

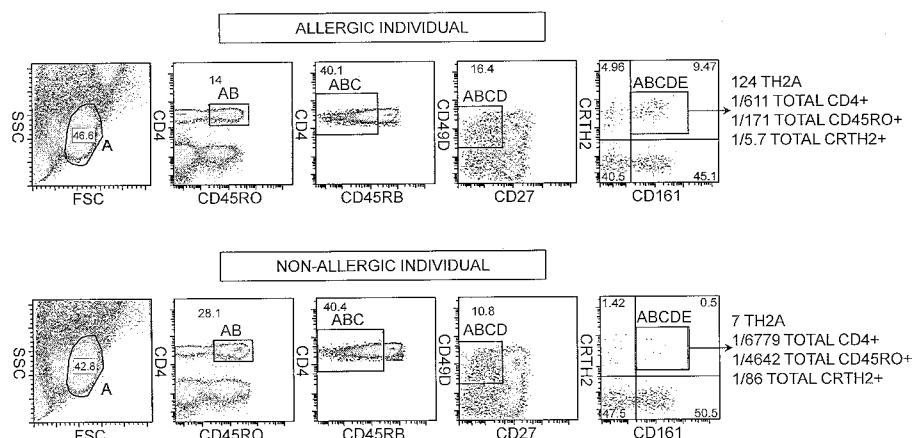


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(54) **Title:** DETECTION OF AN IMMUNE RESPONSE

(57) **Abstract:** Provided herein are methods of detecting and/or monitoring the presence or severity of an immune disorder in a subject, including detecting a frequency of a Th2a subset of CD4+ T cells in a biological sample of the subject. In some embodiments, the detecting includes: (a) detecting a frequency of CD4+ T cells in a biological sample of said subject; (b) detecting a frequency of a Th2a subset of the CD4+ T cells in the biological sample; and (c) comparing the frequency of the Th2a subset with the frequency of the CD4+ T cells.

DETECTION OF AN IMMUNE RESPONSE

William W. Kwok and Erik Wambre

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Related Applications

This application claims the benefit of United States Provisional Patent Application Serial No. 61/446,797, filed February 25, 2011, the disclosure of which is incorporated by reference herein in its entirety.

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Statement of Government Support

This invention was made with Government support under contract number HHSN272200700046C awarded by the National Institutes of Health. The Government has certain rights in the invention.

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Field of the Invention

The present invention is in the field of immunology and immunology-based testing and/or monitoring.

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Background

Allergen specific immunotherapy (SIT) is the only treatment available that treats the cause of an allergy. However, the therapy typically lasts from 3 to 5 years, with a widely-ranging efficacy rate of 50-90%. The ability to monitor the progress of SIT would allow better predictions of efficacy as well as the more precise tailoring to the patient populations being treated with this long and expensive therapy.

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In addition, the ability to accurately detect and/or monitor an immune response could aid in the diagnosis and treatment of immune-related disorders, including allergy and infection.

Summary

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Provided herein are methods of detecting an immune disorder in a subject, including detecting (*e.g., in vitro*) a frequency of an allergen-specific Th2 subset (Th2a) of CD4+ T cells in a biological sample of (or collected from) the subject (*e.g.,* a whole blood sample or a peripheral blood mononuclear cell (PBMC) sample). In some embodiments, the detecting includes: (*a*) detecting a frequency of CD4+ T cells in a biological sample of (or collected from) said subject; (*b*) detecting a frequency of a Th2a subset of the CD4+ T cells in the biological sample; and (*c*) comparing the frequency of the Th2a subset with the frequency of the CD4+ T

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cells. In some embodiments, a higher frequency of Th2a cells as compared to a control subject indicates an immune disorder.

In some embodiments, the CD4+ T cells are total CD4+ T cells. In some embodiments, the CD4+ T cells are total memory CD4+ T cells. In some embodiments, the CD4+ T cells are total CRTH2+ CD4+ T cells.

In some embodiments, a frequency of at least 50, at least 60, or at least 70 Th2a cells per 100,000 total CD4+ cells (or at least 500, at least 600, or at least 700 Th2a cells per one million CD4+ cells) indicates that said subject has an immune disorder. In some embodiments, a frequency of at least 60, 65, 75, 80, 85, 90, 95 or 100 Th2a cells per 100,000 total memory CD4+ T cells. In some embodiments, a frequency of at least 1,000, 2,000, 3,000, 4,000 or 5,000 Th2a cells per 100,000 total CRTH2+ CD4+ T cells indicates that said subject has an immune disorder.

In some embodiments, the immune disorder is an allergic disorder, e.g., seasonal rhinoconjunctivitis, animal dander allergy, food allergy, or venom anaphylaxis.

In some embodiments, the CD4+ Th2a cells are CD161 positive, CRTH2 positive, CD27 negative, CD49d positive and/or CD45RB negative.

Also provided are methods of monitoring immune deviation in a subject (e.g., during a course of an immunotherapy treatment), including detecting a frequency of a Th2a subset of CD4+ T cells in a biological sample of the subject (e.g., a whole blood sample or a peripheral blood mononuclear cell (PBMC) sample). In some embodiments, the monitoring includes: (a) detecting a first frequency of CD4+ Th2a cells as compared to a frequency of total CD4+ cells in a first biological sample of said subject; and then (b) detecting a second frequency of CD4+ Th2a cells as compared to a frequency of total CD4+ cells in a second biological sample of said subject, wherein a lower number for the second frequency of said CD4+ Th2a in step (b) as compared to the first frequency in step (a) indicates immune deviation in said subject.

In some embodiments, the second biological sample is collected from about 2, 4, or 6 weeks to about 1, 2, 3, 4 or 5 years after the first biological sample is collected from the subject.

In some embodiments, the CD4+ Th2a cells are CD161 positive. In some embodiments, the CD4+ Th2a cells are CD161 positive, CRTH2 positive, CD27 negative, CD49d positive and/or CD45RB negative.

In some embodiments, the detecting step is carried out by fluorescent activated cell sorting (FACS) (e.g., with a 4-, 5-, 6-, or 7-color combination).

Also provided is the use of a CD4+ Th2a cell frequency for detecting an immune disorder in a subject (e.g., an allergy), or for monitoring an immune deviation in a subject.

Methods of treating an immune disorder in a subject are further provided, comprising: detecting a Th2a cell frequency in said subject; and then, if the Th2a cell frequency is significant, administering an immunotherapy treatment to said subject.

In some embodiments, the immunotherapy treatment is oral immunotherapy treatment (OIT), sublingual immunotherapy (SLIT), or subcutaneous immunotherapy (SCIT) treatment.

In some embodiments, a significant Th2a cell frequency is greater than about one cell per 500, 1,000, or 5,000 total CD4⁺ T cells or per total CD45RO⁺ (memory) T cells. In some embodiments, a significant Th2a cell frequency is greater than about one cell per 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 total CRTH2⁺ CD4⁺ T cells.

Further provided is the use of a means for detecting CD4⁺ Th2a T cell frequency in a biological sample (*e.g.*, immunoassay, flow cytometry, FACS, etc.) for carrying out a method as taught herein.

Brief Description of the Drawings

Figure 1 presents a FACS plot showing that allergen-specific Th2 cells (Th2a) are a subset of Th2 cells expressing CD161 and CD49d, and lacking CD27 and CD45RB expression.

