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Genetic adjuvants for immunotherapy

Shyam S. Mohapatra

Mukesh Kumar

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(54) **GENETIC ADJUVANTS FOR IMMUNOTHERAPY**

(75) Inventors: **Shyam S. Mohapatra**, Tampa, FL (US); **Mukesh Kumar**, Norwood, MA (US)

(73) Assignee: **University of South Florida**, Tampa, FL (US)

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(52) **U.S. Cl.** **514/44**; 424/93.1

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See application file for complete search history.

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Primary Examiner—Deborah Crouch
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(57) **ABSTRACT**

The present invention pertains to methods and pharmaceutical compositions for modulating an immune response. The method of the present invention involves administration of an effective amount of nucleic acid molecules encoding interleukin-12 (IL-12), interferon-gamma (IFN-γ), or a combination thereof, to a patient in need of such treatment. The pharmaceutical compositions of the invention contain nucleic acid molecules encoding IL-12 and/or IFN-γ and an operably-linked promoter sequence. In another aspect, the present invention concerns expression vectors containing a nucleotide sequence encoding IL-12 and IFN-γ, and an operably-linked promoter sequence. In another aspect, the present invention concerns cells generally modified with a nucleotide sequence encoding IL-12 and IFN-γ.

31 Claims, 5 Drawing Sheets

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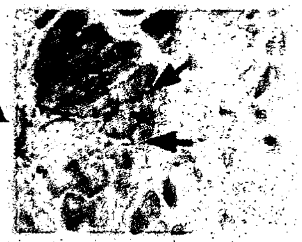
FIG. 1A


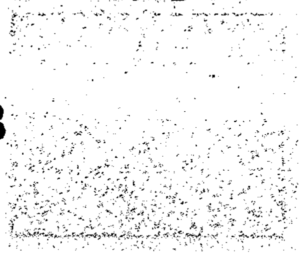
FIG. 1B


FIG. 1C


FIG. 1D

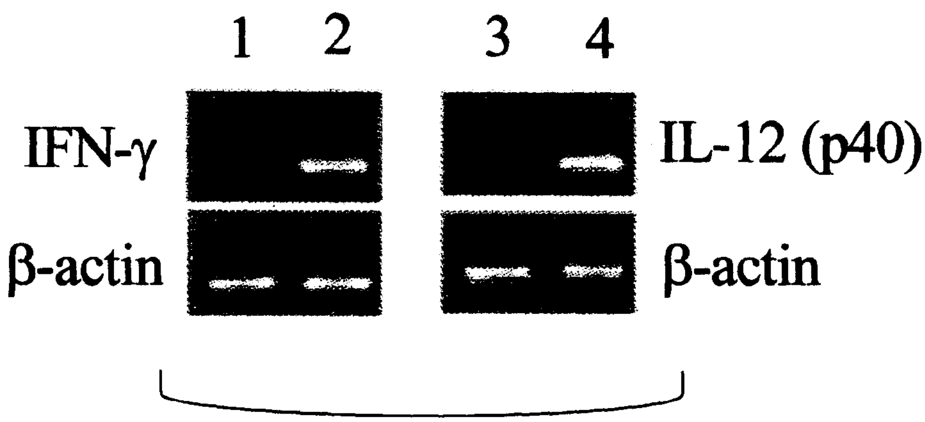



FIG. 1E

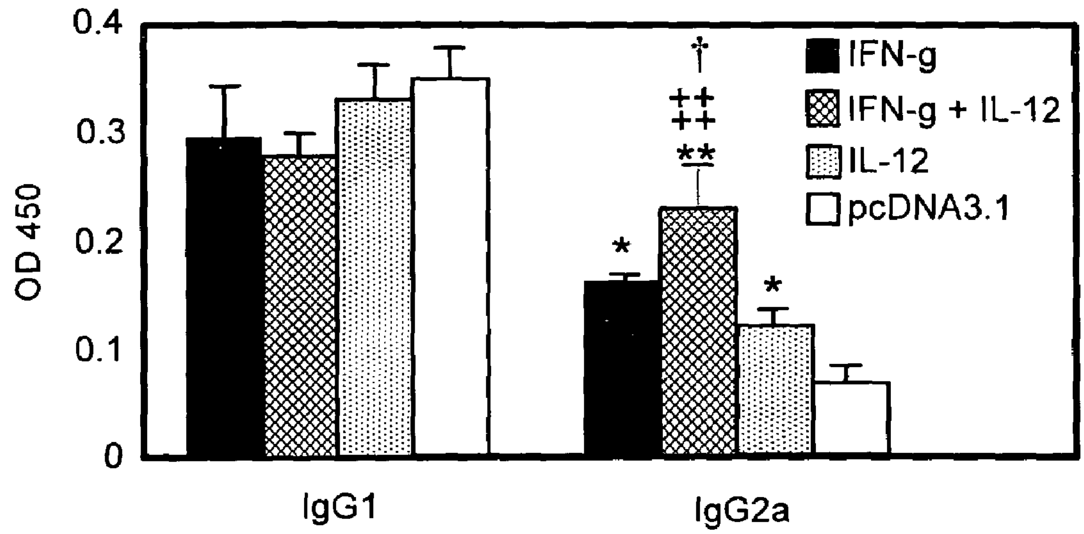

0.4
0.3
0.2
0.1
0
OD 450
IgG1
IgG2a
IFN-g
IFN-g + IL-12
IL-12
pcDNA3.1
†
++
++
**
*
*

FIG. 2A

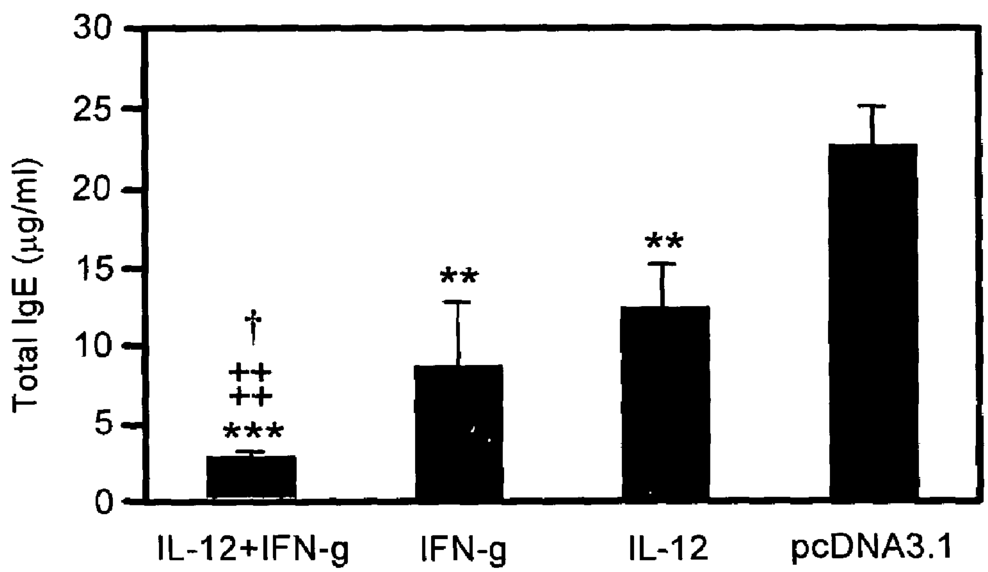

30
25
20
15
10
5
0
Total IgE (μg/ml)
IL-12+IFN-g
IFN-g
IL-12
pcDNA3.1
†
++
++
***
**
**

FIG. 2B

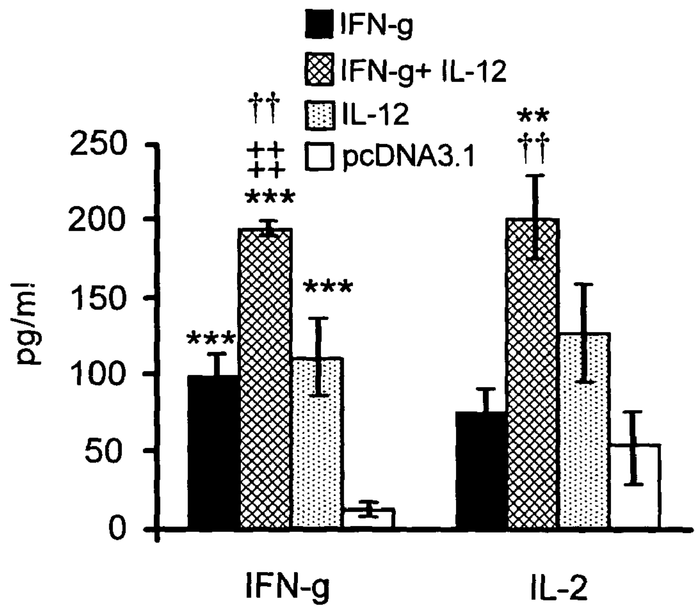


FIG. 3A

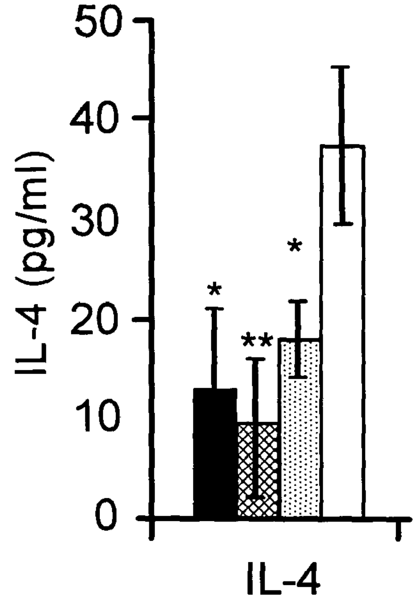


FIG. 3B

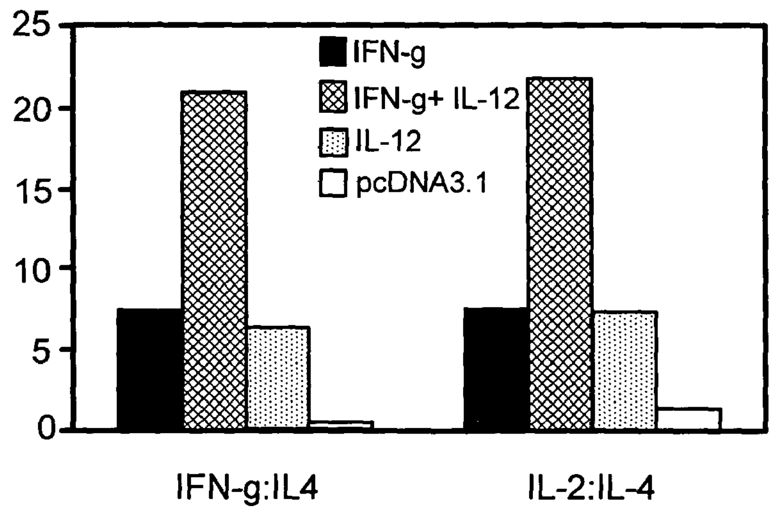


FIG. 3C

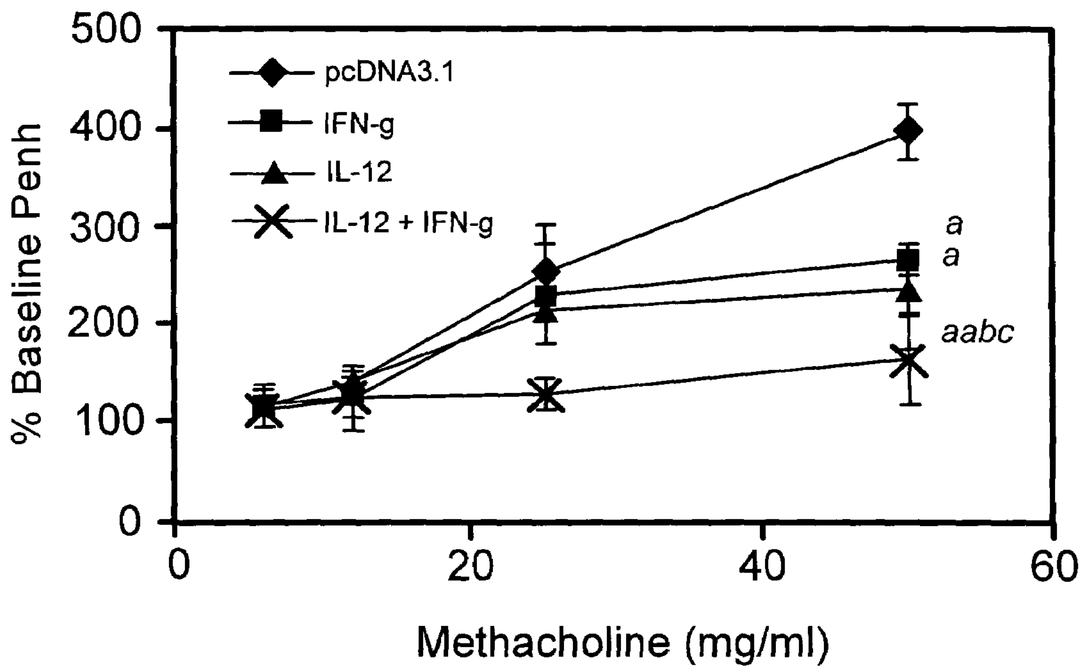

500
400
300
200
100
0
% Baseline Penh
pcDNA3.1
IFN-g
IL-12
IL-12 + IFN-g
a
a
aabc
0
20
40
60
Methacholine (mg/ml)

