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(54) Title: VACCINES AND IMMUNOTHERAPEUTICS COMPRISING IL-15 RECEPTOR ALPHA AND/OR NUCLEIC ACID MOLECULES ENCODING THE SAME, AND METHODS FOR USING THE SAME

(57) Abstract: Compositions, recombinant vaccines and live attenuated pathogens comprising one or more isolated nucleic acid molecules that encode an immunogen in combination with an isolated nucleic acid molecule that encodes IL-15Ra or a functional fragment thereof are disclosed. Methods of inducing an immune response in an individual against an immunogen, using such compositions are disclosed.

**VACCINES AND IMMUNOTHERAPEUTICS COMPRISING IL-15 RECEPTOR ALPHA AND/OR NUCLEIC ACID MOLECULES ENCODING THE SAME, AND METHODS FOR USING THE SAME**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Application No. 61/242,210, filed September 14, 2009, which is hereby incorporated by reference in its entirety.

**FIELD OF THE INVENTION**

The present invention relates to improved vaccines, improved methods for prophylactically and/or therapeutically immunizing individuals against immunogens, and to improved immunotherapeutic compositions and improved immunotherapy methods.

**BACKGROUND OF THE INVENTION**

Immunotherapy refers to modulating a person's immune responses to impart a desirable therapeutic effect. Immunotherapeutics refer to those compositions which, when administered to an individual, modulate the individual's immune system sufficient to ultimately decrease symptoms which are associated with undesirable immune responses or to ultimately alleviate symptoms by increasing desirable immune responses. In some cases, immunotherapy is part of a vaccination protocol in which the individual is administered a vaccine that exposes the individual to an immunogen against which the individual generates an immune response in such cases, the immunotherapeutic increases the immune response and/or selectively enhances a portion of the immune response (such as the cellular arm or the humoral arm) which is desirable to treat or prevent the particular condition, infection or disease.

Vaccine protocols can be improved by the delivery of agents that modulate a person's immune responses to induce an improved immune response. In some vaccination protocols in which the individual is administered a vaccine that exposes the individual to an immunogen against which the individual generates an immune response, an agent is provided that increases the immune response and/or selectively enhances a portion of the immune response (such as the cellular arm or the humoral arm) which is desirable to treat or prevent the particular condition, infection or disease.

Vaccines are useful to immunize individuals against target antigens such as allergens, pathogen antigens or antigens associated with cells involved in human diseases. Antigens associated with cells involved in human diseases include cancer-associated tumor antigens and antigens associated with cells involved in autoimmune diseases.

In designing such vaccines, it has been recognized that vaccines that produce the target antigen in cells of the vaccinated individual are effective in inducing the cellular arm of the immune system. Specifically, live attenuated vaccines, recombinant vaccines which use avirulent vectors, and DNA vaccines each lead to the production of antigens in the cell of the vaccinated individual which results in induction of the cellular arm of the immune system. On the other hand, killed or inactivated vaccines, and sub-unit vaccines which comprise only proteins do not induce good cellular immune responses although they do induce an effective humoral response.

A cellular immune response is often necessary to provide protection against pathogen infection and to provide effective immune-mediated therapy for treatment of pathogen infection, cancer or autoimmune diseases. Accordingly, vaccines that produce the target antigen in cells of the vaccinated individual such as live attenuated vaccines, recombinant vaccines that use avirulent vectors and DNA vaccines are often preferred.

The generation of potent CD8+ T cell responses by DNA vaccine technology is a goal sought by DNA vaccine developers. There are reports that CD8+ T cells contribute to controlling viral replication in both human (Koup et al., 1994; Cao et al., 1995; Musey et al., 1997; Ogg et al., 1998; Betts et al., 1999) and non human primate models (Jin et al., 1999; Schmitz et al., 1999; Barouch et al., 2000; Amara et al., 2001; Shiver et al., 2002) in the HIV model as well as other viral infections.

Many different strategies have been used along with DNA vaccine technology, including improved delivery techniques, enhanced construct design, heterologous prime-boost strategies, and the use of molecular adjuvants. Molecular adjuvants including chemokines and cytokines can be incorporated into a vaccine strategy to skew the immune response towards cellular or humoral immunity. Cytokines such as IL-12 and IL-15 have been effective in enhancing the immune response in both murine and non-human primate models (Morrow and Weiner, 2008).

Interleukin-15 (IL-15) has been shown to play a role in the generation and maintenance of CD8+ T cells as it signals through the common  $\beta\gamma$  chain, which is also utilized by IL-2. IL-15 has been shown to be trans-presented on the surface of antigen presenting cells via IL-15Ra

during the priming of Natural Killer and CD8+T cells (Dubois et al., 2002; Koka et al., 2004; Lucas et al., 2007; Sato et al., 2007). IL-15Ra has also recently been shown to play a role in the regulation of IL-15 secretion (Duitman et al., 2008). This cell surface complex is thought to allow IL-15 to signal thorough the  $\beta\gamma$  receptor on memory CD8+ T cells promoting cell division and survival of these cells (Ladolce et al., 1998; Kennedy et al., 2000; Lodolce et al., 2001; Burkett et al., 2003; Burkett et al., 2004; Sandau et al., 2004; Schluns et al., 2004a; Schluns et al., 2004b). IL-15 and IL-15Ra together as a complex exhibit enhanced stability and secretion compared to either molecule alone (Bergamaschi et al., 2008).

In regards to its employment in vaccination models, the use of plasmid-encoded IL-15 as an HIV-1 vaccine adjuvant has been previously reported to enhance cytolytic and memory CD8+T cell responses in mice (Oh et al., 2003; Kutzler et al., 2005; Zhang et al., 2006; Calarota et al., 2008; Li et al., 2008). Studies in rhesus macaques have also shown the ability of IL-15 to enhance effector functions of CD4+T cells (Picker et al., 2006) and rescued dual IFN- $\gamma$ /TNF responses in both effector CD4+ and CD8+ T cells (Halwani et al., 2008). Importantly, addition of pIL-15 with SIV/HIV antigens in rhesus macaques resulted in enhanced protection after SHIV89.6p challenge (Boyer et al., 2007).

While such vaccines are often effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods that produce an enhanced immune response. There still remains a need for improved strategies to enable effective DNA vaccines, including new adjuvants that enhance the immune response to DNA vaccines.

Likewise, while some immunotherapeutics are useful to modulate immune response in a patient there remains a need for improved immunotherapeutic compositions and methods.

## SUMMARY OF THE INVENTION

The present invention relates to nucleic acid molecule that comprise SEQ ID NO:1 or fragments thereof that encode proteins with IL-15Ra immunomodulatory function and/or IL-15 binding function and/or binding function to other subunits of a IL-15 receptor complex.

The present invention relates to a composition an isolated nucleic acid molecule that encodes an immunogen in combination with an isolated nucleic acid molecule that encodes or IL-15Ra or functional fragments thereof.

The present invention further relates to a composition an isolated nucleic acid molecule that encodes both an immunogen and IL-15Ra or functional fragments thereof.

The present invention relates to injectable pharmaceutical compositions comprising an isolated nucleic acid molecule that encodes an immunogen in combination with an isolated nucleic acid molecule that encodes IL-15Ra or functional fragments thereof.

The present invention relates to injectable pharmaceutical compositions comprising an isolated nucleic acid molecule that encodes both an immunogen and IL-Ra or functional fragments thereof.

In some aspects of the invention, the immunogen is a pathogen antigen, a cancer-associated antigen or an antigen from cells associated with autoimmune disease. In some aspects the pathogen is a pathogen that causes chronic infection.

The present invention further relates to methods of inducing an immune response in an individual against an immunogen, comprising administering to the individual a composition an isolated nucleic acid molecule that encodes an immunogen in combination with an isolated nucleic acid molecule that encodes IL-15Ra or functional fragments thereof.

The present invention further relates to methods of inducing an immune response in an individual against an immunogen, comprising administering to the individual a nucleic acid molecule that encodes an immunogen and IL-15Ra or functional fragments thereof.

The present invention further relates to recombinant vaccines comprising a nucleotide sequence that encodes an immunogen operably linked to regulatory elements, a nucleotide sequences that encode IL-15Ra or functional fragments thereof, and to methods of inducing an immune response in an individual against an immunogen comprising administering such a recombinant vaccine to an individual.

The present invention further relates to a live attenuated pathogen, comprising a nucleotide sequence that encodes IL-15Ra or functional fragments thereof, and to methods of inducing an immune response in an individual against a pathogen comprising administering the live attenuated pathogen to an individual.

## **BRIEF DESCRIPTION OF THE FIGURES**

Figures 1A-1E show results and information related to the generation of an IL-15Ra monoclonal antibody. Figure 1A shows Coomassie staining of the recombinant human IL-15Ra

protein in 2-fold dilutions from 12.0 to .16  $\mu$ g of protein. Figure 1B shows that a commercial anti-human IL-15R $\alpha$  antibody can detect recombinant IL-15R $\alpha$  protein in a direct ELISA. Figure 1C shows the immunization schedule for anti-human IL-15R $\alpha$  monoclonal antibody generation in BALB/c mice. Figure 1D shows that hybridoma supernatants from Clone KK1.23 have high titers of antibody against recombinant IL-15R $\alpha$  protein by ELISA. Figure 1E shows that the purified monoclonal antibody KK1.23 binds recombinant IL-15R $\alpha$  in ELISA and specifically as shown by Western blot analysis.

Figures 2A-2G relates to the construction and expression of the IL-15R $\alpha$  DNA plasmid. Figure 2A depicts how human IL-15R $\alpha$  cDNA was inserted into a pVAX1 expression vector. Figures 2B and 2C show that plasmid IL-15R $\alpha$  expresses the appropriate size protein (~30 kDa) as detected by radioactive *in vitro* translation with the R&D and the KK1.23 monoclonal anti-human IL-15R $\alpha$  antibody, respectively. Figure 2D shows that monoclonal KK1.23 antibody (IgG1 isotype) does not bind non-transfected HeLa cells (20x). Figure 2E shows pTRACER-IL-15R $\alpha$  transfected cells (green) stained with a mouse IgG1 isotype control (20x). Figures 2F and 2G show that the KK1.23 anti-human IL-15R $\alpha$  antibody (red) binds pIL-15R $\alpha$ -pTRACER transfected cells at 20x and at 60x, respectively.

Figures 3A-3C show that the combination of pIL-15 and pIL-15R $\alpha$  augments immune responses compared to either plasmid delivered alone. Figure 3A shows the immunization schedule for groups of BALB/c mice that were injected with DNA formulations containing combinations of vector, antigenic plasmid (HIV-1 gag and pol), pIL-15, and/or pIL-15R $\alpha$ . The combination of pIL-15/pIL-15R $\alpha$  was either given in the same leg or split between two different legs (pIL-15 in one, pIL-15R $\alpha$  in another). Intramuscular immunizations were given with electroporation 3 times, and mice were sacrificed one week following the final boost. Figures 3B and 3C show cellular responses. Splenocytes from immunized mice were used in an IFN- $\gamma$  ELISpot assay. Splenocytes were stimulated overnight with medium (negative control), Concanavalin A (positive control) or antigenic peptide (HIV-1gag and pol pools) and IFN- $\gamma$  spot forming units were counted.

Figures 4A and 4B show that IL-15R $\alpha$  DNA plasmid is immunogenic in a dose-dependent manner without pIL-15. BALB/c mice were immunized as shown in Figure 3A with DNA formulations containing combinations of vector, 5 $\mu$ g of antigenic plasmid (HIV-1gag and pol), and increasing doses of IL-15R $\alpha$  (7.5, 10, or 15 $\mu$ g). IFN- $\gamma$  ELISpot was carried out on

splenocytes stimulated with R10 (negative control), ConA (positive control), and HIV-1 gag peptide pools or HIV-1 pol peptide pools. Data from experiments using HIV-1 gag peptide pools are shown in Figure 4A. Data from experiments using HIV-1 pol peptide pools are shown in Figure 4B.

Figures 5A and 5B show that pIL-15R $\alpha$  augments IFN- $\gamma$  secretion primarily by CD8 $+$  T cells. The contribution of IFN- $\gamma$  secretion by CD8 $+$  T cells was measured by *ex vivo* depletion of CD8 $+$  T cells from the splenocytes of immunized mice using Miltenyi beads. Figure 5 A shows the total antigenic response for whole splenocytes (black bars) and CD8 depleted splenocytes (gray bars) and was measured by IFN- $\gamma$  ELISpot. In Figure 5B, antibody titers against HIV-1gag p24 antigenic protein were determined from sera samples of BALB/c mice immunized with the same constructs and timeline as described in the materials and methods. Dilutions of sera taken at the time of sacrifice were run on an ELISA plate coated with p24 and detected with an anti-mouse IgG-HRP antibody to measure levels of antigen specific IgG. Background responses in diluent wells only were subtracted from the sample OD values before graphing.

Figures 6A to 6C shows that the combination of pIL-15 and pIL-15R $\alpha$  does not enhance memory responses. Mice were immunized three times and rested for 30 weeks before sacrificing. Figure 6A shows IFN- $\gamma$  secretion was measured from splenocytes of immunized mice by IFN- $\gamma$  ELISpot. Figure 6B shows intracellular cytokine staining used to determine the memory response from immunized mice. Splenocytes from immunized mice were stimulated for 5 hours with medium, PMA/Ionomycin, or the dominant and subdominant HIV-1gag and pol antigenic peptides in the presence of Brefeldin A. In Figure 6C, sera was taken from immunized mice and run on an HIV-1gag p24 ELISA. Dilutions of sera were analyzed for antigen specific IgG. Background responses in diluent wells only were subtracted from the sample OD values before graphing.

Figures 7A to 7C show that pIL-15R $\alpha$  can adjuvant in the absence of endogenous IL-15. To explore the mechanism of IL-15R $\alpha$  as an adjuvant, we looked at the ability of human IL-15R $\alpha$  to bind murine IL-15. Figure 7A shows radiolabeled human IL-15R $\alpha$  binds mouse IL-15 and is co-immunoprecipitated with anti-mouse IL-15 antibody. Immunizations were also repeated in control C57BL/6, shown in Figure 7B, and IL-15 knockout mice, shown in Figure 7C, according to the schedule in Figure 3A and IFN- $\gamma$  ELISpots were performed on splenocytes.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

As used herein, the term "IL-15Ra" refers to interleukin 15 receptor alpha protein.