Figure 2 shows that significant numbers of Th2a cells were exclusively observed in PBMC samples from allergic individuals.

Figure 3 provides a schematic representation of an exemplary assay that may be performed to detect Th2a cells in a biological sample.

Figure 4 demonstrates that differences in Th2a cell frequencies can be seen between patients receiving sublingual immunotherapy (SLIT) and patients receiving placebo. Th2a cell frequencies were monitored in grass pollen allergic patients receiving active or placebo tablets in a double blind placebo-controlled study conducted in an allergen exposure chamber. The Th2a cell frequencies were assessed in coded frozen/thawed PBMCs samples at baseline (V3), and after 2 (V6) and 4 (V7) months of sublingual immunotherapy (SLIT). Percentages of Th2a cells were normalized to 100 for each patient at baseline.

Detailed Description of the Preferred Embodiments

Provided herein are methods for the determination of an immune response, including, but not limited to, an allergy response based upon detection of allergy-specific CD4⁺ T cell phenotype and/or the frequency of said phenotype in a patient or patient sample. This determination is useful for, *inter alia*, detecting or monitoring an allergy and/or the effectiveness of immunotherapy.

The disclosures of all United States patent references cited herein are hereby incorporated by reference to the extent they are consistent with the disclosure set forth herein. As used herein in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, the terms "about" and "approximately" as used herein when referring to a measurable value such as an amount of a compound, dose, time, temperature, and the like, is meant to encompass variations of 20%, 10%, 5%, 1%, 0.5%, or even 0.1% of the specified amount. Also, as used herein, "and/or" or "/" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

An "immune response" elicited, detected and/or monitored can be a protective immune response, a cellular immune response, a humoral immune response, a Th1 immune response, a Th2 immune response, or any combination thereof. Detection and/or monitoring of the immune response may be useful, *e.g.*, in monitoring immune function and/or response to an antigen, *e.g.*, for autoimmune disease, a pathogen, cancer, diabetes or multiple sclerosis, or to test the effectiveness of a vaccine or vaccination.

An "immune disorder" is a disease or illness associated with an immune response, including, but not limited to, allergy or allergic disorder, autoimmune disease, pathogen infection, cancer, diabetes, multiple sclerosis, etc.

An "allergy", "allergic disorder", or "allergic disease" is a disorder in which the immune system is hypersensitive to normally harmless environmental substances. These environmental substances that cause allergies are called "allergens." Common allergic diseases include seasonal rhinoconjunctivitis (*e.g.*, allergies to grasses and pollen such as ragweed, timothy grass), allergies to pet dander such as cat dander or dog dander, food allergies such as peanut, dairy and wheat allergies, venom anaphylaxis, and asthma. Production of the "IgE" form of antibody is associated with allergic reaction. The IgE antibody causes excessive activation of mast cells and basophils, resulting in an overreactive inflammatory response, which can be severe enough to result in anaphylactic shock. Thus, in some embodiments the IgE antibody may also be measured, if desired.

An "antigen" as used herein is a molecule or molecule fragment that is able to bind specifically to a major histocompatibility complex (MHC) molecule for presentation to the immune system cells, which complex of molecule or molecule fragment and MHC molecule, in turns, binds to immune system cell receptors (*e.g.*, T cell receptors). An "immunogen" is a particular type of antigen that is able to provoke a humoral and/or cell mediated immune

response if injected on its own. Antigens are usually proteins or polysaccharides, including parts (coats, capsules, cell walls, flagella, fimbriae, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids are normally only antigenic when combined with proteins and polysaccharides. Non-microbial exogenous (non-self) antigens can include pollen, egg white or other food antigens, and proteins from transplanted tissues and organs or on the surface of transfused blood cells. A "vaccine" is an example of immunogenic antigens intentionally administered to induce acquired immunity in the recipient.

Though not wishing to be bound by theory, it is thought that an allergic reaction begins when an MHC class II molecule of an antigen-presenting cell binds to and presents an allergen or portion thereof to CD4⁺ T cells of the immune system, which, in turn, elicit an immune response to that allergen. An "immune response" elicited, detected and/or monitored can be a protective immune response, a cellular immune response, a humoral immune response, a Th1 immune response, a Th2 immune response, or any combination thereof. Detection and/or monitoring of the immune response may be useful, *e.g.*, in monitoring immune function and/or response to an antigen, *e.g.*, for autoimmune disease, cancer, diabetes or multiple sclerosis, or to test the effectiveness of a vaccine or vaccination.

As noted above, common allergens include, but are not limited to, pollens, animal dander (*e.g.*, cat or dog dander), grasses, molds, dusts, antibiotics, stinging insect venoms, and a variety of environmental (including chemicals and metals), drug and food allergens. Common tree allergens include pollens from cottonwood, popular, ash, birch, maple, oak, elm, hickory, and pecan trees; common plant allergens include those from mugwort, ragweed, English plantain, sorrel-dock and pigweed; plant contact allergens include those from poison oak, poison ivy and nettles; common grass allergens include rye grass, Timothy, Johnson, Bermuda, fescue and bluegrass allergens; common allergens also include those from molds or fungi such as *Alternaria*, *Fusarium*, *Hormodendrum*, *Aspergillus*, *Micropolyspora*, *Mucor*, and thermophilic *Actinomyces*; epidermal allergens can be obtained from house or organic dusts (typically fungal in origin), from arthropods such as house mites (*Dermatophagoides pteronyssinus*), or from animal sources such as feathers, cat and dog dander; common food allergens include milk and cheese (diary), egg, wheat, nut (*e.g.*, peanut), seafood (*e.g.*, shellfish), pea, bean and gluten allergens; common environmental allergens include metals (*e.g.*, nickel and gold), chemicals (*e.g.*, formaldehyde, trinitrophenol and turpentine), latex, rubber, fiber (*e.g.*, cotton or wool), burlap, hair dye, cosmetic, detergent and perfume allergens. Common drug allergens include local anesthetic and salicylate allergens; antibiotic allergens include penicillin, tetracycline and

sulfonamide allergens; and common insect allergens include bee, wasp and ant venom, and cockroach calyx allergens.

Symptoms of an allergy include, but are not limited to, sneezing, runny nose and/or nasal congestion, difficulty breathing, cramps, rash, vomiting, etc. Common symptoms of a food allergy can include wheezing and difficulty breathing, itchy skin rashes, including hives, vomiting, diarrhea, nausea, abdominal pain, and swelling around the mouth and in the throat. Food allergies in children can range from mild to severe, though many will outgrow the allergies by age three. Common food allergies in children under 18 years of age include nut (*e.g.*, peanuts), egg, fin fish, strawberry, wheat, and soy.

In some embodiments, detection of the allergic reaction as taught herein is useful for determining if particular symptoms are due to an allergy. Monitoring the allergy as taught herein is also useful to determine if the allergy is decreasing in severity (*e.g.*, the child is outgrowing the allergy, for example, by measuring and comparing an immune deviation at about 0, 1, 2, 3, 4, 5, 6, 7, or 8 years of age).