FIG. 4

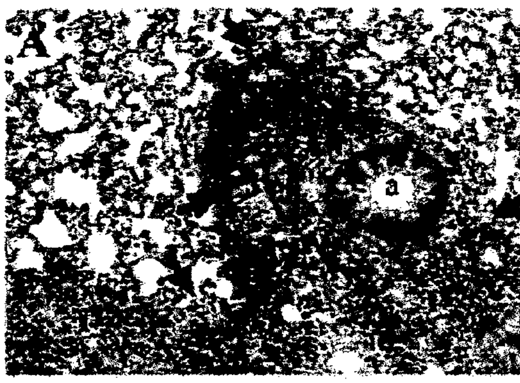


FIG. 5A

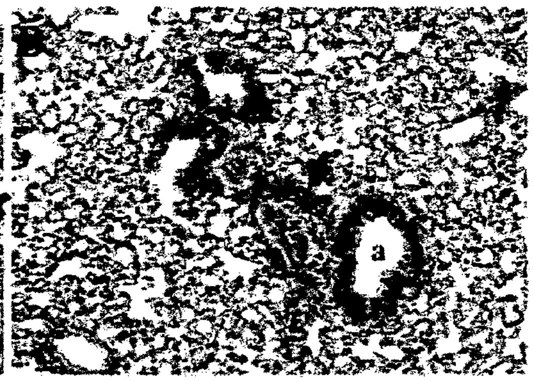


FIG. 5B

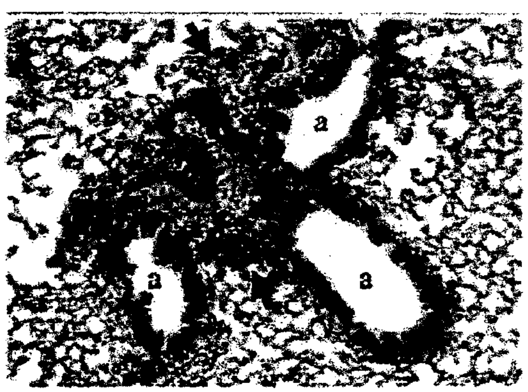


FIG. 5C

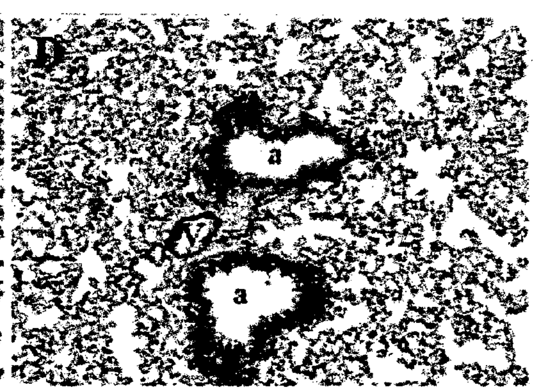


FIG. 5D

GENETIC ADJUVANTS FOR IMMUNOTHERAPY

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of priority of U.S. Provisional Application Ser. No. 60/319,523, filed Sep. 5, 2002, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

BACKGROUND OF INVENTION

The effectiveness of allergen immunotherapy (AIT) is often compromised by the use of high doses of allergens for treatment, which can lead to severe systemic reactions and the inconvenience and discomfort of frequent dosing (Kemp, S. F., *Immunol Allergy Clin North Am,* 2000, 20:571). It has been suggested that the utilization of an adjuvant in conjunction with an allergen vaccine might enhance both the safety and the effectiveness of AIT (Mohapatra, S. S. and San Juan, H. *Immunol Allergy Clin North Am,* 2000, 20:625-642). Two approaches are under investigation. The first is coimmunization with a mixture of antigen(s) and bacterial DNA or immunostimulatory oligodeoxynucleotides (ODNs), which contain CpG motifs (Horner, A. A. et al., *J. Allergy Clin Immunol,* 2000, 106:349-356) and elicit a protective T_H1 response (Roman, M. et al., *Nat Med,* 1997, 3:849-854). The potential of CpG-containing ODNs as adjuvants in AIT is under intense investigation; however, it has been reported that the immunostimulatory activity of these ODNs might be blocked by certain non-CpG motifs (Krieg, A. M. et al., *Proc Natl Acad Sci USA,* 1998, 95:12631-12636; Hacker, H. et al., *EMBO J,* 1998, 17:6230-6240). In addition, these ODNs might not suppress established allergic responses (Peng, Z. et al., *Int Immunol,* 2001, 13:3-11). In a second approach, the potential of plasmid DNA (pDNA)-encoding cytokine(s) as genetic adjuvants has been examined, with varying levels of success, for modulating the immune response stimulated by administered antigen vaccines (Pasquini, S. et al., *Immunol Cell Biol,* 1997, 75:397-401).

Cytokines interferon-γ (IFN-γ) and interleukin-12 (IL-12) are known to mediate T-cell differentiation toward a T_H1-like phenotype (Boehm, U. et al., *Annu Rev Immunol,* 1997, 15:749-795). Despite numerous studies in which pure or recombinant IFN-γ and IL-12 were used, their in vivo use has been limited by the short half-life of these molecules and the associated severe adverse effects (Mohapatra, S. S., *Science,* 1995, 269:1499). Mucosal IFN-γ gene transfer has earlier been shown to inhibit both antigen- and T_H2 cell-induced pulmonary eosinophilia and airway hyper-reactivity (Li, X. M. et al., *J. Immunol.,* 1996, 157:3116-3219). Vaccinia virus-mediated IL-12 gene transfer to the mouse airway abrogated airway eosinophilia and IgE synthesis (Hogan, S. P. et al., *Eur. J. Immunol.,* 1998, 28:413-423). However, the direct effects of these cytokine plasmids as genetic adjuvants in the allergen vaccines used for AIT have not been addressed.

In a previous study, a combination of allergen and IFN-γ effectively redirected allergen-specific T-cell cytokine production toward elevated IFN-γ production in human PBMC cultures (Parronchi, P. et al., *Eur J Immunol,* 1996, 26:697-703). In a murine model of Kentucky blue grass (KBG) allergy, parenteral administration of 1 mg of recombinant allergen induced effective immune deviation (Cao, Y. et al., *Immunology,* 1997, 90:46-51). Cytokine gene transfer studies have been carried out (Li, X. M. et al., *J. Immunol.,* 1996, 157:3116-3219; Hogan, S. P. et al., *Eur. J. Immunol.,* 1998, 28:413-423).

It would be advantageous to have the capability to mediate T-cell differentiation toward a T_H1-like phenotype using IFN-γ and/or IL-12, thereby enhancing the efficacy of allergen vaccines, without limitation by the short half-life of these molecules and the associated severe adverse effects.

BRIEF SUMMARY

The present invention pertains to methods and pharmaceutical compositions for modulating an immune response. The method of the present invention involves administration of an effective amount of nucleic acid molecules encoding interleukin-12 (IL-12), interferon-gamma (IFN-γ), or a combination thereof, to a patient in need of such treatment. In one embodiment, the nucleic acid molecules encoding IL-12 and/or IFN-γ are co-administered with an antigen.

The present invention further concerns pharmaceutical compositions comprising a nucleic acid sequence encoding IL-12 and/or IFN-γ and a pharmaceutically acceptable carrier. Preferably, the composition contains a nucleic acid sequence encoding both IL-12 and IFN-γ, and a pharmaceutically acceptable carrier. Optionally, in addition to a nucleic acid sequence encoding IL-12 and/or IFN-γ, the pharmaceutical composition of the present invention contains an antigen.

In another aspect, the present invention concerns expression vectors containing a nucleotide sequence encoding IL-12 and IFN-γ, and operably-linked promoter sequence. In another aspect, the present invention concerns cells genetically modified with a nucleotide sequence encoding IL-12 and IFN-γ.

BRIEF DESCRIPTION OF DRAWINGS

FIGS. **1**A-**1**E show expression of murine IFN-γ and IL-12 p40 subunit in the mouse muscle. Mice were vaccinated as described in the Methods section. Seven days after the last DNA injection, expression for IFN-γ and IL-12 p40 subunit was checked by immunohistochemistry (FIG. **1**A-**1**D) and RT-PCR (FIG. **1**E). Mice vaccinated with pIFN-γ (FIG. **1**A) and pIL-12 (FIG. **1**C) showed expression of IFN-γ and IL-12 p40 subunits, respectively, as indicated by positive staining (arrows). Mice vaccinated with control empty vector did not show expression for IFN-γ (FIG. **1**B) and IL-12 p40 (FIG. **1**D). In FIG. **1**E, total RNA was isolated from the muscle, and RT-PCR was performed for IFN-γ and IL-12 p40 subunit. Mice vaccinated with pIFN-γ and pIL-12 showed IFN-γ (panel 1, lane 2) and IL-12 p40-specific mRNA amplification (panel 1, lane 4). No amplification was observed in the mice vaccinated with control empty vector (panel 1, lanes 1 and 3), β-actin was used as internal control (lower panel).

FIGS. **2**A-**2**B show analysis of the total IgE and KBG-specific IgG subtypes. Four groups of mice (n=6) were vaccinated as described in the Methods section. On day 21, after immunization with alum and KBG allergen, their serum was analyzed for total IgE (FIG. **2**B) and KBG-specific IgG2a and IgG1 (FIG. **2**A) antibodies by ELISA. Bars represent the means±SDs. *P<0.05; **P<0.01; ***P<0.001 in comparison with pcDNA3.1 group. †P<0.05; ††P<0.01; †††P<0.001 in comparison with pIFN-γ. ‡P<0.05; ‡‡P<0.01; ‡‡‡P<0.001 in comparison with pIL-12 group.

FIGS. **3**A-**3**C show analysis of cytokine production and dominant cytokine pattern following cytokine-encoding DNA vaccination. FIGS. **3**A and **3**B show analysis of cytokine production. Mice (n=6) were vaccinated as described in

the Methods section. On day 7 after KBG and alum immunization, their spleens were cultured in vitro for 48 hours in the presence of KBG allergen and cytokines were measured by ELISA. Bars represent the means±SDs. *P<0.05; **P<0.01; ***P<0.001 in comparison with pcDNA3.1 group. †P<0.05; ††P<0.01; †††P<0.001 in comparison with pIFN-γ (IFN-g) group. ‡P<0.05; ‡‡P<0.01; ‡‡‡P<0.001 in comparison with pIL-12 (IL-12) group. FIG. **3**C shows analysis of the dominant cytokine pattern after cytokine DNA vaccination. Dominant cytokine pattern was determined from the IFN-γ/IL-4 and IL-2/IL-4 ratios. Bars represent means.