As used herein, "functional fragment" is meant to refer to a fragment of IL-15Ra that, when delivered in conjunction with an immunogen, provides an increased immune response compared to the immune that is induced when the immunogen is delivered without the fragment. Fragments are generally 10 or more amino acids in length.

As used herein the term "target protein" is meant to refer to peptides and protein encoded by gene constructs of the present invention that act as target proteins for an immune response. The terms "target protein" and "immunogen" are used interchangeably and refer to a protein against which an immune response can be elicited. The target protein is an immunogenic protein that shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which an immune response is desired. The immune response directed against the target protein will protect the individual against and/or treat the individual for the specific infection or disease with which the target protein is associated.

As used herein, the term "genetic construct" refers to the DNA or RNA molecules that comprise a nucleotide sequence which encodes a target protein or immunomodulating protein. The coding sequence includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the individual to whom the nucleic acid molecule is administered.

As used herein, the term "expressible form" refers to gene constructs that contain the necessary regulatory elements operably linked to a coding sequence that encodes a target protein or an immunomodulating protein, such that when present in the cell of the individual, the coding sequence will be expressed.

As used herein, the term "sharing an epitope" refers to proteins that comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure that is not identical to an epitope of a protein but nonetheless invokes a cellular or humoral immune response that cross-reacts to that protein.

As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins.

As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells.

As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease.

The invention arises from the discovery that when delivered as part of a vaccine, nucleic acid molecules that encode IL-15Ra and functional fragments thereof, and combinations thereof modulate immune responses. Accordingly nucleic acid molecules that encode IL-15Ra and functional fragments thereof, and combinations thereof may be delivered as immunotherapeutics in combination with or as components of a vaccine.

Additionally, the invention arises from the discovery that when delivered as part of a vaccine, nucleic acid molecules that encode IL-15Ra or functional fragments thereof, in combination with nucleic acid molecules that IL-15 or functional fragments thereof modulate immune responses. Accordingly nucleic acid molecules that encode IL-15Ra or functional fragments thereof, in combination with nucleic acid molecules that IL-15 or functional fragments may be delivered as immunotherapeutics in combination with or as components of a vaccine.

Some aspects of the invention provide the use of nucleic acid coding sequences of IL-15Ra protein in therapeutic vaccines, particularly in such cases where a CD8+ memory T cell response is not desired. Such therapeutic vaccines include those in which the antigen target is an antigen expressed in normal as well as disease associated cells whereby short term elimination of antigen-bearing cells has a therapeutic effect without a long term immune response directed to normal cells expressing the antigen. Accordingly, this aspect of the invention is particularly useful in therapeutic vaccines directed toward antigens on cancer cells which are also present on normal cells, therapeutic vaccines directed toward antigens expressed by cells associated with autoimmune disease which are also present on normal cells, and therapeutic vaccines directed toward pathogen antigens involved in chronic infections for which a persistent immune response would be undesirable. Chronic infections refer to those pathogen infections in which the pathogen is not cleared. Examples include but are not limited to HCV, HSV, CMV, chicken

pox, HIV, and the like, as contrasted with acute infections such as polio, small pox, mumps and the like.

Some aspects of the invention provide the use of nucleic acid coding sequences that encodes IL-15Ra protein in combination with nucleic acid coding sequences that encodes IL-15 in therapeutic vaccines, particularly in such cases where an enhanced burst immune response is desired. The combination of nucleic acid coding sequences of IL-15Ra protein and nucleic acid coding sequences that encodes IL-15 provide an additive adjuvant effect upon initial induction of the immune response. The nucleic acid coding sequences that encodes IL-15Ra protein may be administered to the same site as the nucleic acid coding sequences that encodes IL-15 or the nucleic acid coding sequences that encodes IL-15Ra protein and the nucleic acid coding sequences may be delivered to different sites to achieve the additive immune response.

The nucleotide sequence of human IL-15Ra is disclosed as Genbank accession nos. NM172200 and NM002189, which are each incorporated herein by reference. The protein sequence of human IL-15Ra is disclosed as Genbank accession nos. Q13261, NP002180 and NP751950, which are each incorporated herein by reference. In some embodiments of the invention, a nucleic acid coding sequences that encodes IL-15Ra protein is optimized for high levels of expression. In some embodiments of the invention, a nucleic acid coding sequences that encodes IL-15Ra protein are optimized such as in SEQ ID NO:1. In some embodiments of the invention, nucleic acid coding sequences that encodes IL-15Ra protein are non-optimized such as in SEQ ID NO:2.

The nucleotide sequence of human IL-15 is disclosed as Genbank accession nos. NM172174 and NM000585, which are each incorporated herein by reference. The protein sequence of human IL-15 is disclosed as Genbank accession nos. CAA86100, CAA62616, AAI00964, CAA72044, AAH18149 and AAU21241, which are each incorporated herein by reference. In some embodiments of the invention, a nucleic acid coding sequences that encodes IL-15 protein is optimized for high levels of expression. In some embodiments, improved IL-15 constructs such as those described in U.S. Serial No. 10/560,650 (US 20070041941), which is incorporated herein by reference, are used. In some embodiments, improved IL-15 constructs such as those described in U.S. Serial No. 12/160,766, which is incorporated herein by reference, are used. In some embodiments of the invention, a nucleic acid coding sequences that encodes IL-15 protein is SEQ ID NO:3.

In some embodiments of the invention, the nucleic acid coding sequences that encodes IL-15Ra protein and the nucleic acid coding sequence that encodes the target antigen are each on the same plasmid.

In some embodiments of the invention, a composition is provided comprising two plasmids: a first plasmid comprising the nucleic acid coding sequences that encodes IL-15Ra protein; and a second plasmid comprising the nucleic acid coding sequence that encodes the target antigen are each on the same plasmid.

In some embodiments of the invention, two compositions are provided. The first composition comprises a plasmid comprising the nucleic acid coding sequences that encodes IL-15Ra protein, and the second composition comprises a plasmid comprising the nucleic acid coding sequence that encodes the target antigen are each on the same plasmid. The two compositions may be provided in separate containers and packaged as a kit.

In some embodiments of the invention, the nucleic acid coding sequences that encodes IL-15Ra protein, the nucleic acid coding sequences that encodes IL-15, and the nucleic acid coding sequence that encodes the target antigen are each on the same plasmid.

In some embodiments of the invention, the invention provides a composition that comprises two plasmids. The nucleic acid coding sequences that encodes IL-15Ra protein and the nucleic acid coding sequences that encodes IL-15 are on one plasmid and the nucleic acid coding sequence that encodes the target antigen on a second plasmid.

In some embodiments of the invention, the invention provides a composition that comprises three plasmids. The nucleic acid coding sequence that encodes IL-15Ra protein is on a first plasmid, the nucleic acid coding sequence that encodes IL-15 is on a second plasmid and the nucleic acid coding sequence that encodes the target antigen on a third plasmid.

In some embodiments of the invention, the invention provides two compositions, a first composition that comprises one plasmid and a second composition that comprises one plasmid. In some such embodiments, the first composition comprises a plasmid that comprises the nucleic acid coding sequence that encodes IL-15Ra protein and the nucleic acid coding sequence that encodes the target antigen. The second composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes IL-15. In some such embodiments, the first composition comprises a plasmid that comprises the nucleic acid coding sequence that encodes IL-15 protein and the nucleic acid coding sequence that encodes the target antigen. The second

composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes IL-15Ra. In some such embodiments, the first composition comprises a plasmid that comprises the nucleic acid coding sequence that encodes IL-15 protein and the nucleic acid coding sequence that encodes IL-15Ra. The second composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes the target antigen. The multiple compositions may be provided in separate containers that are packaged to form a kit.

In some embodiments of the invention, the invention provides two compositions, a first composition that comprises two plasmids and a second composition that comprises one plasmid. In some such embodiments, the first composition comprises a first plasmid that comprises the nucleic acid coding sequence that encodes IL-15Ra protein and a second plasmid that comprises the nucleic acid coding sequence that encodes the target antigen. The second composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes IL-15. In some such embodiments, the first composition comprises a first plasmid that comprises the nucleic acid coding sequence that encodes IL-15 protein and a second plasmid that comprises the nucleic acid coding sequence that encodes the target antigen. The second composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes IL-15Ra. In some such embodiments, the first composition comprises a first plasmid that comprises the nucleic acid coding sequence that encodes IL-15 protein and a second plasmid that comprises the nucleic acid coding sequence that encodes IL-15Ra. The second composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes the target antigen. The multiple compositions may be provided in separate containers that are packaged to form a kit.

In some embodiments of the invention, the invention provides three compositions, a first composition that comprises one plasmid, a second composition that comprises one plasmid and a third composition that comprises one plasmid. In some such embodiments, the first composition comprises a plasmid that comprises the nucleic acid coding sequence that encodes IL-15Ra protein. The second composition comprises a plasmid that comprises the nucleic acid coding sequence that encodes the target antigen. The third composition comprises a plasmid that comprises the nucleic acid coding sequences that encodes IL-15. The multiple compositions may be provided in separate containers that are packaged to form a kit.

Isolated cDNA that encodes the immunomodulating proteins are useful as a starting material in the construction of constructs that can produce that immunomodulating protein.

Using standard techniques and readily available starting materials, a nucleic acid molecule that encodes an immunomodulating protein may be prepared.

The nucleic acid molecules may be delivered using any of several well known technologies including DNA injection (also referred to as DNA vaccination), recombinant vectors such as recombinant adenovirus, recombinant adenovirus associated virus and recombinant vaccinia virus.

DNA vaccines are described in U.S. Pat. Nos. 5,593,972, 5,739,118, 5,817,637, 5,830,876, 5,962,428, 5,981,505, 5,580,859, 5,703,055, 5,676,594, and the priority applications cited therein, which are each incorporated herein by reference. In addition to the delivery protocols described in those applications, alternative methods of delivering DNA are described in U.S. Pat. Nos. 4,945,050 and 5,036,006, which are both incorporated herein by reference.

Routes of administration include, but are not limited to, intramuscular, intranasally, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as topically, transdermally, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue. Preferred routes of administration include to mucosal tissue, intramuscular, intraperitoneal, intradermal and subcutaneous injection. Genetic constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microparticle bombardment gene guns".

Another route of administration involves the use of electroporation to deliver the genetic construct, as described in U.S. Patent Nos. 5,273,525, 5,439,440, 5,702,359, 5,810,762, 5,993,434, 6,014,584, 6,055,453, 6,068,650, 6,110,161, 6,120,493, 6,135,990, 6,181,964, 6,216,034, 6,233,482, 6,241,701, 6,347,247, 6,418,341, 6,451,002, 6,516,223, 6,567,694, 6,569,149, 6,610,044, 6,654,636, 6,678,556, 6,697,669, 6,763,264, 6,778,853, 6,865,416, 6,939,862 and 6,958,060, which are hereby incorporated by reference.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule. DNA may be introduced into cells, where it is present on a transient basis, in the form of a plasmid or plasmids. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may constitute part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which are administered to subjects. Gene constructs may be part of genomes of

recombinant viral vaccines where the genetic material remains extrachromosomal. Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the target protein or the immunomodulating protein. It is necessary that these elements be operably linked to the sequence that encodes the desired proteins and that the regulatory elements are operable in the individual to whom they are administered.

An initiation codon and a stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (MV) such as the BIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metallothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals, bovine growth hormone polyadenylation (bgh-PolyA) signal and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal that is in pCEP4 plasmid (Invitrogen, San Diego Calif.), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pVAX1, pCEP4 and pREP4 from Invitrogen (San Diego, Calif.) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In some preferred embodiments related to immunization applications, nucleic acid molecule(s) are delivered which include nucleotide sequences that encode a target protein, the immunomodulating protein and, additionally, genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode other cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-10, IL-12 and IL-15 including IL-15 having the signal sequence deleted and optionally including the signal peptide from IgE.

The compositions used in the methods may further comprise one or more of the following proteins and/or nucleic acid molecules encoding such proteins, as set forth in U.S. Serial No. 10/139,423, which corresponds to U.S. Publication No. 20030176378, which is incorporated herein by reference: Major Histocompatibility Complex antigens including Major Histocompatibility Complex Class I antigen or Major Histocompatibility Complex Class II antigen; death domain receptors including, but not limited to, Apo-1, Fas, TNFR-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and DR6; death signals, i.e. proteins that interact with the death domain receptors including, but not limited to FADD, FAP-1, TRADD, RIP, FLICE, and RAIDD; or death signals that include ligands that bind death domain receptors and initiate apoptosis including, but not limited to, FAS-L, and TNF; and mediators that interact with death domain receptors including, but not limited to, FADD, MORT1, and MyD88; toxins including proteins which kill cells such as, but not limited to, insect and snake venoms, bacterial endotoxins such as *Psuedomoneus* endotoxin, double chain ribosome inactivating proteins such as ricin including single chain toxin, and gelonin.

The compositions used in the methods may further comprise one or more of the following proteins and/or nucleic acid molecules encoding such proteins, as set forth in U.S. Serial No. 10/560,650, which corresponds to U.S. Publication No. 20070041941, which is incorporated

herein by reference: IL-15 including fusion proteins comprising non-IL-15 signal peptide linked to IL-15 protein sequences such as fusion proteins comprising an IgE signal peptide linked to IL-15 protein sequences, CD40L, TRAIL; TRAILrecDRC5, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, F461811 or MICA, MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, CD30, CD153 (CD30L), Fos, c-jun, Sp-1, Ap1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, I kB, NIK, SAP K, SAP1, JNK2, JNK1B2, JNK1B1, JNK2B2, JNK2B1, JNK1A2, JNK2A1, JNK3A1, JNK3A2, NF-kappa-B2, p49 splice form, NF-kappa-B2, p100 splice form, NF-kappa-B2, p105 splice form, NF-kappa-B 50K chain precursor, NFkB p50, human IL-1.alpha., human IL-2, human IL-4, murine IL-4, human IL-5, human IL-10, human IL-15, human IL-18, human TNF-.alpha., human TNF-.beta., human interleukin 12, MadCAM-1, NGF IL-7, VEGF, TNF-R, Fas, CD40L, IL-4, CSF, G-CSF, GM-CSF, M-CSF, LFA-3, ICAM-3, ICAM-2, ICAM-1, PECAM, P150.95, Mac-1, LFA-1, CD34, RANTES, IL-8, MIP-1.alpha., E-selecton, CD2, MCP-1, L-selecton, P-selecton, FLT, Apo-1, Fas, TNFR-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4 (TRAIL), DR5, KILLER, TRAIL-R2, TRICK2, DR6, ICE, VLA-1, and CD86 (B7.2).

