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(54) Title: B-ARRESTIN-2 MODULATION IN THE TREATMENT OF ALLERGIC ASTHMA

(57) Abstract: The present invention relates to methods of preventing or treating a condition involving T cell migration. These conditions include allergic asthma. The methods include administering a composition that modulates desensitization to a patient. The composition has the ability to modulate the chemotaxis of T cells. The invention also relates to methods of screening compositions for the ability to modulate desensitization, and chemotaxis of T cells.

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β -Arrestin-2 modulation in the treatment of Allergic Asthma

5 BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods and compositions for treating allergic asthma regulated by T helper cells that produce type 2 cytokines (Th2 cells). In particular, the invention relates to methods and compositions for treating Th2 mediated allergic asthma by administering a composition that reduces Th2 cell chemotaxis or trafficking to the lung by modulation of the translocation of β -arrestin.

15 History of Related Art

Asthma is a complex inflammatory disease that afflicts nearly 15 million Americans and costs over 5 billion dollars a year to treat. Despite research advances, the worldwide prevalence, morbidity and mortality of asthma has increased over the last two decades. In humans, the hallmark feature of allergic asthma is the abnormal expansion in the lung of Th2 cells that produce cytokines. This pathological event leads to the classic symptoms of asthma including airway inflammation, airway hyperresponsiveness, reversible airflow obstruction and airway remodeling. Allergic asthma has an external trigger and is caused by an IgE mediated response to an inhaled allergen, resulting in airway mast cell degranulation, mediator release, and the beginning of the inflammatory cascade. This type of asthma typically appears before the fourth decade of life, and is the most common type of childhood asthma.

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Asthma in general is most often diagnosed from episodes of wheezing, coughing, chest tightness, and shortness of breath. Diagnostic

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pulmonary function tests include peak expiratory flow rate (PEFR) using a peak flow meter, or spirometry, which indicates forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1). Allergic asthma can be diagnosed from the presence of a temporal relationship between exposure to a putative aeroallergen and the development of airway symptoms (either early phase, late phase, or both), and from demonstrable skin test reactivity to the inhaled allergen. In many cases the symptoms of allergic asthma will be accompanied by symptoms of allergic rhinitis (rhinorrhea, sneezing, ocular burning). Allergic asthma can also be diagnosed from the presence of increased IgE levels (e.g., IgE >300 ng/ml in serum), or atopy.

Like other immune cells, T cells in general are functionally dependent on their ability to migrate, localize within tissues and interact with other immune cells. Th2 cells specifically migrate to the lung tissue where they are responsible for the allergic asthma response.

Chemokine receptors are part of the enormous family of heptahelical cell surface receptors known as G protein –coupled receptors (GPCR's). These receptors transduce extracellular signals into intracellular events by activating heterotrimeric G proteins. The dissociation of these G protein subunits activates cell signaling systems such as adenylate cyclases, phospholipases or ion channels, which ultimately results in a physiological response. In the case of chemokine receptors, at least one of these physiological responses is cell migration.

Like other GPCR's, chemokine receptor function is regulated by β -arrestin proteins. β -arrestins, members of the arrestin family of proteins, are designated β -arrestin-1 or β -arrestin-2, are ubiquitously expressed, and regulate the GPCR function through multiple mechanisms. As the name suggest β -arrestin proteins were originally discovered to "arrest" G protein-mediated cell signaling events. Since that time, our understanding of the mechanisms by which β -arrestin modulates GPCR function has expanded

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considerably. In addition to their classical role, β -arrestin proteins also act as adapters that couple GPCR's to a clathrin-coated pit endocytic mechanism, and as scaffolds that link GPCR's to a second wave of cell signaling via mitogen-activated protein kinase (MAPK), and other signaling pathways as well.

In vitro studies have shown that chemotaxis of lymphocytes devoid of β -arrestin-2 or human embryonic kidney (HEK) 293 cells having suppression of β -arrestin-2, demonstrate impaired migration toward the chemotactic factor stromal cell-derived factor 1 α (SDF-1 α), also known as CXCL12.

Although β -arrestin-2 is essential to the normal migration of immune cells in vitro, the ability of β -arrestin-2 to mediate immune cell chemotaxis in vivo and thus its utility as a treatment mechanism, has not been demonstrated.

Currently, persons suffering from allergic asthma are treated by a limited variety of treatments. One is by immunization against the asthma initiating antigen with an antigen-based composition. Antigen immunization limits the antigen-stimulated events of the early phase of allergic asthma at the risk of inducing IgE mediated anaphylaxis. Such immunization does not target the cytokine-mediated events of late phase immune response in allergic asthma. In US patent No. 6,426,336 a method for treating both phases of allergic asthma is disclosed by introducing naked polynucleotides that operatively encode for the asthma-initiating antigen into a host. This method does not prevent the migration of Th2 cells to the lung after exposure to an antigen and relies on knowing what the asthma-initiating antigen is.

SUMMARY OF THE INVENTION

The present invention is based on the inventor's discovery that β -arrestin-2 activity is necessary for chemotaxis of Th2 cells to the lung. The

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present invention also relates to the discovery that blocking of β -arrestin-2 in vivo reduces or eliminates the symptoms of allergic asthma in a mammal.

Accordingly in one aspect, the invention relates to a method for treating Th2 related allergic asthma comprising administering to a mammal
5 in need of therapy for allergic asthma a composition which modulates the expression or activity of β -arrestin-2 on the chemotaxis of Th2 cells to the lung of said human.

Another aspect of the invention relates to a method of screening a compound for activity in a mammal against allergic asthma comprising
10 providing a cell comprising a chemokine receptor and a detectable labeled β -arrestin-2, exposing the cell to the compound, determining the distribution of the arrestin, providing a chemokine that binds to the receptor and redetermining the distribution of the arrestin, the result is then compared to the redistribution of arrestin under the same conditions but without pre-
15 treatment by the compound.

In yet another aspect of the invention, it relates to a method of treating allergic asthma in a mammal preferably a human by modulating desensitization of a cytokine receptor in a selected host cell particularly a TH2 cell, comprising (a) providing a compound that is capable of modulating
20 the activity of β -arrestin-2 binding to the receptor and (b) administering said compound to the mammal such that the compound modulates the activity of the arrestin in the host cell. In a further embodiment of this invention the chemokine receptor is a CCR4 receptor, e.g. a human CCR4 receptor.

A further aspect of the invention comprises administering to a
25 mammal, a compound that modulates the activity of the MAPK cascade in the mammal comprising administering a compound which modulates the activity of β -arrestin-2 such that the MAPK cascade is modulated. In a further embodiment the MAPK is p38 MPAK.

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A further aspect of the invention comprises administering to a mammal, a compound that modulates the activity of the ERK cascade in the mammal comprising administering a compound which modulates the activity of β -arrestin-2 such that the ERK cascade is modulated.

5 A further aspect of the invention comprises administering to a mammal, a compound that modulates the activity of the JNK-3 cascade in the mammal comprising administering a compound which modulates the activity of β -arrestin-2 such that the JNK-3 cascade is modulated.

10 Another aspect of the invention relates to treating allergic asthma in a mammal by administering to the mammal a compound that modulates phosphoralization of a chemokine receptor by a GRK such that binding by β -arrestin-2 to the chemokine receptor is modulated.

BRIEF DESCRIPTION OF THE DRAWINGS

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Fig. 1 *Effect of OVA treatment on airway responsiveness.* Airway responsiveness to methacholine, defined by the time-integrated change in peak airway pressure (APTI) was measured for β -arrestin-2^{-/-} (circles) and wild-type (squares) mice treated with either alum (filled symbol) or OVA (open symbols). Data are means $\pm$ S.E.M.; $n = 9-12$ mice per group. *, effect of OVA treatment was significantly different between wild-type and β -arrestin-2^{-/-} mice, $P < 0.05$.

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Fig. 2 *Effect of OVA treatment on airway inflammation.* a, Effect of genotype and OVA treatment on lung inflammation evaluated by histological analysis of lung sections. Lung sections from wild-type-alum (lower left) and β -arrestin-2^{-/-}-alum mice (lower right) appeared normal with no inflammatory cell infiltration. Lung sections from wild-type-OVA mice (upper left) showed severe cellular infiltration in the interstitium. Lung sections from β -arrestin-2^{-/-}

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β -OVA mice (upper right) showed mild extravasation of inflammatory cells in the interstitium. Representative histological sections shown for $n = 8-11$ mice per group. *b*, Images of cross-sectioned airways together with peribroncho-vascular connective tissue are shown. CD3⁺ T cells were counted in the subtended area, surrounding each bronchiole and in the extravascular connective tissue space. OVA-treated wild-type mice showed an increased number of CD3⁺ T cells in the peribroncho-vascular zone relative to alum treated-mice. No such infiltration of CD3⁺ T cells was observed in OVA-treated β -arrestin-2^{-/-} mice.

c, Effect of genotype and OVA treatment on lung inflammation assessed by identification of cells harvested from whole lung lavage. Filled bars represent wild-type-OVA mice; open bars represent β -arrestin-2^{-/-}-OVA mice; right-rising bars represent wild-type-alum mice; left-rising bars represent β -arrestin-2^{-/-}-alum mice. Data are means $\pm$ s.e.m.; $n = 9-12$ mice per group. **, $P < 0.05$ versus all other groups

Fig. 3 Effect of genotype and OVA treatment on lung cytokine release in whole lung lavage fluid. *a*, Cytokines associated with a T_H2-type response were significantly elevated in wild-type-OVA mice relative to β -arrestin-2^{-/-}-OVA mice and alum-treated mice of either genotype. Filled bars represent wild-type-OVA mice; open bars represent β -arrestin-2^{-/-}-OVA mice. Cytokine levels in alum-treated wild-type and alum-treated β -arrestin-2^{-/-} mice were not different and therefore, combined as shown by cross-hatched bars. Data are means $\pm$ s.e.m.; $n = 11-19$ mice per group and are representative of 3 independent experiments. **, $P < 0.05$ versus all other groups. *b*, Cytokines associated with a T_H1-type response were not significantly elevated by OVA treatment and were similar for wild-type and β -arrestin-2^{-/-} mice. Filled bars represent wild-type-OVA mice; open bars represent β -arrestin-2^{-/-}-OVA mice. Rising-right bars represent alum-treated wild-type mice; left-rising bars

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represent β -arrestin-2^{-/-} mice. Data are means $\pm$ S.E.M.; $n = 7$ -13 mice per group and are representative of 2-3 independent experiments.

Fig. 4 *Effect of OVA treatment on serum immunoglobulin production.*

5 Although OVA-specific-IgE (a) and -IgG₁ (b) levels changed significantly over time, serum immunoglobulin levels in wild-type-OVA mice (filled bars) were not significantly different from those in β -arrestin-2^{-/-}-OVA mice (open bars) when compared at each time point (day 10, 17, 24). These OVA-specific immunoglobulins were not detected in alum-treated mice of either genotype.
10 Measurements were made using the endpoint titer method. Data are means $\pm$ S.D.; $n = 3$ -8 mice per group and are representative of 2 independent experiments. One-way ANOVA and Tukey HSD post-hoc test were used.

Fig. 5 *IgG_{2a} production.* a, Total serum IgG_{2a} levels were not different
15 between wild-type-OVA mice (filled bars) and β -arrestin-2^{-/-}-OVA mice (open bars) at day 10, 17 or 24. Similarly, there was no difference in IgG_{2a} levels in serum from alum-treated wild-type (right-rising bars) and alum-treated β -arrestin-2^{-/-} mice (left-rising bars). Data are means $\pm$ S.E.M.; $n = 4$ -14 mice per group and are representative of 3 independent experiments. b, OVA-
20 specific-IgG_{2a} measurements were not different between wild-type-OVA mice (filled bars) and β -arrestin-2^{-/-}-OVA mice (open bars) at day 24. Measurements were made using the endpoint titer method. Data are means $\pm$ S.D.; $n = 3$ mice per group and are representative of 2 experiments.

25 **Fig. 6** : *Chemotactic responses to macrophage-derived chemokine (MDC).* a, Lung CD4⁺ T cells from β -arrestin-2^{-/-} mice exhibit decreased migration towards MDC. CD4⁺ T cells were isolated on day 24 of the OVA treatment protocol and tested for their ability to chemotax towards 100nM MDC. Shown is the mean chemotactic index and standard error from three

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independent experiments. **6b** shows the MDC level in lavage fluid, and illustrates the significant difference between WT-OVA and combined alum.

Fig. 7 *Effect of LPS treatment on airway responsiveness and inflammation.* **a**, Effect of genotype and LPS treatment on lung inflammation assessed by identification of cells harvested from whole lung lavage. Filled bars represent wild-type-LPS mice; open bars represent β -arrestin-2^{-/-}-LPS mice. Data are means $\pm$ S.E.M.; $n = 13-15$ mice per group. **b**, Airway responsiveness to methacholine, defined by the time-integrated change in peak airway pressure (APTI) was measured for β -arrestin-2^{-/-} (circles) and wild-type (squares) mice treated with LPS (open symbols) or untreated (filled symbols). Data are means $\pm$ S.E.M.; $n = 13-15$ mice per group. *, LPS treatment caused a similar significant increase in APTI in wild-type and β -arrestin-2^{-/-} mice. $P < 0.05$.