FIG. **4** shows measurement of the airway hyperresponsiveness in KBG-sensitized and -challenged mice after cytokine DNA vaccination. Naive mice (n=4) were vaccinated as described in the Methods section and sensitized with the allergen 7 days later. Ten days after the sensitization, animals were challenged intranasally 3 times with 50 μg of KBG allergen. Airway reactivity to inhaled methacholine (6 to 50 mg/mL) was measured 24 hours later. Results are expressed as means±SDs of enhanced pause values. a, P<0.05; aa, P<0.01 in comparison with pcDNA3.1 group. b and c, P<0.05 in comparison with pIFN-γ (IFN-g) and pIL-12 (IL-12) groups, respectively.

FIGS. **5**A-**5**D show assessment of the lung inflammation in KBG-sensitized and -challenged mice after cytokine DNA vaccination. Lung tissue was removed from the different groups of mice (n=4) 24 hours after the last intranasal allergen challenge and was stained with hematoxylin and eosin. A representative photomicrograph from each group is shown (FIG. **5**A: pcDNA3.1; FIG. **5**B: pIFN-γ, FIG. **5**C: pIL-12; FIG. **5**D: pIFN-γ+pIL-12). Arrows indicated cellular infiltration. a, airway; v, vessel.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO:1 is a forward primer for the murine IL-12 p40 subunit.

SEQ ID NO:2 is a reverse primer for the murine IL-12 p40 subunit.

SEQ ID NO:3 is a forward primer to generate the plasmid pc40.

SEQ ID NO:4 is a reverse primer to generate the plasmid pc40.

SEQ ID NO:5 is a forward primer for murine IL-12 p35 subunit.

SEQ ID NO:6 is a reverse primer for murine IL-12 p35 subunit.

SEQ ID NO:7 is a nucleotide sequence encoding the human IL-12 p35 subunit.

SEQ ID NO:8 is the amino acid sequence of the human IL-12 p35 subunit.

SEQ ID NO:9 is the nucleotide sequence of the human IL-12 p40 subunit.

SEQ ID NO:10 is the amino acid sequence of the human IL-12 p40 subunit.

SEQ ID NO:11 is the nucleotide sequence encoding human IFN-γ.

SEQ ID NO:12 is the amino acid sequence of human IFN-γ.

DETAILED DISCLOSURE

The present invention pertains to adjuvantation using nucleic acid molecules encoding IL-12, IFN-γ, or both IL-12 and IFN-γ. In one embodiment, the nucleic acid molecules encoding IL-12 and/or IFN-γ are co-administered with an antigen of the pathogen in order to modulate an immune response to a pathogen (one or more) in a patient (human or non-human mammal). Preferably, effective amounts of nucleic acid sequences encoding both IL-12 and IFN-γ are administered simultaneously or sequentially to a patient in need of such treatment. More preferably, effective amounts of nucleic acid sequences encoding both IL-12 and IFN-γ are administered simultaneously or sequentially to a patient, and an antigen is administered simultaneously or sequentially to the patient.

As described herein, the "immune response" modulated in the patient can be, for example, a cytokine immune response and/or a humoral immune response (e.g., antigen-specific). As used herein, the term "modulate", and grammatical variations thereof, is intended to mean strengthening (i.e., augmenting) or weakening (i.e., lessening, inhibiting) of an immune response within a patient. In one embodiment, the immune response of the patient is modulated such that production of IgE antibodies is inhibited. In further embodiments, the cytokine profiles of the patient's cells are altered to produce more T_H1-like cytokines (e.g., interleukin-2 (IL-2) and IFN-γ) and less T_H2-like cytokines ((interleukin-4) IL-4). The altered cytokine profile is more apparent in patients administered nucleic acid sequences encoding both IL-12 and IFN-γ. Depending upon the particular antigen that the patient is administered or otherwise exposed to (e.g., an allergen), the nucleic acid sequences encoding IL-12 and/or IFN-γ may inhibit or prevent airway hyper-responsiveness and inflammation.

Administration of nucleic acid molecules encoding IL-12 and/or IFN-γ, or biologically active fragments or homologs thereof, according to the subject invention, may be used to modulate the immune response in order to prevent or treat immune-related or inflammatory-related conditions such as, but not limited to, allergies, allergic rhinitis, atopic dermatitis, asthma, allergic sinusitis, pulmonary fibrosis, and cancer.

In other embodiments, the nucleic acid molecules used in the methods, vectors, cells, and compositions of the present invention encode IL-12-like molecules and/or IFN-γ-like molecules. As used herein, the terms "IL-12-like molecule" and "IFN-γ-like molecule" refers to polypeptides exhibiting IL-12-like activity and IFN-γ-like activity, respectively, when the nucleic acid molecule encoding the polypeptide is expressed, as can be determined in vitro or in vivo. For purposes of the subject invention, IL-12-like activity and IFN-γ-like activity refer to those polypeptides having one or more of the functions of the native IL-12 or IFN-γ cytokine, such as the capability to alter the cytokine profile of a patient's cells to produce more T_H1-like cytokines (e.g., interleukin-2 (IL-2) and IFN-γ) and less T_H2-like cytokines ((interleukin-4 (IL-4)). An example of an IL-12-like molecule is interleukin-23 (IL-23). Examples of IFN-γ-like molecules are interferon-alpha (IFN-α) and interferon-beta (IFN-β), including biologically active fragments thereof.

Following administration, the efficacy of the cytokine-encoding nucleic acid sequences can be assessed by specific cytokine production or antibody production, for example. One of ordinary skill in the art can assess these parameters using conventional methods.

A used herein, the term "antigen" is intended to mean one or more immunostimulatory agents capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. Antigens can be molecules or portions of molecules including, but not limited to, proteins or fragments thereof (e.g., proteolytic fragments), peptides (e.g., synthetic peptides, polypeptides), glycopro-

teins, carbohydrates (e.g., polysaccharides), lipids, glycolipids, hapten conjugates, recombinant nucleotides (e.g., recombinant DNA), whole organisms (killed or attenuated) or portions thereof, toxins and toxoids (e.g., tetanus, diphtheria, cholera) and/or organic molecules. Particular examples of antigens for use in the present invention include allergens, such as Kentucky blue grass (KBG) allergen.

The antigen can be obtained or derived from a variety of pathogens or organisms, such as bacteria (e.g., *bacillus,* Group B *streptococcus,* Bordetella, Listeria, *Bacillus anthracis, S. pneumoniae, N. meningiditis, H. influenza*), viruses (e.g., hepatitis, measles, poliovirus, human immunodeficiency virus, influenza virus, parainfluenza virus, respiratory syncytial virus), mycobacteria (*M. tuberculosis*), parasites (Leishmania, Schisotosomes, Tranpanosomes, toxoplasma, pneumocystis) and fungi (e.g., *Candida, Cryptococcus, Coccidiodes, Aspergillus*), against which an immune response is desired in a patient. The antigen of a pathogen can be obtained using skills known in the art. For example, the antigen can be isolated (purified, essentially pure) directly from the pathogen, derived using chemical synthesis, or obtained using recombinant methodology. In addition, the antigen can be obtained from commercial sources. A suitable antigen for use in the present invention can include at least one B and/or T cell epitope (e.g., T helper cell or cytolytic T cell epitope). Other suitable antigens useful in the compositions of the present invention can be determined by those of skill in the art.

The IL-12-encoding nucleic acid sequence and IFN-γ-encoding nucleic acid sequence can be within the same nucleic acid molecule or separate nucleic acid molecules. Furthermore, it will be understood that a nucleic acid sequence can be a multimer comprising repeating units of the IL-12-encoding nucleic acid sequence and/or IFN-γ-encoding nucleic acid sequence (i.e., homopolymers or heteropolymers) for enhanced expression of the IL-12 and/or IFN-γ coding sequences, or biologically active fragments or homologs thereof.

As used herein, the term "co-administer", and grammatical variations thereof, is intended to mean administration of agents (e.g., nucleic acids, antigens, etc.) simultaneously or sequentially.

The nucleic acid sequences encoding IL-12 and/or IFN-γ used in the methods, expression vectors, and pharmaceutical compositions of the present invention are preferably isolated. According to the present invention, an isolated nucleic acid molecule or nucleic acid sequence is a nucleic acid molecule or sequence that has been removed from its natural milieu. As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has been purified. An isolated nucleic acid molecule or sequence useful in the present composition can include DNA, RNA, or any derivatives of either DNA or RNA. An isolated nucleic acid molecule or sequence can be double stranded (i.e., containing both a coding strand and a complementary strand) or single stranded.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases are used interchangeably herein. As used herein, a "coding" nucleic acid sequence refers to a nucleic acid sequence that encodes at least a portion of a peptide or protein (e.g., a portion of an open reading frame), and can more particularly refer to a nucleic acid sequence encoding a peptide or protein which, when operably-linked to a transcription control sequence (e.g., a promoter sequence), can express the peptide or protein. A translation initiation codon can be inserted as necessary, making methionine the first amino acid in the sequence. Optionally, the IL-12 and/or IFN-γ encoding nucleic acid sequences used in the subject invention include a sequence encoding a signal peptide upstream of the cytokine(s)-encoding sequence(s), thereby permitting secretion of the IL-12 and/or IFN-γ, or a biologically active fragment thereof, from a host cell.

The term "operably-linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably-linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence. For example, a coding sequence is operably-linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence, and the promoter sequence can still be considered "operably-linked" to the coding sequence. Each nucleotide sequence coding for IL-12 or IFN-γ will typically have its own operably-linked promoter sequence.

The term "vector" is used to refer to any molecule (e.g., nucleic acid, plasmid, or virus) used to transfer coding information (e.g., nucleic acid sequence encoding IL-12 and/or IFN-γ) to a host cell. The term "expression vector" refers to a vector that is suitable for use in a host cell (e.g., patient's cell) and contains nucleic acid sequences which direct and/or control the expression of heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present. A great variety of expression vectors can be used to produce IL-12 and/or IFN-γ, or biologically active fragments thereof. IL-12 and/or IFN-γ-encoding nucleic acid sequences can be modified according to methods known in the art to provide optimal codon usage for expression in a particular expression system.

In another aspect, the present invention includes pharmaceutical compositions comprising a nucleic acid sequence encoding IL-12 and/or IFN-γ and a pharmaceutically acceptable carrier. Preferably, the composition contains a nucleic acid sequence encoding both IL-12 and IFN-γ, and a pharmaceutically acceptable carrier. The nucleic acid sequence encoding IL-12 and/or IFN-γ may be contained within an expression vector, such as a DNA plasmid or viral vector (e.g., retrovirus, modified herpes virus, herpes virus, adenovirus, adeno-associated virus, and the like). Where a combination of nucleic acid sequences encoding IL-12 and IFN-γ are to be administered to a patient, sequences may be contained within one vector (e.g., a DNA plasmid) or separate vectors (e.g., separate plasmids), or types of vectors. Optionally, the pharmaceutical composition of the present invention further includes an antigen.

The pharmaceutical compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Furthermore, as used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. The pharmaceutically acceptable carrier can include diluents, adjuvants, and vehicles, as well as implant carriers, and inert, non-toxic solid or liquid fillers, diluents, or encapsulating material that does not react with the active ingredients of the invention. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions. The carrier can be a solvent or dispersing medium containing, for example, etha-

nol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. Formulations are described in a number of sources that are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Sciences* (Martin E W [1995] Easton, Pa., Mack Publishing Company, 19^{th} ed.) describes formulations which can be used in connection with the subject invention. Formulations suitable for parental administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powder, granules, tablets, etc. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the subject invention can include other agents conventional in the art having regard to the type of formulation in question. The pharmaceutical composition can be adapted for various forms of administration. Administration can be continuous or at distinct intervals as can be determined by a person skilled in the art.

