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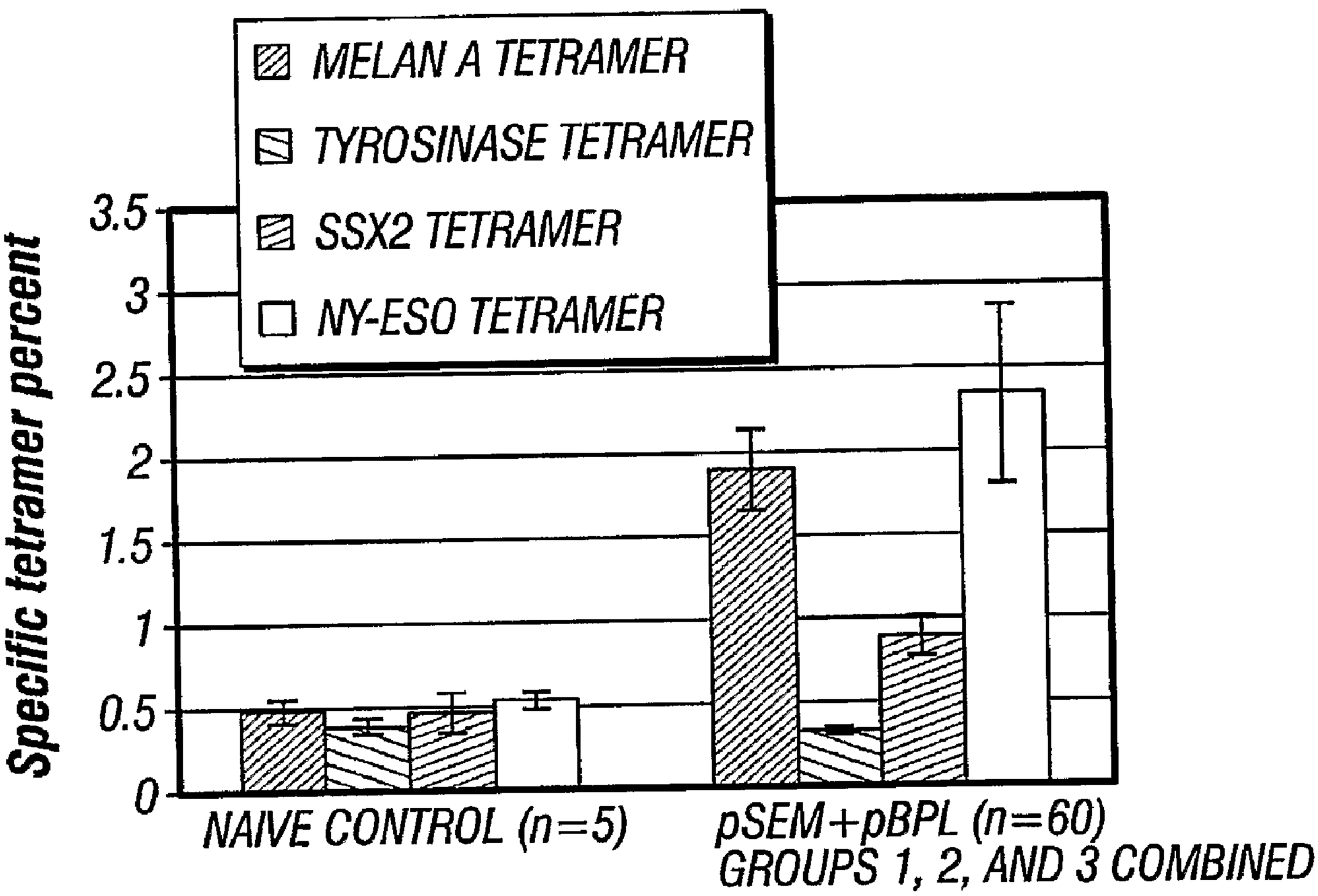

(57) Abrégé/Abstract:
The present invention provides a method of treating a cell proliferative disease such as cancer by providing to a subject in need thereof an immunogenic composition comprising plasmid and peptide(s) or analogues thereof. In embodiments of the present

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invention there is provided methods and compositions for inducing, entraining, and/or amplifying the immune response to MHC class-I restricted epitopes of carcinoma antigens to generate an effective anti-cancer immune response.

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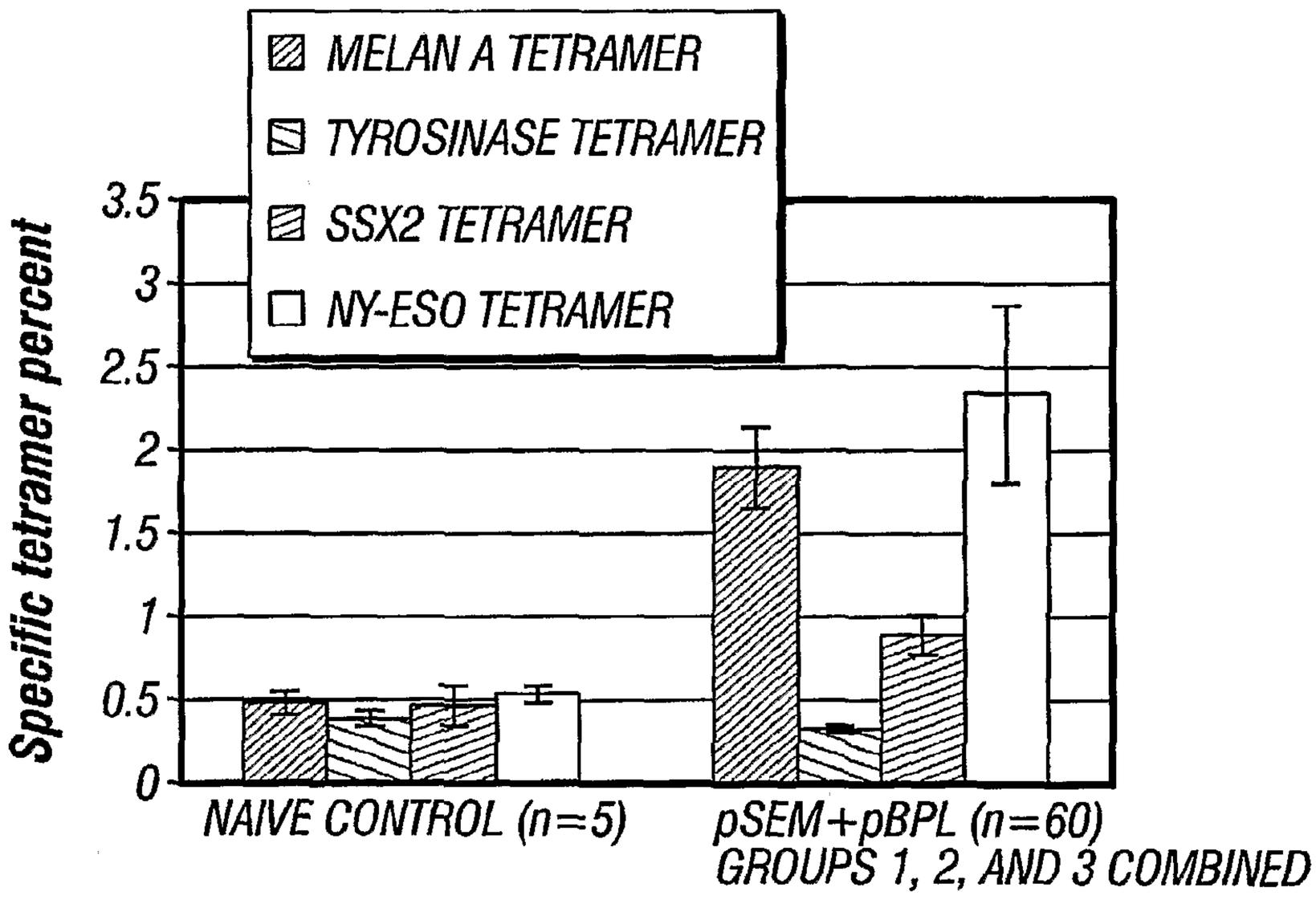

(57) Abstract: The present invention provides a method of treating a cell proliferative disease such as cancer by providing to a subject in need thereof an immunogenic composition comprising plasmid and peptide(s) or analogues thereof. In embodiments of the present invention there is provided methods and compositions for inducing, entraining, and/or amplifying the immune response to MHC class-I restricted epitopes of carcinoma antigens to generate an effective anti-cancer immune response.

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MULTIVALENT ENTRAIN-AND-AMPLIFY IMMUNOTHERAPEUTICS FOR CARCINOMA

BACKGROUND OF THE INVENTION

[0001] The present application claims the benefit of the filing date of U.S. Provisional Patent Application Serial No. 60/691,581, filed on June 17, 2005, the entire text of which is incorporated herein by reference without disclaimer.

Field of the Invention

[0002] The invention disclosed herein relates to methods and compositions for inducing an MHC class-I restricted immune response, controlling the nature and magnitude of the response, particularly a multivalent response, and promoting effective immunologic intervention in pathogenic processes. Disclosed herein are methods and compositions for inducing an immune response against various combinations of tumor-associated antigens, which can promote effective immunologic intervention in pathogenic processes.

Description of the Related Art

[0003] The American Cancer Society has estimated that over one million people get cancer each year, and that approximately one out of every two American men and one out of every three American women will have some type of cancer at some point during their lifetime.

[0004] Normal body cells grow, divide, and die in an orderly fashion. In cell proliferative diseases such as cancer, cells, instead of dying, continue to grow out of control and divide. Although there are many kinds of cancer, they usually start because of out-of-control growth of abnormal cells.

[0005] Usual treatment options for cancer include surgery, radiation therapy, and chemotherapy. A fourth branch of treatment, which is referred to as immunotherapy, has more recently become established. Immunotherapies are designed to help the immune system recognize cancer cells, and/or to strengthen a response against cancer cells in order to destroy the cancer. Immunotherapies include active and passive immunotherapies. Active immuotherapies attempt to stimulate the body's own immune system to fight the disease. Passive immunotherapies generally do not rely on the patient's immune system

to attack the disease; instead, they use immune system components (such as antibodies) created outside of the patient's body.

[0006] The immune system can be categorized into two discrete effector arms, innate and adaptive immunity. Innate immunity involves numerous cellular components and soluble factors that respond immediately, but generally to foreign stimuli. Adaptive immunity is customized to respond specifically to precise epitopes from foreign agents. The adaptive immune response is further divided into two effector arms known as the humoral and cellular immune systems. The humoral arm is centered on the production of antibodies by B-lymphocytes while the cellular arm involves the cytolytic activity of cytotoxic T lymphocytes.

[0007] Cytotoxic T lymphocytes (CTL) do not recognize epitopes on the targeted antigens themselves. Rather, CTL detect fragments of antigens that are displayed on the surface of cells. As a result antigens are visible to CTL only after they have been processed by the cell and displayed on the surface of the cell. The antigen processing and display system of cells has been well established. CTL recognize short peptide antigens, which are displayed on the surface in non-covalent association with class I major histocompatibility complex molecules (MHC). These class I peptides are in turn derived from the degradation of cytosolic proteins.

[0008] Despite various types of cancer treatments, a continuing need exists for additional and more effective treatment alternatives. One such alternative envisions methodologies of medical treatment that require or benefit from an ability to initiate, stimulate, and/or enhance an immune response by immunization. These methodologies include those depending upon the creation of an immune response against a desired antigenic polypeptide and those that depend upon the initiation or modulation of an innate immune response. Thus one approach in the treatment of cancer is the manipulation of the immune system by use of a therapeutic anticancer vaccine.

[0009] To generate a vaccine or other immunogenic composition, an antigen or epitope against which an immune response can be mounted is introduced into a subject. Although neoplastic cancer cells are derived from and therefore are substantially identical to normal cells on a genetic level, many neoplastic cells are known to present tumor-associated antigens (TuAAs). These antigens can be used by a subject's immune system to recognize and attack the neoplastic cells as foreign. Unfortunately, neoplastic cells generally appear to be ignored by the host's immune system.

[0010] A number of different strategies have been developed in the art in an attempt to generate vaccines with activity against neoplastic cells; however, an effective and marketable product has not emerged. The present invention therefore serves to overcome the deficiencies in the art and provides a plurality of immunogenic compositions, disclosed herein, for targeting cancer or tumor cells.

SUMMARY OF THE INVENTION

[0011] The present invention relates to methods and compositions for inducing, entraining, and/or amplifying the immune response to MHC class-I restricted epitopes of carcinoma antigens to generate an effective anti-cancer immune response.

[0012] Embodiments of the disclosed invention are directed to the use of combinations of tumor-associated antigens (TuAAs) for the immunotherapy of patients with various types of cancer. In preferred embodiments, the TuAAs are antigens expressed by the cancer cell itself. Examples of such TuAAs are Melan-A, tyrosinase, SSX-2, NY-ESO-1, and PRAME. In alternate embodiments, the TuAAs are antigens associated with non-cancerous components of the tumor, such as tumor-associated neovasculature or other stroma. An example of such an antigen is PSMA – though, in prostate cancer PSMA is expressed by cancerous cells. In particularly preferred embodiments both types of antigen are targeted. Different aspects of the invention include the immunogenic compositions, their collection into defined products, and methods for their use.

[0013] Some specific embodiments relate to an immunogenic product comprising a plurality of compositions comprising one or more nucleic acid compositions and one or more peptide compositions; wherein the one or more nucleic acid compositions are capable of expressing one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; wherein the one or more peptide compositions consist essentially of said one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; and wherein the one or more peptides correspond to the epitopes expressed by the selected nucleic acids.

[0014] In some embodiments of the immunogenic product the one or more nucleic acid compositions comprise a plasmid selected from the group consisting of

pSEM, pBPL and pRP12. In some embodiments the peptide compositions comprise a peptide selected from the group consisting of SSX-2_{41-49} (SEQ ID NO. 1), its analogue KVSEKIFYV (SEQ ID NO. 5); NY-ESO-1 $_{157-165}$ (SEQ ID NO. 2), its analogue SNvaLMWITQV (SEQ ID NO. 6); $PRAME_{425-433}$ (SEQ ID NO. 3), its analogue S(Nva)LQHLIG(Nle) (SEQ ID NO. 7); $PSMA_{288-297}$ (SEQ ID NO. 4), its analogue GLPSIPVHPV (SEQ ID NO. 8); Melan-A_{26-35} (SEQ ID NO. 9), , its analogue ENvaAGIGILTV(SEQ ID NO. 11); $tyrosinase_{369-377}$(SEQ ID NO. 10), and its analogue yMdgtmsqNva(SEQ ID NO. 12). In some embodiments the plurality of compositions comprise: a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing a PRAME class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing a PSMA class I MHC restricted epitope, or analogue thereof; a peptide consisting essentially of said SSX-2 epitope, or analogue thereof; a peptide consisting essentially of said NY-ESO-1 epitope, or analogue thereof; a peptide consisting essentially of said PRAME epitope, or analogue thereof; and a peptide consisting essentially of said PSMA epitope, or analogue thereof.

[0015] In some embodiments of the immunogenic product the included nucleic acid molecules are part of the same composition. In some embodiments the nucleic acid molecules are the same. In some embodiments the nucleic acid molecule comprises a sequence encoding the liberation sequence of pBPL (SEQ ID NO. 13). In some embodiments the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pBPL (SEQ ID NO. 16). In some embodiments the nucleic acid molecule is pBPL (SEQ ID NO. 20). In some embodiments the nucleic acid molecule comprises a sequence encoding the liberation sequence of pRP12 (SEQ ID NO. 14). In some embodiments the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pRP12 (SEQ ID NO. 17). In some embodiments the nucleic acid molecule is pRP12 (SEQ ID. 21). In some embodiments the SSX-2 epitope is $SSX-_{241-49}$ (SEQ ID NO. 1). In some embodiments the NY-ESO-1 epitope is NY-ESO-$1_{157-165}$ (SEQ ID NO. 2). In some embodiments the PRAME epitope is $PRAME_{425-433}$ (SEQ ID NO. 3). In some embodiments the PSMA epitope is $PSMA_{288-297}$ (SEQ ID NO. 4). In some embodiments the SSX-2 analogue is KVSEKIFYV (SEQ ID NO. 5). In some embodiments the NY-ESO-1 analogue is SNvaLMWITQV (SEQ ID NO. 6). In some

embodiments the PRAME analogue in is S(Nva)LQHLIG(Nle) (SEQ ID NO. 7). In some embodiments the PSMA analogue in is GLPSIPVHPV (SEQ ID NO. 8).

[0016] In some embodiments of the immunogenic product the plurality of compositions comprise: a nucleic acid molecule capable of expressing a Melan-A class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing a Tyrosinase class I MHC restricted epitope, or analogue thereof; a peptide consisting essentially of said Melan-A epitope, or analogue thereof; and a peptide consisting essentially of said Tyrosinase epitope, or analogue thereof. In some embodiments the nucleic acid molecules are part of the same composition. In some embodiments the nucleic acid molecules are the same. In some embodiments the nucleic acid molecule comprises a sequence encoding the liberation sequence of pSEM (SEQ ID NO. 15). In some embodiments the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pSEM (SEQ ID NO. 18). In some embodiments the nucleic acid molecule is pSEM (SEQ ID NO. 19). In some embodiments the Melan-A epitope is Melan-A_{26-35} (SEQ ID NO. 9). In some embodiments the Tyrosinase epitope is $Tyrosinase_{369-377}$ (SEQ ID NO. 10). In some embodiments the immunogenic product further comprises: a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; and a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof. In some embodiments the immunogenic product also comprises a peptide consisting essentially of an NY-ESO-1 epitope. In some embodiments the immunogenic product also comprises a peptide consisting essentially of an SSX-2 epitope.

[0017] Embodiments of the current invention relate to compositions and methods for entraining and amplifying a T cell response. Such methods include an entraining 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 the composition is delivered to the lymphatic system (for example to a lymph node). The entrainment step can include one or more deliveries of that composition, for example, spread out over a period of time or in a continuous fashion over a period of time. The methods can further include an amplification step comprising administering a composition comprising a peptide immunogen such as $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1), $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2), PRAME $_{425-433}$ (SEQ ID NO. 3), $PSMA_{288-297}$ (SEQ ID NO. 4), Melan-A_{26-35} (SEQ ID NO. 9), $Tyrosinase_{369-377}$ (SEQ ID NO. 10),

and analogues thereof (as represented by SEQ. ID NOS. 5, 6, 7, 8, 11, and 12) having substantial similarity to the corresponding TuAA epitopes encoded by the nucleic acid composition. 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.

[0018] Further embodiments include those in which the disclosed plasmids are used individually or in any combination. The peptide compositions corresponding to these epitopes and part of the amplification portion of the immunization strategy can be native sequences or peptide analogues substantially similar to the native epitope sequence. The peptides can be incorporated into the amplification protocol individually or in combinations of 2, 3, 4, or more of the immunogens.

[0019] Still other embodiments can include alternate epitopes (such as those described in the U.S. Patent Application Serial No. 10/117,937, entitled "Epitope Sequences," filed on April 4, 2002 (Publication No. 20030220239 A1), which is hereby expressly incorporated by reference) substituted in similar combination as the epitopes expressed in the pSEM (SEQ ID NO. 19), pBPL (SEQ ID NO. 20), and pRP12 (SEQ ID NO. 21) plasmids and corresponding peptide immunogens administered as the amplification portion of the immunization strategy.

[0020] Embodiments of the invention can encompass, for example, two monovalent plasmids expressing single immunogens in place of one bivalent plasmid expressing both immunogens; a trivalent plasmid expressing three immunogens in place of one bivalent and one monovalent plasmid; a trivalent plasmid and one monovalent plasmid in place of a tetravalent plasmid; or two bivalent plasmids in place of a tetravalent plasmid. Embodiments can also encompass the use of the various plasmid combinations as part of the entrain step of the entrain-and-amplify immunization strategy.

[0021] Embodiments of the inventions can encompass a polypeptide or otherwise conjugated peptide that can be cleaved into individual peptides in the lymph and its use in the amplification step of the entrain-and-amplify immunization strategy.

[0022] Embodiments of the current invention 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, in a non-limiting example, 1-2 entraining doses. The method can include administering a plurality of entraining doses, wherein said doses are administered over a course of one to about 7 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, but is not limited to such.

[0023] Other embodiments relate to a method of treating carcinoma comprising a step of administering to a patient in need thereof a plurality of compositions including a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing a PRAME class I MHC restricted epitope, or analogue thereof; and a nucleic acid molecule capable of expressing a PSMA class I MHC restricted epitope, or analogue thereof. Another embodiment relates to the above-method further comprising a step of administering one or more peptides selected from the group epitopes or analogues consisting essentially of SSX-2, NY-ESO-1, PRAME, and PSMA.

[0024] A method of treating cancer comprising administering an immunogenic product comprising a plurality of compositions comprising one or more nucleic acid compositions and one or more peptide compositions; wherein the one or more nucleic acid compositions are capable of expressing one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; wherein the one or more peptide compositions consist essentially of said one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; and wherein the one or more peptides correspond to the epitopes expressed by the selected nucleic acids. Some embodiments relate to the use of the above method wherein the cancer is a breast cancer,

an ovarian cancer, a pancreatic cancer, a prostate cancer, a colon cancer, a bladder cancer, a lung cancer, a liver cancer, a stomach cancer, a testicular cancer, an uterine cancer, a brain cancer, a lymphatic cancer, a skin cancer, a bone cancer, a kidney cancer, a rectal cancer, a melanoma, a glioblastoma, or a sarcoma.

[0025] Still other embodiments relate to a method of treating cancer comprising a step of administering to a patient in need thereof a plurality of compositions comprising: a nucleic acid molecule capable of expressing a Melan-A class I MHC restricted epitope, or analogue thereof; a nucleic acid molecule capable of expressing a Tyrosinase class I MHC restricted epitope, or analogue thereof; a peptide consisting essentially of said Melan-A epitope, or analogue thereof; and a peptide consisting essentially of said Tyrosinase epitope, or analogue thereof. Other embodiments relate to the use of the method where the cancer to be treated to is glioblastoma or melanoma. Yet other embodiments include a further step of administering to a patient in need thereof a composition comprising: a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; and a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof; a peptide consisting essentially of said NY-ESO-1 epitope or analogue thereof; and a peptide consisting essentially of said SSX-2 epitope or analogue thereof.

[0026] Other embodiments relate to sets of immunogenic compositions for inducing an immune response in a mammal including, in a non-limiting manner, 1-6 entraining doses and at least one amplifying dose. In such embodiments, 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 by a 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, (e.g., a lymph node and the like). The nucleic acid can be a plasmid. The epitope can be a class I HLA epitope. 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 but is not limited to such. The immunogen can include a fragment of a target-associated antigen that can include an epitope cluster.

[0027] Further embodiments relate to the method of use of the entrain-and-amplify therapeutic compositions, tetravalent, bivalent, and/or monovalent plasmids and

corresponding peptide immunogens, in the treatment of carcinoma, including melanoma, comprising administration via lymph node injection (*i.e.*, directly into the organs where the immune responses are initiated and amplified) according to an optimized immunization schedule.

[0028] Yet further embodiments related to the manufacture of medicaments comprising the compositions of the invention. One embodiment relates to the manufacture of a medicament suitable for administration to the lymphatic system of a subject. Another embodiment relates to the manufacture of a medicament suitable for inducing an anti-cancer immune response in a subject. Another embodiment relates to the manufacture of a medicament that entrains and amplifies a T cell response in a subject. Another embodiment relates to the manufacture of a medicament suitable for treating carcinoma in a subject. Another embodiment relates to the use of one or more nucleic acid compositions capable of expressing one or more class I MHC restricted epitopes, or an analog thereof, and one or more peptide compositions corresponding to the said class I MHC restricted epitopes or analogues thereof, in the manufacture of a medicament suitable for inducing an anti-cancer immune response in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] **FIG. 1.** Tetramer analysis of pSEM/pBPL primed animals prior to peptide boost. Group 1, 2, and 3 animals (n=60) were primed with four injections of the pSEM/pBPL plasmid mixture on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes. Tetramer analysis was performed on day 25, 10 days following the final plasmid injection and compared to untreated naïve littermate controls (n=5). Tetramer values (Melan A, Tyrosinase, SSX-2, NY-ESO-1) represent the average +/- SEM.