Fig. 8. *Quantitation of T lymphocytes in the lung.* Using calibrated digital images, the perimeter of the bronchiolar basal lamina of each histological profile (represented in Figure 2b) was traced and the length was measured. The results were expressed as the number of CD3⁺ T cells per millimeter of airway basal lamina for each profile. To quantitate the number of CD4⁺ T cells, lung from OVA-treated mice were processed and cells were identified using GK1.5-PE (anti-CD4) (BD Pharmingen) and FACS analysis. The mean ($\pm$ S.E.M.) for each treatment/genotype group is shown; $n = 7-10$ mice per group. *, $P < 0.05$ versus all other groups

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have determined the role of β -arrestin-2 in the mediation of allergic asthma and the physiologic effects it has on the lungs especially the development of allergic asthma symptoms. The present

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inventors have determined that β -arrestin-2 is a target for modulating desensitization of receptors especially the chemokine receptors by administering compounds which have such a modulating effect on the activity in vivo of β -arrestin-2 especially in relationship to the chemokine receptors and especially those in TH2 cells.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, immunology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook *et al*, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (2000)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984); *Using Antibodies: A Laboratory Manual: Portable Protocol No. 1*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1998); *Using Antibodies: A Laboratory Manual*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1999).

Unless otherwise stated, the following terms used in the specification and claims have the meanings given below:

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

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A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in the initiation of DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence that is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3'

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direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels
5 detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters
10 contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational
15 control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that
20 communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

25 The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

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The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension
5 product, which is complementary to a nucleic acid strand, is induced, *i.e.*, in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing
10 agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

15 The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary
20 nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and
25 thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

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A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian
5 cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the
10 eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

15 Two DNA sequences are "substantially homologous" when at least about 65% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in
20 sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al.*, supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

25 "Arrestin" means all types of naturally occurring and engineered variants of arrestin, including, but not limited to, visual arrestin (sometimes referred to as Arrestin 1), cone arrestin (sometimes referred to as arrestin-4), β -arrestin 1 (sometimes referred to as Arrestin 2), and β -arrestin-2 (sometimes referred to as Arrestin 3).

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"Internalization" of a GPCR is the translocation of a GPCR from the cell surface membrane to an intracellular vesicular membrane, where it may be inaccessible to substances remaining outside the cell.

"Carboxyl-terminal tail" means the carboxyl-terminal tail of a GPCR following membrane span 7. The carboxyl-terminal tail of many GPCRs begins shortly after the conserved NPXXY motif that marks the end of the seventh transmembrane domain (*i.e.* what follows the NPXXY motif is the carboxyl-terminal tail of the GPCR). The carboxyl-terminal tail may be relatively long (approximately tens to hundreds of amino acids), relatively short (approximately tens of amino acids), or virtually non-existent (less than approximately ten amino acids). As used herein, "carboxyl-terminal tail" shall mean all three variants (whether relatively long, relatively short, or virtually non-existent), and may or may not contain palmitoylated cysteine residue(s).

"Class A receptor" preferably do not translocate together with arrestin proteins to endocytic vesicles or endosomes in association with arrestin-GFP in HEK-293 cells.

"Class B receptors" preferably do translocate together with arrestin proteins to endocytic vesicles or endosomes associated with arrestin-GFP in HEK-293 cells.

"DACs" mean any desensitization active compounds. Desensitization active compounds are any compounds that influence or modulate the GPCR desensitization mechanism by either stimulating or inhibiting the process. DACs may influence the GPCR desensitization pathway by acting on any cellular component of the process, as well as any cellular structure implicated in the process, including but not limited to: arrestins, GRKs, GPCRs, phosphoinositide 3-kinase, AP-2 protein, clathrin, protein phosphatases, and the like. DACs may include, but are not limited to, compounds that inhibit arrestin translocating to a GPCR, compounds that inhibit arrestin binding to a GPCR, compounds that stimulate arrestin

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translocating to a GPCR, compounds that stimulate arrestin binding to a GPCR, compounds that inhibit GRK phosphorylation of a GPCR, compounds that stimulate GRK phosphorylation of a GPCR, compounds that stimulate or inhibit GRK binding to a GPCR, compounds that inhibit protein phosphatase dephosphorylation of a GPCR, compounds that stimulate protein phosphatase dephosphorylation of a GPCR, compounds that prevent GPCR internalization or recycling to the cell surface, compounds that regulate the release of arrestin from a GPCR, antagonists of a GPCR, inverse agonists and the like. DACs may inhibit or stimulate the GPCR desensitization process and may not bind to the same ligand binding site of the GPCR as traditional agonists and antagonists of the GPCR. DACs may act independently of the GPCR, *i.e.*, they do not have high specificity for one particular GPCR or one particular type of GPCRs. DACs may bind the same site(s) as agonist or antagonist but do not desensitize the receptor (perhaps by not altering the receptor to be properly phosphorylated or bind to arrestin or any other protein). DACs may bind to allosteric sites on the receptor and inhibit or enhance desensitization.

"Label" or "labeled" means any molecule capable of detection by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to, fluorescence, phosphorescence, and bioluminescence and radioactive decay. Labels include, but are not limited to, GFP, luciferase, β -galactosidase, rhodamine-conjugated antibody, and the like. It also includes radioisotopes, epitope tags, affinity labels, enzymes, fluorescent groups, chemiluminescent groups, and the like. Labels include molecules which are directly or indirectly detected as a function of their interaction with other molecule(s).

"GFP" means Green Fluorescent Protein which refers to various naturally occurring forms of GFP which may be isolated from natural sources or genetically engineered, as well as artificially modified GFPs. GFPs are

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well known in the art. See, for example, U.S. Patent Nos. 5,625,048; 5,777,079; and 6,066,476. It is well understood in the art that GFP is readily interchangeable with other fluorescent proteins, isolated from natural sources or genetically engineered, including but not limited to, yellow
5 fluorescent proteins (YFP), red fluorescent proteins (RFP), cyan fluorescent proteins (CFP), blue fluorescent proteins, luciferin, UV excitable fluorescent proteins, or any wave-length in between. As used herein, "GFP" shall mean all fluorescent proteins known in the art.

The phrase "pharmaceutically acceptable" refers to molecular entities
10 and compositions that are physiologically tolerable and do not typically produce a significant allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human such that it could not pass FDA or other regulatory body approval.

The phrase "therapeutically effective amount" is used herein to mean
15 an amount sufficient to prevent, and preferably reduce some feature of pathology such as for example, reduced allergic response, allergic rhinitis, etc.

By "mammal" is meant livestock animals (e.g., ungulates, such as cattle, buffalo, horses, sheep, pigs and goats), as well as rodents (e.g., mice,
20 hamsters, rats and guinea pigs), canines, felines, primates including humans, lupine, camelid, cervidae, rodent, avian and ichthyies.

"GPCR" means G protein-coupled receptor and includes GPCRs naturally occurring in nature, as well as GPCRs which have been modified. Such modified GPCRs are described in U.S.S.N. 09/993,844 and U.S.S.N.
25 10/054,616.

"Abnormal GPCR desensitization" and "abnormal desensitization" mean that the GPCR desensitization pathway is disrupted such that the balance between active receptor and desensitized receptor is altered with respect to wild-type conditions. Either there is more active receptor than

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normal or there is more desensitized receptor than wild-type conditions. Abnormal GPCR desensitization may be the result of a GPCR that is constitutively active or constitutively desensitized, leading to an increase above normal in the signaling of that receptor or a decrease below normal in the signaling of that receptor.

5 "Biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject; wherein said sample can be blood, serum, a urine sample, a fecal sample, a tumor sample, a cellular wash, an oral sample, sputum, biological fluid, a tissue extract, freshly harvested cells, or cells which have
10 been incubated in tissue culture.

"Concurrent administration", "administration in combination", "simultaneous administration", or "administered simultaneously" mean that the compounds are administered at the same point in time or sufficiently
15 close in time that the results observed are essentially the same as if the two or more compounds were administered at the same point in time.

"Desensitized GPCR" means a GPCR that presently does not have ability to respond to agonist and activate conventional G protein signaling.

"Desensitization pathway" means any cellular component of the desensitization process, as well as any cellular structure implicated in the desensitization process and subsequent processes, including but not limited to, arrestins, GRKs, GPCRs, AP-2 protein, clathrin, protein phosphatases, and the like. In the methods of assaying of the present invention, the polypeptides may be detected, for example, in the cytoplasm, at a cell
20 membrane, in clathrin-coated pits, in endocytic vesicles, endosomes, any stages in between, and the like.

"GPCR signaling" means GPCR induced activation of G proteins. This may result in, for example, cAMP production.

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"G protein-coupled receptor kinase" (GRK) includes any kinase that has the ability to phosphorylate a GPCR.

"*Homo sapiens* GPCR" means a naturally occurring GPCR in a *Homo sapiens*.

5 The term "pharmaceutically acceptable carrier," as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent.

10 "Modulation" includes at least an up-regulation or down-regulation of the expression, or an increase or decrease in activity of a protein. Modulation of a protein includes the up-regulation, down-regulation, increase or decrease in activity of a protein or compound that regulates a protein. Modulation also includes the regulation of the gene, the mRNA, or any other step in the synthesis of the protein of interest.

15 An "overexpressed" protein refers to a protein that is expressed at levels greater than wild-type expression levels.

A "chemokine receptor" is a series of receptors all of which have in common the binding of chemokine as a natural agonist. An example of such receptor is the CCR4 receptor.

20

GPCR's, desensitization in relationship to allergic asthma

The exposure of a GPCR such as the chemokine receptor to agonist such as a chemokine produces rapid attenuation of its signaling ability that involves uncoupling of the receptor from its cognate heterotrimeric G-protein.

25 The cellular mechanism mediating agonist-specific or homologous desensitization is a two-step process in which agonist-occupied receptors are phosphorylated by a G protein-coupled receptor kinases (GRKs) and then bind a β -arrestin-2 protein or other appropriate arrestin.

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It is known that after agonists bind GPCRs, G-protein coupled receptor kinases (GRKs) phosphorylate intracellular domains of GPCRs. After phosphorylation, an arrestin protein associates with the GRK-phosphorylated receptor and uncouples the receptor from its cognate G protein. The interaction of the arrestin with the phosphorylated GPCR terminates GPCR signaling and produces a non-signaling, desensitized receptor.

The arrestin bound to the desensitized GPCR targets the GPCR to clathrin-coated pits or other cellular machinery for endocytosis (*i.e.*, internalization) by functioning as an adaptor protein, which links the GPCR to components of the endocytic machinery, such as adaptor protein-2 (AP-2) and clathrin. The internalized GPCRs are dephosphorylated and are recycled back to the cell surface desensitized, or are retained within the cell and degraded. The stability of the interaction of arrestin with the GPCR is one factor that dictates the rate of GPCR dephosphorylation, recycling, and resensitization. The involvement of GPCR phosphorylation and dephosphorylation in the desensitization process has been exemplified in U.S.S.N. 09/933,844, filed November 5, 2001, the disclosure of which is hereby incorporated by reference in its entirety.

Seven distinct GRK genes are known, named GRK1 through GRK7, that were classified into three distinct groups. GRK6 is a member of the GRK4 subfamily of GRKs, which also contains GRK4 and GRK5. Multiple GRK enzymes are found in regions around the body including in Th2 cells.

Chemokine receptors, like other members of the G protein-coupled receptor (GPCR) family, are regulated via activation-dependent phosphorylation by a family of G protein-coupled receptor kinases (GRKs). While several *in vitro* studies were focused on the role of GRKs in Chemokine receptor regulation, no data on physiological significance of this

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regulation and *in vivo* specificity of chemokine receptor/GRK interaction are currently available especially in relation to allergic asthma.

The decrease in GRK levels or activity could enhance the behavioral effects of chemokine and their receptors related to allergic asthma.

5 Therefore, the data herein demonstrates that modulating the amount or activity of GRK by either pharmacological or genetic approaches would be useful in allergic asthma disease, by blocking or modulating the effects of β -arrestin-2 especially in Th2 cells.

10 **Methods of testing a compound for ability to modulate β -arrestin-2**

The present invention is also related to methods of testing a compound for the ability to modulate β -arrestin-2. For example, the test compound may be administered to a wild-type non-human animal; and the wild-type non-human animal exposed to the compound will be compared to
15 the locomotor response of the non-human transgenic animal that has a disrupted gene. Tests involving a labeled β -arrestin-2 and monitoring the translocation of the arrestin after administration of a test compound can also give indication when a compound modulates the activity of β -arrestin-2 especially relating to chemokine receptors and in Th2 cells. Such testing is
20 well known in the art. Methods of testing for DAC's can be found for example in PCT application US03/20838 disclosing methods for a DAC which modulates GRK6 (and thus the related arrestin and can be adapted for use in this invention by one skilled in the art. Also methods for testing DAC's active against multiple receptors is disclosed in us Serial
25 No.10/633,438 incorporated herein by reference which also discloses methods which can be adapted to test for activity against a single receptor.