The administration of the IL-12 and/or IFN-γ-encoding nucleic acid sequences are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight, and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. A therapeutically effective amount of IL-12 and/or IFN-γ-encoding nucleic acid molecules is that amount necessary to provide a therapeutically effective amount of the corresponding polypeptide(s), when expressed in vivo. The amount of IL-12 and/or IFN-γ must be effective to achieve a modulated immune response, including but not limited to total prevention of (e.g., protection against) pathogen infection and/or allergic response, and to improved survival rate or more rapid recovery, or improvement or elimination of symptoms associated with pathogen infection or allergic response, and other indicators as are selected as appropriate measures by those skilled in the art. In accordance with the present invention, a suitable single dose size is a dose that is capable of preventing or alleviating (reducing or eliminating) a symptom in a patient when administered one or more times over a suitable time period. One of skill in the art can readily determine appropriate single dose sizes for systemic administration based on the size of a mammal and the route of administration.

Mammalian species which benefit from the disclosed compositions and methods include, and are not limited to, apes, chimpanzees, orangutans, humans, monkeys; domesticated animals (e.g., pets) such as dogs, cats, guinea pigs, hamsters, Vietnamese pot-bellied pigs, rabbits, and ferrets; domesticated farm animals such as cows, buffalo, bison, horses, donkey, swine, sheep, and goats; exotic animals typically found in zoos, such as bear, lions, tigers, panthers, elephants, hippopotamus, rhinoceros, giraffes, antelopes, sloth, gazelles, zebras, wildebeests, prairie dogs, koala bears, kangaroo, opossums, raccoons, pandas, hyena, seals, sea lions, elephant seals, otters, porpoises, dolphins, and whales. As used herein, the term "patient" is intended to include such human and non-human mammalian species. Nucleic acid molecules encoding IL-12 and/or IFN-γ, or biologically active fragments thereof, can be administered to patients of the same species or different species from which the nucleic acid molecules naturally exist, for example.

The nucleic acid sequences encoding IL-12 and/or IFN-γ (and pharmaceutical compositions containing them) can be administered to a patient by any route that results in modulated immune response. For example, the genetic material can be administered intravenously (I.V.), intramuscularly (I.M.), subcutaneously (S.C.), intradermally (I.D.), orally, intranasally, etc.

Examples of intranasal administration can be by means of a spray, drops, powder or gel and also described in U.S. Pat. No. 6,489,306, which is incorporated herein by reference in its entirety. One embodiment of the present invention is the administration of the invention as a nasal spray. Alternate embodiments include administration through any oral or mucosal routes, sublingual administration, and even eye drops. However, other means of drug administrations are well within the scope of the present invention.

The present invention also encompasses combination therapy. By combination therapy is meant that nucleic acid sequences encoding IL-12 and/or IFN-γ can be administered in combination with other biologically active agents, such as antigens, other immunomodulators or immunostimulatory molecules, such as interferons or interleukens, and antimicrobial agents, such as antibiotics, antifungal drugs, antiviral drugs, etc.

The present invention can be conjugated with chitosan or chitosan derivatives. For example, DNA chitosan nanospheres can be generated, as described by Roy, K. et al. (*Nat Med,* 1999, 5:387). Chitosan allows increased bioavailability of the nucleic acid sequences because of protection from degradation by serum nucleases in the matrix and thus has great potential as a mucosal gene delivery system. Chitosan also has many beneficial effects, including anticoagulant activity, wound-healing properties, and immunostimulatory activity, and is capable of modulating immunity of the mucosa and bronchus-associated lymphoid tissue. In one embodiment of the present invention, chitosan derived nanoparticles are used as adjuvants or conjugates.

IL-12 is a heterodimeric cytokine that has a molecular weight of 75 kDa and is composed of disulfide-bonded 40 kDa and 35 kDa subunits. As used herein, "interleukin-12" and "IL-12" refer to interleukin 12 protein, its individual subunits, multimers of its individual subunits, biologically active fragments of IL-12, and biologically active homologs of "interleukin-12" and "IL-12", such as mammalian homologs. As defined herein, biologically active fragments of IL-12 are fragments that, for example, modulate an immune response to an antigen in a patient who has been administered or otherwise exposed to the antigen. As also defined herein, biologically active fragments or homologs of "interleukin-12" and "IL-12" include modified IL-12 protein such that the resulting IL-12 product has immune response modulation activity similar to the IL-12 described herein (e.g., the ability to modulate an immune response to an antigen, when administered with the antigen, in a patient, relative to in vivo conditions in the absence of the cytokine or relative to administration of the antigen alone). Biologically active homologs or fragments of "interleukin-12" also include nucleic acid sequences (e.g., DNA, RNA) and portions thereof, which encode a protein or peptide having the IL-12 function or activity described herein (e.g., the ability to modulate an immune response to an antigen, when administered with the

antigen, in a patient). In addition, the term includes a nucleotide sequence which, through the degeneracy of the genetic code, encodes a similar peptide gene product as IL-12 and has the IL-12 activity described herein. For example, a homolog of "interleukin-12" and "IL-12" includes a nucleotide sequence which contains a "silent" codon substitution (e.g., substitution of one codon encoding an amino acid for another codon encoding the same amino acid) or an amino acid sequence which contains a "silent" amino acid substitution (e.g., substitution of one acidic amino acid for another acidic amino acid).

An exemplified nucleotide sequence encodes the human IL-12 p35 subunit (Accession No: NM_000882, NCBI database, which is hereby incorporated by reference in its entirety):

1 tttcattttg ggccgagctg gaggcggcgg ggccgtcccg gaacggctgc ggccgggcac
61 cccgggagtt aatccgaaag cgccgcaagc cccgcgggcc ggccgcaccg cacgtgtcac
121 cgagaagctg atgtagagag agacacagaa ggagacagaa agcaagagac cagagtcccg
181 ggaaagtcct gccgcgcctc gggacaatta taaaaatgtg gccccctggg tcagcctccc
241 agccaccgcc ctcacctgcc gcggccacag gtctgcatcc agcggctcgc cctgtgtccc
301 tgcagtgccg gctcagcatg tgtccagcgc gcagcctcct ccttgtggct accctggtcc
361 tcctggacca cctcagtttg gccagaaacc tccccgtggc cactccagac ccaggaatgt
421 tcccatgcct tcaccactcc caaaacctgc tgagggccgt cagcaacatg ctccagaagg
481 ccagacaaac tctagaattt taccettgca cttctgaaga gattgatcat gaagatatca
541 caaaagataa aaccagcaca gtggaggcct gtttaccatt ggaattaacc aagaatgaga
601 gttgcctaaa ttccagagag acctctttca taactaatgg gagttgcctg gcctccagaa
661 agacctcttt tatgatggcc ctgtcaatta gtagtattta tgaagacttg aagatgtacc
721 aggtggagtt caagaccatg aatgcaaagc ttctgatgga tcctaagagg cagatctttc
781 tagatcaaaa catgctggca gttattgatg agctgatgca ggccctgaat ttcaacagtg
841 agactgtgcc acaaaaatcc tcccttgaag aaccggattt ttataaaact aaaatcaagc
901 tctgcatact tcttcatgct ttcagaattc gggcagtgac tattgataga gtgatgagct
961 atctgaatgc ttcctaaaaa gcgaggtccc tccaaaccgt tgtcattttt ataaaacttt
1021 gaaatgagga aactttgata ggatgtggat taagaactag ggagggggaa agaaggatgg
1081 gactattaca tccacatgat acctctgatc aagtattttt gacatttact gtggataaat
1141 tgtttttaag ttttactgaa tgaattgcta agaagggaaa atatccatcc tgaaggtgtt
1201 tttcattcac tttaatagaa gggcaaatat ttataagcta tttctgtacc aaagtgtttg
1261 tggaaacaaa catgtaagca taacttattt taaaatattt atttatataa cttggtaatc
1321 atgaaagcat ctgagctaac ttatatttat ttatgttata tttattaaat tatttatcaa
1381 gtgtatttga aaaatatttt taagtgttct aaaaataaaa gtattgaatt aaagtgaaaa
1441 aaaa (SEQ ID NO:7)

MWPPGSASQPPPSPAAATGLHPAARPVSLQCRLSMCPARSLLLVATLVLLDHLSLAR NLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKLHAFRIRAVTIDRVMSYLNAS (SEQ ID NO:8)

A further exemplified nucleotide sequence encodes the human IL-12 p40 subunit (Accession No: NM_002187, NCBI database, which is hereby incorporated by reference in its entirety):

1 ctgtttcagg gccattggac tctccgtcct gcccagagca agatgtgtca ccagcagttg
61 gtcatctctt ggttttccct ggtttttctg gcatctcccc tcgtggccat atgggaactg
121 aagaaagatg tttatgtcgt agaattggat tggtatccgg atgcccctgg agaaatggtg
181 gtcctcacct gtgacacccc tgaagaagat ggtatcacct ggaccttgga ccagagcagt
241 gaggtcttag gctctggcaa aaccctgacc atccaagtca aagagtttgg agatgctggc
301 cagtacacct gtcacaaagg aggcgaggtt ctaagccatt cgctcctgct gcttcacaaa
361 aaggaagatg gaatttggtc cactgatatt ttaaaggacc agaaagaacc caaaaataag
421 acctttctaa gatgcgaggc caagaattat tctggacgtt tcacctgctg gtggctgacg
481 acaatcagta ctgatttgac attcagtgtc aaaagcagca gaggctcttc tgacccccaa
541 ggggtgacgt gcggagctgc tacactctct gcagagagag tcagagggga caacaaggag
601 tatgagtact cagtggagtg ccaggaggac agtgcctgcc cagctgctga ggagagtctg
661 cccattgagg tcatggtgga tgccgttcac aagctcaagt atgaaaacta caccagcagc
721 ttcttcatca gggacatcat caaacctgac ccacccaaga acttgcagct gaagccatta
781 aagaattctc ggcaggtgga ggtcagctgg gagtaccctg acacctggag tactccacat
841 tcctacttct ccctgacatt ctgcgttcag gtccagggca agagcaagag agaaaagaaa
901 gatagagtct tcacggacaa gacctcagcc acggtcatct gccgcaaaaa tgccagcatt
961 agcgtgcggg cccaggaccg ctactatagc tcatcttgga gcgaatgggc atctgtgccc
1021 tgcagttagg ttctgatcca ggatgaaaat ttggaggaaa agtggaagat attaagcaaa
1081 atgtttaaag acacaacgga atagacccaa aaagataatt tctatctgat ttgctttaaa
1141 acgttttttt aggatcacaa tgatatcttt gctgtatttg tatagttaga tgctaaatgc
1201 tcattgaaac aatcagctaa tttatgtata gattttccag ctctcaagtt gccatgggcc
1261 ttcatgctat ttaaatattt aagtaattta tgtatttatt agtatattac tgttatttaa
1321 cgtttgtctg ccaggatgta tggaatgttt catactctta tgacctgatc catcaggatc
1381 agtccctatt atgcaaaatg tgaatttaat tttatttgta ctgacaactt ttcaagcaag
1441 gctgcaagta catcagtttt atgacaatca ggaagaatgc agtgttctga taccagtgcc
1501 atcatacact tgtgatggat gggaacgcaa gagatactta catggaaacc tgacaatgca
1561 aacctgttga gaagatccag gagaacaaga tgctagttcc catgtctgtg aagacttcct
1621 ggagatggtg ttgataaagc aatttagggc cacttacact tctaagcaag tttaatcttt

1681 ggatgcctga attttaaaag ggctagaaaa aaatgattga ccagcctggg aaacataaca

1741 agaccccgtc tctacaaaaa aaatttaaaa ttagccaggc gtggtggctc atgcttgtgg

1801 tcccagctgt tcaggaggat gaggcaggag gatctcttga gcccag-gagg tcaaggctat

1861 ggtgagccgt gattgtgcca ctgcatacca gcctaggtga cagaat-gaga ccctgtctca

1921 aaaaaaaaaa tgattgaaat taaaattcag ctttagcttc catggcagtc ctcacccccca

1981 cctctctaaa agacacagga ggatgacaca gaaacaccgt aagt-gtctgg aaggcaaaaa

2041 gatcttaaga ttcaagagag aggacaagta gttatggcta aggacatgaa attgtcagaa

2101 tggcaggtgg cttcttaaca gccctgtgag aagcagacag atgcaaa-gaa aatctggaat

2161 cccttctca ttagcatgaa tgaacctgat acacaattat gaccagaaaa tatggctcca

2221 tgaaggtgct acttttaagt aatgtatgtg cgctctgtaa agtgattaca tttgtttcct

2281 gtttgtttat ttatttattt atttttgcat tctgaggctg aactaataaa aactct-tctt

2341 tgtaatc (SEQ ID NO:9)

