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(54) Title: METHODS FOR CONTROLLING INFLAMMATORY AND IMMUNOLOGICAL DISEASES

(57) Abstract: The present invention provides methods for generating mucosal tissue homing immunosuppressive T-cells comprising treating naïve T-cells with retinoids and/or retinoid agonists. Methods are also provided for treating a mammal having an inflammatory or immunological disease by administering a therapeutically effective dose of retinoids and/or retinoid agonists. Additional methods are also provided for boosting the immune system of a mammal by inhibiting the generation of immunosuppressive T-cells by administering a therapeutically effective dose of a retinoid receptor antagonist to the mammal.

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## METHODS FOR CONTROLLING INFLAMMATORY AND IMMUNOLOGICAL DISEASES

### GOVERNMENT RIGHTS

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**[0001]** This invention was made with Government support under Grants AI063064 and AI074745 awarded by the National Institute of Allergy and Infectious Diseases. The Government has certain rights in this invention.

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### BACKGROUND OF THE INVENTION

**[0002]** The present invention relates generally to methods for controlling inflammatory and immunological diseases and more particularly to methods of administering retinoids for generation of immunosuppressive T cells for  
15 controlling inflammatory and immunological diseases.

**[0003]** Preformed vitamin A in food is absorbed in the form of retinol, which can be made into retinal and retinoic acid in the body (Chambon, P., 1996, *Faseb J* 10: 940; Semba, R.D., 1994, *Clin. Infect. Dis.* 19: 489; Zhang, C. and Duvic, M., 2003, *Dermatol. Ther.* 16:322; Mark, M. et al., 2006, *Annu. Rev. Pharmacol. Toxicol.* 46: 451). Also retinol can be generated from provitamin A carotenoids  
20

such as beta-carotene. Three metabolites of vitamin A, 11-cis-retinal, all-trans-retinoic acid (ATRA) and 9-cis-RA, mediate the major biological functions of the vitamin (Mark, M. et al., 2006, *Annu. Rev. Pharmacol. Toxicol.* 46: 451; Duester, G., 2000, *Eur. J. Biochem.* 267: 4315). Vitamin A has pleiotropic functions in the body. 11-cis-retinal functions as chromophores in light absorption for vision, and retinoic acid participates in bone formation, reproduction, and differentiation of many cell types during embryogenesis. In this regard, retinoid deficiency or over-doses can cause teratogenesis (Collins, M.D. and Mao, G.E., 1999, *Annu. Rev. Pharmacol. Toxicol.* 39: 399). Vitamin A plays important roles in fighting against pathogens. One such a function is its role in formation of epithelial linings of the body including eyes, membranes of mucosal tissues (respiratory/urinary/intestinal tracts) and skin, breakage of which leads to invasion by pathogens. Through their effects on cell differentiation and death, retinoids inhibit or reverse the carcinogenic process in some types of cancers in oral cavity, head and neck, breast, skin, liver and blood cells (Soprano, D.R. et al., 2004, *Annu. Rev. Nutr.* 24: 201; Okuno, M. et al., 2004, *Curr Cancer Drug Targets* 4: 285).

**[0004]** All-trans-RA and 9-cis-RA function as the ligands for retinoid nuclear receptors that act as transcription factors for gene expression. As retinoid nuclear receptors, RA receptor (RAR) isotypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and the retinoid X receptor

(RXR) isotypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) have been identified (Mark, M. et al., 2006, Annu. Rev. Pharmacol. Toxicol. 46: 451). Each receptor has multiple isoforms, generated through alternative mRNA splicing and/or transcription (Chambon, P., 1996, Faseb J 10: 940). ATRA preferentially binds RARs, whereas 9-cis-RA  
5 binds equally well to both RARs and RXRs (Mark, M. et al., 2006, Annu. Rev. Pharmacol. Toxicol. 46: 451; Szondy, Z. et al., 1998, Cell Death Differ 5:4). The two groups of retinoid nuclear receptors form RXR/RAR heterodimers, which can function as either transcriptional repressors or activators (Bourguet, W. et al., 2000, Trends Pharmacol Sci 21: 381). In the absence of RAR ligands, apo-  
10 receptors recruit corepressors such as nuclear receptor corepressor (N-CoR) or silencing mediator for retinoid and thyroid hormone receptor (SMRT), which then recruit histone deacetylases (HDACs). Binding of RAR agonists to the receptors induces active conformation of the receptors. This decreases the affinity for corepressors, and creates a binding surface for histone acetyltransferase (HAT)  
15 co-activators, such as CREB-binding protein (CBP) and p160 (e.g. TIF2). Transcriptional coactivators are involved in chromatin remodeling (decondensation due to histone acetylation) or recruitment of the basal transcription machinery. However, RXR agonists alone cannot dissociate co-repressors from heterodimers of RAR–RXR, and retinoid receptor antagonists  
20 prevent the formation of the holo-conformation (Clarke, N. et al., 2004, Expert

Rev Mol Med 6: 1).