Method of identifying compounds

The present invention relates to methods of identifying compounds that modulate β -arrestin-2 associated activity especially in TH2 cells in vivo. A cell is provided which includes a GPCR (such as a chemokine receptor) that binds β -arrestin-2, and a β -arrestin-2, wherein one of the molecules is detectably labeled and the GPCR is overexpressed. The cell is contacted with a candidate modulator. The cellular distribution of the, GPCR or arrestin in the presence of the compound is compared to the cellular distribution in the absence of the compound. The difference between the cellular distribution of the, GPCR or arrestin in the presence or absence of the compound(s) can also be correlated to modulation of GRK activity or other compounds in the arrestin cycle and cascade. Such methods are described herein, and in U.S.S.N. 09/993,844 filed on November 5, 2001, U.S.S.N. 10/054,616 filed on January 22, 2002, and U.S.S.N. 10/101,235 filed on March 19, 2002, which are hereby incorporated by reference in their entirety.

In an embodiment, a GRK or a β -arrestin-2 is overexpressed. The molecule is labeled and may be localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes. The detectable molecule may be a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group. The molecule may be detectably labeled due to its interaction with another molecule, which may be detectably labeled.

The present invention further relates to methods of inhibiting desensitization of the chemokine receptor in a cell especially the TH2 cell in vivo. These methods may include contacting the cell with a compound. The compound may be an antisense oligonucleotide, or another compound as described herein. The antisense oligonucleotide may inhibit expression of a nucleic acid encoding GRK, or another gene that affects β -arrestin-2 activity.

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Methods of detection

Methods of detecting the intracellular location of the detectably labeled arrestin, the intracellular location of a detectably labeled GPCR, the intracellular location of a detectably labeled GRK, or interaction of the detectably labeled molecule with a GPCR or any other cell structure, including for example, the concentration of arrestin, GRK, or GPCR at a cell membrane, colocalization of arrestin with GPCR in endosomes, and concentration of arrestin or GPCR in clathrin-coated pits, and the like, will vary dependent upon the detectable molecule(s) used.

One skilled in the art readily will be able to devise detection methods suitable for the detectable molecule(s) used. For optically detectable molecules, any optical method may be used where a change in the fluorescence, bioluminescence, or phosphorescence may be measured due to a redistribution or reorientation of emitted light. Such methods include, for example, polarization microscopy, Transfluor, BRET, BRET-2, FRET, evanescent wave excitation microscopy, and standard or confocal microscopy.

In an embodiment arrestin may be conjugated to GFP and the arrestin-GFP conjugate may be detected by confocal microscopy. In another preferred embodiment, arrestin may be conjugated to a GFP and the GPCR or GRK may be conjugated to an immunofluorescent molecule, and the conjugates may be detected by confocal microscopy. In an additional preferred embodiment, arrestin may be conjugated to a GFP and the carboxy-terminus of the GPCR may be conjugated to a luciferase and the conjugates may be detected by bioluminescence resonance emission technology. In a further preferred embodiment arrestin may be conjugated to a luciferase and GPCR may be conjugated to a GFP, and the conjugates may be detected by bioluminescence resonance emission technology.

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In an embodiment, the localization pattern of the detectable molecule is determined. In a further preferred embodiment, alterations of the localization pattern of the detectable molecule may be determined. The localization pattern may indicate cellular localization of the detectable molecule. Certain methods of detection are described in U.S.S.N. 10/095,620, filed March 12, 2002, which claims priority to U.S. Provisional Patent Application No: 60/275,339, filed March 13, 2001, the contents of which are incorporated by reference in their entirety.

Molecules may also be detected by their interaction with another detectably labeled molecule, such as an antibody.

Conjugates

The cells used in the methods of assaying of the present invention may comprise a conjugate of a GRK protein or a β -arrestin-2 protein and a detectable molecule, and the like. The detectable molecule allows detection of molecules interacting with the detectable molecule, as well as the molecule itself.

All forms of GRKs, and β -arrestin-2's naturally occurring and engineered variants, may be used in the present invention. GRKs may interact to a detectable level with all forms of GPCRs. Arrestins may interact with all levels of desensitization and MAPK cascades.

Detectable molecules i.e. labels, that may be used include, but are not limited to, molecules that are detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to bioluminescence, phosphorescence, and fluorescence. These detectable molecules should be a biologically compatible molecule and should not compromise the biological function of the molecule and must not compromise the ability of the detectable molecule to be detected. Preferred detectable molecules are

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optically detectable molecules, including optically detectable proteins, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. More preferred detectable molecules are inherently fluorescent molecules, such as fluorescent proteins, including, for example, Green Fluorescent Protein (GFP). The detectable molecule may be conjugated to the GRK protein by methods as described in Barak *et al.* (U.S. Patent Nos. 5,891,646 and 6,110,693). The detectable molecule may be conjugated at the front-end, at the back-end, or in the middle.

The GPCRs may also be conjugated with a detectable molecule. Preferably, the carboxyl-terminus of the GPCR is conjugated with a detectable molecule. If the GPCR is conjugated with a detectable molecule, proximity of the GPCR with the GRK may be readily detected. In addition, if the GPCR is conjugated with a detectable molecule, compartmentalization of the GPCR with the GRK may be readily confirmed. The detectable molecule used to conjugate with the GPCRs may include those as described above, including, for example, optically detectable molecules, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. Preferred optically detectable molecules may be detected by immunofluorescence, luminescence, fluorescence, and phosphorescence.

For example, the GPCRs may be antibody labeled with an antibody conjugated to an immunofluorescence molecule or the GPCRs may be conjugated with a luminescent donor. In particular, the GPCRs may be conjugated with, for example, luciferase, for example, Renilla luciferase, or a rhodamine-conjugated antibody, for example, rhodamine-conjugated anti-HA mouse monoclonal antibody. Preferably, the carboxyl-terminal tail of the GPCR may be conjugated with a luminescent donor, for example, luciferase. The GPCR, preferably the carboxyl-terminal tail, also may be conjugated

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with GFP as described in L. S. Barak *et al.* Internal Trafficking and Surface Mobility of a Functionally Intact β 2-Adrenergic Receptor-Green Fluorescent Protein Conjugate, *Mol. Pharm.* (1997) 51, 177 - 184.

5 **Cell types and substrates**

 The cells of the present invention may express at least one GRK, β -arrestin-2, and GPCR, wherein at least one of the molecules is detectably labeled. Cells useful in the present invention include eukaryotic and prokaryotic cells, including, but not limited to, bacterial cells, yeast cells,
10 fungal cells, insect cells, nematode cells, plant cells, and animal cells. Suitable animal cells include, but are not limited to, HEK cells, HeLa cells, COS cells, and various primary mammalian cells. An animal model expressing a conjugate of a GRK6 and a detectable molecule throughout its tissues or within a particular organ or tissue type, may also be used in the
15 present invention.

 A substrate may have deposited thereon a plurality of cells of the present invention. The substrate may be any suitable biologically substrate, including but not limited to, glass, plastic, ceramic, semiconductor, silica, fiber optic, diamond, biocompatible monomer, or biocompatible polymer
20 materials.

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Expression of proteins

Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control
5 sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading
10 frame upstream of the DNA sequence.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include
15 derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col EI, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, *e.g.*, the numerous derivatives of phage λ , *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful
20 in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Any of a wide variety of expression control sequences -- sequences
25 that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the lac system, the trp system, the TAC system, the TRC system, the LTR system,

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the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast α -mating factors, and other sequences known to control the
5 expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*,
10 *Bacillus*, *Streptomyces*, fungi such as yeasts, plant cells, nematode cells, and animal cells, such as HEK-293, CHO, RI.I, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

15 It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without
20 undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic
25 markers, will also be considered.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential

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secondary structures. Suitable unicellular hosts will be selected by consideration of, *e.g.*, their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

It is further intended that modified GRK and β -arrestin-2 analogs may be prepared from nucleotide sequences of the protein complex/subunit derived within the scope of the present invention. Analogs, such as fragments, may be produced, for example, by pepsin digestion. Other analogs, such as muteins, can be produced by standard site-directed mutagenesis of coding sequences. Analogs exhibiting " β -arrestin-2 activity" or " β -arrestin-2 antagonist activity" such as small molecules, whether functioning as promoters or inhibitors, may be identified by known *in vivo* and/or *in vitro* assays.

As mentioned above, a DNA sequence encoding a modified β -arrestin-2 can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the β -arrestin-2 amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, *e.g.*, Edge, *Nature*, 292:756 (1981); Nambair *et al.*, *Science*, 223:1299 (1984); Jay *et al.*, *J. Biol. Chem.*, 259:6311 (1984). Synthetic DNA sequences allow convenient construction of genes which will express β -

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arrestin-2 analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native or modified β -arrestin-2 genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis.

5 A general method for site-specific incorporation of unnatural amino acids into proteins is described in Christopher J. Noren, Spencer J. Anthony-Cahill, Michael C. Griffith, Peter G. Schultz, Science, 244:182-188 (April 1989). This method may be used to create analogs with unnatural amino acids.

10

Method of evaluating treatments of β -arrestin-2 -associated disease

Provided in the present invention are methods of evaluating treatments of β -arrestin-2 associated disease. The compound that modulates β -arrestin-2 will be administered to a wild-type non-human animal. The responses of this animal will be compared to the responses of the animal that has not been treated with the modulating compound. Means of examining the responses are described in the examples.

15

Method of treating or diagnosing a disease

20 The present invention is related to methods of treating or diagnosing a allergic asthma. The disease treatment may involve administering a compound that modulates β -arrestin-2. The compound may directly or indirectly modulate β -arrestin-2. the modulation is such that such modulation modulates the chemotaxis of Th2 cells to the lung when the lung is challenged with antigen. The compound may be an antisense molecule or an immunoglobulin. The methods of disease diagnosis relate to the detection of the β -arrestin-2 protein, nucleic acid, or activity in a sample. Such methods include detection using immunoglobulins, nucleic acids, and antisense molecules.

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The methods of disease treatment of the present invention include the concurrent administration of the compound that modulates β -arrestin-2 with an additional compound. The additional compound may directly or indirectly affect asthma allergic response pathway. Such compounds include anti-
5 allergens or antigen-encoding polynucleotides or the like. The compound that modulates β -arrestin-2 may increase the effectiveness of the additional compound. The concurrent administration of the compound that modulates β -arrestin-2 may decrease the amount of the additional compound required by the patient.

10

Disease treatment

The present invention relates to methods of treating a human or non-human subject suffering from a β -arrestin-2 -related disease, such as allergic asthma. Such treatment can be performed either by administering to
15 a subject in need of such treatment, an amount of the compound identified by the present method sufficient to treat the disease, or at least to lessen the symptoms thereof.

Diagnostic and Therapeutic Treatments

20

The possibilities of both diagnostic and therapeutic treatments that are raised by the existence of β -arrestin-2's effect on the Th2 cell chemotaxis to the lung derive from the fact that these factors appear to participate in direct and causal protein-protein interaction between a ligand thereto, and those factors that thereafter initiate an intracellular signal. As
25 discussed earlier and elaborated further on herein, the present invention contemplates pharmaceutical intervention in the cascade of reactions in which the β -arrestin-2 or GPCRs especially Chemokine receptors and the MAPK cascade are implicated, to modulate the activity of TH2 cell chemotaxis associated with allergic asthma.

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Thus, in instances where it is desired to reduce or inhibit the activity of β -arrestin-2 resulting from a particular stimulus or factor, an appropriate inhibitor of the GRK could be introduced to block the phosphorylation of the GPCR by the GRK. Correspondingly, instances in which insufficient
5 activation of a G protein or second messenger is taking place could be remedied by introduction of additional quantities of the GRK or its chemical or pharmaceutical cognates, analogs, fragments and the like. Instances in which excess activation of a G protein or second messenger is taking place could be remedied by introduction of decreased quantities of the GRK or its
10 chemical or pharmaceutical cognates, analogs, fragments and the like.

The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in a mixture, a pharmaceutically acceptable excipient (carrier) and a compound that modulates a β -arrestin-2,
15 as described herein as an active ingredient. In an embodiment, the composition comprises a drug capable of modulating the phosphorylation of the Chemokine GPCR by a GRK.

Pharmaceutical compositions

20 The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments or small molecules as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection
25 can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the

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composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents that enhance the effectiveness of the active ingredient.

5 A β -arrestin-2 modulating compound obtained by the methods disclosed herein can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such
10 organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

15 The therapeutic compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material
20 calculated to produce the desired therapeutic effect in association with the required diluent (*i.e.*, carrier, or vehicle).

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compositions lies preferably within a range of circulating concentrations
25 that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any composition used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a

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circulating plasma concentration range that includes the IC₅₀ (*i.e.*, the concentration of the test composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in
5 plasma may be measured, for example, by high performance liquid chromatography.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the
10 subject's immune system to utilize the active ingredient, and degree of modulation of β -arrestin-2 activity desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.001 to 30, preferably about 0.01 to about 25, and
15 more preferably about 0.1 to 20 milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other
20 administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to,
25 the severity of the disease or condition, disorder, or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the composition(s) can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is

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5 treated with the composition in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of the composition used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

10 The therapeutic compositions may further include an effective amount of the β -arrestin-2 modulating compound and one or more other active ingredients.

15 The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

20 Pharmaceutically acceptable base addition salts for use with modulatory compounds are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, *e.g.*, Berge *et al.*, "Pharmaceutical Salts," J. Pharma. Sci., 1977, 66: 1-19). The base addition salts of acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the

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conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are known in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids (e.g., hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid); with organic carboxylic, sulfonic, sulfo- or phospho-acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

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Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations.

5 Carbonates or hydrogen carbonates are also possible.

The antisense compounds and other modulatory compounds described herein can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound or other modulatory compound to a suitable pharmaceutically acceptable diluent or carrier. Use
10 of the compounds and methods of the invention may also be useful prophylactically.