[0030] **FIG. 2.** Melan-A/Tyrosinase, SSX-2/NY-ESO-1 tetramer analysis was performed on day 39 demonstrating a tetravalent immune response in individual animals. Animals were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of SSX2 $_{41\text{-}49}$ A42V (SEQ ID. NO. 5) in the left lymph node and Tyrosinase$_{369\text{-}377}$ V377Nva (SEQ ID. NO. 12) on in the right lymph node on days 28 and 32 (25 μg/day). Representative animals (n=3) from Group 2 are shown and compared to tetramer values for an untreated naïve littermate control.

[0031] **FIG. 3.** Tetramer analysis of pSEM/pBPL primed, SSX-2/Tyrosinase boosted animals. Melan-A/Tyrosinase, SSX-2/NY-ESO-1 tetramer analysis was

performed on day 39, 7 days following the last peptide injection. Group 1 animals (n=10) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) followed by a boost with a plasmid mixture of pBPL+pSEM on days 28 and 32 (100 μg/day). Group 2 and 3 animals (n=50) were primed with a plasmid mixture of pBPL+pSEM similar to Group 1 followed by a peptide boost consisting of SSX-2_{41-49} A42V (SEQ ID. NO. 5) in the left lymph node and Tyrosinase$_{369-377}$ V377Nva (SEQ ID. NO. 12) in the right lymph node on days 28 and 32 (25 μg/day). Average tetramer values (Melan A, Tyrosinase, SSX-2, and NY-ESO-1) were compared to untreated naïve littermate controls (n=5) and represent the average +/- SEM.

[0032] FIGs. 4A - 4B. IFN-γ ELISpot analysis following a first peptide boost **(FIG. 4A)**. ELISPOT analysis was performed on day 41. Group 1 animals (n=3 sacrificed) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) followed by a boost with a plasmid mixture of pBPL+pSEM on days 28 and 32 (100 μg/day). Group 2 and 3 animals (n=6 sacrificed) were primed with a plasmid mixture of pBPL+pSEM similar to Group 1 followed by a peptide boost consisting of SSX-2_{41-49} A42V (SEQ ID. NO. 5) in the left lymph node and Tyrosinase$_{369-377}$ V377Nva (SEQ ID. NO. 12) in the right lymph node on days 28 and 32 (25 μg/day). Antigen specific (Melan A, Tyrosinase, SSX-2, and NY-ESO-1) interferon-γ spot forming cells per spleen were compared to untreated naïve littermate controls (n=3); **FIG. 4A**. IFN-γ ELISpot analysis was performed in triplicate, values represent average +/- Stdev. Peptide stimulating concentration was at 10μg/ml and incubated for 42hrs. **FIG. 4B.** - IFN-γ ELISpot analysis following the second peptide boost. ELISPOT analysis was performed by sacrificing representative animals on day 63. Group 1 animals (n=3 sacrificed) received injections of a mixture of pBPL+pSEM on Days 1, 4, 15, 18, 28, 32, 49, and 53 (100 μg/day). Group 2 animals (n=4 sacrificed) received injections of a mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) followed by a peptide boost consisting of SSX-2_{41-49} A42V (SEQ ID. NO. 5) in the left lymph node and Tyrosinase $_{369-377}$ V377Nva (SEQ ID. NO. 12) in the right lymph node on days 28, 32, 49, and 53 (25 μg/day). Group 3 animals (n=7 sacrificed) received injections of a mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of SSX-2_{41-49} A42V (SEQ ID. NO. 5) in the left lymph node and Tyrosinase$_{369-377}$ V377Nva (SEQ ID. NO. 12) in the right lymph node on

days 28 and 32 (25μg/day) and a second peptide boost consisting of NY-ESO-$1_{157-165}$ L158Nva (SEQ ID. NO. 6), C165V (12.5 μg on days 49 and 53) in the left lymph node and Melan A_{26-35} A27Nva (SEQ ID. NO. 11) (25 μg on days 49 and 53) in the right lymph node. Antigen specific (Melan A, Tyrosinase, SSX-2, and NY-ESO-1) interferon-γ spot forming cells per spleen were compared to a untreated naïve littermate control **(FIG. 4B)**. IFN-γ ELISpot analysis was performed in triplicate, values represent average +/- SEM. Peptide stimulating concentration was at 10μg/ml and incubated for 42hrs.

[0033] FIG. 5. Depicts tetramer levels, IFN-γ ELISPOT and carboxy-fluorescein diacetate, succinimidyl ester (CFSE) histograms from *in vivo* studies where animals were challenged with human melanoma tumor cells expressing all four tumor associated antigens. Naïve control (**top left panel**); two animals with tetravalent immunity (**top right panel and lower left panel**); and an animal with a monovalent response to Melan A (**lower right panel**).

[0034] FIG. 6. Tetramer analysis of the "original" versus the "expanded" protocol. Animals were injected based on a "original protocol" (Groups 1-3) or an "expanded protocol" (Groups 4-6) with 4 injections of D1 (pRP12) plasmid (4mg/ml) in the right inguinal lymph node and 4 injections of D2 (pBPL) plasmid (4mg/ml) in left inguinal lymph node. Animals were subsequently boosted with PSMA, SSX-2, PRAME and NY-ESO-1 peptides. Animals were primed with D1 (pRP12) plasmid and D2 (pBPL) plasmid (4mg/ml) on days 1, 4, 15, and 18, followed by boosting with $PSMA_{288-297}$ (I297V) peptide (RLN) (SEQ ID. NO. 8) and SSX-2_{41-49} (A42V) peptide (LLN) (SEQ ID. NO. 5) on days 29 and 31 for the original protocol; and boosting with $PRAME_{425-433}$ (L426Nva, L433Nle)) peptide (RLN) (SEQ ID. NO. 7) and NY-ESO-$1_{157-165}$ (L158Nva, C165V) peptide (LLN) (SEQ ID. NO. 6) on days 42, 45 for the expanded protocol (Groups 4-6). Values represent average +/- SEM from individual animals after peptide boost and are compared to untreated naïve littermate controls (n=5).

[0035] FIG. 7. Tetravalent immune response from a representative animal in Group 1 (FIG. 6). Following plasmid priming, PSMA peptide (25μg) and PRAME peptide (20μg) were injected into the right lymph node. Twenty-five micrograms each of SSX-2 and NY-ESO-1 peptides were injected into the left lymph node. Data shown measures immune response by both tetramer and ELISpot assays.

[0036] **FIG. 8.** IFN-γ ELISPOT analysis of the "original" versus the "expanded" protocol. Total antigen specific (SSX-2, NY-ESO-1, PRAME, and PSMA) interferon-γ spot forming cells per spleen are shown comparing the "original" and "expanded" protocols comprised of low, medium and high peptide boosts. IFN-γ ELISpot analysis was performed in triplicate, values represent average +/- SEM after peptide boost. Splenocytes ($3x10^5$ cells per well) were stimulated, *ex vivo* in 96 well ELISpot plates, with peptide (SSX-2, NY-ESO-1, PRAME, and PSMA) at a concentration of 10μg/ml for 72hrs. Values are extrapolated from total nucleated splenocytes and normalized per spleen from each animal.

[0037] **FIG. 9.** ^{51}Cr cytotoxicity assays. **FIG. 9** depicts CTL response to $PRAME_{425-433}$ (SEQ ID. NO. 3), $PSMA_{288-297}$ (SEQ ID. NO. 4), NY-ESO-$1_{157-165}$ (SEQ ID. NO. 2) and SSX-2 $_{41-49}$ (SEQ ID. NO. 1) after DNA prime and peptide boost and one round of *in vitro* stimulation in immunized mice. Data are presented as follows: the x-axis shows the effector to target ratio; the y-axis shows the corresponding percentage specific lysis.

[0038] **FIG. 10.** Immune response elicited by two cycles of therapeutic regimens of the PP (PRAME and PSMA) regimen and the NS (NY-ESO-1 and SSX-2) regimen showing peptide dominance of PRAME.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0039] Embodiments of the present invention are based upon the induction of active immunity (therapeutic vaccination) preferably co-targeted against multiple molecules expressed by cancer cells and by the underlying neovasculature. This approach preferably involves targeted delivery of both recombinant DNA (plasmid) and synthetic peptides directly into the lymph nodes, thereby eliciting a strong cell-mediated immune response with the potential to ultimately interfere with the survival and/or viability of tumor cells within primary and metastatic lesions.

[0040] The methodology of the present invention includes the combined use of recombinant DNA plasmid and synthetic peptides, preferably administered using a prime (plasmid) / boost (peptide) approach via lymph node injection according to an optimized immunization schedule. In preferred embodiments, the lymph node injection is directly into the organism where the immune responses are initiated and amplified.

Embodiments of the current invention can be administered to patients with tumor tissue that express HLA-A2, particularly HLA-A*0201. It has been observed that by using this immunization protocol that not only can the plasmid initiate an immune response, it biases the response and its subsequent amplification toward an effector as opposed to a regulatory character. Without this prior nucleic acid-based immunization, the repeated administration of peptide leads to a response ever more dominated by regulatory T cells. The long-lived bias toward an effector response is termed entrainment.

[0041] The disclosed embodiments relating to entrain-and-amplify therapeutics for carcinoma and melanoma can be used to achieve a multivalent attack, offering the advantage of increasing the sensitivity of the tumor to attack. If more than a single antigen on a tumor cell is targeted, the effective concentration of antitumor agent is increased accordingly. Attack on stroma associated with the tumor, such as vasculature, can also increase the accessibility of the tumor cells to the agent(s) targeting them. Thus, even an antigen that is also expressed on some normal tissue can receive greater consideration as a target antigen if the other antigens to be targeted in a multivalent attack are not also expressed by that tissue.

[0042] Practice of such immunization protocols involving disparate forms of immunogen requires 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.

[0043] 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 indicated immunization protocol. 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 Serial No. 60/580,969, filed on June 17, 2004; U.S. Patent Application Serial No. 11/155,288 (Publication No 20060008468) filed June 17, 2005, and U.S. Patent Application Serial No. 11/323,964 filed December 29, 2005, all entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOSTICS FOR VARIOUS TYPES OF CANCERS"; and U.S. Provisional Patent Application Serial No. 60/580,964, and U.S. Patent Application Serial No. 11/155,928 (Publication No. 20050287068), both entitled "IMPROVED EFFICACY OF ACTIVE IMMUNOTHERAPY BY INTEGRATING DIAGNOSTIC WITH THERAPEUTIC METHODS", each of which is hereby incorporated by reference in its entirety.

[0044] Embodiments of the current invention encompass peptides incorporated into the amplification protocol individually or in combinations of 2, 3, 4, or more of the immunogens. Reasons for using less than all peptide epitopes include but are not limited to the following: 1) sub-optimal expression of any of the antigens; 2) the patient does not express, or no longer expresses, the corresponding antigen; 3) a less robust response is being generated to one or another of the epitopes, in which case such peptide(s) can be given in the absence of the others in order to obtain a more balanced response; 4) and a peptide can be discontinued if it is generating some sort of immunotoxicity.

I. Therapeutic Peptides and Plasmids of the Present Invention

A. Therapeutic Peptides and Analogues thereof

[0045] The present invention contemplates the use of multiple molecules expressed by cancer cells and by the neovasculature as therapeutics in the treatment of cancer. Such molecules include tumor-associated antigens (TuAAs) which are antigens expressed by the cancer cell itself or associated with non-cancerous components of the tumor, such as tumor-associated neovasculature or other stroma. TuAAs help to match a patient's cancer condition or type with an appropriate immunotherapeutic agent or

regimen. Non-limiting examples of TuAAs contemplated in the present invention include SSX-2, NY-ESO-1, PRAME, PSMA (prostate-specific membrane antigen), Melan-A, and tyrosinase. Therefore, in particular embodiments of the present invention, there is provided peptides, peptide analogues or epitopes of TuAAs (Table 1) as cancer therapeutics. In alternate embodiments of the present invention, the peptides can comprise the native sequence or be analogues of NY-ESO-1, SSX-2, Melan-A, tyrosinase, PRAME and PSMA, such as those disclosed in U.S. Provisional Application Serial Nos. 60/581,001, 60/580,962, and 60/691,889 and their corresponding Patent Application Serial Nos. 11/156,253 (Publication No. 20060063913), 11/155,929, (Publication No. 20060094661), 11/156,369 (Publication No. 20060057673), __/____ (Attorney Docket No. MANNK.052A filed on the same date as the present application), __/____ (Attorney Docket No. MANNK.052A2 filed on the same date as the present application) and __/____ (Attorney Docket No. MANNK.052A3 filed on the same date as the present application); and U.S. Patent Application Serial Nos. 11/156,369 (Publication No. 20060057673) and 11/156,253 (Publication No. 20060063913); each of which is hereby incorporated by reference in its entirety.

[0046] Tyrosinase, a melanin biosynthetic enzyme, is predominantly expressed in melanocytes with high levels often observed in melanomas. Therefore, tyrosinase is considered one of the most specific markers of melanocytic differentiation. It is also expressed in glial cells, which like melanocytes, develop from the neuroectoderm. Tyrosinase is thus also a useful TuAA for glioblastomas, including glioblastoma multiform. Further details of tyrosinase as a TuAA is disclosed in U.S. Patent No. 5,747,271, incorporated herein by reference in its entirety. In particular embodiments of the present invention there is provided the tyrosinase$_{369\text{-}377}$ epitope represented herein by SEQ. ID NO: 10 (Table 1).

[0047] Another TuAA employed in the present invention is Melan-A, also known as MART-1 (Melanoma Antigen Recognized by T cells). Melan-A/MART-1 is a melanin biosynthetic protein also expressed at high levels in melanomas. Melan-A/MART-1 is disclosed as a TuAA in U.S. Patent Nos. 5,994,523; 5,874,560; and 5,620,886, each of which is incorporated herein by reference in its entirety. In preferred embodiments of the present invention there is provided the Melan-A TuAA, Melan-A$_{26\text{-}35}$, represented herein by SEQ. ID NO: 9 (Table 1).

[0048] SSX-2, also known as Hom-Mel-40, is a member of a family of highly conserved cancer-testis (CT) antigens (Gure, A.O. *et al.*, *Int. J. Cancer* 72:965-971, 1997, which is incorporated herein by reference in its entirety). Cancer-testis antigens are found in a variety of tumors, but are generally absent from normal adult tissues except testis. Expression of different members of the SSX family has been found in various tumor cell lines. SSX-2 as a TuAA is disclosed in U.S. Patent No. 6,025,191, which is hereby incorporated by reference in its entirety. In particular embodiments of the present invention there is provided $SSX\text{-}2_{41\text{-}49}$ (SEQ. ID NO: 1) and an analogue thereof, SSX-2 Analogue (SEQ. ID NO: 5), Table 1.

[0049] NY-ESO-1, also known as CTAG-1 (Cancer-Testis Antigen-1) and CAG-3 (Cancer Antigen-3), is a cancer-testis antigen found in a wide variety of tumors. NY-ESO-1 as a TuAA is disclosed in U.S. Patent No. 5,804,381, which is incorporated herein by reference in its entirety. In preferred embodiments, the present invention provides epitopes of NY-ESO-1 and analogues thereof, as represented by SEQ. ID NO: 2 and SEQ. ID NO: 6 respectively (Table 1).

[0050] Another TuAA contemplated in the present invention is PRAME, also known as MAPE, DAGE, and OIP4. PRAME is known in the art as a cancer-testis (CT) antigen. However, unlike many CT antigens, such as: MAGE, GAGE and BAGE, it is expressed in acute myeloid leukemias. PRAME as a TuAA is disclosed in U.S. Patent No. 5,830,753, incorporated herein by reference in its entirety. In preferred embodiments, the present invention provides epitopes of PRAME and analogues thereof, as represented by SEQ. ID NO: 3 and SEQ. ID NO: 7 respectively (Table 1).

[0051] Yet another TuAA employed in the present invention is the prostate-specific membrane antigen (PSMA). PSMA is found to be highly expressed in prostate cancer cells. However, PSMA expression is also noted in normal prostate epithelium and in the neovasculature of non-prostatic tumors. PSMA as an anti-neovasculature preparation is disclosed in U.S. Provisional Patent Application Serial No. 60/274,063, and U.S. Patent Application Serial Nos. 10/094,699 (Publication No. 20030046714) and 11/073,347 (Publication No. 20050260234); each of which is incorporated herein by reference in its entirety. PSMA as a TuAA is described in U.S. Patent No. 5,538,866 incorporated herein by reference in its entirety. In preferred embodiments, the present invention provides epitopes of PSMA and analogues thereof, as represented by SEQ. ID NO: 4 and SEQ. ID NO: 8 respectively (Table 1).

TABLE 1. PARTIAL LISTING OF SEQ. ID NOS.

SEQ. ID NO.	IDENTITY	SEQUENCE
1	SSX-2_{41-49}	KASEKIFYV
2	NY-ESO-$1_{157-165}$	SLLMWITQC
3	$PRAME_{425-433}$	SLLQHLIGL
4	$PSMA_{288-297}$	GLPSIPVHPI
5	SSX-2 Analogue	KVSEKIFYV
6	NY-ESO-1 Analogue	SNvaLMWITQV
7	PRAME Analogue	SNvaLQHLIGNle
8	PSMA Analogue	GLPSIPVHPV
9	Melan-A_{26-35}	ELAGIGILTV

10	Tyrosinase$_{369-377}$	YMDGTMSQV
11	Melan-A Analogue	ENvaAGIGILTV
12	Tyrosinase Analogue	YMDGTMSQNva
13	pBPL plasmid liberation sequence	*IKASEKIFYV***SLLM WITQC***KASEKIFY VK*
14	pRP12 plasmid liberation sequence	KR-**SLLQHLIGL**-GDAAY-**SLLQHLIGL**-ISPEKEEQYIA-**SLLQHLIGL**-KRPSIKR-**GLPSIPVHPV**
15	pSEM plasmid liberation sequence	MLLAVLYCL-ELAGIGILTV-YMDGTMSQV
16	pBPL encoded immunogenic polypeptide	MSLLMWITQCKA SEKIFYVGLPSIPV HPIGLPSIPVHPIK ASEKIFYVSLLMW ITQCKASEKIFYV KASEKIFYVRCGA RGPESRLLEFYLA MPFATPMEAELA RRSLAQDAPPLPV PGVLLKEFTVSGN ILTIRLTAADHRQ LQLSISSCLQQLSL LMWITQCFLPVFL AQPPSGQRR
17	pRP12 encoded immunogenic polypeptide	MNLLHETDSAVA TARRPRWLCAGA LVLAGGFFLLGFL FGWFIKSAQLAG AKGVILYSDPAD YFAPGVKSYPDG WNLPGGGVQRG NILNLNGAGDPLT PGYPANEYAYRR GIAEAVGLPSIPV HPIALQSLLQHLIG LSNLTHVLYPVPL ESYEDIHGTLHLE RLAYLHARLRELL CELGRPSMVWLS ANPCPHCGDRTF YDPEPILCPCFMP NKRSLLQHLIGLG

		DAAYSLLQHLIGL ISPEKEEQYIASLL QHLIGLKRPSIKR *GLPSIPVHPV*
18	pSEM encoded immunogenic polypeptide	MLLAVLYCLELA GIGILTVYMDGT MSQVGILTVILGV LLLIGCWYCRRR NGYRALMDKSLH VGTQCALTRRCP QEGFDHRDSKVS LQEKNCEPV

[0052] The antigens of the invention, as discussed above, may be employed in various therapeutic regimens in treating a disease such as, but not limited to, cancer.

B. Immunogenic Compositions Comprising Plasmids in Combination with Peptides

[0053] As discussed above, the present invention provides immunogenic compositions for the treatment of cancer comprising plasmid(s) used in combination with synthetic peptide(s). Such an immunogenic protocol elicits a strong cell-mediated immune response to target a particular cancer thereby eliminating, eradicating or ameliorating the cancer in a subject. Preferred plasmids employed in the present invention are the pRP12 plasmid (SEQ ID NO. 21) (U.S. Provisional Patent Application No. 60/691,579 and the corresponding U.S Patent Application Serial No._____ (Attorney Docket No. MANNK.053A filed on the same date as the present application) both entitled "METHODS AND COMPOSITIONS TO ELICIT MULTIVALENT IMMUNE RESPONSES AGAINST DOMINANT AND SUBDOMINANT EPITOPES EXPRESSED ON CANCER CELLS AND TUMOR STROMA"), the pBPL plasmid (SEQ ID NO. 20), and the pSEM plasmid (SEQ ID NO. 19) disclosed in U.S. Provisional Patent Application No. 60/691,579 and U.S. Patent Application Serial No. 10/292,413 (Publication No. 20030228634) respectively; each of which is incorporated herein by reference in its entirety (Note that in those documents pSEM is referred to as pMA2M). Additional plasmids that can be used are disclosed in these references and in U.S. Patent Application Serial No. 10/225,568 (Publication No. 20030138808).

[0054] Thus, in various embodiments immunotherapeutic products comprise assemblages of immunogenic compositions. Such assemblages can comprise 1, 2, or 3

plasmids as a set of individual compositions or a single composition can comprise two or more plasmids. Such assemblages can also comprise multiple peptides corresponding to the epitopes expressed by the plasmids. Similarly, they can be provided as compositions comprising individual or multiple peptides. In some embodiments, an entraining plasmid or plasmids will be sold together with the corresponding amplifying peptides. In other embodiments, the multiple plasmids will be sold together, but without corresponding peptides. In still other embodiments sets of corresponding peptides will be sold together without the plasmid, for example, for subsequent rounds of amplification of the entrained response.

[0055] Therefore, in one particular embodiment of the present invention there is provided an assemblage comprising the pBPL plasmid (described in detail in U.S. Application Serial No. 10/292,413 (Publication No. 20030228634), entitled "EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS AND METHODS FOR THEIR DESIGN," which is hereby expressly incorporated by reference in its entirety) expressing the NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) and SSX-2_{41-49} (SEQ ID NO. 1) epitopes and the pRP12 plasmid (described in U.S. Provisional Application No. 60/691,579 U.S Patent Application Serial No._____ (Atty. Docket No. MANNK.053A, filed on the same date as the present application) both entitled "METHODS AND COMPOSITIONS TO ELICIT MULTIVALENT IMMUNE RESPONSES AGAINST DOMINANT AND SUBDOMINANT EPITOPES EXPRESSED ON CANCER CELLS AND TUMOR STROMA," which are hereby expressly incorporated by reference in their entirety) expressing the $PRAME_{425-433}$ (SEQ ID NO. 3) and $PSMA_{288-297}$ (SEQ ID NO. 4) epitopes. The liberation sequence for the pBPL and pRP12 plasmids are represented herein as SEQ ID NO.13 and 14 respectively, and are also disclosed in U.S. Patent Application Serial No. 10/212,413 (Publication No. 20030228634), incorporated herein by reference. The plasmids encode the epitopes in such a manner that they can be expressed and presented by pAPC.

[0056] In another particular embodiment of the present invention there is provided an assemblage comprising the pSEM plasmid, (described in detail and referred to as pMA2M in U.S. Patent Application Serial No. 10/292,413 (Publication No. 20030228634) incorporated herein by reference) expressing the Melan-A_{26-35} (SEQ ID NO. 9) and $tyrosinase_{369-377}$ (SEQ ID NO. 10) epitopes. The peptide analogues Melan-A_{26-35} A27Nva (SEQ ID NO. 11) and $tyrosinase_{369-377}$ V377Nva (SEQ ID NO. 12) are

disclosed in U.S. Patent Application Serial No. 11/156,369, and U.S. Provisional Patent Application Serial No. 60/691,889, both entitled "EPITOPE ANALOGS", each of which is hereby incorporated by reference in its entirety. The liberation sequence of this plasmid is represented herein as SEQ ID No.15 and is also disclosed in U.S. Provisional Patent Application No. 60/691,579, filed on June 17, 2005; and U.S. Patent Application __/_____, (Attorney Docket NO. MANNK.053A) filed on the same date as the present application, both entitled "METHODS AND COMPOSITIONS TO ELICIT MULTIVALENT IMMUNE RESPONSES AGAINST DOMINANT AND SUBDOMINANT EPITOPES, EXPRESSED ON CANCER CELLS AND TUMOR STROMA." The pSEM plasmid encodes the Melan-A and tyrosinase epitopes in a manner that allows for their expression and presentation by pAPCs.