The compositions used in the methods may further comprise one or more of the following proteins and/or nucleic acid molecules encoding such proteins, as set forth in U.S. Serial No. 10/560,653, which corresponds to U.S. Publication No. 20070104686, which is incorporated herein by reference: Fos, c-jun, Sp-1, Ap-1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, I kB, Inactive NIK, SAP K, SAP-1, JNK, interferon response genes, NFkB, Bax, TRAIL, TRAILrec, TRAILrecDRC5, TRAIL-R3, TRAIL-R4, RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, MICA, MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, TAP1, and TAP2.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate cells receiving the genetic construct for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the genetic construct. The drug gancyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk, thus, providing the means for the selective destruction of cells with the genetic construct.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons

may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs that are functional in the cells.

In some embodiments, gene constructs may be provided to in order to produce coding sequences for the immunomodulatory proteins described herein linked to IgE signal peptide.

One method of the present invention comprises the steps of administering nucleic acid molecules intramuscularly, intranasally, intraperitoneally, subcutaneously, intradermally, or topically or by lavage to mucosal tissue selected from the group consisting of inhalation, vaginal, rectal, urethral, buccal and sublingual.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a polynucleotide function enhancer or a genetic vaccine facilitator agent. Polynucleotide function enhancers are described in U.S. Pat. Nos. 5,593,972 and 5,962,428, which are each incorporated herein by reference. Genetic vaccine facilitator agents are described in U.S. Pat. No. 5,739,118, which is incorporated herein by reference. The co-agents that are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules. In addition, other agents which may function transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with a polynucleotide function enhancer include growth factors, cytokines and lymphokines such as  $\alpha$ -interferon, gamma-interferon, GM-CSF, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-10, IL-12 and IL-15 as well as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), LPS analog including monophosphoryl Lipid A (WL), muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the genetic construct. In some embodiments, an immunomodulating protein may be used as a polynucleotide function enhancer. In some embodiments, the nucleic acid molecule is provided in association with poly(lactide-co-glycolide) (PLG), to enhance delivery/uptake.

The pharmaceutical compositions according to the present invention comprise about 1 nanogram to about 2000 micrograms of DNA. In some preferred embodiments, pharmaceutical compositions according to the present invention comprise about 5 nanogram to about 1000 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain

about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 to about 200 microgram DNA.

The pharmaceutical compositions according to the present invention are formulated according to the mode of administration to be used. In cases where pharmaceutical compositions are injectable pharmaceutical compositions, they are sterile, pyrogen free and particulate free. An isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation.

According to some embodiments of the invention, methods of inducing immune responses against an immunogen are provided by delivering a combination of the immunogen and IL-15R $\alpha$  or functional fragments thereof to an individual. The vaccine may be a live attenuated vaccine, a recombinant vaccine or a nucleic acid or DNA vaccine.

The present invention is useful to elicit enhanced immune responses against a target protein, i.e. proteins specifically associated with pathogens, allergens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

According to some aspects of the present invention, DNA or RNA that encodes a target protein and immunomodulating protein is introduced into the cells of tissue of an individual where it is expressed, thus producing the encoded proteins. The DNA or RNA sequences encoding the target protein and immunomodulating protein are linked to regulatory elements

necessary for expression in the cells of the individual. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct.

In some embodiments, expressible forms of sequences that encode the target protein and expressible forms of sequences that encode both immunomodulating proteins are found on the same nucleic acid molecule that is delivered to the individual.

In some embodiments, expressible forms of sequences that encode the target protein occur on a separate nucleic acid molecule from expressible forms of sequences that encode the immunomodulatory protein. In some embodiments, expressible forms of sequences that encode the target protein and expressible forms of sequences that encode one or more of the immunomodulatory proteins occur on a one nucleic acid molecule that is separate from the nucleic acid molecule that contain expressible forms of sequences that encode the immunomodulating protein]. Multiple different nucleic acid molecules can be produced and delivered according to the present invention.

The nucleic acid molecule(s) may be provided as plasmid DNA, the nucleic acid molecules of recombinant vectors or as part of the genetic material provided in an attenuated vaccine. Alternatively, in some embodiments, the target protein and immunomodulating protein may be delivered as a protein in addition to the nucleic acid molecules that encode them or instead of the nucleic acid molecules which encode them.

Genetic constructs may comprise a nucleotide sequence that encodes a target protein or an immunomodulating protein operably linked to regulatory elements needed for gene expression. According to the invention, combinations of gene constructs that include one construct that comprises an expressible form of the nucleotide sequence that encodes a target protein and one construct that includes an expressible form of the nucleotide sequence that encodes an immunomodulating protein are provided. Delivery into a living cell of the DNA or RNA molecule(s) that include the combination of gene constructs results in the expression of the DNA or RNA and production of the target protein and one or more immunomodulating proteins. An enhanced immune response against the target protein results.

The present invention may be used to immunize an individual against pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize

an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryote such as gonorrhea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens that include a stage in the life cycle where they are intracellular pathogens. Table 1 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences that encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 2. Those skilled in the art can readily identify and distinguish those pathogens which cause chronic infections from those which are cleared post infection, i.e. acute infection.

## TABLES

### TABLE 1 - Viruses

#### Picornavirus Family

##### Genera:

Rhinoviruses: (Medical) responsible for ~50% cases of the common cold.

Etheroviruses: (Medical) includes polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus.

Aphthoviruses: (Veterinary) these are the foot and mouth disease viruses.

Target antigens: VP1, VP2, VP3, VP4, VPG

#### Calicivirus Family

##### Genera:

Norwalk Group of Viruses: (Medical) these viruses are an important causative agent of epidemic gastroenteritis.

#### Togavirus Family

##### Genera:

Alphaviruses: (Medical and Veterinary) examples include Sindbis virus, Ross River virus and Venezuelan Eastern & Western Equine encephalitis viruses.

Reovirus: (Medical) Rubella virus.

#### Flariviridae Family

Examples include: (Medical) dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses. West Nile virus (Genbank NC001563, AF533540, AF404757, AF404756, AF404755, AF404754, AF404753, AF481864, M12294, AF317203, AF196835, AF260969, AF260968, AF260967, AF206518 and AF202541)

Representative Target antigens: E NS5 C

Hepatitis C Virus: (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a flavivirus. Most similarity is with togavirus family.

**Coronavirus Family: (Medical and Veterinary)**

Infectious bronchitis virus (poultry)

Porcine transmissible gastroenteric virus (pig)

Porcine hemagglutinating encephalomyelitis virus (pig)

Feline infectious peritonitis virus (cats)

Feline enteric coronavirus (cat)

Canine coronavirus (dog)

SARS associated coronavirus

The human respiratory coronaviruses cause about 40% of cases of common cold. EX.

224E, OC43 Note - coronaviruses may cause non-A, B or C hepatitis

Target antigens: E1 - also called M or matrix protein E2 - also called S or Spike protein  
E3 - also called BE or hemagglutin-esterose glycoprotein (not present in all coronaviruses) N - nucleocapsid

**Rhabdovirus Family**

Genera:

Vesiculovirus, Lyssavirus: (medical and veterinary) rabies;

Target antigen: G protein, N protein

**Filoviridae Family: (Medical)**

Hemorrhagic fever viruses such as Marburg and Ebola virus

**Paramyxovirus Family:**

Genera:

Paramyxovirus: (Medical and Veterinary) Mumps virus, New Castle disease virus  
(important pathogen in chickens)

Morbillivirus: (Medical and Veterinary) Measles, canine distemper

Pneumovirus: (Medical and Veterinary) Respiratory syncytial virus

**Orthomyxovirus Family (Medical) The Influenza virus****Bunyavirus Family**

Genera:

Bunyavirus: (Medical) California encephalitis, La Crosse

Phlebovirus: (Medical) Rift Valley Fever

Hantavirus: Puremala is a hemahagin fever virus

Nairvirus (Veterinary) Nairobi sheep disease

Also many unassigned bungaviruses

**Arenavirus Family (Medical) LCM, Lassa fever virus****Reovirus Family**

Genera:

Reovirus: a possible human pathogen

Rotavirus: acute gastroenteritis in children

Orbiviruses: (Medical and Veterinary) Colorado Tick fever,

Lebombo (humans) equine encephalosis, blue tongue

**Retrovirus Family**

Sub-Family:

Oncorivinal: (Veterinary) (Medical) feline leukemia virus, HTLV and HTLVII

Lentivirinal: (Medical and Veterinary) HIV, feline immunodeficiency virus, equine infections, anemia virus

**Spumavirinal Papovavirus Family**

Sub-Family: Polyomaviruses: (Medical) BKU and JCU viruses

Sub-Family: Papillomavirus: (Medical) many viral types associated with cancers or malignant progression of papilloma.

Adenovirus (Medical) EX AD7, ARD., O.B. - cause respiratory disease - some adenoviruses such as 275 cause enteritis

Parvovirus Family (Veterinary)

Feline parvovirus: causes feline enteritis

Feline panleucopeniavirus

Canine parvovirus

Porcine parvovirus

Herpesvirus Family

Sub-Family:

alphaherpesviridue

Genera:

Simplexvirus (Medical)

HSV1 (Genbank X14112, NC001806),

HSVII (NC001798)

Varicella zoster: (Medical Veterinary)

Pseudorabies

varicella zoster

Sub-Family

betaherpesviridae

Genera:

Cytomegalovirus (Medical)

HCMV

Muromegalovirus

Sub-Family.

Gammaherpesviridae

Genera:

Lymphocryptovirus (Medical)

EBV - (Burkitt's lymphoma)

Poxvirus Family

Sub-Family:

Chordopoxviridae (Medical - Veterinary)

Genera:

Variola (Smallpox)

Vaccinia (Cowpox)

Parapoxvirus - Veterinary

Auipoxvirus - Veterinary

Capripoxvirus

Leporipoxvirus

Suipoxvirus

Sub-Family:

Entemopoxviridue

Hepadnavirus Family

Hepatitis B virus

## Unclassified Hepatitis delta virus

## TABLE 2

## Bacterial pathogens

Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal.

Pathogenic gram-negative cocci include: meningococcal; and gonococcal.

Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella, melioidosis; salmonella; shigellosis; haemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus mortiliformis and spirillum; listeria monocytogenes; erysipelothrix rhusiopathiae; diphtheria, cholera, anthrax; donovanosis (granuloma inguinale); and bartonellosis.

Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria.

Pathogenic spirochetal diseases include: syphilis; - treponematoses: yaws, pinta and endemic syphilis; and leptospirosis.

Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma, and chromomycosis; and dermatophytosis.

Rickettsial infections include rickettsial and rickettsioses.

Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections.

Pathogenic eukaryotes

Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material that encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as the coding sequence for the target. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material that encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 1 and 2 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed

against human immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis C virus (HCV), West Nile Virus (WNV) or hepatitis B virus (HBV).

Another aspect of the present invention provides a method of conferring a protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence that encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides; which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein that is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as myb, myc, fyn, and the translocation gene bcr/abl, ras, src, P53, neu, trk and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins that are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-IA and folate binding proteins or PSA.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as

epidemiology allow for the determination of probability and risk assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases.

Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) that are involved in the disease have been characterized. These TCRs include V. $\beta$ .-3, V. $\beta$ .-14, 20 V. $\beta$ .-17 and Va-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M. D., et al.,

1991 Proc. Nat. Acad. Sci. USA 88:10921-10925; Piliard, X., et al, 1991 Science 253:325-329; Williams, W. V., et al., 1992 J Clin. Invest. 90:326-333; each of which is incorporated herein by reference. In MS, several specific variable regions of TCRs that are involved in the disease have been characterized. These TCRs include VfP and Va-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K. W., et al., 1990 Science 248:1016-1019; Oksenberg, J. R., et al, 1990 Nature 345:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs that are involved in the disease have been characterized. These TCRs include V.beta.-6, V.beta.-8, V.beta.-14 and Va-16, Va-3C, Va-7, Va-14, Va-15, Va-16, Va-28 and Va-12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, et al 1987 Sequence of Proteins of Immunological Interest U.S. Department of Health and Human Services, Bethesda Md., which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V. K., et al, 1990 Proc. Natl. Acad Sci. USA 87:1066, which is incorporated herein by reference.

In addition to using expressible forms of immunomodulating protein coding sequences to improve genetic vaccines, the present invention relates to improved attenuated live vaccines and improved vaccines that use recombinant vectors to deliver foreign genes that encode antigens. Examples of attenuated live vaccines and those using recombinant vectors to deliver foreign antigens are described in U.S. Pat. Nos.: 4,722,848; 5,017,487; 5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368; 5,424,065; 5,451,499; 5,453,364; 5,462,734; 5,470,734; and 5,482,713, which are each incorporated herein by reference. Gene constructs are provided which include the nucleotide sequence that encodes an IL-R15 $\alpha$  or functional fragments thereof, wherein the nucleotide sequence is operably linked to regulatory sequences that can function in the vaccine to effect expression. The gene constructs are incorporated in the attenuated live vaccines and recombinant vaccines to produce improved vaccines according to the invention.

The present invention provides an improved method of immunizing individuals that comprises the step of delivering gene constructs to the cells of individuals as part of vaccine compositions which include DNA vaccines, attenuated live vaccines and recombinant vaccines. The gene constructs comprise a nucleotide sequence that encodes an IL-15 RECEPTOR ALPHA or functional fragments and that is operably linked to regulatory sequences that can function in the vaccine to effect expression. The improved vaccines result in an enhanced cellular immune response.

## EXAMPLE

Mice were co-immunized with pIL-15 and pIL-15R $\alpha$  to determine enhanced immune response generated using HIV-1 DNA vaccine antigens. Data show that although the IL-15 and IL-15R $\alpha$  combination indeed enhanced the overall cellular immune response, surprisingly the IL-15R $\alpha$  plasmid augmented immune responses in an IL-15 independent manner. Importantly, the induced memory response was only maintained in mice co-vaccinated with pIL-15 as well as the pIL-15R $\alpha$ , but not the IL-15R $\alpha$  alone. These studies for the first time demonstrate that the IL-15R $\alpha$  protein alone can function as an adjuvant with a limited immune expansion phenotype.