In certain embodiments, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of
15 limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. It could also be inhaled as a spray or
20 nebulizer of a solid where determined to be useful.

For topical application, the compositions may be combined with a carrier so that an effective dosage is delivered, based on the desired activity.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams,
25 gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional

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means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica);
5 disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable
10 vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or
15 fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer, salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give
20 controlled release of the active composition.

The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may
25 take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

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For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray, presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, 5 dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the composition and a suitable powder base such as lactose or 10 starch.

The compositions may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compositions may be formulated with suitable 15 polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing 20 the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. 25 These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according

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to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into
5 association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

In one embodiment of the present invention, the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions,
10 creams, jellies and liposomes. While basically similar in nature, these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

15 The compositions of the present invention may be prepared and formulated as emulsions. See, e.g., Idson, in *Pharmaceutical Dosage Forms* v. 1, p. 199 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York); Rosoff, in *Pharmaceutical Dosage Forms*, v. 1, p. 245; Block in *Pharmaceutical Dosage Forms*, v. 2, p. 335; Higuchi *et al.*, in
20 *Remington's Pharmaceutical Sciences* 301 (Mack Publishing Co., Easton, Pa., 1985). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as
25 minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and

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the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple
5 emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets
10 constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form
15 through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four
20 categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms* v. 1, p. 199 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York).

Synthetic surfactants, also known as surface active agents, have
25 found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, v. 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, v. 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has

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been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations.

Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in
5 Pharmaceutical Dosage Forms).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin
10 and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers, especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, non-swelling clays (e.g., bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium
15 aluminum silicate), pigments and nonpolar solids (e.g., carbon or glyceryl tristearate).

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants,
20 hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, v.1 p.385 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York)).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers, such as polysaccharides (e.g., acacia, agar, alginic
25 acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (e.g., carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (e.g., carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the

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dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers (*e.g.*, tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene) or reducing agents (*e.g.*, ascorbic acid and sodium metabisulfite), and antioxidant synergists (*e.g.*, citric acid, tartaric acid, and lecithin).

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, v. 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, v. 1, p. 245 (Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York); Idson, in *Pharmaceutical Dosage Forms*). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, v. 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing

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an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in *Controlled Release of Drugs: Polymers and Aggregate Systems*, 185-215 (Rosoff, M., Ed., 1989, VCH Publishers, New York). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, 271 (Mack Publishing Co., Easton, Pa., 1985).

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprates (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with co-surfactants. The co-surfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules.

Microemulsions may, however, be prepared without the use of co-surfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to,

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water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono-,
5 di-, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based
10 microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et al.*, Pharm. Res., 1994, 11:1385-90; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13: 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug
15 absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, 1994; Ho *et al.*, J. Pharm. Sci., 1996, 85: 138-143). Often microemulsions may form spontaneously when their components are
20 brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion
25 compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids and other active agents from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids and other active

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agents within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories—surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Crit. Rev. Therap. Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, are useful because of their specificity and the duration of action. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*. Selection of the appropriate liposome depending on the agent to be encapsulated would be evident given what is known in the art.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to

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use a liposome that is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include: (a) liposomes obtained from natural phospholipids are biocompatible and biodegradable; (b) liposomes
5 can incorporate a wide range of water and lipid soluble drugs; (c) liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

10 Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are
15 emptied into the cell where the active agent may act.

Another embodiment also contemplates the use of liposomes for topical administration. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased
20 accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin. Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have
25 been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes that interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an

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endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, Biochem. Biophys. Res. Comm., 1987, 147:980-985).

5 Liposomes that are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the
10 exogenous gene was detected in the target cells (Zhou *et al.*, J. Controlled Release, 1992, 19: 269-74).

Another contemplated liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl
15 phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example,
20 soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

"Sterically stabilized" liposomes, which refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such
25 specialized lipids are also contemplated. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside GM1, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to

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be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, FEBS Lett., 1987, 223: 42; Wu *et al.*, Can. Res., 1993, 53: 3765).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. See, *e.g.*, Sunamoto *et al.* (Bull. Chem. Soc. Jpn., 1980, 53: 2778) described liposomes comprising a nonionic detergent, 2C12 15G, that contains a PEG moiety. Illum *et al.* (FEBS Lett., 1984, 167: 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (FEBS Lett., 1990, 268: 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029: 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by, *e.g.*, Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both

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to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*). Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further
5 derivatized with functional moieties on their surfaces.

Methods of encapsulating compounds in liposomes is also known in the art. See, WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and
10 asserts that the contents of such liposomes may include an antisense RNA.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the
15 hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, p.285 (Marcel Dekker, Inc., New York, N.Y., 1988, p. 285)).

If the surfactant molecule is not ionized, it is classified as a nonionic
20 surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters,
25 sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

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If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, 285 (Marcel Dekker, Inc., New York, N.Y., 1988).

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of the desired compound to the skin of animals including humans. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

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Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Another embodiment of the invention contemplates pharmaceutical compositions comprising surfactants. Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, Crit. Rev. Therap. Drug Carrier Systems, 1991, 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40: 252).

Another embodiment contemplates the use of various fatty acids and their derivatives to act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprinate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C1-10 alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, and the like) (Lee *et al.*, 1991; Muranishi, Crit. Rev. Therap. Drug Carrier Systems, 1990, 7: 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44: 651-4).

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The compositions comprising the active agents of the invention may further comprise bile salts. The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, N.Y., 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, 1991; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, 1990; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263: 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79: 579-83).

The invention further contemplates compositions comprising chelating agents. Chelating agents can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers for use when the active agent is an antisense agent, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a

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divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618: 315-39). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee *et al.*, 1991; Muranishi, 1990; Buur *et al.*, J. Control Rel., 1990, 14: 43-51).

The invention also contemplates pharmaceutical compositions comprising active agents and non-chelating non-surfactants. Non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants, but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, 1990). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, 1991); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39: 621-6).

The pharmaceutical compositions disclosed herein may also comprise excipients. In contrast to carrier compounds, these excipients include a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more active agents to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with an active agent and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen

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phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with compounds of the invention can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of contemplated active agents may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with contemplated active agents can be used.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions

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of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

EXAMPLES AND EXPERIMENTAL

Methods

Because chemotaxis is crucial to the process of inflammation, we theorized that mice lacking β -arrestin-2 might be protected from developing allergic-asthmatic inflammation. To model allergic asthma in mice we employed a standard method consisting of sensitization and challenge to ovalbumin (OVA). This mouse model of allergic asthma mimics several features of human asthma.

Animals.

Male and female β -arrestin-2 deficient (β -arrestin-2^{-/-}) and littermate wild-type mice (back-crossed for six generations onto the C57BL/6 background) were used for all experiments at 8 to 12 weeks of age. All experiments were conducted in accordance with the NIH guidelines for the care and use of animals and with approval from the Duke University Animal Care and Use Committee.

Immunization and airway challenge.

Mice were immunized intraperitoneally (i.p.) on days 0, 7 and 14 with 10 μ g Grade V OVA (A5503, Sigma-Aldrich, St. Louis, MO) adsorbed to 200 μ g of alum adjuvant (Pierce, Rockford, IL) diluted in saline. Sham-immunized

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animals received 200 µg of saline-diluted alum i.p. Secondary challenge consisted of a 60 min exposure to 1% (w/v) OVA in saline on days 21, 22 and 23. All mice were exposed to aerosol in a 60 L Hinners-style exposure chamber connected to the outlet of a 6-jet atomizer that delivered an aerosol of particles with a mean diameter of 0.3 µm (TSI Instruments, St. Paul, MN). The term "OVA treatment" is used hereafter to refer to mice treated according to the OVA sensitization and OVA aerosol challenge protocol described above.

Endotoxin exposures.

Lipopolysaccharide (LPS) for aerosolization was purchased as lyophilized purified *Escherichia coli* 0111:B4 (L2630, Sigma, St. Louis, MO). LPS was solubilized in sterile saline to a concentration of 5 mg/ml, stored at -20°C, and diluted further in saline to the appropriate concentration on the day of the experiment. LPS was aerosolized by a 6-jet atomizer (TSI Instruments, St. Paul, MN), which generated particles with a mean diameter of 0.3 µm, and directed into a 60 L Hinners-style exposure chamber for 2 ½ h. At regular intervals, LPS concentrations were determined by sampling the aerosol through a side port on the chamber. Endotoxin concentrations were assayed with the chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD) as previously described. The average endotoxin concentration used was $5.53 \pm 0.5 \mu\text{g}/\text{m}^3$.

Airway Responsiveness.

The day after the final aerosol challenge airway responsiveness to methacholine was measured as previously described. In brief, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) diluted 50% with saline and surgically prepared with a tracheal cannula and a jugular vein catheter. Mice were paralyzed with doxacurium chloride (0.25 mg/kg) and ventilated with 100% oxygen at a constant volume of 8-10 ml/kg and a frequency of 125 breaths/min. These ventilator settings

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resulted in an average resting peak airway pressure of 7.8 ± 0.2 cmH₂O and were previously shown to provide normal arterial blood gases. Measurement of airway pressure was made at a side port of the tracheal cannula connected to a Validyne differential pressure transducer. The time-integrated
5 change in peak airway pressure (airway pressure time index (APTI)) was calculated for a 30 sec period beginning immediately after methacholine injection.

Whole Lung Lavage.

After *in vivo* measurements, mice were sacrificed by sodium
10 pentobarbital overdose. Lungs were lavaged as previously described. Differential cell counts were performed on cytopsin preparations (Cytospin 2, Shandon, Pittsburgh, PA) stained with Hema3 Protocol stain (Biochemical Sciences Inc., N.J) and 200 cells were classified using standard morphologic criteria. Lavage fluid cytokine levels were determined in unconcentrated
15 lavage fluid using commercially available cytokine ELISA kits (Quantikine, R&D Systems, Minneapolis, MN). The detection limits were as follows: IL-4 (2 pg/ml), IL-5 (7 pg/ml), IL-13 (1.5 pg/ml), IL-12 (<4 pg/ml) and IFN- γ (<2 pg/ml).

Serum immunoglobulin titers.

20 At three time points in the sensitization protocol (day 10, day 17 and after *in vivo* measurements on day 24), blood was harvested from the inferior vena cava of β -arrestin-2^{-/-} and wild-type mice. Serum was separated by centrifugation and stored at -20°C until analyzed. OVA-specific serum IgE, IgG₁, IgG_{2a} levels were determined using ELISA methodology and geometric
25 mean titer analysis. Briefly, 96-well plates (NUNC MaxiSorp, Nalge Nunc International, Denmark) were coated with 100 μ l/well of 100 μ g/ml ovalbumin (A5503, Sigma-Aldrich, St. Louis, MO) in 0.1M carbonate coating buffer [pH 9.6] and incubated overnight at 4°C. The wells were washed with 0.5% Tween20/PBS and blocked with 1% BSA/PBS which also served as the

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assay diluent. After 1.5 hours, wells were washed 3 times and samples (serially diluted 1:2 12 times beginning with 1:64) were added across the plate and incubated for 1.5 hours at 37°C. After washing, the primary antibody (sheep anti-mouse IgE (Calbiochem) was added and incubated a further 1.5 hrs, washed and incubated a second time with the detection antibody (peroxidase conjugated rabbit anti-sheep IgG (Calbiochem). For detection of OVA-IgG₁ the primary antibody was biotin-conjugated anti-mouse IgG₁ with avidin-horseradish peroxidase at 2.5 µg/ml (A3151, Sigma-Aldrich). For detection of OVA-IgG_{2a} the primary antibody was horseradish-peroxidase-conjugated anti-mouse IgG_{2a} (Pharmingen). After a further wash, all plates were developed with tetramethylbenzidine substrate solution, stopped with 2N H₂SO₄ and read at 450 nm. Naïve serum from non-sensitized C57BL/6J mice was assayed on each plate to calculate a standard curve and titers were calculated at 2-fold over the asymptote of the naïve curve.

Isolation of lymphocytes. All lymphocyte isolation was carried out in polypropylene tubes. Spleens were isolated from OVA-treated mice and disrupted in a dounce homogenizer in 1 ml RPMI 1640 medium (Gibco Gaithersburg Md). Following centrifugation at 370 x g for 10 minutes, red blood cells (RBCs) were lysed in Lysis buffer (0.14M NH₄Cl, 0.017M Tris pH 7.4) and the cells pelleted. The cells were washed in RPMI, filtered through a 70 micron strainer (BD Falcon), and counted.

Lungs were isolated and disrupted in HBSS containing 0.5U/ml heparin through a 200 gauge stainless steel mesh. Lymphocytes from either OVA-treated wild-type or OVA-treated *β-arrestin-2*^{-/-} mice were pooled. The cells were centrifuged at 370 x g for 10 minutes at 4°C and resuspended in 15 ml of a 33% Percoll solution (Sigma, St. Louis, Mo) diluted in HBSS containing 100U/ml heparin. The cells were centrifuged at 500 x g for 15 minutes at room temperature. The pelleted cells were resuspended in lysis

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buffer repeatedly until no RBC remained. The cells were then washed in HBSS and resuspended in RPMI containing 10% FBS to be counted.

