METHODS TO ELICIT, ENHANCE AND SUSTAIN IMMUNE RESPONSES AGAINST MHC CLASS I-RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSES

Cross Reference to Related Applications

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/640,402, filed on December 29, 2004, entitled METHODS TO ELICIT, ENHANCE AND SUSTAIN IMMUNE RESPONSES AGAINST MHC CLASS I-RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSES; the disclosure of which is incorporated herein by reference in its entirety.

Background of the Invention

Field of the Invention

[0002] Embodiments of the invention disclosed herein relate to methods and compositions for inducing a MHC class I-restricted immune response and controlling the nature and magnitude of the response, promoting effective immunologic intervention in pathogenic processes. More particularly embodiments relate to immunogenic compositions, their nature and the order, timing, and route of administration by which they are effectively used.

Description of the Related Art

The Major Histocompatibility Complex and T Cell Target Recognition

[0003] T lymphocytes (T cells) are antigen-specific immune cells that function in response to specific antigen signals. B lymphocytes and the antibodies they produce are also antigen-specific entities. However, unlike B lymphocytes, T cells do not respond to antigens in a free or soluble form. For a T cell to respond to an antigen, it requires the antigen to be bound to a presenting complex known as the major histocompatibility complex (MHC).

[0004] MHC proteins provide the means by which T cells differentiate native or "self" cells from foreign cells. MHC molecules are a category of immune receptors that present potential peptide epitopes to be monitored subsequently by the T cells. There are two types of MHC, class I MHC and class II MHC. CD4+ T cells interact with class II

MHC proteins and predominately have a helper phenotype while CD8+ T cells interact with class I MHC proteins and predominately have a cytolytic phenotype, but each of them can also exhibit regulatory, particularly suppressive, function. Both MHC are transmembrane proteins with a majority of their structure on the external surface of the cell. Additionally, both classes of MHC have a peptide binding cleft on their external portions. It is in this cleft that small fragments of proteins, native or foreign, are bound and presented to the extracellular environment.

[0005] Cells called antigen presenting cells (APCs) display antigens to T cells using the MHC. T cells can recognize an antigen, if it is presented on the MHC. This requirement is called MHC restriction. If an antigen is not displayed by a recognizable MHC, the T cell will not recognize and act on the antigen signal. T cells specific for the peptide bound to a recognizable MHC bind to these MHC-peptide complexes and proceed to the next stages of the immune response.

[0006] Peptides corresponding to nominal MHC class I or class II restricted epitopes are among the simplest forms of antigen that can be delivered for the purpose of inducing, amplifying or otherwise manipulating the T cell response. Despite the fact that peptide epitopes have been shown to be effective in vitro at re-stimulating in vivo primed T cell lines, clones, or T cell hybridomas, their in vivo efficacy has been very limited. This is due to two main factors:

(1) The poor pharmacokinetic (PK) profile of peptides, caused by rapid renal clearance and/or *in vivo* degradation, resulting in limited access to APC;

(2) The insufficiency of antigen-induced T cell receptor (TCR)-dependent signaling alone (signal 1) to induce or amplify a strong and sustained immune response, and particularly a response consisting of Tc1 or Th1 cells (producing IFN-γ and TNF-alpha). Moreover, use of large doses of peptide or depot adjuvants, in order to circumvent the limited PK associated with peptides, can trigger a variable degree of unresponsiveness or "immune deviation" unless certain immune potentiating or modulating adjuvants are used in conjunction.

Summary of the Invention

[0007] Embodiments of the present invention include methods and compositions for manipulating, and in particular for inducing, entraining, and/or amplifying, the immune response to MHC class I restricted epitopes.

[0008] Some embodiments relate to methods of immunization. The methods can include, for example, delivering to a mammal a first composition that includes an immunogen, the immunogen can include or encode at least a portion of a first antigen; and administering a second composition, which can include an amplifying peptide, directly to a lymphatic system of the mammal, wherein the peptide corresponds to an epitope of said first antigen, wherein the first composition and the second composition are not the same. The methods can further include the step of obtaining, assaying for or detecting and effector T cell response.

[0009] The first composition can include a nucleic acid encoding the antigen or an immunogenic fragment thereof. The first composition can include a nucleic acid capable of expressing the epitope in a pAPC. The nucleic acid can be delivered as a component of a protozoan, bacterium, virus, or viral vector. The first composition can include an immunogenic polypeptide and an immunopotentiator, for example. The immunopotentiator can be a cytokine, a toll-like receptor ligand, and the like. Adjuvants can include an immunostimulatory sequence, an RNA, and the like.

[0010] The immunogenic polypeptide can be an amplifying peptide. The immunogenic polypeptide can be a first antigen. The immunogenic polypeptide can be delivered as a component of a protozoan, bacterium, virus, viral vector, or virus-like particle, or the like. The adjuvant can be delivered as a component of a protozoan, bacterium, virus, viral vector, or virus-like particle, or the like. The second composition can be adjuvant-free and immunopotentiator-free. The delivering step can include direct administration to the lymphatic system of the mammal. The direct administration to the lymphatic system of the mammal can include direct administration to a lymph node or lymph vessel. The direct administration can be to two or more lymph nodes or lymph vessels. The lymph node can be, for example, inguinal, axillary, cervical, and tonsilar lymph nodes. The effector T cell response can be a cytotoxic T cell response. The effector T cell response can include production of a pro-inflammatory cytokine, and the cytokine

can be, for example, (gamma) γ-IFN or TNFα (alpha). The effector T cell response can include production of a T cell chemokine, for example, RANTES or MIP-1α, or the like.

[0011] The epitope can be a housekeeping epitope or an immune epitope, for example. The delivering step or the administering step can include a single bolus injection, repeated bolus injections, for example. The delivering step or the administering step can include a continuous infusion, which for example, can have duration of between about 8 to about 7 days. The method can include an interval between termination of the delivering step and beginning the administering step, wherein the interval can be at least about seven days. Also, the interval can be between about 7 and about 14 days, about 17 days, about 20 days, about 25 days, about 30 days, about 40 days, about 50 days, or about 60 days, for example. The interval can be over about 75 days, about 80 days, about 90 days, about 100 days or more.

[0012] The first antigen can be a disease-associated antigen, and the disease-associated antigen can be a tumor-associated antigen, a pathogen-associated antigen. Embodiments include methods of treating disease utilizing the described method of immunizing. The first antigen can be a target-associated antigen. The target can be a neoplastic cell, a pathogen-infected cell, and the like. For example, any neoplastic cell can be targeted. Pathogen-infected cells can include, for example, cells infected by a bacterium, a virus, a protozoan, a fungus, and the like, or affected by a prion, for example.

[0013] The effector T cell response can be detected by at least one indicator for example, a cytokine assay, an Elispot assay, a cytotoxicity assay, a tetramer assay, a DTH-response, a clinical response, tumor shrinkage, tumor clearance, inhibition of tumor progression, decrease pathogen titre, pathogen clearance, amelioration of a disease symptom, and the like. The methods can further include obtaining, detecting or assaying for an effector T cell response to the first antigen.

[0014] Further embodiments relate to methods of immunization that include delivering to a mammal a first composition including a nucleic acid encoding a first antigen or an immunogenic fragment thereof; administering a second composition, including a peptide, directly to the lymphatic system of the mammal, wherein the peptide corresponds to an epitope of the first antigen. The methods can further include obtaining, detecting or assaying for an effector T cell response to the antigen.

[0015] Also, embodiments relate to methods of augmenting an existing antigen-specific immune response. The methods can include administering a composition that includes a peptide, directly to the lymphatic system of a mammal, wherein the peptide corresponds to an epitope of the antigen, and wherein the composition was not used to induce the immune response. The methods can further include obtaining, detecting or assaying for augmentation of an antigen-specific immune response. The augmentation can include sustaining the response over time, reactivating quiescent T cells, expanding the population of antigen-specific T cells, and the like. In some aspects, the composition does not include an immunopotentiator.

[0016] Other embodiments relate to methods of immunization which can include delivering to a mammal a first composition comprising an immunogen, the immunogen can include or encode at least a portion of a first antigen and at least a portion of a second antigen; administering a second composition including a first peptide, and a third composition including a second peptide, directly to the lymphatic system of the mammal, wherein the first peptide corresponds to an epitope of the first antigen, and wherein the second peptide corresponds to an epitope of the second antigen, wherein the first composition can be not the same as the second or third compositions. The methods further can include obtaining, detecting or assaying for an effector T cell response to the first and second antigens. The second and third compositions each can include the first and the second peptides. The second and third compositions can be part of a single composition.

[0017] Still further embodiments relate to methods of generating an antigen-specific tolerogenic or regulatory immune response. The methods can include periodically administering a composition, including an adjuvant-free peptide, directly to the lymphatic system of a mammal, wherein the peptide corresponds to an epitope of the antigen, and wherein the mammal can be epitopically naïve. The methods further can include obtaining, detecting and assaying for a tolerogenic or regulatory T cell immune response. The immune response can assist in treating an inflammatory disorder, for example. The inflammatory disorder can be, for example, from a class II MHC-restricted immune response. The immune response can include production of an immunosuppressive cytokine, for example, IL-5, IL-10, or TGB-ß, and the like.

[0018] Embodiments relate to methods of immunization that include administering a series of immunogenic doses directly into the lymphatic system of a mammal wherein the series can include at least 1 entraining dose and at least 1 amplifying dose, and wherein the entraining dose can include a nucleic acid encoding an immunogen and wherein the amplifying dose can be free of any virus, viral vector, or replication-competent vector. The methods can further include obtaining an antigen-specific immune response. The methods can include, for example, 1 to 6 or more entraining doses. The method can include administering a plurality of entraining doses, wherein the doses are administered over a course of one to about seven days. The entraining doses, amplifying doses, or entraining and amplifying doses can be delivered in multiple pairs of injections, wherein a first member of a pair can be administered within about 4 days of a second member of the pair, and wherein an interval between first members of different pairs can be at least about 14 days. An interval between a last entraining dose and a first amplifying dose can be between about 7 and about 100 days, for example.

[0019] Other embodiments relate to sets of immunogenic compositions for inducing an immune response in a mammal including 1 to 6 or more entraining doses and at least one amplifying dose, wherein the entraining doses can include a nucleic acid encoding an immunogen, and wherein the amplifying dose can include a peptide epitope, and wherein the epitope can be presented or is presentable by pAPC expressing the nucleic acid. The one dose further can include an adjuvant, for example, RNA. The entraining and amplifying doses can be in a carrier suitable for direct administration to the lymphatic system, a lymph node and the like. The nucleic acid can be a plasmid. The epitope can be a class I HLA epitope, for example, one listed in Tables 1-4. The HLA preferably can be HLA-A2. The immunogen can include an epitope array, which array can include a liberation sequence. The immunogen can consist essentially of a target-associated antigen. The target-associated antigen can be a tumor-associated antigen, a microbial antigen, any other antigen, and the like. The immunogen can include a fragment of a target-associated antigen that can include an epitope cluster.

[0020] Further embodiments can include sets of immunogenic compositions for inducing a class I MHC-restricted immune response in a mammal including 1-6 entraining doses and at least one amplifying dose, wherein the entraining doses can include an immunogen or a nucleic acid encoding an immunogen and an immunopotentiator, and

wherein the amplifying dose can include a peptide epitope, and wherein the epitope can be presented by pAPC. The nucleic acid encoding the immunogen further can include an immunostimulatory sequence which can be capable of functioning as the immunopotentiating agent. The immunogen can be a virus or replication-competent vector that can include or can induce an immunopotentiating agent. The immunogen can be a bacterium, bacterial lysate, or purified cell wall component. Also, the bacterial cell wall component can be capable of functioning as the immunopotentiating agent. The immunopotentiating agent can be, for example, a TLR ligand, an immunostimulatory sequence, a CpG-containing DNA, a dsRNA, an endocytic-Pattern Recognition Receptor (PRR) ligand, an LPS, a quillaja saponin, tucaresol, a pro-inflammatory cytokine, and the like. In some preferred embodiments for promoting multivalent responses the sets can include multiple entraining doses and/or multiple amplification doses corresponding to various individual antigens, or combinations of antigens, for each administration. The multiple entrainment doses can be administered as part of a single composition or as part of more than one composition. The amplifying doses can be administered at disparate times and/or to more than one site, for example.

[0021] Other embodiments relate to methods of generating various cytokine profiles. In some embodiments of the instant invention, intranodal administration of peptide can be effective in amplifying a response initially induced with a plasmid DNA vaccine. Moreover, the cytokine profile can be distinct, with plasmid DNA induction/peptide amplification generally resulting in greater chemokine (chemoattractant cytokine) and lesser immunosuppressive cytokine production than either DNA/DNA or peptide/peptide protocols.

[0022] An amplifying peptide used in the various embodiments corresponds to an epitope of the immunizing antigen. In some embodiments, correspondence can include faithfully iterating the native sequence of the epitope. In some embodiments, correspondence can include the corresponding sequence can be an analogue of the native sequence in which one or more of the amino acids have been modified or replaced, or the length of the epitope altered. Such analogues can retain the immunologic function of the epitope (i.e., they are functionally similar). In preferred embodiments the analogue has similar or improved binding with one or more class I MHC molecules compared to the native sequence. In other preferred embodiments the analogue has similar or improved

immunogenicity compared to the native sequence. Strategies for making analogues are widely known in the art. Exemplary discussions of such strategies can be found in U.S. Patent Application Nos. 10/117,937 (Pub. No. 2003-0220239 A1), filed on April 4, 2002; and 10/657,022 (Publication No. 20040180354), filed on September 5, 2003, both entitled EPITOPE SEQUENCES; and U.S. Provisional Patent Application No. 60/581,001, filed on June 17, 2004 and U.S. Patent Application No. 11/156,253 (Pub. No. No. _____), filed on June 17, 2005, both entitled SSX-2 PEPTIDE ANALOGS; and U.S. Provisional Patent Application No. 60/580,962 and U.S. Patent Application No. 11/155,929 (Pub. No. _____), filed on June 17, 2005, both entitled NY-ESO PEPTIDE ANALOGS; each of which is hereby incorporated by reference in its entirety.

[0023] Still further embodiments relate to uses of a peptide in the manufacture of an adjuvant-free medicament for use in an entrain-and-amplify immunization protocol. The compositions, kits, immunogens and compounds can be used in medicaments for the treatment of various diseases, to amplify immune responses, to generate particular cytokine profiles, and the like, as described herein. Embodiments relate to the use of adjuvant-free peptide in a method of amplifying an immune response.

[0024] Embodiments are directed to methods, uses, therapies and compositions related to epitopes with specificity for MHC, including, for example, those listed in Tables 1-4. Other embodiments include one or more of the MHCs listed in Tables 1-4, including combinations of the same, while other embodiments specifically exclude any one or more of the MHCs or combinations thereof. Tables 3-4 include frequencies for the listed HLA antigens.

[0025] Some embodiments relate to methods of generating an immune response. The methods can include delivering to a mammal a first composition (composition 1) which can include an immunogen that includes or encodes at least a portion of a first antigen (antigen A) and at least a portion of a second antigen (antigen B); and administering a second composition (composition 2) which can include a first peptide (peptide A), and a third composition (composition 3) that can include a second peptide (peptide B), directly to the lymphatic system of the mammal, wherein peptide A corresponds to an epitope of the antigen A, and wherein the peptide B corresponds to an epitope of antigen B, wherein composition 1 is not the same as composition 2 or composition 3. The methods can further include obtaining an effector T cell response to one or both of the antigens.

[0026] In some aspects composition 2 and composition 3 each can include peptide A and peptide B. Peptides A and B can be administered to separate sites, or to the same site including at different times, for example. Composition 1 can include a nucleic acid molecule encoding both antigen A and antigen B, or portions thereof. Also, composition 1 can include two nucleic acid molecules one encoding antigen A or portion thereof and one encoding antigen B or portion thereof, for example.

[0027] The first and second antigens can be any antigen. Preferably, the first and second antigens are melanoma antigens, CT antigens, carcinoma-associated antigens, a CT antigen and a stromal antigen, a CT antigen and a neovasculature antigen, a CT antigen and a differentiation antigen, a carcinoma-associated antigen and a stromal antigen, and the like. Various antigen combinations are provided in U.S. Application No. 10/871,708 (Pub. No. 20050118186), filed on June 17, 2004, entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS; and U.S. Provisional Application No. 60/640,598, filed on December 29, 2004, and in U.S. Application No. __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.049A) filed on the same date as the instant application, both also entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS, each of which is incorporated herein by reference in its entirety. Preferably the antigen, including antigen A or B can be SSX-2, Melan-A, Tyrosinase, PSMA, PRAME, NY-ESO-1, or the like. Many other antigens are known to those of ordinary skill in the art. It should be understood that in this and other embodiments, more than two compositions, immunogens, antigens, epitopes and/or peptides can be used. For example, three, four, five or more of any one or more of the above can be used.

[0028] Other embodiments relate to methods of generating an immune response, which can include, for example, delivering to a mammal a first composition (composition 1) that includes an immunogen (immunogen 1), which immunogen 1 can include or encode at least a portion of a first antigen (antigen A) and a second composition (composition 2) which can include a second immunogen (immunogen 2) that can include or encode at least a portion of a second antigen (antigen B); and administering a third composition (composition 3) that can include a first peptide (peptide A), and a fourth composition (composition 4) that can include a second peptide (peptide B), directly to the lymphatic

system of the mammal, wherein peptide A corresponds to an epitope of antigen A, and wherein peptide B corresponds to an epitope of antigen B, wherein composition 1 is not the same as composition 2 or composition 3.

[0029] In some aspects composition 2 is not the same as composition 3, for example. Composition 1 and composition 3 can be delivered to a same site, for example, the site can be an inguinal lymph node. Also, compositions 2 and 4 can be delivered to a different site than compositions 1 and 3, for example, to another inguinal lymph node.

[0030] Still further embodiments relate to methods of generating an immune response that can include, for example, delivering a first composition that includes means for entraining an immune response to a first antigen and a second antigen; and administering a second composition that includes a first peptide, and a third composition that includes a second peptide, directly to the lymphatic system of the mammal, wherein the first peptide corresponds to an epitope of the first antigen, and wherein the second peptide corresponds to an epitope of the second antigen, wherein the first composition is not the same as the second or third compositions. The means for entraining an immune response can include, for example, means for expressing the antigens or portions thereof.

[0031] Also, some embodiments relate to methods of immunization, which can include, for example, delivering to a mammal a first composition that includes an immunogen, which immunogen can include or encode at least a portion of a first antigen and at least a portion of a second antigen; and a step for amplifying the response to the antigens. Preferably, the step for amplifying the response to the antigens can include administering a first peptide that corresponds to the at least a portion of a first antigen to a secondary lymphoid organ and administering a second peptide corresponding to the at least a portion of a second antigen to a different secondary lymphoid organ.

Brief Description of the Drawings

[0032] Figure 1 A-C: Induction of immune responses by intra-lymphatic immunization.

[0033] Figure 2 depicts examples of protocols for controlling or manipulating the immunity to MHC class I-restricted epitopes by targeted (lymph node) delivery of antigen.