[0057] In a further particular embodiment of the current invention there is provided an assemblage comprising the pBPL plasmid (described above) expressing the NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) and SSX-2_{41-49} (SEQ ID NO. 1) epitopes, and the pSEM plasmid (described above) (SEQ ID NO. 19) expressing the Melan-A_{26-35} (SEQ ID NO. 9) and tyrosinase$_{369-377}$ (SEQ ID NO. 10) epitopes. The peptide analogues Melan-A_{26-35} A27Nva (SEQ ID NO 11), tyrosinase$_{369-377}$ V377Nva (SEQ ID NO 12), SSX-2_{41-49} A42V (SEQ ID NO. 5), and NY-ESO-$1_{157-165}$ L158Nva, C165V (SEQ ID NO 6)are described in U.S. Provisional Application Serial No. 60/580,962; U.S. Patent Application Serial No. 11/155,929; U.S. Provisional Application Serial No. 60/581,001; U.S. Patent Application Serial No. 11/156,253; U.S. Patent Application Serial No. 11/156,369 and U.S. Provisional Patent Application Serial No. 60/691,889, each of which is hereby incorporated by reference in its entirety. The plasmids, pSEM (SEQ ID NO. 19) and pBPL (SEQ ID NO. 20), encode the respective epitopes (Melan-A, tyrosinase, NY-ESO-1, and SSX-2) in a manner that they can be expressed and presented by pAPCs.

[0058] Another particular embodiment of the current invention relates to the assemblage comprising the pRP12 plasmid (described above) expressing the PSMA$_{288-297}$ (SEQ ID NO. 4) and PRAME$_{425-433}$ (SEQ ID NO. 3) epitopes and the pSEM plasmid (described above) expressing the Melan-A_{26-35} (SEQ ID NO. 9) and tyrosinase$_{369-377}$ (SEQ ID NO. 10) epitopes. The peptide analogues Melan-A_{26-35} A27Nva (SEQ ID NO. 11), tyrosinase$_{369-377}$ V377Nva (SEQ ID NO. 12), PRAME$_{425-433}$ L426Nva, I433Nle (SEQ ID NO. 7), and PSMA$_{288-297}$ I297V (SEQ ID NO. 8) are described in U.S. Provisional Application Serial No. 60/580,962; U.S. Patent Application Serial No. 11/155,929; U.S.

Provisional Application Serial No. 60/581,001; U.S. Patent Application Serial No. 11/156,253; U.S. Patent Application Serial No. 11/156,369 and U.S. Provisional Patent Application Serial No. 60/691,889, each of which is hereby incorporated by reference in its entirety. Both plasmids encode their respective epitopes, (Melan-A, tyrosinase, PRAME, and PSMA), in such a manner that they can be expressed and presented by pAPCs.

[0059] In further embodiments, each of the assemblages above include the peptides corresponding (that is capable of amplifying the response to) to the epitopes expressed by those plasmids. Other particular embodiments comprise an individual plasmid and one or both corresponding peptides. (Although the specific plasmids referred to herein are described as bivalent, they can also be amplified in a monovalent fashion).

[0060] As referred to herein, a PP therapeutic regimen entails administration of plasmid and peptide to target the PRAME and PSMA antigens. Similarly, an MT regimen targets Melan-A/tyrosinase antigens and an NS therapeutic regimen targets NY-ESO-1 and SSX-2 antigens.

II. Cell Proliferative Diseases and Methods of Screening

[0061] The immunogenic compositions of the present invention, comprising a plasmid and one or more peptides or analogues thereof, can be administered in treating a cell proliferative disease such as cancer, in a subject. Cancers that may be treated using the immunogenic composition of the present invention include, for example, melanoma, lung cancer including: non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC), hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, leukemia, neuroblastoma, head and neck cancer, breast cancer, pancreatic cancer, renal cancer, bone cancer, testicular cancer, ovarian cancer, mesothelioma, cervical cancer, gastrointestinal cancer, lymphoma, colon cancer, bladder cancer and/or cancers of the blood, brain, skin, eye, tongue, gum. It is also anticipated that the immunogenic compositions of the present invention may be used to treat cell proliferative diseases other than cancer. Other cell proliferative diseases contemplated in the present invention may include, for example, dysplasias, pre-neoplastic lesions (*e.g.,* adenomatous hyperplasia, prostatic intraepithelial neoplasia, cervical dysplasia, colon polyposis), or carcinoma *in situ*, but is not limited to such.

[0062] Cells or tissue obtained from patients predisposed to, or having a cancer, can be screened in order to better determine the appropriate immunotherapeutic

regimen to administer to the patient. Such screening can include the steps of assaying the patient's tumor tissue for two or more expressed tumor associated antigens (TuAAs) in a preselected panel of antigens to develop an antigen profile for the tumor. An immunotherapeutic regimen can then be selected based on the antigen profile obtained. The regimen selected can comprise administering at least one immunotherapeutic agent targeting two, three, four, or more of the expressed antigens. The immunotherapeutic agent can comprise or encode an epitope restricted by the patient's class I MHC type, for each of two or more antigens expressed by the tumor. The antigen expression can be detected on neoplastic cells, or tumor-associated stromal cells, or both.

[0063] Immunotherapeutic regimens provided in the present invention include: the PP regimen where the target antigens are PRAME and PSMA; this regimen co-targets the vasculature and a cancer testes antigen. Another regimen provided by the present invention is the MT regimen where the target antigens are Melan-A and tyrosinase; this regimen targets tissue specific antigens associated with melanoma and glioblastoma. NS regimen of the invention relates to target antigens NY-ESO-1 and SSX-2 which are cancer testes antigens found with varying frequency in a wide variety of cancers. In other particular embodiments, of the invention, the regimens: PPNS (co-targeting PRAME, PSMA, NYESO-1, SSX2), NSMT (co-targeting NYESO-1, SSX2, Melan A and Tyrosinase) or PPMT (co-targeting PRAME, PSMA, Melan A, Tyrosinase) are provided.

[0064] A screening method employed in the present invention may include the steps of: assaying a patient's tumor tissue to detect one or more expressed polypeptides in a preselected panel, wherein the panel comprises two, or three, or four or more TuAAs and at least one lineage specific marker; and confirming the cancer diagnosis based on the assay. The panel can comprise of at least 2, 3, 4 or more TuAAs selected from the group consisting of NY-ESO-1, PRAME, PSMA, tyrosinase, melan-A/MART-1, and SSX protein. In some instances, the lineage specific marker can be a TuAA; alternatively, the lineage specific marker is not a TuAA. For example, in the case of melanoma and/or glioblastoma, the lineage specific marker can be tyrosinase, melan-A/MART-1, or gp100; in the case of prostate cancer, the lineage specific marker can be, PSA or PSMA.

[0065] As tumor antigen expression tends to be heterogeneous, any particular biopsy sample is likely not to give a complete indication of all the antigens expressed. Thus, it is not necessary that a patient's profile contain all of the antigens for treatment.

The screening methods employed in the present invention may include an assay of a tumor tissue of the corresponding presumptive type for expression of a preselected panel of antigens. In some instances, a panel of TuAAs assembled for one tumor type can be used to screen other tumor types that can express at least some of the same antigens and an expression profile developed.

[0066] The immunogenic compositions of the present invention can be administered to patients with tumor tissue that express HLA-A2, particularly HLA-A*0201.

[0067] Exemplary methodology for obtaining a profile of antigen expression of a particular tumor that can be used to determine which antigen or combination of antigens are useful in treating a particular cancer can be is found in U.S. Provisional Application Serial No. 60/580,969, filed June 17, 2004; U.S. Patent Application Serial No. 11/155,288 (Publication No. 20060008468), filed June 17, 2005; and U.S. Patent Application Serial No. 11/323,964, also filed on June 17, 2005, all entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOSTICS FOR VARIOUS TYPES OF CANCERS"; each incorporated herein by reference in its entirety. Specific antigenic combinations of particular benefit in directing an immune response against particular cancers are disclosed in U.S. Provisional Application Serial No. 60/479,554, filed on June 17, 2003, U.S. Patent Application Serial No. 10/871,708 (Publication No. 20050118186), filed on June 17, 2004, (both entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS"), and PCT Patent Application Publication No. WO 2004/112825, filed June 17, 2004; each of which is incorporated herein by reference in its entirety.

III. Entraining-and-Amplifying Therapeutics For Administration

[0068] In a preferred embodiment, the present invention provides a composition comprising the combined use of recombinant DNA plasmid and synthetic peptides, administered using a prime (plasmid)/boost (peptide) approach. Such a composition may be delivered via lymph node injection according to an optimized immunization schedule. Embodiments of the current invention can be administered to patients with tumor tissue that expresses HLA-A2, particularly HLA-A*0201. Therefore, the immunogenic compositions comprising a plasmid and one or more peptides or analogues thereof can be administered in treating a cancer in a subject. The disclosed

embodiments of the present invention relate to entrain-and-amplify therapeutics for carcinoma, including melanoma, that can be used to achieve a multivalent attack, offering the advantage of increasing the sensitivity of the tumor to attack.

[0069] Therefore, in particular embodiments, the present invention provides multivalent entraining-and-amplifying therapeutics for the treatment of cancer. Such multivalent therapeutics may target more than one antigen on a tumor cell. In instances where more than a single antigen on a tumor cell is targeted, the effective concentration of antitumor therapeutic is increased accordingly. Attack on stroma associated with the tumor, such as vasculature, can increase the accessibility of the tumor cells to the agent(s) targeting them. Thus, even an antigen that is also expressed on some normal tissue can receive greater consideration as a target antigen if the other antigens to be targeted in a multivalent attack are not also expressed by that tissue.

A. Bivalent entrain-and-amplify therapeutic

[0070] An embodiment of the present invention relates to a bivalent entrain-and-amplify therapeutic for melanoma. Therefore, in the current invention there is provided an assemblage comprising the pSEM plasmid and peptides corresponding to Melan-$A_{26\text{-}35}$ (SEQ ID NO. 9) and tyrosinase$_{369\text{-}377}$ (SEQ ID NO. 10) epitopes administered as the MT regimen against melanoma. In preferred embodiments, the peptide analogues Melan-$A_{26\text{-}35}$ A27Nva (SEQ ID NO. 11) and/or tyrosinase$_{369\text{-}377}$ V377Nva (SEQ ID NO. 12) are utilized in the amplification step. The entrain-and-amplify protocol employed in the present invention is as disclosed above.

[0071] The pSEM plasmid assemblage can be delivered in a manner similar to that discussed above for the tetravalent entrain-and-amplify therapeutic for melanoma. Melanoma patients can be screened according to the methods disclosed herein and the MT regimen utilized with patients whose tumor antigen profile includes Melan-A and/or tyrosinase. Administration of the peptide boost can involve one or both of the antigens expressed by the plasmids.

[0072] Similarly the PP and NS regimens can be used for bivalent therapy using assemblages comprising pRP12 and peptides corresponding to the $PSMA_{288\text{-}297}$ (SEQ ID NO. 4) and $PRAME_{425\text{-}433}$ (SEQ ID NO. 3) epitopes, and pBPL and peptides corresponding to the NY-ESO-$1_{157\text{-}165}$ (SEQ ID NO. 2) and SSX-$2_{41\text{-}49}$ (SEQ ID NO. 1) epitopes, respectively. These bivalent regimens can be combined to create treatments of

higher valency, selected embodiments of which are described below, and various sets of immunogenic compositions assembled to support them.

B. Tetravalent Entraining-and-Amplifying Therapeutics

[0073] One embodiment of the current invention relates to a tetravalent entrain-and-amplify therapeutic for carcinoma. Therefore, in one particular embodiment of the present invention there is provided an assemblage comprising the pBPL plasmid expressing the NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) and SSX-2_{41-49} (SEQ ID NO. 1) epitopes and the pRP12 plasmid expressing the $PRAME_{425-433}$ (SEQ ID NO. 3) and $PSMA_{288-297}$ (SEQ ID NO. 4) epitopes (referred to herein as the PP regimen), each administered as the entraining immunogens of an immunization strategy. An "entraining" immunogen as contemplated in the present invention includes in many embodiments an induction that confers particular stability on the immune profile of the induced lineage of T cells.

[0074] Additionally, four peptide compositions corresponding to the NY-ESO-1, SSX-2, PRAME and PSMA epitopes are administered as the amplification portion of the same immunization strategy as that of the entraining immuogen. In a preferred embodiment, the peptide analogues NY-ESO-$1_{157-165}$ L158Nva, C165V (SEQ ID NO. 6); SSX-2_{41-49} A42V (SEQ ID NO. 5) ; $PSMA_{288-297}$ I297V (SEQ ID NO. 8) ; and/or $PRAME_{425-433}$ L426Nva, L433Nle (SEQ ID NO. 7) are utilized in the amplification step. As contemplated in the present invention, the term "amplifying or amplification", as of a T cell response, includes in many embodiments 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.

[0075] The entrain-and-amplify protocol employed in the present invention is described in greater detail in U.S. Provisional Application No. 60/640,402, U.S. Patent Application Serial No. 10/871,707 (Publication No. 20050079152), and U.S. Patent Application Serial No. 11/323,572, each entitled "METHODS TO ELICIT, ENHANCE AND SUSTAIN IMMUNE RESPONSES AGAINST MHC CLASS I-RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSES" each of which is incorporated herein by reference in their entirety.

[0076] In preferred embodiments of the present invention, the plasmids are administered intranodally as an entraining immunogen to the inguinal lymph nodes, one to the left side and one to the right. Subsequently, the peptides are sequentially administered intranodally as amplifying immunogens, two on separate days to the left

node and the other two on separate days to the right node. It is preferred, but not required, that the peptides be administered to the same lymph node that received the plasmid encoding the corresponding epitopes.

[0077] Carcinoma patients, especially those with ovarian, colorectal, pancreatic, or renal cell carcinoma, can be screened according to the methods disclosed herein and PP and or NS therapeutic regimens administered to patients whose tumor profile includes PRAME, PSMA, NY-ESO-1, and/or SSX-2. It is noted that the NY-ESO-1 epitope is also found in LAGE 1a/s, so the presence of this antigen in a profile can also be considered in the tumor profile. As tumor antigen expression tends to be heterogeneous, any particular biopsy sample is likely not to give a complete indication of all the antigens expressed. Thus, it is not necessary that a patient's profile contain all four of the antigens for that patient to be a candidate for treatment with therapeutics of the invention. However, it is preferred that the profile contain 2, 3, or 4 of the antigens.

C. Tetravalent Entraining-and-Amplifying Therapeutics for Melanoma

[0078] An embodiment of the present invention relates to a tetravalent entrain-and-amplify therapeutic for melanoma, comprising the plasmids pSEM and pBPL and the corresponding peptides. The pSEM plasmid encodes the A27L analogue of the Melan-A_{26-35} (SEQ ID NO. 9) epitope and the native tyrosinase (tyrosinase$_{369-377}$ (SEQ ID NO. 10)) epitope sequence (referred to herein as the MT regimen). The pBPL plasmid encodes NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) and SSX-2_{41-49} (SEQ ID NO. 1) native sequences. The assemblage comprising the plasmid is administered as the entraining portion of an immunization strategy against melanoma. Additionally, four peptide compositions corresponding to the NY-ESO-1, SSX-2, Melan-A and tyrosinase epitopes are administered as the amplification portion of the same immunization strategy. In a preferred embodiment, the peptide analogues Melan-A_{26-35} A27Nva (SEQ ID NO. 11), tyrosinase$_{369-377}$ V377Nva (SEQ ID NO. 12), SSX-2_{41-49} A42V (SEQ ID NO. 5), and NY-ESO-$1_{157-165}$ L158Nva, C165V (SEQ ID NO. 6) are utilized in the amplification step.

[0079] For treatment of a cancer such as melanoma, the plasmids are administered intranodally to the inguinal lymph nodes as entraining immunogens. Subsequently the peptides are administered intranodally, preferably one to the left node, the other to the right on any particular day as amplifying immunogens. Melanoma patients can be screened according to the methods disclosed herein and the appropriate regimens administered to patients whose tumor antigen profile includes Melan-A and/or

tyrosinase. Administration of the peptide boost can involve 2, 3, or 4 of the antigens expressed by the plasmids.

D. Tetravalent Entraining-and-Amplifying Therapeutics for Glioblastoma

[0080] In a further particular embodiment of the present invention there is provided a tetravalent entrain-and-amplify therapeutic applicable to melanoma that is applied to other cancers such as glioblastoma. One such embodiment relates to the composite pRP12 plasmid (described above) expressing the $PSMA_{288-297}$ (SEQ ID NO. 4) and $PRAME_{425-433}$ (SEQ ID NO. 3) epitopes and the pSEM plasmid (described above) expressing the Melan-A_{26-35} (SEQ ID NO. 9) and $tyrosinase_{369-377}$ (SEQ ID NO. 10) epitopes administered as the entraining portion of an immunization strategy. Additionally, four peptide compositions corresponding to the PSMA, PRAME, Melan-A and tyrosinase epitopes are administered as the amplification portion of the same immunization strategy. In a preferred embodiment, the peptide analogues Melan-A_{26-35} A27Nva (SEQ ID NO. 11) , $tyrosinase_{369-377}$ V377Nva (SEQ ID NO. 12), $PRAME_{425-433}$ L426Nva, I433Nle (SEQ ID NO. 7), and $PSMA_{288-297}$ I297V (SEQ ID NO. 8) are utilized in the amplification step.

[0081] Cancer patients can be screened according to the methods disclosed herein and the PP and/or MT regimens administered to patients whose tumor antigen profile includes PRAME, PSMA, Melan-A and/or tyrosinase. Administration of the peptide boost can involve 2, 3, or 4 of the antigens expressed by the plasmids.

IV. Methods of Delivering Compositions of the Present Invention

[0082] In the present invention, the preferred administration of the immunogenic composition comprising recombinant DNA plasmid as a prime and synthetic peptide(s) as a boost, is via lymph node injection. The plasmid (prime) may be administered separately from the peptide (boost). Embodiments of the present invention can encompass two monovalent plasmids expressing single immunogens in place of one bivalent plasmid expressing both immunogens. In other embodiments, a trivalent plasmid expressing three immunogens in place of one bivalent and one monovalent plasmid may be employed. In some instances a trivalent plasmid and one monovalent plasmid in place of a tetravalent plasmid; or two bivalent plasmids in place of a tetravalent plasmid may be employed. Whichever combination of the compositions of the invention is employed, lymph node injection is preferred as it allows for delivery directly into the organs where

the immune responses are initiated and amplified according to an optimized immunization schedule.

[0083] To introduce the immunogenic composition into the lymphatic system of the patient the composition is preferably directed to a lymph vessel, lymph node, the spleen, or other appropriate portion of the lymphatic system. In some embodiments each component is administered as a bolus. In other embodiments one or more components are delivered by infusion, generally over several hours to several days. Preferably, the composition is directed to a lymph node such as an inguinal or axillary node by inserting a catheter or needle to the node and maintaining the catheter or needle throughout the delivery. Suitable needles or catheters are available made of metal or plastic (*e.g.*, polyurethane, polyvinyl chloride (PVC), TEFLON, polyethylene, and the like). In inserting the catheter or needle into the inguinal node for example, the inguinal node is punctured under ultrasonographic control using a Vialon™ Insyte W™ cannula and catheter of 24G3/4 (Becton Dickinson, USA) which is fixed using Tegaderm™ transparent dressing (Tegaderm™, St. Paul, MN, USA). This procedure is generally done by an experienced radiologist. The location of the catheter tip inside the inguinal lymph node is confirmed by injection of a minimal volume of saline, which immediately and visibly increases the size of the lymph node. The latter procedure allows confirmation that the tip is inside the node. This procedure can be performed to ensure that the tip does not slip out of the lymph node and can be repeated on various days after implantation of the catheter. In the event that the tip does slip out of location inside the lymph node, a new catheter can be implanted.

[0084] The therapeutic composition(s) of the present invention may be administered to a patient in a manner consistent with standard vaccine delivery protocols that are well known to one of ordinary skill in the art. Methods of administering immunogenic compositions of the present invention comprising plasmids and peptides or peptide analogues of TuAAs include, without limitation, transdermal, intranodal, perinodal, oral, intravenous, intradermal, intramuscular, intraperitoneal, and mucosal administration, delivery by injection or instillation or inhalation. A particularly useful method of vaccine delivery to elicit a CTL response is disclosed in Australian Patent No. 739189; U.S. Patent Nos. 6,994,851 and 6,977,074 both entitled "A METHOD OF INDUCING A CTL RESPONSE".

[0085] Various parameters need to be taken into account in delivering or administering an immunogenic composition to a subject. In addition, a dosage regimen and immunization schedule may be employed. Generally the amount of the components in the therapeutic composition will vary from patient to patient and from antigen to antigen, depending on such factors as: the activity of the antigen in inducing a response; the flow rate of the lymph through the patient's system; the weight and age of the subject; the type of disease and/or condition being treated; the severity of the disease or condition; previous or concurrent therapeutic interventions; the capacity of the individual's immune system to synthesize antibodies; the degree of protection desired; the manner of administration and the like, all of which can be readily determined by the practitioner.

[0086] In general the therapeutic composition may be delivered at a rate of from about 1 to about 500 microliters/hour or about 24 to about 12000 microliters/day. The concentration of the antigen is such that about 0.1 micrograms to about 10,000 micrograms of the antigen will be delivered during 24 hours. The flow rate is based on the knowledge that each minute approximately about 100 to about 1000 microliters of lymph fluid flows through an adult inguinal lymph node. The objective is to maximize local concentration of vaccine formulation in the lymph system. Some empirical investigation on patients may be necessary to determine the most efficacious level of infusion for a given vaccine preparation in humans.

[0087] In particular embodiments, the immunogenic composition of the present invention may be administered as a plurality of sequential doses. Such a plurality of doses may be 2, 3, 4, 5, 6 or more doses as is needed. In further embodiments of the present invention, it is contemplated that the doses of the immunogenic composition would be administered within about seconds or minutes of each other into the right or left inguinal lymph nodes. For example, the plasmid (prime) may first be injected into the right lymph node followed within seconds or minutes by a second plasmid into the left inguinal lymph node. In other instances the combination of one or more plasmids expressing one or more immunogens may be administered. It is preferred that the subsequent injection following the first injection into the lymph node be within at about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more minutes but not greater than about 30, 40, 50, or 60 minutes of the first injection. Similar considerations apply to the administration of two peptides individually to the right and left lymph nodes. It may be desirable to administer the plurality of doses of the immunogenic composition of the invention at an interval of

days, where several days (1, 2, 3, 4, 5, 6, or 7, or more days) lapse between subsequent administrations. In other instances it may be desirable for subsequent administration(s) of the compositions of the invention to be administered via bilateral inguinal lymph node injection within about 1, 2, 3, or more weeks or within about 1, 2, 3, or more months following the initial dose administration.

[0088] Administration may be in any manner compatible with the dosage formulation and in such amount as will be therapeutically effective. An effective amount or dose of an immunogenic composition of the present invention is that amount needed to provide a desired response in the subject to be treated.

V. EXAMPLES

[0089] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the methodology disclosed in the examples which follow represent methodologies discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that one can make many changes to the specific disclosed embodiments and still obtain a like or similar result within the spirit and scope of the invention.

EXAMPLE 1

EXPERIMENTAL PROCEDURE

Animals

[0090] Since the immune response against human T cell epitopes cannot be studied in original nonclinical models due to the inherent MHC-restriction of immunity, a genetically manipulated mouse model was chosen that expresses the human A*0201 gene (Pascolo *et al.*, 1997), which is frequently expressed in the human population. In contrast to immune deficient mice (the basis for xenograft models), the A*0201 transgenic model (HHD) is immune competent, thus allowing the evaluation of active immunotherapeutic strategies.