Chemotaxis Assays. 1×10^6 splenocytes or 1×10^5 lung lymphocytes were placed in 100 μ l RPMI containing 10% FBS in the upper chamber of a transwell (6.5mm diameter, 5 micron pore size (Costar). The lower chamber of the transwell contained 100nM macrophage-derived chemokine (MDC) (R & D, Minneapolis, Mn) in 600 μ l RPMI 10% FBS. After incubation at 37°C for 90 minutes, the migrated cells from the lower chamber were collected. The cells were stained with the following antibodies: 17A2-FITC (anti-CD3) (BD Pharmingen), GK1.5-PE (anti-CD4) (BD Pharmingen), and anti-CD45R-TC (Caltag) and analyzed using a Coulter Epics XL flow cytometer. The chemotactic index was calculated as the number of cells migrating towards MDC/number of cells migrating to medium alone. All assays were performed in duplicate. *β -arrestin-2^{-/-}* mice and their littermate-matched controls were tested simultaneously.

Histopathology.

Immediately after collection of lavage fluid, ice-cold 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in 1 $\times$ PBS (pH 7.4) was instilled through the tracheal cannula at a constant pressure of 25 cm H₂O to inflation-fix the lung. Specimens were immersed in 4% paraformaldehyde at 4°C overnight, and dehydrated in a graded series of ethanol solutions. Tissue was embedded in paraffin. Sections were cut at 5 μ m thickness and mounted onto positively charged slides (Super Frost Plus, Fisher Scientific). For determination of inflammatory cells hematoxylin- and eosin-stained slides were semi-quantitatively scored in a blinded fashion. For determination of CD3⁺ T cells, tissue sections underwent high temperature antigen-unmasking procedure as follows. After dewaxing and rehydration, the sections were immersed in 1.6 l of 1mM EDTA, pH 8.0, in a pressure cooker that was then closed and slowly, over a period of 3 to 4 minutes,

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brought to boiling temperature. After a total time of 5 minutes, the pressure **cooker** was cooled under running tap water and opened, and the slides were transferred to PBS (pH 7.5) at room temperature for 5 minutes. Endogenous peroxidase was destroyed with 0.3% H₂O₂ for 30 minutes at room temperature, followed by a rinse in blocking normal rabbit serum. The primary antibody used was a rat monoclonal anti-CD3 antibody from Novocastra Laboratories Ltd. (Newcastle, UK). Slides were incubated with either active antibody (1:250) or with normal rat IgG as a negative control. The secondary antibody was biotin-conjugated rabbit anti-rat IgG (Vectors Laboratories Inc., Burlingame, CA), addition of which was followed by treatment with standard avidin-biotin horseradish peroxidase. Peroxidase activity was detected with 3,3'-diaminobenzidine (DAB) (Vectors Laboratories Inc.) and hydrogen peroxide, after which a hematoxylin counterstain was performed.

Statistical Analysis.

Multivariate analysis of variance was used to determine differences in airway responsiveness. A Student's t-test was used to determine differences when only two experimental groups existed. Otherwise, a one-way analysis of variance, in combination with a Tukey honestly significant difference (HSD) post-hoc test, was used to determine differences among groups. For all tests, significance was indicated when $P < 0.05$.

Results:

To examine the role of β -arrestin-2 in the pathogenesis of asthma, we used an OVA model of allergic asthma and an endotoxin model of non-allergic asthma. We measured physiologic and biologic variables including airway responsiveness, airway inflammatory cell infiltration, T cell

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chemotaxis, cytokine levels in whole lung lavage fluid and serum immunoglobulin levels.

Airway responsiveness. OVA treatment resulted in a dramatic increase in airway responsiveness in wild-type mice (Fig. 1) as measured by airway pressure time index (APTI). In marked contrast, β -arrestin-2^{-/-} mice
5 treated with OVA showed no such increase in airway responsiveness compared to either wild-type-alum or β -arrestin-2^{-/-}-alum (control) mice.

Lung inflammation. Histological analysis showed that only mild extravasation of inflammatory cells, including CD3⁺ T lymphocytes, occurred
10 in the airways of β -arrestin-2^{-/-} mice treated with OVA (β -arrestin-2^{-/-}-OVA), whereas severe cellular infiltration occurred in the interstitium of wild-type-OVA mice (Fig. 2a, b). Quantitation of CD3⁺ cells per millimeter of basement membrane or CD4⁺ cells harvested from lung show that β -arrestin-2^{-/-} mice experience significantly reduced T lymphocyte infiltration into the lung (Fig.
15 8). Differential cell counts from lavage fluid supported the histological data revealing that lymphocyte and eosinophil infiltration into the lungs of β -arrestin-2^{-/-}-OVA mice was markedly reduced compared to wild-type-OVA mice and not different from control mice of either genotype (alum treated)(Fig. 2c). The lack of T lymphocytes in the lung of β -arrestin-2^{-/-}-
20 OVA mice suggests that the migration of T cells to the lung is impaired in the absence of β -arrestin-2.

Cytokine levels. Activated T cells migrate to the lungs and release inflammatory cytokines to orchestrate the allergic inflammatory response. Consistent with the demonstrated lack of lymphocytes in the airways of β -
25 arrestin-2^{-/-}-OVA mice was their diminished level of T_H2 cytokines. Analysis of lavage fluid from wild-type-OVA mice showed a significant increase in the levels of IL-4, IL-5 and IL-13, relative to the respective cytokine level in lavage fluid from β -arrestin-2^{-/-}-OVA mice and alum controls (Fig. 3a). To determine if β -arrestin-2^{-/-} mice have a T_H1-skewed response, which might

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thereby inhibit the development of T_H2 lymphocytes, we measured T_H1-type cytokines in lung lavage fluid (18). Levels of IL-12 and IFN- γ were not different between wild-type and β -arrestin-2^{-/-} mice, and the level of these cytokines was unaffected by OVA-treatment (Fig. 3b).

5 *Immunoglobulin production.* The absence of the classic features of a T_H2-mediated asthmatic response in OVA-treated β -arrestin-2^{-/-} mice indicates that β -arrestin-2 exerts its regulatory effect early in the progression of allergic asthma. β -arrestin-2 may regulate the handling of aeroallergen, a series of events that occurs prior to, and is necessary for, T cell stimulation and allergen-specific immunoglobulin production. However, the serum levels
10 of OVA-specific IgE and OVA-specific IgG₁ were significantly elevated in β -arrestin-2^{-/-}-OVA mice and the magnitude of this antigen-specific antibody production was not significantly different from the response observed in wild-type-OVA mice (Fig. 4a,b). Thus, β -arrestin-2^{-/-} mice are competent in their
15 ability to present antigen, to generate antigen-specific T cell responses and to undergo immunoglobulin isotype switching. Measurement of IgG_{2a}, a serum immunoglobulin typical of a T_H1-type response was not different between wild-type and β -arrestin-2^{-/-} mice (Fig. 5 a,b). These data show that the lack of a T_H2-type response in β -arrestin-2^{-/-} mice is not due to
20 enhanced induction of the T_H1 arm of the T helper cell pathway for development.

CD4⁺ T cell chemotaxis. MDC is a potent T_H2 cell chemoattractant produced by activated lung macrophages in allergic asthma. To investigate whether CD4⁺ T cell migration to the lung is impaired in β -arrestin-2^{-/-} mice,
25 we tested the ability of splenocytes, thoracic lymph node cells and lung lymphocytes from OVA-treated wild-type and β -arrestin-2^{-/-} mice to respond to MDC. Only lung lymphocytes, and not spleen or lymph node T cells (data not shown) migrated toward MDC. While CD4⁺ T cells harvested from lung of OVA-treated wild-type mice migrated well towards MDC, CD4⁺ T cells

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lacking β -arrestin-2 were significantly impaired in their migration toward MDC (Figure 6). These findings suggest that there is a specific defect in CD4⁺ lung T cell migration towards MDC in OVA-treated β -arrestin-2^{-/-} mice.

5 *Non-allergic asthma model.* To further investigate the specificity of the impaired asthmatic response in β -arrestin-2^{-/-} mice, we subjected mice to a model of endotoxin-mediated asthma, or non-allergic asthma. Endotoxin, a LPS, is a major component of the outer cell membrane of all gram-negative bacteria and even small amounts of this molecule are immune stimulatory. Subjects with endotoxic asthma demonstrate lung
10 neutrophilic inflammation and airway hyperresponsiveness and this response is coordinated by alveolar macrophages and epithelial cells rather than T cells. Both wild-type and β -arrestin-2^{-/-} mice exposed to aerosolized LPS developed lung neutrophilic inflammation and increased airway
15 inflammatory processes, or the ability of airways to respond to methacholine, in β -arrestin-2^{-/-} mice.

Discussion of results

20 When treated with OVA, wild-type mice develop symptoms of allergic asthma. In stark contrast, these symptoms, including airway inflammation and airway hyperresponsiveness, do not appear in similarly treated β -arrestin-2^{-/-} mice. These results suggest that β -arrestin-2 is essential to the development of allergic asthma and that it exerts its' regulatory effect at a proximal step in the inflammatory cascade.

25 Numerous immune cell types, including antigen presenting cells, T lymphocytes, B lymphocytes, eosinophils and mast cells, interact in a highly coordinated fashion to respond to allergens. Although the nature and sequence of these interactions are not entirely delineated, the infiltration of activated T_H2 cells into the lung is a primary pathological event underlying

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allergic asthma. Without these cells, the symptoms of asthma, including airway inflammation, airway hyperresponsiveness and reversible airflow obstruction, do not occur.

The initial event in the development of allergic asthma is the processing of inhaled allergens. This involves the capture, modification and presentation of allergen to T and B cells. Once activated, T cells proliferate and differentiate to a T_H2 phenotype defined by the release of type 2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13). Release of these cytokines at germinal centers within peripheral lymphoid tissue supports B cell-mediated production of allergen-specific antibodies. In addition, type 2 cytokines released from T cells that have migrated to the lung activate and recruit mast cells and eosinophils, the primary effector cells of the allergic asthmatic response.

Our study shows that antigen processing, presentation and activation of T cells are intact in *β-arrestin-2*^{-/-} mice since their serum levels of OVA-specific IgE and IgG₁ are comparable to those in wild-type mice. Although there is a trend for a slight delay in the kinetics of the immunoglobulin response in *β-arrestin-2*^{-/-} mice, immunoglobulin levels are the same on day 24 when physiologic and biologic measurements are made. Despite this normal development of OVA-IgE and -IgG₁ in OVA-treated *β-arrestin-2*^{-/-} mice, the classic features of a T_H2-mediated asthmatic response, including airway inflammation, airway hyperresponsiveness, and elevated T_H2 cytokines, are absent in these mice. Therefore, the defect in the allergic asthmatic response in *β-arrestin-2*^{-/-} mice lies between the generation of a T_H2 response and airway inflammation. Our data showing defective CD4⁺ T cell migration to the T_H2 chemoattractant MDC suggests that the defect is present at the level of T_H2 cell trafficking to the lung.

Other potential mechanisms underlying the phenotype observed in *β-arrestin-2*^{-/-} mice have been evaluated, but ruled out. Enhanced induction of

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T_H1-type cytokines may inhibit the development of a T_H2-type response. However, we find no evidence to support this notion since IgG_{2a}, IL-12 and IFN- γ , (factors indicative of a T_H1 response) are not elevated in β -arrestin-2^{-/-} mice relative to wild-type mice.

5 Another possible explanation for the failure of β -arrestin-2^{-/-} mice to develop asthma is mast cell dysfunction. Antigen cross-linking of cell surface IgE receptors (FcRI) stimulates mast cell release of mediators including histamine, prostaglandins, leukotrienes and cytokines. Collectively, these agents perpetuate airway inflammation and airway hyperresponsiveness and
10 thus, appear pivotal to the development of allergic asthma. However, the ability to respond to OVA and to develop allergic asthma is preserved in mast cell deficient mice. Thus, the failure of β -arrestin-2^{-/-} mice to develop asthma cannot be explained by mast cell dysfunction.

 T cell function is crucial to the development of allergic asthma. When
15 T cells are non-functional or absent, allergic inflammation, airway hyperresponsiveness and lung cytokine production are prevented in response to OVA treatment. These results are nearly identical to the impaired allergic asthmatic response we observed in β -arrestin-2^{-/-} mice. Because β -arrestin-2^{-/-} mice have normal levels of CD3⁺ T splenocytes and
20 normal architecture of spleen follicles (data not shown), we suggest that T cell dysfunction, rather than reduction in T cell number, is the root of the impaired allergic asthmatic response in β -arrestin-2^{-/-} mice. Since the manifestation of allergic asthma requires T cell proliferation, differentiation and migration, any one of these processes may be regulated by β -arrestin-2
25 and, thus, dysregulated in mice completely lacking that protein.

 This study shows that β -arrestin-2 positively regulates T cell chemotaxis to the lung inflammatory chemokine MDC. We hypothesize that this diminished T cell migration is the primary impairment in β -arrestin-2^{-/-}

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mice and is largely responsible for their protection against the development of allergic asthma.

Multiple *in vitro* reports demonstrate that β -arrestin regulates chemokine receptor signaling and internalization. Other *in vitro* reports, including our previous work, show that CXCR4-mediated migration is impaired in T cells lacking β -arrestin-2 and in HEK 293 cells having suppressed expression of β -arrestin-2. Although these reports examined only CXCR4, a receptor that primarily mediates T cell migration during homeostatic conditions, our data demonstrate that β -arrestin-2 also positively regulates CCR4, the receptor for lung inflammatory chemokines MDC and thymus- and activation-regulated chemokine.