MCHQQLVISWFSLVFLASPLVAI-WELKKDVYVVELDWYPDAPGEMVVLTCDTPEED GITWTLDQSSEVLGSGKTLTIQVKEF-GDAGQYTCHKGGEVLSHSLLLLHKKEDGIWS TDILKDQKEPKNKTFLRCEAKNYSGR-FTCWWLTTISTDLTFSVKSSRGSSDPQGVTC GAATLSAERVRGDNKEYEYSVEC-QEDSACPAAEESLPIEVMVDAVHKLKYENYTSS FFIRDIIKPDPPKNLQLKPLKN-SRQVEVSWEYPDTWSTPHSYFSLT-FCVQVQGKSKRE KKDRVFTDKTSATVICRKNA-SISVRAQDRYYSSSWSEWASVPCS (SEQ ID NO:10)

IFN-γ is a 14-18 kDalton 143 amino acid glycosylated protein that is a potent multifunctional cytokine. As used herein, "interferon-gamma" and "IFN-γ" refer to IFN-γ protein, biologically active fragments of IFN-γ, and biologically active homologs of "interferon-gamma" and "IFN-γ", such as mammalian homologs. As defined herein, biologically active fragments of IFN-γ are fragments that, for example, modulate an immune response to an antigen in a patient who has been administered or otherwise exposed to the antigen. As also defined herein, biologically active fragments or homologs of "interferon-gamma" and "IFN-γ" include modified IFN-γ protein such that the resulting IFN-γ product has immune response modulating activity similar to the IFN-γ described herein (e.g., the ability to modulate an immune response to an antigen, when administered with the antigen, in a patient, relative to in vivo conditions in the absence of the cytokine or relative to administration of the antigen alone). Biologically active homologs or fragments of "interferon-gamma" also include nucleic acid sequences (e.g., DNA, RNA) and portions thereof, which encode a protein or peptide having the IFN-γ function or activity described herein (e.g., the ability to modulate an immune response to an antigen in a patient that has been administered or otherwise exposed to the antigen). In addition, the term includes a nucleotide sequence which through the degeneracy of the genetic code encodes a similar peptide gene product as IFN-γ and has the IFN-γ activity described herein. For example, a homolog of "interferon-gamma" and "IFN-γ" includes a nucleotide sequence which contains a "silent" codon substitution (e.g., substitution of one codon encoding an amino acid for another codon encoding the same amino acid) or an amino acid sequence which contains a "silent" amino acid substitution (e.g., substitution of one acidic amino acid for another acidic amino acid).

A further exemplified nucleotide sequence encodes human IFN-γ (Accession No. NM_000619), NCBI database, which is hereby incorporated by reference in its entirety):

1 cacattgttc tgatcatctg aagatcagct attagaagag aaagatcagt taagtccttt

61 ggacctgatc agcttgatac aagaactact gatttcaact tctttggctt aat-tctctcg

121 gaaacgatga aatatacaag ttatatcttg gcttttcagc tctgcatcgt tttgggttct

181 cttggctgtt actgccagga cccatatgta aaagaagcag aaaaccttaa gaaatatttt

241 aatgcaggtc attcagatgt agcggataat ggaactcttt tcttaggcat tttgaagaat

301 tggaaagagg agagtgacag aaaaataatg cagagccaaa ttgtctcctt ttacttcaaa

361 ctttttaaaa actttaaaga tgaccagagc atccaaaaga gtgtggagac catcaaggaa

421 gacatgaatg tcaagttttt caatagcaac aaaaagaaac gagatgactt cgaaaagctg

481 actaattatt cggtaactga cttgaatgtc caacgcaaag caatacatga actcatccaa

541 gtgatggctg aactgtcgcc agcagctaaa acagggaagc gaaaaag-gag tcagatgctg

601 tttcgaggtc gaagagcatc ccagtaatgg ttgtcctgcc tgcaatattt gaattttaaa

661 tctaaatcta tttattaata tttaacatta tttatatggg gaatatattt ttagact-cat

721 caatcaaata agtatttata atagcaactt ttgtgtaatg aaaatgaata tctattaata

781 tatgtattat ttataattcc tatatcctgt gactgtctca cttaatcctt tgttttctga

841 ctaattaggc aaggctatgt gattacaagg ctttatctca ggggccaact aggcagccaa

901 cctaagcaag atcccatggg ttgtgtgttt atttcacttg atgatacaat gaa-cacttat

961 aagtgaagtg atactatcca gttactgccg gtttgaaaat atgcctgcaa tctgagccag

1021 tgctttaatg gcatgtcaga cagaacttga atgtgtcagg tgaccctgat gaaaacatag

1081 catctcagga gatttcatgc ctggtgcttc caaatattgt tgacaactgt gactgtaccc

1141 aaatggaaag taactcattt gttaaaatta tcaatatcta atatatatga ataaagtgta

1201 agttcacaac aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa (SEQ ID NO: 11)

The corresponding amino acid sequence for human IFN-γ (Accession No. NP_000610), NCBI database, is hereby incorporated by reference in its entirety):

1 MKYTSYILAF QLCIVLGSLG CYCQDPYVKE AEN-LKKYFNA GHSDVADNGT LFLGILKNWK

61 EESDRKIMQS QIVSFYFKLF KNFKDDQSIQ KSVE-TIKEDM NVKFFNSNKK KRDDFEKLTN

121 YSVTDLNVQR KAIHELIQVM AELSPAAKTG KRKRSQMLFR GRRASQ (SEQ ID NO:12)

The nucleotide sequences encoding IL-12 and/or IFN-γ used in the subject invention include "homologous" or "modified" nucleotide sequences. Modified nucleic acid sequences will be understood to mean any nucleotide sequence obtained by mutagenesis according to techniques well known to persons skilled in the art, and exhibiting modifications in relation to the normal sequences. For example, mutations in the regulatory and/or promoter sequences for the expression of a polypeptide that result in a modification of the level of expression of a polypeptide according to the invention provide for a "modified nucleotide sequence". Likewise,

substitutions, deletions, or additions of nucleic acids to the polynucleotides of the invention provide for "homologous" or "modified" nucleotide sequences. In various embodiments, "homologous" or "modified" nucleic acid sequences have substantially the same biological or serological activity as the native (naturally occurring) IL-12 and/or IFN-γ peptide. A "homologous" or "modified" nucleotide sequence will also be understood to mean a splice variant of the polynucleotides of the instant invention or any nucleotide sequence encoding a "modified polypeptide" as defined below.

A homologous nucleotide sequence, for the purposes of the present invention, encompasses a nucleotide sequence having a percentage identity with the bases of the nucleotide sequences of between at least (or at least about) 20.00% to 99.99% (inclusive). The aforementioned range of percent identity is to be taken as including, and providing written description and support for, any fractional percentage, in intervals of 0.01%, between 20.00% and 99.99%. These percentages are purely statistical and differences between two nucleic acid sequences can be distributed randomly and over the entire sequence length.

In various embodiments, homologous sequences exhibiting a percentage identity with the bases of the nucleotide sequences of the present invention can have 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identity with the polynucleotide sequences of the instant invention. Homologous nucleic acid sequences and amino acid sequences include mammalian homologs of the human IL-12 and/or IFN-γ nucleic acid sequences and amino acid sequences, including homologs of biologically active fragments, such as biologically active subunits.

Both protein and nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman *Proc. Natl. Acad. Sci. USA,* 1988, 85(8):2444-2448; Altschul et al. *J. Mol. Biol.,* 1990, 215(3): 403-410; Thompson et al. *Nucleic Acids Res.,* 1994, 22(2): 4673-4680; Higgins et al. *Methods Enzymol.,* 1996, 266:383-402; Altschul et al. *J. Mol. Biol.,* 1990, 215(3):403-410; Altschul et al. *Nature Genetics,* 1993, 3:266-272).

Identity and similarity of related nucleic acid molecules and polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; York (1988); Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; York (1993); Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Jersey (1994); Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; York (1991); and Carillo et al., SIAM J. Applied Math., 48:1073 (1988).

The methods, pharmaceutical compositions, and vectors of the present invention can utilize biologically active fragments of nucleic acid sequences encoding IL-12 and/or IFN-γ. Representative fragments of the polynucleotide sequences according to the invention will be understood to mean any polynucleotide fragment having at least 8 or 9 consecutive nucleotides, preferably at least 12 consecutive nucleotides, and still more preferably at least 15 or at least 20 consecutive nucleotides of the sequence from which it is derived. The upper limit for such fragments is the total number of nucleotides found in the full-length sequence (or, in certain embodiments, of the full length open reading frame (ORF) identified herein).

In other embodiments, fragments can comprise consecutive nucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, and up to one nucleotide less than the full-length IL-12 and/or IFN-γ coding sequences. In some embodiments, fragments comprise biologically active subunits of IL-12 and/or IFN-γ (e.g., p35 and/or p40 subunit of IL-12), or biologically active fragments of such subunits.

It is also well known in the art that restriction enzymes can be used to obtain biologically active fragments of the nucleic acid sequences, such as those encoding IL-12 and/or IFN-γ. For example, Bal31 exonuclease can be conveniently used for time-controlled limited digestion of DNA (commonly referred to as "erase-a-base" procedures). See, for example, Maniatis et al. [1982] *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, New York; Wei et al. [1983] *J. Biol. Chem.* 258:13006-13512.

The methods of the subject invention also contemplate the administration of cells that have been genetically modified to produce both IL-12 and IFN-γ, or biologically active fragments thereof. Such genetically modified cells can be administered alone or in combinations with different types of cells. Thus, genetically modified cells of the invention can be co-administered with other cells, which can include genetically modified cells or non-genetically modified cells. Genetically modified cells may serve to support the survival and function of the co-administered cells, for example.

The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a cell of the subject invention by intentional introduction of exogenous nucleic acids by any means known in the art (including for example, direct transmission of a polynucleotide sequence from a cell or virus particle, transmission of infective virus particles, and transmission by any known polynucleotide-bearing substance) resulting in a permanent or temporary alteration of genotype. The nucleic acids may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful polynucleotides in addition to those encoding IL-12 and IFN-γ, or biologically active fragments thereof. A translation initiation codon can be inserted as necessary, making methionine the first amino acid in the sequence. The term "genetic modification" is not intended to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like. The genetic modification may confer the ability to produce IL-12 and IFN-γ, or biologically active fragments thereof, wherein the cell did not previously have the capability, or the modification may increase the amount of IL-12 and IFN-γ produced by the cell, e.g., through increased expression.

Exogenous nucleic acids and/or vectors encoding IL-12 and/or IFN-γ, or biologically active fragments thereof, can be introduced into a cell by viral vectors (retrovirus, modified herpes virus, herpes virus, adenovirus, adeno-associated virus, and the like) or direct DNA transfection (lipofection, calcium phosphate transfection, DEAE-dextran, electropora-

tion, and the like), microinjection, cationic lipid-mediated transfection, transduction, scrape loading, ballistic introduction and infection (see, for example, Sambrook et al. [1989] *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Preferably, the exogenous nucleic acid sequence encoding IL-12 and/or IFN-γ, or biologically active fragments thereof, is operably linked to a promoter sequence that permits expression of the nucleic acid sequence in a desired tissue within the patient. The promoters can be inducible or tissue specific as necessary.

The genetically modified cell may be chosen from eukaryotic or prokaryotic systems, for example bacterial cells (Gram negative or Gram positive), yeast cells, animal cells, plant cells, and/or insect cells using baculovirus vectors. In some embodiments, the genetically modified cell for expression of the nucleic acid sequences encoding IL-12 and/or IFN-γ, or biologically active fragments thereof, are human or non-human mammal cells.

Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989) and in Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988), and in Watson et al., Recombinant DNA, Scientific American Books, New York and in Birren et al. (eds) Genome Analysis: A Laboratory Manual Series, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659; and 5,272,057; and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in PCR Protocols: A Guide To Methods And Applications, Academic Press, San Diego, Calif. (1990). In situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al., *Blood,* 1996, 87:3822).

MATERIALS AND METHODS

Animals. Female B6D2F1 mice, 6 to 8 weeks old, from Jackson Laboratory (Bar Harbor, Me.) were maintained in pathogen-free conditions at the animal center at the James A. Haley Veterans Hospital. All procedures were reviewed and approved by the committee on animal research at the James A. Haley VA Medical Center and the University of South Florida College of Medicine.

Vaccination protocol. Four groups of naive mice (n=12) were intramuscularly vaccinated 3 times at intervals of 2 days, each in its right quadriceps muscle, with 100 μg of pIFN-γ, 100 μg of pIL-12, or a mixture of pIL-12 and pIFN-γ (50 μg of each), along with subcutaneous injection of 50 μg per mouse of crude KBG extract given at the back of the animal. Control mice each received 100 μg of pcDNA3.1 plasmid and 50 μg of KBG allergen extract. Seven days after the last DNA and KBG vaccinations, control and experimental groups of mice were immunized intraperitoneally with 10 μg of KBG allergen and 1 mg of alum.

Construction of the pIL-12 and pIFN-γ plasmids. To clone murine IL-12, the IL-12 p40 subunit was amplified from a mouse cDNA library as an NheI-XhoI cassette through use of the following set of primers: forward primer 5'-dCCA GGC AGC TAG CAG CAA AGC AA-3' (SEQ ID NO:1) and reverse primer 5'-dTCC CTC GAG GCA TCC TAG GAT CGG AC-3' (SEQ ID NO:2). The amplified product was ligated to the mammalian expression vector pcDNA 3.1 (INVITROGEN, San Diego, Calif.) at the corresponding sites. The resulting plasmid, pcP40, was used as the template to amplify the p40 subunit and bovine growth hormone (BGH) poly A sequences derived from the pcDNA 3.1 vector as an HindIII-KpnI cassette and ligated to pcDNA 3.1 at the corresponding sites to generate the plasmid pc40. The following primers were used: 5'-dACC CAA GCT TGC TAG CAG CAA A-3' (SEQ ID NO:3) and 5'-dGAA GCC ATA GAG GGT ACC GCA TC-3' (SEQ ID NO:4). Through use of forward primer 5'-dTGC GGA TCC AGC ATG TGT CAA T-3' (SEQ ID NO:5) and reverse primer 5'-dGCA GAG GGC CTC GAG CTT TCA G-3' (SEQ ID NO:6), the p35 subunit of murine IL-12 was amplified as a BamHI-XhoI fragment and cloned into pcDNA3.1 to generate to pcP35 vector. With pcP35 used as a template, the cytomegalovirus (CMV) promoter and p35 subunit were as an EcoRI-EcoRV cassette and ligated to the corresponding site in the vector pc40. The resulting plasmid, pIL-12, had each of the 2 subunits of IL-12 under a separate CMV promoter and a BGH gene poly A sequence. Murine IFN-γ was cloned in pcDNA3.1; the construction of this plasmid has been described elsewhere (Kumar, M. et al., *Vaccine,* 1999, 18:558-567). Large-scale plasmid preparation was preformed through use of a QIAGEN kit (QIAGEN, Valencia, Calif.).

Immunohistochemistry. Groups of naive mice were vaccinated intramuscularly 3 times with 100 μg of pIFN-γ, pIL-12, or pcDNA3.1 control vector at intervals of 2 days each. Seven days after the last vaccination, the mice were killed and their right quadriceps muscles were removed and subjected to paraffin embedding, as described previously (Kumar, M. et al., *Vaccine,* 1999, 18:558-567). Immunostaining for IFN-γ and IL-12 was preformed through use of polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif.) against murine IFN-γ and the murine IL-12 p40 subunit, as described previously (Matsuse, H. et al., *J. Immunol.,* 2000, 164:6583-6592).

Splenocyte culture and assay for cytokines. Seven days after the KBG immunization, one half of the animals in each group were killed and their spleens removed aseptically. Single-cell suspensions were cultured with 1 mg/mL KBG allergen for 48 hours. Supernatants were collected from the cultures, and the production of IFN-γ, IL-2, and IL-4 was determined by ELISA (R&D Systems, Minneapolis, Minn.), according to the manufacturer's instructions.

Antibody assays. Mice were bled 21 days after KBG immunization, and their sera were collected. Total IgE and antigen-specific IgG1 and IgG2a were estimated by ELISA through use of purified antimouse mABs (PHARMINGEN, San Diego, Calif.). Microtiter plates (COSTAR, Cambridge, Mass.) were coated overnight with 0.1 μg per well of purified KBG allergen in a bicarbonate buffer (0.05 mol/L, pH 9.6) or antimouse IgE mAbs. Wells were washed with washing buffer (0.5% Tween-20 in PBS, pH 7.4) and blocked with 200 μL per well of PBS (pH 7.4) containing 1% BSA for 1 hour at 37° C. After 3 washes, serum samples were added at 100 μL per well and incubated for 2 hours at 37° C. After washing, biotinylated antimouse IgG1, IgG2a, and IgE antibodies were added to each well and incubated at 37° C. for 1 hour. Streptavidin-peroxidase conjugate (1:10,000 dilution, SIGMA CHEMICALS, St Louis, Mo.) was added after washing, and the wells were further incubated at 37° C. for 1 hour. Finally, the plates were washed and color was developed by the addition of substrate tetramethyl benzidine (PHARMINGEN) at room temperature for 30 minutes. The reaction was stopped and the absorbance read at 450 nm through use of an automated ELISA reader.

Pulmonary function. To assess pulmonary function, naive mice (n=4) were vaccinated 3 times as described in connection with the vaccination protocol. Seven days after the last cytokine pDNA and KBG vaccinations, animals were sensitized intraperitoneally with 10 μg of KBG allergen and 1 mg of alum. Ten days later, these mice were challenged intranasally with 50 μg of KBG allergen once daily for 3 consecutive days. Airway responsiveness was measured with a whole-body plethysmograph (BUXCO ELECTRONICS, Troy, N.Y.) in conscious, unrestrained mice at 24 hours after allergen challenge; response to methacholine was expressed as enhanced pause, as previously described (Matsuse, H. et al., *J. Immunol.,* 2000, 164:6583-6592).

Histologic analysis. Mice were killed with an overdose (0.6 g/kg) of pentobarbital (NEMBUTAL, Abbott Laboratories, North Chicago, Ill.) 24 hours after the final allergen challenge, and lung sections were subjected to paraffin embedding. Lung inflammation was assessed after the sections were stained with hematoxylin and eosin; this was followed by scoring for severity of inflammation on a scale of 0 to 3 (0=minimum degree of inflammation; 3=the maximum), as described previously (Kumar, M. et al., *Vaccine,* 1999, 18:558-567). Pathologic scores were expressed as means±SDs. The slides were coded and scored in a blinded fashion twice each by 3 different individuals. Intraobserver variation was <5%.

Statistical analysis. Pairs of groups were compared through use of Student t tests. Differences between groups were considered significant at P<0.05. Values for all measurements are expressed as means±SDs.

EXAMPLE 1

Plasmid Constructs and Expression of IL-12 and IFN-γ

The 2 subunits of murine IL-12, p35 and p40, were cloned into the same pcDNA3.1 vector. The cloning strategy was such that each subunit was under the transcriptional control of an individual CMV immediate-early promoter and also had its own BGH poly A sequences derived from the vector pcDNA3.1. Mice given pIFN-γ or pIL-12 exhibited expression of the IFN-γ or IL-12 p40 subunit, respectively, in their muscle, as observed by immunohistochemical staining of the muscle tissues (FIGS. **1**A and **1**C). No immunostaining was observed in a control group of mice that received the empty vector pcDNA3.1 (FIGS. **1**B and **1**D). The results of RT-PCR analyses of muscle mRNAs revealed that mice given either pIFN-γ or pIL-12 (FIG. **1**E, lane 2 and lane 4), but not the control group of mice (FIG. **1** E, lane 1 and lane 3), expressed IFN-γ and IL-12 p40 subunit-specific mRNAs, respectively.

EXAMPLE 2

Cytokine Genetic Adjuvants Inhibit Production of IgE Antibodies and Enhanced Production of IgG2a Antibodies

Four groups of mice (n=6) were vaccinated 3 times, as described in connection with the vaccination protocol. Sera were collected on day 21 after KBG immunization for an antibody assay. As shown in FIGS. **2**A and **2**B, mice given cytokine plasmid(s) as an adjuvant exhibited significantly lower total IgE levels than control mice (P<0.01). The group of mice given both pIL-12 and pIFN-γ constructs revealed a significantly lower amount of total IgE than mice given pIL-12 (P<0.01) or pIFN-γ (P<0.05) alone (FIG. **2**B). There was also an increase in antigen-specific IgG2a levels (P<0.01) in mice given pIFN-γ plus pIL-12 as an adjuvant in comparison with the control mice and the mice receiving either plasmid alone (FIG. **2**A). However, no significant difference was observed for antigen-specific IgG1 antibody levels among the cytokine pDNA-vaccinated and control groups of mice (FIG. **2**A). These results indicate that administration of cytokine pDNA adjuvants along with allergen vaccines resulted in a shift in the antibody production from the IgE type to the IgG2a type.

EXAMPLE 3

Cytokine Genetic Adjuvants Alter the Cytokine Profiles of Splenocytes

Four groups of mice (n=6) were vaccinated 3 times as described in connection with the vaccination protocol. Spleens were removed on day 7, after KBG immunization, and cultured in vitro for assessment of T_H1-like cytokines IL-2 and IFN-γ or T_H2-like cytokine IL-4. Mice given cytokine pDNA adjuvants produced more T_H1-like cytokines than did the controls (FIGS. **3**A-**3**C). Mice given combined pIFN-γ and pIL-12 produced higher amounts of IFN-γ (194.64 pg/mL) than mice given pIFN-γ (100.14 pg/mL) or pIL-12 (111.87 pg/mL) alone. Control mice produced only 11.71 pg/mL IFN-γ (FIG. **3**A). Mice vaccinated with pDNA cytokine(s) adjuvant produced more IL-2 than did control mice. IL-2 levels after vaccinations were as follows: pIFN-γ, 73.68 pg/mL; pIL-12, 128.43 pg/mL; pIFN-γ plus pIL-12, 202.57 pg/mL; and pcDNA3.1 (control), 51.88 pg/mL (FIG. **3**A). In contrast, control mice, which were vaccinated only with the empty vector plasmid, produced more IL-4 (37.55 pg/mL) than the groups injected with the pDNA cytokine adjuvant, which produced 9.71 pg/mL (pIFN-γ plus pIL-12), 13.05 pg/mL (pIFN-γ) and 17.99 pg/mL (pIL-12) of IL-4 (FIG. **3**B). No significant difference was observed in IL-4 production among the pIFN-γ, pIL-12, and pIFN-γ plus pIL-12 treatment groups.

To examine the dominant pattern of cytokine responses, IFN-γ:IL-4 and IL-2:IL-4 ratios were compared among different groups of mice (FIG. **3**C). The IFN-γ:IL-4 ratios in the mice vaccinated with pIFN-γ, pIL-12, and pIFN-γ plus pIL-12 as adjuvants were 7.48, 6.33, and 20.86, respectively, whereas the empty pcDNA3.1-vaccinated control exhibited a ratio of only 0.31. The ratios of IL-2:IL-4 in pIFN-γ, IL-12, pIFN-γ plus IL-12, and pcDNA3.1 controls were 7.50, 7.32, 21.71, and 1.4, respectively. These results indicate that the net cytokine balance shifted in favor of the T_H1-like response in cytokine plasmid-vaccinated mice; however, this shift was greater in the group vaccinated with the adjuvant that was a combination of pIFN-γ and pIL-12 plasmid DNAs.