[0091] Therefore, female H-2 class I-negative (knockout) HLA-A2.1-transgenic HHD mice, 8-12 weeks of age, were used in these studies. The animals were housed under pathogen-free conditions.

Methodology

[0092] The bivalent pSEM plasmid (non-replicating recombinant DNA) encoding for the tumor-associated antigens Melan-A_{26-35} (SEQ ID NO. 9) and $Tyrosinase_{369-377}$ (SEQ ID NO. 10) and pBPL bivalent plasmid encoding for tumor-associated antigens SSX-2_{41-49} (SEQ ID NO. 1) and NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) were evaluated regarding the ability to prime a Tc1 (gamma interferon-producing) immune response in Examples 2-6. The Melan-A_{26-35} (A27Nva; ENvaAGIGILTV (SEQ ID NO. 11)) peptide analogue, $Tyrosinase_{369-377}$ (V377Nva; YMDGTMSQNva (SEQ ID NO. 12)) peptide analogue, NY-ESO-$1_{157-165}$ (L158Nva, C165V; S(Nva)LMWITQV (SEQ ID NO. 6)) peptide analogue, and SSX-2_{41-49} (A42V; KVSEKIFY (SEQ ID NO. 5)) peptide analogue were used for subsequent boosting.

[0093] 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). The Melan A_{26-35} (A27Nva) (SEQ ID NO. 11) analogue was formulated in PBS at 1.0mg/ml concentration. Similarly, the $Tyrosinase_{369-377}$ (V377Nva) (SEQ ID NO. 12) analogue was formulated in PBS at 1.0mg/ml concentration. The SSX-2_{41-49} (A42V) (SEQ ID NO. 5) analogue was formulated in PBS at 1.0mg/ml concentration while the NY-ESO-$1_{157-165}$ (L158Nva, C165V) (SEQ ID NO. 6) 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.

Intranodal Delivery of Plasmids and Peptides

[0094] The dose preparations were administered via bilateral inguinal intranodal injection on Days 1, 4, 15, 18, 29, 32, 49, and 53 of the study. Mice were anesthetized by inhalation of isoflurane and surgeries were conducted under aseptic conditions. Following preparation for surgery, an incision 0.5-1 cm in length was made in the inguinal fold exposing the inguinal lymph node. A maximum volume of 25 µL (25 µg in a 1 mg/mL solution) of plasmid or peptide was injected directly into the right and left inguinal lymph node using a 0.5 mL insulin syringe. The incision was closed with sterile 6.0 nylon skin sutures.

Plasmid (prime) / Peptide (boost) Immunization Schedule

[0095] Three groups of female HHD animals were immunized as described above with a mixture of pSEM/pBPL (100 µg/day) to the bilateral inguinal lymph nodes.

Group 1 (n=10 mice) received plasmid injections on Days 1, 4, 15, 18, 28, 32, 49, and 53; Group 2 and Group 3 (n=25 mice per group) received plasmid injections on Days 1, 4, 15, and 18 respectively as shown in Table 2 (below).

[0096] Animals from Group 2 were boosted in the right lymph node with Tyrosinase V377Nva (SEQ ID NO. 12) (25 μg/day) and in the left lymph node with SSX-2 A42V (SEQ ID NO. 5) (25 μg/day) peptides on days 28, 32, 49, and 53. Group 3 animals were boosted in the right lymph node with Tyrosinase V377Nva (SEQ ID NO. 12) (25 μg/day) and in the left lymph node with SSX-2 A42V (SEQ ID NO. 5) (25 μg/day) peptides on days 28 and 32 then were boosted in the right lymph node with NY-ESO-1 L158Nva, C165V (SEQ ID NO. 6) (12.5 μg/day) and in the left lymph node with Melan A A27Nva (SEQ ID NO. 11) (25 μg/day) peptides on days 49 and 53 as also shown in Table 2.

Table 2. Immunization Schedule

Group	N*	Plasmids (prime)			Peptide (boost)		
		Plasmids	Days	Each Dose	Peptide and Lymph Node (R/L)	Days	Each Dose
1	10	pSEM + pBPL	1, 4, 15, 18, 28, 32, 49, 53	100 μg	--	--	--
2	25	pSEM + pBPL	1, 4, 15, 18	100 μg	Tyrosinase (R)	28, 32, 49, 53	25 μg
					SSX-2 (L)	28, 32, 49, 53	25 μg
3	25	pSEM + pBPL	1, 4, 15, 18	100 μg	Tyrosinase (R)	28, 32	25 μg
					SSX-2 (L)	28, 32	25 μg
					NY-ESO-1 (R)	49, 53	12.5 μg
					Melan A (L)	49, 53	25 μg

Tetramer analysis

[0097] Enumeration of $CD8^+$ antigen-specific T cells requires cognate recognition of the T cell receptor (TCR) by a Class I MHC / peptide complex. This can be accomplished using Class I MHC tetramers which are composed of a complex of four HLA MHC Class I molecules each bound to the specific peptide and conjugated with a fluorescent protein. Thus tetramer assays allow quantitation of the total T cell population specific for a given peptide complexed in a particular MHC molecule. Flow cytometry is employed in quantifying binding of cells with the appropriate T cell receptor to the labeled tetramers. Furthermore, since binding does not depend on functional pathways, this population includes all specific $CD8^+$ T-cells regardless of functional status.

[0098] The CTL response was measured in animals immunized as described in the above plasmid/peptide immunization schedule, 7 days following the last plasmid (Day 25) and peptide immunizations (Days 39 and 60). Mononuclear cells were isolated from peripheral blood after density centrifugation (Lympholyte Mammal, Cedarlane Labs), and stained with HLA-A*0201 SSX-2 (KASEKIFYV (SEQ ID NO. 1))-PE MHC tetramer (Beckman Coulter, T02001), HLA-A*0201 NY-ESO (SLLMWITQC (SEQ ID NO. 2))-APC MHC tetramer (Beckman Coulter, T02001), HLA-A*0201 Melan A (ELAGIGILTV (SEQ ID NO. 9))-PE MHC tetramer (Beckman Coulter, T02001), HLA-A*0201 Tyrosinase (YMDGTMSQV (SEQ ID NO. 10))-APC MHC tetramer (Beckman Coulter, T02001). These cells were then co-stained using FITC conjugated rat anti-mouse CD8a (Ly-2) monoclonal antibody (BD Biosciences, 553031). Data were collected using a BD FACS Calibur flow cytometer and analyzed using Cellquest software by gating on the lymphocyte population and calculating the percent of tetramer positive cells within the $CD8^+$ CTL population.

Interferon-γ (IFN- γ) ELISpot assay

[0099] Instead of measuring cytotoxicity, the $CD8^+$ CTL response can be assessed by measuring IFN-γ production by specific effector cells in an ELISPOT assay. In this assay, antigen-presenting cells (APC) are immobilized on the plastic surface of a microtiter well and effector cells are added at various effector:target ratios. The binding of APCs by antigen-specific effector cells triggers the production of cytokines including IFN- γ by the effector cells. The cells can be stained to detect the presence of intracellular IFN- γ and the number of positively staining foci (spots) counted under a microscope.

[0100] Spleens were isolated on days 27 and 62 from euthanized animals, subjected to the plasmid/peptide immunization schedule as described above. The mononuclear cells, after density centrifugation (Lympholyte Mammal, Cedarlane Labs, Burlington, NC), were resuspended in HL-1 medium. Splenocytes ($5x10^5$, or $3x10^5$ cells per well) were incubated with 10μg of Melan-A_{26-35} (SEQ ID NO. 9) , $Tyrosinase_{369-377}$ (SEQ ID NO.) , $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1) , or $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2) natural peptide in triplicate wells of a 96 well filter membrane plates (Multiscreen IP membrane 96-well plate, Millipore, Boston, MA). Samples were incubated for 42 hours at 37°C with 5% CO_2 and 100% humidity prior to development. Mouse IFN-γ coating antibody (IFN-γ

antibody pair, U-CyTech Biosciences, The Netherlands) was used as a coating reagent prior to incubation with splenocytes, followed by the accompanied biotinylated detection antibody. GABA conjugate and proprietary substrates from U-CyTech 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.

^{51}Chromium-release assay

[0101] The chromium release assay, is a well known assay for evaluating CTL activity. Briefly, target cells expressing antigen on their surface are labeled with a radioactive isotope of chromium (^{51}Cr). Patient cells are then mixed with the target cell and incubated for several hours. Lysis of antigen-expressing cells release ^{51}Cr into the medium. Cell-specific lysis is calculated by comparing lysis of target cells expressing the antigen(s) of interest or control antigen(s) in the presence or absence of patient effector cells, and is usually expressed as the % specific lysis.

EXAMPLE 2

IMMUNIZATION WITH PLASMIDS PSEM AND PBPL PRIOR TO PEPTIDE BOOST

[0102] The purpose of this study was to determine whether immunization with the plasmids pSEM and pBPL could induce a tetravalent response against the four tumor associated antigens SSX-2_{41-49} (SEQ ID NO. 1) , NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) , Melan-A_{26-35} (SEQ ID NO. 9) and Tyrosinase$_{369-377}$ (SEQ ID NO. 10).

[0103] Three groups of female HHD animals (H-2 class I-negative (knockout) HLA-A2.1-transgenic HHD mice, 8-12 weeks of age) were immunized with a mixture of pSEM/pBPL (100 μg/day) to the bilateral inguinal lymph nodes. Group 1 (n=10 mice) received plasmid injections on Days 1, 4, 15, 18, 28, 32, 49, and 53; Group 2 and Group 3 (n=25 mice per group) received plasmid injections on Days 1, 4, 15, and 18 respectively (Table 2; above). On day 25, blood was collected from the immunized animals, and $CD8^+$ T cell analysis was performed using a tetramer assay as discussed elsewhere herein. Responses were compared to naïve littermate control mice (n=5).

[0104] **FIG. 1** shows tetramer data from animals that were primed with four injections of a mixture of the pSEM and pBPL bivalent plasmids (n=60), which are designed to encode for Melan-A_{26-35} (SEQ ID NO. 9) / tyrosinase$_{369-377}$ (SEQ ID NO. 10) ,

and NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) / SSX-2_{41-49} (SEQ ID NO. 1) respectively, prior to peptide boost. Animals primed with four injections of the pSEM/pBPL plasmid mixture at a daily dose of 100 μg exhibited a trivalent SSX-2, NY-ESO-1, and Melan-A response (Groups 1-3, n=60 total) but failed to generate any tyrosinase specific CTLs as measured by tetramer assay. In addition, Melan-A and NY-ESO-1 were revealed to be the dominant epitopes expressed by the bivalent plasmids pSEM and pBPL respectively, as shown in **FIG.1**.

EXAMPLE 3

INDIVIDUAL IMMUNIZATION WITH PLASMID PRIMED SSX-2/TYROSINASE

[0105] It was assessed whether boosting with the subdominant epitope peptides alone following plasmid priming was sufficient to achieve a tetravalent immune response. Therefore, animals from Group 2 above, were boosted with the sub-dominant epitopes, tyrosinase V377Nva (SEQ ID NO. 12) and SSX-2 A42V (SEQ ID NO. 5) peptide analogues and immune responses were compared to a naïve control.

[0106] Animals were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of SSX-2_{41-49} A42V (SEQ ID NO. 1) in the left lymph node and $Tyrosinase_{369-377}$ V377Nva (SEQ ID NO. 12) on in the right lymph node on days 28 and 32 (25 μg/day). On day 39, seven days following the last peptide injection, blood was collected from the immunized animals, and $CD8^+$ T cell analysis was performed using a tetramer assay as discussed elsewhere herein.

[0107] **FIG. 2** shows the tetravalent responses from peripheral blood on day 39 following the Tyrosinase and SSX-2 peptide boost, generated in three representative immunized animals as compared to a selected naïve control animal using a tetramer flow cytometry assay. For example, animal 2 demonstrated tetramer responses specific to SSX-2 (5.8%), NY-ESO-1 (4.1%), tyrosinase (8.7%) and Melan A (10.8%). These data taken together, represent specific CTL responses comprised of 29.4% of the total $CD8^+$ T cell repertoire. Furthermore, the results show that boosting with the subdominant epitope peptides alone, following plasmid priming, was sufficient to achieve a tetravalent immune response.

EXAMPLE 4

IMMUNIZATION WITH PLASMID PRIMED SSX-2/TYROSINASE

[0108] In order to generate a more balanced tetravalent immune response, animals were boosted with the sub-dominant peptide epitopes Tyrosinase$_{369-377}$ (V377Nva) (SEQ ID NO. 12) and SSX-2$_{41-49}$ (A42V) (SEQ ID NO. 5) (Groups 2 and 3, n=50) and immune responses were compared to animals boosted with a mixture of pSEM/pBPL plasmid (Group 1, n=10) or naïve controls (n=10).

[0109] Melan-A/Tyrosinase, SSX2/NY-ESO-1 tetramer analysis (as described above), was performed on day 39, seven days following the last peptide injection. Group 1 animals (n=10) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) followed by a boost with a plasmid mixture of pBPL+pSEM on days 28 and 32 (100 μg/day) in bilateral inguinal lymph nodes. Group 2 and 3 animals (n=50) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of SSX2$_{41-49}$ A42V (SEQ ID NO. 5) in the left lymph node and Tyrosinase$_{369-377}$ V377Nva (SEQ ID NO. 12) in the right lymph node on days 28 and 32 (25 μg/day).

[0110] Average tetramer values for Melan A, Tyrosinase, SSX2, and NY-ESO-1 were compared to untreated naïve littermate controls (n=5) and represent the average +/- SEM. **FIG. 3** shows the immune responses prior to and following the tyrosinase and SSX-2 boost for Groups 2 and 3 (n=50) compared to Group 1 (n=10; plasmid alone).

[0111] Following the plasmid boost, the predominant immune response was Melan-A and NY-ESO-1 specific (Group 1), as observed in FIG. 1. On the other hand, animals primed with the plasmid mixture and boosted with the subdominant peptides boosted their tyrosinase response >2 fold and SSX-2 response >2.5 fold, thereby establishing a balanced tetravalent immune response **FIG. 3**.

[0112] Thus, the data shows that a balanced tetravalent immune response was achieved by boosting with the sub-dominant epitope peptides, Tyrosinase$_{369-377}$ (V377Nva) (SEQ ID NO. 12), and SSX-2$_{41-49}$ (A42V) (SEQ ID NO. 5).

EXAMPLE 5

IFN-γ ELISPOT ANALYSIS OF FIRST AND SECOND PEPTIDE BOOST

[0113] The tetramer data obtained in the above Examples was confirmed by measuring the frequency of interferon gamma producing (IFNγ) cells following the peptide boost in select animals from Groups 2. IFN-γ ELISpot analysis was conducted following the first peptide boost **(FIG. 4A)** and a second peptide boost **(FIG. 4B)**

[0114] ELISPOT analysis, as described elsewhere herein, was performed by sacrificing representative animals on day 41, nine days following the last peptide boost. Group 1 animals (n=3 sacrificed) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) followed by a boost with a plasmid mixture of pBPL+pSEM on days 28 and 32 (100 μg/day) in bilateral inguinal lymph nodes. Group 2 animals (n=6 sacrificed) were primed with a plasmid mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of $SSX2_{41-49}$ A42V (SEQ ID NO. 5) in the left lymph node and $Tyrosinase_{369-377}$ V377Nva (SEQ ID NO. 12) in the right lymph node on days 28 and 32 (25 μg/day). Antigen specific (Melan A, Tyrosinase, SSX2, and NY-ESO-1) interferon-γ spot forming cells per spleen were compared to untreated naïve littermate controls (n=3), **FIG. 4A**.

[0115] Following the second peptide boost, ELISPOT analysis was performed by sacrificing representative animals on day 63, ten days following the second peptide boost. Group 1 animals (n=3 sacrificed) received injections of a mixture of pBPL+pSEM on days 1, 4, 15, 18, 28, 32, 49, and 53 (100 μg/day) in bilateral inguinal lymph nodes. Group 2 animals (n=4 sacrificed) received injections of a mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of $SSX2_{41-49}$ A42V in the left lymph node and $Tyrosinase_{369-377}$ V377Nva in the right lymph node on days 28, 32, 49, and 53 (25 μg/day). Group 3 animals (n=4 sacrificed) received injections of a mixture of pBPL+pSEM on days 1, 4, 15, and 18 (100 μg/day) in bilateral inguinal lymph nodes followed by a peptide boost consisting of $SSX2_{41-49}$ A42V (SEQ ID NO. 5) in the left lymph node and $Tyrosinase_{369-377}$ V377Nva (SEQ ID NO. 12) in the right lymph node on days 28 and 32 (25 μg/day) and a second peptide boost consisting of NY-ESO-$1_{157-165}$ L158Nva, C165V (SEQ ID NO. 6) (12.5 μg on Days 49 and 53) in the left lymph node and Melan A_{26-35} A27Nva (SEQ ID NO. 11) (25 μg on Days 49 and 53) in the right lymph node. Antigen specific (Melan A,

Tyrosinase, SSX2, and NY-ESO-1) interferon-γ spot forming cells per spleen were compared to a untreated naïve littermate control **FIG. 4B**.

[0116] **FIG. 4A** shows that animals primed with the plasmid mixture and boosted with tyrosinase and SSX-2 peptides ((Group 2, n=6) demonstrated a robust tetravalent response of 2 to 8 fold higher more IFNγ producing cells than plasmid alone treated animals (Group 1, n=3). In addition, when the animals received a second boost of either the subdominant epitope peptides, SSX-2 and Tyrosinase (Group 2), or the dominant epitope peptides, NY-ESO-1 and Melan A (Group 3), a tetravalent response was maintained as compared to animals that were primed and boosted with the pSEM and pBPL plasmid combination alone (Group 1) **(FIG. 4B)**. A more balanced immune response against all four antigens was achieved simply by boosting with the subdominant epitope analogues SSX-2 and Tyrosinase.

[0117] Overall, the data obtained from the above Examples (2-5), depict the successful generation of a tetravalent immune response in animals immunized with the NS and/or MT regimens of the present invention. A comparison of the immune responses (tetramer and IFN-γ ELISPOT analysis) in naïve animals or animals boosted with a mixture of pSEM/pBPL plasmid alone (Group 1) to animals boosted with the sub-dominant peptide epitopes tyrosinase and SSX-2 (Groups 2 and 3) on days 28 and 32 confirmed the successful generation of a tetravalent immune response in animals immunized with this regimen. Similar results were obtained following the second peptide boost on days 49 and 53 in where Group 3 (n=25) was boosted with the dominant epitope peptides, Melan $A_{26\text{-}35}$ (A27Nva) (SEQ ID NO. 11) and NY-ESO-$1_{157\text{-}165}$ (L158Nva, C165V) (SEQ ID NO. 6) and Group 2 (n=25) was boosted again with the sub-dominant epitope peptides Tyrosinase$_{369\text{-}377}$ (V377Nva) (SEQ ID NO. 12) and SSX-$2_{41\text{-}49}$ (A42V) (SEQ ID NO. 5).

EXAMPLE 6

GENERATION OF AN IMMUNE RESPONSE TO HUMAN MELANOMA

[0118] The carboxy-fluorescein diacetate, succinimidyl ester (CFSE) assay provides a simple and sensitive means for fluorescently labeling cells. This method allows for the analysis of antigen specific and non-specific T cell proliferation.

[0119] The CFSE methodology was employed to evaluate the efficacy of the immunization protocols. Animals, selected based on their tetramer levels, were analyzed for their ability to clear human CFSE labeled melanoma tumor cells in the lung.

[0120] On day 61, two animals from each group (Group 1, 2, and 3) were selected based on high tetramer levels, and injected intravenously with CFSE labeled tumor cells. More precisely, human 624.38 cultured melanoma tumor cells ($10x10^6$), expressing all four tumor associated antigens for SSX-2, NY-ESO-1, Tyrosinase, and Melan A, were stained with CFSEhi (Vybrant CFDA SE cell tracer kit, Molecular Probes) fluorescence (1.0 μM for 15 minutes) and co-injected intravenously into Group 1, 2, or 3 immunized mice (N = 2/group) or into naïve HHD mice (N=2) with an equal ratio of 624.28 HLA-A2 negative control cells stained with CFSElo fluorescence (0.1 μM). Animals received a second injection of target cells two hours later.

[0121] The specific elimination of human target cells was measured on day 62, approximately 14 hours after the injection of target cells, by sacrificing the mice, removing lung tissue, and measuring CFSEhi relative to CFSElo fluorescence (FL1 channel) by flow cytometry. The formula used to calculate the percent specific lysis is shown below.

[0122] [(1-%CFSEhi / %CFSElo) in immunized - (1-%CFSEhi / %CFSElo) in naïve] x 100

[0123] **FIG. 5** shows tetramer levels, IFNγ ELISPOT results, and two peak CFSE histograms from a naïve control (top left panel), two animals with tetravalent immunity (top right and lower left panel), and an animal with a monovalent response to Melan A (lower right panel). As expected, the naïve control animal was unable to clear the target cells as demonstrated by the maintenance of an equal ratio of both histogram peaks as was the case in the animal demonstrating the monovalent immune response. On the other hand, animals displaying an immune response to all four antigens were much more capable of clearing the human melanoma tumor target cells with 71% and 95% specific lysis.

EXAMPLE 7

GENERATION OF AN IMMUNE RESPONSE BY A ORIGINAL VS. EXPANDED PROTOCOL

[0124] It was assessed whether immunization with the plasmids D1 (pRP12) and D2 (pBPL) could induce a tetravalent response in HHD-1 mice against four tumor-

associated antigens: $PSMA_{288-297}$ (SEQ ID NO. 4), $PRAME_{425-433}$ (SEQ ID NO. 3), $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1), and $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2).

[0125] Two different boosting strategies were tested with regard to their ability to enhance the desired immune responses. The first approach (the "original" protocol) utilized a single injection of each peptide during the boosting procedure. The second approach (the "expanded" protocol) tested two injections of each peptide. Three dosage levels of each peptide (low, mid, and high) were tested in an effort to determine a dose-response relationship and to help define the optimum peptide concentration.

[0126] Six groups of 10 female HHD-1 animals/group were immunized with plasmids D1 and D2 injected directly into the bilateral inguinal lymph nodes. Animals from Groups 1-3 were boosted using the "original" protocol, and Groups 4-6 animals were boosted using the "expanded" protocol.

[0127] Animals on the "original protocol" (Groups 1-3, n=10 per group) received 4 injections of D1 (pRP12 (SEQ ID NO. 21)) plasmid (100 μg per dose) in the right inguinal lymph node and 4 injections of D2 (pBPL (SEQ ID NO. 20)) plasmid (100 μg/dose) in left inguinal lymph node on days 1, 4, 15 and 18. This was followed by a boost with $PSMA_{288-297}$ (I297V) (SEQ ID NO. 8) in the right lymph node and $SSX\text{-}2_{41-49}$ (A42V) (SEQ ID NO. 5)in the left lymph node on day 29, and with $PRAME_{425-433}$ (L426Nva, L433Nle) (SEQ ID NO. 7) in the right lymph node and $NY\text{-}ESO\text{-}1_{157-165}$ (L158Nva, C165V) (SEQ ID NO. 6) in the left lymph node on day 32.

[0128] Animals on the "expanded protocol" (Groups 4-6, n=10 per group) received 4 injections of D1 (pRP12 (SEQ ID NO. 21)) plasmid (100 μg/dose) in right inguinal lymph node and D2 (pBPL (SEQ ID NO. 20)) plasmid (100 μg/dose) in left inguinal lymph node on days 1, 4, 15, and 18. The animals were subsequently boosted with $PSMA_{288-297}$ (I297V) (SEQ ID NO. 8) in the right lymph node and $SSX\text{-}2_{41-49}$ (A42V) (SEQ ID NO. 5) in the left lymph node on days 29 and 32 and with $PRAME_{425-433}$ (L426Nva, L433Nle) (SEQ ID NO. 7) in the right lymph node and $NY\text{-}ESO\text{-}1_{157-165}$ (L158Nva, C165V) (SEQ ID NO. 6) in the left lymph node on days 43 and 46.