β -arrestin proteins act as scaffolds to link GPCR activation to at least three MAPK cascades – the extracellular signal-regulated kinase (ERK) cascade, the c-Jun N-terminal kinase 3 (JNK3) cascade and the p38 MAPK cascade. A recent report indicates that β -arrestin-2 activation of the p38 MAPK cascade is required for CXCR4-mediated migration of HEK 293 cells to SDF-1 α . β -arrestin-2 may similarly regulate CCR4, the receptor for MDC, through mechanisms involving MAPK or other signaling pathways. Although MAPKs regulate the phosphorylation of nuclear transcription factors, recent studies indicate that β -arrestin-MAPK scaffolds are preferentially targeted to cytosolic substrates. Such cytosolic substrates may be proteins involved in cell chemotaxis. Thus, β -arrestin-2, through its' action as a scaffold protein, may positively regulate T cell chemotaxis.

Alternatively, in keeping with their classically described role, β -arrestins may regulate chemotaxis through termination of chemokine receptor signaling. This signal termination, or chemokine receptor desensitization, may be crucial to the directional sensing, and thus migration, of T cells.

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Although we feel that β -arrestin-2-regulation of T cell chemotaxis is the major mechanism responsible for the profound protection against allergic asthma observed in the β -arrestin-2^{-/-} mice, β -arrestin-2 regulation of other T cell functions, or modulation of cells other than lymphocytes, may make a
5 contribution to the dramatic phenotype observed in these mice.

β -arrestin-2 may regulate T cell differentiation. Yamashita et al., showed that T cell antigen receptor-mediated activation of the Ras/ ERK1/2 pathway enhances IL-4R signaling and is required for the differentiation of naïve T cells into T_H2 cells. Although neither T cell receptors, nor IL-4
10 receptors are GPCRs, there is evidence in the literature of β -arrestin-2 modulation of non-GPCR-mediated events through transactivation of non-heptahelical receptors or through intracellular modulation of downstream signaling pathways resulting from antigen or cytokine receptor stimulation. Although differentiation of T cells to the T_H2 lineage may be reduced in β -
15 *arrestin-2*^{-/-} mice, our results suggest that this does not occur during the antigen sensitization phase since the release of IL-4 from T_H2 cells is required for the production of allergen-specific IgE by B cells and this function is not impaired in β -*arrestin-2*^{-/-} mice.

Alternatively, β -*arrestin-2*^{-/-} could regulate non-hematopoietic cells in the lung such as airway smooth muscle or airway epithelial cells. Although
20 we have not ruled out these possibilities, our data indirectly suggest that neither of these cells is significantly regulated by β -*arrestin-2*^{-/-}. Airway responses to methacholine challenge were not different between β -*arrestin-2*^{-/-} and wild-type mice when treated with alum or LPS suggesting that β -
25 *arrestin-2* does not regulate airway smooth muscle cell contraction. Similarly, β -*arrestin-2* does not likely regulate the production of lung chemokines by airway epithelial cells in response to inhaled allergen. T cells harvested from lung of β -*arrestin-2*^{-/-} mice demonstrated impaired chemotaxis. Had this

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been paired with reduced chemotactic signal, these T cells would not likely have reached the lung.

The fact that LPS-exposed β -arrestin-2^{-/-} mice developed lung inflammation (neutrophilic) and increased airway responsiveness comparable to similarly treated wild-type mice demonstrates that there is no universal impairment in inflammatory cell migration in β -arrestin-2^{-/-} mice. This demonstration, of a functionally intact innate immune system, is critical to the pursuit of β -arrestin-2 as a potential therapeutic target for treating allergic asthma.

The absence of β -arrestin-2 prevents the development of allergic asthma without compromising the function of the innate immune system. β -arrestin-2 regulation of T cell migration to the lung is thought to be the primary mechanism underlying this protection. Because β -arrestin-2 exerts its regulatory effect proximal to the recruitment of activated T cells into the lung it is an attractive therapeutic target for treating allergic asthma.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The following documents, as well as any documents referenced in the foregoing text, should be considered as incorporated by reference in their entirety.

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CLAIMS**We Claim:**

- 5 1. A method of preventing or treating a condition involving T cell migration comprising administering to a mammal in need thereof a therapeutically effective amount of a composition that modulates desensitization, wherein the composition has the ability to modulate the chemotaxis of T cells.
- 10 2. The method of claim 1, wherein the condition is allergic asthma.
3. The method of claim 1, wherein the T cells are Th2 cells.
- 15 4. The method of claim 1, wherein the composition modulates the activity of β -arrestin-2.
5. The method of claim 1, wherein the compound that modulates the expression of β -arrestin-2.
- 20 6. The method of claim 1, wherein the composition modulates the activity or expression of a GRK.
7. The method of claim 1, wherein the composition binds to a chemokine receptor.
- 25 8. A method of preventing or treating a chemokine receptor related disease comprising administering to a mammal in need thereof a therapeutically effective amount of a composition that modulates desensitization, wherein the composition has the ability to modulate
- 30 chemotaxis of T cells.

9. The method of claim 8, wherein the che
disease is allergic asthma.
10. The method of claim 8, wherein the composition modulates the
5 activity of β -arrestin-2.
11. The method of claim 8, wherein the compound that modulates the
expression of β -arrestin-2.
- 10 12. The method of claim 8, wherein the composition modulates the
activity or expression of a GRK.
13. The method of claim 8, wherein the composition binds to a
chemokine receptor.
- 15 14. A method of preventing or treating inflammation or an inflammatory
disease comprising administering to a mammal in need thereof a
therapeutically effective amount of a composition that modulates
desensitization, wherein the composition has the ability to modulate
20 chemotaxis.
15. A method of preventing or treating an immune disease comprising
administering to a mammal in need thereof a therapeutically effective
amount of a composition that modulates desensitization, wherein the
25 composition has the ability to modulate chemotaxis.
16. A method of preventing or treating a disease in a mammal by
modulating the desensitization of a chemokine receptor comprising:
 - a) providing a therapeutically effective amount of a compound
30 that is capable of modulating the activity of β -arrestin-2 binding
to the receptor; and

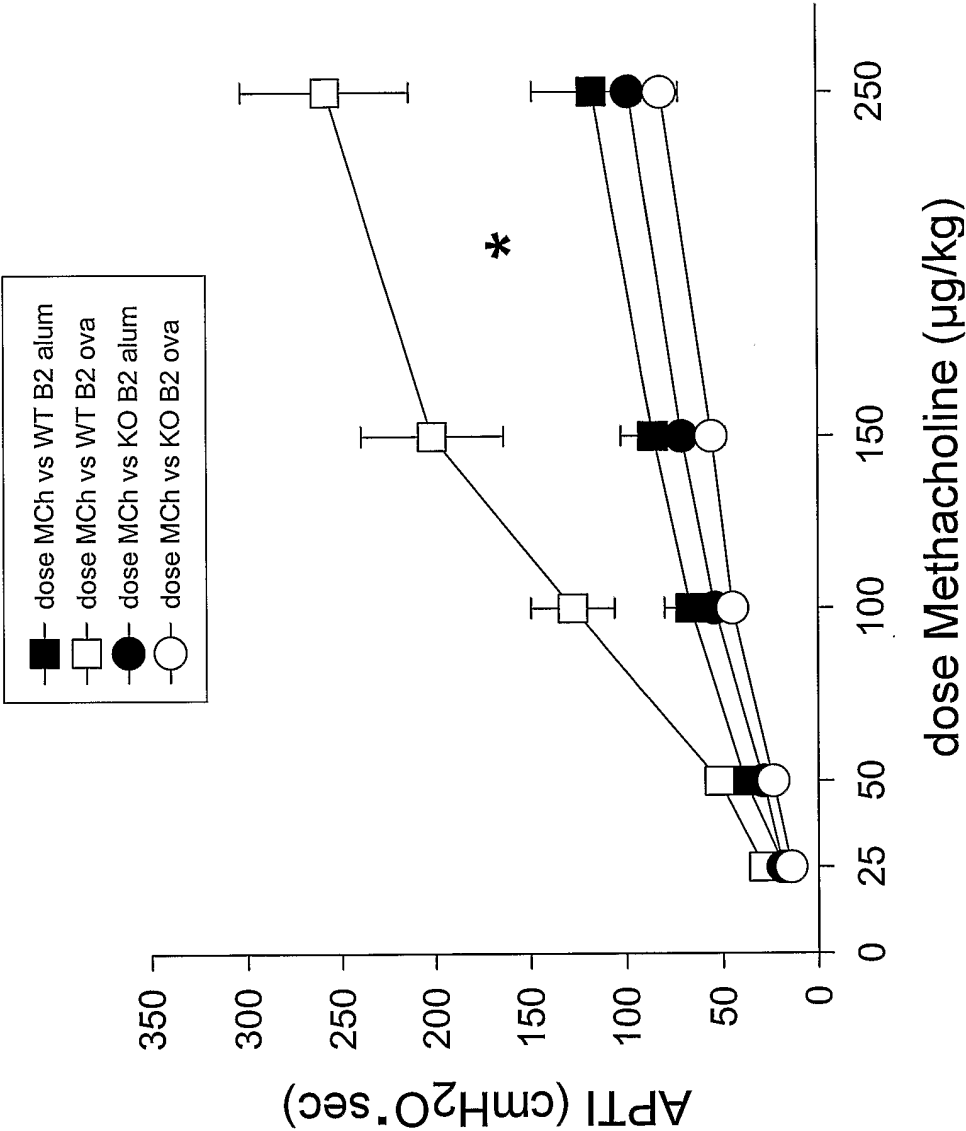
- b) administering the compound to the mammal, wherein the compound modulates the activity of the β -arrestin-2 in the mammal.

- 5 17. A method according to claim 16 wherein the compound blocks the activity of a GRK that phosphorylates the chemokine receptor.
18. A method according to claim 16 wherein the chemokine receptor is CCR4.
- 10 19. A method of treating a MAPK related disease in a mammal comprising administering to a mammal a therapeutically effective amount of a compound that modulates desensitization, wherein the composition has the ability to modulate the MAPK cascade.
- 15 20. The method of claim 19, wherein the composition modulates the activity or expression of β -arrestin 2.
- 20 21. A method according to claim 19 wherein the MAPK cascade is the P39 MAPK cascade.
22. A method according to claim 19 wherein the MAPK cascade is the ERK cascade.
- 25 23. A method according to claim 19 wherein the MAPK cascade is the JNK-3 cascade.
- 30 24. A method of treating allergic asthma in a mammal comprising administering to the mammal a therapeutically effective amount of a composition that modulates the phosphorylation of a chemokine receptor by a GRK, and wherein the binding of β -arrestin-2 to the chemokine receptor is modulated.

25. A composition of matter comprising a therapeutic amount of a pharmaceutically acceptable compound capable of modulating β -arrestin-2 in a mammal such that the compound inhibits the chemotaxis of Th2 cells to the lung upon allergic challenge to the mammal.
26. A method of testing a compound for the ability to modulate β -arrestin-2 in a mammal comprising:
- a) providing a cell comprising a chemokine receptor and a detectably labeled β -arrestin-2,
 - b) exposing the cell to the compound,
 - c) providing a chemokine that binds to the chemokine receptor
 - d) determining the distribution or translocation of the β -arrestin-2, and
 - e) comparing the distribution or translocation of the β -arrestin-2 to the distribution or translocation of β -arrestin-2 under the same conditions but without pre-treatment by the compound.