**[0005]** Many functions of retinoids have been identified in regulation of immune responses. As positive roles in regulation of the immune system, retinoids enhance the numbers and effector functions of neutrophils, NK cells, B cells, and Th2 cells (Stephensen, C.B., 2001, Annu Rev Nutr 21:167; Cantorna, M.T. et al., 1994, J Immunol 152: 1515; Racke, M.K. et al., 1995, J Immunol 154: 450; Hoag, K.A. et al., 2002, J Nutr 132:3736). Retinoids induce gut homing receptor expression in T cells and B cell Ig switch to IgA, and, therefore, is an important regulator of mucosal immunity. Vitamin A is required for antibody responses to T-dependent and bacterial polysaccharide antigens (Pasatiempo A.M. et al., 1990, Faseb J 4:2518; Smith, S.M. and Hayes, C.E., 1987, Proc Natl Acad Sci U S A 84:5878), normal IgA levels in the intestinal fluid (Sirisinha, S. et al., 1980, Clin Exp Immunol 40: 127), prevention of activation-induced T cell apoptosis, and normal phagocytic functions and resistance to bacterial pathogens. On the other hand, retinoids decrease macrophage numbers in marrow and spleen. Vitamin A supplementation decreases inflammatory responses and tissue damage (Shams, N.B. et al., 1994, Cornea 13:156). Vitamin A supplementation decreased serum concentrations of inflammatory cytokines such as TNF- $\alpha$  and IL-1 but increased suppressive cytokines such as IL-10 (Szondy, Z. et al., 1998, Cell Death Differ 5:4). Consistently,

supplementation with vitamin A suppressed inflammation in experimental autoimmune encephalomyelitis. Paradoxically, vitamin A deficiency is linked to both defective delayed type hypersensitivity and excessive Th1 cell function. Vitamin A is also linked to both lower and higher incidences of several types of  
5 cancers in humans (Fontham, E.T., 1990, Int J Epidemiol 19: S32).

**[0006]** Regulatory T cells were initially identified by their suppressive activity on immune response to self-antigens, but it was found later that they can also suppress immune responses to alloantigens (Wood, K.J. and Sakaguchi, S., 2003, Nat Rev Immunol 3: 199) and pathogens (Hori, S. et al., 2002, Proc Natl  
10 Acad Sci U S A 99:8213). The existence of regulatory T cells that induce immune tolerance to self antigens was first reported many decades ago. Although regulatory T cells can be induced from non-regulatory T cells in the periphery, the presence of naturally generated regulatory cells is firmly established. The key feature of naturally generated regulatory T cells is that they express FoxP3 (the  
15 product of the mouse *FoxP3* gene) or FOXP3 (the human version)(Ziegler, S.F., 2006, Annu Rev Immunol 24:209). The term "FoxP3" will be used for both human FOXP3 and mouse FoxP3 in this application. FoxP3 is a member of the forkhead/winged-helix family of transcriptional regulators. FoxP3 confers T cells with regulatory function and increases the expression of CTLA-4 and CD25, but  
20 decreases IL-2 production by acting as a transcriptional repressor. FoxP3 binds

to and suppresses nuclear factor of activated T cells (NFAT) and nuclear factor-kappaB (NFKB) (Bettelli, E.M. et al, 2005, Proc Natl Acad Sci U S A 102:5138). In addition to the natural FoxP3<sup>+</sup> cells of thymic origin, regulatory T cells can also be generated in response to antigens in the periphery.

5   **[0007]**     Regulatory T cells play critical roles in regulation of immune response not only to self-antigens, alloantigens and tumor antigens but also to foreign antigens including pathogens in certain conditions. In animal models such as mice and rats, CD4<sup>+</sup>CD25<sup>+</sup> T cells (commonly called Tregs; enriched with FoxP3<sup>+</sup> cells) have been shown to suppress a number of antigen-induced autoimmune  
10   diseases: diabetes, autoimmune encephalomyelitis, thyroiditis (, inflammatory bowel disease and gastritis to name a few. They can also prevent graft rejection and graft-versus-host disease. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by inappropriate regulation of self-reactive B and T cells. In pediatric SLE patients, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T  
15   cells was significantly decreased in patients with active SLE compared with normal controls and patients with inactive SLE. In collagen-induced arthritis, a popular model of rheumatoid arthritis, CD25<sup>+</sup> cell depletion lead to a significantly more severe disease with higher antibody titers against collagen than that of control mice. In infection by pathogens, regulatory T cells are involved in limiting  
20   the magnitude of immune responses to pathogens. This suppression, however, is

a double edged-sword for hosts: While regulatory T cells function to limit tissue damage in the hosts, they can also help pathogens to avoid the immune system, leading to chronic infection.