[0129] Blood was collected from each group in both protocols, 7 days following the last peptide boost, and $CD8^+$ T cell analysis was performed using a tetramer assay **(FIG. 6)**. Responses were compared to naïve littermate control mice (n=5). SSX-2,

NY-ESO-1, PRAME, and PSMA tetramer values are shown comparing the original and expanded protocols comprised of low, medium and high peptide boosts in **FIG. 6**.

[0130] Animals primed with four injections of D1 and D2 plasmid and subsequently boosted with the peptide analogues PSMA, PRAME, NY-ESO-1, and SSX-2 demonstrated immune responses to all four antigens, as assessed by tetramer analysis (**FIG. 6**), that was dominated by immune responses to PRAME and PSMA. In addition, tetravalent immune responses elicited by this immunization strategy was demonstrated in individual animals (**FIG 7**). The responses were observed to be independent of boosting regimen (original vs. expanded). In addition, no apparent dose-response was observed, although the high dose group (25μg peptide) in each therapeutic protocol yielded the highest response rate. Furthermore, the tetramer data indicated that PRAME and PSMA were the dominant epitopes following immunization of the animals.

EXAMPLE 8

IFN- Γ ELISPOT OF AN IMMUNE RESPONSE BY A ORIGINAL VS. EXPANDED PROTOCOL

[0131] To confirm the results observed with the tetramer assay, an interferon-γ (IFN-γ) ELISpot assay was conducted. Animals from each group in Example 7, were sacrificed 22 days following the last peptide boost and spleens were removed for IFN-γ ELISPOT analysis.

[0132] Spleens were isolated on day 68 from euthanized animals and the mononuclear cells, after density centrifugation (Lympholyte Mammal, Cedarlane Labs, Burlington, NC), were resuspended in HL-1 medium. Splenocytes (3×10^5 or 1.5×10^5 cells per well) were incubated with 10μg of $PSMA_{288-297}$ (SEQ ID NO. 4), $PRAME_{425-433}$ (SEQ ID NO. 3), $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1), or $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2), natural peptide in triplicate wells of a 96 well filter membrane plates (Multi-screen IP membrane 96-well plate, Millipore, MA). Samples were incubated for 72 hours at 37°C with 5% CO_2 and 100% humidity prior to development. Mouse IFN-γ coating antibody (IFN-γ antibody pair, U-CyTech Biosciences, The Netherlands) was used as coating reagent prior to incubation with splenocytes, followed by the accompanied biotinylated detection antibody. GABA conjugate and proprietary substrates from U-CyTech Biosciences 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.

[0133] The IFNγ ELISPOT results shown in **FIG. 7** correlate well with the tetramer data (FIG.6) and confirm a robust immune response to $PRAME_{425-433}$ (SEQ ID NO. 3), $PSMA_{288-433}$ (SEQ ID NO. 4), $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1), and $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2) elicited by the "original" therapeutic protocol. The "expanded" protocol did not appear to offer any apparent advantage over the "original" protocol as measured by IFN-γ ELISPOT analysis.

EXAMPLE 9

TETRAVALENT IMMUNE RESPONSE GENERATED BY THE PP/NS THERAPEUTIC REGIMEN

[0134] It was assessed whether a tetravalent immune response can be elicited by first boosting with the subdominant epitopes PSMA and SSX-2 followed by boosting with the dominant epitopes PRAME and NY-ESO-1. A representative animal from Group 1 ("original protocol"; high dose) received 4 injections of D1 (pRP12 (SEQ ID NO. 21)) plasmid (100 μg/dose) in the right inguinal lymph node and 4 injections of D2 (pBPL (SEQ ID NO. 20)) plasmid (100 μg/dose) in left inguinal lymph node on days 1, 4, 15 and 18. This was followed by a boost with peptides, $PSMA_{288-297}$ (I297V) (SEQ ID NO. 8) in the right lymph node (25 μg) and $SSX\text{-}2_{41-49}$ (A42V) (SEQ ID NO. 5)in the left lymph node (25 μg) on day29 and with $PRAME_{425-433}$ (L426Nva, L433Nle) (SEQ ID NO. 7)in the right lymph node (20 μg) and $NY\text{-}ESO\text{-}1_{157-165}$ (L158Nva, C165V) (SEQ ID NO. 6) in the left lymph node (25 μg) on day 32. The data (**FIG. 8**) shows a tetravalent immune response as measured by two separate assays, tetramer and ELISpot analyses.

EXAMPLE 10

^{51}CHROMIUM-RELEASE ASSAY MEASURING CTL ACTIVITY TO PRAME, PSMA, NY-ESO AND SSX-2

[0135] CTL response to $PRAME_{425-433}$ (SEQ ID NO. 3), $PSMA_{288-297}$ (SEQ ID NO. 4), $NY\text{-}ESO\text{-}1_{157-165}$ (SEQ ID NO. 2) and $SSX\text{-}2_{41-49}$ (SEQ ID NO. 1), using ^{51}Cr cytotoxicity assays, after DNA prime and peptide boost and one round of *in vitro* stimulation in immunized mice was assessed. CTLs were generated by *ex vivo* stimulation of splenocytes harvested from immunized mice (N=6) 22 days after the completion of the peptide immunization regimens.

[0136] Briefly, mice were sacrificed and the spleens were removed. The spleens were homogenized and the cell suspension was strained to yield a single-cell suspension. Quantities of $5x10^6$ cells/well were plated in 24 well tissue culture plates and $1.5x10^6$ peptide-pulsed, γ-irradiated and LPS (lipopolysaccharide) blasted B cells were added to each well. Mouse recombinant IL-2 was also added at a concentration of 1 ng/ml. The cells were incubated for 4 days for the PRAME group and 6 days for each of the PSMA, SSX-2 and NY-ESO-1 groups.

[0137] After the *ex vivo* stimulation, CTLs were collected from the plates, washed, and plated into 96 well U-bottom micro-titer assay plates at concentrations of 10^6, $3.3x10^5$, and $1.1x10^5$ cells/well in a total of 100 µL per well. To assess peptide specific lysis, T2 cells were labeled with ^{51}Cr and pulsed with 20 µg/mL of each peptide (SSX-2, NY-ESO-1, PSMA, or PRAME) at 37°C for 1.5 hours. After the incubation, the cells were washed and resuspended. Ten thousand ^{51}Cr-labeled and peptide-pulsed T2 cells were added to each well. The cells were then incubated at 37°C for 4 hours.

[0138] After incubation, supernatants were harvested and the cytolytic activity was measured in triplicate samples using a gamma counter. The corrected percent lysis was calculated for each concentration of effector cells, using the mean cpm for each replicate of wells **(FIG. 8)**. Percent specific lysis was calculated using the following formula: Percent release =100 x (Experimental release-spontaneous release) /(Maximum release - spontaneous release). Data are presented as follows: the x-axis shows the effector to target ratio; the y-axis shows the corresponding percentage specific lysis.

[0139] The results **(FIG. 8)** show ^{51}Chromium release assay (CRA) data for CTL from each group against T2 cells pulsed with $PRAME_{425-433}$ (SEQ ID NO. 3) (panel 1), $PSMA_{288-297}$ (SEQ ID NO. 4) (panel 2), NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) (panel 3), or SSX-2_{41-49} (SEQ ID NO. 1) (panel 4) peptides as targets. Specific lysis values were compared to un-pulsed T2 control cells. Given that the ELISA analysis (data not shown) indicated that immunogenicity of the PRAME group is very strong and to avoid antigen-induced cell deaths, the CRA for the PRAME group was pursued following a 4-day IVS protocol. The CRA was done following 6 days IVS for the other peptide groups. It was found that after *in vitro* re-stimulation, T cells isolated from all immunized groups specifically killed T2 cells pulsed with peptide in contrast with those from naïve animals. CTL responses to $PRAME_{425-433}$ (SEQ ID NO. 3), $PSMA_{288-297}$ (SEQ ID NO. 4), SSX-2_{41-}

$_{49}$ (SEQ ID NO. 1) and NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) were induced in all groups, as assessed by ^{51}Cr cytotoxicity assays. These CTLs had no effect on T2 control cells without peptide. The results demonstrated that T2 target cell lysis by the CTLs isolated from immunized mice is peptide specific. Compared to the "original" protocol, the "expanded" protocol offered no significant enhancement of the lysis percentage, further suggesting that the "original" protocol is sufficient for eliciting a substantial immune response against multiple antigens. Furthermore, due to the increased sensitivity of the CRA assay, the specific NY-ESO-1 responses from each group were more prevalent as compared to the tetramer and ELISPOT assays.

EXAMPLE 11

EMPLOYING MULTIPLE THERAPEUTIC CYCLES

[0140] It was assessed whether immunization with the plasmids D1 (pRP12 (SEQ ID NO. 21)) and D2 (pBPL (SEQ ID NO. 20)) could maintain robust immune responses in HHD-1 mice against four tumor-associated antigens: $PSMA_{288-297}$ (SEQ ID NO. 4), $PRAME_{425-433}$ (SEQ ID NO. 3), SSX-2_{41-49} (SEQ ID NO. 1), and NY-ESO-$1_{157-165}$ (SEQ ID NO. 2) after more than one cycle of a therapeutic regimen of the present invention.

[0141] Male and female HHD-1 mice were immunized with plasmids D1 and D2 injected directly into the bilateral inguinal lymph nodes followed by peptide boost with $PSMA_{288-297}$ (SEQ ID NO. 4), $PRAME_{425-433}$ (SEQ ID NO. 3), SSX-2_{41-49} (SEQ ID NO. 1), and NY-ESO-$1_{157-165}$ (SEQ ID NO. 2). Animals received 4 injections of D1 (pRP12 (SEQ ID NO. 21)) plasmid in the right inguinal lymph node and 4 injections of D2 (pBPL (SEQ ID NO. 20)) plasmid in left inguinal lymph node on days 1, 4, 15 and 18. This was followed by a boost with $PSMA_{288-297}$ (I297V) (SEQ ID NO. 8) in the right lymph node and SSX-2_{41-49} (A42V) (SEQ ID NO. 5) in the left lymph node on day 29, and with $PRAME_{425-433}$ (L426Nva, L433Nle) (SEQ ID NO. 7) in the right lymph node and NY-ESO-$1_{157-165}$ (L158Nva, C165V) (SEQ ID NO. 6) in the left lymph node on day 32. The second prime (plasmid) / boost (peptide) therapeutic cycle was repeated following a rest period of 14 days.

[0142] Animals were injected with plasmid vehicle (N=16 animals/group); peptide vehicle (N=16 animals/group); plasmid (400μg total dose) at high dose (N=16 animals/group); peptide (25μg total dose) at high dose (N=16 animals/group); plasmid (400μg total dose) at high dose + peptide (5μg total dose) at low dose (N=16

animals/group); plasmid at low dose (100μg total dose) + peptide (25μg total dose) at high dose (N=14 animals/group); or plasmid (400μg total dose) at high dose + peptide (25μg total dose) at high dose (N=16 animals/group) and compared to the naïve control group N=7 animals/group.

[0143] Animals from each group, were sacrificed 14 days following the last peptide boost and spleens were removed for IFN-γ ELISPOT analysis **(FIG. 9)**.

[0144] The data show that animals can generate robust immune responses following two cycles of therapeutic regimens of the PP (PRAME and PSMA) regimen and the NS (NY-ESO-1 and SSX-2) regimen.

[0145] Overall, the data obtained in Examples 7-11 shows significant T cell, but no significant antibody responses, following the PP/NS therapeutic immunization protocol. No peptide-specific antibodies were detected in the serum of immunized mice using an ELISA assay following one complete therapeutic cycle (data not shown). Furthermore, antigen-specific T cell responses encompassed effector and memory T cells (IFNγ cytokine producing, cytolytic and tetramer binding) with PRAME and PSMA leading and SSX-2 and NY-ESO-1 trailing in magnitude. In addition the results indicate, that while expanding the therapeutic protocol may not achieve higher T cell immunity, reordering of subdominant relative to the dominant peptides within a therapeutic cycle may be needed to improve on immunity against NY-ESO-1 or any other subdominant epitope.

[0146] In addition to those already disclosed in this application, the following applications are hereby expressly incorporated by reference in their entireties. Useful methods for using the disclosed analogs in inducing, entraining, maintaining, modulating and amplifying class I MHC-restricted T cell responses, and particularly effector and memory CTL responses to antigen, are described in U.S. Patent Nos. 6,994,851 (2/7/06) and 6,977,074 (12/20/2005) both entitled "A Method of Inducing a CTL Response"; U.S. Provisional Application No. 60/479,393, filed on June 17, 2003, entitled "METHODS TO CONTROL MHC CLASS I-RESTRICTED IMMUNE RESPONSE"; and U.S. Patent Application No. 10/871,707 (Pub. No. 2005 0079152) and Provisional U.S. Patent Application No. 60/640,402 filed on December 29, 2004, both entitled "Methods to elicit, enhance and sustain immune responses against MHC class I-restricted

epitopes, for prophylactic or therapeutic purpose". The analogs can also be used in research to obtain further optimized analogs. Numerous housekeeping epitopes are provided in U.S. Application Nos. 10/117,937, filed on April 4, 2002 (Pub. No. 20030220239 A1), and 10/657,022 (20040180354), and in PCT Application No. PCT/US2003/027706 (Pub. No. WO04022709A2), filed on September 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 on March 7, 2002; and 60/409,123, filed on September 5, 2002; each of which applications is entitled "Epitope Sequences". The analogs can further be used in any of the various modes described in those applications. Epitope clusters, which may comprise or include the instant analogs, are disclosed and more fully defined in U.S. Patent Application No. 09/561,571, filed on April 28, 2000, entitled EPITOPE CLUSTERS. Methodology for using and delivering the instant analogs is described in U.S. Patent applications 09/380,534 and 6977074 (Issued December 20, 2005) and in PCT Application No. PCTUS98/14289 (Pub. No. WO9902183A2), each entitled A "METHOD OF INDUCING A CTL RESPONSE". Beneficial epitope selection principles for such immunotherapeutics are disclosed in U.S. Patent Application Nos. 09/560,465, filed on April 28, 2000, 10/026,066 (Pub. No. 20030215425 A1), filed on December 7, 2001, and 10/005,905 filed on November 7, 2001, all entitled "Epitope Synchronization in Antigen Presenting Cells"; 6 , 861 , 234 (issued 01-Mar-2005; app. # 09/561,074) , 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, entitled "Anti-Neovasculature Preparations for Cancer"; Application Nos. 10/117,937 (Pub. No. 20030220239 A1) and PCTUS02/11101 (Pub. No. WO02081646A2), both filed on April 4, 2002, and both entitled "EPITOPE SEQUENCES"; and Application Nos. 10/657,022 and PCT Application No. PCT/US2003/027706 (Pub. No. WO04022709A2), both filed on September 5, 2003, and both entitled "EPITOPE SEQUENCES". Aspects of the overall design of vaccine plasmids are disclosed in U.S. Patent Application Nos. 09/561,572, filed on April 28, 2000, entitled "Expression Vectors Encoding Epitopes of Target-Associated Antigens" and 10/292,413 (Pub. No.20030228634 A1), filed on November 7, 2002, entitled "Expression Vectors Encoding Epitopes of Target-Associated Antigens and Methods for their Design"; 10/225,568 (Pub No. 2003-0138808), filed on August 20, 2002, PCT Application No. PCT/US2003/026231 (Pub. No. WO 2004/018666), filed on August 19,

2003, both entitled "EXPRESSION VECTORS ENCODING EPITOPES OF TARGET-ASSOCIATED ANTIGENS"; and U.S. Patent No. 6,709,844, entitled "AVOIDANCE OF UNDESIRABLE REPLICATION INTERMEDIATES IN PLASMID PROPAGATION". Specific antigenic combinations of particular benefit in directing an immune response against particular cancers are disclosed in Provisional U.S. patent Application No. 60/479,554, filed on June 17, 2003 and U.S. Patent Application No. 10/871,708, filed on June 17, 2004 and PCT Patent Application No. PCT/US2004/019571 (Pub. No. WO 2004/112825), all entitled "Combinations of tumor-associated antigens in vaccines for various types of cancers". 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 No. 10/094,699 (Pub. No. 20030046714 A1), filed March 7, 2002, entitled "Anti-Neovasculature Preparations for Cancer". Methods to trigger, maintain, and manipulate immune responses by targeted administration of biological response modifiers are disclosed U.S. Provisional Application No. 60/640,727, filed on December 29, 2004. Methods to bypass CD4+ cells in the induction of an immune response are disclosed in U.S. Provisional Application No. 60/640,821, filed on December 29, 2004. Exemplary diseases, organisms and antigens and epitopes associated with target organisms, cells and diseases are described in U.S. Application No. 6977074 (issued December 20, 2005) filed February 2, 2001 and entitled "METHOD OF INDUCING A CTL RESPONSE". Exemplary methodology is found in U.S. Provisional Application No. 60/580,969, filed on June 17, 2004, and U.S. Patent Application No. 2006-0008468-A1, published on January 12, 2006, both entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOTISTICS FOR VARIOUS TYPES OF CANCERS". Methodology and compositions are also disclosed in U.S. Provisional Application No. 60/640,598, filed on December 29, 2004, entitled "COMBINATIONS OF TUMOR-ASSOCAITED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCER". The integration of diagnostic techniques to assess and monitor immune responsiveness with methods of immunization including utilizing the instant analogs is discussed more fully in Provisional U.S. Patent Application No. 60/580,964 filed on June 17, 2004 and U.S. Patent Application No. US-2005-0287068-A1 , published on December 29, 2005) both entitled "Improved efficacy of active immunotherapy by integrating diagnostic with therapeutic methods". The immunogenic polypeptide encoding vectors are disclosed in U.S. Patent Application No. 10/292,413 (Pub. No.

20030228634 A1), filed on November 7, 2002, entitled Expression Vectors Encoding Epitopes of Target-Associated Antigens and Methods for their Design, and in U.S. Provisional Application No. 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". Additional useful disclosure, including methods and compositions of matter, is found in U.S. Provisional Application No 60/691,581, filed on June 17, 2005, entitled "Multivalent Entrain-and-Amplify Immunotherapeutics for Carcinoma". Further methodology, compositions, peptides, and peptide analogs are disclosed in U.S. Provisional Application Nos. 60/581,001 and 60/580,962, both filed on June 17, 2004, and respectively entitled "SSX-2 PEPTIDE ANALOGS" and "NY-ESO PEPTIDE ANALOGS." Each of the applications and patents mentioned in the above paragraphs is hereby incorporated by reference in its entirety for all that it teaches. Additional analogs, peptides and methods are disclosed in U.S. Patent Application Publication No 20060063913, entitled "SSX-2 PEPTIDE ANALOGS"; and U.S. Patent Publication No. 2006-0057673 A1, published on March 16, 2006, entitled "EPITOPE ANALOGS"; and PCT Application Publication No. WO/2006/009920, entitled "EPITOPE ANALOGS"; all filed on June 17, 2005. Further methodology and compositions are disclosed in U.S. Provisional Application No. 60/581,001, filed on June 17, 2004, entitled "SSX-2 PEPTIDE ANALOGS", and to U.S. Provisional Application No. 60/580,962, filed on June 17, 2004, entitled "NY-ESO PEPTIDE ANALOGS"; each of which is incorporated herein by reference in its entirety. As an example, without being limited thereto each reference is incorporated by reference for what it teaches about class I MHC-restricted epitopes, analogs, the design of analogs, uses of epitopes and analogs, methods of using and making epitopes, and the design and use of nucleic acid vectors for their expression. Other applications that are expressly incorporated herein by reference are: U.S. Patent Application Serial No. 11/156,253 (Publication No. 20060063913), filed on June 17, 2005, entitled “SSX-2 PEPTIDE ANALOGS”; U.S. Patent Application Serial No. 11/155,929, filed on June 17, 2005, entitled “NY-ESO-1 PEPTIDE ANALOGS” (Publication No. 20060094661); U.S. Patent Application Serial No. 11/321,967, filed on December 29, 2005, entitled “METHODS TO TRIGGER, MAINTAIN AND MANIPULATE IMMUNE RESPONSES BY TARGETED ADMINISTRATION OF BIOLOGICAL RESPONSE MODIFIERS INTO LYMPHOID ORGANS”; U.S. Patent Application Serial No. 11/323,572, filed on December 29, 2005, entitled “METHODS TO

ELICIT ENHANCE AND SUSTAIN IMMUNE REPONSES AGAINST MCH CLASS I RESTRICTED EPITOPES, FOR PROPHYLACTIC OR THERAPEUTIC PURPOSES"; U.S. Patent Application Serial No. 11/323,520, filed December 29, 2005, entitled "METHODS TO BYPASS CD4+ CELLS IN THE INDUCTION OF AN IMMUNE RESPONSE"; U.S. Patent Application Serial No. 11/323,049, filed December 29, 2005, entitled "COMBINATION OF TUMOR-ASSOCIATED ANTIGENS IN COMPOSITIONS FOR VARIOUS TYPES OF CANCERS"; U.S. Patent Application Serial No. 11,323,964, filed December 29, 2005, entitled "COMBINATIONS OF TUMOR-ASSOCIATED ANTIGENS IN DIAGNOSTICS FOR VARIOUS TYPES OF CANCERS"; U.S. Provisional Application Serial No. 60/691,889, filed on June 17, 2005 entitled "EPITOPE ANALOGS."

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME	1	DE	2
CONTENANT LES PAGES	1	À	50

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME	1	OF	2
CONTAINING PAGES	1	TO	50

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED:

1. An immunogenic product comprising a plurality of compositions comprising one or more nucleic acid compositions and one or more peptide compositions; wherein the one or more nucleic acid compositions are capable of expressing one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; wherein the one or more peptide compositions consist essentially of said one or more class I MHC restricted epitopes, or an analog thereof, selected from the group consisting of an SSX-1 epitope, an NY-ESO-1 epitope, a PRAME epitope, a PSMA epitope, a tyrosinase epitope, and a Melan-A epitope; and wherein the one or more peptides correspond to the epitopes expressed by the selected nucleic acids.

2. The immunogenic product of claim 1 wherein said one or more nucleic acid compositions comprise a plasmid selected from the group consisting of pSEM, pBPL and pRP12.

3. The immunogenic product of claim 1 wherein said one or more peptide compositions comprise a peptide selected from the group consisting of $SSX\text{-}2_{41\text{-}49}$ (SEQ ID NO. 1), its analogue KVSEKIFYV (SEQ ID NO. 5); $NY\text{-}ESO\text{-}1_{157\text{-}165}$ (SEQ ID NO. 2), its analogue SNvaLMWITQV (SEQ ID NO. 6); $PRAME_{425\text{-}433}$ (SEQ ID NO. 3), its analogue S(Nva)LQHLIG(Nle) (SEQ ID NO. 7); $PSMA_{288\text{-}297}$ (SEQ ID NO. 4), its analogue GLPSIPVHPV (SEQ ID NO. 8); Melan-$A_{26\text{-}35}$ (SEQ ID NO. 9), , its analogue ENvaAGIGILTV(SEQ ID NO. 11); $tyrosinase_{369\text{-}377}$(SEQ ID NO. 10), and its analogue YMDGTMSQNva(SEQ ID NO. 12).

4. The immunogenic product of claim 1 wherein said plurality of compositions comprise:

a) a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof;

b) a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof;

c) a nucleic acid molecule capable of expressing a PRAME class I MHC restricted epitope, or analogue thereof;

d) a nucleic acid molecule capable of expressing a PSMA class I MHC restricted epitope, or analogue thereof;

e) a peptide consisting essentially of said SSX-2 epitope, or analogue thereof;

f) a peptide consisting essentially of said NY-ESO-1 epitope, or analogue thereof;

g) a peptide consisting essentially of said PRAME epitope, or analogue thereof; and

h) a peptide consisting essentially of said PSMA epitope, or analogue thereof.

5. The product of claim 4 wherein the nucleic acid molecules of a) and b) are part of the same composition.

6. The product of claim 5 wherein the nucleic acid molecules are the same.

7. The product of claim 6 wherein the nucleic acid molecule comprises a sequence encoding the liberation sequence of pBPL(SEQ ID NO. 13) .