[0034] Figure 3 represents a visual perspective on representative wells corresponding to the data described in Figure 4.

[0035] Figure 4 depicts the magnitude of immune response resulting from application of protocols described in Figure 2, measured by ELISPOT and expressed as number (frequency) of IFN-γ (gamma) producing T cells recognizing the peptide

[0036] Figure 5 shows the cytotoxic profile of T cells generated by targeted delivery of antigen, as described in Figure 2.

[0037] Figure 6 depicts the cross-reactivity of MHC class I-restricted T cells generated by the protocol depicted in the Figure 2.

[0038] Figure 7A shows the profile of immunity, expressed as ability of lymphocytes to produce members of three classes of biological response modifiers (pro-inflammatory cytokines, chemokines or chemo-attractants, and immune regulatory or suppressor cytokines), subsequent to application of the immunization protocols described in the Figure 2.

[0039] Figure 7B shows cell surface marker phenotyping by flow cytometry for T cell generated by the immunization protocols described in Figure 2. Repeated administration of peptide to the lymph nodes induces immune deviation and regulatory T cells.

[0040] Figure 8A and B show the frequency of specific T cells measured by tetramer, in mice immunized with DNA, peptide or an entrain/amplify sequence of DNA and peptide.

[0041] Figure 8C shows the specific cytotoxicity occurring *in vivo*, in various lymphoid and non-lymphoid organs, in mice immunized with DNA ("pSEM"), peptide ("ELA" = ELAGIGILTV (SEQ ID NO:1)) or an entrain/amplify sequence of DNA and peptide.

[0042] Figure 9A shows the persistence / decay of circulating tetramer stained T cells in animals immunized with peptide and amplified with peptide, along with the recall response following a peptide boost.

[0043] Figure 9B shows the persistence / decay of circulating tetramer stained T cells in animals entrained with DNA and amplified with peptide, along with the recall response following a peptide amplification.

[0044] Figure 9C shows the persistence / decay of circulating tetramer stained T cells in animals immunized with DNA and amplified with DNA, along with the recall response following a peptide boost.

[0045] Figure 10A shows the expansion of antigen-specific CD8+ T cells using various two-cycle immunization protocols.

[0046] Figure 10B shows the expansion of antigen-specific CD8+ T cells using various three-cycle immunization protocols.

[0047] Figure 10C shows the expansion of circulating antigen-specific T cells detected by tetramer staining, in animals primed using various protocols and amplified with peptide.

[0048] Figure 10D shows the expansion of antigen-specific T cells subsequent to various immunization regimens and detected by tetramer staining, in lymphoid and non-lymphoid organs.

[0049] Figure 11A shows an example of a schedule of immunizing mice with plasmid DNA and peptides

[0050] Figure 11B shows the immune response determined by ELISPOT analysis triggered by various immunization protocols (alternating DNA and peptide in respective or reverse order).

[0051] Figure 12A shows *in vivo* depletion of antigenic target cells, in blood and lymph nodes, in mice immunized with plasmid and peptide.

[0052] Figure 12B shows *in vivo* depletion of antigenic target cells, in spleen and lungs, in mice immunized with plasmid and peptide.

[0053] Figure 12C shows a summary of the results presented in 12A,B.

[0054] Figure 12D shows a correlation between frequency of specific T cells and *in vivo* clearance of antigenic target cells in mice immunized by the various protocols.

[0055] Figure 13A shows the schedule of immunizing mice with plasmid DNA and peptides, as well as the nature of measurements performed in those mice.

[0056] Figure 13B describes the schedule associated with the protocol used for determination of *in vivo* clearance of human tumor cells in immunized mice.

[0057] Figure 13C shows *in vivo* depletion of antigenic target cells (human tumor cells) in lungs of mice immunized with plasmid and peptide.

[0058] Figure 14A shows the immunization protocol used to generate the anti SSX-2 response shown in 14B.

[0059] Figure 14B shows the expansion of circulating SSX-2 specific T cells subsequent to applying a DNA entraining / peptide amplification regimen, detected by tetramer staining.

[0060] Figure 15A shows the *in vivo* clearance of antigenic target cells in spleens of mice that underwent various entrain-and-amplify protocols to simultaneously immunize against epitopes of Melan A (ELAGIGILTV (SEQ ID NO:1)) and SSX2 (KASEKIFYV (SEQ ID NO:2)).

[0061] Figure 15B shows the *in vivo* clearance of antigenic target cells in the blood of mice that underwent various entrain-and-amplify protocols to simultaneously immunize against epitopes of Melan A (ELAGIGILTV (SEQ ID NO:1)) and SSX2 (KASEKIFYV (SEQ ID NO:2)).

[0062] Figure 15C summarizes the results shown in detail in Figs 15A,B.

[0063] Figure 16 shows the expansion of the circulating antigen-specific CD8+ T cells measured by tetramer staining, in mice undergoing two cycles of various entrain-and-amplify protocols.

[0064] Figure 17A and B show the persistence of circulating antigen-specific T cells in animals undergoing two cycles of entrain-and-amplify protocols consisting of DNA/DNA/peptide (A) or DNA/peptide/peptide (B).

[0065] Figure 18 shows long-lived memory in animals undergoing two cycles of an entrain-and-amplify protocol consisting of DNA/DNA/DNA.

[0066] Figure 19 shows a clinical practice schema for enrollment and treatment of patients with DNA / peptide entrain-and-amplify protocols.

[0067] Figure 20 depicts a schedule of immunization using two plasmids: pCBP expressing SSX2 41-49 and pSEM expressing Melan A 26-35(A27L).

[0068] Figure 21 shows specific cytotoxicity induced by administration of two plasmids as a mixture versus administration to individually to separate sites.

[0069] Figure 22 depicts the addition of peptide boost steps to the immunization protocol described in Figure 20.

[0070] Figure 23 presents data showing that peptide boost rescues the immunogenicity of a less dominant epitope even when the vectors and peptides respectively, are used as a mixture.

[0071] Figures 24 A and B depict alternative immunization protocols to induce strong, multivalent responses in clinical practice.

[0072] Figure 25 depicts a plasmid capable of eliciting multivalent responses.

[0073] Figure 26 presents a protocol for initiating an immune response with a multivalent plasmid and rescue of the response to a subdominant epitope by intranodal administration of peptide.

[0074] Figure 27A shows the frequency of specific T cells obtained by priming with multivalent plasmid and amplification of response against a dominant (Melan-A) epitope by intranodal administration of peptide.

[0075] Figure 27B shows the frequency of specific T cells obtained by priming with multivalent plasmid and amplification of response against a subdominant epitope (Tyrosinase 369-377) by intranodal administration of peptide.

[0076] Figure 28A shows the specific cytotoxicity obtained by priming with multivalent plasmid and amplification of response against a dominant (Melan-A) epitope by intranodal administration of peptide.

[0077] Figure 28B shows the specific cytotoxicity obtained by priming with multivalent plasmid and amplification of response against a subdominant epitope (Tyrosinase 369-377) by intranodal administration of peptide.

[0078] Figure 29 depicts an immunization protocol priming with a multivalent plasmid and amplifying the response against a dominant and a subdominant epitope, simultaneously.

[0079] Figure 30A shows the frequency of Melan-A specific T cells obtained by priming with multivalent plasmid and amplification of response against a dominant (Melan-A) epitope and a subdominant (Tyrosinase) epitope by intranodal administration of peptide.

[0080] Figure 30B shows the frequency of Tyrosinase specific T cells obtained by priming with multivalent plasmid and amplification of response against a dominant (Melan-A)epitope and a subdominant (Tyrosinase) epitope by intranodal administration of peptide.

[0081] Figure 30C shows the frequency of both Melan-A and Tyrosinase specific T cells in mice primed with pSEM and amplified with both Melan-A and tyrosinase peptides. Results from two individual mice are shown.

[0082] Figure 31 shows *in vivo* cytotoxicity data for T cells co-initiated and amplified by a multivalent plasmid followed by intranodal administration of peptides, corresponding to a dominant (Melan A 26-35) and a subdominant (Tyrosinase 369-377) epitope, as a mixture.

[0083] Figure 32: Dual multi-color tetramer analysis of pSEM/pBPL immunized animals prior to amplification.

[0084] Figure 33: Dual multi-color tetramer analysis of the immune response of mice induced with a mixture of the plasmids pSEM and pBPL, and amplified with SSX2 and Tyrosinase peptide epitope analogues.

[0085] Figure 34: Dual multi-color tetramer analysis of the immune response of 3 individual mice induced with a mixture of the plasmids pSEM and pBPL, and amplified with SSX2 and Tyrosinase peptide epitope analogues.

[0086] Figure 35A: IFN-γ ELISpot analysis after the 1st round of amplification

[0087] Figure 35B: IFN-γ ELISpot analysis after the 2nd rounds of amplification

[0088] Figure 36: CFSE *in vivo* challenge with human melanoma tumor cells expressing all four tumor associated antigens. Panels A-D each show tetramer analysis, IFN-γ ELISpot analysis, and in vivo tumor cell killing individual mice following completion of the protocol. Panel A shows data from a naïve control mouse, panels B-C show data from two mice, from group 3 and 2, respectively, achieving substantial tetravalent immunity, and panel D shows data from a mouse from group 3, whose immunity was substantially monovalent.

[0089] Figure 37 depicts a global method to induce multivalent immunity.

Detailed Description of the Preferred Embodiment

[0090] Embodiments of the present invention provide methods and compositions, for example, for generating immune cells specific to a target cell, for directing an effective immune response against a target cell, or for affecting/treating inflammatory disorders. The methods and compositions can include, for example, immunogenic compositions such as vaccines and therapeutics, and also prophylactic and

therapeutic methods. Disclosed herein is the novel and unexpected discovery that by selecting the form of antigen, the sequence and timing with which it is administered, and delivering the antigen directly into secondary lymphoid organs, not only the magnitude, but the qualitative nature of the immune response can be managed.

[0091] Some preferred embodiments relate to compositions and methods for entraining and amplifying a T cell response. For example such methods can include an entrainment step where a composition comprising a nucleic acid encoded immunogen is delivered to an animal. The composition can be delivered to various locations on the animal, but preferably is delivered to the lymphatic system, for example, a lymph node. The entrainment step can include one or more deliveries of the composition, for example, spread out over a period of time or in a continuous fashion over a period of time. Preferably, the methods can further include an amplification step comprising administering a composition comprising a peptide immunogen. The amplification step can be performed one or more times, for example, at intervals over a period of time, in one bolus, or continuously over a period of time. Although not required in all embodiments, some embodiments can include the use of compositions that include an immunopotentiator or adjuvant.

[0092] Each of the disclosures of the following applications, including all methods, figures, and compositions, is incorporated herein by reference in its entirety: U.S. Provisional Application No. 60/479,393, filed on June 17, 2003, entitled METHODS TO CONTROL MHC CLASS I-RESTRICTED IMMUNE RESPONSE; U.S. Application No. 10/871,707 filed on June 17, 2004 (Pub. No. 20050079152), U.S. Provisional Application No. 60/640,402, filed on December 29, 2004, and U.S. Application No. __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.047A), filed on the same date as this application, all three of which are entitled "METHODS TO ELICIT, ENHANCE AND SUSTAIN IMMUNE RESPONSES AGAINST MHC CLASS I-RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSES"; U.S. Application No. 10/871,708 (Pub. No. 20050118186), filed on June 17, 2004, entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS"; and Provisional Application No. 60/640,598, filed on December 29, 2004, and U.S. Patent Application No. __/___,___ (Pub. No. _____), (Attorney Docket No. MANNK.049A), filed on the same date as this

application, both of which are entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS," and each of which are incorporated by reference in its entirety Also, the following applications include methods and compositions that can be used with the instant methods and compositions. Plasmid and principles of plasmid design are disclosed in US Patent Application No. 10/292,413 (Pub. No. 20030228634 A1), entitled "EXPRESSION VECTORS ENCODING EPITOPES OF TARGET ASSOCIATED ANTIGENS AND METHODS FOR THEIR DESIGN," which is hereby incorporated by reference in its entirety; additional methodology, compositions, peptides, and peptide analogues are disclosed in U.S. Provisional Application No 60/581,001, filed on June 17, 2004, U.S. Application No. 11/156,253 (Pub. No. _____), entitled "SSX-2 PEPTIDE ANALOGS"; each of which is incorporated herein by reference in its entirety; U.S. Provisional Application No. 60/580,962, filed on June 17, 2004, U.S. Application No. 11/155,929 (Pub. No. _____), filed on June 17, 2005, entitled "NY-ESO PEPTIDE ANALOGS"; each of which is incorporated herein by reference in its entirety; and U.S. Application Nos. 10/117,937 (Pub. No. 20030220239), filed on April 4, 2002, and 10/657,022 (Pub. No. 20040180354), filed on September 5, 2003, both of which are entitled EPITOPE SEQUENCES, and each of which is hereby incorporated by reference in its entirety.

[0093] In some embodiments, depending on the nature of the immunogen and the context in which it is encountered, the immune response elicited can differ in its particular activity and makeup. In particular, while immunization with peptide can generate a cytotoxic/cytolytic T cell (CTL) response, attempts to further amplify this response with further injections can instead lead to the expansion of a regulatory T cell population, and a diminution of observable CTL activity. Thus compositions conferring high MHC/peptide concentrations on the cell surface within the lymph node, without additional immunopotentiating activity, can be used to purposefully promote a regulatory or tolerogenic response. In contrast immunogenic compositions providing ample immunopotentiation signals (e.g.,. toll-like receptor ligands [or the cytokine/autocrine factors they would induce]) even if providing only limiting antigen, not only induce a response, but entrain it as well, so that subsequent encounters with ample antigen (e.g., injected peptide) amplifies the response without changing the nature of the observed activity. Therefore, some embodiments relate to controlling the immune response profile,

for example, the kind of response obtained and the kinds of cytokines produced. Some embodiments relate to methods and compositions for promoting the expansion or further expansion of CTL, and there are embodiment that relate to methods and compositions for promoting the expansion of regulatory cells in preference to the CTL, for example.

[0094] The disclosed methods are advantageous over many protocols that use only peptide or that do not follow the entrain-and-amplify methodology. As set forth above, many peptide-based immunization protocols and vector-based protocols have drawbacks. Nevertheless, if successful, a peptide based immunization or immune amplification strategy has advantages over other methods, particularly certain microbial vectors, for example. This is due to the fact that more complex vectors, such as live attenuated viral or bacterial vectors, may induce deleterious side-effects, for example, in vivo replication or recombination; or become ineffective upon repeated administration due to generation of neutralizing antibodies against the vector itself. Additionally, when harnessed in such a way to become strong immunogens, peptides can circumvent the need for proteasome-mediated processing (as with protein or more complex antigens, in context of "cross-processing" or subsequent to cellular infection). That is because cellular antigen processing for MHC-class I restricted presentation is a phenomenon that inherently selects dominant (favored) epitopes over subdominant epitopes, potentially interfering with the immunogenicity of epitopes corresponding to valid targets. Finally, effective peptide based immunization simplifies and shortens the process of development of immunotherapeutics.

[0095] Thus, effective peptide-based immune amplification methods, particularly including those described herein, can be of considerable benefit to immunotherapy (such as for cancer and chronic infections) or prophylactic vaccination (against certain infectious diseases). Additional benefits can be achieved by avoiding simultaneous use of cumbersome, unsafe, or complex adjuvant techniques, although such techniques can be utilized in various embodiments described herein.

Definitions:

[0096] Unless otherwise clear from the context of the use of a term herein, the following listed terms shall generally have the indicated meanings for purposes of this description.

[0097] PROFESSIONAL ANTIGEN-PRESENTING CELL (pAPC) – a cell that possesses T cell costimulatory molecules and is able to induce a T cell response. Well characterized pAPCs include dendritic cells, B cells, and macrophages.

[0098] PERIPHERAL CELL – a cell that is not a pAPC.

[0099] HOUSEKEEPING PROTEASOME – a proteasome normally active in peripheral cells, and generally not present or not strongly active in pAPCs.

[0100] IMMUNOPROTEASOME – a proteasome normally active in pAPCs; the immunoproteasome is also active in some peripheral cells in infected tissues or following exposure to interferon.

[0101] EPITOPE – a molecule or substance capable of stimulating an immune response. In preferred embodiments, epitopes according to this definition include but are not necessarily limited to a polypeptide and a nucleic acid encoding a polypeptide, wherein the polypeptide is capable of stimulating an immune response. In other preferred embodiments, epitopes according to this definition include but are not necessarily limited to peptides presented on the surface of cells, the peptides being non-covalently bound to the binding cleft of class I MHC, such that they can interact with T cell receptors (TCR). Epitopes presented by class I MHC may be in immature or mature form. "Mature" refers to an MHC epitope in distinction to any precursor ("immature") that may include or consist essentially of a housekeeping epitope, but also includes other sequences in a primary translation product that are removed by processing, including without limitation, alone or in any combination, proteasomal digestion, N-terminal trimming, or the action of exogenous enzymatic activities. Thus, a mature epitope may be provided embedded in a somewhat longer polypeptide, the immunological potential of which is due, at least in part, to the embedded epitope; likewise, the mature epitope can be provided in its ultimate form that can bind in the MHC binding cleft to be recognized by TCR.

[0102] MHC EPITOPE – a polypeptide having a known or predicted binding affinity for a mammalian class I or class II major histocompatibility complex (MHC) molecule. Some particularly well characterized class I MHC molecules are presented in Tables 1-4.

[0103] HOUSEKEEPING EPITOPE – In a preferred embodiment, a housekeeping epitope is defined as a polypeptide fragment that is an MHC epitope, and that is displayed on a cell in which housekeeping proteasomes are predominantly active. In

another preferred embodiment, a housekeeping epitope is defined as a polypeptide containing a housekeeping epitope according to the foregoing definition, that is flanked by one to several additional amino acids. In another preferred embodiment, a housekeeping epitope is defined as a nucleic acid that encodes a housekeeping epitope according to the foregoing definitions. Exemplary housekeeping epitopes are provided in U.S. Patent Application Nos. 10/117,937, filed on April 4, 2002 (Pub. No. 20030220239 A1), 11/067,159 (Pub. No. 2005-0221440 A1), filed February 25, 2005, 11/067,064 (Pub. No. 2005-0142144 A1), filed February 25, 2005, and 10/657,022 (Pub. No. 2004-0180354 A1), filed September 5, 2003, and in PCT Application No. PCT/US2003/027706 (Pub. No. WO 2004/022709 A2), filed 9/5/2003; and U.S. Provisional Application Nos. 60/282,211, filed on April 6, 2001; 60/337,017, filed on November 7, 2001; 60/363,210 filed March 7, 2002; and 60/409,123, filed on September 6, 2002. Each of the listed applications is entitled EPITOPE SEQUENCES. Each of the applications mentioned in this paragraph is incorporated herein by reference in its entirety.