## MATERIALS AND METHODS

### Western Blot Analysis

Western blotting analysis was performed according to standard protocols. 3 $\mu$ g per well of recombinant IL-15R $\alpha$  or VPR protein (Abgent) was run on a SDS-PAGE gel (CambreX, Rockland, ME), blotted on nitrocellulose membrane, and probed with either the R&D or the KK1.23 anti-human IL-15R $\alpha$  antibody. The signal was amplified using an anti-mouse IgG-HRP (Zymed) and detected with ECL (GE Healthcare, Chalfont St. Giles, United Kingdom).

### DNA Plasmids

DNA vaccine constructs expressing HIV-1gag and HIV-1pol (Kim et al., 1998) and human IL-15 (Kutzler et al., 2005) were prepared as previously described. The open reading frame of human IL-15R $\alpha$  was moved into pVAX1 and pTRACER vectors (Invitrogen, Carlsbad, CA). Restriction enzyme digestion using EcoRI and BamHI or NheI and EcoRI (New England Biolabs, Beverly, MA) were used, respectively. Positive clones were verified by sequence analysis.

### In-vitro Translation assay

The TNT- T7 Quick Coupled Transcription/Translation Reticulocyte Lysate system (Promega, WI) and [ $^{35}$ S] methionine were used to create labeled IL-15R $\alpha$  protein product. pVAX vector alone (negative control) or pVAX vector containing IL-15R $\alpha$  and [ $^{35}$ S] methionine were added to the reaction mix according to the instructions supplied by the manufacturer. The reaction was carried out at 30oC for 1 hour. Labeled proteins were immunoprecipitated using 5 $\mu$ g purified monoclonal anti- IL-15R $\alpha$  antibody (R&D Systems) or Clone KK1.23 at 4oC with rotation overnight in RIPA buffer. Approximately 5mg of protein G-Sepharose beads (GE Healthcare) (50 $\mu$ L of 100mg/mL stock) was added to each

immunoprecipitation reaction, and the samples were incubated at 4°C with rotation for 2 hours. The beads were washed three times with binding buffer containing high salt and bovine serum albumin and finally suspended in 2x sample buffer. The immunoprecipitated protein complexes were eluted from sepharose beads by boiling for 5 minutes and were run on a 12% SDS-PAGE gel (CambreX). The gel was fixed and treated with amplifying solution (GE Healthcare), and dried for 2 hours in a gel drier (Bio-Rad, Hercules, CA). The dried gel was exposed to X-ray film at -80° and developed using the Kodak automatic developer (Kodak, Rochester, NY).

### **Indirect Immunofluorescent Assay**

The indirect immunofluorescent assay for confirmation of the pIL-15R $\alpha$  plasmid expression was conducted by the following protocol previously described (Ramanathan et al., 2002). HeLa cells (ATCC, Rockville, MD) grown in slide chambers (BD Biosciences, Bedford, MA) at a density of 100,000 cells per chamber in complete DMEM plus 10%FBS (Hyclone, Logan, UT) and antibiotic-antimycotic (GIBCO, Invitrogen, Carlsbad, CA) were allowed to adhere overnight. Cells were transfected with pIL-15R $\alpha$ TRACER or pVAX-1 (1 $\mu$ g/well) using FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to manufacturer's protocol. Twenty-four hours after transfection, cells were washed with PBS and fixed on slides using 2% PFA/PBS for 1 hour at RT. Slides were incubated with 5 $\mu$ g Clone KK1.23 mouse antihuman IL-15R $\alpha$  made in our laboratory or IgG1 Isotype control (R&D systems, Minneapolis, MN) for 90 minutes at 37 degrees. Anti mouse IgG-Rhodamine conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at 1:200 and the slides were incubated for 45 minutes at RT. Following, DAPI (Molecular Probes, Invitrogen) stain for 10 minutes at RT, slides were mounted in Fluoromount G medium (Electron Microscopy Sciences, Hatfield, PA) and analyzed using the Phase 3 Image Pro Program for fluorescent microscopy (Media Cybernetics, Bethesda, MD).

### **Plasmid Immunization and Mice**

The tibialis anterior muscle of 6 to 8 week old female BALB/c (Jackson Laboratory, Bar Harbor, ME), C57BL/6 (Taconic, Germantown, NY), or IL-15 knockout (Taconic) (Kennedy et al., 2000) mice were injected 3 times, 2 weeks apart, and electroporated as previously described (Khan et al., 2003; Laddy et al., 2008) using the CELLECTRA® adaptive constant current device (VGX Pharmaceuticals, The Woodlands, TX). For all experiments in mice, the animals were immunized with either 35 $\mu$ g of pVAX1, 5 $\mu$ g of HIV-1 antigenic plasmid (gag, pol), 10 $\mu$ g

of pIL-15, and/or 7.5, 10, or 15 $\mu$ g of pIL-15Ra (n=3-7 per group). Co-administration of various gene plasmids involved mixing the designated DNA plasmids before injection in 0.25% bupivacaine-HCL (Sigma) in isotonic citrate buffer (Kim et al., 1998; Kutzler et al., 2005) to a final volume of 40 $\mu$ l. All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institutes of Health and the University of Pennsylvania.

#### **Method for mouse sacrifice, sample collection and tissue harvest**

At timepoints designated in the immunization schedule, the animals were sedated using an analgesic and blood, was taken before animals were sacrificed by cervical dislocation. The spleen from each mouse were harvested and pooled (per experimental group) into a 15ml conical containing R10 medium (RPMI1640 plus 10% fetal bovine serum, antibiotic/antimycotic, and B-Mercaptoethanol). In a sterile tissue culture hood, the pooled spleen/medium mixture from each experimental group was crushed into a single cell suspension using a stomacher apparatus (Seward 80, Metrohm, Riverview, FL). The cell/tissue stroma were put through a 40-micron cell strainer and washed with R10, pelleted and incubated for 5 minutes at room temperature in ACK lysing buffer (Lonza, Switzerland) to lyse red blood cells. The splenocytes were then counted and utilized in immune assays described below.

#### **IFN- $\gamma$ ELISPOT Assay**

IFN- $\gamma$  ELISPOT was performed as previously described (Kutzler et al., 2005) to determine antigen specific cytokine secretion from immunized mice. Briefly, ELISpot 96-well plates are coated with anti-mouse IFN- $\gamma$  capture antibody and incubated for 24 hours at 4° C (R&D Systems). 2x10<sup>5</sup> splenocytes from immunized mice were added to each well of an ELISpot plate and stimulated overnight at 37° C, 5% CO<sub>2</sub>, in the presence of R10 (negative control), concanavalin A (positive control), or specific peptide (HIV-1 gag or pol) antigens (10 $\mu$ g/ml). HIV-1 Consensus Clade B subtype HIV-1 gag and pol 15-mer peptides spanning the entire respective protein, overlapping by 11 amino acids, were acquired from by the AIDS Reagent and Reference Repository (Frederick, MD). For CD8 depletion experiments, CD8+ T cells were removed from total splenocytes by positive magnetic selection using an anti-CD8a (Ly-2) antibody (Miltenyi Biotech, Germany) according to manufacturer's protocol. Following 24 hours of stimulation, the plates were washed and incubated at 4° C overnight with biotinylated anti-mouse IFN- $\gamma$  antibody (R&D Systems). The plates were washed and incubated with

streptavidinalkaline phosphatase (R&D Systems) for 2 hours at room temperature. The plate was washed, and 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT) (the Chromogen Color Reagent, R&D Systems) was added. The plate was rinsed with distilled water, and dried at room temperature. Spots were counted by an automated ELISpot reader (CTL Limited, Inc. Cleveland, OH). Raw values are determined and multiplied by a factor of five so that data is represented as spot forming cells per million splenocytes. Background values in the R10 wells of each group were subtracted from peptide-stimulated wells before graphing.

**Intracellular Cytokine Staining** HIV-1 specific T cell responses were also determined by intracellular cytokine staining using the Cytofix/Cytoperm Kit and standard protocol (BD Biosciences). Splenocytes from immunized mice were stimulated for 5 hours in the presence of 1ul/ml GolgiPlug (BD Biosciences) with R10 and DMSO (negative control), 10ng/ml PMA and 250ng/ml ionomycin (positive control), or HIV-1 consensus Clade B gag or pol 15-mer peptides. Prior to surface staining, cells were stained with the LIVE/DEAD fixable violet kit (Molecular Probes, Invitrogen) at 37° for 10 minutes and Fc block (BD) was added for 15 minutes at 4° to block Fc receptors. All antibodies were purchased from BD Biosciences and used at 1ul/test. Prior to permeabilization/fixation cells were stained with CD4-Alexa700 and CD8-PerCP for 30 minutes at 4°. CD3-PECy5 and IFN- $\gamma$  PE-Cy7 were included in the intracellular stain for 45 minutes at 4°. Data from 50,000 live CD3+ lymphocyte gated events were acquired using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc., Ashland, OR). Responses from the negative control wells were subtracted from the antigenic stimulations prior to graphing.

#### **Analysis of HIV-1Gag binding antibodies**

ELISA was used to determine HIV-1 Gag-specific antibodies IgG in mouse sera as described (Ogawa et al., 1989; Mestecky et al., 2004). EIA/RIA plates (Corning Costar, Cambridge, MA) were coated with 1 $\mu$ g/ml of recombinant HIV-1IIIB gag p24 (Immunodiagnostics, Woburn, MA) diluted in PBS (Mediatech, Herndon, VA) at a final volume of 100ul per well and incubated overnight at 4° C. Plates were washed with PBS/Tween (0.05% Tween 20) and blocked against non-specific binding with 200ul of blocking buffer/diluent (3%BSA in PBS) for 2 hours at room temperature. The plates were washed and dilutions of pooled sera from immunized mice were added in triplicate (100ul per well), at dilutions from 1

to 10 to 1 to 1600 and incubated at room temperature for 2 hours. Bound antibodies were detected with horseradish peroxidase-labeled goat anti-mouse IgG (H+L) (Zymed) and developed with substrate TMB H2O2 (Sigma-Aldrich). The color reaction was stopped with 2N H2SO4, and the absorbance at 450 nm read in an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

## RESULTS

### Generation of anti-human IL-15R $\alpha$ Antibody

A monoclonal antibody was generated against human IL-15R $\alpha$ , as commercially available antibodies are deficient in the ability to detect expression of the IL-15R $\alpha$  plasmid (pIL-15R $\alpha$ ) on cells. Recombinant human IL-15R $\alpha$  was generated as follows:

Recombinant human IL-15R $\alpha$  protein was generated by Abgent (San Diego, CA). The open reading frame of human IL-15R $\alpha$  (a generous gift from Thomas Waldmann (NCI, NIH, Bethesda, MD)) was cloned into high expressing bacterial vector, pET21a (EMD Biosciences, Gibbstown, NJ). Competent cells were transformed, amplified in E.coli, and recombinant protein was purified using a Ni-NTA column. The accuracy of the purified protein was confirmed by direct ELISA using anti-human IL-15R $\alpha$  antibody (R&D Systems, Minneapolis, MN).

To confirm the size of the newly generated IL-15R $\alpha$  protein, decreasing dilutions of purified protein were run on a SDS-PAGE gel and stained with coomassie blue dye (Figure 1A). As shown in Figure 1A, the generated protein runs at approximately 30kDa, the expected size. This protein was tested for the ability to bind to commercially available antibody as an indication of its correct integrity. Figure 1B shows an ELISA assay with plates captured with recombinant IL-15R $\alpha$  or VPR protein, a negative control. VPR was used as it was produced by a similar method to the IL-15R $\alpha$  protein. Figure 1B shows that the commercially available anti-human IL-15R $\alpha$  antibody can detect the generated recombinant protein.

To generate an antibody against IL-15R $\alpha$ , recombinant human IL-15R $\alpha$  protein was injected into BALB/c mice as in Figure 1C, and as follows:

Recombinant human IL-15R $\alpha$  protein was injected into BALB/c mice (n=4) for monoclonal antibody generation. 5 $\mu$ g of total protein emulsified in complete Freund's adjuvant (first immunization only) or incomplete Freund's adjuvant (subsequent immunizations) was given per injection (Sigma, St. Louis, MO). 50 $\mu$ l was injected subcutaneously into each flank and 100  $\mu$ l was injected into the peritoneum. Mice were given a final boost of 35ug protein in

sterile PBS intravenously three days prior to fusion. Antibody levels in the sera were determined by direct ELISA using recombinant IL-15R $\alpha$  protein and anti-mouse IgG-HRP (Zymed, San Francisco, CA). One mouse with a 1:8,000 titer of antibody against IL-15R $\alpha$  was sacrificed at its spleen removed for fusion with a myeloma cell line. 1,500 hybridoma supernatants were screened by ELISA, 8 positive clones were expanded, and one was purified by an ammonium sulfate column, antibody KK1.23. Monoclonal antibodies were generated and purified by Julia Conicello of the Wistar Institute Hybridoma Facility (Philadelphia, PA)...

After screening approximately 1,500 hybridoma supernatants by ELISA, one hybridoma KK1.23 exhibited titers of antibody (> 1 to 12,800) as shown in Figure 1D. This hybridoma was subsequently cloned, expanded, and purified. Purified antibody KK1.23 is specific for human IL-15R $\alpha$  as shown by Western blot analysis in Figure 1E. In addition, KK1.23 appears to bind to human IL-15R $\alpha$  with a higher affinity than the commercially available antibody (Figure 1E).

### **pIL-15R $\alpha$ expresses bioactive protein**

An IL-15R $\alpha$  expression vector was created that was suitable for use in vaccination studies. The human IL-15R $\alpha$  ORF was cloned into the pVAX1 expression vector as shown in Figure 2A, under the control of the CMV promoter. To assess the appropriate expression of the IL-15R $\alpha$  plasmid, an in vitro translation assay was carried out. The S35 radiolabeled protein is shown in Figures 2B & 2C migrating at roughly 30.0kD, whereas the control plasmid, pVAX, did not yield any detectable protein product as expected. The commercial R&D (2B) or the KK1.23 (2C) antibody against human IL-15R $\alpha$  was utilized to immunoprecipitate the radiolabeled protein.