8. The product of claim 7 wherein the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pBPL (SEQ ID NO. 16) .

9. The product of claim 8 wherein the nucleic acid molecule is pBPL (SEQ ID NO. 20).

10. The product of claim 4 wherein the nucleic acid molecules of c) and d) are part of the same composition.

11. The product of claim 10 wherein the nucleic acid molecules are the same.

12. The product of claim 10 wherein the nucleic acid molecule comprises a sequence encoding the liberation sequence of pRP12 (SEQ ID NO. 14) .

13. The product of claim 12 wherein the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pRP12 (SEQ ID NO. 17) .

14. The product of claim 12 wherein the nucleic acid molecule is pRP12 (SEQ ID. 21).

15. The product of claim 1 wherein the SSX-2 epitope is SSX-2_{41-49} (SEQ ID NO. 1).

16. The product of claim 1 wherein the NY-ESO-1 epitope is NY-ESO-$1_{157-165}$ (SEQ ID NO. 2).

17. The product of claim 1 wherein the PRAME epitope is $PRAME_{425-433}$ (SEQ ID NO. 3).

18. The product of claim 1 wherein the PSMA epitope is $PSMA_{288-297}$ (SEQ ID NO. 4).

19. The product of claim 1 wherein said SSX-2 analogue in e) is KVSEKIFYV (SEQ ID NO. 5).

20. The product of claim 1 wherein said NY-ESO-1 analogue in f) is SNvaLMWITQV (SEQ ID NO. 6).

21. The product of claim 1 wherein said PRAME analogue in g) is S(Nva)LQHLIG(Nle) (SEQ ID NO. 7).

22. The product of claim 1 wherein said PSMA analogue in h) is GLPSIPVHPV (SEQ ID NO. 8).

23. A method of treating cancer comprising administering the product of claim 1 to a patient in need thereof.

24. The method of claim 23, wherein the cancer is a breast cancer, an ovarian cancer, a pancreatic cancer, a prostate cancer, a colon cancer, a bladder cancer, a lung cancer, a liver cancer, a stomach cancer, a testicular cancer, an uterine cancer, a brain cancer, a lymphatic cancer, a skin cancer, a bone cancer, a kidney cancer, a rectal cancer, a melanoma, a glioblastoma, or a sarcoma.

25. A method of treating carcinoma comprising a step of administering to a patient in need thereof a plurality of compositions comprising:

i) a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof;

ii) a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof;

iii) a nucleic acid molecule capable of expressing a PRAME class I MHC restricted epitope, or analogue thereof; and

vi) a nucleic acid molecule capable of expressing a PSMA class I MHC restricted epitope, or analogue thereof.

26. The method of claim 25 further comprising a step of administering one or more peptides selected from the group epitopes or analogues consisting essentially of SSX-2, NY-ESO-1, PRAME, and PSMA.

27. The immunogenic product of claim 1 wherein said plurality of compositions comprise:

i) a nucleic acid molecule capable of expressing a Melan-A class I MHC restricted epitope, or analogue thereof;

ii) a nucleic acid molecule capable of expressing a Tyrosinase class I MHC restricted epitope, or analogue thereof;

iii) a peptide consisting essentially of said Melan-A epitope, or analogue thereof; and

vi) a peptide consisting essentially of said Tyrosinase epitope, or analogue thereof.

28. The product of claim 27 wherein the nucleic acid molecules of i) and ii) are part of the same composition.

29. The product of claim 28 wherein the nucleic acid molecules are the same.

30. The product of claim 29 wherein the nucleic acid molecule comprises a sequence encoding the liberation sequence of pSEM (SEQ ID NO. 15) .

31. The product of claim 30 wherein the nucleic acid comprises a sequence encoding the immunogenic polypeptide of pSEM (SEQ ID NO. 18) .

32. The product of claim 31 wherein the nucleic acid molecule is pSEM.

33. The product of claim 27 wherein the Melan-A epitope is Melan-A_{26-35} (SEQ ID NO. 9).

34. The product of claim 27 wherein the Tyrosinase epitope is $Tyrosinase_{369-377}$ (SEQ ID NO. 10).

35. The product of claim 27 further comprising:

i) a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; and

ii) a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof.

36. The product of claim 35 further comprising a peptide consisting essentially of an NY-ESO-1 epitope.

37. The product of claim 35 or 36 further comprising a peptide consisting essentially of an SSX-2 epitope.

38. A method of treating cancer comprising a step of administering to a patient in need thereof a plurality of compositions comprising:

i) a nucleic acid molecule capable of expressing a Melan-A class I MHC restricted epitope, or analogue thereof;

ii) a nucleic acid molecule capable of expressing a Tyrosinase class I MHC restricted epitope, or analogue thereof;

iii) a peptide consisting essentially of said Melan-A epitope, or analogue thereof; and

iv) a peptide consisting essentially of said Tyrosinase epitope, or analogue thereof.

39. The method of claim 38, wherein said cancer is glioblastoma.

40. The method of claim 38, wherein said cancer is melanoma.

41. The method of claim 38 further comprising a step of administering to a patient in need thereof a composition comprising:

i) a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof; and

ii) a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof

iii) a peptide consisting essentially of said NY-ESO-1 epitope or analogue thereof; and

vi) a peptide consisting essentially of said SSX-2 epitope or analogue thereof.

42. The immunogenic product of claim 1 wherein said plurality of compositions comprise:

a) a nucleic acid molecule capable of expressing an SSX-2 class I MHC restricted epitope, or analogue thereof;

b) a nucleic acid molecule capable of expressing an NY-ESO-1 class I MHC restricted epitope, or analogue thereof;

c) a peptide consisting essentially of said SSX-2 epitope, or analogue thereof;

d) a peptide consisting essentially of said NY-ESO-1 epitope, or analogue thereof.

43. The immunogenic product of claim 1 wherein said plurality of compositions comprise:

a) a nucleic acid molecule capable of expressing a PRAME class I MHC restricted epitope, or analogue thereof;

b) a nucleic acid molecule capable of expressing a PSMA class I MHC restricted epitope, or analogue thereof;

c) a peptide consisting essentially of said PRAME epitope, or analogue thereof; and

d) a peptide consisting essentially of said PSMA epitope, or analogue thereof.

44. The use of the immunogenic product of claim 1 in the manufacture of a medicament suitable for administration to the lymphatic system of a subject.

45. The use of the immunogenic product of claim 1 in the manufacture of a medicament suitable for inducing an anti-cancer immune response in a subject.

46. The use of the immunogenic product of claim 1 in the manufacture of a medicament that entrains and amplifies a T cell response in a subject.

47. The use of the immunogenic product of claim 1 in the manufacture of a medicament for treating carcinoma in a subject.

48. The use of one or more nucleic acid compositions capable of expressing one or more class I MHC restricted epitopes, or an analog thereof, and one or more peptide compositions corresponding to the said class I MHC restricted epitopes or analogues thereof, in the manufacture of a medicament suitable for inducing an anti-cancer immune response in a subject.

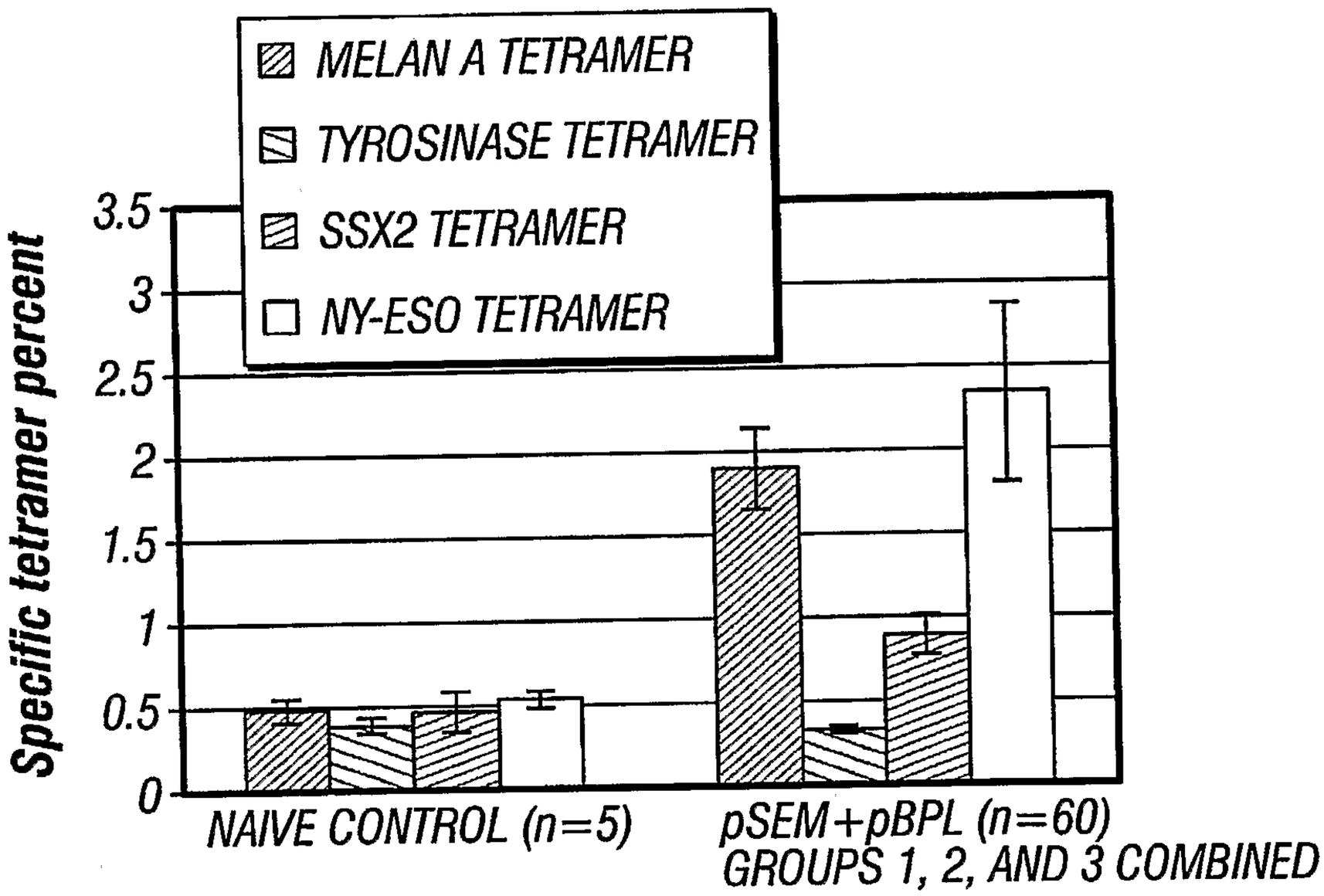

MELAN A TETRAMER
TYROSINASE TETRAMER
SSX2 TETRAMER
NY-ESO TETRAMER
Specific tetramer percent
3.5
3
2.5
2
1.5
1
0.5
0
NAIVE CONTROL (n=5)
pSEM+pBPL (n=60)
GROUPS 1, 2, AND 3 COMBINED

FIG. 1

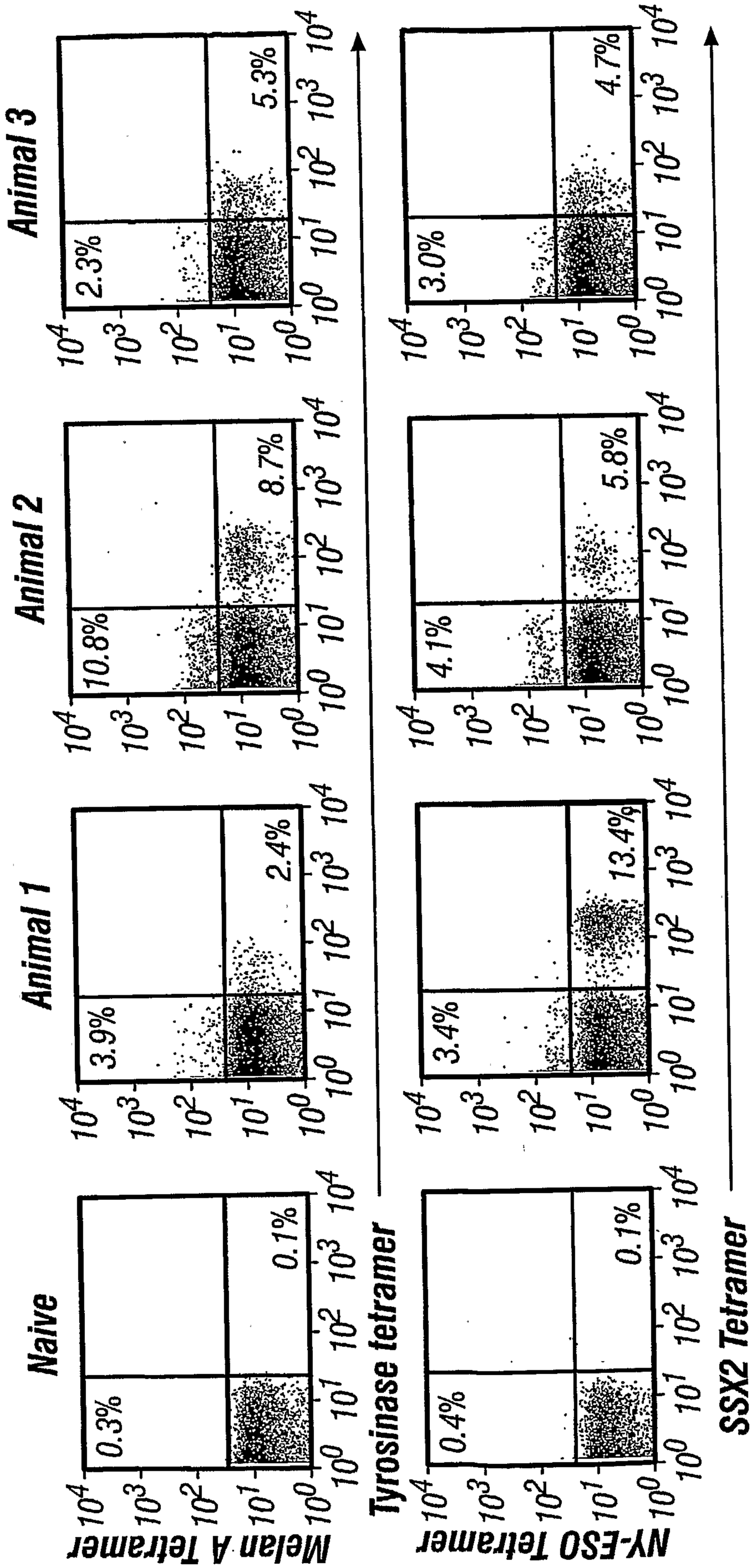

Animal 3
Animal 2
Animal 1
Naive
2.3%
5.3%
3.0%
4.7%
10.8%
8.7%
4.1%
5.8%
3.9%
2.4%
3.4%
13.4%
0.3%
0.1%
0.4%
0.1%
Melan A Tetramer
Tyrosinase tetramer
NY-ESO Tetramer
SSX2 Tetramer

FIG. 2

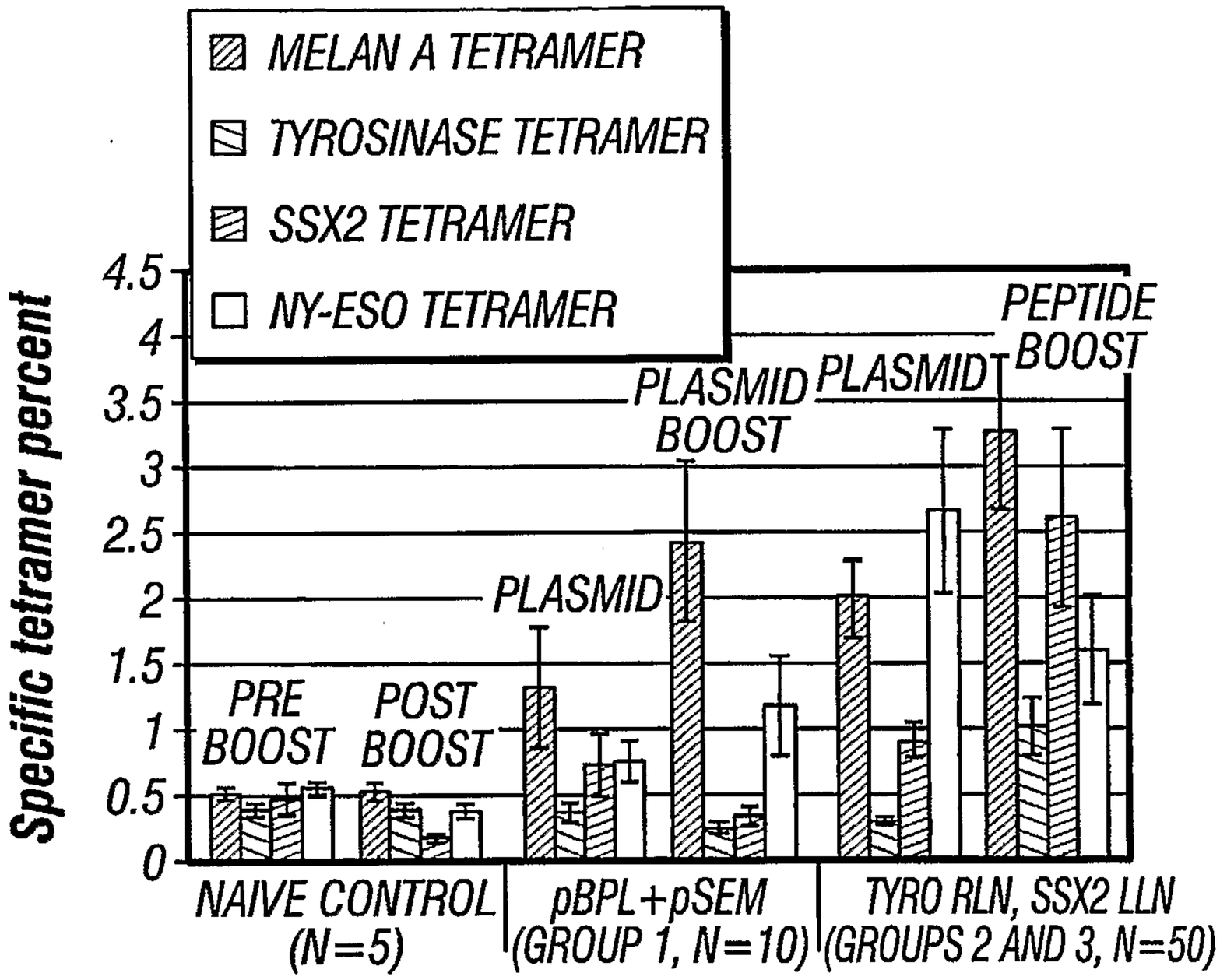

MELAN A TETRAMER
TYROSINASE TETRAMER
SSX2 TETRAMER
NY-ESO TETRAMER
Specific tetramer percent
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PRE BOOST
POST BOOST
PLASMID
PLASMID BOOST
PLASMID
PEPTIDE BOOST
NAIVE CONTROL (N=5)
pBPL+pSEM (GROUP 1, N=10)
TYRO RLN, SSX2 LLN (GROUPS 2 AND 3, N=50)

FIG. 3

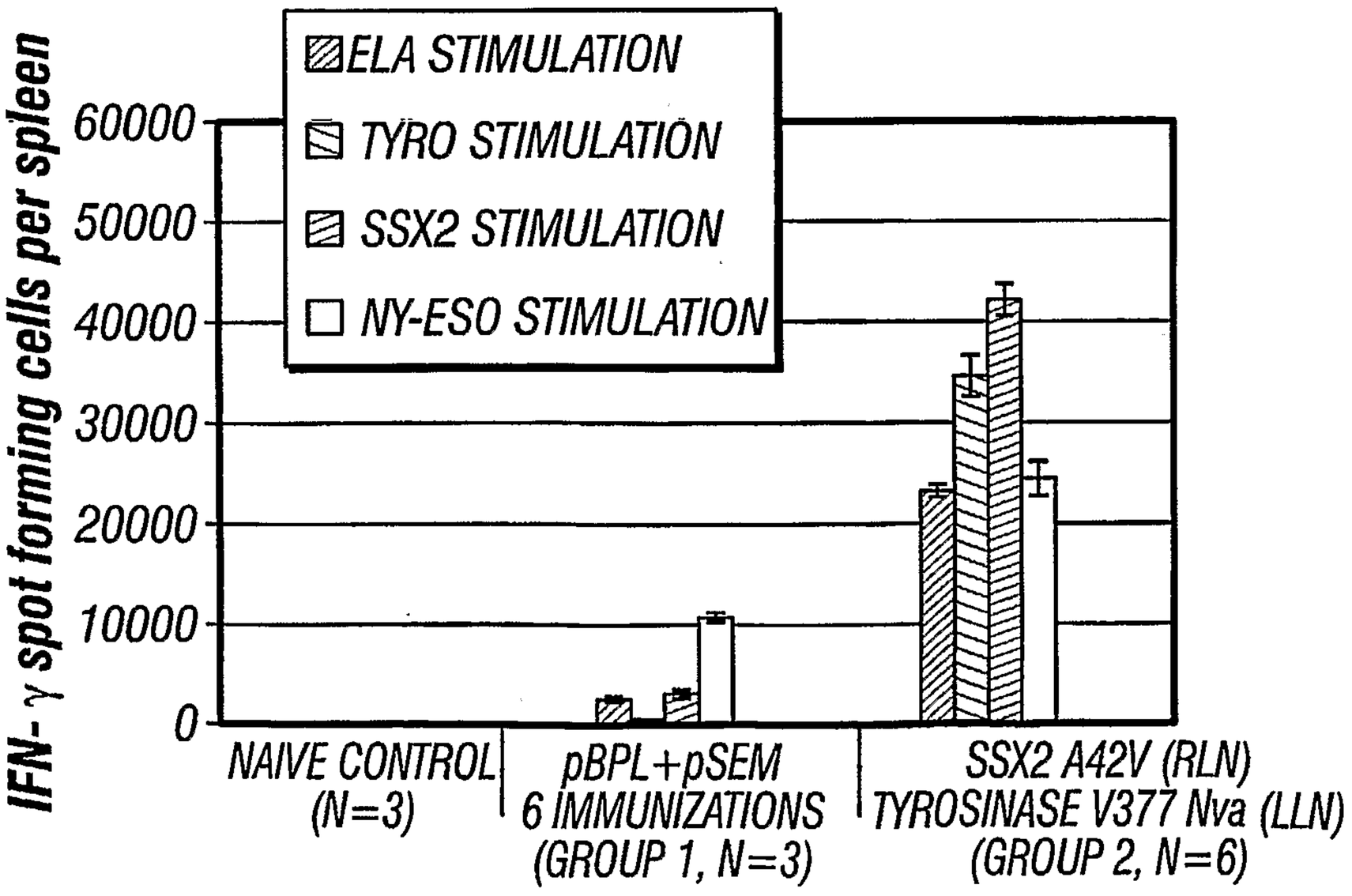

ELA STIMULATION
TYRO STIMULATION
SSX2 STIMULATION
NY-ESO STIMULATION
IFN-γ spot forming cells per spleen
60000
50000
40000
30000
20000
10000
0
NAIVE CONTROL (N=3)
pBPL+pSEM 6 IMMUNIZATIONS (GROUP 1, N=3)
SSX2 A42V (RLN) TYROSINASE V377 Nva (LLN) (GROUP 2, N=6)