[0104] IMMUNE EPITOPE – In a preferred embodiment, an immune epitope is defined as a polypeptide fragment that is an MHC epitope, and that is displayed on a cell in which immunoproteasomes are predominantly active. In another preferred embodiment, an immune epitope is defined as a polypeptide containing an immune epitope according to the foregoing definition that is flanked by one to several additional amino acids. In another preferred embodiment, an immune epitope is defined as a polypeptide including an epitope cluster sequence, having at least two polypeptide sequences having a known or predicted affinity for a class I MHC. In yet another preferred embodiment, an immune epitope is defined as a nucleic acid that encodes an immune epitope according to any of the foregoing definitions.

[0105] TARGET CELL – In a preferred embodiment, a target cells is a cell associated with a pathogenic condition that can be acted upon by the components of the immune system, for example, a cell infected with a virus or other intracellular parasite, or a neoplastic cell. In another embodiment, a target cell is a cell to be targeted by the vaccines and methods of the invention. Examples of target cells according to this definition include but are not necessarily limited to: a neoplastic cell and a cell harboring an intracellular parasite, such as, for example, a virus, a bacterium, or a protozoan. Target cells can also include cells that are targeted by CTL as a part of an assay to determine or confirm proper

epitope liberation and processing by a cell expressing immunoproteasome, to determine T cell specificity or immunogenicity for a desired epitope. Such cells can be transformed to express the liberation sequence, or the cells can simply be pulsed with peptide/epitope.

[0106] TARGET-ASSOCIATED ANTIGEN (TAA) – a protein or polypeptide present in a target cell.

[0107] TUMOR-ASSOCIATED ANTIGENS (TuAA) – a TAA, wherein the target cell is a neoplastic cell.

[0108] HLA EPITOPE – a polypeptide having a known or predicted binding affinity for a human class I or class II HLA complex molecule. Particularly well characterized class I HLAs are presented in Tables 1-4.

[0109] ANTIBODY – a natural immunoglobulin (Ig), poly- or monoclonal, or any molecule composed in whole or in part of an Ig binding domain, whether derived biochemically, or by use of recombinant DNA, or by any other means. Examples include inter alia, F(ab), single chain Fv, and Ig variable region-phage coat protein fusions.

[0110] SUBSTANTIAL SIMILARITY – this term is used to refer to sequences that differ from a reference sequence in an inconsequential way as judged by examination of the sequence. Nucleic acid sequences encoding the same amino acid sequence are substantially similar despite differences in degenerate positions or minor differences in length or composition of any non-coding regions. Amino acid sequences differing only by conservative substitution or minor length variations are substantially similar. Additionally, amino acid sequences comprising housekeeping epitopes that differ in the number of N-terminal flanking residues, or immune epitopes and epitope clusters that differ in the number of flanking residues at either terminus, are substantially similar. Nucleic acids that encode substantially similar amino acid sequences are themselves also substantially similar.

[0111] FUNCTIONAL SIMILARITY – this term is used to refer to sequences that differ from a reference sequence in an inconsequential way as judged by examination of a biological or biochemical property, although the sequences may not be substantially similar. For example, two nucleic acids can be useful as hybridization probes for the same sequence but encode differing amino acid sequences. Two peptides that induce cross-reactive CTL responses are functionally similar even if they differ by non-conservative amino acid substitutions (and thus may not be within the substantial similarity definition). Pairs of antibodies, or TCRs, that recognize the same epitope can be functionally similar to

each other despite whatever structural differences exist. Testing for functional similarity of immunogenicity can be conducted by immunizing with the "altered" antigen and testing the ability of an elicited response, including but not limited to an antibody response, a CTL response, cytokine production, and the like, to recognize the target antigen. Accordingly, two sequences may be designed to differ in certain respects while retaining the same function. Such designed sequence variants of disclosed or claimed sequences are among the embodiments of the present invention.

[0112] EXPRESSION CASSETTE – a polynucleotide sequence encoding a polypeptide, operably linked to a promoter and other transcription and translation control elements, including but not limited to enhancers, termination codons, internal ribosome entry sites, and polyadenylation sites. The cassette can also include sequences that facilitate moving it from one host molecule to another.

[0113] EMBEDDED EPITOPE – in some embodiments, an embedded epitope is an epitope that is wholly contained within a longer polypeptide; in other embodiments, the term also can include an epitope in which only the N-terminus or the C-terminus is embedded such that the epitope is not wholly in an interior position with respect to the longer polypeptide.

[0114] MATURE EPITOPE – a peptide with no additional sequence beyond that present when the epitope is bound in the MHC peptide-binding cleft.

[0115] EPITOPE CLUSTER – a polypeptide, or a nucleic acid sequence encoding it, that is a segment of a protein sequence, including a native protein sequence, comprising two or more known or predicted epitopes with binding affinity for a shared MHC restriction element. In preferred embodiments, the density of epitopes within the cluster is greater than the density of all known or predicted epitopes with binding affinity for the shared MHC restriction element within the complete protein sequence. Epitope clusters are disclosed and more fully defined in U.S. Patent Application No. 09/561,571, filed April 28, 2000, entitled EPITOPE CLUSTERS, which is incorporated herein by reference in its entirety.

[0116] LIBERATION SEQUENCE – a designed or engineered sequence comprising or encoding a housekeeping epitope embedded in a larger sequence that provides a context allowing the housekeeping epitope to be liberated by processing

activities including, for example, immunoproteasome activity, N terminal trimming, and/or other processes or activities, alone or in any combination.

[0117] CTLp – CTL precursors are T cells that can be induced to exhibit cytolytic activity. Secondary in vitro lytic activity, by which CTLp are generally observed, can arise from any combination of naïve, effector, and memory CTL in vivo.

[0118] MEMORY T CELL – A T cell, regardless of its location in the body, that has been previously activated by antigen, but is in a quiescent physiologic state requiring re-exposure to antigen in order to gain effector function. Phenotypically they are generally CD62L- CD44hi CD107α- IGN-γ - LTβ- TNF-α- and is in G0 of the cell cycle.

[0119] EFFECTOR T CELL – A T cell that, upon encountering antigen antigen, readily exhibits effector function. Effector T cells are generally capable of exiting the lymphatic system and entering the immunological periphery. Phenotypically they are generally CD62L- CD44hi CD107α+ IGN-γ+ LTβ+ TNF-α+ and actively cycling.

[0120] EFFECTOR FUNCTION – Generally, T cell activation generally, including acquisition of cytolytic activity and/or cytokine secretion.

[0121] INDUCING a T cell response – Includes in many embodiments the process of generating a T cell response from naïve, or in some contexts, quiescent cells; activating T cells.

[0122] AMPLIFYING A T CELL RESPONSE – Includes in many embodiment a process for increasing the number of cells, the number of activated cells, the level of activity, rate of proliferation, or similar parameter of T cells involved in a specific response.

[0123] ENTRAINMENT – Includes in many embodiments an induction that confers particular stability on the immune profile of the induced lineage of T cells. In various embodiments, the term "entrain" can correspond to "induce," and/or "initiate."

[0124] TOLL-LIKE RECEPTOR (TLR) – Toll-like receptors (TLRs) are a family of pattern recognition receptors that are activated by specific components of microbes and certain host molecules. As part of the innate immune system, they contribute to the first line of defense against many pathogens, but also play a role in adaptive immunity.

[0125] TOLL-LIKE RECEPTOR (TLR) LIGAND – Any molecule capable of binding and activating a toll-like receptor. Examples include, without limitation: poly IC A synthetic, double-stranded RNA know for inducing interferon. The polymer is made of one

strand each of polyinosinic acid and polycytidylic acid, double-stranded RNA, unmethylated CpG oligodeoxyribonucleotide or other immunostimulatory sequences (ISSs), lipopolysacharide (LPS), β-glucans, and imidazoquinolines, as well as derivatives and analogues thereof.

[0126] IMMUNOPOTENTIATING ADJUVANTS – Adjuvants that activate pAPC or T cells including, for example: TLR ligands, endocytic-Pattern Recognition Receptor (PRR) ligands, quillaja saponins, tucaresol, cytokines, and the like. Some preferred adjuvants are disclosed in Marciani, D.J. Drug Discovery Today 8:934-943, 2003, which is incorporated herein by reference in its entirety.

[0127] IMMUNOSTIMULATORY SEQUENCE (ISS) – Generally an oligodeoxyribonucleotide containing an unmethlylated CpG sequence. The CpG may also be embedded in bacterially produced DNA, particularly plasmids. Further embodiments include various analogues; among preferred embodiments are molecules with one or more phosphorothioate bonds or non-physiologic bases.

[0128] VACCINE – In preferred embodiments a vaccine can be an immunogenic composition providing or aiding in prevention of disease. In other embodiments, a vaccine is a composition that can provide or aid in a cure of a disease. In others, a vaccine composition can provide or aid in amelioration of a disease. Further embodiments of a vaccine immunogenic composition can be used as therapeutic and/or prophylactic agents.

[0129] IMMUNIZATION – a process to induce partial or complete protection against a disease. Alternatively, a process to induce or amplify an immune system response to an antigen. In the second definition it can connote a protective immune response, particularly proinflammatory or active immunity, but can also include a regulatory response. Thus in some embodiments immunization is distinguished from tolerization (a process by which the immune system avoids producing proinflammatory or active immunity) while in other embodiments this term includes tolerization.

Table 1

Class I MHC Molecules

Class I

Human

HLA-A1
HLA-A*0101
HLA-A*0201
HLA-A*0202
HLA-A*0203
HLA-A*0204
HLA-A*0205
HLA-A*0206
HLA-A*0207
HLA-A*0209
HLA-A*0214
HLA-A3
HLA-A*0301
HLA-A*1101
HLA-A23
HLA-A24
HLA-A25
HLA-A*2902
HLA-A*3101
HLA-A*3302
HLA-A*6801
HLA-A*6901
HLA-B7
HLA-B*0702
HLA-B*0703
HLA-B*0704
HLA-B*0705
HLA-B8
HLA-B13
HLA-B14
HLA-B*1501 (B62)
HLA-B17
HLA-B18
HLA-B22
HLA-B27
HLA-B*2702
HLA-B*2704
HLA-B*2705
HLA-B*2709
HLA-B35
HLA-B*3501
HLA-B*3502
HLA-B*3701

HLA-B*3801
HLA-B*39011
HLA-B*3902
HLA-B40
HLA-B*40012 (B60)
HLA-B*4006 (B61)
HLA-B44
HLA-B*4402
HLA-B*4403
HLA-B*4501
HLA-B*4601
HLA-B51
HLA-B*5101
HLA-B*5102
HLA-B*5103
HLA-B*5201
HLA-B*5301
HLA-B*5401
HLA-B*5501
HLA-B*5502
HLA-B*5601
HLA-B*5801
HLA-B*6701
HLA-B*7301
HLA-B*7801
HLA-Cw*0102
HLA-Cw*0301
HLA-Cw*0304
HLA-Cw*0401
HLA-Cw*0601
HLA-Cw*0602
HLA-Cw*0702
HLA-Cw8
HLA-Cw*1601 M
HLA-G

Murine (Mouse)

$H2\text{-}K^{d}$
$H2\text{-}D^{d}$
$H2\text{-}L^{d}$
$H2\text{-}K^{b}$
$H2\text{-}D^{b}$
$H2\text{-}K^{k}$
$H2\text{-}K^{kml}$
$Qa\text{-}1^{a}$
Qa-2
H2-M3

Rat
RT1.A[a]
RT1.A[l]

Bovine (Cow)
Bota-A11
Bota-A20

Chicken
B-F4
B-F12
B-F15
B-F19

Chimpanzee
Patr-A*04
Patr-A*11
Patr-B*01
Patr-B*13
Patr-B*16

Baboon
Papa-A*06

Macaque
Mamu-A*01

Swine (Pig)
SLA (haplotype d/d)

Virus homolog
hCMV class I homolog UL18

Table 2

Class I MHC Molecules

Class I

Human

HLA-A1
HLA-A*0101
HLA-A*0201
HLA-A*0202
HLA-A*0204
HLA-A*0205
HLA-A*0206
HLA-A*0207
HLA-A*0214
HLA-A3
HLA-A*1101
HLA-A24
HLA-A*2902
HLA-A*3101
HLA-A*3302
HLA-A*6801
HLA-A*6901
HLA-B7
HLA-B*0702
HLA-B*0703
HLA-B*0704
HLA-B*0705
HLA-B8
HLA-B14
HLA-B*1501 (B62)
HLA-B27
HLA-B*2702
HLA-B*2705
HLA-B35
HLA-B*3501
HLA-B*3502
HLA-B*3701
HLA-B*3801
HLA-B*39011
HLA-B*3902
HLA-B40
HLA-B*40012 (B60)
HLA-B*4006 (B61)
HLA-B44
HLA-B*4402
HLA-B*4403
HLA-B*4601

HLA-B51
HLA-B*5101
HLA-B*5102
HLA-B*5103
HLA-B*5201
HLA-B*5301
HLA-B*5401
HLA-B*5501
HLA-B*5502
HLA-B*5601
HLA-B*5801
HLA-B*6701
HLA-B*7301
HLA-B*7801
HLA-Cw*0102
HLA-Cw*0301
HLA-Cw*0304
HLA-Cw*0401
HLA-Cw*0601
HLA-Cw*0602
HLA-Cw*0702
HLA-G

Murine

$H2\text{-}K^{d}$
$H2\text{-}D^{d}$
$H2\text{-}L^{d}$
$H2\text{-}K^{b}$
$H2\text{-}D^{b}$
$H2\text{-}K^{k}$
$H2\text{-}K^{kml}$
Qa-2

Rat

$RT1.A^{a}$
$RT1.A^{l}$

Bovine

Bota-A11
Bota-A20

Chicken

B-F4
B-F12
B-F15
B-F19

Virus homolog

hCMV class I homolog UL18

Table 3

Estimated gene frequencies of HLA-A antigens

Antigen	CAU		AFR		ASI		LAT		NAT	
	Gf[a]	SE[b]	Gf	SE	Gf	SE	Gf	SE	Gf	SE
A1	15.1843	0.0489	5.7256	0.0771	4.4818	0.0846	7.4007	0.0978	12.0316	0.2533
A2	28.6535	0.0619	18.8849	0.1317	24.6352	0.1794	28.1198	0.1700	29.3408	0.3585
A3	13.3890	0.0463	8.4406	0.0925	2.6454	0.0655	8.0789	0.1019	11.0293	0.2437
A28	4.4652	0.0280	9.9269	0.0997	1.7657	0.0537	8.9446	0.1067	5.3856	0.1750
A36	0.0221	0.0020	1.8836	0.0448	0.0148	0.0049	0.1584	0.0148	0.1545	0.0303
A23	1.8287	0.0181	10.2086	0.1010	0.3256	0.0231	2.9269	0.0628	1.9903	0.1080
A24	9.3251	0.0395	2.9668	0.0560	22.0391	0.1722	13.2610	0.1271	12.6613	0.2590
A9 unsplit	0.0809	0.0038	0.0367	0.0063	0.0858	0.0119	0.0537	0.0086	0.0356	0.0145
A9 total	11.2347	0.0429	13.2121	0.1128	22.4505	0.1733	16.2416	0.1382	14.6872	0.2756
A25	2.1157	0.0195	0.4329	0.0216	0.0990	0.0128	1.1937	0.0404	1.4520	0.0924
A26	3.8795	0.0262	2.8284	0.0547	4.6628	0.0862	3.2612	0.0662	2.4292	0.1191
A34	0.1508	0.0052	3.5228	0.0610	1.3529	0.0470	0.4928	0.0260	0.3150	0.0432
A43	0.0018	0.0006	0.0334	0.0060	0.0231	0.0062	0.0055	0.0028	0.0059	0.0059
A66	0.0173	0.0018	0.2233	0.0155	0.0478	0.0089	0.0399	0.0074	0.0534	0.0178
A10 unsplit	0.0790	0.0038	0.0939	0.0101	0.1255	0.0144	0.0647	0.0094	0.0298	0.0133
A10 total	6.2441	0.0328	7.1348	0.0850	6.3111	0.0993	5.0578	0.0816	4.2853	0.1565
A29	3.5796	0.0252	3.2071	0.0582	1.1233	0.0429	4.5156	0.0774	3.4345	0.1410
A30	2.5067	0.0212	13.0969	0.1129	2.2025	0.0598	4.4873	0.0772	2.5314	0.1215
A31	2.7386	0.0221	1.6556	0.0420	3.6005	0.0761	4.8328	0.0800	6.0881	0.1855
A32	3.6956	0.0256	1.5384	0.0405	1.0331	0.0411	2.7064	0.0604	2.5521	0.1220
A33	1.2080	0.0148	6.5607	0.0822	9.2701	0.1191	2.6593	0.0599	1.0754	0.0796
A74	0.0277	0.0022	1.9949	0.0461	0.0561	0.0096	0.2027	0.0167	0.1068	0.0252
A19 unsplit	0.0567	0.0032	0.2057	0.0149	0.0990	0.0128	0.1211	0.0129	0.0475	0.0168
A19 total	13.8129	0.0468	28.2593	0.1504	17.3846	0.1555	19.5252	0.1481	15.8358	0.2832
AX	0.8204	0.0297	4.9506	0.0963	2.9916	0.1177	1.6332	0.0878	1.8454	0.1925

[a]Gene frequency.
[b]Standard error.