To confirm expression of the plasmid IL-15R $\alpha$ , an immunofluorescence assay was performed using the KK1.23 antibody from Example I, above. The ORF of human IL-15R $\alpha$  was cloned into the pTRACER expression vector, which also encodes for the green fluorescent protein (GFP) reporter. Therefore, cells fluorescing green (Figure 2E-G) also express pIL-15R $\alpha$ . The KK1.23 anti-human IL-15R $\alpha$  is detected using anti-mouse IgG-PE (Red). The untransfected control is shown in Figure 2D, and the isotype control in Figure 2E. The data illustrates both the ability of the pIL-15R $\alpha$  plasmid to express as well as the ability of the anti-human pIL-15R $\alpha$  antibody to detect the translated protein product. The pIL-15R $\alpha$  plasmid encodes for a conformationally accurate and surface localized protein.

### **Combining pIL-15 and pIL-15R $\alpha$ as an Adjuvant**

To examine the ability of pIL-15R $\alpha$  to enhance immune responses as compared with pIL-15, BALB/c mice were immunized intramuscularly in the tibialis anterior muscle accompanied by in vivo electroporation, according to the schedule shown in Figure 3A. Mice were immunized with either pVAX control vector, or 5 $\mu$ g of antigenic constructs (HIV-1 gag, HIV-1 pol) with 10 $\mu$ g of pIL-15, 15 $\mu$ g of pIL-15R $\alpha$ , or both pIL-15 and pIL-15R $\alpha$  in a final volume of 40 $\mu$ l. These doses were predetermined to give optimal responses in preliminary studies (data not shown). As shown in Figure 3B, immunization with antigenic constructs alone resulted in 2,300 spot forming cells (SFC)/106 splenocytes as measured by IFN- $\gamma$  ELISpot. The addition of pIL-15 enhanced the response to 3,800 SFC, while co-immunization with pIL-15R $\alpha$  and pIL-15 exhibited the most dramatic increase over the antigenic group alone, resulting in 5,900 SFC. These results support the idea that the formation of the IL-15/IL-15R $\alpha$  immune complex can serve as a more potent adjuvant than IL-15 alone.

To determine whether this immune complex was truly being formed in vivo, another immunization group was added in which the pIL-15 and pIL-15R $\alpha$  were injected (with antigen) in separate legs. In this split delivery method, plasmid-delivered IL-15 and IL-15R $\alpha$  would be unable to form an immune complex. The co-immunization of pIL-15 and IL-15R $\alpha$  in separate legs was found to elicit levels of IFN- $\gamma$  similar to those observed with the same combination delivered in the same leg (4,562 vs. 4,072 SFC, respectively). It is noted that the immunization group with antigenic construct and pIL-15R $\alpha$  also augmented antigen specific IFN- $\gamma$  secretion to approximately 3500 SFC (Figure 3B). To confirm these results, we immunized a new set of mice with increasing doses of pIL-15R $\alpha$  plasmid in conjunction with antigenic constructs to see if the pIL-15R $\alpha$  would induce responses in a dose-dependent fashion. As shown in Figure 4, the inclusion of pIL-15R $\alpha$  did enhance the induced IFN- $\gamma$  secretion in a dose dependent fashion, in measured responses against pGag (Panel A) or pPol (Panel B). Regardless of the HIV-1 antigenic construct used, co-immunization with pIL-15R $\alpha$  augmented cellular immune responses by 1.5 to 2 fold at the highest dose used. Notably, IL-15R $\alpha$  appears to enhance antigen specific immune responses even in the absence of IL-15.

To further confirm the adjuvant properties of pIL-15R $\alpha$ , the effector functions of CD4+ and CD8+ T cells after vaccination were examined. CD8+ T cells were depleted from splenocytes of mice immunized with each vaccine combination previously mentioned, above, prior to carrying out the IFN- $\gamma$  ELISpot assay. As shown in Figure 5A, the depletion of CD8+ T

cells from the splenocytes of mice immunized with either pIL-15, pIL-15Ra or the combination of both significantly decreased the amount of IFN- $\gamma$  secretion detected. There was no difference between the CD4+ T cell contribution (grey bars) in any of the immunized groups, compared to the total responses observed in whole splenocytes (black bars). Taken together, the combination of pIL-15Ra and pIL-15 in a vaccination strategy greatly enhance the immune response over either construct delivered alone. This additive effect primarily acts on CD8+T cells, as the effect was lost with the depletion of this cell population.

To determine whether pIL-15Ra would also have an effect on humoral immune responses, antibody responses elicited through each vaccination strategy were also measured by ELISA. Sera from immunized mice was assayed to measure the levels of IgG antibodies against the HIV-1 Gag (p24) protein (Figure 5B). While the combination of pIL-15 and pIL-15Ra was the best at eliciting cellular immunity, mice immunized with either pIL-15 or pIL-15Ra alone had the highest titers of HIV-1 specific antibodies (1:1600) compared to mice immunized with pVAX (not detected), pGag alone or the combination (1:800).

#### **The pIL-15Ra adjuvant does not appear to enhance CD8+T cell memory**

Mice were immunized three times as previously mentioned, herein; however, instead of sacrificing these animals one week post the third immunization, they were allowed to rest for approximately 30 weeks to be sure the responses observed would be contributed primarily by the memory population. As shown in Figure 6A, the responses after a significant rest period were still quite robust. The mice immunized with the antigenic construct alone had responses around 1700 SFC. The highest responses were clearly in groups of mice co-immunized with pIL-15, ~2800 SFC for both pIL-15 and pIL-15/pIL-15Ra combination. In mice co-immunized with pIL-15Ra in the absence of pIL-15, an adjuvant affect was no longer observed (~1700 SFC). The same trends were also observed by intracellular cytokine staining and flow cytometry (Figure 6B) where the level of IFN- $\gamma$  production by CD8+ T cells was most pronounced in mice coimmunized with pIL-15. The addition of pIL-15Ra in the vaccination strategy was observed to have little effect on memory responses, whereas pIL-15 was observed to have a large effect. Thus, supporting the theory that while pIL-15Ra was a robust adjuvant early after vaccination, over time IL-15 appears to be a better inducer of memory CD8+ T cells.

The memory antibody response was similar to that observed during the effector phase. As shown in Figure 6C, mice immunized with pIL-15 had detectable antibodies against HIV-1 Gag

(p24) at dilutions out to 1:1600, whereas all other groups, including the combination of pIL-15/pIL-15R $\alpha$  diluted out at 1:400. While pIL-15 shows to be an effective adjuvant in the generation of humoral as well as cellular memory responses; pIL-15R $\alpha$ , on the other hand, appears to play role in accelerating the acute immune response to antigen.

### **pIL-15R $\alpha$ Adjuvant without IL-15**

To test whether human IL-15R $\alpha$  protein could be augmenting immune responses in vaccinated mice by forming complexes with endogenous murine IL-15, or independently of IL-15, vaccinations were studied in IL-15 knockout mice. Initially, as a control, translated human IL-15R $\alpha$  protein was tested for binding to mouse IL-15. As shown in Figure 7A, S35 radiolabeled human IL-15R $\alpha$  protein incubated with murine IL-15 was able to be immunoprecipitated with an anti-mouse IL-15 antibody suggesting the ability of murine IL-15 to bind to human IL-15R $\alpha$ .

The ability of pIL-15R $\alpha$  to adjuvant in the absence of murine IL-15 was examined. Accordingly, the same vaccination studies in IL-15 knockout mice were conducted, which lack endogenous IL-15 and as a result have a deficiency in NK and memory CD8+T cells (Kennedy et al., 2000). Figure 7C shows that pIL-15R $\alpha$  adjuvants the immune responses in the absence of endogenous murine IL-15 in the knockout mice, as determined by IFN- $\gamma$  ELISpot. Furthermore, the combination of pIL-15 and pIL-15R $\alpha$  fails to further enhance the immune response by either adjuvant administered alone as initially observed in BALB/c mice. The IL-15-/ mice were generated on a C57/BL6 background from Taconic. Therefore, to verify we would get similar responses in the appropriate background control mice as were observed in BALB/c, the same experiments were repeated. Figure 7B shows the results from the control mice immunized with the identical schedule and shows the same trend as BALB/c immunized mice.

## **CONCLUSION**

Coimmunization of the IL-15R $\alpha$  construct together with a human IL-15 and HIV-1 antigenic DNA constructs resulted in levels of IFN- $\gamma$  secretion that were 2.5 fold more potent than immunization with the antigenic constructs alone (Figure 3B). The IFN- $\gamma$  secretion was attributable to CD8+ T cells as the depletion of these cells prior to plating on the ELISpot resulted in 10 fold less IFN- $\gamma$  secretion. The increased potency observed with the combined delivery of IL-15/IL-15R $\alpha$  is not likely due to the formation of a stable complex in transfected cells as injecting these pIL-15 and pIL-15R $\alpha$  into separate legs (with antigen) also elicited

immune responses similar to delivery in the same leg. It is therefore more likely that the enhanced response observed with the co-delivery of pIL-15/pIL-15R $\alpha$  is an additive effect of two independent adjuvants. Co-delivery of these two adjuvants did not appear to further enhance humoral immune responses as measured by IgG antibodies in the sera.

The long-term effects of the combined pIL-15/pIL-15R $\alpha$  on the immune response was also examined. In order to observe the memory responses, 30 weeks after the third immunization was allowed to pass prior to carrying out immune analysis. The results showed that although the combination of these two adjuvants elicits potent CD8+ T cell responses in the early phase of an immune response, the effect on memory T cells is primarily observed only in mice immunized along with pIL-15. The inclusion of pIL-15 was needed for an enhanced memory immune response over the antigen alone. Similarly, IL-15R $\alpha$  initially elicited immune responses equal to or greater than that IL-15, but it did not help to sustain the memory response. Therefore, while IL-15R $\alpha$  expanded burst size, burst size in the absence of the IL-15 signal for memory was not enough to sustain a long-term response.

In a surprising observation, the delivery of the antigenic plasmid with pIL-15R $\alpha$  also augmented cellular immune responses, and equal to those elicited by pIL-15. To be certain, immunizations were performed with increasing amounts of pIL-15R $\alpha$  and dose-dependent responses were observed. It may be that the human IL-15R $\alpha$  protein was able to bind to endogenous murine IL-15 and transpresent it in a similar fashion since murine IL-15 is ~73% identical to the human IL-15 (Anderson et al., 1995a). To test this hypothesis, IL-15 knockout mice, which lack any endogenous IL-15, were immunized. An approximate 2 fold increase in the IL-15 knockout mice immunized with pIL-15R $\alpha$  over antigen alone was observed. Due to the difficulty of obtaining IL-15 $^{-/-}$  female mice in large numbers between the ages of 6-8 weeks, the pIL-15 group from these experiments were excluded. However, the enhanced effect of the combination of pIL-15/pIL-15R $\alpha$  was no longer observed. It should be noted that control C57/BL6 mice exhibited the same trends that were seen in BALB/c mice (albeit lower total spot counts and higher background in the pVAX group) and that the IL-15 knockout mice had overall lower responses even compared to the C57/BL6 mice. These mice have been previously described to have somewhat defective host defense responses including the inability to protect against a vaccinia challenge (Kennedy et al., 2000). Regardless of the overall lower immune responses, the adjuvanting effect of pIL-15R $\alpha$  was still observed in the absence of any

endogenous IL-15. While not intending to be bound by this theory, considering the full complement of results, it is believed that IL-15R $\alpha$  can serve as a novel adjuvant capable of eliciting responses independently of IL-15. This amplification of the immune response appears particularly focused on immune expansion during the acute phase rather than the memory phase of the host T cell response.

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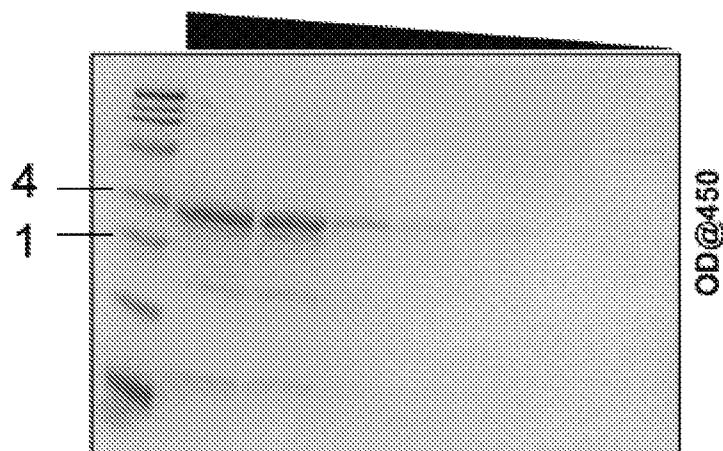
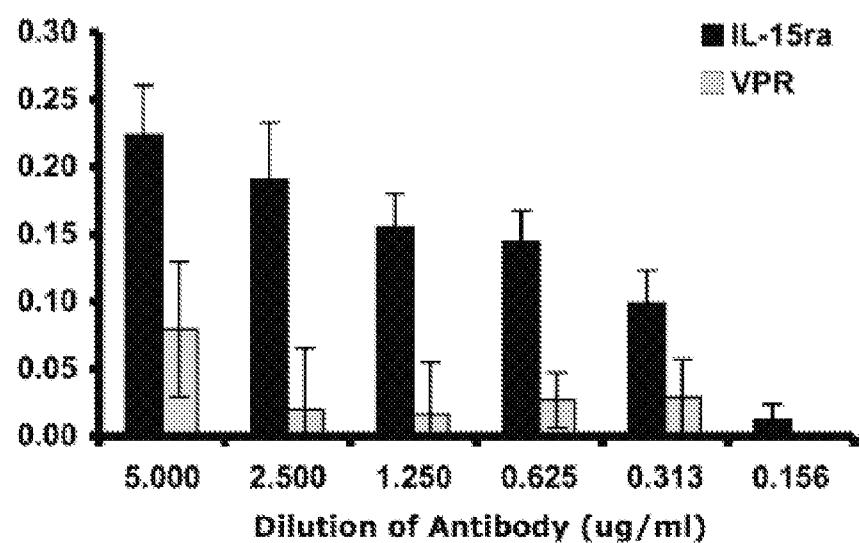
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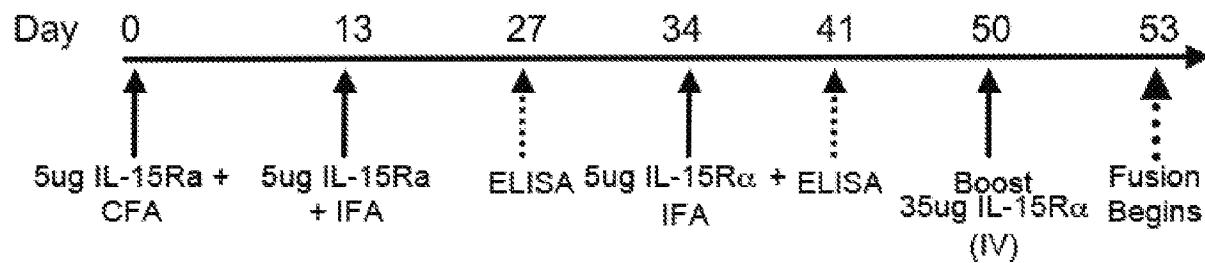
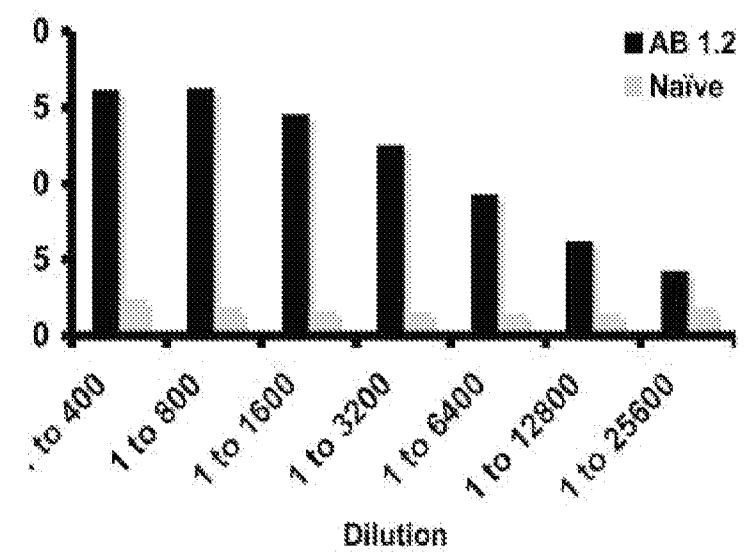
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**CLAIMS**