"CD4+ T cells," also known as "helper T cells" or "Th," are a type of white blood cell and express the CD4 protein on their surface. Activated CD4+ T cells differentiate into at least the two major subtypes, "Type 1" ("Th1") and "Type 2" ("Th2"), as well as "Type 17" ("Th17") and "T regulatory" ("Treg"). In some embodiments, CRTH2 and/or lack of CD27 expression is used as a Th2 marker. The analysis of markers such as CRTH2 and/or CD27 within allergen-specific CD4+ T cells allows the determination of the allergen-specific T cells subset (Th1 or Th2). This, in turn, may be used to predict the effectiveness of allergen-specific immunotherapy in a patient during desensitization based upon the patient's HLA genotype. For example, after excluding monocytes, macrophages, dendritic cells and B cells (CD14 and CD19 positive cells), CD4+ T lymphocytes may be analyzed.

"Memory CD4+ T cells" as used herein is a subtype of CD4+ T cells that are also CD45RO positive.

"CRTH2+ T cells" as used herein is a subtype of CD4+ T cells that are also CRTH2 positive.

As taught herein, the Th2 response, and a subtype of Th2, in particular, is associated with an allergic reaction. This subtype is referred to herein as "Th2a." CD4+ T cells having a Th2a phenotype have one or more of the following marker profiles: CRTH2+, CD27-, CD161+, CD49d+, CD45RB-. The presence of a significant number of Th2a cells (*i.e.*, frequency) is associated with an allergy or other immune disorder.

In particular embodiments, a frequency of 0, 1, 2, or 5, up to 20, 30, 40, or 49 Th2a cells per 100,000 CD4⁺ T cells indicates the absence of an immune disorder, such as an allergy, whereas a frequency of at least 50, at least 60, at least 70, or at least 80 Th2a cells per 100,000 CD4⁺ T cells indicates an immune disorder, such as an allergy. In some embodiments, a frequency of 50, 60, 70, 80, 90, or 100, up to about 300, 400, 500, 600, 700, 800, 900, or 1,000 Th2a cells per 100,000 CD4⁺ T cells (*e.g.*, memory T cells, CRTH2⁺ T cells) indicate an immune disorder, such as an allergy.

Allergen-specific CD4⁺ Th2 cells ("Th2a") according to some embodiments can be identified as CD4^{pos} CRTH2^{pos} CD27^{neg} CD49d^{pos} CD45RB^{neg} and CD161^{pos} (*i.e.*, having a significant number of CD161^{pos} cells) whereas non-allergen-specific CD4⁺ Th2 cells can be identified as having CD4^{pos} CRTH2^{pos} CD161^{neg} (*i.e.*, having a comparatively lower frequency of CD161^{pos} cells as taught herein) . The overall number and/or percentage of Th2a cells in the Th2 phenotype may in some embodiments correlate with the severity of the allergy or other immune disorder in a subject. A decrease in the number and/or percentage of Th2a cells in the Th2 phenotype may indicate an immune deviation, for example, in response to immunotherapy.

Detection of one or more of these markers may be performed using techniques commonly known in the art. *See, e.g.*, U.S. Patent No. 7,713,688 to Collins and U.S. Patent No. 6,933,148 to Collins et al., which are incorporated by reference herein.

CD4 is a glycoprotein expressed on the surface of T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. T cells expressing CD4 are also known as CD4⁺ T cells. CD4 is a co-receptor that assists the T cell receptor (TCR) to activate its T cell following an interaction with an antigen presenting cell. Using its portion that resides inside the T cell, CD4 amplifies the signal generated by the TCR by recruiting an enzyme, known as the tyrosine kinase lck, which is essential for activating many molecules involved in the signaling cascade of an activated T cell. CD4 also interacts directly with MHC class II molecules on the surface of the antigen presenting cell using its extracellular domain.

CRTH2 (Chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes) is a cognate receptor for prostaglandin (PG) D₂, and is suggested to play a functional role in Th2-dependent allergic inflammation. The high expression levels of CRTH2 in Th2 lymphocytes, basophils and eosinophils imply a major role of CRTH2 in allergic diseases. CRTH2 is a reliable marker for the detection of human Th2.

CD45RO is expressed on CD4⁺ and CD8⁺ T memory cells as well as on CD4⁺ effector T cells. CD45RO is also expressed on monocytes, macrophages, and granulocytes.

CD27 is a member of the TNF-receptor superfamily. This receptor is required for generation and long-term maintenance of T cell immunity. It binds to ligand CD70, and plays a key role in regulating B-cell activation and immunoglobulin synthesis. It is thought to be down-regulated on terminally differentiated effector cells, which could be in that state because of repeated antigenic stimulation.

CD161 is a C-type lectin receptor (NKR-P1A). The function of CD161 is undefined, but it may play a role in T cell migration from circulation into tissue, or may act as a co-stimulatory receptor. CD161 is a marker of human IL-17-producing T cells and is induced by retinoic acid-related orphan receptor C (RORC).

CD49d, or integrin alpha 4 (VLA4), is thought to be involved in lymphocyte migration from circulation into tissue by strengthening lymphocyte adhesion to endothelial cells.

CD45RB, is an isoform of CD45 with exon 5 splicing (encodes B determinant). It is involved in TCR and BCR signaling. As T cells become activated and progress from naïve to memory cells, CD45RB expression is normally downregulated. Additionally, functionally distinct CD4⁺ T cell subsets, which secrete differing cytokine profiles, can be separated by CD45RB intensity.

In some embodiments, markers are detected using a suitable immunological technique, *e.g.*, flow cytometry for membrane-bound markers, immunohistochemistry for intracellular markers, and enzyme-linked immunoassay for markers secreted into the medium. The expression of protein markers can also be detected at the mRNA level by, *e.g.*, reverse transcriptase-PCR using marker-specific primers. *See, e.g.*, U.S. Patent No. 5,843,780, which is incorporated by reference herein.

In some embodiments, the assay makes use of flow cytometry (*e.g.*, FACS) for analyzing the phenotype and/or frequency of CD4⁺ T cells. CD4⁺ T cells in some embodiments are analyzed with a gating tool configured to detect and gate based upon predetermined markers and/or molecule detection.

T cells can be provided in a biological sample from a subject. Suitable samples can include, for example, blood, lymph, lymph nodes, spleen, liver, kidney, pancreas, tonsil, thymus, joints, synovia, and other tissues in which T cells may be found. T cells may be isolated, for example, as peripheral blood mononuclear cells (PBMC). PBMC can be partially purified, for example, by centrifugation (*e.g.*, from a buffy coat), by density gradient centrifugation (*e.g.*, through a Ficoll-Hypaque), by panning, affinity separation, cell sorting (*e.g.*, using antibodies specific for one or more cell surface markers), and other techniques that provide enrichment of

PBMC and/or T cells. *See, e.g.*, U.S. Patent No. 7,713,688 to Collins and U.S. Patent No. 6,933,148 to Collins et al., which are incorporated by reference herein.

In some embodiments, and for ease of use, the biological sample provided for the testing disclosed herein is whole blood without prior purification and/or enrichment of PBMC and/or T cells. In some embodiments, only a small sample is required for testing as described herein, *e.g.*, between 0.1, 0.5, 1 or 2, and 3, 5, 8 or 10 milliliters of a whole blood sample.