Table 4

Estimated gene frequencies for HLA-B antigens

Antigen	CAU		AFR		ASI		LAT		NAT	
	Gf[a]	SE[b]	Gf	SE	Gf	SE	Gf	SE	Gf	SE
B7	12.1782	0.0445	10.5960	0.1024	4.2691	0.0827	6.4477	0.0918	10.9845	0.2432
B8	9.4077	0.0397	3.8315	0.0634	1.3322	0.0467	3.8225	0.0715	8.5789	0.2176
B13	2.3061	0.0203	0.8103	0.0295	4.9222	0.0886	1.2699	0.0416	1.7495	0.1013
B14	4.3481	0.0277	3.0331	0.0566	0.5004	0.0287	5.4166	0.0846	2.9823	0.1316
B18	4.7980	0.0290	3.2057	0.0582	1.1246	0.0429	4.2349	0.0752	3.3422	0.1391
B27	4.3831	0.0278	1.2918	0.0372	2.2355	0.0603	2.3724	0.0567	5.1970	0.1721
B35	9.6614	0.0402	8.5172	0.0927	8.1203	0.1122	14.6516	0.1329	10.1198	0.2345
B37	1.4032	0.0159	0.5916	0.0252	1.2327	0.0449	0.7807	0.0327	0.9755	0.0759
B41	0.9211	0.0129	0.8183	0.0296	0.1303	0.0147	1.2818	0.0418	0.4766	0.0531
B42	0.0608	0.0033	5.6991	0.0768	0.0841	0.0118	0.5866	0.0284	0.2856	0.0411
B46	0.0099	0.0013	0.0151	0.0040	4.9292	0.0886	0.0234	0.0057	0.0238	0.0119
B47	0.2069	0.0061	0.1305	0.0119	0.0956	0.0126	0.1832	0.0159	0.2139	0.0356
B48	0.0865	0.0040	0.1316	0.0119	2.0276	0.0575	1.5915	0.0466	1.0267	0.0778
B53	0.4620	0.0092	10.9529	0.1039	0.4315	0.0266	1.6982	0.0481	1.0804	0.0798
B59	0.0020	0.0006	0.0032	0.0019	0.4277	0.0265	0.0055	0.0028	0[c]	
B67	0.0040	0.0009	0.0086	0.0030	0.2276	0.0194	0.0055	0.0028	0.0059	0.0059
B70	0.3270	0.0077	7.3571	0.0866	0.8901	0.0382	1.9266	0.0512	0.6901	0.0639
B73	0.0108	0.0014	0.0032	0.0019	0.0132	0.0047	0.0261	0.0060	0[c]	
B51	5.4215	0.0307	2.5980	0.0525	7.4751	0.1080	6.8147	0.0943	6.9077	0.1968
B52	0.9658	0.0132	1.3712	0.0383	3.5121	0.0752	2.2447	0.0552	0.6960	0.0641
B5 unsplit	0.1565	0.0053	0.1522	0.0128	0.1288	0.0146	0.1546	0.0146	0.1307	0.0278
B5 total	6.5438	0.0435	4.1214	0.0747	11.1160	0.1504	9.2141	0.1324	7.7344	0.2784
B44	13.4838	0.0465	7.0137	0.0847	5.6807	0.0948	9.9253	0.1121	11.8024	0.2511
B45	0.5771	0.0102	4.8069	0.0708	0.1816	0.0173	1.8812	0.0506	0.7603	0.0670
B12 unsplit	0.0788	0.0038	0.0280	0.0055	0.0049	0.0029	0.0193	0.0051	0.0654	0.0197
B12 total	14.1440	0.0474	11.8486	0.1072	5.8673	0.0963	11.8258	0.1210	12.6281	0.2584
B62	5.9117	0.0320	1.5267	0.0404	9.2249	0.1190	4.1825	0.0747	6.9421	0.1973
B63	0.4302	0.0088	1.8865	0.0448	0.4438	0.0270	0.8083	0.0333	0.3738	0.0471
B75	0.0104	0.0014	0.0226	0.0049	1.9673	0.0566	0.1101	0.0123	0.0356	0.0145
B76	0.0026	0.0007	0.0065	0.0026	0.0874	0.0120	0.0055	0.0028	0	
B77	0.0057	0.0010	0.0119	0.0036	0.0577	0.0098	0.0083	0.0034	0[c]	
B15 unsplit	0.1305	0.0049	0.0691	0.0086	0.4301	0.0266	0.1820	0.0158	0.0059	0.0059
B15 total	6.4910	0.0334	3.5232	0.0608	12.2112	0.1344	5.2967	0.0835	0.0715	0.0206
									7.4290	0.2035
B38	2.4413	0.0209	0.3323	0.0189	3.2818	0.0728	1.9652	0.0517	1.1017	0.0806
B39	1.9614	0.0188	1.2893	0.0371	2.0352	0.0576	6.3040	0.0909	4.5527	0.1615
B16 unsplit	0.0638	0.0034	0.0237	0.0051	0.0644	0.0103	0.1226	0.0130	0.0593	0.0188
B16 total	4.4667	0.0280	1.6453	0.0419	5.3814	0.0921	8.3917	0.1036	5.7137	0.1797
B57	3.5955	0.0252	5.6746	0.0766	2.5782	0.0647	2.1800	0.0544	2.7265	0.1260
B58	0.7152	0.0114	5.9546	0.0784	4.0189	0.0803	1.2481	0.0413	0.9398	0.0745
B17 unsplit	0.2845	0.0072	0.3248	0.0187	0.3751	0.0248	0.1446	0.0141	0.2674	0.0398
B17 total	4.5952	0.0284	11.9540	0.1076	6.9722	0.1041	3.5727	0.0691	3.9338	0.1503
B49	1.6452	0.0172	2.6286	0.0528	0.2440	0.0200	2.3353	0.0562	1.5462	0.0953
B50	1.0580	0.0138	0.8636	0.0304	0.4421	0.0270	1.8883	0.0507	0.7862	0.0681
B21 unsplit	0.0702	0.0036	0.0270	0.0054	0.0132	0.0047	0.0771	0.0103	0.0356	0.0145
B21 total	2.7733	0.0222	3.5192	0.0608	0.6993	0.0339	4.3007	0.0755	2.3680	0.1174
B54	0.0124	0.0015	0.0183	0.0044	2.6873	0.0660	0.0289	0.0063	0.0534	0.0178
B55	1.9046	0.0185	0.4895	0.0229	2.2444	0.0604	0.9515	0.0361	1.4054	0.0909
B56	0.5527	0.0100	0.2686	0.0170	0.8260	0.0368	0.3596	0.0222	0.3387	0.0448
B22 unsplit	0.1682	0.0055	0.0496	0.0073	0.2730	0.0212	0.0372	0.0071	0.1246	0.0272
B22 total	2.0852	0.0217	0.8261	0.0297	6.0307	0.0971	1.3771	0.0433	1.9221	0.1060

Antigen	CAU		AFR		ASI		LAT		NAT	
	Gf[a]	SE[b]	Gf	SE	Gf	SE	Gf	SE	Gf	SE
B60	5.2222	0.0302	1.5299	0.0404	8.3254	0.1135	2.2538	0.0553	5.7218	0.1801
B61	1.1916	0.0147	0.4709	0.0225	6.2072	0.0989	4.6691	0.0788	2.6023	0.1231
B40 unsplit	0.2696	0.0070	0.0388	0.0065	0.3205	0.0230	0.2473	0.0184	0.2271	0.0367
B40 total	6.6834	0.0338	2.0396	0.0465	14.8531	0.1462	7.1702	0.0963	8.5512	0.2168
BX	1.0922	0.0252	3.5258	0.0802	3.8749	0.0988	2.5266	0.0807	1.9867	0.1634

[a]Gene frequency. [b]Standard error. [c]The observed gene count was zero.

Table 5

Listing of CT genes*:

CT Identifier	Transcript / Transcript family	Family Members/CT Identifier (Synonyms)
CT1	MAGEA	MAGEA1/CT1.1, MAGEA2/CT1.2, MAGEA3/CT1.3, MAGEA4/CT1.4, MAGEA5/CT1.5, MAGEA6/CT1.6, MAGEA7/CT1.7, MAGEA8/CT1.8, MAGEA9/CT.9, MAGEA10/CT1.10, MAGEA11/CT1.11, MAGEA12/CT1.12
CT2	BAGE	BAGE/CT2.1, BAGE2/CT2.2, BAGE3/CT2.3, BAGE4/CT2.4, BAGE5/CT2.5
CT3	MAGEB	MAGEB1/CT3.1, MAGEB2/CT3.2, MAGEB5/CT3.3, MAGEB6/CT3.4
CT4	GAGE1	GAGE1/CT4.1, GAGE2/CT4.2, GAGE3/CT4.3, GAGE4/CT4.4, GAGE5/CT4.5, GAGE6/CT4.6, GAGE7/CT4.7, GAGE8/CT4.8
CT5	SSX	SSX1/CT5.1, SSX2/CT5.2a, SSX2/CT5.2b, SSX3/CT5.3, SSX4/CT5.4
CT6	NY-ESO-1	NY-ESO-1/CT6.1, LAGE-1a/CT6.2a, LAGE-1b/CT6.2b
CT7	MAGEC1	MAGEC1/CT7.1, MAGEC3/CT7.2
CT8	SYCP1	SYCP1/CT8

CT9	BRDT	BRDT/CT9
CT10	MAGEE1	MAGEE1/CT10
CT11	CTp11/SPANX	SPANXA1/CT11.1, SPANXB1/CT11.2, SPANXC/CT11.3, SPANXD/CT11.4
CT12	XAGE-1/GAGED	XAGE-1a/CT12.1a, XAGE-1b/CT12.1b, XAGE-1c/CT12.1c, XAGE-1d/CT12.1d, XAGE-2/CT12.2, XAGE-3a/CT12.3a, XAGE-3b/CT12.3b, XAGE-4/CT12.4
CT13	HAGE	HAGE/CT13
CT14	SAGE	SAGE/CT14
CT15	ADAM2	ADAM2/CT15
CT16	PAGE-5	PAGE-5/CT16.1, CT16.2
CT17	LIP1	LIP1/CT17
CT18	NA88	NA88/CT12
CT19	IL13RA1	IL13RA1/CT19
CT20	TSP50	TSP50/CT20
CT21	CTAGE-1	CTAGE-1/CT21.1, CTAGE-2/CT21.2
CT22	SPA17	SPA17/CT22
CT23	OY-TES-1	OY-TES-1/CT23
CT24	CSAGE	CSAGE/CT24.1, TRAG3/CT24.2
CT25	MMA1/DSCR8	MMA-1a/CT25.1a, MMA-1b/CT25.1b
CT26	CAGE	CAGE/CT26
CT27	BORIS	BORIS/CT27

CT28	HOM-TES-85	HOM-TES-85/CT28
CT29	AF15q14/ D40	D40/CT29
CT30	E2F-like/HCA661	HCA661/CT30
CT31	PLU-1	PLU-1/CT31
CT32	LDHC	LDHC/CT32
CT33	MORC	MORC/CT33
CT34	SGY-1	SGY-1/CT34
CT35	SPO11	SPO11/CT35
CT36	TPX1	TPX-1/CT36
CT37	NY-SAR-35	NY-SAR-35/CT37
CT38	FTHL17	FTHL17/CT38
CT39	NXF2	NXF2/CT39
CT40	TAF7L	TAF7L/CT40
CT41	TDRD1	TDRD1/CT41.1, NY-CO-45/CT41.2
CT42	TEX15	TEX15/CT42
CT43	FATE	FATE/CT43
CT44	TPTE	TPTE/CT44
---	PRAME	(MAPE, DAGE)

See Scanlan et al., "The cancer/testis genes: Review, standardization, and commentary," *Cancer Immunity*, Vol. 4, p. 1 (23 January 2004), which is incorporated herein by reference in its entirety.

[0130] The following discussion sets forth the present understanding or belief of the operation of aspects of the invention. However, it is not intended that this discussion limit the patent to any particular theory of operation not set forth in the claims.

[0131] Effective immune-mediated control of tumoral processes or microbial infections generally involves induction and expansion of antigen-specific T cells endowed with multiple capabilities such as migration, effector functions, and differentiation into memory cells. Induction of immune responses can be attempted by various methods and involves administration of antigens in different forms, with variable effect on the magnitude and quality of the immune response. One limiting factor in achieving a control of the immune response is targeting pAPC able to process and effectively present the resulting epitopes to specific T cells.

[0132] A solution to this problem is direct antigen delivery to secondary lymphoid organs, a microenvironment abundant in pAPC and T cells. The antigen can be delivered, for example, either as polypeptide or as an expressed antigen by any of a variety of vectors. The outcome in terms of magnitude and quality of immunity can be controlled by factors including, for example, the dosage, the formulation, the nature of the vector, and the molecular environment. Embodiments of the present invention can enhance control of the immune response. Control of the immune response includes the capability to induce different types of immune responses as needed, for example, from regulatory to pro-inflammatory responses. Preferred embodiments provide enhanced control of the magnitude and quality of responses to MHC class I-restricted epitopes which are of major interest for active immunotherapy.

[0133] Previous immunization methods displayed certain important limitations: first, very often, conclusions regarding the potency of vaccines were extrapolated from immunogenicity data generated from one or from a very limited panel of ultra sensitive read-out assays. Frequently, despite the inferred potency of a vaccination regimen, the clinical response was not significant or was at best modest. Secondly, subsequent to immunization, T regulatory cells, along with more conventional T effector cells, can be generated and/or expanded, and such cells can interfere with the function of the desired immune response. The importance of such mechanisms in active immunotherapy has been recognized only recently.

[0134] Intranodal administration of immunogens provides a basis for the control of the magnitude and profile of immune responses. The effective in vivo loading of pAPC accomplished as a result of such administration, enables a substantial magnitude of immunity, even by using an antigen in its most simple form—a peptide epitope—otherwise generally associated with poor pharmocokinetics. The quality of response can be further controlled via the nature of immunogens, vectors, and protocols of immunization. Such protocols can be applied for enhancing/modifying the response in chronic infections or tumoral processes.

[0135] Immunization has traditionally relied on repeated administration of antigen to augment the magnitude of the immune response. The use of DNA vaccines has resulted in high quality responses, but it has been difficult to obtain high magnitude responses using such vaccines, even with repeated booster doses. Both characteristics of the response, high quality and low magnitude, are likely due to the relatively low levels of epitope loading onto MHC achieved with these vectors. Instead it has become more common to boost such vaccines using antigen encoded in a live virus vector in order to achieve the high magnitude of response needed for clinical usefulness. However, the use of live vectors can entail several drawbacks including potential safety issues, decreasing effectiveness of later boosts due to a humoral response to the vector induced by the prior administrations, and the costs of creation and production. Thus, use of live vectors or DNA alone, although eliciting high quality responses, may result in a limited magnitude or sustainability of response.

[0136] Disclosed herein are embodiments that relate to protocols and to methods that, when applied to peptides, rendered them effective as immune therapeutic tools. Such methods circumvent the poor PK of peptides, and if applied in context of specific, and often more complex regimens, result in robust amplification and/or control of immune response. In preferred embodiments, direct administration of peptide into lymphoid organs results in unexpectedly strong amplification of immune responses, following a priming agent that induces a strong, moderate or even mild (at or below levels of detection by conventional techniques) immune response consisting of Tc1 cells. While preferred embodiments of the invention can employ intralymphatic administration of antigen at all stages of immunization, intralymphatic administration is the most preferred mode of administration for adjuvant-free peptide. Peptide amplification utilizing

intralymphatic administration can be applied to existing immune responses that may have been previously induced. Previous induction can occur by means of natural exposure to the antigen or by means of commonly used routes of administration, including without limitation subcutaneous, intradermal, intraperitoneal, intramuscular, and mucosal.

[0137] Also as shown herein, optimal initiation, resulting in subsequent expansion of specific T cells, can be better achieved by exposing the naive T cells to limited amounts of antigen (as can result from the often limited expression of plasmid-encoded antigen) in a rich co-stimulatory context (such as in a lymph node). That can result in activation of T cells carrying T cell receptors that recognize with high affinity the MHC - peptide complexes on antigen presenting cells and can result in generation of memory cells that are more reactive to subsequent stimulation. The beneficial co-stimulatory environment can be augmented or ensured through the use of immunopotentiating agents and thus intralymphatic administration, while advantageous, is not in all embodiments required for initiation of the immune response. In embodiments involving the use of epitopic peptide for induction/entrainment it is preferred that a relatively low dosage of peptide (as compared to an amplifying dose or to a MHC-saturating concentration) be used so that presentation is limited, especially if using direct intralymphatic administration. Such embodiments will generally involve inclusion of an immunopotentiator to achieve entrainment.

[0138] While the poor pharmacokinetics of free peptides has prevented their use in most routes of administration, direct administration into secondary lymphoid organs, particularly lymph nodes, has proven effective when the level of antigen is maintained more or less continuously by continuous infusion or frequent (for example, daily) injection. Such intranodal administration for the generation of CTL is taught in U.S. Patent Application Nos. 09/380,534, 09/776,232 (Pub. No. 20020007173 A1), now U.S. Patent No. 6,977,074, and __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.001CP2C1), filed on December 19, 2005), and in PCT Application No. PCTUS98/14289 (Pub. No. WO9902183A2), each entitled METHOD OF INDUCING A CTL RESPONSE, each of which is hereby incorporated by reference in its entirety. In some embodiments of the instant invention, intranodal administration of peptide was effective in amplifying a response initially induced with a plasmid DNA vaccine. Moreover, the cytokine profile was distinct, with plasmid DNA induction/peptide amplification generally resulting in

greater chemokine (chemoattractant cytokine) and lesser immunosuppressive cytokine production than either DNA/DNA or peptide/peptide protocols.

[0139] Thus, such DNA induction/peptide amplification protocols can improve the effectiveness of compositions, including therapeutic vaccines for cancer and chronic infections. Beneficial epitope selection principles for such immunotherapeutics are disclosed in U.S. Patent Application Nos. 09/560,465, 10/026,066 (Pub. No. 20030215425 A1), 10/005,905, filed November 7, 2001, 10/895,523 (Pub. No. 2005-0130920 A1), filed July 20, 2004, and 10/896,325 (Pub No. _____), filed July 20, 2004, all entitled EPITOPE SYNCHRONIZATION IN ANTIGEN PRESENTING CELLS; 09/561,074, now U.S. Patent No. 6,861,234, and 10/956,401 (Pub. No. 2005-0069982 A1), filed on October 1, 2004, both entitled METHOD OF EPITOPE DISCOVERY; 09/561,571, filed April 28, 2000, entitled EPITOPE CLUSTERS; 10/094,699 (Pub. No. 20030046714 A1), filed March 7, 2002, 11/073,347, (Pub. No. _____), filed June 30, 2005, each entitled ANTI-NEOVASCULATURE PREPARATIONS FOR CANCER; and 10/117,937 (Pub. No. 20030220239 A1), filed April 4, 2002, 11/067,159 (Pub. No. 2005-0221440A1), filed February 25, 2005, 10/067,064 (Pub. No. 2005-0142114 A1), filed February 25, 2005, and 10/657,022 (Publication No. 2004-0180354 A1), and PCT Application No. PCT/US2003/027706 (Pub. No. WO 04/022709 A2), each entitled EPITOPE SEQUENCES, and each of which is hereby incorporated by reference in its entirety. Aspects of the overall design of vaccine plasmids are disclosed in U.S. Patent Application Nos. 09/561,572, filed April 28, 2000, and 10/225,568 (Pub. No. 2003-0138808 A1), filed August 20, 2002, both entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS and U.S. Patent Application Nos. 10/292,413 (Pub. No.20030228634 A1), 10/777,053 (Pub. No. 2004-0132088 A1), filed on February 10, 2004, and 10/837,217 (Pub. No. _____), filed on April 30, 2004, all entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS AND METHODS FOR THEIR DESIGN; 10/225,568 (Pub No. 2003-0138808 A1), PCT Application No. PCT/US2003/026231 (Pub. No. WO 2004/018666) and U.S. Patent No. 6,709,844 and U.S. Patent Application No. 10/437,830 (Pub. No. 2003-0180949 A1), filed on May 13, 2003, each entitled AVOIDANCE OF UNDESIRABLE REPLICATION INTERMEDIATES IN PLASMID PROPAGATION, each of which is hereby incorporated by reference in its entirety. Specific antigenic combinations of

particular benefit in directing an immune response against particular cancers are disclosed in provisional U.S. Provisional Application No. 60/479,554, filed on June 17, 2003, U.S. Patent Application No. 10/871,708 (Pub. No. 2005-0118186 A1), filed on June 17, 2004, PCT Patent Application No. PCT/US2004/019571 (Pub. No. WO 2004/112825), U.S. Provisional Application No. 60/640,598, filed December 29, 2005, and U.S. Patent Application No __/___,___ (Pub. No. _____), (Attorney Docket No. MANNK.049A), filed on the same date as this application, all entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN VACCINES FOR VARIOUS TYPES OF CANCERS, each of which is also hereby incorporated by reference in its entirety. The use and advantages of intralymphatic administration of BRMs are disclosed in provisional U.S. Patent Application No. 60/640,727, filed December 29, 2005 and U.S. Patent Application No. __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.046A), filed on the same date as this application, both entitled Methods to trigger, maintain and manipulate immune responses by targeted administration of biological response modifiers into lymphoid organs, each of which is incorporated herein by reference in it entirety. Additional methodology, compositions, peptides, and peptide analogues are disclosed in U.S. Patent Application No. 09/999,186, filed November 7, 2001, entitled METHODS OF COMMERCIALIZING AN ANTIGEN; and U.S. Provisional U.S. Patent Application No. 60/640,821, filed December 29, 2005 and Application No. __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.048A), filed on the same date as this application, both entitled METHODS TO BYPASS CD4+ CELLS IN THE INDUCTION OF AN IMMUNE RESPONSE, each of which is hereby incorporated by reference in its entirety.