1. A composition comprising: an isolated nucleic acid molecule that encodes an immunogen; and an isolated nucleic acid molecule that encodes IL-15R $\alpha$  or functional fragments thereof.
2. The composition of claim 1 wherein said immunogen is a pathogen antigen, a cancer associated antigen or an antigen associated with cells involved in autoimmune diseases.
3. The composition of claim 2 wherein said immunogen is a pathogen antigen from a pathogen that causes chronic infection.
4. The composition of any of claims 1-3 wherein said isolated nucleic acid molecule that encodes IL-15R $\alpha$  comprises a nucleic acid coding sequence that encodes IL-15Ra having a sequence as set forth in SEQ ID NO:1.
5. The composition of any of claims 1-4 further comprising a nucleic acid sequence that encodes IL-15 or a functional fragment thereof.
6. The composition of any of claims 1-5 wherein said nucleic acid molecules are plasmids
7. An injectable pharmaceutical composition comprising the composition of any of claims 1-6.
8. A method of inducing an immune response in an individual against an immunogen comprising administering to said individual a composition according to any of claims 1-7.
9. A recombinant vaccine comprising a nucleotide sequence that encodes an immunogen operably linked to regulatory elements, and a nucleotide sequence that encodes IL-15R $\alpha$  or a functional fragment thereof.
10. The recombinant vaccine of claim 9 wherein said immunogen is a pathogen antigen, a cancer-associated antigen or an antigen associated with cells involved in autoimmune diseases.
11. The recombinant vaccine of claim 10 wherein said immunogen is a pathogen antigen from a pathogen that causes chronic infection.

12. The recombinant vaccine of any of claims 9-11 wherein said isolated nucleic acid molecule that encodes IL-15R $\alpha$  comprises anucleic acid coding sequence that encodes IL-15Ra having a sequence as set forth in SEQ ID NO:1.
13. The recombinant vaccine of any of claims 9-12 further comprising a nucleic acid sequence that encodes IL-15 or a functional fragment thereof.
14. A method of inducing an immune response in an individual against an immunogen comprising administering to said individual a recombinant vaccine of any of claims 9-13.
15. A live attenuated pathogen comprising a nucleotide sequence that encodes IL-15R $\alpha$  or a functional fragment thereof.
16. The live attenuated pathogen of claim 15 wherein said pathogen is an attenuated strain of a pathogen that causes chronic infection.
17. The live attenuated pathogen of any of claims 15 or 16 wherein the nucleic acid coding sequence that encodes IL-15R $\alpha$  is SEQ ID NO:1.
18. The live attenuated pathogen of any of claims 15-17 further comprising a nucleic acid sequence that encodes IL-15 or a functional fragment thereof.
19. A method of inducing an immune response in an individual against an immunogen comprising administering to said individual a live attenuated pathogen of any of claims 15-18.
20. A nucleic acid molecule comprising SEQ ID NO: 1 or a fragment thereof that has IL-15R $\alpha$  immunomodulatory function, IL-15 binding function, binding function to other subunits of a IL-15 receptor complex, or a combination thereof.

**Figure 1A****Figure 1B**

**Figure 1C****Figure 1D**

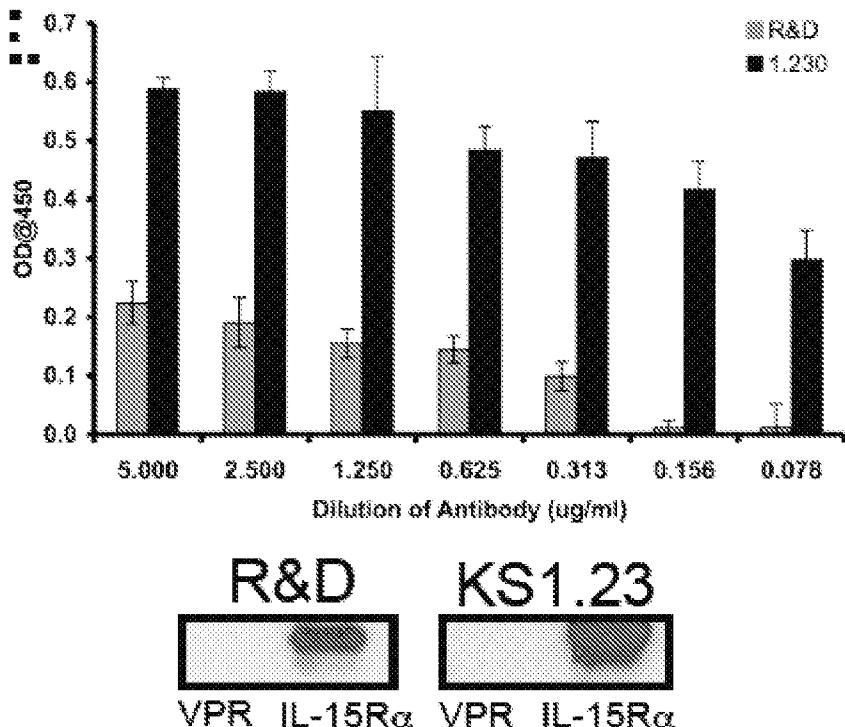
**Figure 1E**

Figure 2A

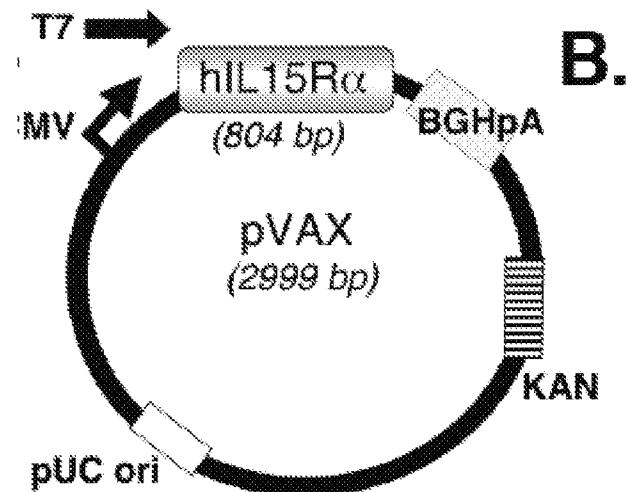
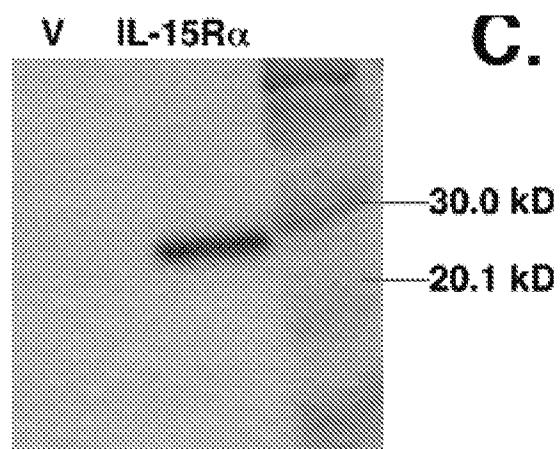
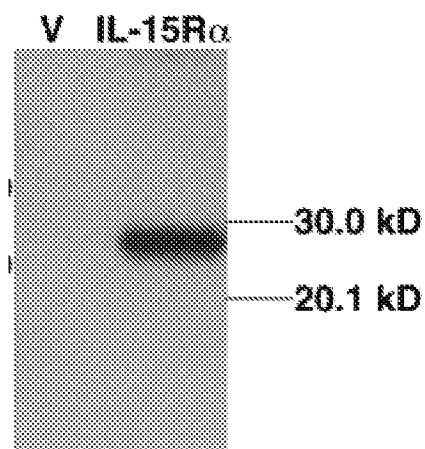
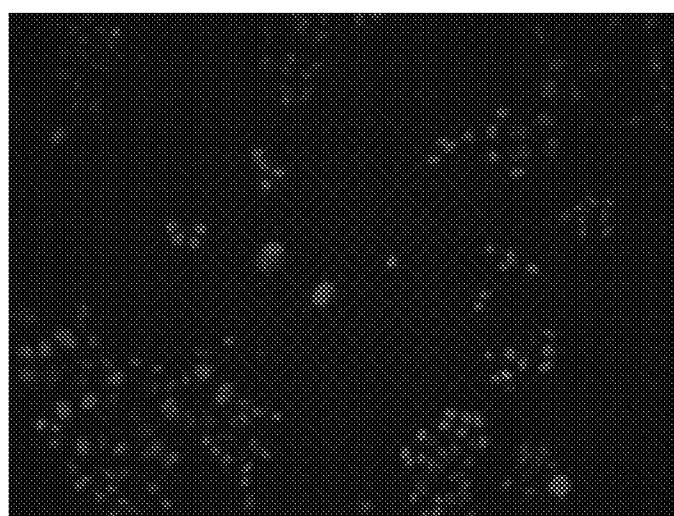
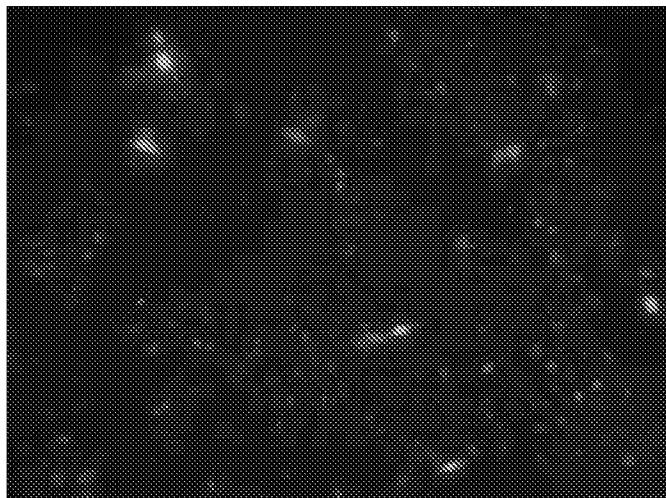