Though not required, in some embodiments MHC class II tetramer staining may also be used in order to detect the CD4⁺ T cell phenotype and/or frequency based upon a predetermined MHC class II molecule-antigen complex. Major histocompatibility complex (MHC) class II tetramers allow the direct visualization of antigen specific CD4⁺ T cells by flow cytometry. *See* U.S. Patent No. 7,094,555 to Kwok et al., which is incorporated by reference herein. This method relies on the highly specific interaction between the peptide-loaded MHC class II molecule and its corresponding T-cell receptor. While the affinity of a single MHC/peptide molecule is low, cross-linking MHC/peptide complexes with streptavidin increases the affinity of the interaction, enabling their use as staining reagents.

In some embodiments, detecting the CD4⁺ phenotype may also include detecting cytokine production. Cytokines associated with Th2 include interleukin-4, interleukin-5, interleukin-6, interleukin-10, and interleukin-13. In contrast, cytokines associated with Th1 are associated with a normal, non-allergic response to an antigen. Cytokines associated with Th1 include interferon- γ and tumor necrosis factor- α .

As taught herein, in some embodiments monitoring a Th2 response in a subject, and Th2a, in particular, is useful in immunotherapy. "Specific immunotherapy" or "allergen-specific immunotherapy" is the administration of gradually increasing doses of crude extracts of allergens, making subjects tolerant to them (*see, e.g.*, Francis et al., Peptide-based vaccination: where do we stand? *Curr Opin Allergy and Clin Immunology* 2005, 5:537-543).

Successful allergen-specific immunotherapy is associated with "immune deviation," *i.e.*, the switch from the allergen-specific Th2 response typical for allergic patients (*e.g.*, CD4⁺ CRTH2⁺ CD27⁻) to a more Th1/Treg response characteristic for non-allergic individuals (*e.g.*, CD4⁺ CRTH2⁻ CD27⁺). *See, e.g.*, U.S. Patent Application Publication No. 2009/0214580 to Houghten et al.

As taught herein, allergen-specific CD4⁺ Th2 cells ("Th2a") according to some embodiments can be identified as CD4^{pos} CRTH2^{pos} CD27^{neg} CD49d^{pos} CD45RB^{neg} CD161^{pos} (*i.e.*, having a significant number of CD161^{pos} cells), whereas non-allergen-specific CD4⁺ Th2 cells can be identified as CD4^{pos} CRTH2^{pos} CD161^{neg} (*i.e.*, having a comparatively lower

number of CD161^{pos} cells as taught herein). The overall number and/or percentage of Th2a cells in the Th2 phenotype may in some embodiments correlate with the severity of the allergy in a subject. In some embodiments, a decrease in the number and/or percentage of Th2a cells in the Th2 phenotype may indicate an immune deviation in response to immunotherapy (*e.g.*, CD4^{pos} CRTH2^{pos} CD27^{neg} CD49d^{pos} CD45RB^{neg} CD161^{pos} frequency lower than 30, 40 or 50 per 100,000 of CD4+ cells, and/or lower than 30, 40, or 50 per 100,000 of memory CD4+ T cells, and/or lower than 500, 1,000, or 2,000 per 100,000 of CRTH2+ T cells).

In some embodiments, a significant Th2a cell frequency indicating an immune disorder is greater than about one cell per 500, 1,000, or 5,000 total CD4+ T cells or per total CD45RO+ (memory) T cells. In some embodiments, a significant Th2a cell frequency is greater than about one cell per 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 total CRTH2+ CD4+ T cells.

In some embodiments, immune deviation is measured by an increase in the ratio of Th1/Th2 response, *e.g.*, by 1, 5, or 10, to 20, 15 or 100-fold. In some embodiments, immune deviation is measured by the change in the ratio of Th2a/CD4 T cell response, and/or Th2A/memory CD4+ T cell response, and/or Th2A/CRTH2+ T cell response, and/or Th2a/Th1 response, *e.g.*, by 1, 5, or 10, to 20, 15 or 100-fold.

In some embodiments, immune deviation may also be measured by the change in the ratio of IgG to IgE antibodies specific for the allergen being administered. In some embodiments, immune deviation is measured by an increase in the IgG/IgE ratio, *e.g.*, in which this ratio increases by 1, 5, or 10, to 20, 15 or 100-fold.

Immune deviation may be monitored in a subject undergoing immunotherapy by detecting the phenotype and/or frequency of Th2 and/or Th2a cells in the subject, and then again after one or more administrations of the antigens during the course of treatment. Treatment may be continued, discontinued, or modified according to the indication that one or more treatment options (*e.g.*, different antigens or mixtures thereof, different adjuvants, different modes of administration, etc.) may be more effective than others for the subject based on the detected immune deviation.

Immune deviation monitoring may be performed as desired. In some embodiments, a sample is collected during or prior to the start of immunotherapy, and a subsequent sample or samples are collected about 2 weeks, about 1, 2, 3, 4, 5, or 6 months, about 1 year, etc., afterwards. The frequency of allergy-specific CD4+ T cells is determined and compared with the frequency found in previously-collected samples in order to determine whether or not the subject is showing an immune deviation. In some embodiments, the frequency of a given sample is

measured close to the time of collection; however, in some embodiments, samples may also be frozen for determination at a later time, if desired.

"Subjects" that may be treated and/or monitored by methods of the present invention include both human subjects for medical purposes and animal subjects for veterinary and laboratory purposes. Other suitable animal subjects are, in general, mammalian subjects such as primates, bovines, ovines, caprines, porcines, equines, felines, canines, lagomorphs, rodents (e.g., rats and mice), etc. Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile, adult and geriatric subjects.

"Treating" refers to any type of treatment or prevention that imparts a benefit to a subject afflicted with or at risk of developing an immune-related disease or illness, including, but not limited to, allergies or an allergic reaction to an antigen of interest, including improvement in the condition of the subject (e.g., in one or more symptoms), delay in the onset of symptoms or slowing the progression of symptoms, etc. As used herein, "treatment" is not necessarily meant to imply cure or complete abolition of symptoms, but refers to any type of treatment that imparts a benefit to a patient, for example, a patient afflicted with or at risk for an allergy or allergic reaction.

"Treatment effective amount", "prevention effective amount", "amount effective to treat", "amount effective to prevent" or the like as used herein means an amount of the material or composition sufficient to produce a desirable effect upon a patient afflicted with an immune disorder such as an allergy. This includes improvement in the condition of the patient (e.g., in one or more symptoms), delay in the onset or progression of the disease, etc.

A "control subject" as used herein is a subject that is known to not have an immune disorder (e.g., and allergy). In some embodiments, the Th2a subset frequency of a control subject may be used to compare with a test subject to determine if the test subject has a larger Th2a frequency, which larger frequency may indicate an immune disorder as taught herein.

Some embodiments of present invention are explained in greater detail in the following non-limiting examples.

Example 1: Determination of allergen-specific Th2 cell phenotype. Microarray analysis was used to evaluate the gene expression profiles of allergen-specific CD4+ cells between allergic and non-allergic individuals. It was found that CD161 is over-expressed in allergen-specific CD4+ cells from allergic individuals as compared to non-allergic individuals.