[0140] Other relevant disclosures are present in U.S. Patent Application No. 11/156,369 (Pub. No. _____), and U.S. Provisional Patent Application No. 60/691,889, both filed on June 17, 2005, both entitled EPITOPE ANALOGS, and each of which is incorporated herein by reference in its entirety. Also relevant are, U.S. Provisional Patent App. Nos. 60/691,579, filed on June 17, 2005, entitled METHODS AND COMPOSITIONS TO ELICIT MULTIVALENT IMMUNE RESPONSES AGAINST DOMINANT AND SUBDOMINANT EPITOPES, EXPRESSED ON CANCER CELLS AND TUMOR STROMA, and 60/691,581, filed on June 17, 2005, entitled

MULTIVALENT ENTRAIN-AND-AMPLIFY IMMUNOTHERAPEUTICS FOR CARCINOMA, each of which is incorporated herein by reference in its entirety.

[0141] Surprisingly, repeated intranodal injection of peptide according to a traditional prime-boost schedule resulted in reducing the magnitude of the cytolytic response compared to response observed after initial dosing alone. Examination of the immune response profile shows this to be the result of the induction of immune regulation (suppression) rather than unresponsiveness. This is in contrast to induce-and-amplify protocols encompassing DNA-encoded immunogens, typically plasmids. Direct loading of pAPC by intranodal injection of antigen generally diminishes or obviates the need for adjuvants that are commonly used to correct the pharmacokinetics of antigens delivered via other parenteral routes. The absence of such adjuvants, which are generally proinflammatory, can thus facilitate the induction of a different (i.e., regulatory or tolerogenic) immune response profile than has previously been observed with peptide immunization. Since the response, as shown in the examples below, is measured in secondary lymphoid organs remote from the initial injection site, such results support the use methods and compositions according to of the embodiments of the invention for modifying (suppressing) ongoing inflammatory reactions. This approach can be useful even with inflammatory disorders that have a class II MHC-restricted etiology, either by targeting the same antigen, or any suitable antigen associated with the site of inflammation, and relying on bystander effects mediated by the immunosuppressive cytokines.

[0142] Despite the fact that repeated peptide administration results in gradually decreasing cytolytic immune response, induction with an agent such as non-replicating recombinant DNA (plasmid) had a substantial impact on the subsequent doses, enabling robust amplification of immunity to epitopes expressed by the recombinant DNA and peptide, and entraining its cytolytic nature. In fact, when single or multiple administrations of recombinant DNA vector or peptide separately achieved no or modest immune responses, inducing with DNA and amplifying with peptide achieved substantially higher responses, both as a rate of responders and as a magnitude of response. In the examples shown, the rate of response was at least doubled and the magnitude of response (mean and median) was at least tripled by using a recombinant DNA induction / peptide –amplification protocol. Thus, preferred protocols result in induction of immunity (Tc1 immunity) that is able to deal with antigenic cells in vivo, within lymphoid and non-lymphoid organs. One

limiting factor in most cancer immunotherapy is the limited susceptibility of tumor cells to immune-mediated attack, possibly due to reduced MHC/peptide presentation. In preferred embodiments, robust expansion of immunity is achieved by DNA induction / peptide amplification, with a magnitude that generally equals or exceeds the immune response generally observed subsequent to infection with virulent microbes. This elevated magnitude can help to compensate for poor MHC/peptide presentation and does result in clearance of human tumor cells as shown in specialized pre-clinical models such as, for example, HLA transgenic mice.

[0143] Such induce-and-amplify protocols involving specific sequences of recombinant DNA entrainment doses, followed by peptide boosts administered to lymphoid organs, are thus useful for the purpose of induction, amplification and maintenance of strong T cell responses, for example for prophylaxis or therapy of infectious or neoplastic diseases. Such diseases can be carcinomas (e.g., renal, ovarian, breast, lung, colorectal, prostate, head-and-neck, bladder, uterine, skin), melanoma, tumors of various origin and in general tumors that express defined or definable tumor associated antigens, such as oncofetal (e.g., CEA, CA 19-9, CA 125, CRD-BP, Das-1, 5T4, TAG-72, and the like), tissue differentiation (e.g., Melan-A, tyrosinase, gp100, PSA, PSMA, and the like), or cancer-testis antigens (e.g., PRAME, MAGE, LAGE, SSX2, NY-ESO-1, and the like; see Table 5). Cancer-testis genes and their relevance for cancer treatment are reviewed in Scanlon et al., Cancer Immunity 4:1-15, 2004, which is hereby incorporated by reference in its entirety). Antigens associated with tumor neovasculature (e.g., PSMA, VEGFR2, Tie-2) are also useful in connection with cancerous diseases, as is disclosed in U.S. Patent Application Nos. 10/094,699 (Pub. No. 20030046714 A1) and 11/073,347 (Pub. No. _____), filed on June 30, 2005, entitled ANTI-NEOVASCULATURE PREPARATIONS FOR CANCER, each of which is hereby incorporated by reference in its entirety. The methods and compositions can be used to target various organisms and disease conditions. For example, the target organisms can include bacteria, viruses, protozoa, fungi, and the like. Target diseases can include those caused by prions, for example. Exemplary diseases, organisms and antigens and epitopes associated with target organisms, cells and diseases are described in U.S. Application No. 09/776,232 (Pub. No. 20020007173 A1), now U.S. Patent No. 6,977,074, which is incorporated herein by reference in its entirety. Among the infectious diseases that can be addressed are those

caused by agents that tend to establish chronic infections (HIV, herpes simplex virus, CMV, Hepatitis B and C viruses, papilloma virus and the like) and/or those that are connected with acute infections (for example, influenza virus, measles, RSV, Ebola virus). Of interest are viruses that have oncogenic potential – from the perspective of prophylaxis or therapy – such as papilloma virus, Epstein Barr virus and HTLV-1. All these infectious agents have defined or definable antigens that can be used as basis for designing compositions such as peptide epitopes.

[0144] Preferred applications of such methods (See, e.g., Figure 19) include injection or infusion into one or more lymph nodes, starting with a number (e.g., 1 to 10, or more, 2 to 8, 3 to 6, preferred about 4 or 5) of administrations of recombinant DNA (dose range of 0.001 - 10 mg/kg, preferred 0.005-5mg/kg) followed by one or more (preferred about 2) administrations of peptide, preferably in an immunologically inert vehicle or formulation (dose range of 1 ng/kg - 10 mg/kg, preferred 0.005-5 mg/kg). Because dose does not necessarily scale linearly with the size of the subject, doses for humans can tend toward the lower, and doses for mice can tend toward the higher, portions of these ranges. The preferred concentration of plasmid and peptide upon injection is generally about 0.1μg/ml-10 mg/ml, and the most preferred concentration is about 1mg/ml, generally irrespective of the size or species of the subject. However, particularly potent peptides can have optimum concentrations toward the low end of this range, for example between 1 and 100 μg/ml. When peptide only protocols are used to promote tolerance doses toward the higher end of these ranges are generally preferred (e.g., 0.5-10 mg/ml). This sequence can be repeated as long as necessary to maintain a strong immune response in vivo. Moreover, the time between the last entraining dose of DNA and the first amplifying dose of peptide is not critical. Preferably it is about 7 days or more, and can exceed several months. The multiplicity of injections of the DNA and/or the peptide can be reduced by substituting infusions lasting several days (preferred 2-7 days). It can be advantageous to initiate the infusion with a bolus of material similar to what might be given as an injection, followed by a slow infusion (24-12000 μl/day to deliver about 25-2500 μg/day for DNA, 0.1 - 10,000 μg/day for peptide). This can be accomplished manually or through the use of a programmable pump, such as an insulin pump. Such pumps are known in the art and enable periodic spikes and other dosage profiles, which can be desirable in some embodiments.

[0145] The invention has generally been described a single cycle of immunization comprising administration of one or initiating doses followed the administration of one or more amplifying doses. Further embodiments of the invention entail repeated cycles of immunization. Such repeated cycles can be used to further augment the magnitude of the response. Also, when a multivalent response is sought not all individuals will necessarily achieve a substantial response to each of the targeted antigens as the result of a single cycle of immunization. Cycles of immunization can be repeated until a particular individual achieves an adequate response to each targeted antigen. The individual cycles of immunization can also be modified to achieve a more balanced response by adjusting the order, timing, or number of doses of each individual component that are given. Multiple cycles of immunization can also be used to maintain the response over time, for example to sustain an active effector phase of the response to be substantially co-extensive in time with, and as may be advantageous for, the treatment of a disease or other medical condition.

[0146] It should be noted that while this method successfully makes use of peptide, without conjugation to proteins, addition of adjuvant, etc., in the amplification step, the absence of such components is not required. Thus, conjugated peptide, adjuvants, immunopotentiators, etc. can be used in embodiments. More complex compositions of peptide administered to the lymph node, or with an ability to home to the lymphatic system, including peptide-pulsed dendritic cells, suspensions such as liposome formulations, aggregates, emulsions, microparticles, nanocrystals, composed of or encompassing peptide epitopes or antigen in various forms, can be substituted for free peptide in the method. Conversely, peptide boost by intranodal administration can follow priming via any means / or route that achieves induction of T memory cells even at modest levels.

[0147] In order to reduce occurrence of resistance due to mosaicism of antigen expression, or to mutation or loss of the antigen, it is advantageous to immunize to multiple, preferably about 2-4, antigens concomitantly. Any combination of antigens can be used. A profile of the antigen expression of a particular tumor can be used to determine which antigen or combination of antigens to use. Exemplary methodology is found in U.S. Provisional Application No. 60/580,969, filed on June 17, 2004, U.S. Patent Application No. 11/155,288 filed June 17, 2005, and U.S. Patent Application No. __/___,___ (Pub. No. _____) (Attorney Docket No. MANNK.050CP1) filed on even date with the instant

application, all entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOTISTICS FOR VARIOUS TYPES OF CANCERS; and each of which is hereby incorporated by reference in its entirety. Specific combinations of antigens particularly suitable to treatment of selected cancers are disclosed in U.S. Provisional Patent Applications No. 60/479,554 and U.S. Patent Applications No. 10/871,708 (Pub. No. 2005-0118186 A1) and PCT Application No. PCT/US2004/019571, cited and incorporated by reference above. To trigger immune responses to a plurality of antigens or to epitopes from a single antigen, these methods can be used to deliver multiple immunogenic entities, either individually or as mixtures. When immunogens are delivered individually, it is preferred that the different entities be administered to different lymph nodes or to the same lymph node(s) at different times, or to the same lymph node(s) at the same time. This can be particularly relevant to the delivery of peptides for which a single formulation providing solubility and stability to all component peptides can be difficult to devise. A single nucleic acid molecule can encode multiple immunogens. Alternatively, multiple nucleic acid molecules encoding one or a subset of all the component immunogens for the plurality of antigens can be mixed together so long as the desired dose can be provided without necessitating such a high concentration of nucleic acid that viscosity becomes problematic.

[0148] In preferred embodiments the method calls for direct administration to the lymphatic system. In preferred embodiments this is to a lymph node. Afferent lymph vessels are similarly preferred. Choice of lymph node is not critical. Inguinal lymph nodes are preferred for their size and accessibility, but axillary and cervical nodes and tonsils can be similarly advantageous. Administration to a single lymph node can be sufficient to induce or amplify an immune response. Administration to multiple nodes can increase the reliability and magnitude of the response. For embodiments promoting a multivalent response and in which multiple amplifying peptides are therefor used, it can be preferable that only a single peptide be administered to any particular lymph node on any particular occasion. Thus one peptide can be administered to the right inguinal lymph node and a second peptide to the left inguinal lymph node at the same time, for example. Additional peptides can be administered to other lymph nodes even if they were not sites of induction, as it is not essential that initiating and amplifying doses be administered to the same site, due to migration of T lymphocytes. Alternatively any additional peptides can be administered a few days later, for example, to the same lymph nodes used for the previously

administered amplifying peptides since the time interval between induction and amplification generally is not a crucial parameter, although in preferred embodiments the time interval can be greater than about a week. Segregation of administration of amplifying peptides is generally of less importance if their MHC-binding affinities are similar, but can grow in importance as the affinities become more disparate. Incompatible formulations of various peptides can also make segregated administration preferable.

[0149] Patients that can benefit from such methods of immunization can be recruited using methods to define their MHC protein expression profile and general level of immune responsiveness. In addition, their level of immunity can be monitored using standard techniques in conjunction with access to peripheral blood. Finally, treatment protocols can be adjusted based on the responsiveness to induction or amplification phases and variation in antigen expression. For example, repeated entrainment doses preferably can be administered until a detectable response is obtained, and then administering the amplifying peptide dose(s), rather than amplifying after some set number of entrainment doses. Similarly, scheduled amplifying or maintenance doses of peptide can be discontinued if their effectiveness wanes, antigen-specific regulatory T cell numbers rise, or some other evidence of tolerization is observed, and further entrainment can be administered before resuming amplification with the peptide. The integration of diagnostic techniques to assess and monitor immune responsiveness with methods of immunization is discussed more fully in Provisional U.S. Patent Application No. 60/580,964, which was filed on June 17, 2004 and U.S. Patent Application No. 11/155,928 (Pub. No. _____), filed June 17, 2005, both entitled IMPROVED EFFICACY OF ACTIVE IMMUNOTHERAPY BY INTEGRATING DIAGNOSTIC WITH THERAPEUTIC METHODS, each of which is hereby incorporated by reference in its entirety.

[0150] Practice of many of the methodological embodiments of the invention involves use of at least two different compositions and, especially when there is more than a single target antigen, can involve several compositions to be administered together and/or at different times. Thus embodiments of the invention include sets and subsets of immunogenic compositions and individual doses thereof. Multivalency can be achieved using compositions comprising multivalent immunogens, combinations of monovalent immunogens, coordinated use of compositions comprising one or more monovalent

immunogens or various combinations thereof. Multiple compositions, manufactured for use in a particular treatment regimen or protocol according to such methods, define an immunotherapeutic product. In some embodiments all or a subset of the compositions of the product are packaged together in a kit. In some instances the inducing and amplifying compositions targeting a single epitope, or set of epitopes, can be packaged together. In other instances multiple inducing compositions can be assembled in one kit and the corresponding amplifying compositions assembled in another kit. Alternatively compositions may be packaged and sold individually along with instructions, in printed form or on machine-readable media, describing how they can be used in conjunction with each other to achieve the beneficial results of the methods of the invention. Further variations will be apparent to one of skill in the art. The use of various packaging schemes comprising less than all of the compositions that might be employed in a particular protocol or regimen facilitates the personalization of the treatment, for example based on tumor antigen expression, or observed response to the immunotherapeutic or its various components, as described in_ U.S. Provisional Application No. 60/580,969, filed on June 17, 2004, U.S. Patent Application No. 11/155,288 (Pub. No. _____). filed June 17, 2005, and U.S. Patent Application No. __/___,___ (Attorney Docket No. MANNK.050CP1) filed 12/29/05, all entitled COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOTISTICS FOR VARIOUS TYPES OF CANCERS; and Provisional U.S. Patent Application No. 60/580,964, and U.S. Patent Application No. 11/155,928 (Pub. No. _____), both entitled IMPROVED EFFICACY OF ACTIVE IMMUNOTHERAPY BY INTEGRATING DIAGNOSTIC WITH THERAPEUTIC METHODS, each of which is incorporated by reference in its entirety above.

[0151] In some embodiments, the numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term "about." Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by a particular embodiment. In some embodiments, the numerical parameters should be construed in light

of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable. The numerical values presented in some embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0152] In some embodiments, the terms "a" and "an" and "the" and similar referents used in the context of describing a particular embodiment of the invention (especially in the context of certain of the following claims) may be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0153] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0154] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans may employ such variations as appropriate, and the invention may be practiced otherwise than specifically

described herein. Accordingly, many embodiments of this invention include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0155] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above cited references and printed publications are herein individually incorporated by reference in their entirety.

[0156] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed may be within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0157] The following examples are for illustrative purposes only and are not intended to limit the scope of the invention or its various embodiments in any way.

Example 1. Highly effective induction of immune responses by intra-lymphatic immunization.

[0158] Mice carrying a transgene expressing a chimeric single-chain version of a human MHC class I (A*0201, designated "HHD"; see Pascolo et al. J. Exp. Med. 185(12):2043-51, 1997, which is hereby incorporated herein by reference in its entirety) were immunized by intranodal administration as follows. Five groups of mice (n=3) were immunized with plasmid expressing Melan-A 26-35 A27L analogue (pSEM) for induction and amplified one week later, by employing different injection routes: subcutaneous (sc), intramuscular (im) and intralymphatic (in, using direct inoculation into the inguinal lymph nodes). The schedule of immunization and dosage is shown in Figure 1A. One week after the amplification, the mice were sacrificed; the splenocytes were prepared and stained using tagged anti-CD8 mAbs and tetramers recognizing Melan-A 26-35 -specific T cell receptors. Representative data are shown in Figure 1B: while subcutaneous and intramuscular administration achieved frequencies of tetramer+CD8+ T cells around or less than 1%,

intralymphatic administration of plasmid achieved a frequency of more than 6%. In addition, splenocytes were stimulated ex vivo with Melan-A peptide and tested against 51Cr-labeled target cells (T2 cells) at various E:T ratios (Figure 1C). The splenocytes from animals immunized by intralymph node injection showed the highest level of in vitro lysis at various E:T ratios, using this standard cytotoxicity assay.

Example 2. Effects of the order in which different forms of immunogen are administered.

[0159] HHD mice were immunized by intranodal administration of plasmid (pSEM) or peptide (Mel A; ELAGIGILTV; SEQ ID NO:1) in various sequences. The immunogenic polypeptide encoded by pSEM is disclosed in U.S. Patent application 10/292,413 (Pub. No. 20030228634 A1) entitled Expression Vectors Encoding Epitopes of Target-Associated Antigens and Methods for their Design incorporated herein by reference in its entirety above.

[0160] The protocol of immunization (Figure 2) comprised:

i) Induction Phase/Inducing doses: bilateral injection into the inguinal lymph nodes of 25 μl (microliters) of sterile saline containing either 25 μg (micrograms) of plasmid or 50 μg (micrograms) of peptide, at day 0 and day 4.

ii) Amplifying doses: as described above in Example 1 and initiated at 2 weeks after the completion of the induction phase.