Figure 2B

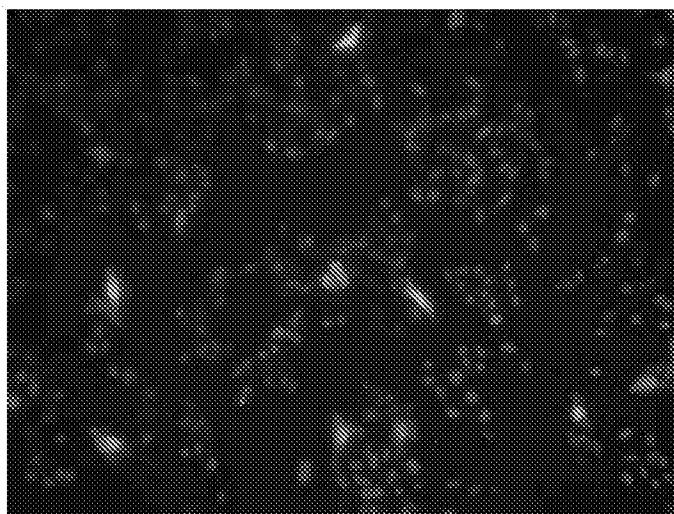
**C.**

**Figure 2C****Figure 2D**

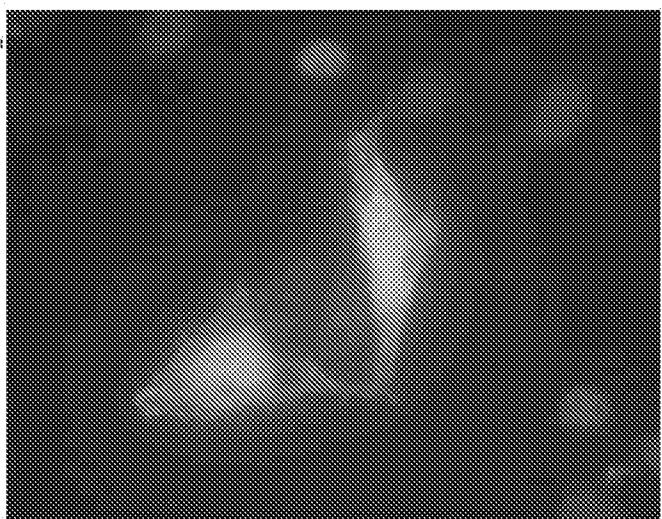
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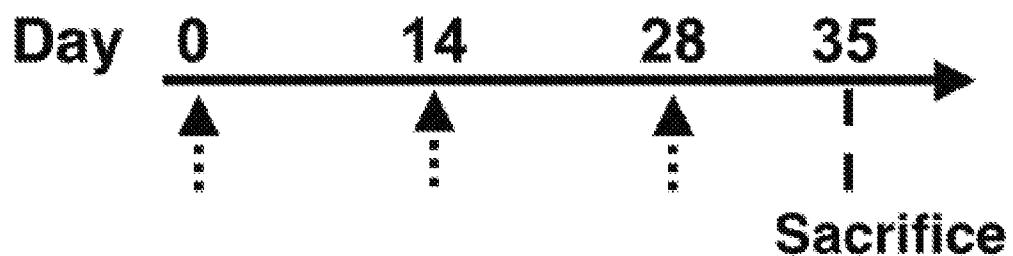
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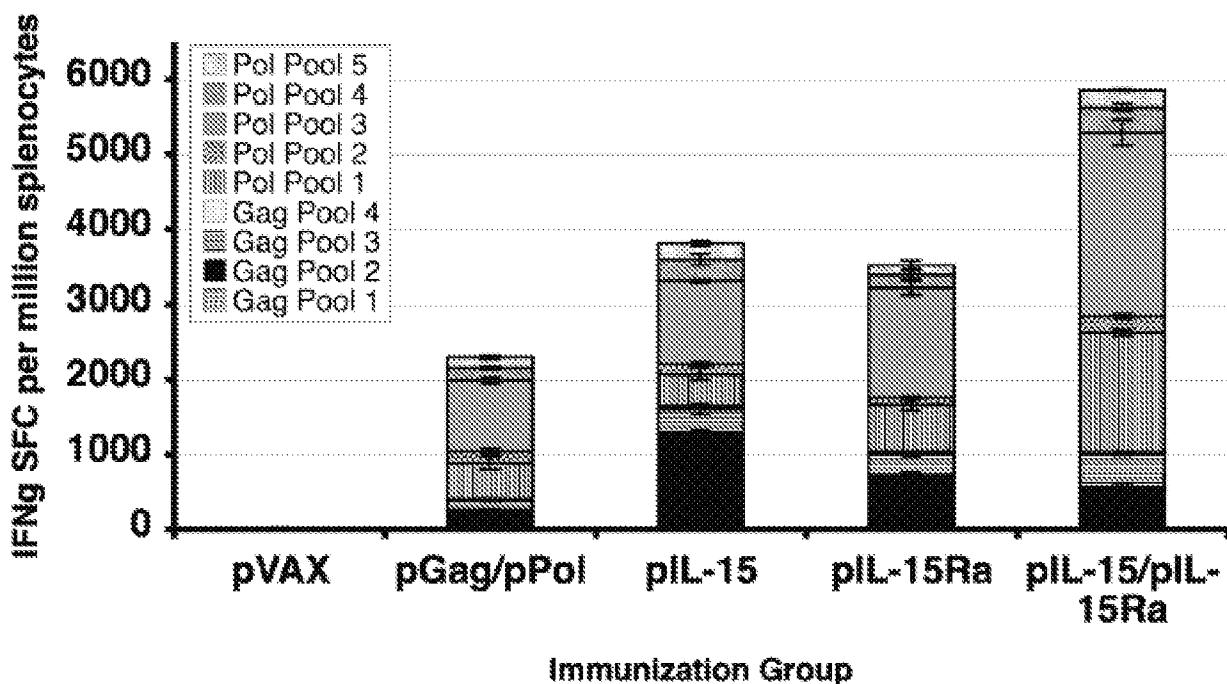
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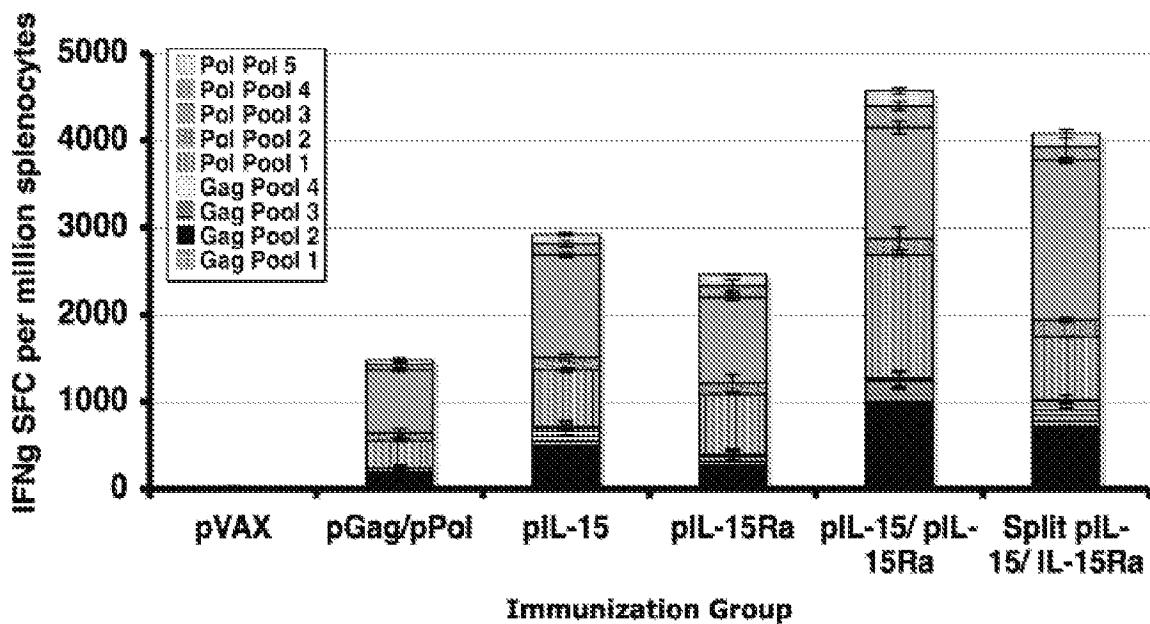
**Figure 3A**

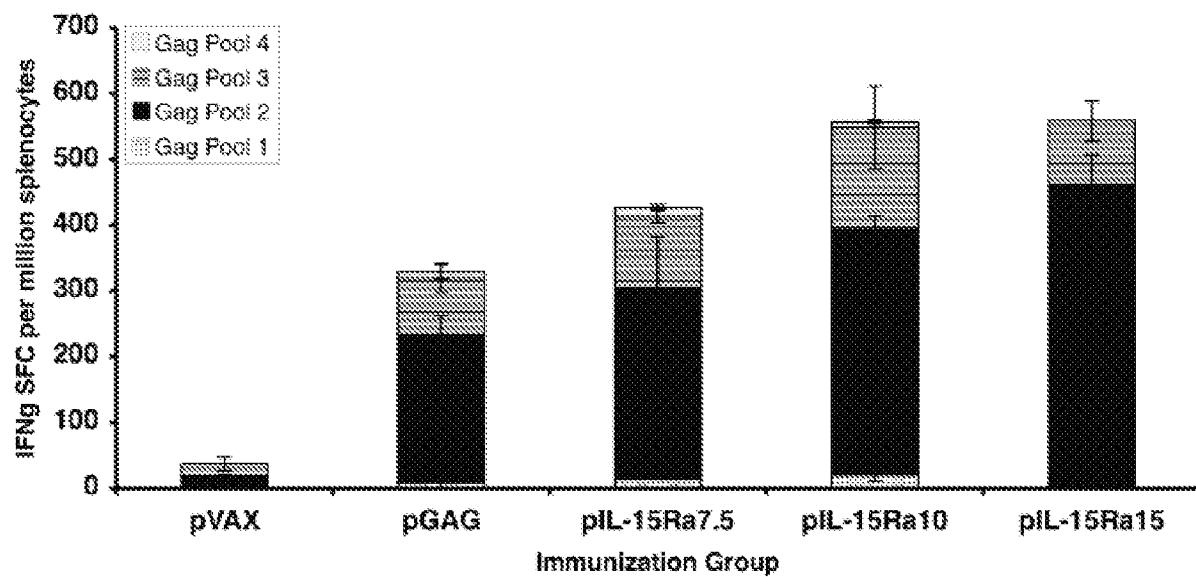
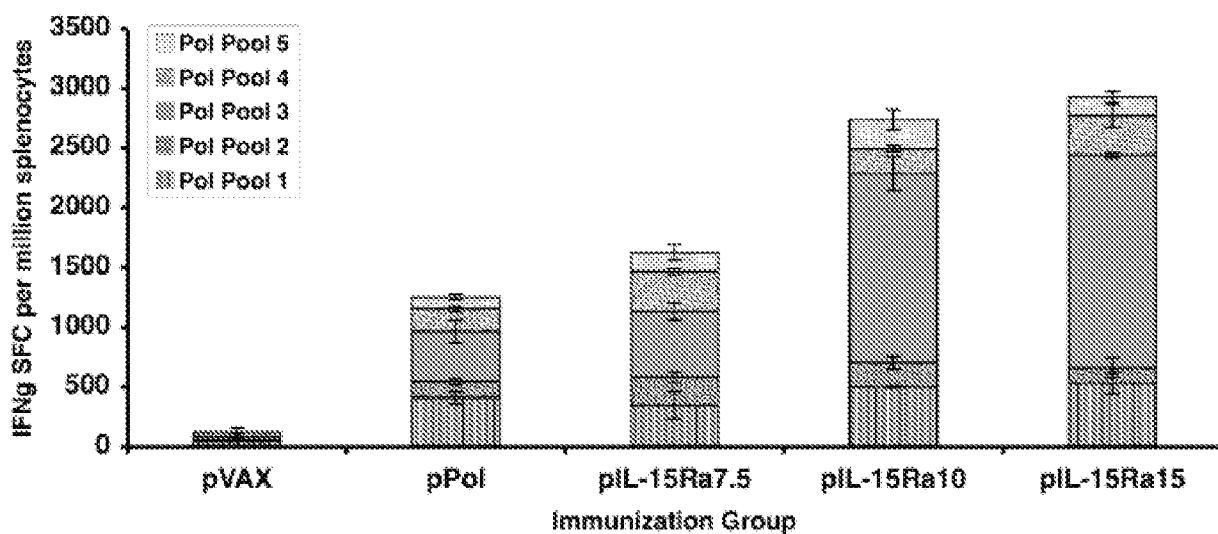