Allergen-specific Th2 cells (Th2a) are a subset of Th2 cells having expression of CD161, CD49d and lacking CD27 and CD45RB expression. As shown in **Figure 1** and **Figure 2**, significant numbers of Th2a cells were exclusively observed in allergic individuals.

5 **Example 2: FACS detection of CD4+ Th2a cell frequency.** The assay is designed to identify and characterize allergen-specific CD4+ T cells by flow cytometry in either a whole blood sample or isolated PBMCs. A schematic of a representative assay is presented in **Figure 4**.

For this purpose, a 4- to 6-color combination may be used. Exemplary color combinations are presented in **Table 1**.

Table 1. Exemplary 4- to 6- color combinations for FACS analysis of allergen-specific CD4+ T cells.

4-colors	5-colors		6-colors	7-colors
anti-CD27 + anti-CD45RB	anti-CD27 + anti-CD45RB	anti-CD27	anti-CD27	anti-CD27
anti-CD161	anti-CD161	anti-CD161	anti-CD161	anti-CD161
anti-CD4	anti-CD4	anti-CD4	anti-CD4	anti-CD4
anti-CRTH2	anti-CRTH2	anti-CRTH2	anti-CRTH2	anti-CRTH2
	anti-CD49d	anti-CD45RB	anti-CD45RB	anti-CD45RB
			anti-CD49d	anti-CD49d
				anti-CD45RO

5 CD4+ T lymphocytes are analyzed using CD4 expression. Allergen-specific CD4+ Th2 (Th2a) cells are identified as CD4^{pos} CRTH2^{pos} CD27^{neg} CD49d^{pos} CD45RB^{neg} CD161^{pos}, whereas non-allergen-specific CD4+ Th2 cells are identified as CD4^{pos} CRTH2^{pos} CD161^{neg}.

For whole blood assay, peripheral blood samples are collected aseptically into a sterile
 20 evacuated blood collection tube with anticoagulant. Specimen should be stored at room temperature until processing (preferably less than 48 hours). The specimen should be homogenized by gentle agitation prior to pipetting.

One ml of whole blood per test is alloquated into 15ml conical tubes and stained with antibodies cocktail for 45 minutes at room temperature, protected from light. Then 100μl of
 25 fixative solution is added into each tube and gently vortexed for approximately 5 seconds.

Ten ml of lysing solution (room temperature) is added to each tube and vortexed vigorously, and then incubated at room temperature for 10 minutes, protected from light. The tubes are then centrifuged for 5 minutes at 200g, and aspirate the supernatant. The lysing steps are then repeated.

5 The cell pellet is resuspended with 400 μ l of PBS, and the cells are transferred into a (12 x 75 mm) FACS tube. Data is then acquired on the flow cytometer. If not analyzed within one hour, processed samples are stored between 2-8°C, protected from light, and analyzed within 6 hours.

0 For PBMC assay, one million freshly isolated or frozen PBMCs are alloquated into a (12 x 75 mm) FACS tube and stained with antibodies cocktail for 45 minutes or 20 mintues at room temperature, protected from light. Cells are washed twice with 1 ml of PBS and the cell pellet is resuspended with 400 μ l or 200 μ l of PBS. Data is then acquired on the flow cytometer. If not analyzed within one hour, processed samples are stored between 2-8 °C, protected from light, and analyzed within 6 hours.

5 The flow cytometer is equipped to detect Forward Scatter, Side Scatter and a minimum of four fluorochromes are used: FITC, PE, PercP and APC.

Create Dot Plots as follows in order to detect allergen-specific CD4+ Th2 (Th2a) cells using a six- or seven-colors flow cytometer:

1. Create Dot Plot 1 as Forward Scatter vs Side Scatter.
- 10 2. Create Dot Plot 2 as CD4 vs Side Scatter or CD45RO.
3. Create Dot Plot 3 as CD4 vs CD45RB.
4. Create Dot Plot 4 as CD49d vs CD27.
5. Create Dot Plot 5 as CRTH2 vs CD161.
6. Optionally, create Dot Plot 6 as CRTH2 vs CD161.

15 Create regions as follows:

1. Dot Plot 1 – Create an amorphous/polygonal Region A on Dot Plot 1 to include all lymphocytes and eliminate red blood cell debris, aggregates, monocyte and granulocytes.
2. Dot Plot 2 – Create a rectilinear Region B on Dot Plot 2 to include all CD4^{pos} and T lymphocytes (*e.g.*, CD45RO^{pos} T lymphocytes) while excluding CD4^{neg} (and optionally CD45RO^{neg}) cells.
- 30 3. Dot Plot 3 – Create a rectilinear Region C on Dot Plot 3 to include all CD4^{pos}CD45RB^{neg} events.
4. Dot Plot 4 – Create a rectilinear Region D on Dot Plot 4 to include all CD49d^{pos}CD27^{neg} events.

5. Dot Plot 5 – Create rectilinear Region E on Dot Plot 5 to include all CD161^{pos}CRTH2^{pos} events.

Create gates as follows:

1. Dot Plot 1 – Ungated to display all events.
2. Dot Plot 2 – Assign "A" to Dot Plot 2 to display all lymphocytes while eliminating red blood cell debris, aggregates, monocyte and granulocytes.
3. Dot Plot 3 – Assign "A" and "B" (AB) to Dot Plot 3 to display all CD4^{pos} (and optionally CD45RO^{pos}) T lymphocytes while excluding CD4^{neg} (and optionally CD45RO^{neg}) cells.
4. Dot Plot 4 – Assign "A", "B" and "C" (ABC) to Dot Plot 4 to display all CD4^{pos} (and optionally CD45RO^{pos}) CD45RB^{neg} lymphocytes.
5. Dot Plot 5 – Assign "A", "B", "C" and "D" (ABCD) to Dot Plot 5 to display all CD4^{pos} (and optionally CD45RO^{pos}) CD45RB^{neg} CD27^{neg} CD49d^{pos} lymphocytes.
6. Dot Plot 6 – Assign "A", "B", "C", "D" and "E" (ABCDE) to Dot Plot 6 to display all CD4^{pos} (and optionally CD45RO^{pos}) CD45RB^{neg} CD27^{neg} CD49d^{pos} CD161^{pos} CRTH2^{pos} lymphocytes.

To calculate Th2a cell frequencies per total CD4+ T cells, determine the total number of CD4^{pos} T cells in Dot Plot 3; then determine the total of Th2a cells in Dot Plot 6 (in gate ABCDE). Th2a cell frequency per total CD4+ T cells = (# of Th2a cells) / (# of CD4+ T cells).

To calculate Th2a cell frequencies per total memory CD4+ T cells, determine the total number of CD4^{pos} CD45RO^{pos} T cells in Dot Plot 3 (Gate AB) ; then determine the total of Th2a cells in Dot Plot 6 (in gate ABCDE). Th2a cell frequency per total memory CD4+ T cells = (# of Th2a cells) / (# of CD4+ CD45RO+ T cells).

To calculate Th2a cell frequencies per total CRTH2+ CD4+ CD45RO+ T cells, determine the total number of CRTH2^{pos} T cells in CD4^{pos} CD45RO^{pos} T cells (Gate AB) ; then determine the total of Th2a cells in Dot Plot 6 (in gate ABCDE). Th2a cell frequency per total CRTH2+ CD4+ T cells = (# of Th2a cells) / (# of CD4+ CD45RO+ CRTH2+ T cells).