EXAMPLE 4

Cytokine Genetic Adjuvants Prevent the Development of Airway Hyperresponsiveness in Allergen-Sensitized and -Challenged Mice

Mice (n=4) were vaccinated, sensitized, and challenged as described in connection with pulmonary function. The airways of control mice that received the empty vector as vaccine adjuvant were significantly more reactive to 50 mg/mL of methacholine than those of the mice that received pIFN-γ and/or pIL-12, as shown in FIG. **4**. Mice vaccinated with pIFN-γ plus pIL-12 exhibited the least reactivity to the inhaled methacholine challenge in comparison with the

pcDNA3.1 (P<0.01), pIFN-γ, and pIL-12 groups (P<0.05). These results suggest that the vaccination of mice with combined pIFN-γ and pIL-12 as an adjuvant significantly reduces airway reactivity.

EXAMPLE 5

Combination of IFN-γ and IL-12 Plasmid DNAs Prevents Lung Inflammation in the Allergen-Sensitized Mice

Mice (n=4) were vaccinated, sensitized, and challenged as described in connection with pulmonary function. Lung inflammation was examined 24 hours after the final allergen challenge. Representative pathologic features are shown in FIGS. **5**A-**5**D. The group of mice receiving the combined constructs as an adjuvant (FIG. **5**D) exhibited less epithelial damage and less infiltration of mononuclear cells and polymorphs in the interstitial and peribronchovascular region than was seen in the control group (FIG. **5**A) and the other vaccinated groups (FIGS. **5**B and **5**C). A semi-quantitative analysis using a scoring system for inflammatory cells in the lung is shown in Table 1. Groups of mice that received pIFN-γ, pIL-12, or a combination of the two exhibited reduced pulmonary inflammation in comparison with the empty vector (pcDNA3.1) control. The group of mice that received the combination of pIFN-γ and pIL-12 showed significantly less (P<0.05) pulmonary inflammation than the pIFN-γ and pIL-12 groups. No statistically significant difference was found between the pIFN-γ and pIL-12 groups.

TABLE 1

Quantification of pulmonary inflammation in mice after allergen vaccination with various cytokine adjuvants

Pathology	pcDNA3.1	Adjuvants pIFN-γ	pIL-12	pIFN-γ + pIL-12
Epithelial damage	2.8 ± 0.12	2.23 ± 0.23*	2.3 ± 0.16*	1.36 ± 0.48*†‡
Interstitial-alveolar infiltrate	2.73 ± 0.20	2.4 ± 0.25*	2.56 ± 0.42	1.76 ± 0.36*†‡
Peribroncho-vascular infiltrate	2.83 ± 0.26	2.16 ± 0.23*	2.16 ± 0.32*	1.26 ± 0.20*†‡

Each value represents the mean±SD of 5 fields from 6 individual lung sections from each mouse in a group (n=4). Values were considered significant when the P value was less than 0.05 (*†‡). Statistical differences are indicated as follows: * in comparison with pcDNA3.1 control; † in comparison with pIFN-γ control; ‡ in comparison with pIL-12 control.

Previously, it was shown in a mouse model that effective parenteral vaccination of mice with grass allergens required subcutaneous injection of a high dose (250 μg to 1 mg per mouse) of allergens (Cao, Y. et al., *Immunology,* 1997, 90:46-51). By corollary, an even higher dose of allergen mixture would be required to induce an effective immune deviation from an allergic response. The results of this study demonstrate that in a mouse, a substantially lower dosage (50 μg) of allergens, when administered along with a pDNA cytokine(s) adjuvant, can induce effective immune deviation and a protective airway response in comparison with allergens alone. Furthermore, a comparison of pIFN-γ and/or pIL-12 as adjuvants indicates that the combination of pIFN-γ and pIL-12 is a more effective adjuvant than either of these plasmids alone. Although the effect of these adjuvants in mice with an established allergic response was not examined, a number of studies have reported the effectiveness of IFN-γ and of IL-12 in modulating established allergic responses (Li, X. M. et al., *J. Immunol,* 1996, 157:3216-3219; Hogan, S. P. et al., *Eur J Immunol,* 1998, 28:413-423; Dow, S. W. et al., *Hum Gene Ther,* 1999, 10:1905-1914; Maecker, H. T. et al., *J Immunol,* 2001, 166:959-965). The results of the study, therefore, have significant implications for "time-honored" AIT, which might be rendered more safe and effective by including these cytokine plasmids as adjuvants.

A major finding of this study is that a vaccine adjuvant comprising both pIFN-γ and pIL-12 inhibited total IgE levels in mice with a concomitant increase of allergen-specific IgG2a antibody production. The level of IgE synthesis in mice vaccinated with a cocktail adjuvant was significantly (P<0.001) more suppressed than that of levels obtained by vaccination with pIFN-γ or pIL-12 alone as an adjuvant. Both systemic and local IFN-γ gene delivery has been shown to decrease serum IgE levels (Dow, S. W. et al., *Hum Gene Ther,* 1999, 10:1905-1914), whereas the role of the IL-12 protein as an adjuvant on serum IgE levels has been controversial (Kips, J. C. et al., *Am J Respir Crit Care Med,* 1996, 153:535-539; Brusselle, G. G. et al., *Am. J Respir Cell Mol Biol,* 1997, 17:767-771; Sur, S. et al., *J Immunol,* 1996, 157:4173-4180; Yoshimoto, T. et al., *Proc Natl Acad Sci USA,* 1997, 94:3948-3953). The results of this study indicate that an adjuvant comprising the IFN-γ and IL-12 plasmids enhances the production of IFN-γ. This finding is in agreement with a report that mice vaccinated with IL-12 exhibited enhanced local IFN-γ production (Schwarze, J. et al., *J Allergy Clin Immunol,* 1998, 102:86-93).

The deviation of the cytokine production profile from T_H2-like to T_H1-like is a significant marker of the effectiveness of a vaccine. Analysis of the cytokine pattern of mice after vaccination with allergens and different plasmid adjuvants revealed that the group of mice receiving pIFN-γ plus pIL-12 as the adjuvant had a more profound T_H1-like effect—i.e., it produced higher levels of IFN-γ and IL-2 than either of the cytokine plasmids alone. Analysis of the IL-4 expression from the splenocytes of different groups of mice revealed no significant difference among the groups of mice vaccinated with pIFN-γ, pIL-12, or a combination of the two. A predominance of the T_H1-like response seen with cytokine adjuvants is consistent with the finding of Hogan et al. (Hogan, S. P. et al., *Eur J Immunol,* 1998, 28:413-423), who showed, using the vaccinia virus as a gene delivery vehicle, that IL-12 gene transfer to murine lung inhibited T_H2 cytokine production, which was mediated through the production of IFN-γ. Because allergic individuals produce limited amounts of IFN-γ, an adjuvant providing the expression of both IFN-γ and IL-12 induces a more effective T_H1-like response.

In patients with allergic asthma, it was shown that airway hyperresponsiveness correlates with the severity of disease and is therefore a major clinical feature of allergic asthma. A direct instillation into the respiratory tract or an intraperitoneal administration of either rIL-12 or rIFN-γ inhibits allergic airway inflammation and in some cases suppresses airway hyperresponsiveness (Gavett, S. H. et al., *J Exp Med,* 1995, 182:1527-1536; Iwamoto, I. et al., *J Exp Med,* 1993, 177:573-576). However, these protocols require a regular or daily dosing over periods ranging from 5 to 13 days to produce significant protective effects. Furthermore, gene transfer studies using IFN-γ and IL-12 have been shown to suppress airway hyperreactivity (Li, X. M. et al., *J Immunol,* 1996, 157:3216-3219; Hogan, S. P. et al., *Eur J Immunol,* 1998, 28:413-423). The results of this investigation indicate that allergen vaccine formulations that include allergens and

pIFN-γ, pIL-12, or a combination of both cytokines as an adjuvant significantly decrease airway hyperresponsiveness and that a combination of pIFN-γ and pIL-12 induces the highest reduction in airway responsiveness. There appears to be a synergistic effect when both cytokine plasmids (pIFN-γ and pIL-12) are administered to the mice. A parenteral administration of only 50 μg of allergen-induced massive cellular infiltration and airway obstruction in the lung tissue of mice in the present study. However, allergens given with pIFN-γ, pIL-12, or a combination as an adjuvant limited not only the cellular infiltration but also epithelial cell damage. Consistent with previous observations, either pIFN-γ or pIL-12 as an adjuvant decreased eosinophilic cellular infiltration (Li, X. M. et al., *J Immunol,* 1996, 157:3216-3219; Hogan, S. P. et al., *Eur J Immunol,* 1998, 28:413-423), whereas treatment with a combination of pIFN-γ and pIL-12 as an adjuvant was most effective.

Not wishing to be bound by theory, one mechanism for the effectiveness of pIFN-g and pIL-12 as an adjuvant may be that CpG sequences, which are present in the backbone plasmid pcDNA3.1, nonspecifically contribute to the endogenous IL-12/IFN-g production (Roman, M. et al., *Nat Med,* 1997, 3:849-854). One possibility is that CpG sequences present in the backbone plasmid, pcDNA3.1, nonspecifically contribute to the endogenous IL-12/IFN-γ production (Roman, M. et al., *Nat Med,* 1997, 3:849-854). However, this is unlikely, because all groups received an equal dose of plasmid and mice receiving both pIFN-γ and pIL-12 received an adjusted dose (50 μg each, for a total of 100 μg). The empty vector, which also contained CpGs, had no cytokine-inducing or immunomodulatory effect. Furthermore, the higher effectiveness of combined pIFN-γ and pIL-12 in comparison with either of the plasmids alone (100 μg) might be attributed to the CpG motifs in the coding sequences; however, the cDNA inserts are mammalian sequences, which have a low frequency of CpG dinucleotides, are mostly methylated, and do not have immunostimulatory activity (Hemmi, H. et al., *Nature,* 2000, 408:740-750). Nonetheless, a search for a canonical hexamer purine-purine-CG-pyrimidine-pyrimidine as a possible core CpG motif led to the identification of GACGTT, GGCGTC, GGCGTT sequences in IL-12, and AACGCT, AGCGCT sequences in IFN-γ. However, whether these sequences in the mouse cDNA are capable of binding to the mouse Toll-like receptor 9 and cause immune stimulation, like the CpG motifs present in the bacterial DNA, remains to be investigated (Hemmi, H. et al., *Nature,* 2000, 408:740-750).