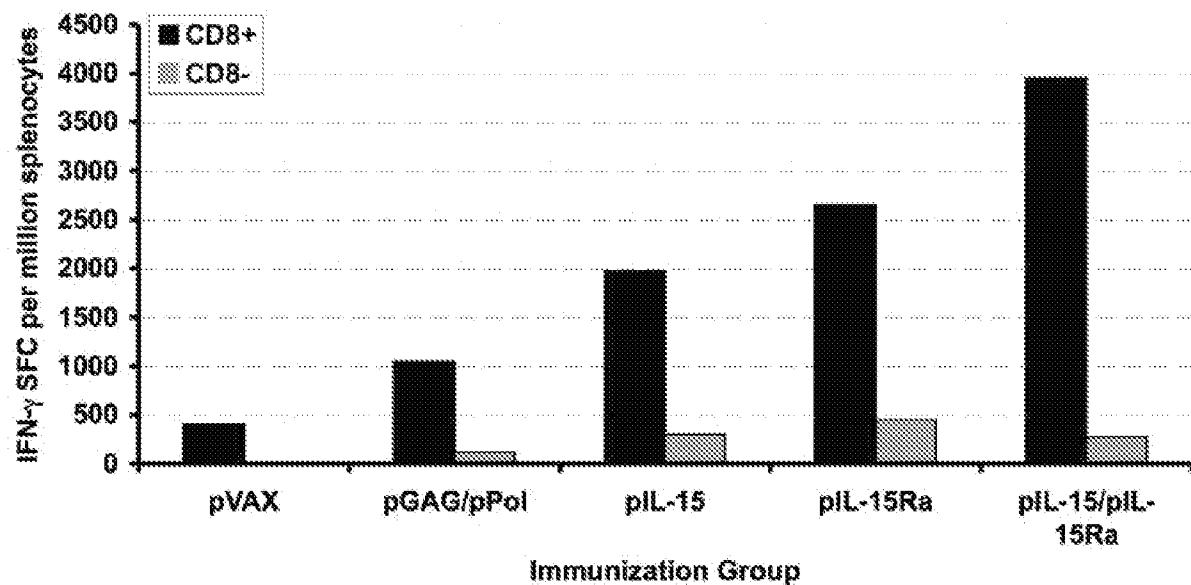
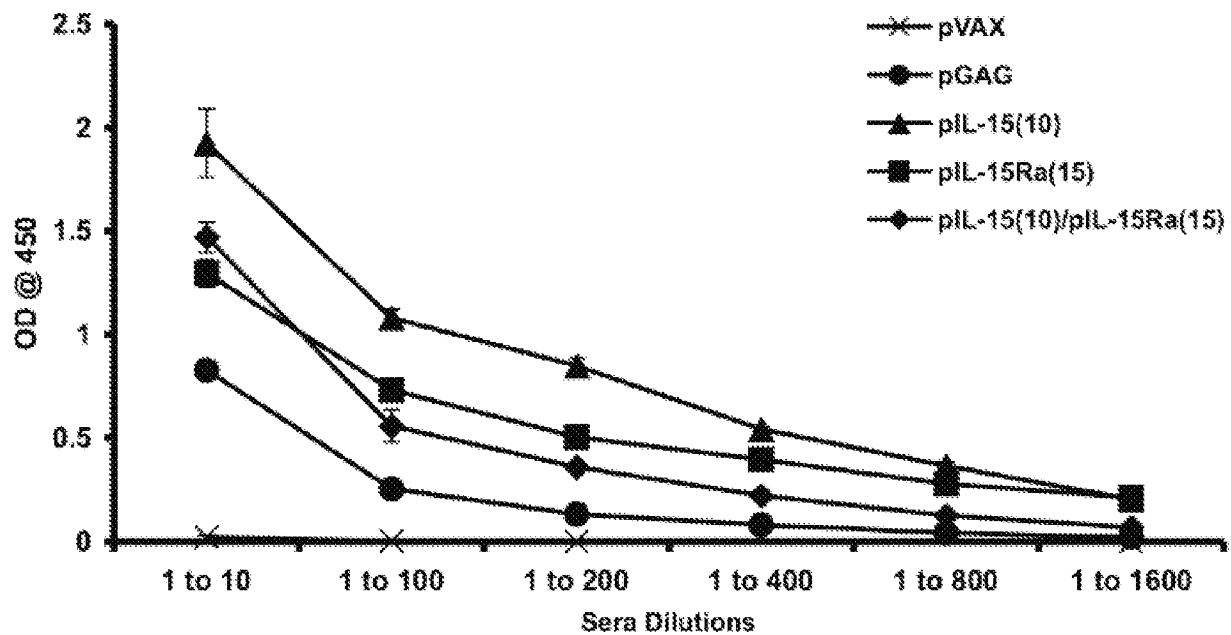
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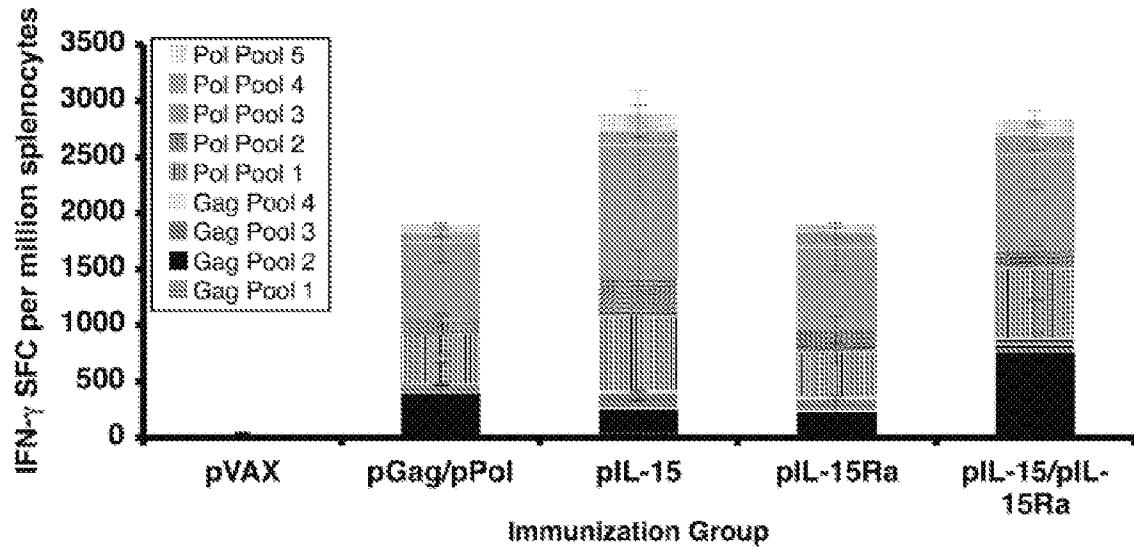
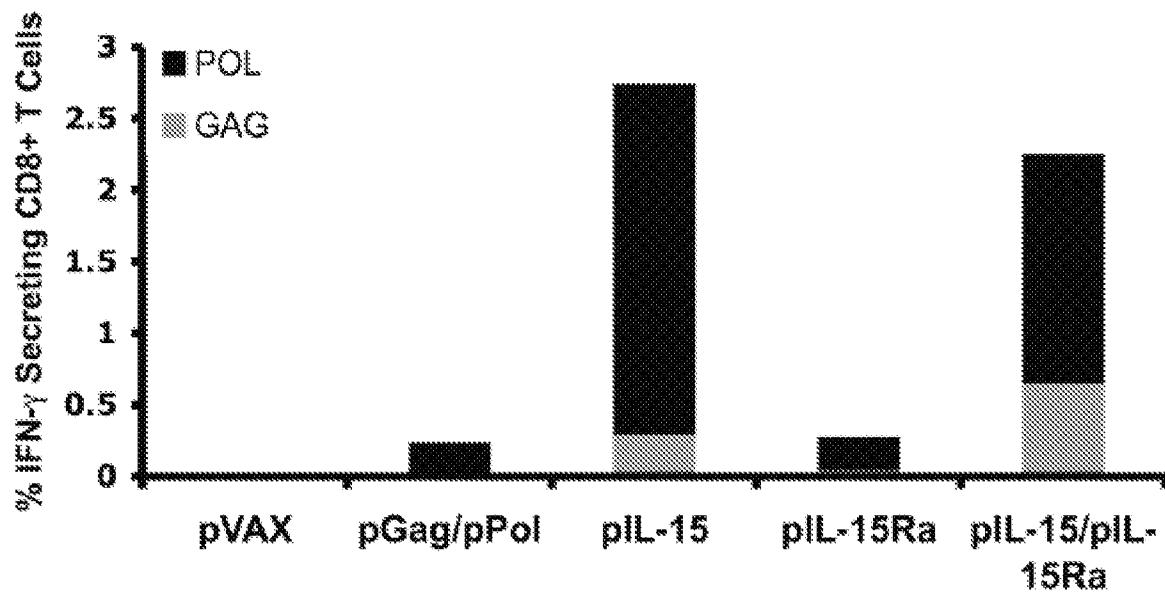


**Figure 3C**



**Figure 4A****Figure 4B**

**Figure 5A****Figure 5B**

**Figure 6A****Figure 6B**

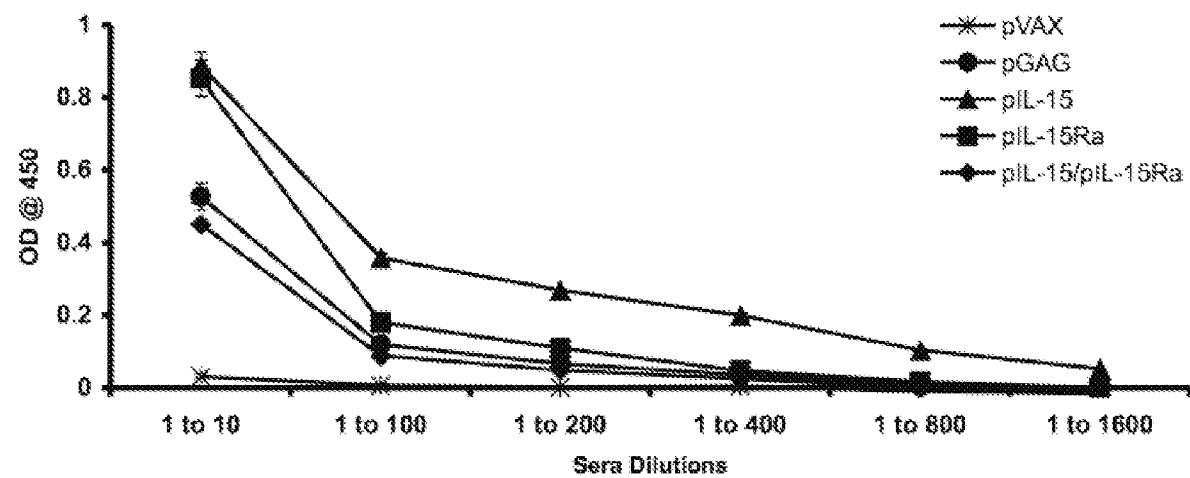
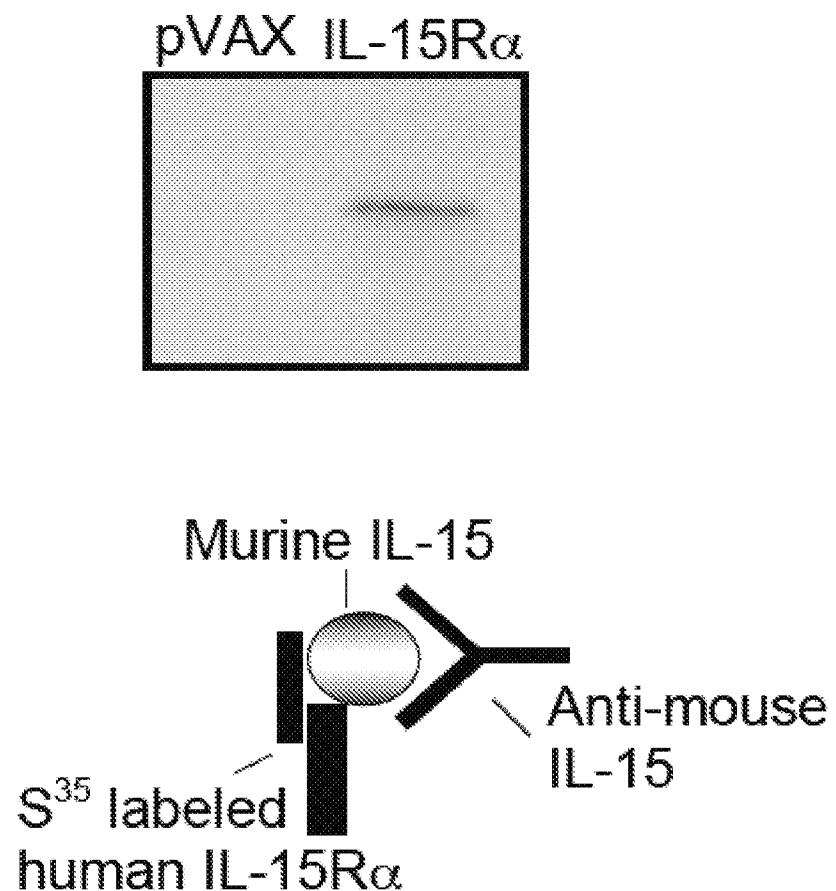
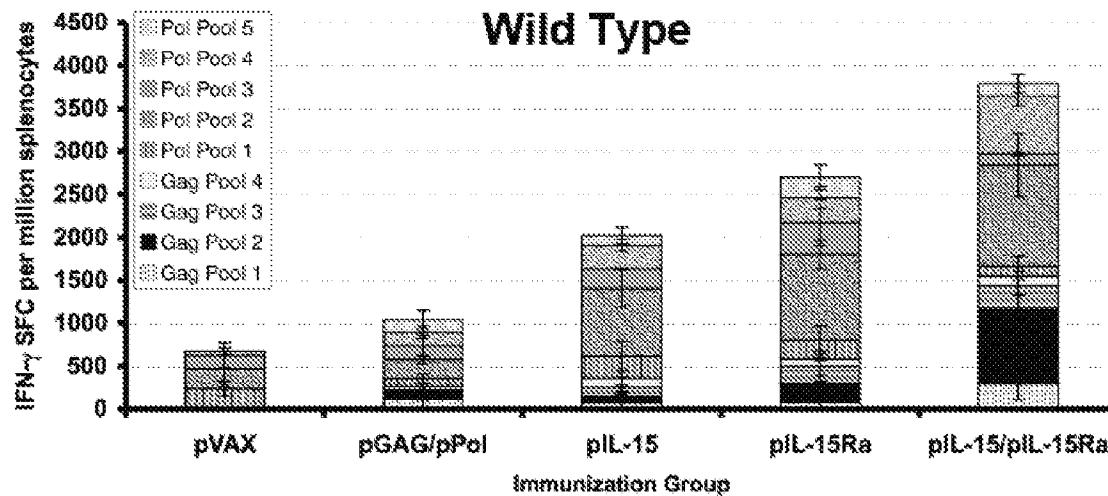
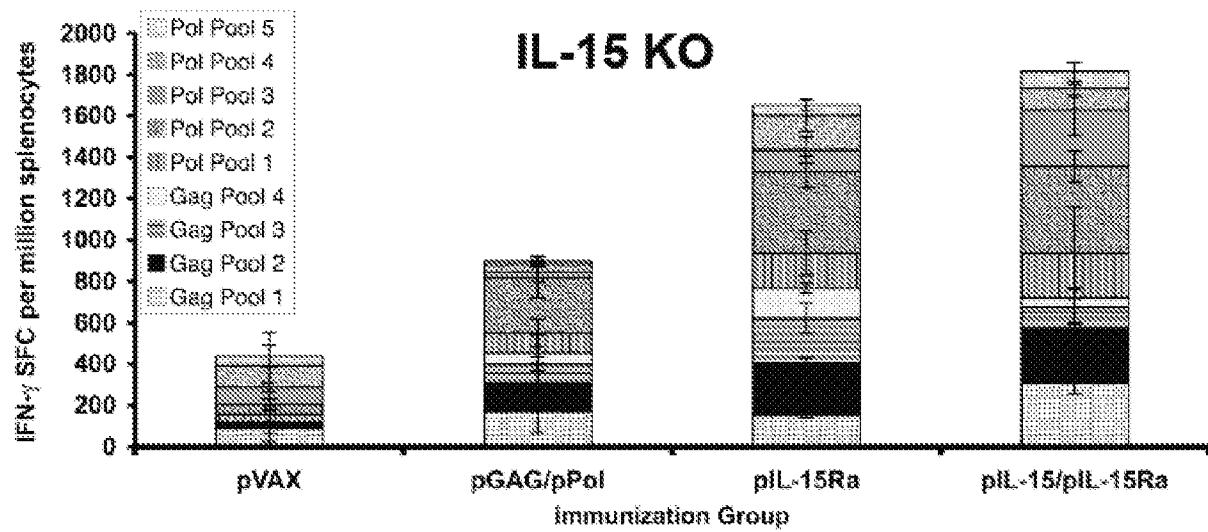
**Figure 6C**

Figure 7A



**Figure 7B****Figure 7C**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/48827

A. CLASSIFICATION OF SUBJECT MATTER  
IPC(8) - A61K 45/00; A61K 39/00; A61K 39/02  
USPC - 424/278.1; 424/184.1; 424/234.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 45/00; A61K 39/00; A61K 39/02

USPC: 424/278.1; 424/184.1; 424/234.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
USPC: 424/277.1, 424/185.1Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
PubWest, Google Scholar: live attenuated, vaccin\$4, recombinant, IL-15a, chronic infection, HCV, HSV, CMV, chicken pox, HIV, immunogen\$2, antigen\$2, pathogen antigen\$2

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | US 2006/0263857 A1 (LEFRANCOIS et al.) 17 May 2006 (17.05.2006); abstract          | 1, 9                  |
| Y         |  | 2-3, 10-11, 15-16     |
| Y         | US 2009/0227664 A1 (PAVLAKIS et al.) 10 Sep 2009 (10.09.2009); para [0038]         | 2-3, 10-11, 15-16     |
| A         | WO 2009/002562 A1 (PAVLAKIS et al.) 27 Jun 2008 (27.06.2008); SEQ ID NO:1          | 4, 12, 17, 20         |

 Further documents are listed in the continuation of Box C. 

\* Special categories of cited documents:  
 "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier application or patent but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
 "&" document member of the same patent family

|   |  |
|---|--|
| Date of the actual completion of the international search<br>23 December 2010 (23.12.2010)  | Date of mailing of the international search report<br>14 JAN 2011                          |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-3201 | Authorized officer:<br>Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 10/48827

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 5-8, 13, 14, 18, and 19  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.