Example 3: Th2a cell frequencies are monitored in grass pollen allergic patients receiving allergen-specific immunotherapy. Th2a cell frequencies were monitored in grass pollen allergic patients receiving active or placebo tablets in a double blind placebo-controlled study conducted in an allergen exposure chamber. The Th2a cell frequencies were assessed in coded frozen/thawed PBMCs samples at baseline (V3), and after 2 (V6) and 4 (V7) months of

sublingual immunotherapy (SLIT). Percentages of Th2a cells were normalized to 100 for each patient at baseline.

As shown in **Figure 4**, differences are seen between the patients receiving the active tablets versus the placebo group after 2 and 4 months of treatment, demonstrating immune
5 deviation with treatment in at least some of the patients.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

0

THAT WHICH IS CLAIMED IS:

1. A method of detecting an immune disorder in a subject, comprising:
(a) detecting a frequency of CD4+ T cells in a biological sample of said subject;
(b) detecting a frequency of a Th2a subset of the CD4+ T cells in the biological sample;
and
(c) comparing the frequency of the Th2a subset with the frequency of the CD4+ T cells,
wherein a higher frequency of the Th2a subset as compared to a Th2a frequency in a
control subject indicates said immune disorder.
2. The method of claim 1, wherein the CD4+ T cells are total CD4+ T cells, total
memory CD4+ T cells, or total CRTH2+ CD4+ T cells.
3. The method of claim 1 or claim 2, wherein a frequency of:
at least 50, at least 60, or at least 70 Th2a cells per 100,000 total CD4+ cells;
at least 75, at least 80, or at least 85 Th2a cells per 100,000 total memory CD4+ T cells;
or
at least 3,000, at least 4,000 or at least 5,000 Th2a cells per 100,000 total CRTH2+
CD4+ T cells,
indicates that said subject has an immune disorder.
4. The method of any one of claims 1-3, wherein said immune disorder is an allergic
disorder.
5. The method of any one of claims 1-3, wherein said immune disorder is an allergic
disorder selected from: seasonal rhinoconjunctivitis, animal dander allergy, food allergy, and
venom anaphylaxis.
6. The method of any one of claims 1-5, wherein said Th2a subset of the CD4+ T
cells are CD161 positive, CD49d positive, CD27 negative and CD45RB negative.
7. The method of any one of claims 1-5, wherein said CD4+ Th2a cells are CD161
positive, CRTH2 positive, CD27 negative, CD49d positive and CD45RB negative.

8. The method of any one of claims 1-7, wherein said detecting step is carried out by fluorescent activated cell sorting (FACS).
9. The method of any one of claims 1-8, wherein said biological sample is a whole blood sample or a peripheral blood mononuclear cell (PBMC) sample.
10. The method of claim 9, wherein said blood sample has a volume of from 0.05 to 10 milliliters.
11. The method of claim 9, wherein said blood sample has a volume of from 0.1 to 5 milliliters.
12. The method of any one of claims 1-11, wherein said subject is a human subject.
13. The method of claim 12, wherein said subject is between 0 and 5 years of age, and wherein said immune disorder is a food allergy.
14. A method of monitoring immune deviation in a subject, comprising:
 - (a) detecting a first frequency of CD4+ Th2a cells as compared to a frequency of CD4+ T cells in a first biological sample of said subject; and then
 - (b) detecting a second frequency of CD4+ Th2a cells as compared to a frequency of CD4+ T cells in a second biological sample of said subject,wherein a lower number for the second frequency of said CD4+ Th2a in step (b) as compared to the first frequency in step (a) indicates immune deviation in said subject.
15. The method of claim 14, wherein the CD4+ T cells are total CD4+ T cells, total memory CD4+ T cells, or total CRTH2+ CD4+ T cells.
16. The method of claim 14 or claim 15, wherein the second biological sample is collected from about 2 weeks to about 1, 2, 3, 4 or 5 years after the first biological sample is collected from the subject.
17. The method of any one of claims 14-16, wherein said CD4+ Th2a cells are CD161 positive, CD49d positive, CD27 negative and CD45RB negative.

18. The method of any one of claims 14-16, wherein said CD4+ Th2a cells are CD161 positive, CRTH2 positive, CD27 negative, CD49d positive and CD45RBnegative.

19. The method of any one of claims 14-18, wherein one or both of said detecting steps is carried out by fluorescent activated cell sorting (FACS).

20. The method of any one of claims 14-19, wherein said biological sample is a whole blood sample or a peripheral blood mononuclear cell (PBMC) sample.

21. The method of claim 20, wherein said blood sample has a volume of from 0.05 to 10 milliliters.

22. The method of claim 20, wherein said blood sample has a volume of from 0.1 to 5 milliliters.

23. The method of any one of claims 14-22, wherein said monitoring is carried out during the course of an immunotherapy treatment.

24. The method of claim 23, wherein said immunotherapy treatment is oral immunotherapy treatment (OIT), sublingual immunotherapy (SLIT), or subcutaneous immunotherapy (SCIT) treatment.

25. The method of any one of claims 14-24, wherein said subject is a human subject.

26. The method of claim 25, wherein said subject is between 0 and 5 years of age, and said monitoring is carried out to monitor a food allergy in said subject.

27. The use of a CD4+ Th2a cell frequency for detecting an immune disorder in a subject.

28. The use of claim 27, wherein said immune disorder is an allergy.

29. The use of a CD4+ Th2a cell frequency for monitoring an immune deviation in a subject.

30. The use of a means for detecting CD4+ Th2a cell frequency for carrying out a method of any of claims 1-29.

31. A method of treating an immune disorder in a subject, comprising:
detecting a Th2a cell frequency in said subject; and then, if the Th2a cell frequency is significant,
administering an immunotherapy treatment to said subject.

32. The method of claim 31, wherein a frequency of:
at least one Th2a cell per 3,000 total CD4+ cells;
at least one Th2a cell per 3,000 total memory CD4+ T cells; or
at least one Th2a cell per 50 total CRTH2+ CD4+ T cells,
indicates that the Th2a cell frequency is significant.

33. The method of claim 31, wherein a frequency of:
at least one Th2a cell per 1,000 total CD4+ cells;
at least one Th2a cell per 500 total memory CD4+ T cells; or
at least one Th2a cell per 25 total CRTH2+ CD4+ T cells,
indicates that the Th2a cell frequency is significant.

34. The method of any one of claims 31-33, wherein said immunotherapy treatment is oral immunotherapy treatment (OIT), sublingual immunotherapy (SLIT), or subcutaneous immunotherapy (SCIT) treatment.