FIG. 4A

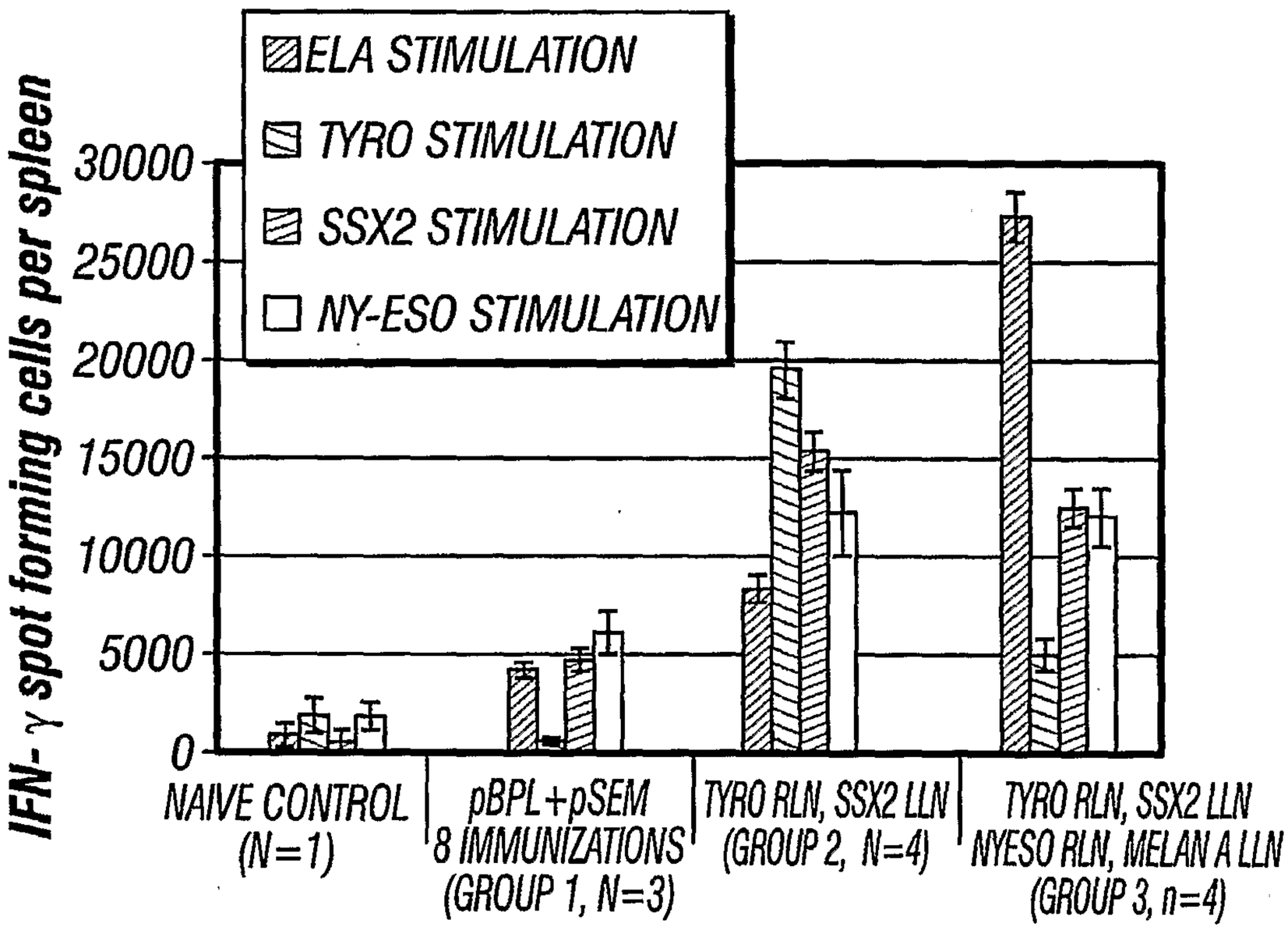

ELA STIMULATION
TYRO STIMULATION
SSX2 STIMULATION
NY-ESO STIMULATION
IFN-γ spot forming cells per spleen
30000
25000
20000
15000
10000
5000
0
NAIVE CONTROL (N=1)
pBPL+pSEM 8 IMMUNIZATIONS (GROUP 1, N=3)
TYRO RLN, SSX2 LLN (GROUP 2, N=4)
TYRO RLN, SSX2 LLN NYESO RLN, MELAN A LLN (GROUP 3, n=4)

FIG. 4B

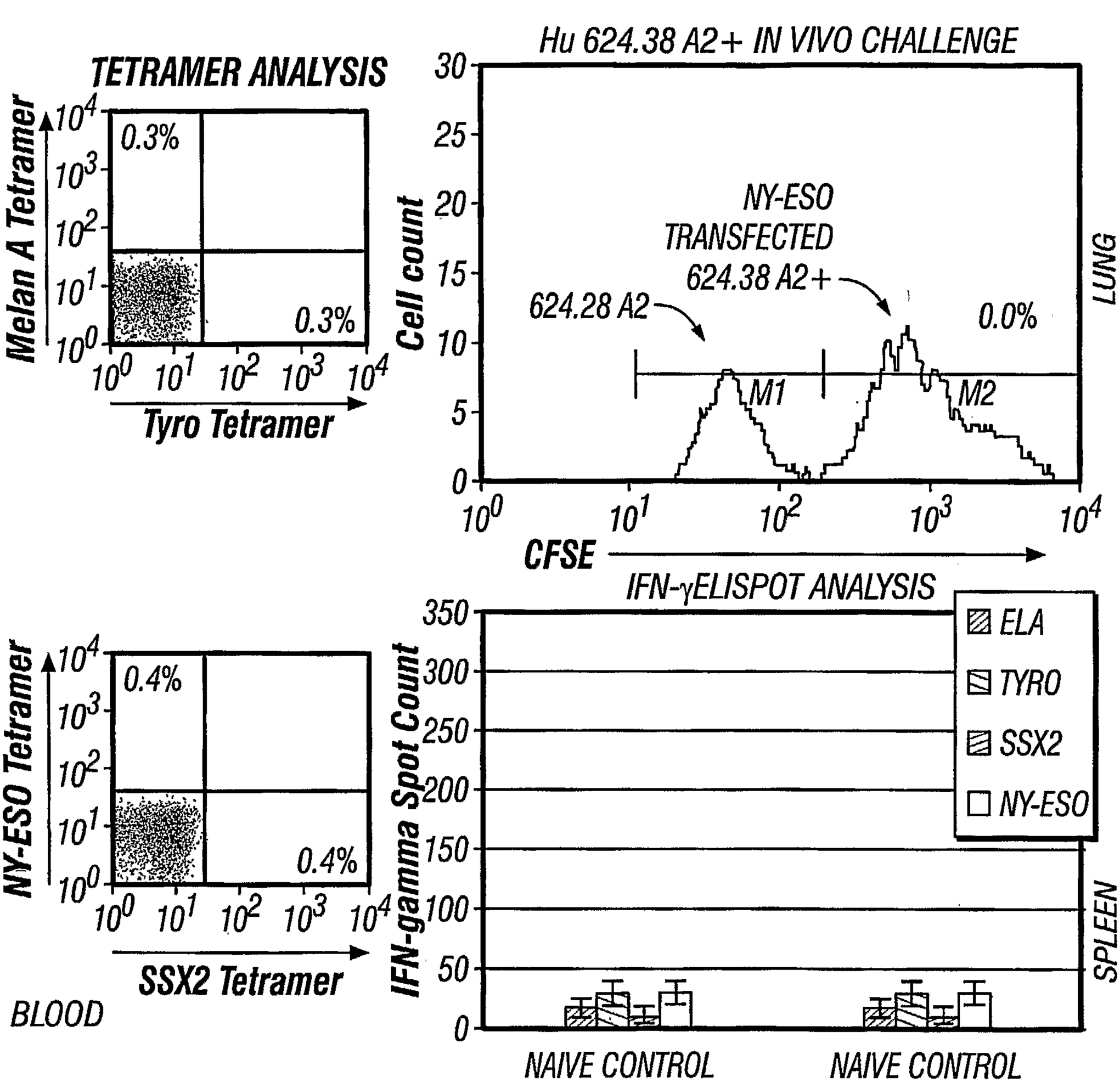

TETRAMER ANALYSIS
Melan A Tetramer
10^4
10^3
10^2
10^1
10^0
0.3%
0.3%
10^0 10^1 10^2 10^3 10^4
Tyro Tetramer
Hu 624.38 A2+ IN VIVO CHALLENGE
Cell count
30
25
20
15
10
5
0
NY-ESO
TRANSFECTED
624.38 A2+
624.28 A2
0.0%
M1
M2
LUNG
10^0 10^1 10^2 10^3 10^4
CFSE
NY-ESO Tetramer
10^4
10^3
10^2
10^1
10^0
0.4%
0.4%
10^0 10^1 10^2 10^3 10^4
SSX2 Tetramer
BLOOD
IFN-γELISPOT ANALYSIS
IFN-gamma Spot Count
350
300
250
200
150
100
50
0
ELA
TYRO
SSX2
NY-ESO
SPLEEN
NAIVE CONTROL
NAIVE CONTROL

FIG. 5

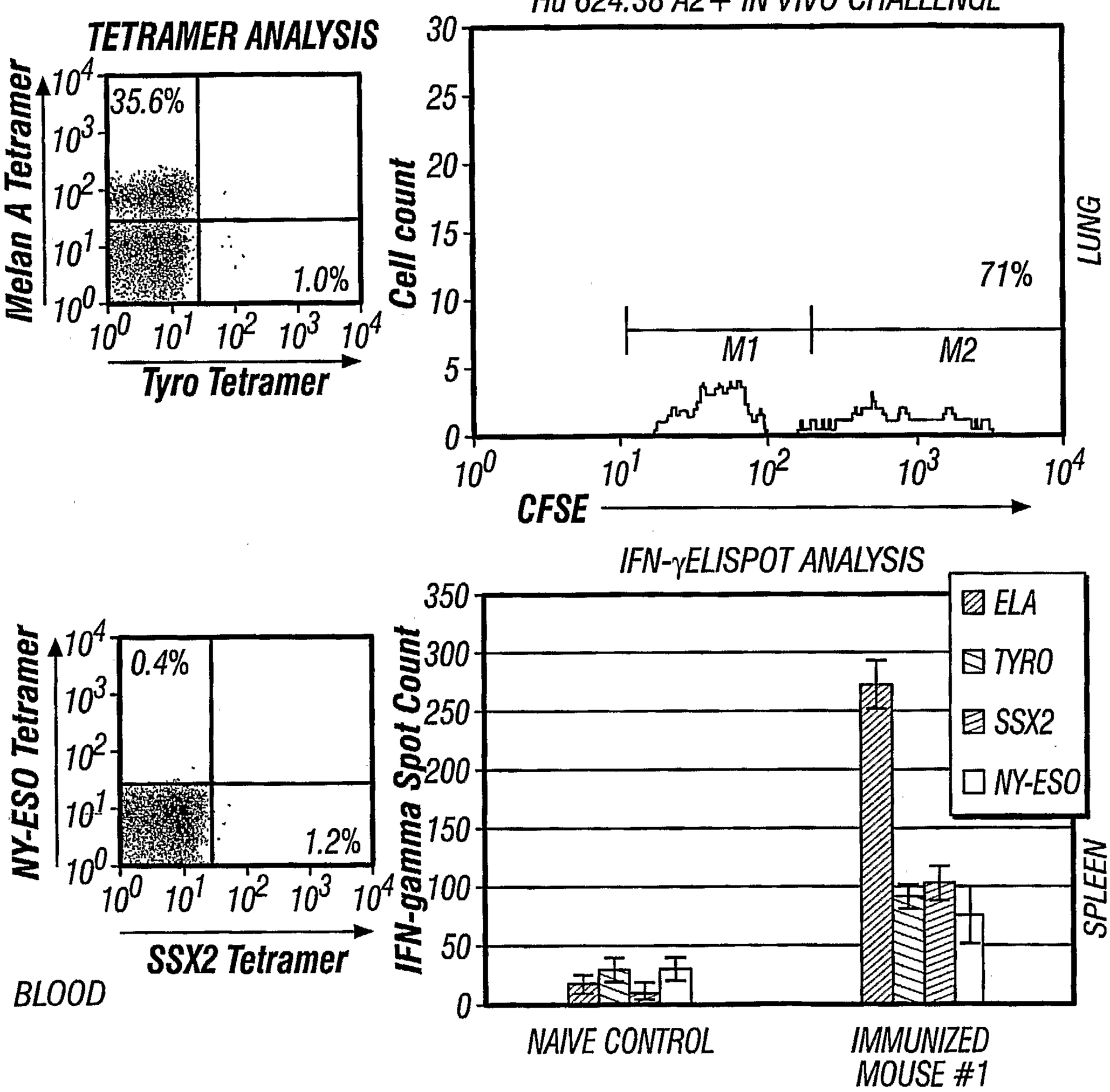


FIG. 5
(Continued)

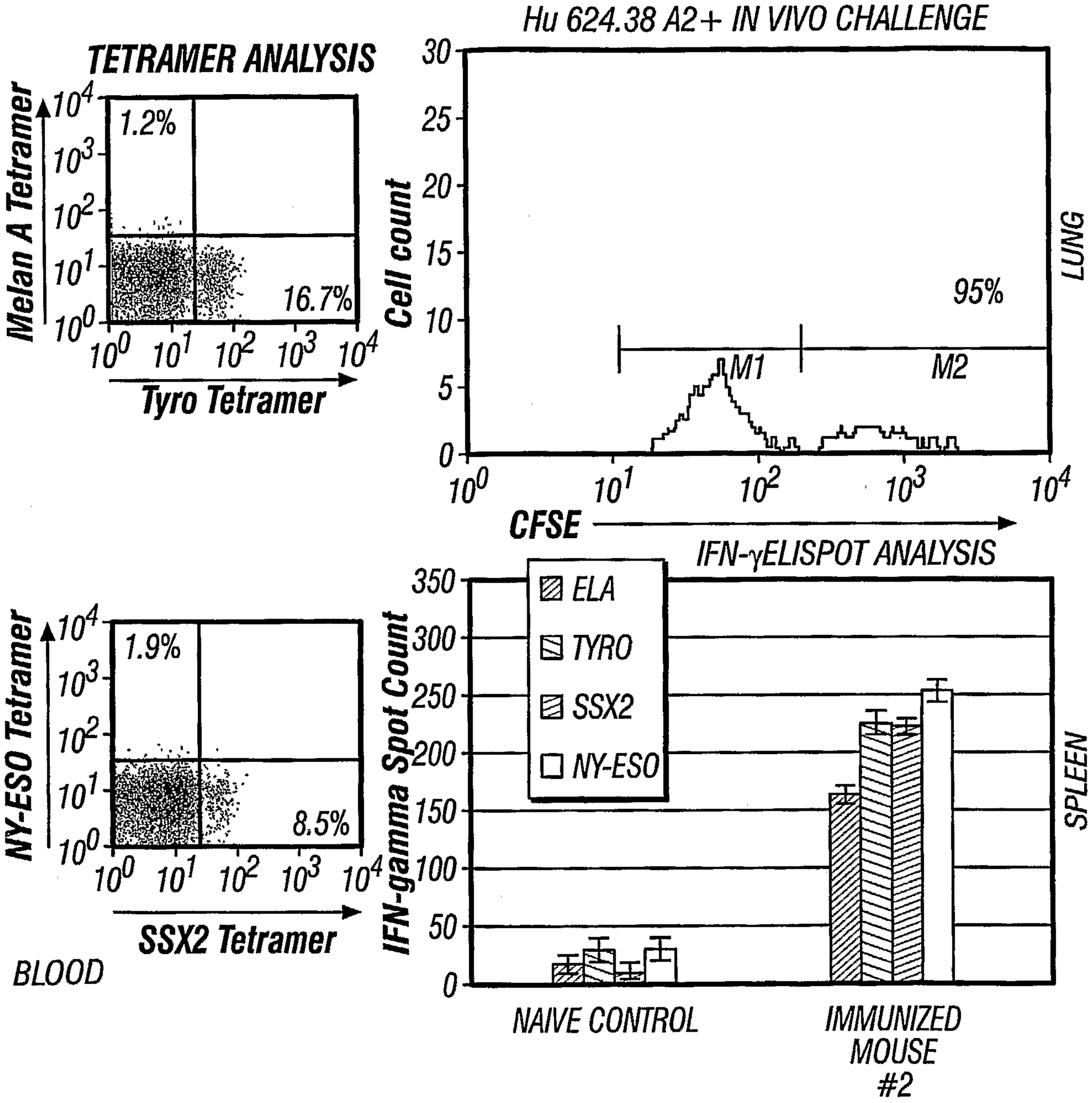


FIG. 5
(Continued)

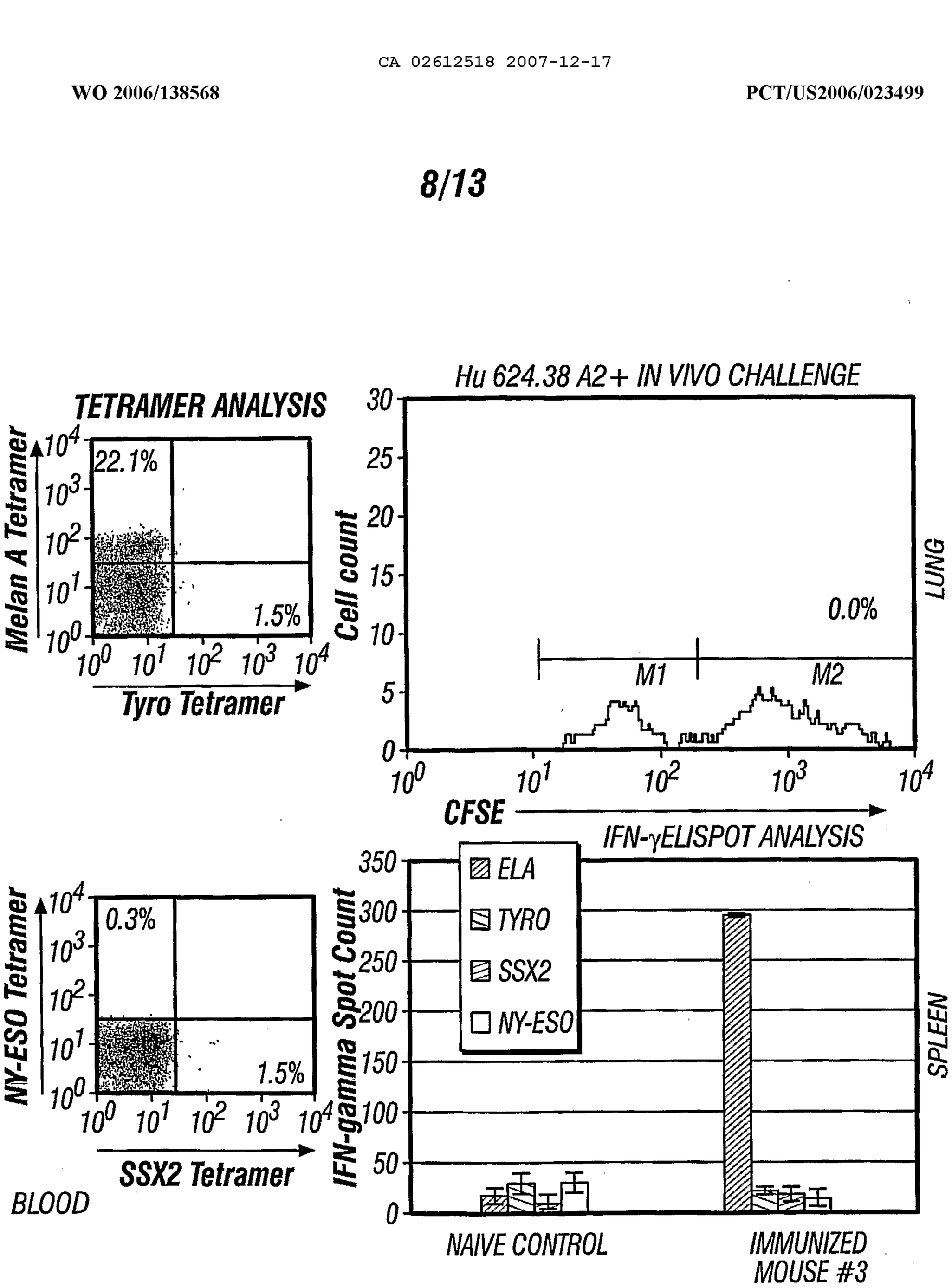


FIG. 5
(Continued)

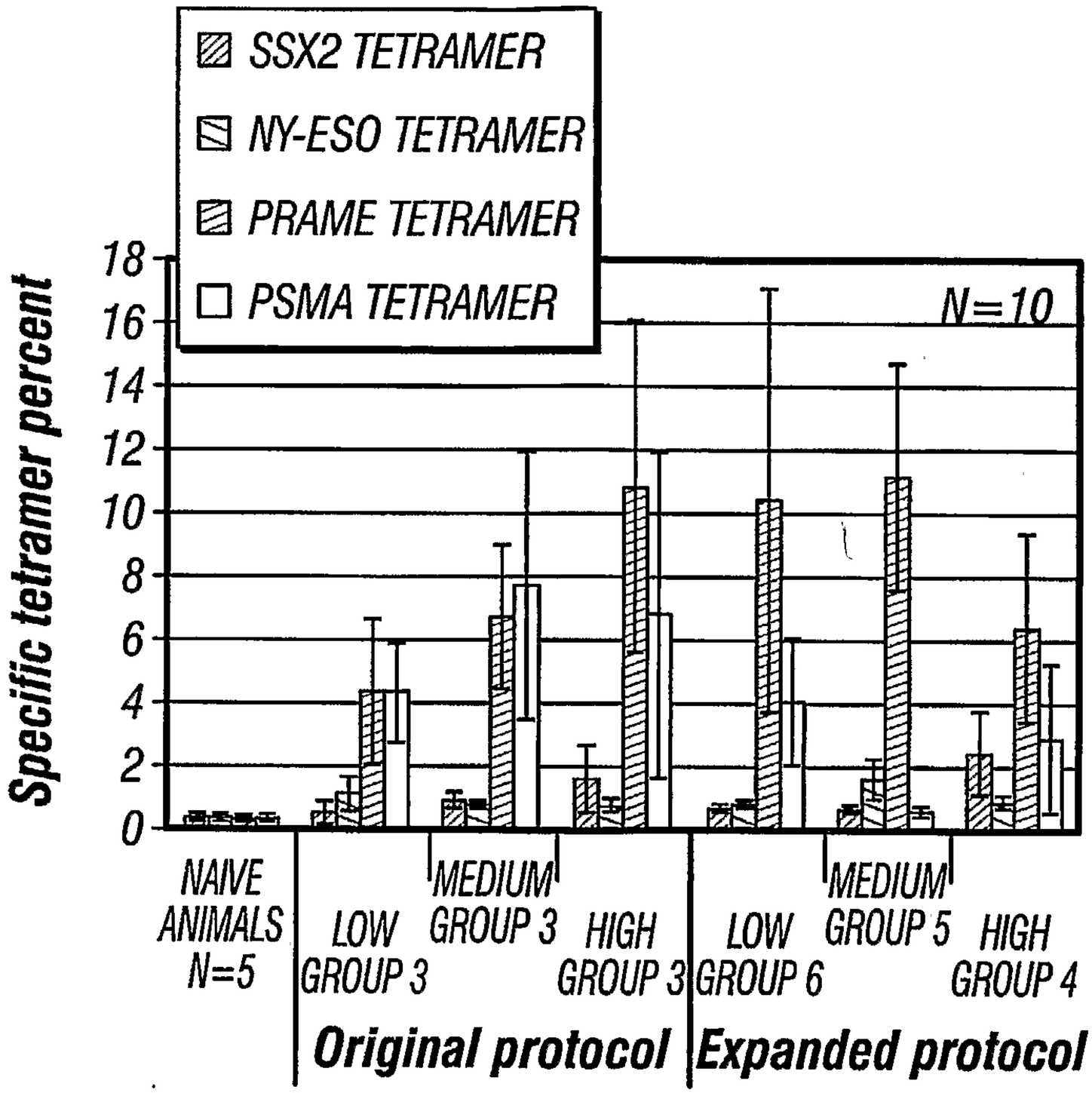

SSX2 TETRAMER
NY-ESO TETRAMER
PRAME TETRAMER
PSMA TETRAMER
Specific tetramer percent
18
16
14
12
10
8
6
4
2
0
N=10
NAIVE ANIMALS N=5
LOW GROUP 3
MEDIUM GROUP 3
HIGH GROUP 3
LOW GROUP 6
MEDIUM GROUP 5
HIGH GROUP 4
Original protocol
Expanded protocol