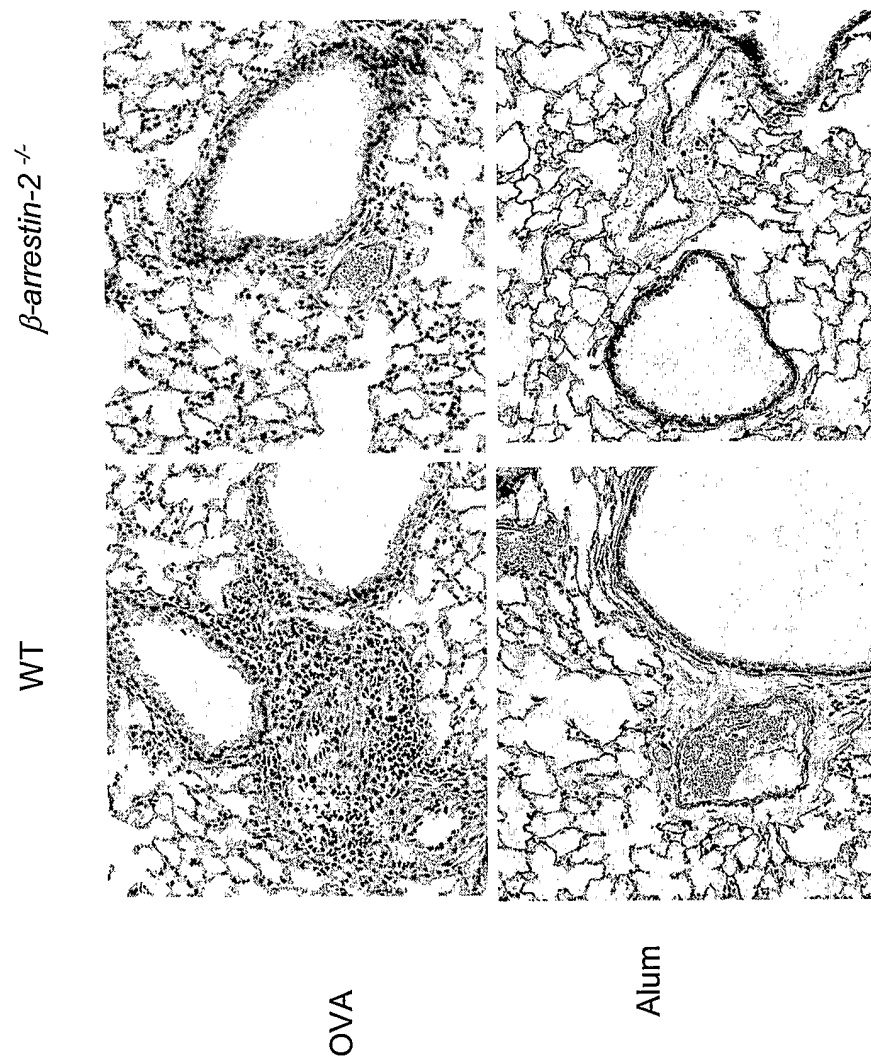
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Fig. 1



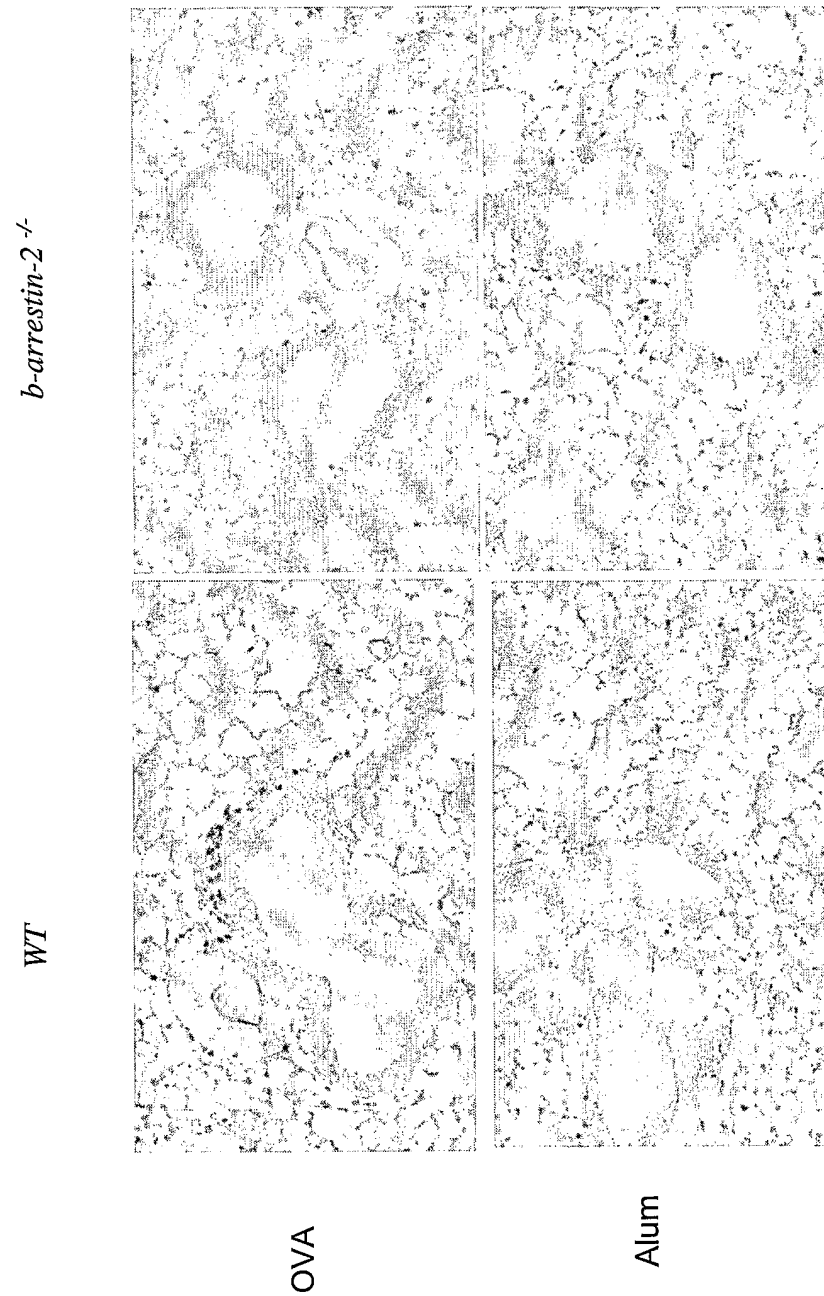
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Fig. 2a



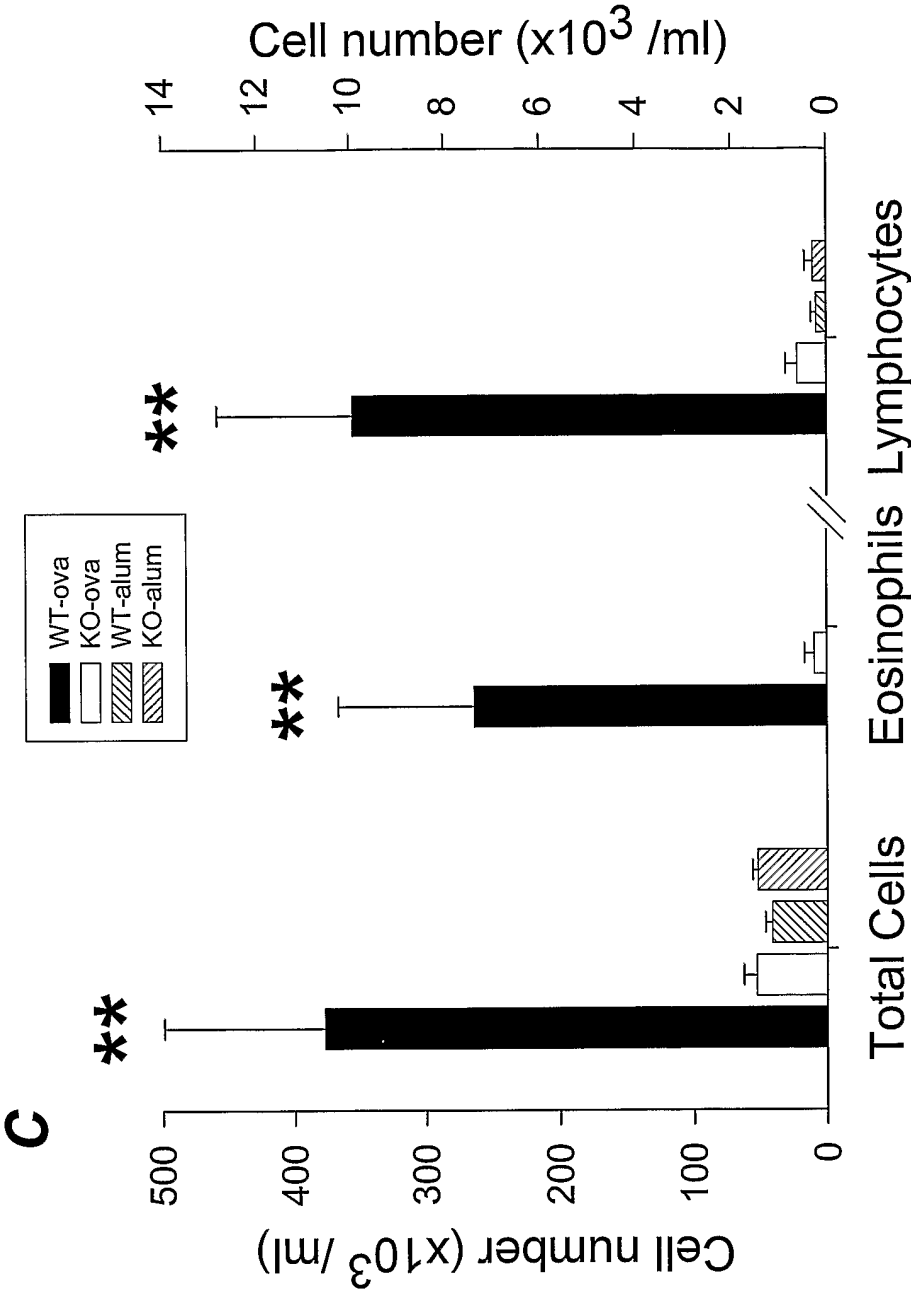
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Fig. 2b



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Fig. 2c



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Fig. 3 A

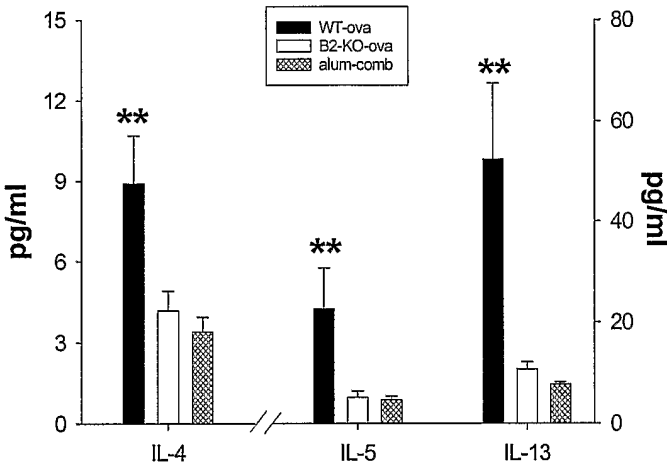
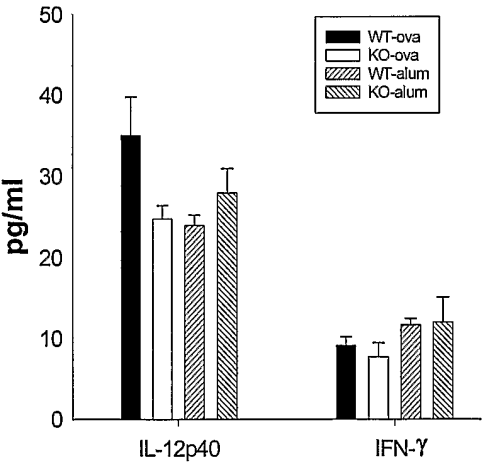
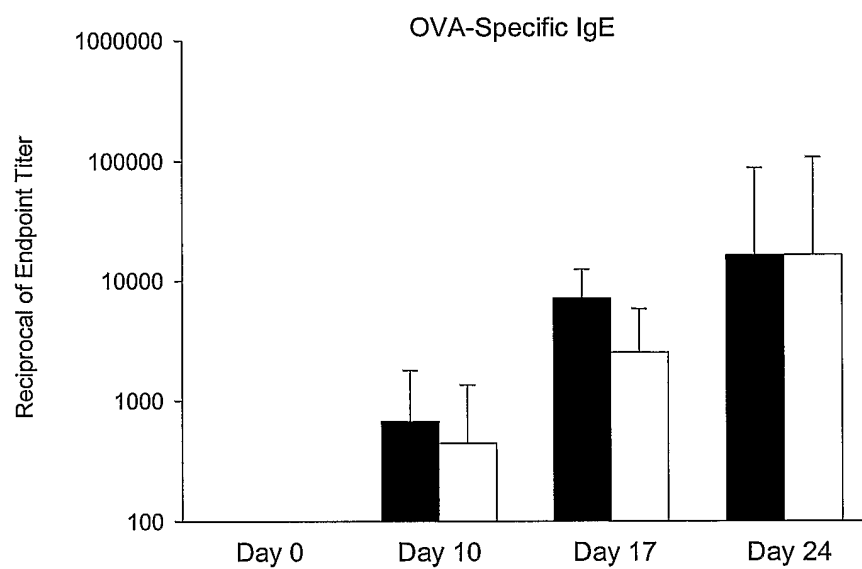
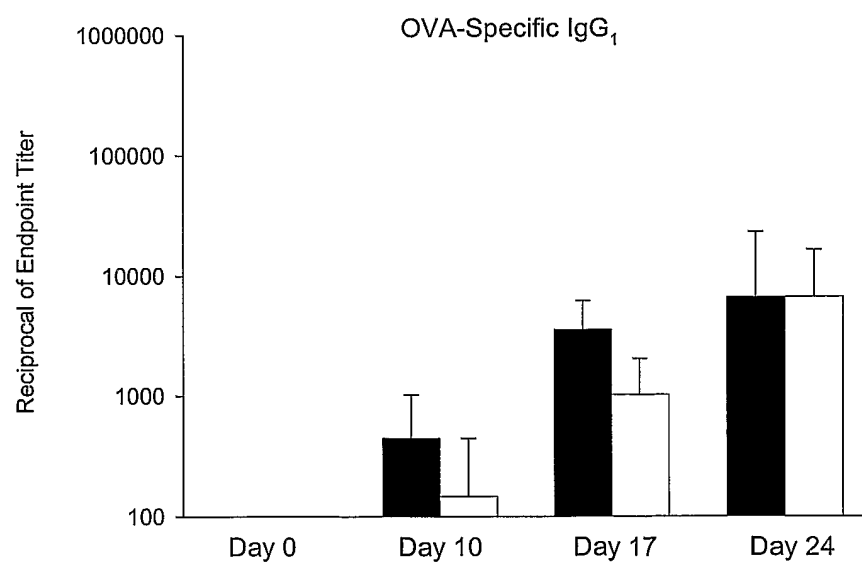