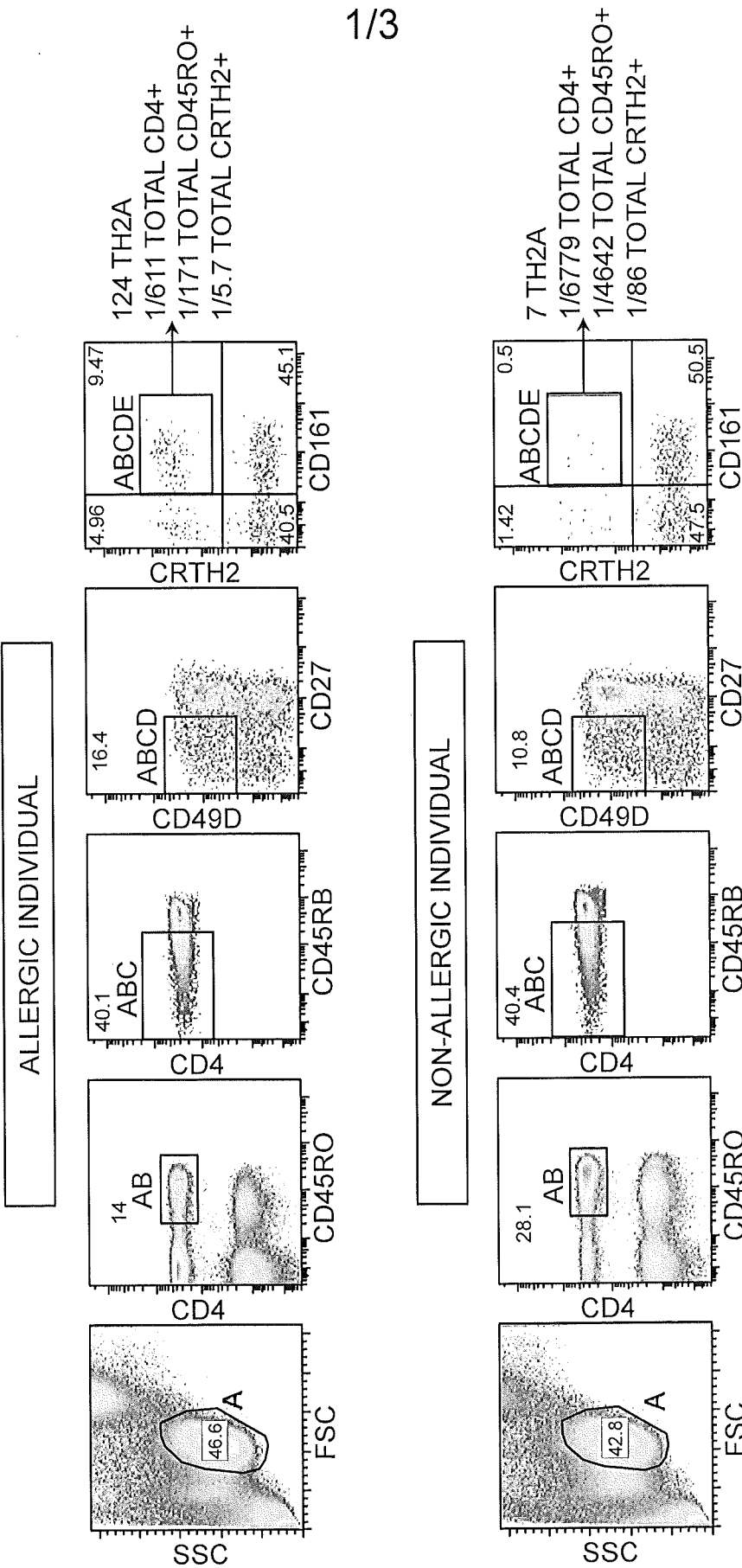


FIG. 1

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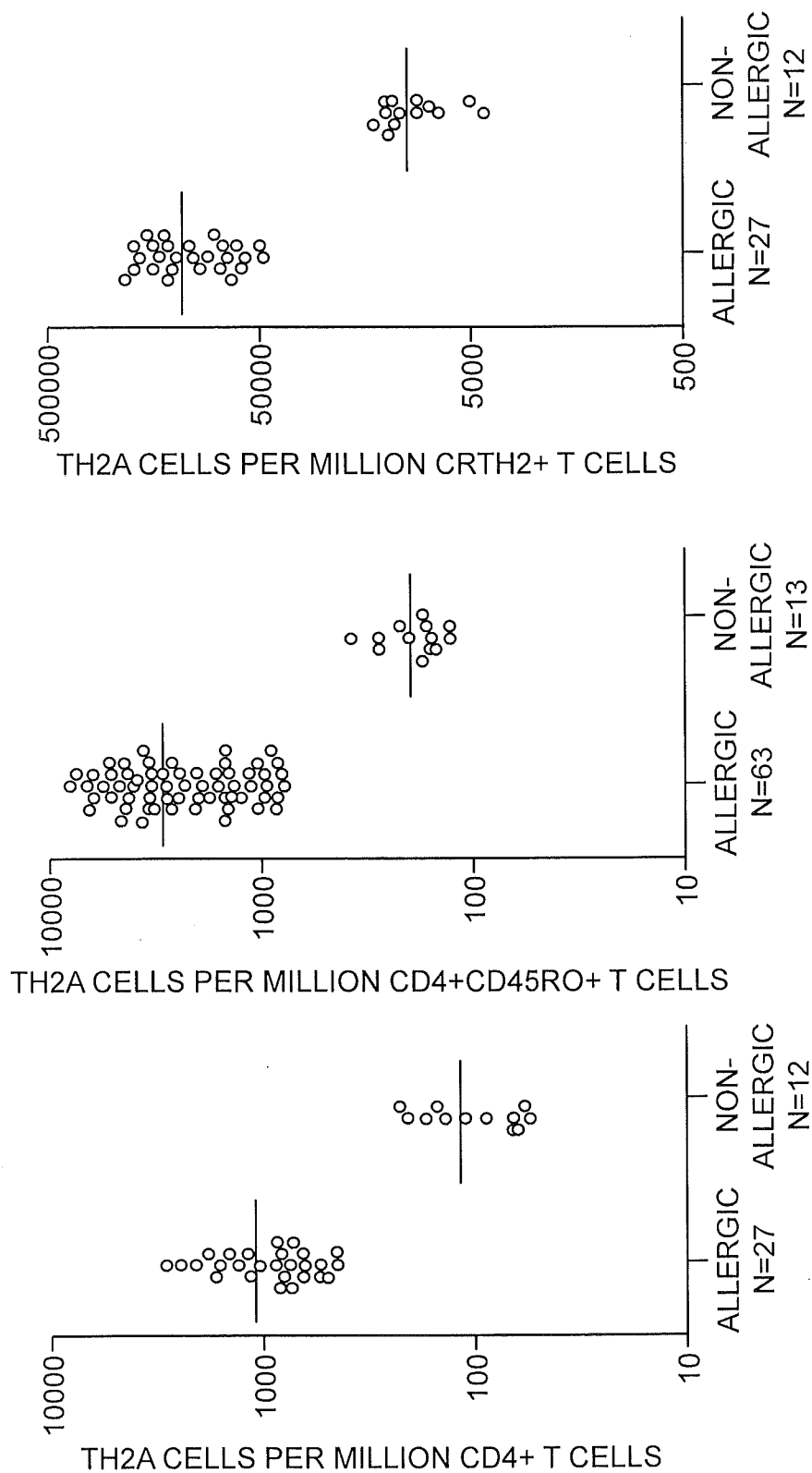