Alternatively, the synergy between pIFN-γ and pIL-12 on the induction of a T_H1-dominant state and the inhibition of airway inflammation may be in their cellular mechanism of action. First, although IL-12 is the primary determinant of the T_H1 differentiation, IFN-γ endogenously synthesized during the priming of naive CD4-positive T cells both accelerates and enhances the T_H1 differentiating effects of IL-12 (Wenner, C. A. et al., *J Immunol,* 1996, 56:1442-1447; Bradley, L. M. et al., *J Immunol,* 1996, 157:1350-1358). Thus, IFN-γ presumably synergizes with T-cell receptor engagement and induces the expression of functional IL-12 receptors on naive T cells. This idea is consistent with the report that the presence of IFN-γ and IFN-α in cultures enhanced IL-12Rβ2 expression on CD4-positive and CD8-positive T cells and that the enhancing effect of IFN-γ was independent of endogenous IL-12 or IFN-α (Wu, C. Y. et al., *Eur J Immunol,* 2000, 30:1364-1374). Second, the highest amount of IL-2 produced by a combined vaccination with pIFN-γ and pIL-12 might synergize with IFN-γ effects and contribute to a more effective T_H1-like response. IL-2 regulates expression of the IL-12β2 receptor on natural killer cells (Wu, C. Y. et al., *Eur J Immunol,* 2000, 30:1364-1374). It is to be noted however, that these 2 possibilities are not mutually exclusive. Irrespective of the mechanism involved, the results lead to the conclusion that in a mouse model of grass allergy, the administration of cytokine plasmids encoding IFN-γ and IL-12 as an adjuvant enhances the therapeutic effectiveness of grass allergen vaccines.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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ggggtgacgt gcggagctgc tacactctct gcagagagag tcagagggga caacaaggag    600

tatgagtact cagtggagtg ccaggaggac agtgcctgcc cagctgctga ggagagtctg    660

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agaccccgtc tctacaaaaa aaatttaaaa ttagccaggc gtggtggctc atgcttgtgg   1800

tcccagctgt tcaggaggat gaggcaggag gatctcttga gcccaggagg tcaaggctat   1860

ggtgagccgt gattgtgcca ctgcatacca gcctaggtga cagaatgaga ccctgtctca   1920
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aaaaaaaaaa tgattgaaat taaaattcag ctttagcttc catggcagtc ctcacccccca 1980

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gatcttaaga ttcaagagag aggacaagta gttatggcta aggacatgaa attgtcagaa 2100

tggcaggtgg cttcttaaca gccctgtgag aagcagacag atgcaaagaa aatctggaat 2160

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tgaaggtgct acttttaagt aatgtatgtg cgctctgtaa agtgattaca tttgtttcct 2280

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<213> ORGANISM: Homo sapiens

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Val Glu Leu Asp Trp Tyr Pro Asp Ala Pro Gly Glu Met Val Val Leu
        35                  40                  45

Thr Cys Asp Thr Pro Glu Glu Asp Gly Ile Thr Trp Thr Leu Asp Gln
    50                  55                  60

Ser Ser Glu Val Leu Gly Ser Gly Lys Thr Leu Thr Ile Gln Val Lys
65                  70                  75                  80

Glu Phe Gly Asp Ala Gly Gln Tyr Thr Cys His Lys Gly Gly Glu Val
                85                  90                  95

Leu Ser His Ser Leu Leu Leu Leu His Lys Lys Glu Asp Gly Ile Trp
            100                 105                 110

Ser Thr Asp Ile Leu Lys Asp Gln Lys Glu Pro Lys Asn Lys Thr Phe
        115                 120                 125

Leu Arg Cys Glu Ala Lys Asn Tyr Ser Gly Arg Phe Thr Cys Trp Trp
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Leu Thr Thr Ile Ser Thr Asp Leu Thr Phe Ser Val Lys Ser Ser Arg
145                 150                 155                 160

Gly Ser Ser Asp Pro Gln Gly Val Thr Cys Gly Ala Ala Thr Leu Ser
                165                 170                 175

Ala Glu Arg Val Arg Gly Asp Asn Lys Glu Tyr Glu Tyr Ser Val Glu
            180                 185                 190

Cys Gln Glu Asp Ser Ala Cys Pro Ala Ala Glu Glu Ser Leu Pro Ile
        195                 200                 205

Glu Val Met Val Asp Ala Val His Lys Leu Lys Tyr Glu Asn Tyr Thr
    210                 215                 220

Ser Ser Phe Phe Ile Arg Asp Ile Ile Lys Pro Asp Pro Pro Lys Asn
225                 230                 235                 240

Leu Gln Leu Lys Pro Leu Lys Asn Ser Arg Gln Val Glu Val Ser Trp
                245                 250                 255

Glu Tyr Pro Asp Thr Trp Ser Thr Pro His Ser Tyr Phe Ser Leu Thr
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Phe Cys Val Gln Val Gln Gly Lys Ser Lys Arg Glu Lys Lys Asp Arg
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-continued

Val Phe Thr Asp Lys Thr Ser Ala Thr Val Ile Cys Arg Lys Asn Ala
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Ser Ile Ser Val Arg Ala Gln Asp Arg Tyr Tyr Ser Ser Ser Trp Ser
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gaaacgatga aatatacaag ttatatcttg gcttttcagc tctgcatcgt tttgggttct 180

cttggctgtt actgccagga cccatatgta aaagaagcag aaaaccttaa gaaatatttt 240

aatgcaggtc attcagatgt agcggataat ggaactcttt tcttaggcat tttgaagaat 300

tggaaagagg agagtgacag aaaaataatg cagagccaaa ttgtctcctt ttacttcaaa 360

ctttttaaaa actttaaaga tgaccagagc atccaaaaga gtgtggagac catcaaggaa 420

gacatgaatg tcaagttttt caatagcaac aaaaagaaac gagatgactt cgaaaagctg 480

actaattatt cggtaactga cttgaatgtc caacgcaaag caatacatga actcatccaa 540

gtgatggctg aactgtcgcc agcagctaaa acagggaagc gaaaaaggag tcagatgctg 600

tttcgaggtc gaagagcatc ccagtaatgg ttgtcctgcc tgcaatattt gaattttaaa 660

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caatcaaata agtatttata atagcaactt ttgtgtaatg aaaatgaata tctattaata 780

tatgtattat ttataattcc tatatcctgt gactgtctca cttaatcctt tgttttctga 840

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cctaagcaag atcccatggg ttgtgtgttt atttcacttg atgatacaat gaacacttat 960

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tgctttaatg gcatgtcaga cagaacttga atgtgtcagg tgaccctgat gaaaacatag 1080

catctcagga gatttcatgc ctggtgcttc caaatattgt tgacaactgt gactgtaccc 1140

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20 25 30

Asn Leu Lys Lys Tyr Phe Asn Ala Gly His Ser Asp Val Ala Asp Asn
35 40 45

Gly Thr Leu Phe Leu Gly Ile Leu Lys Asn Trp Lys Glu Glu Ser Asp
50 55 60

-continued

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Arg Lys Ile Met Gln Ser Gln Ile Val Ser Phe Tyr Phe Lys Leu Phe
65                  70                  75                  80

Lys Asn Phe Lys Asp Asp Gln Ser Ile Gln Lys Ser Val Glu Thr Ile
                85                  90                  95

Lys Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys Arg
            100                 105                 110

Asp Asp Phe Glu Lys Leu Thr Asn Tyr Ser Val Thr Asp Leu Asn Val
        115                 120                 125

Gln Arg Lys Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser
    130                 135                 140

Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Arg
145                 150                 155                 160

Gly Arg Arg Ala Ser Gln
                165
```

What is claimed is:

1. A method for modulating an immune response, comprising co-administering to a mammal:

an effective amount of a nucleic acid sequence encoding p35 and p40 subunits of human IL-12, and a promoter sequence operably linked to the nucleic acid sequence encoding the p35 and p40 subunits;

an effective amount of a nucleic acid sequence encoding human IFN-γ, and a promoter sequence operably linked to the nucleic acid sequence encoding human IFN-γ; and

an antigen, such that the co-administering results in an increase of IFN-γ and IL-2 production, an increase of IgG2a specific to the antigen, a decrease of IL-4 production, and reduced serum IgE.

2. The method of claim **1**, wherein the co-administering results in expression of the p35 and p40 subunits, the p35 subunit comprising the amino acid sequence of SEQ ID NO:8, and the p40 subunit comprising the amino acid sequence of SEQ ID NO:10.

3. The method of claim **1**, wherein the co-administering results in expression of the human IFN-γ, and wherein the human IFN-γ comprises the amino acid sequence of SEQ ID NO:12.

4. The method of claim **1**, wherein the nucleic acid sequence encoding the p35 and the p40 subunits of the human IL-12 comprises SEQ ID NO:7 and SEQ ID NO:9.

5. The method of claim **1**, wherein the nucleic acid sequence encoding the human IFN-γ comprises SEQ ID NO:11.

6. The method of claim **1**, wherein the nucleic acid sequences are administered with a pharmaceutically acceptable carrier.

7. The method of claim **1**, wherein the nucleic acid sequences and promoter sequences are administered within a viral vector.

8. The method of claim **1**, wherein the antigen is selected from the group consisting of a protein, peptide, glycoprotein, carbohydrate, lipid, glycolipid, hapten conjugate, recombinant, nucleotides, killed or attenuated organism, toxin, toxoid, and organic molecule.

9. The method of claim **1**, wherein the antigen is administered to the mammal with the nucleic acid sequences and a pharmaceutically acceptable carrier.

10. The method of claim **1**, wherein the mammal is human.

11. A method for modulating an immune response, comprising co-administering to a mammal:

an effective amount of a plasmid comprising a nucleic acid sequence encoding p35 and p40 subunits of human IL-12, and a promoter sequence operably linked to the nucleic acid sequence encoding the p35 and p40 subunits;

an effective amount of a plasmid comprising a nucleic acid sequence encoding human IFN-γ, and a promoter sequence operably linked to the nucleic acid sequence encoding the human IFN-γ; and

an antigen, such that the co-administering results in an increase of IFN-γ and IL-2 production, an increase of IgG2a specific to the antigen, a decrease of IL-4 production, and reduced serum IgE.

12. The method of claim **11**, wherein the antigen comprises an allergen.

13. The method of claim **11**, wherein the antigen comprises Kentucky blue grass (KBG) allergen extract.

14. The method of claim **11**, wherein the operably linked promoter sequences comprise cytomegalovirus (CMV) promoters.

15. The method of claim **11**, wherein the antigen comprises Kentucky blue grass (KBG) allergen extract, and the operably linked promoter sequences comprise cytomegalovirus (CMV) promoters.

16. The method of claim **11**, wherein the mammal is human.

17. The method of claim **11**, wherein the co-administering results in expression of the p35 and the p40 subunits, the p35 subunit comprising the amino acid sequence of SEQ ID NO:8, and the p40 subunit comprising the amino acid sequence of SEQ ID NO:10.

18. The method of claim **11**, wherein the mammal suffers from a condition selected from the group consisting of allergy, allergic rhinitis, atopic dermatitis, asthma, allergic sinusitis, pulmonary fibrosis, and cancer.

19. The method of claim **11**, wherein the plasmids are administered by a route selected from the group consisting of intramuscularly, orally, and intranasally.

20. The method of claim **1**, wherein the nucleic acid sequence encoding the p35 and p40 subunits of the human IL-12 and the nucleic acid sequence encoding the human IFN-γ are co-administered to the mammal through a mucosal route.

21. The method of claim **1**, wherein the nucleic acid sequence encoding the p35 and p40 subunits of the human IL-12 and the nucleic sequence encoding the human IFN-γ are co-administered to the mammal intranasally.

22. The method of claim **11**, wherein the plasmids are co-administered to the mammal through a mucosal route.

23. The method of claim **11**, wherein the plasmids are co-administered to the mammal intranasally.

24. The method of claim **1**, wherein the mammal suffers from a condition selected from the group consisting of allergy, allergic rhinitis, atopic dermatitis, asthma, allergic sinusitis, pulmonary fibrosis, and cancer.

25. The method of claim **11**, wherein the nucleic acid sequence encoding the p35 and p40 subunits of human IL-12 comprises SEQ ID NO: 7 and SEQ ID NO: 9.

26. The method of claim **11**, wherein the nucleic acid sequence encoding human IFN-γ comprises SEQ ID NO: 11.

27. The method of claim **1**, wherein the nucleic acid sequences are administered by a route selected from the group consisting of intramuscularly, orally, and intranasally.

28. The method of claim **1**, wherein said co-administering is carried out intramuscularly.

29. The method of claim **11**, wherein said co-administering is carried out intramuscularly.

30. The method of claim **1**, wherein the nucleic acid sequences are conjugated with chitosan to form nanoparticles.

31. The method of claim **11**, wherein the plasmids are conjugated with chitosan to form nanoparticles.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,595,303 B1
APPLICATION NO. : 10/655873
DATED : September 29, 2009
INVENTOR(S) : Shyam S. Mohapatra and Mukesh Kumar

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page, (57) Abstract,
Line 13, "cells generally modified" should read --cells genetically modified--.

Column 3,
Line 32, "a, airway;" should read --*a*, airway;--.

Column 7,
Line 9, "parental administration" should read --parenteral administration--.

Column 9,
Line 38, "ctgtcaatta" should read --ctgtgcctta--.
Line 54, "ttttactgaa" should read --ttttcatgaa--.

Column 10,
Line 5, "YKTKLHA" should read --YKTKIKLCILLHA--.

Column 33,
Lines 61-62, "recombinant, nucleotides," should read --recombinant nucleotides,--.

Signed and Sealed this

Second Day of February, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office