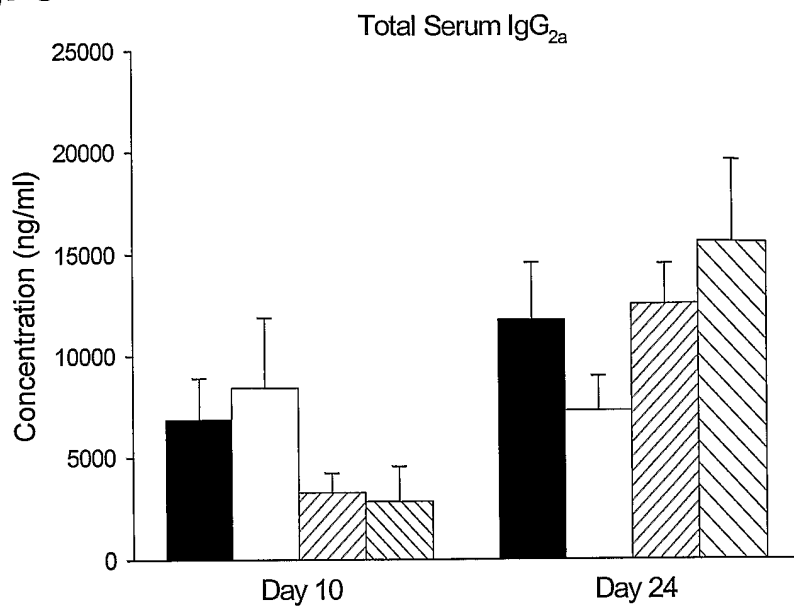
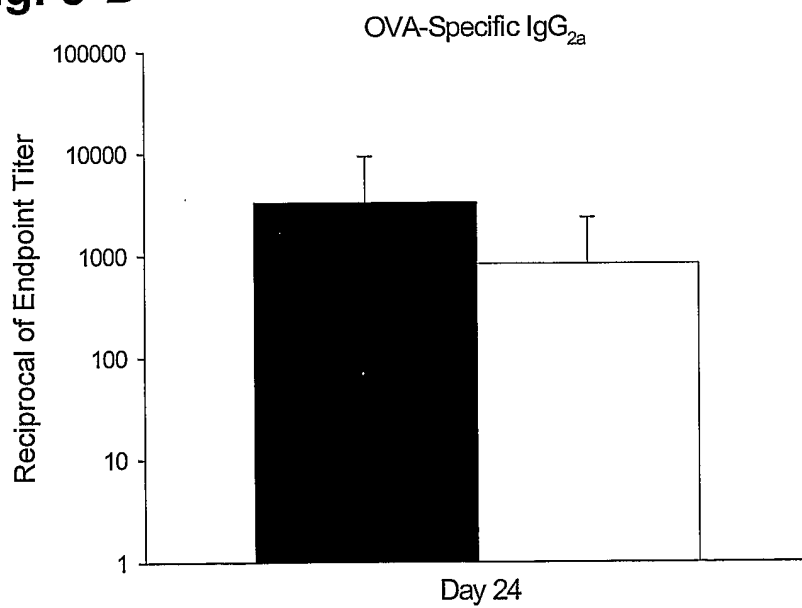
Fig. 3 B



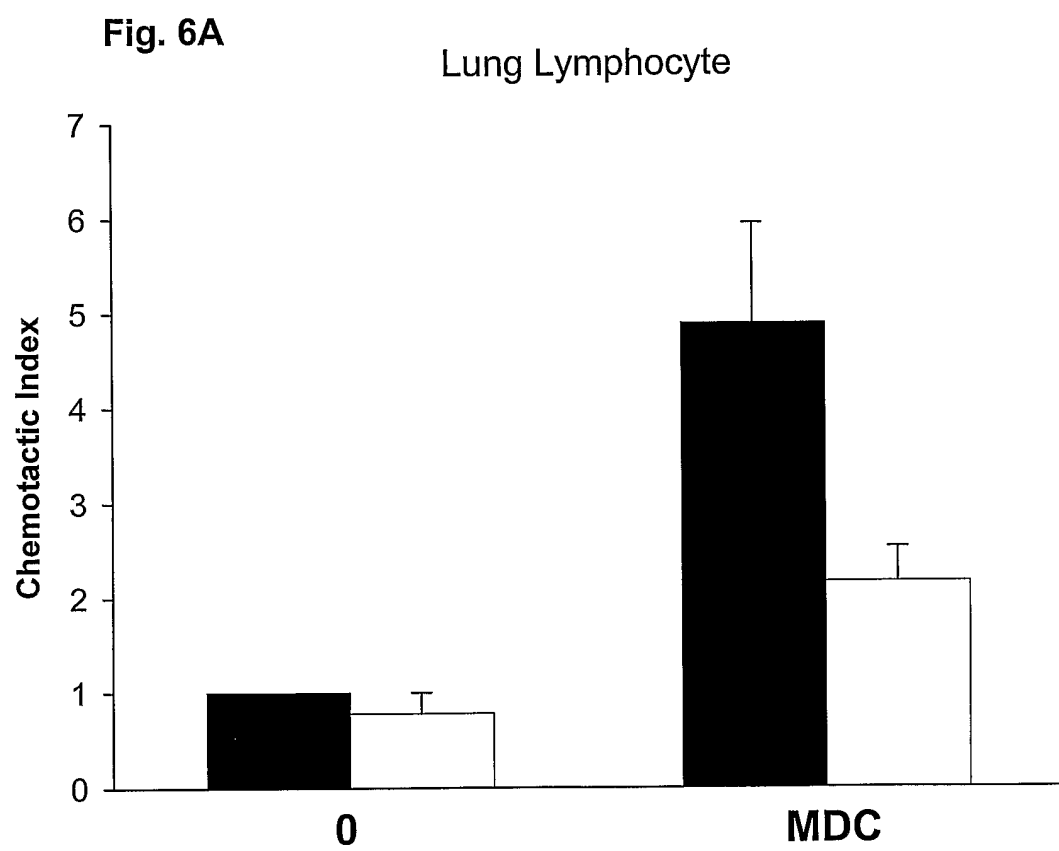
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Fig. 4 A**Fig. 4 B**

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Fig. 5 A**Fig. 5 B**

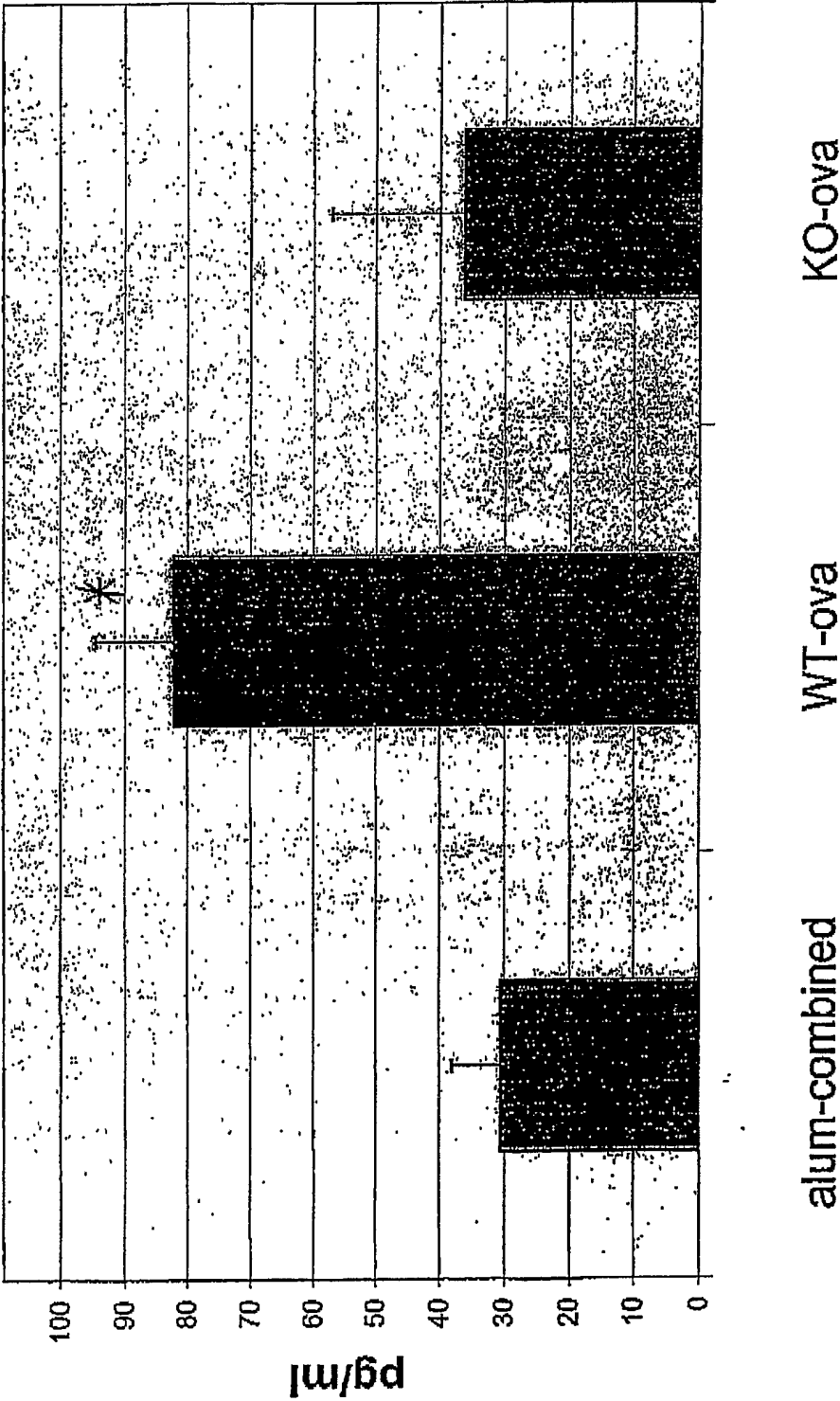
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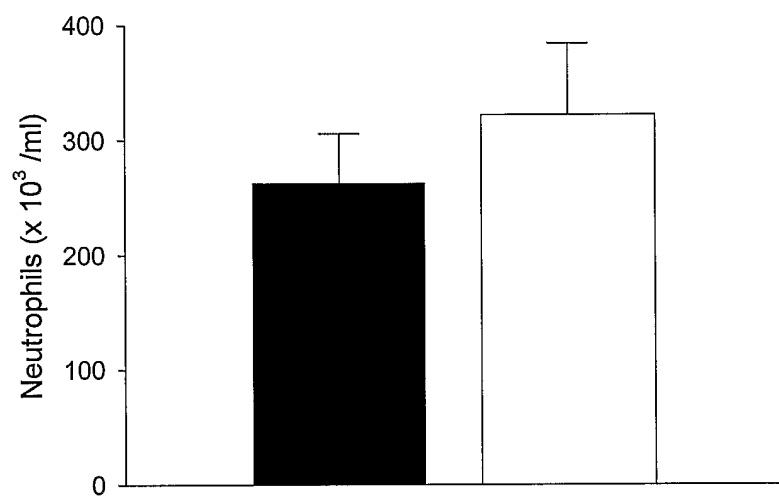
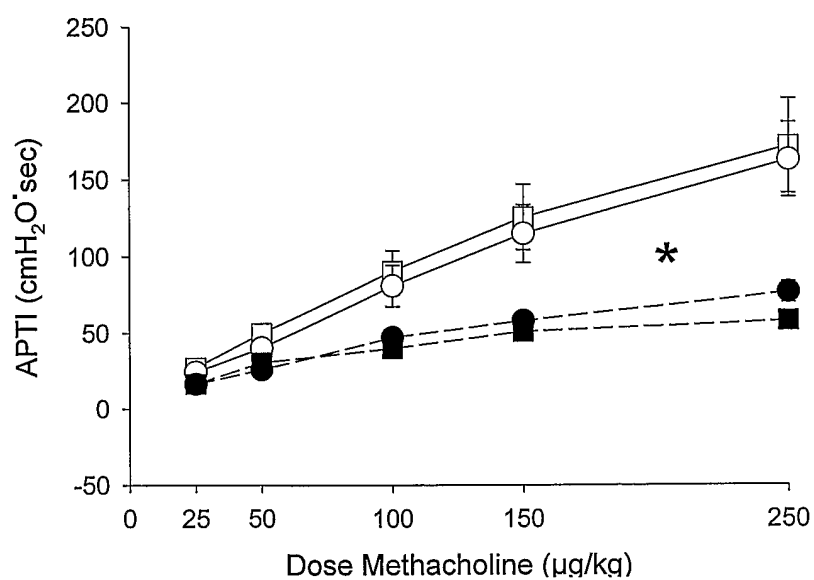
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Fig. 6b

MDC Level in Lavage Fluid



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Fig. 7A**Fig.7 B**

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Fig. 8

	CD3(+) cells per mm of Basement Membrane	CD4(+) T cells harvested from Lung
WT-ova	31.04 ± 1.36	21,400/lung
KO-ova	2.08 ± 1.57	6,900/lung
WT-alum	2.96 ± 0.85	
KO-alum	2.21 ± 0.74	
KO-alum/saline	2.27 ± 0.15	