FIG. 2

3/3

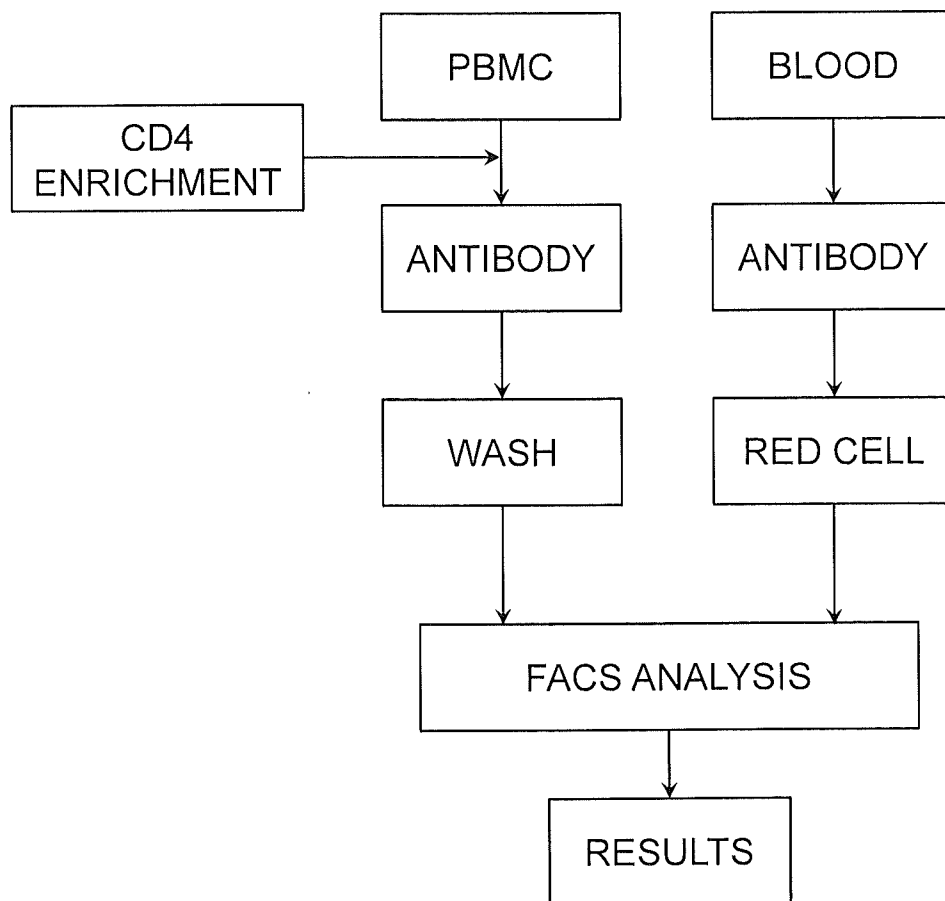


FIG. 3

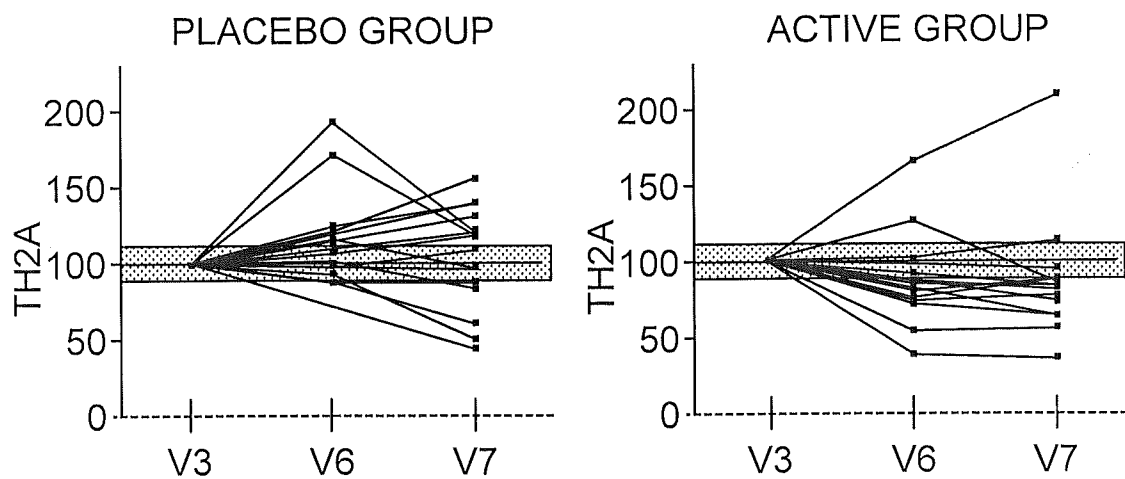


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/026592

A. CLASSIFICATION OF SUBJECT MATTER *G01N 33/50 (2006.01)*
G01N 33/52 (2006.01)

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)

G01N 21/00, 21/64, 33/00, 33/48, 33/53, 35/00, 33/50, 33/52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Esp@cenet, VINITI.RU, EAPO, Enters PubMed, USPTO DB, PatSearch

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG, Yui-Hsi et al. A novel subset of CD4+ T _H 2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. J. Exp. Med., 2010, 207(11), pp.2479-2491 [online] [Retrieved 2012-04-16] Retrieved from the Internet: <URL: http://jem.rupress.org/content/207/11/2479.full.pdf+html >, especially, pp. 2479, 2480, p. 2482, col. 2, – p. 2483, p. 2484, col. 2, p. 2485, fig. 5, p. 2487, col. 2, p. 2488	1-4, 6-9, 12, 14-20, 23-25, 27-31
Y		5, 10-11, 13, 21-22, 26
Y	RU 2302635 C1 (GOSUDARSTVENNOE UCHREZHDIENIE NAUCHNY TSENTR ZDOROVIA DETEY RAMN) 10.07.2007, abstract, p. 6, example 2	5, 10-11, 21-22
Y	NOVIKOV P.D. et al. The principles of evaluation of immune status and diagnostic of immunodeficiency diseases. Immunopathology, allergology, infectology, 2005, №2, pp. 8-22 [online] [Retrieved 2012-04-17] Retrieved from the Internet: <URL: http://www.immunopathology.com/ru/article.php?article=166 >, especially, pp. 10-13, 16-17, col. 1, p. 20, col. 2 – p. 21, col. 1	13, 26

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier document but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

25 April 2012 (25.04.2012)

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/026592

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CONTI F. et al. Treatment of allergic manifestations with transfer factor (TF). Physiologic regulating medicine, 2006, 1, pp. 31-34 [online] [Retrieved 2012-04-16] Retrieved from the Internet: <URL: http://gunacanada.com/wp-content/uploads/2011/03/2006-5Conti.pdf >	1-34
A	AKDIS M. et al. Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance between Allergen-specific T Regulatory 1 and T Helper 2 Cells. J. Exp. Med., 2004, 199(11), pp.1567-1575[online] [Retrieved 2012-04-16] Retrived from the Internet: <URL: http://jem.rupress.org/content/199/11/1567.full.pdf+html >	1-34
A	DONG, Chen et al. T cells: the usual subsets. Nature Reviews Immunology, 2010, Poster, [online] [Retrived 2012-04-16] Retrived from the Internet: <URL: http://docs.abcam.com/pdf/immunology/t_cells_the_usual_subsets.pdf >	1-34
A	COSMI Lorenzo et al. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. Eur. J. Immunol., 2000, 30(10), pp. 2972-2979	1-34