FIG. 6

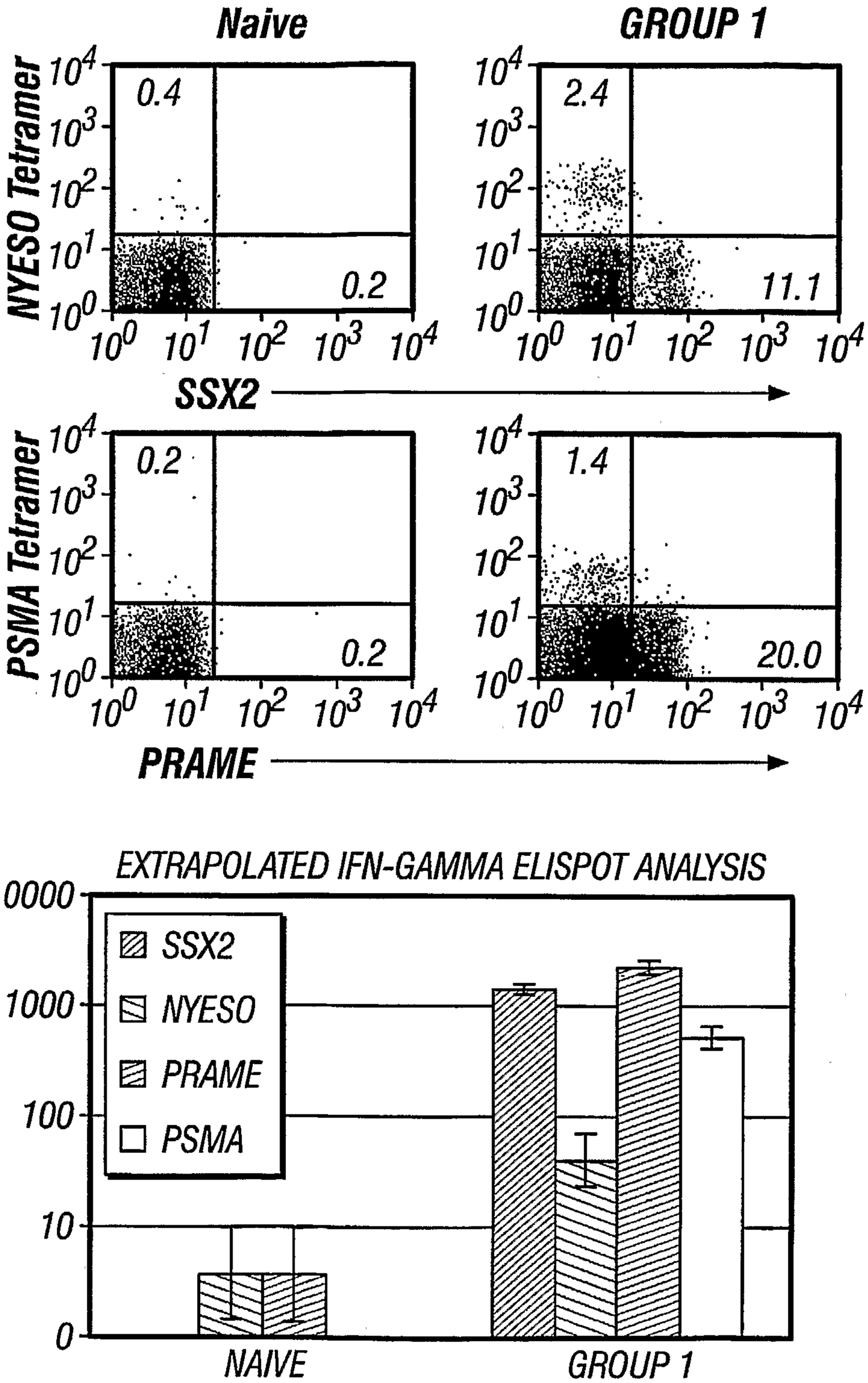

Naive
GROUP 1
NYESO Tetramer
PSMA Tetramer
SSX2
PRAME
0.4
0.2
2.4
11.1
0.2
0.2
1.4
20.0
EXTRAPOLATED IFN-GAMMA ELISPOT ANALYSIS
IFNγ spot forming Cells Per spleen
SSX2
NYESO
PRAME
PSMA
NAIVE
GROUP 1

FIG. 7

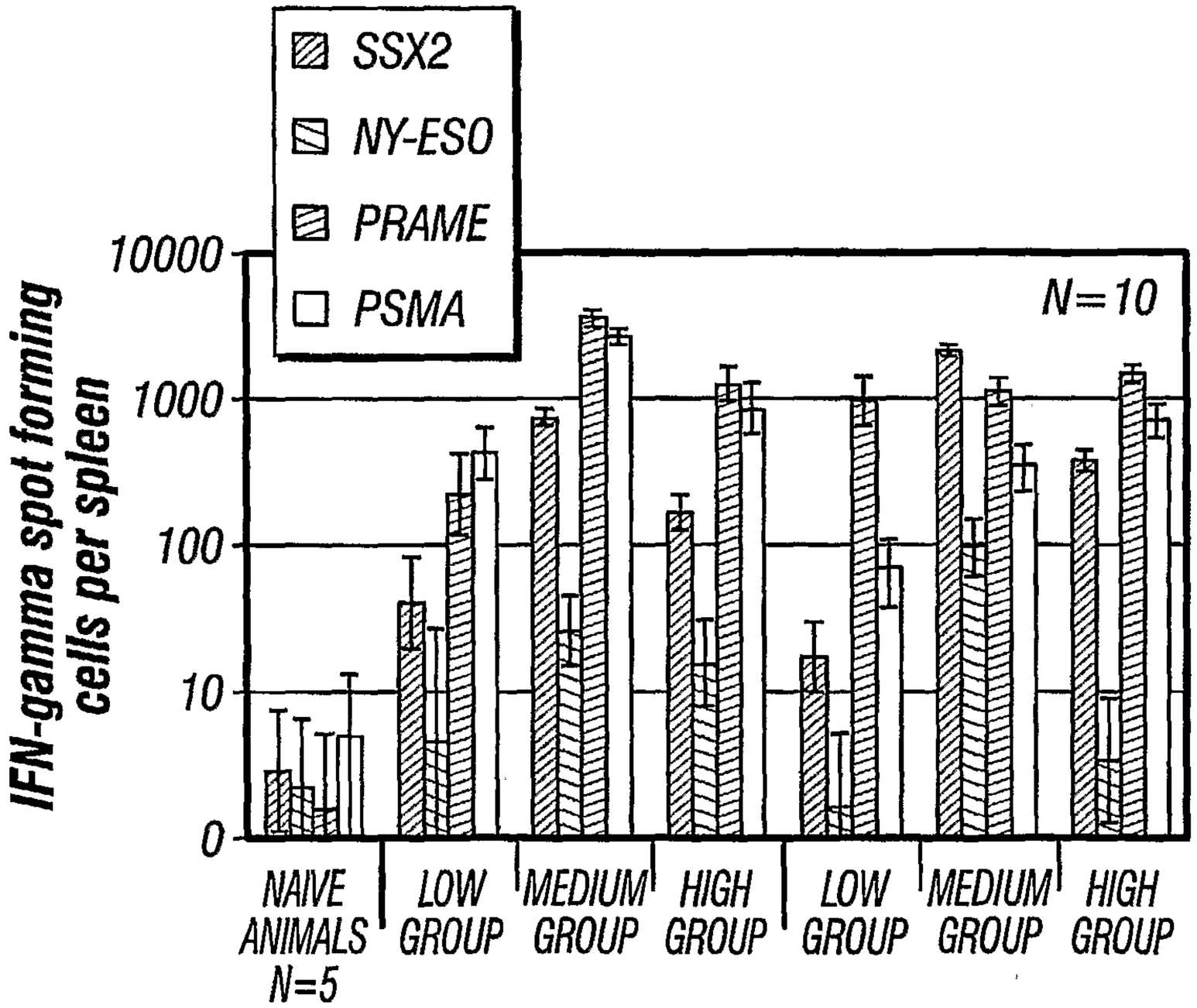

SSX2
NY-ESO
PRAME
PSMA
N=10
IFN-gamma spot forming cells per spleen
10000
1000
100
10
0
NAIVE ANIMALS N=5
LOW GROUP
MEDIUM GROUP
HIGH GROUP
LOW GROUP
MEDIUM GROUP
HIGH GROUP
Original protocol
Expanded protocol

FIG. 8

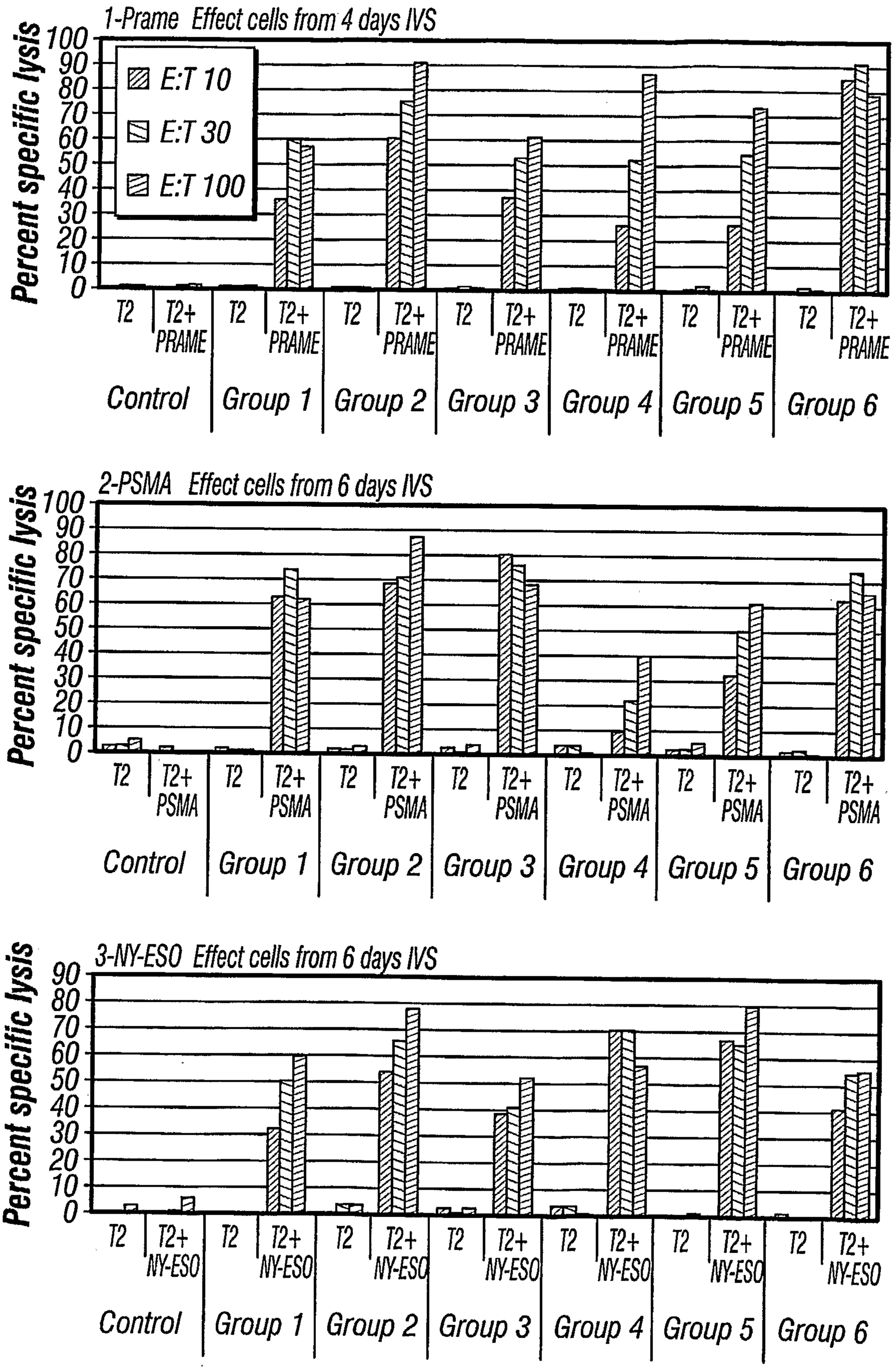

1-Prame Effect cells from 4 days IVS
Percent specific lysis
100
90
80
70
60
50
40
30
20
10
0
E:T 10
E:T 30
E:T 100
T2
T2+ PRAME
Control
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
2-PSMA Effect cells from 6 days IVS
Percent specific lysis
T2+ PSMA
3-NY-ESO Effect cells from 6 days IVS
Percent specific lysis
T2+ NY-ESO

FIG. 9

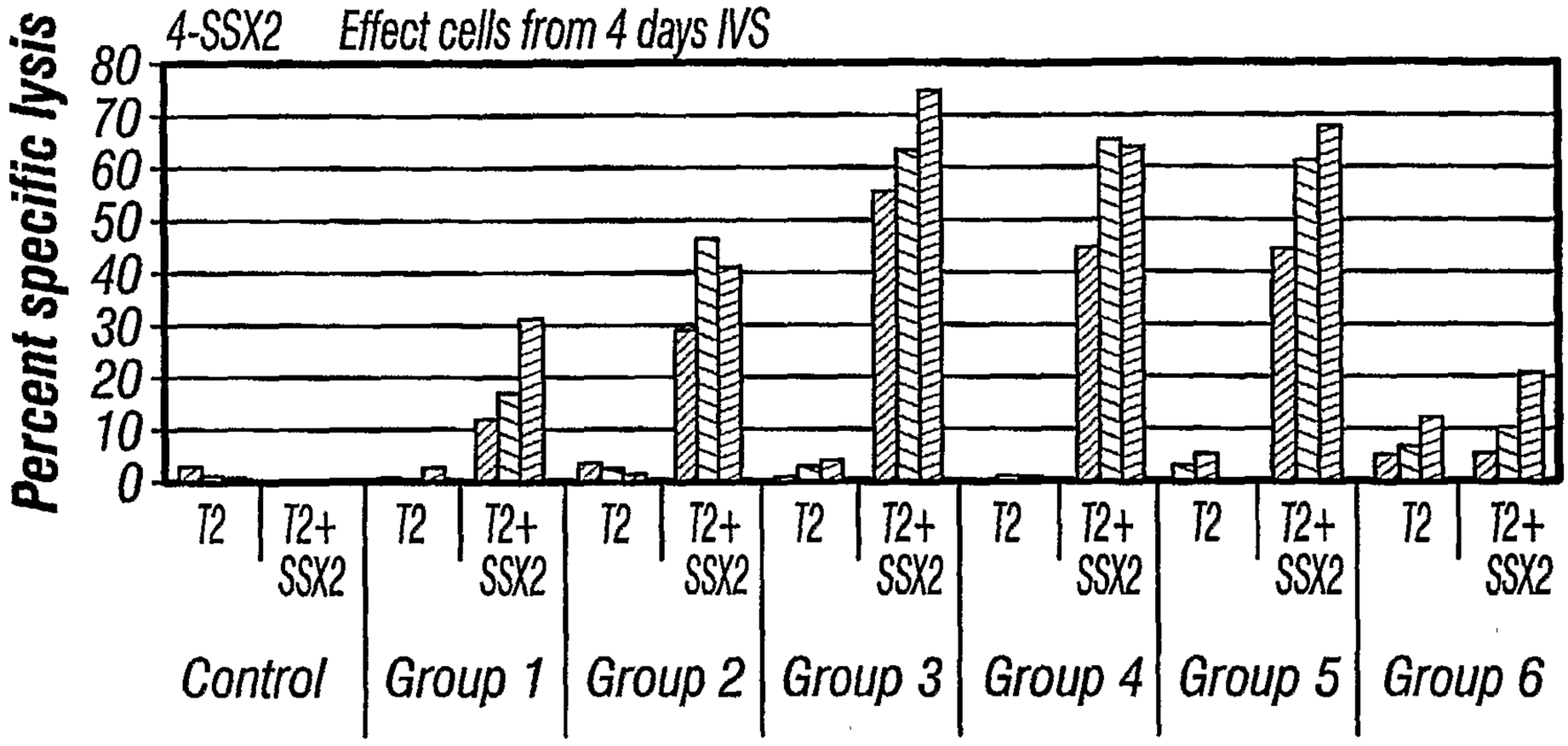


FIG. 9
(Continued)

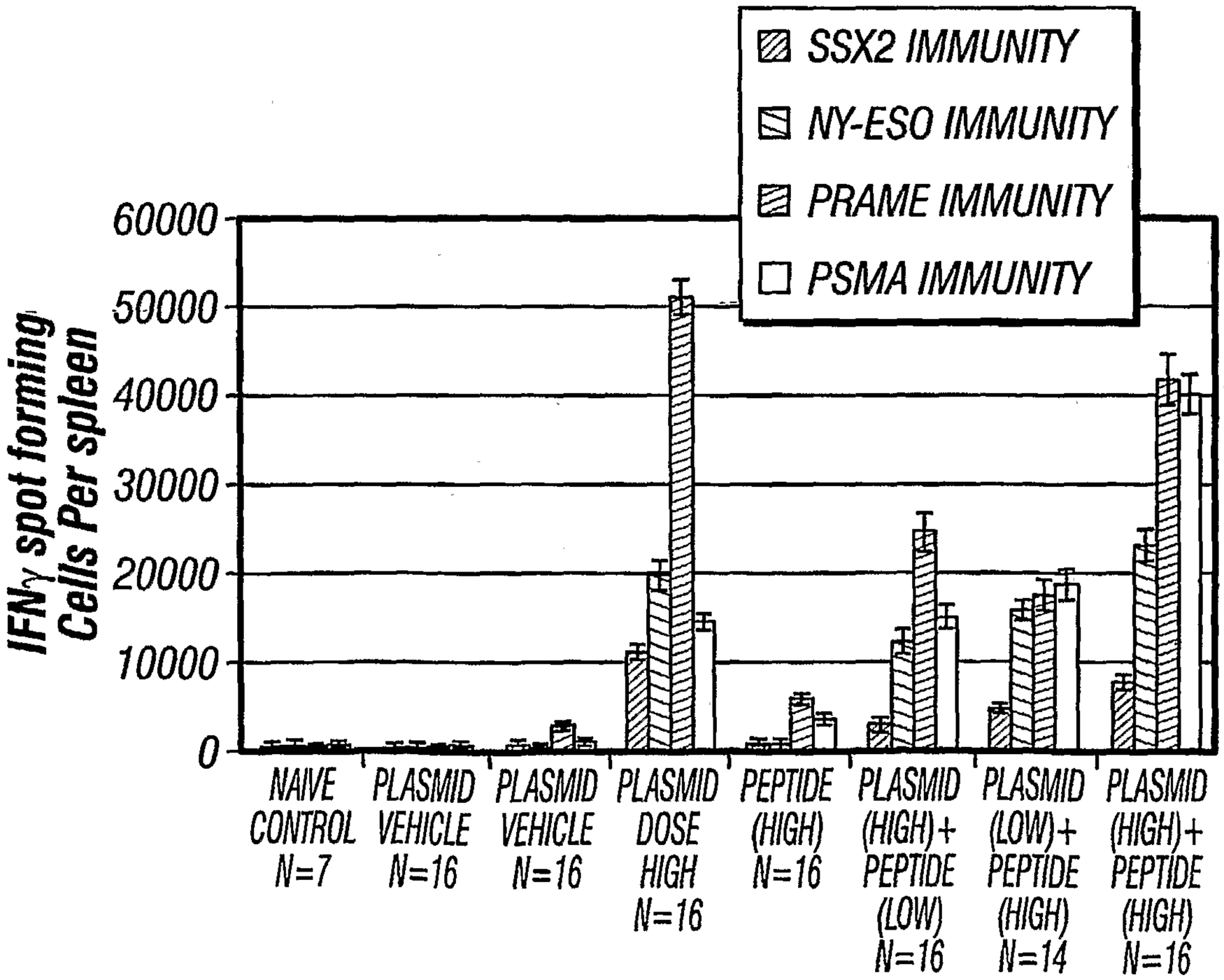
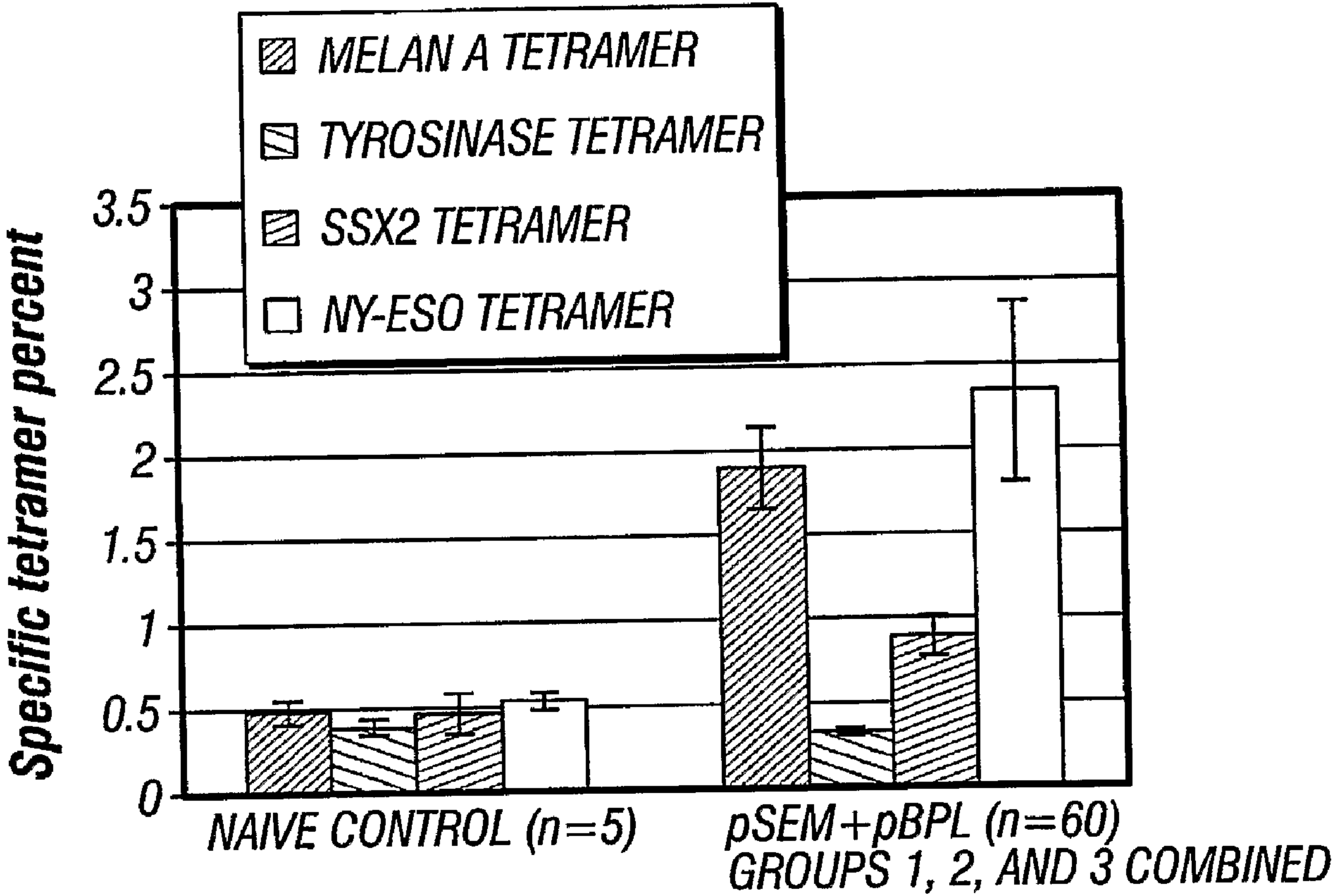


FIG. 10


MELAN A TETRAMER
TYROSINASE TETRAMER
SSX2 TETRAMER
NY-ESO TETRAMER
Specific tetramer + per percent
3.5
3
2.5
2
1.5
1
0.5
0
NAIVE CONTROL (n=5)
pSEM+pBPL (n=60)
GROUPS 1, 2, AND 3 COMBINED