[0161] The immune response was measured by standard techniques, after the isolation of splenocytes and in vitro stimulation with cognate peptide in the presence of pAPC. It is preferable that the profile of immune response be delineated by taking into account results stemming from multiple assays, facilitating assessment of various effector and regulatory functions and providing a more global view of the response. Consideration can be given to the type of assay used and not merely their number; for example, two assays for different proinflammatory cytokines is not as informative as one plus an assay for a chemokine or an immunosuppresive cytokine.

Example 3. ELISPOT analysis of mice immunized as described in Example 2.

[0162] ELISPOT analysis measures the frequency of cytokine-producing, peptide-specific, T cells. Figure 3 presents representative examples in duplicates; and

Figure 4 presents a summary of data expressed individually as number of cytokine producing cells / 106 responder cells. The results show that, in contrast to mice immunized with peptide, plasmid-immunized or plasmid-entrained / peptide-amplified mice developed elevated frequencies of IFN-γ (gamma)-producing T cells recognizing the Melan-A peptide. Four out of four mice, entrained with plasmid and amplified with peptide, displayed frequencies in excess of 1/2000. In contrast, two out of four mice immunized throughout the protocol with plasmid, displayed frequencies in excess of 1/2000. None of the mice using only peptide as an immunogen mounted elevated response consisting in IFN-γ-producing T cells. Indeed, repeated administration of peptide diminished the frequency of such cells, in sharp contrast to peptide administered after entrainment with plasmid.

Example 4. Analysis of cytolytic activity of mice immunized as described in Example 2.

[0163] Pooled splenocytes were prepared (spleens harvested, minced, red blood cells lysed) from each group and incubated with LPS-stimulated, Melan-A peptide–coated syngeneic pAPC for 7 days, in the presence of rIL-2. The cells were washed and incubated at different ratios with 51Cr-tagged T2 target cells pulsed with Melan-A peptide (ELA), for 4 hours. The radioactivity released in the supernatant was measured using a γ (gamma)-counter. The response was quantified as % lysis = (sample signal – background) / (maximal signal – background) x 100, where background represents radioactivity released by target cells alone when incubated in assay medium, and the maximal signal is the radioactivity released by target cells lysed with detergent. Figure 5 illustrates the results of the above-described cytotoxicity assay. The levels of cytolytic activity achieved, after in vitro stimulation with peptide, was much greater for those groups that had received DNA as the inducing dose in vivo than those that had received peptide as the inducing dose. Consistent with the ELISPOT data above, induction of an immune response with a DNA composition led to stable, amplifiable effector function, whereas immunization using only peptide resulted in a lesser response, the magnitude of which further diminished upon repeated administration.

Example 5. Cross-reactivity

[0164] Splenocytes were prepared and used as above in Example 4 against target cells coated with three different peptides: the Melan-A analogue immunogen and

those representing the human and murine epitopes corresponding to it. As shown in Figure 6, similar cytolytic activity was observed on all three targets, demonstrating cross-reactivity of the response to the natural sequences.

Example 6. Repeated administration of peptide to the lymph nodes induces immune deviation and regulatory T cells.

[0165] The cytokine profile of specific T cells generated by the immunization procedures described above (and in figure 2), was assessed by ELISA or Luminex®. (Luminex® analysis is a method to measure cytokine produced by T cells in culture in a multiplex fashion.) Seven-day supernatants of mixed lymphocyte cultures generated as described above were used for measuring the following biological response modifiers: MIP-1α, RANTES and TGF-β (capture ELISA, using plates coated with anti-cytokine antibody and specific reagents such as biotin-tagged antibody, streptavidin-horse radish peroxidase and colorimetric substrate; R&D Systems). The other cytokines were measured by Luminex®, using the T1/T2 and the T inflammatory kits provided by specialized manufacturer (BD Pharmingen).

[0166] The data in Figure 7A compare the three different immunization protocols and show an unexpected effect of the protocol on the profile of immune response: whereas plasmid entrainment enabled the induction of T cells that secrete pro-inflammatory cytokines, repeated peptide administration resulted in generation of regulatory or immune suppressor cytokines such as IL-10, TGF-beta and IL-5. It should be appreciated that the immunization schedule used for the peptide-only protocol provided periodic rather than continuous presence of the epitope within the lymphatic system that instead prolongs the effector phase of the response. Finally, plasmid entrainment followed by peptide amplification resulted in production of elevated amounts of the T cell chemokines MIP-1α and RANTES. T cell chemokines such as MIP-1α and RANTES can play an important role in regulating the trafficking to tumors or sites of infection. During immune surveillance, T cells specific for target-associated antigens may encounter cognate ligand, proliferate and produce mediators including chemokines. These can amplify the recruitment of T cells at the site where the antigen is being recognized, permitting a more potent response. The data were generated from supernatants obtained from bulk cultures (means + SE of duplicates, two independent measurements).

[0167] Cells were retrieved from the lung interstitial tissue and spleen by standard methods and stained with antibodies against CD8, CD62L and CD45RB, along with tetramer agent identifying Melan-A-specific T cells. The data in Figure 7B represent gated populations of CD8+ Tetramer + T cells (y axis CD45RB and x axis CD62L).

[0168] Together, the results demonstrate immune deviation in animals injected with peptide only (reduced IFN-gamma, TNF-alpha production, increased IL-10, TGF-beta and IL-5, robust induction of CD62L- CD45Rblow CD8+ tetramer+ regulatory cells).

Example 7. Highly effective induction of immune responses by alternating non-replicating plasmid (entrainment) with peptide (amplification) administered to the lymph node.

[0169] Three groups of HHD mice, transgenic for the human MHC class I HLA.A2 gene, were immunized by intralymphatic administration against the Melan-A tumor associated antigen. Animals were primed (induced) by direct inoculation into the inguinal lymph nodes with either pSEM plasmid (25μg/lymph node) or ELA peptide (ELAGIGILTV (SEQ ID NO:1), Melan A 26-35 A27L analogue) (25μg/lymph node) followed by a second injection three days later. After ten days, the mice were boosted with pSEM or ELA in the same fashion followed by a final boost three days later to amplify the response (see Figure 11A for a similar immunization schedule), resulting in the following induce & amplify combinations: pSEM + pSEM, pSEM + ELA, and ELA + ELA (12 mice per group). Ten days later, the immune response was monitored using a Melan-A specific tetramer reagent (HLA-A*0201 MART1 (ELAGIGILTV (SEQ ID NO:1))-PE, Beckman Coulter). Individual mice were bled via the retro-orbital sinus vein and PBMC were isolated using density centrifugation (Lympholyte Mammal, Cedarlane Labs) at 2000rpm for 25 minutes. PBMC were co-stained with a mouse specific antibody to CD8 (BD Biosciences) and the Melan-A tetramer reagent and specific percentages were determined by flow cytometery using a FACS caliber flow cytometer (BD). The percentages of Melan-A specific CD8+ cells, generated by the different prime/boost combinations, are shown in Figures 8A and 8B. The plasmid-prime / peptide-boost group (pSEM + ELA) elicited a robust immune response with an average tetramer percentage of 4.6 between all the animals. Responder mice were defined to have tetramer percentages of 2 or greater which represented a value equivalent to the average of the unimmunized control group plus 3 times the standard deviation (SE). Such values are considered very robust responses in the

art and can usually be achieved only by using replicating vectors. The pSEM + ELA immunization group contained 10 out of 12 mice that were found to be responders and this represented a statistically significant difference as compared to the control group (p (Fisher) = 0.036). The other two immunization series, pSEM + pSEM and ELA + ELA, yielded 6 out of 12 responders but had p values greater than 0.05 rendering them less statistically significant. To measure the immunity of these mice, animals were challenged with peptide coated target cells in vivo. Splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL ELA peptide for 2 hours. These cells were then stained with CFSEhi fluorescence (4.0μM for 15 minutes) and intravenously co-injected into immunized mice with an equal ratio of control splenocytes that had not been incubated with peptide, stained with CFSElo fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing spleen, lymph node, PBMC, and lung from challenged animals (5 mice per group) and measuring CFSE fluorescence by flow cytometry. The results are shown in Figure 8C. In the pSEM + ELA prime/boost group, 4 out of 5 mice demonstrated a robust immune response and successfully cleared roughly 50% of the targets in each of the tissues tested. Representative histograms for each experimental groups are showed as well (PBMC).

Example 8. Peptide boost effectively reactivates the immune memory cells in animals induced with DNA and rested until tetramer levels were close to baseline.

[0170] Melan-A tetramer levels were measured in mice (5 mice per group) following immunization, as described in Figure 9A. By 5 weeks after completion of the immunization schedule, the tetramer levels had returned close to baseline. The animals were boosted at 6 weeks with ELA peptide to determine if immune responses could be restored. Animals receiving prior immunizations of pSEM plasmid (DNA/DNA, Figure 9C) demonstrated an unprecedented expansion of Melan-A specific CD8+ T cells following the ELA amplification, with levels in the range of greater than 10%. On the other hand, animals receiving prior injections of ELA peptide (Figure 9A) derived little benefit from the ELA boost as indicated by the lower frequency of tetramer staining cells. Mice that received DNA followed by peptide as the initial immunization exhibited a significant, but intermediate, expansion upon receiving the peptide amplification, as compared to the other

groups. (Figure 9B). These results clearly demonstrate a strong rationale for a DNA/DNA-entrainment and peptide-amplification immunization strategy.

Example 9. Optimization of immunization to achieve high frequencies of specific T cells in lymphoid and non-lymphoid organs.

[0171] As described in Figure 9A-C, mice that were subjected to an entraining immunization with a series of two clusters of plasmid injections followed by amplification with peptide yielded a potent immune response. Further evidence for this is shown in Figures 10A-C which illustrate the tetramer levels prior to (Figure 10A) and following peptide administration (Figure 10B). Tetramer levels in individual mice can be clearly seen and represent up to 30% of the total CD8+ population of T cells in mice receiving the DNA/DNA/Peptide immunization protocol. These results are summarized in the graph in Figure 10C. In addition, high tetramer levels are clearly evident in blood, lymph node, spleen, and lung of animals receiving this refined immunization protocol (Figure 10D).

[0172] Multiple further experiments have been carried out to characterize the phenotype of CTL generated by this protocol. The immune profile initiated in such conditions was imprinted, since peptide boost resulted in substantial, expansion of a CD43+, CD44+, CD69+, CD62L-, CD45RBdim, peptide-MHC class I-specific T cell population. These specific T cells colonized non-lymphoid organs and, upon additional specific stimulation, rapidly acquired the expression of CD107α and IFN-γ, in a fashion dependent on the density of stimulating peptide complexes.

Example 10. A precise administration sequence of plasmid and peptide immunogen determines the magnitude of immune response.

[0173] Six groups of mice (n=4) were immunized with plasmid expressing Melan-A 26-35 A27L analogue (pSEM) or Melan-A peptide using priming and amplification by direct inoculation into the inguinal lymph nodes. The schedule of immunization is shown in Figure 11A (doses of 50μg of plasmid or peptide / lymph node, bilaterally). Two groups of mice were initiated using plasmid and amplified with plasmid or peptide. Conversely, two groups of mice were initiated with peptide and amplified with peptide or plasmid. Finally, two groups of control mice were initiated with either peptide or plasmid but not amplified. At four weeks after the last inoculation, the spleens were

harvested and splenocyte suspensions prepared, pooled and stimulated with Melan-A peptide in ELISPOT plates coated with anti-IFN-γ antibody. At 48 hours after incubation, the assay was developed and the frequency of cytokine-producing T cells that recognized Melan-A was automatically counted. The data were represented in Fig 5B as frequency of specific T cells / 1 million responder cells (mean of triplicates + SD). The data showed that reversing the order of initiating and amplifying doses of plasmid and peptide has a substantial effect on the overall magnitude of the response: while plasmid entrainment followed by peptide amplification resulted in the highest response, initiating doses of peptide followed by plasmid amplification generated a significantly weaker response, similar to repeated administration of peptide.

Example 11. Correlation of immune responses with the protocol of immunization and *in vivo* efficacy--manifested by clearing of target cells within lymphoid and non-lymphoid organs.

[0174] To evaluate the immune response obtained by the entrain-and-amplify protocol, 4 groups of animals (n=7) were challenged with Melan-A coated target cells in vivo. Splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL ELA peptide for 2 hours. These cells were then stained with CFSEhi fluorescence (4.0μM for 15 minutes) and intravenously co-injected into immunized mice with an equal ratio of control splenocytes stained with CFSElo fluorescence (0.4μM). Eighteen hours later the specific elimination of target cells was measured by removing spleen, lymph node, PBMC, and lung from challenged animals and measuring CFSE fluorescence by flow cytometry. Figures 12A and 12B show CFSE histogram plots from tissues of unimmunized control animals or animals receiving an immunization protocol of peptide/peptide, DNA/peptide, or DNA/DNA (two representative mice are shown from each group). The DNA-entrain/peptide-amplify group demonstrated high levels of specific killing of target cells in lymphoid as well as non-lymphoid organs (Figure 12C) and represented the only immunization protocol that demonstrated a specific correlation with tetramer levels (Figure 12D, r2 = 0.81 or higher for all tissues tested).

Example 12. Clearance of human tumor cells in animals immunized by the refined entrain- and -amplify protocol.

[0175] Immunity to the Melan-A antigen was further tested by challenging mice with human melanoma tumor cells following immunization with the refined protocol. Figure 13A shows the refined immunization strategy employed for the 3 groups tested. Immunized mice received two intravenous injections of human target cells, 624.38 HLA.A2+, labeled with CFSEhi fluorescence mixed with an equal ratio of 624.28 HLA.A2- control cells labeled with CFSElo as illustrated in Figure 13B. Fourteen hours later, the mice were sacrificed and the lungs (the organ in which the human targets accumulate) were analyzed for the specific lysis of target cells by flow cytometry. Figure 13C shows representative CFSE histogram plots derived from a mouse from each group. DNA-entrainment followed by a peptide-amplification clearly immunized the mice against the human tumor cells as demonstrated by nearly 80% specific killing of the targets in the lung. The longer series of DNA-entrainment injections also led to a further increased frequency of CD8+ cells reactive with the Melan-A tetramer.

Example 13. DNA-entraining, peptide-amplification strategy results in robust immunity against an SSX2-derived epitope, KASEKIFYV ($SSX2_{41-49}$).

[0176] Animals immunized against the SSX2 tumor associated antigen using the immunization schedule defined in Figure 14A, demonstrated a robust immune response. Figure 14B shows representative tetramer staining of mice primed (entrained) with the pCBP plasmid and boosted (amplified) with either the SSX241-49 K41F or K41Y peptide analogue. These analogues are cross-reactive with T cells specific for the SSX241-49 epitope. These examples illustrate that the entrain-and-amplify protocol can elicit a SSX2 antigen specificity that approaches 80% of the available CD8 T cells. The pCBP plasmid and principles of its design are disclosed in US Patent Application No. 10/292,413 (Pub. No. 20030228634 A1) entitled Expression Vectors Encoding Epitopes of Target-Associated Antigens and Methods for their Design, which is hereby incorporated by reference in its entirety. Additional methodology, compositions, peptides, and peptide analogues are disclosed in U.S. Provisional Application No 60/581,001, filed on June 17, 2004, and U.S. Application No. 11/156,253, filed June 17, 2005, both entitled SSX-2 PEPTIDE ANALOGS; each of which is incorporated herein by reference in its entirety. Further

methodology, compositions, peptides, and peptide analogues are disclosed in U.S. Provisional Application No. 60/580,962, filed on June 17, 2004, and U.S. Application No. 11/155,929, filed June 17, 2005, each entitled NY-ESO PEPTIDE ANALOGS; and each of which is incorporated herein by reference in its entirety.

Example 14. The Entrain-and-Amplify strategy can be used to elicit immune responses against epitopes located on different antigens simultaneously.

[0177] Four groups of HHD mice (n=6) were immunized via intra lymph node injection with either pSEM alone; pCBP alone; pSEM and pCBP as a mixture; or with pSEM in the left LN and pCBP in the right LN. These injections were followed 10 days later with either an ELA or SSX2 peptide boost in the same fashion. All immunized mice were compared to unimmunized controls. The mice were challenged with HHD littermate splenocytes coated with ELA or SSX2 peptide, employing a triple peak CFSE *in vivo* cytotoxicity assay that allows the assessment of the specific lysis of two antigen targets simultaneously. Equal numbers of control-$CFSE^{lo}$, SSX2-$CFSE^{med}$, and ELA-$CFSE^{hi}$ cells were intravenously infused into immunized mice, and 18 hours later the mice were sacrificed and target cell elimination was measured in the spleen (Figure 15A) and blood (Figure 15B) by CFSE fluorescence using a flow cytometer. Figures 15A and 15B show the percent specific lysis of the SSX2 and Melan-A antigen targets from individual mice and Figure 15C summarizes the results in a bar graph format. Immunizing the animals with a mixture of two vaccines generated immunity to both antigens and resulted in the highest immune response, representing an average SSX2 percent specific lysis in spleen of 30+/-11 and 97+/-1 for Melan-A.

[0178] Variations on inducing multivalent responses, including responses to subdominant epitopes, are further exemplified in examples 24-34.

Example 15. Repeated cycles of DNA entrainment and peptide amplification achieve and maintain strong immunity.

[0179] Three groups of animals (n=12) received two cycles of the following immunization protocols: DNA/DNA/DNA; DNA/peptide/peptide; or DNA/DNA/peptide. Melan-A tetramer levels were measured in the mice following each cycle of immunization and are presented in Figure 16. The initial DNA/DNA/peptide immunization cycle resulted

in an average of 21.1+/-3.8 percent tetramer+ CD8+ T cells—nearly 2 fold higher than the other two groups. Following the second cycle of entrain-and-amplify immunization the average tetramer percentage for the DNA/DNA/peptide group increased by 54.5% to 32.6+/-5.9—2.5-fold higher than the DNA/peptide/peptide levels and 8.25-fold higher than the DNA/DNA/DNA group levels. In addition, under these conditions, the other immunization schedules achieved little increase in the frequency of tetramer positive T cells.

Example 16. Long-lived memory T cells triggered by immune inducing and amplifying regimens, consisting in alternating plasmid and peptide vectors.

[0180] Four HHD transgenic animals (3563, 3553, 3561 and 3577) received two cycles of the following entrain-and-amplify protocol: DNA/DNA/peptide. The first cycle involved immunization on days –31, -28, -17, -14, -3, 0; the second cycle involved immunizations on day 14, 17, 28, 31, 42 and 45. Mice were boosted with peptide on day 120. Melan-A tetramer levels were measured in the mice at 7-10 days following each cycle of immunization and periodically until 90 days after the second immunization cycle. The arrows in the diagram correspond to the completion of the cycles. (Figure 17A). All four animals mounted a response after the last boost (amplification), demonstrating persistence of immune memory rather than induction of tolerance.

