is a herpes simplex virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, Epstein-Barr virus, influenza virus, measles virus, or human immunodeficiency virus.

87. The nucleic acid of claim 70, wherein the heterologous polypeptide comprises an HPV E7 antigen.

88. The nucleic acid of claim 70, wherein the heterologous polypeptide comprises an HPV E6 antigen.

89. The nucleic acid of claim 88, wherein the HPV E6 antigen is HPV 16 E6.

90. The nucleic acid of claim 65, wherein the heterologous polypeptide comprises an MHC-binding epitope of a protein produced by a human pathogen.

91. The nucleic acid of claim 90, wherein the human pathogen is a virus, bacterium, mycoplasm, fungus, or protozoan.

92. The nucleic acid of claim 65, wherein the heterologous polypeptide comprises an MHC-binding epitope of a tumor associated antigen.

93. The nucleic acid of claim 92, wherein the tumor associated antigen is MAGE1, MAGE2, MAGE3, BAGE, GAGE, PRAME, SSX-2, Tyrosinase, MART-1, NY-ESO-1, gp100, TRP-1, TRP-2, A2 melanotope, BCR/ABL, Proeinase-3/Myeloblastin, HER2/neu, CEA, P1A, HK2, PAPA, PSA, PSCA, PSMA, pg75, MUM-1, MUC-1, E6, E7, GnT-V, Beta-catenin, CDK4, or P15.

94. A plasmid comprising the nucleic acid of claim 65.

95. An expression vector comprising the nucleic acid of claim 65.

96. The expression vector of claim 95, wherein the expression vector is a mammalian expression vector.

97. The nucleic acid of claim 76, wherein the virus is a herpes simplex virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, Epstein-Barr virus, influenza virus, measles virus, or human immunodeficiency virus.

98. The nucleic acid of claim 77, wherein the heterologous polypeptide comprises an HPV E7 antigen.

99. The nucleic acid of claim 77, wherein the heterologous polypeptide comprises an HPV E6 antigen.

100. The nucleic acid of claim 99, wherein the HPV E6 antigen is HPV 16 E6.

101. The nucleic acid of claim 72, wherein the heterologous polypeptide comprises an MHC-binding epitope of a protein produced by a human pathogen.

102. The nucleic acid of claim 101, wherein the human pathogen is a virus, bacterium, mycoplasm, fungus, or protozoan.

103. The nucleic acid of claim 72, wherein the heterologous polypeptide comprises an MHC-binding epitope of a tumor associated antigen.

104. The nucleic acid of claim 103, wherein the tumor associated antigen is MAGE1, MAGE2, MAGE3, BAGE, GAGE, PRAME, SSX-2, Tyrosinase, MART-1, NY-ESO-1, gp100, TRP-1, TRP-2, A2 melanotope, BCR/ABL, Proeinase-3/Myeloblastin, HER2/neu, CEA, P1A, HK2, PAPA, PSA, PSCA, PSMA, pg75, MUM-1, MUC-1, E6, E7, GnT-V, Beta-catenin, CDK4, or P15.

105. A plasmid comprising the nucleic acid of claim 72.

106. An expression vector comprising the nucleic acid of claim 72.

107. The expression vector of claim 106, wherein the expression vector is a mammalian expression vector.

108. The nucleic acid of claim 83, wherein the virus is a herpes simplex virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, Epstein-Barr virus, influenza virus, measles virus, or human immunodeficiency virus.

109. The nucleic acid of claim 84, wherein the heterologous polypeptide comprises an HPV E7 antigen.

110. The nucleic acid of claim 84, wherein the heterologous polypeptide comprises an HPV E6 antigen.

111. The nucleic acid of claim 110, wherein the HPV E6 antigen is HPV 16 E6.

112. The nucleic acid of claim 79, wherein the heterologous polypeptide comprises an MHC-binding epitope of a protein produced by a human pathogen.

113. The nucleic acid of claim 112, wherein the human pathogen is a virus, bacterium, mycoplasm, fungus, or protozoan.

114. The nucleic acid of claim 79, wherein the heterologous polypeptide comprises an MHC-binding epitope of a tumor associated antigen.

115. The nucleic acid of claim 114, wherein the tumor associated antigen is MAGE1, MAGE2, MAGE3, BAGE, GAGE, PRAME, SSX-2, Tyrosinase, MART-1, NY-ESO-1, gp100, TRP-1, TRP-2, A2 melanotope, BCR/ABL, Proeinase-3/Myeloblastin, HER2/neu, CEA, P1A, HK2, PAPA, PSA, PSCA, PSMA, pg75, MUM-1, MUC-1, E6, E7, GnT-V, Beta-catenin, CDK4, or P15.

116. A plasmid comprising the nucleic acid of claim 79.

117. An expression vector comprising the nucleic acid of claim 79.

118. The expression vector of claim 117, wherein the expression vector is a mammalian expression vector.