[0181] Five HHD transgenic animals (3555, 3558, 3566, 3598 and 3570) received two cycles of the following entrain-and-amplify protocol: DNA/peptide/peptide. As before, the first cycle consisted in immunization on days –31, -28, -17, -14, -3, 0; the second cycle consisted in immunizations on day 14, 17, 28, 31, 42 and 45.. Mice were boosted with peptide on day 120. Melan-A tetramer levels were measured in the mice at 7-10 days following each cycle of immunization and periodically until 90 days after the second immunization cycle (Figure 17B). By comparison this entrain-and-amplify protocol substituting peptide for the later DNA injections in each cycle resulted, in this experiment, in diminished immune memory or reduced responsiveness.

Example 17. Long-lived memory T cells with substantial expansion capability are generated by intranodal DNA administration.

[0182] Seven HHD transgenic animals received two cycles of the following immunization protocol: DNA/DNA/DNA. The first cycle involved immunization on days –31, -28, -17, -14, -3, 0; the second cycle involved immunizations on day 14, 17, 28, 31, 42 and 45. Mice were boosted with peptide on day 120. Melan-A tetramer levels were measured in the mice at 7-10 days following each cycle of immunization and periodically until 90 days after the second immunization cycle. (Figure 18). All seven animals showed borderline % frequencies of tetramer+ cells during and after the two immunization cycles but mounted strong responses after a peptide boost, demonstrating substantial immune memory.

Example 18. Various combinations of antigen plus immunopotentiating adjuvant are effective for entrainment of a CTL response.

[0183] Intranodal administration of peptide is a very potent means to amplify immune responses triggered by intralymphatic administration of agents (replicative or non-replicative) comprising or in association with adjuvants such as TLRs.

[0184] Subjects (such as mice, humans, or other mammals) are entrained by intranodal infusion or injection with vectors such as plasmids, viruses, peptide plus adjuvant (CpG, dsRNA, TLR ligands), recombinant protein plus adjuvant (CpG, dsRNA, TLR ligands), killed microbes or purified antigens (e.g., cell wall components that have immunopotentiating activity) and amplified by intranodal injection of peptide without adjuvant. The immune response measured before and after boost by tetramer staining and other methods shows substantial increase in magnitude. In contrast, a boost utilizing peptide without adjuvant by other routes does not achieve the same increase of the immune response.

Example 19. Intranodal administration of peptide is a very potent means to amplify immune responses triggered by antigen plus immunopotentiating adjuvant through any route of administration.

[0185] Subjects (such as mice, humans, or other mammals) are immunized by parenteral or mucosal administration of vectors such as plasmids, viruses, peptide plus

adjuvant (CpG, dsRNA, TLR ligands), recombinant protein plus adjuvant (CpG, dsRNA, TLR ligands), killed microbes or purified antigens (e.g., cell wall components that have immunopotentiating activity) and amplified by intranodal injection of peptide without adjuvant. The immune response measured before and after boost by tetramer staining and other methods shows substantial increase in magnitude. In contrast, a boost utilizing peptide without adjuvant by other routes than intranodal does not achieve the same increase of the immune response.

Example 20. Tolerance Breaking using an Entrain-and-Amplify Immunization protocol.

[0186] In order to break tolerance or restore immune responsiveness against self-antigens (such as tumor-associated antigens) subjects (such as mice, humans, or other mammals) are immunized with vectors such as plasmids, viruses, peptide plus adjuvant (CpG, dsRNA, TLR mimics), recombinant protein plus adjuvant (CpG, dsRNA, TLR mimics), killed microbes or purified antigens and boosted by intranodal injection with peptide (corresponding to a self epitope) without adjuvant. The immune response measured before and after boost by tetramer staining and other methods shows substantial increase in the magnitude of immune response ("tolerance break").

Example 21. Clinical practice for entrain-and–amplify immunization.

[0187] Patients are diagnosed as needing treatment for a neoplastic or infectious disease using clinical and laboratory criteria; treated or not using first line therapy; and referred to evaluation for active immunotherapy. Enrollment is made based on additional criteria (antigen profiling, MHC haplotyping, immune responsiveness) depending on the nature of disease and characteristics of the therapeutic product. The treatment (Figure 19) is carried out by intralymphatic injection or infusion (bolus, programmable pump, or other means) of vector (plasmids) and protein antigens (peptides) in a precise sequence. The most preferred protocol involves repeated cycles encompassing plasmid entrainment followed by amplifying dose(s) of peptide. The frequency and continuation of such cycles can be adjusted depending on the response measured by immunological, clinical and other means. The composition to be administered can be monovalent or polyvalent, containing multiple vectors, antigens, or epitopes. Administration can be to one or multiple lymph nodes

simultaneously or in staggered fashion. Patients receiving this therapy demonstrate amelioration of symptoms.

Example 22. Clinic practice for induction of immune deviation or de-activation of pathogenic T cells.

[0188] Patients with autoimmune or inflammatory disorders are diagnosed using clinical and laboratory criteria, treated or not using first line therapy, and referred to evaluation for active immunotherapy. Enrollment is made based on additional criteria (antigen profiling, MHC haplotyping, immune responsiveness) depending on the nature of disease and characteristics of the therapeutic product. The treatment is carried out by intralymphatic injection or infusion (bolus, programmable pump or other means) of peptide devoid of T1-promoting adjuvants and/or together with immune modulators that amplify immune deviation. However, periodic bolus injections are the preferred mode for generating immune deviation by this method. Treatments with peptide can be carried weekly, biweekly or less frequently (e.g., monthly), until a desired effect on the immunity or clinical status is obtained. Such treatments can involve a single administration, or multiple closely spaced administrations as in figure 2, group 2. Maintenance therapy can be afterwards initiated, using an adjusted regimen that involves less frequent injections. The composition to be administered can be monovalent or polyvalent, containing multiple epitopes. It is preferred that the composition be free of any component that would prolong residence of peptide in the lymphatic system. Administration can be to one or multiple lymph nodes simultaneously or in staggered fashion and the response monitored by measuring T cells specific for immunizing peptides or unrelated epitopes ("epitope spreading"), in addition to pertinent clinical methods.

Example 23. Immunogenic Compositions (e.g., Viral Vaccines)

[0189] Six groups (n=6) of HLA-A2 transgenic mice are injected with 25 ug of plasmid vector bilaterally in the inguinal lymph nodes, according to the following schedule: day 0, 3, 14 and 17. The vector encodes three A2 restricted epitopes from HIV gag (SLYNTVATL (SEQ ID NO:3), VLAEAMSQV (SEQ ID NO:4), MTNNPPIPV (SEQ ID NO:5)), two from pol (KLVGKLNWA (SEQ ID NO:6), ILKEPVHGV (SEQ ID NO:7)) and one from env (KLTPLCVTL (SEQ ID NO:8)). Two weeks after the last cycle of

entrainment, mice are injected with mixtures encompassing all these five peptides (5ug/peptide/node bilaterally three days apart). In parallel, five groups of mice are injected with individual peptides (5ug/peptide/node bilaterally three days apart). Seven days later the mice are bled and response is assessed by tetramer staining against each peptide. Afterwards, half of the mice are challenged with recombinant Vaccinia viruses expressing env, gag or pol (103 TCID50/mouse) and at 7 days, the viral titer is measured in the ovaries by using a conventional plaque assay. The other half are sacrificed, the splenocytes are stimulated with peptides for 5 days and the cytotoxic activity is measured against target cells coated with peptides. As controls, mice are injected with plasmid or peptides alone. Mice entrained with plasmid and amplified with peptides show stronger immunity against all five peptides, by tetramer staining and cytotoxicity.

[0190] More generally, in order to break tolerance, restore immune responsiveness or induce immunity against non-self antigens such as viral, bacterial, parasitic or microbial, subjects (such as mice, humans, or other mammals) are immunized with vectors such as plasmids, viruses, peptide plus adjuvant (CpG, dsRNA, TLR mimics), recombinant protein plus adjuvant (CpG, dsRNA, TLR mimics), killed microbes or purified antigens (such as cell wall components) and boosted by intranodal injection with peptide (corresponding to a target epitope) without adjuvant. The immune response measured before and after boost by tetramer staining and other methods shows substantial increase in the magnitude of immune response. Such a strategy can be used to protect against infection or treat chronic infections caused by agents such as HBV, HCV, HPV, CMV, influenza virus, HIV, HTLV, RSV, etc.

Example 24. Schedule of immunization with two plasmids:
pCBP expressing SSX2 41-49 and pSEM expressing Melan-A 26-35 (A27L).

[0191] Two groups of HHD mice (n=4) were immunized via intralymph node injection with either pSEM and pCBP as a mixture; or with pSEM in the left inguinal lymph node and pCBP in the right inguinal lymph node, twice, at day 0 and 4 as shown in Figure 20. The amount of the plasmid was 25μg/plasmid/dose. Two weeks later, the animals were sacrificed, and cytotoxicity was measured against T2 cells pulsed or not with peptide.

Example 25. Vector segregation rescues the immunogenicity of the less dominant epitope.

[0192] Animals immunized as described in Example 24, were sacrificed and the splenocytes pooled by group and stimulated with one of the two peptides, Melan-A 26-35 (A27L) or SSX2 41-49, in parallel. The cytotoxicity was measured by incubation with 51Cr-loaded, peptide-pulsed T2 target cells. Data in Figure 21 show mean of specific cytotoxicity (n=4/group) against various target cells.

[0193] The results show that use of the plasmid mixture interfered with the response elicited by pCBP plasmid; however, segregating the two plasmids relative to site of administration rescued the activity of pCBP. Co-administration of different vectors carrying distinct antigens results in establishment of a hierarchy in regard to immunogenicity. Vector segregation rescues the immunogenicity of the less dominant component, resulting in a multivalent response.

Example 26. Addition of peptide amplification steps to the immunization protocol.

[0194] Four groups of HHD mice (n=6) were immunized via intralymph node injection with either pSEM and pCBP as a mixture; or with pSEM in the left inguinal lymph node and pCBP in the right inguinal lymph node, twice, at day 0 and 4 as shown in Figure 22. As control, mice were immunized with either pSEM or pCBP plasmid alone. The amount of the plasmid was 25μg/plasmid/dose. Two weeks later at days 14 and 17, the animals were boosted with Melan-A and/or SSX2 peptides, mirroring the plasmid immunization in regard to dose and combination. Two weeks later at day 28, the animals were challenged with splenocytes stained with CFSE and pulsed or not with Melan-A (ELA) or SSX2 peptide, for evaluation of in vivo cytotoxicity.

Example 27. Peptide boost rescues the immunogenicity of a less dominant epitope even when the vectors and peptides respectively, are used as a mixture.

[0195] Animals were immunized as described in Example 26 and challenged with HHD littermate splenocytes coated with ELA or SSX2 peptide, employing a triple peak CFSE *in vivo* cytotoxicity assay that allows the assessment of the specific lysis of two antigen targets simultaneously. Equal numbers of control-$CFSE^{lo}$, SSX2-$CFSE^{med}$, and ELA-$CFSE^{hi}$ cells were intravenously infused into immunized mice and 18 hours later the mice were sacrificed and target cell elimination was measured in the spleen (Figure 23) by

CFSE fluorescence using flow cytometry. The figure shows the percent specific lysis of the SSX2 and Melan-A antigen targets from individual mice, the mean and SEM for each group.

[0196] Interestingly, immunizing the animals with a mixture of two vaccines comprising plasmids first and peptides afterwards, generated immunity to both antigens and resulted in the highest immune response, representing an average SSX2 percent specific lysis in spleen of 30±11 and 97±1 for Melan-A. Thus, as illustrated in Figure 23, peptide boost can rescue the immunogenicity of a less dominant epitope even when the vectors and peptides respectively are used as a mixture.

Example 28. Clinical practice for entrain-and–amplify immunization.

[0197] Two scenarios are shown in Figure 24 for induction of strong multivalent responses: in the first one (A), use of peptides for amplification restores multivalent immune responses even if plasmids and peptides are used as mixtures. In the second scenario (B), segregation of plasmid and peptide components respectively, allows induction of multivalent immune responses. It is preferred that peptide be administered to the same lymph node to which the entraining plasmid for the common epitope is administered. However this is not absolutely required since T memory cells lose CD62L expression and thus colonize other lymphoid organs. The time interval between entrainment and amplification shown in figure 24 is convenient, but is not considered critical. Substantially shorter intervals are less preferred but much longer intervals are quite acceptable.

Example 29. A single plasmid eliciting a multivalent response.

[0198] The plasmid pSEM, described in Figure 25 and the table below, encompasses within an open reading frame ("synchrotope polypeptide coding sequence") multiple peptides from two different antigens (Melan-A and tyrosinase) adjoined together. Thus it has potential to express, and induce immunization against, more than a single epitope. The peptide sequences encoded are the following: Tyrosinase 1-9; Melan-A/MART-1 26-35(A27L); Tyrosinase 369-377; and Melan-A/MART-1 31-96.

[0199] The cDNA sequence for the polypeptide in the plasmid is under the control of promoter/enhancer sequence from cytomegalovirus (CMVp) which allows efficient transcription of messenger for the polypeptide upon uptake by antigen presenting cells. The bovine growth hormone polyadenylation signal (BGH polyA) at the 3' end of the encoding sequence provides signal for polyadenylation of the messenger to increase its stability as well as translocation out of nucleus into the cytoplasm. To facilitate plasmid transport into the nucleus, a nuclear import sequence (NIS) from Simian virus 40 has been inserted in the plasmid backbone. One copy of a CpG immunostimulatory motif is engineered into the plasmid to further boost immune responses. Lastly, two prokaryotic genetic elements in the plasmid are responsible for amplification in E. coli, kanamycin resistance gene (Kan R) and the pMB bacterial origin of replication. Further description of pSEM can be found in U.S. Patent Application No. 10/292,413, where it is named variously pMA2M and pVAXM3, incorporated by reference above.

Genetic Element Name	**Description**
CMV Enhancer/ promoter	Entire Cytomegalovirus Immediate Early gene enhancer and promoter region
BGH Polyadenylation region	Bovine growth Hormone Polyadenylation region. Contains consensus sequences that are known to extend message 1/2 life
Kanamycin Resistance Gene	Transposon Tn10 gene capable of conferring Kanamycin drug resistance to bacterial host cells (TOP10) used to clone and ferment the plasmid
PMB Origin of Replication (reverse orientation)	PMB origin of replication is a similar but slightly different plasmid bacterial origin than ColE1. It is a high copy ori capable of supporting 100-1500 copies of plasmid DNA/ bacterial cell. We reversed its orientation in relation to the stock pVAX plasmid to eliminate the production of unwanted replication intermediates in our plasmid vaccine constructs. See U.S. Patent No. 6,709,844, which is incorporated herein by reference in its entirety.
ISS sequence (naturally occurs in *E. coli*)	The sequence GTCGTT is a highly preferred and naturally occurring CpG sequence reported to be capable of eliciting an anti-bacterial DNA adjuvant response in human immune cells.
Second ISS sequence (synthetic/ ACL I site)	The sequence AACGTT is an ACLI site and a preferred CpG sequence reported to be capable of eliciting an anti-bacterial DNA adjuvant response in murine immune cells.
Nuclear Import sequence (NIS), SV40 72 bp repeat	The SV40 72 base pair repeat is reported to act as an efficient Nuclear Import Sequence (NIS) allowing higher levels of transcription from plasmids entering the target eukaryotic cells. The entire SV40 origin of replication is not included in the NIS and should not support episomal replication in mammalian cells.

Example 30. Protocol to "rescue" or amplify an immune response against a subdominant epitope subsequent to initiation by using a multivalent vector.

[0200] A notorious limitation of vectors co-expressing epitopes of therapeutic relevance is that within the newly engineered context, one epitope will assume a dominant role in regard to induction of immunity, whereas the others will be subdominant (particularly when such epitopes bind to the same MHC restriction elements).

[0201] In Figure 26, such a protocol is described: eight groups of HHD mice (n=4) were immunized via intralymph node injection with pSEM, on days 0, 3, 14 and 17. The amount of the plasmid was 25μg of plasmid/dose. On days 28 and 31, the mice were intranodally administered amplifying peptides corresponding to either Melan-A 26-35 (Figure 27A) or tyrosinase 369-377 (Figure 27B), also at 25μg of peptide/dose. The immune response was measured by tetramer staining of CD8+ T cells in the peripheral blood at two weeks after the completion of immunization, using Melan-A or tyrosinase specific reagents.

[0202] The results in Figure 27 show that while priming with pSEM elicited a significant response against Melan-A, the response against tyrosinase was not detectable. In parallel, animals immunized with peptide only showed no detectable tetramer response to either epitope. Together, these data demonstrate that the Melan-A epitope assumed an immune dominant role relative to the tyrosinase epitope. After the boost with tyrosinase ("natural peptide") however, the immune response against tyrosinase (Figure 27B, first grouping) was of similar magnitude compared to the levels achieved against Melan-A (Figure 27A, the second and fourth groupings), in animals immunized with Melan-A peptide subsequent to pSEM priming.

[0203] In summary, intralymphatic administration of tyrosinase peptide rescued the immune response initiated by pSEM against this epitope, overcoming its subdominance relative to the Melan-A epitope in context of the vector (pSEM) used for initiating the response.

Example 31. Protocol to "rescue" or amplify an immune response against a subdominant epitope subsequent to initiation by using a multivalent vector: evaluation of cytotoxic immunity.

[0204] The immunization was carried out as described in Example 30: eight groups of HHD mice (n=4) were immunized via intralymph node injection with pSEM, on days 0, 3, 14 and 17. The amount of the plasmid was 25μg /dose. On days 28 and 31, the mice were immunized with peptides corresponding to either Melan-A 26-35 (Figure 28A) or tyrosinase 369-377 (Figure 28B) epitopes, administered into the lymph nodes (25μg of peptide/dose). Immunity was assessed by cytotoxicity assay 14 days after the completion of immunization, following ex vivo restimulation of splenocytes with Melan-A or tyrosinase epitope peptides. In brief, splenocytes were prepared (spleens harvested, minced, red blood cells lysed) and incubated with LPS-stimulated, Melan-A (Figure 28A) or tyrosinase (Figure 28B) peptide–coated syngeneic pAPC for 7 days, in the presence of rIL-2. The cells were washed and incubated at different ratios with 51Cr-labeled Melan-A+, tyrosinase+ 624.38 target cells, for 4 hours. The radioactivity released into the supernatant was measured using a γ (gamma)-counter. The response was quantified as % lysis = (sample signal – background) / (maximal signal – background) x 100, where background represents radioactivity released by target cells alone when incubated in assay medium, and the maximal signal is the radioactivity released by target cells lysed with detergent.

[0205] As in the Example 30, the results in Figure 28 demonstrate the rescue / amplification of immunity by intranodal peptide boost, against an epitope (tyrosinase) that is subdominant in the context of the immune initiating vector (pSEM).

Example 32. Protocol to co-induce and amplify immune responses against two epitopes – one dominant and one subdominant within the context of initiating vector - simultaneously.

[0206] In the previous two examples rescue of the response to the subdominant epitope was demonstrated in the absence of amplification of the response to the dominant epitope. Next, simultaneous amplification of both responses was attempted.

[0207] In Figure 29, such a protocol is described: four groups of HHD mice (n=6) were immunized via intra lymph node injection with pSEM, on days 0, 3, 14 and 17. The amount of the plasmid was 25μg /dose. On days 28 and 31, the mice were simultaneously immunized with peptides corresponding to the Melan A 26-35 (left inguinal

lymph node) and tyrosinase 369-377 (right inguinal lymph node) epitopes, at 25μg of peptide/dose. The immune response was measured by tetramer staining of CD8+ T cells in the peripheral blood at two weeks after the completion of immunization, using Melan A (Figure 30A) or Tyrosinase (11B) specific reagents. The data were represented as mean % tetramer+ cells within the CD8+ subset. Animals primed with the pSEM plasmid and amplified with peptide analogues Melan A 26-35 A27Nva {E(Nva)AGIGILTV; SEQ ID NO:9} (left lymph node) and Tyrosinase 369-377 V377Nva {YMDGTMSQ(Nva); SEQ ID NO:10} (Right lymph node) showed a multivalent immune response specific to each epitope as measured by multi-color tetramer staining (Figure 30C). Dot plots were gated on total CD8 positive cells from peripheral blood and represent duel immune reponses in individual mice. Tetramer levels were calculated as the percent of CD8 positive T cells.

[0208] The results in Figure 30 show that by co-administration of Melan A and tyrosinase peptides, one could co-amplify the immune response against both Melan A and tyrosinase epitopes that have a dominant / subdominant relationship in context of the immune initiating vector (pSEM).

Example 33. Co-induction and amplification of cytolytic responses against two epitopes – one dominant and one subdominant – within the context of initiating vector using mixtures of peptides.

[0209] To further explore simplified product formulations, an alternate method was tested, integrating use of a bivalent plasmid expressing a dominant and a subdominant epitope, followed by amplification of response to each epitope by administration of a mixture of dominant and subdominant peptides, rather than separate administration of peptides – as described in the previous example.

[0210] Six groups of HHD mice (n=6) were immunized as described in the previous examples with pSEM plasmid (or not immunized respectively), and boosted with peptides (as a mixture between Melan-A + various tyrosinase peptides), in the lymph nodes, at a dose of 12.5μg /peptide/dose, using the following schedule: plasmid on days 0, 3; peptide days 14 and 17 with a repeat of this cycle two weeks later. The tyrosinase peptides used were: Tyr 369-377, as above; Tyr 1-9, which is encoded by the plasmid but not presented by transformed cells; and Tyr 207-215, which is not encoded by the plasmid.

[0211] The immune response was measured two weeks after the completion of immunization regimen, by CFSE assay, as described above. Briefly: splenocytes were isolated from littermate control HHD mice and incubated with 20μg/mL ELA or 20ug/ml of tyrosinase peptide for 2 hours. These cells were then stained with $CFSE^{hi}$ and $CFSE^{med}$ fluorescence and co-injected intravenously into immunized mice with an equal ratio of control splenocytes stained with $CFSE^{lo}$ fluorescence. Eighteen hours later spleens were removed and specific elimination of target cells was measured using flow cytometry and calculating % *in vivo* specific lysis by the following formula:

$\{[1-(\%CFSE^{hi\ or\ med}/\ \%CFSE^{lo}\ )] - [1-\ (\%CFSE^{hi\ \ or\ med}Control\ /\ \%CFSE^{lo}$ Control)]}x100

[0212] wherein each % term in the equation represents the proportion of the total sample represented by each peak.

[0213] Overall, the results displayed in Figure 31 (% *in vivo* specific lysis against Melan-A epitope coated or tyrosinase epitope coated splenocytes; with x axis depicting the peptides used for boost) show that co-amplification of immunity against the dominant (Melan-A) and subdominant (tyrosinase 369-377) epitopes occurred using a mixture of the peptides in the amplification stage of a regimen of plasmid initiation / peptide amplification. In addition, use of peptides alone did not result in effective response. For this combination of peptides significant responses were obtained to both epitopes. However, it should be noted that expectations of success from mixtures of peptides are greater when the MHC-binding affinities of the various peptides are similar, and lessen as the affinities become more disparate.

Example 34. Induction of a Response with Higher Order Multivalency

[0214] In this study immunity was induced with two bivalent plasmids and amplified with four peptide epitope analogues. The plasmid pSEM was used to induce immunity to Melan-A and tyrosinase epitopes and the response amplified using the analogues Melan-A (A27Nva) and Tyrosinase (V377Nva) as before. Immunity was also induced to the epitopes SSX2 41-49, NY-ESO-1 157-165 using the plasmid pBPL. The immunogenic polypeptide encoded by pBPL is disclosed in U.S. Patent application 10/292,413 (Pub. No. 20030228634 A1) entitled EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS AND METHODS FOR THEIR

DESIGN incorporated herein by reference in its entirety above. Amplification used the peptide epitope analogues SSX2 41-49 (A42V) and NY-ESO-1 157-165 (L158Nva, C165V). Further discussion of epitope analogues is provided in the epitope analogues applications cited and incorporated by reference above. These analogues generally have superior affinity and stability of binding to MHC as compared to the natural sequence, but are cross-reactive with TCR recognizing the natural sequence.

[0215] Three groups of female HHD-A2 mice were immunized with a mixture of pSEM/pBPL (100 μg each plasmid/day; 25 μl/injected node) administered bilaterally to the inguinal lymph nodes. Group 1 (n=10) received plasmid only, throughout the protocol, with injections on Days 1, 4, 15, 18, 28, 32, 49, and 53. Group 2 and Group 3 (n=25 each group) received plasmid injections on Days 1, 4, 15, and 18 and peptides on subsequent days. On day 25, blood was collected from the immunized animals, and $CD8^+$ T cell were analyzed by flow cytometry using an MHC- tetramer assay. Responses were compared to naïve littermate control mice (n=5).

[0216] The mice in Group 2 were boosted by administering the peptides Tyrosinase V377Nva (25 μg/day) to the right lymph node and with SSX2 A42V (25 μg/day) to the left lymph node on days 28, 32, 49, and 53. Group 3 animals were boosted by administering the peptides Tyrosinase V377Nva (25 μg/day) to right lymph node and SSX2 A42V (25 μg/day) to the left lymph node on days 28 and 32 followed by NY-ESO-1 L158Nva, C165V (12.5 μg/day) to the right lymph node and Melan-A A27Nva (25 μg/day) to the left lymph node on days 49 and 53. All injections were 25 μl/injected node. On days 39 and 60, blood was collected from each group, and CD8+ T cell analysis was performed using a tetramer assay. Responses were compared to naïve littermate control mice (n=5).

[0217] On days 41 and 63, selected animals from each group were sacrificed and spleens were removed for IFNγ ELISPOT analysis on splenocyte cell suspensions.

[0218] On day 62, selected animals from each group received, via intravenous injection, CFSE-labeled 624.38 human melanoma cells expressing all four tumor associated antigens and used as targets for SSX2, NY-ESO-1, Tyrosinase, and Melan A specific CTLs in immunized mice.

[0219] Plasmids were formulated in clinical buffer (127mM NaCl, 2.5mM Na_2HPO_4, 0.88mM KH_2PO_4, 0.25mM Na_2EDTA, 0.5% ETOH, in H_2O; 2 mg/ml each plasmid, 4 mg/ml total). The Melan-A 26-35 (A27Nva), Tyrosinase 369-377 (V377Nva),

and SSX2 41-49 (A42V) analogues were formulated in PBS at 1.0mg/ml. The NY-ESO 157-165 (L158Nva, C165V) peptide analogue was prepared for immunization in PBS containing 5% DMSO at a concentration of 0.5mg/ml. Cytometry data were collected using a BD FACS Calibur flow cytometer and analyzed using CellQuest software by gating on the lymphocyte population. PBMCs were co-stained with FITC conjugated rat anti-mouse CD8a (Ly-2) monoclonal antibody (BD Biosciences, 553031) and an MHC tetramer: HLA-A*0201 SSX2 (KASEKIFY (SEQ ID NO:11))-PE MHC tetramer (Beckman Coulter, T02001), HLA-A*0201 NY-ESO (SLLMWITQC) (SEQ ID NO:12)-APC MHC tetramer (Beckman Coulter, T02001), HLA-A*0201 Melan-A (ELAGIGILTV (SEQ ID NO:1))-PE MHC tetramer (Beckman Coulter, T02001), or HLA-A*0201 Tyrosinase (YMDGTMSQV (SEQ ID NO:13))-APC MHC tetramer (Beckman Coulter, T02001).

[0220] An IFN- γ ELISpot assay was carried out as follows. Spleens were removed on Days 27 and 62 from euthanized animals, and the mononuclear cells isolated by density centrifugation (Lympholyte Mammal, Cedarlane Labs), and resuspended in HL-1 medium. Splenocytes (5 or 3 $x10^5$ cells per well) were incubated with 10μg of Melan-A 26-35 A27L, Tyrosinase 369-377, SSX2 41-49, or NY-ESO-1 157-165 peptide in triplicate wells of a 96 well filter membrane plates (Multiscreen IP membrane 96-well plate, Millipore). Samples were incubated for 42 hours at 37°C with 5% CO_2 and 100% humidity prior to development. Mouse IFN-γ coating antibody was used to coat the filters prior to incubation with splenocytes and biotinylated detection antibody was added to develop signal after lysing and washing the cells off of the filter with water (IFN-γ antibody pair, Ucytech). GABA conjugate and proprietary substrates from Ucytech were used for IFN-γ spot development. The CTL response in immunized animals was measured 24 hours after development on the AID International plate reader using ELISpot Reader software version 3.2.3 calibrated for IFN-γ spot analysis.

[0221] An in vivo cytotoxicity assay was carried out on Day 61 as follows. Human 624.38 (HLA $A*0201^{pos}$) cultured melanoma tumor cells were stained with $CFSE^{hi}$ (Vybrant CFDA SE cell tracer kit, Molecular Probes) fluorescence (1.0 μM for 15 minutes) and 624.28 HLA-A2 (HLA $A*0201^{neg}$) stained with $CFSE^{lo}$ fluorescence (0.1 μM for 15 minutes). Two mice from each group (Group 1, 2, and 3) selected on the basis of high tetramer levels and 2 naïve control mice received $20x10^6$ $CFSE^{hi}$-labeled 624.38 (HLA $A*0201^{pos}$) human melanoma cells mixed with an equal number of $CFSE^{lo}$-labeled 624.28

(HLA A*0201^{neg}) via intravenous injection split in two aliquots delivered 2 hours apart. The specific elimination of HLA A*0201^{pos} human target cells was measured after approximately 14 hours by sacrificing the mice, removing lung tissue, making a single cell suspension, and measuring CFSE fluorescence by flow cytometry. Percent specific lysis was calculated as shown above.

[0222] The immune response obtained was assessed at various points in the protocol. Figure 32 shows the response obtained as judged by tetramer analysis 7 days after the 4th of the plasmid injections, which were common to all three groups. Substantial responses were observed to all but the tyrosinase epitope. Melan-A 26-35 and NY-ESO-1 157-165 were revealed to be dominant epitopes. In order to generate a more balanced tetravalent immune response, the response to the sub-dominant epitopes was amplified by administration of the tyrosinase V377Nva and SSX2 A42V peptide epitope analogues to groups 2 and 3. Group 1 received another round of immunization with the plasmid mixture. As seen in figure 33 further immunization with the plasmids (group 1) only boosted the response to the dominant epitopes. In contrast, administration of peptides corresponding to the two subdominant epitopes resulted in substantial and more balanced responses to all four epitopes. Figure 34 shows the response of selected individual animals demonstrating that a truly tetravalent response can be generated. IFN-γ ELISpot analysis of a subset of mice sacrificed on day 27 confirmed the general pattern observed from the tetramer data (fig. 35A). Another cohort of mice was sacrificed on day 62 following a further round of amplification that concluded on day 59 and subjected to IFN-γ ELISpot analysis (fig. 35b). For group 1 this final round of immunization again used the plasmid mixture and the pattern of response remained similar to that observed following the earlier rounds. Using only those peptides corresponding to the subdominant epitopes (group 2) maintained a relatively balanced response to the four epitopes. Peptides corresponding to all four epitopes were administered to group 3. A degree of the dominance of the melan-A epitope re-emerged at the apparent expense of the response to the tyrosinase epitope, though a significant response to that epitope was still observed. It should be noted that because the general responsiveness of the cohorts of animals sacrificed at the two time points differed, the absolute magnitude of the responses depicted in figures 35 A and B are not directly comparable. In vivo cytolytic activity was also assessed by challenge with CFSE labeled human tumor cells expressing all four of the targeted antigens. These tumor cells were a

derivative of the cell line 624.38, which naturally expresses SSX2, PRAME, tyrosinase, and melan-A, that had been transformed using a plasmid vector to stably express NY-EOS-1 as well. As would be expected in a naïve mouse, with only background levels of tetramer or IFN-γ response by ELISpot analysis, there is no specific depletion of HLA-$A2^+$ tumor cells as compared to the HLA-$A2^-$ controls (fig. 36A). However in mice with substantially tetravalent responses specific depletion was observed, and the more balanced response achieved the better result. Compare the epitope specific responses seen by tetramer and ELISpot analysis for figure 36B (71% specific lysis) and 36C (95% specific lysis). No specific lysis was also observed for a mouse with a substantially monovalent response. In vivo cytotoxicity due to a monovalent response was seen above (in Example 7), but the target cells in that experiment had significantly greater epitope expression. Thus, a multivalent response was here seen here to overcome the protective effect of low target antigen expression levels.

Example 35. A global method to induce multivalent immunity.

[0223] The method can comprise the following steps (depicted in Figure 37):

[0224] Identification of epitopes from different antigens or the same antigen. Such epitopes can have a relationship of dominance / subdominance (e.g., due to expression or presentation to widely different extents, TCR repertoire bias, etc.) relative to each other, or can be co-dominant in their native context.

[0225] Retrieving the sequence associated with such epitopes and engineering expression vectors that encompass within the same reading frame or within the same vector, such epitopes. The new context, can create or alter the relationship of immune dominance / subdominance relative to each other as compared to their natural context.

[0226] Immunization with the vector, resulting in initiating a response that can be dominated by one specificity (dominant epitope) relative to others.

[0227] Amplifying the response to subdominant epitopes by administering a corresponding peptide. The peptide can be the native sequence or be an analogue of it. The peptide can be administered alone or concurrently with other peptides corresponding to dominant and/or subdominant epitopes, at the same site, or more preferred at separate sites.

[0228] Any of the methods described in the examples and elsewhere herein can be and are modified to include different compositions, antigens, epitopes, analogues, etc. For example, any other cancer antigen can be used. Also, many epitopes can be interchanged, and the epitope analogues, including those disclosed, described, or incorporated herein can be used. The methods can be used to generate immune responses, including multivalent immune responses against various diseases and illnesses.

[0229] Many variations and alternative elements of the invention have been disclosed. Still further variations and alternate elements will be apparent to one of skill in the art. Various embodiments of the invention can specifically include or exclude any of these variation or elements.

[0230] Each reference cited herein is hereby incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A method of immunization comprising:

delivering to a mammal a first composition comprising a first immunogen, the first immunogen comprising or encoding at least a portion of a first antigen and a second composition comprising a second immunogen, the second immunogen comprising or encoding at least a portion of a second antigen; and subsequently

administering a third composition comprising a first peptide directly to the lymphatic system of the mammal, wherein the first peptide corresponds to an epitope of said first antigen, wherein said third composition is not the same as the first or second compositions.

2. The method of Claim 1, wherein the first and second compositions are the same.

3. The method of Claim 2, wherein a single macromolecule comprises said first and second immunogen.

4. The method of Claim 1, further comprising administering, subsequent to said delivering step, a fourth composition comprising a second peptide directly to the lymphatic system of the mammal, wherein the second peptide corresponds to an epitope of said second antigen, wherein said fourth composition is not the same as the first or second compositions.

5. The method of Claim 4, wherein said third and fourth compositions each comprise the first and the second peptides.

6. The method of Claim 4, wherein said first and second compositions are delivered to separate sites.

7. The method of Claim 4, wherein said first and second peptides are administered to separate sites.

8. The method of Claim 4, wherein said first immunogen is delivered to a same site as said first peptide is administered to.

9. The method of Claim 4, said first and second peptides are administered at about the same time.

10. The method of Claim 4, said first and second peptides are administered on different days.

11. The method of Claim 1, wherein said first antigen is selected from the group consisting of Tyrosinase, Melan-A, SSX-2, NY-ESO-1, PRAME, PSMA, VEGFR2, VEGF-A, and PLK1.

12. The method of Claim 1, wherein administering directly to the lymphatic system comprises administration to an inguinal lymph node.

13. The method of Claim 1, wherein immunization comprises induction of a CTL response.

14. The method of Claim 1, wherein the delivering step comprises delivery of an epitopic peptide that is the same as the first peptide of the administering step, and wherein the third composition differs from the first or second composition at least by comprising a larger dose of the epitopic peptide.

15. The method of Claim 1, wherein the delivering step comprises delivering an immunopotentiator.

16. The method of Claim 15, wherein the immunopotentiator is delivered with at least one of the first composition and the second composition.

17. A method of immunization comprising:

delivering to a mammal means for entraining an immune response to multiple antigens; and subsequently

administering one or more peptides directly to the lymphatic system of the mammal, wherein each of said peptides corresponds to an epitope of one of said antigens, wherein a composition used in the administering step is not the same as any composition used in the delivering step.

18. The method of Claim 17, wherein said means entrain an immune response to 3 or 4 antigens.

19. A method of immunization comprising:

delivering to a mammal one or more compositions comprising or encoding at least a portion of multiple antigens; and

a subsequent step for amplifying the response to said antigens.

20. A method of treatment comprising repeated cycles of immunizations according to the method of claim 1.

21. The method of Claim 20, wherein cycle repetition continues for sufficient time to maintain an immune response effective to achieve a medical need.

22. The method of Claim 21, wherein cycle repetition improves multivalency of an immune response.

23. A set of immunogenic compositions for inducing an immune response in a mammal comprising 1or more entraining doses for each of 2 or more antigens and at least one amplifying dose, wherein the entraining doses for each antigen comprise an immunogen or a nucleic acid encoding said immunogen wherein the immunogen comprises at least a portion of said antigen; and an immunopotentiator; and wherein the amplifying dose comprises a peptide epitope.

24. The set of Claim 23, wherein at least one composition is multivalent.

25. The set of Claim 23, wherein the nucleic acid encoding the immunogen further comprises an immunostimulatory sequence with serves as the immunopotentiating agent.

26. The set of Claim 23, wherein the immunopotentiating agent is selected from the group consisting of a TLR ligand, an immunostimulatory sequence, a CpG-containing DNA, a dsRNA, an endocytic-Pattern Recognition Receptor (PRR) ligand, an LPS, a quillaja saponin, tucaresol, and a pro-inflammatory cytokine.

27. The set of Claim 23, wherein the doses are adapted for intranodal delivery.

28. The set of Claim 27, wherein at least one of the entraining doses comprises a nucleic acid.

29. The set of Claim 28, wherein a one-day dose of nucleic acid is about 25-2500μg.

30. The set of Claim 27, wherein the amplifying dose is about 5-5000 μg of peptide per kg of the intended recipient.