**[0008]** CD4<sup>+</sup>CD25<sup>+</sup> T cells (FoxP3<sup>+</sup>) T cells are highly enriched in tumors, including melanoma, cervical carcinoma, gastrointestinal tract cancer, gastric cancer, lung cancer, ovarian cancer, colorectal cancer , breast cancer, pancreas adeno carcinoma, head and neck cancer, hepatocellular carcinoma, and leukemia and lymphoma (adult T cell leukemia/lymphoma, Hodgkin's lymphoma, and chronic lymphocytic leukemia). A single injection of anti-CD25 (interleukin 2 receptor- $\alpha$ ) monoclonal antibody (PC61) resulted in the regression of tumors in mice. Tumor transplants in nude mice were eradicated by transferring CD25<sup>+</sup> cell-depleted, but not total, spleen cells. Depletion of CD25<sup>+</sup> T cells also induced anti-tumor-immunity against murine colorectal tumor CT26. Thus, FoxP3<sup>+</sup> regulatory T cells represent another barrier to effective tumor immunity in animals and humans.

**[0009]** Thus it would be desirable to have a method for controlling inflammatory and immunological diseases by generating immunosuppressive T cells where the immunosuppressive T cells target normal and inflamed mucosal tissues. It would also be desirable to provide methods of blocking immunosuppressive T cells to boost immune responses to pathogens and

cancers.

## SUMMARY OF THE INVENTION

5   **[0010]**     In one aspect of the present invention there is provided a method for generating mucosal tissue homing immunosuppressive T-cells comprising treating naive T-cells with a retinoid or a retinoid receptor agonist. The retinoid may be retinol palmitate, retinyl palmitate, retinol acetate, beta-carotene or combinations thereof. Alternatively, the retinoid may be all-trans-, 9-cis-, 11-cis  
10   retinoic acid or combinations thereof. The retinoid receptor agonist is AM-580, BMS641, BMS961, CD666, TTNPB, ATRA, Ro25-7386, methoprene acid or combinations thereof.

**[0011]**     In another aspect of the present invention there is provided a method for treating a mammal having an inflammatory or immunological disease  
15   comprising administering to the mammal a therapeutically effective dose of a retinoid or a retinoid receptor agonist.

**[0012]**     In a further aspect of the present invention there is provided a method for treating a mammal having an inflammatory or immunological disease comprising the steps of obtaining naïve T-cells from the mammal, culturing the  
20   naïve T-cells with a retinoid or a retinoid receptor agonist, generating

immunosuppressive T-cells and administering the immunosuppressive T-cells to the mammal.

**[0013]** In yet another aspect of the present invention there is provided a method for boosting the immune response in a mammal comprising  
5 administering to the mammal a therapeutically effective dose of a retinoid receptor antagonist.

**[0014]** These and other features, aspects and advantages of the present invention will become better understood with reference to the following drawings, description and claims.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0015]** Figure 1A shows the RT-PCR analysis of retinoic acid induced FoxP3 gene expression in CD4 T cells;

**[0016]** Figure 1B is a histogram showing flow cytometry analysis of retinoic  
15 acid induced FoxP3 gene expression in CD4 T cells;

**[0017]** Figure 1C is a graph showing dose-dependent induction of FoxP3 by ATRA;

**[0018]** Figure 2 shows the induction of RAR $\alpha$  by ATRA in CD4 T cells;

**[0019]** Figure 3A is a histogram showing the RAR $\alpha$  dependent induction of  
20 FoxP3<sup>+</sup> cells;

**[0020]** Figure 3B is a FACS dot plot showing the  $RAR\alpha$  dependent induction of FoxP3<sup>+</sup> cells;

**[0021]** Figure 4A is a FACS dot plot showing the cooperation of ATRA and TGF- $\beta$ 1 in induction of FoxP3<sup>+</sup> T cells;

5 **[0022]** Figure 4B is a graph showing  $RAR\alpha$ -independent induction of FoxP3 by TGF- $\beta$ 1;

**[0023]** Figure 5A is bar graph showing that ATRA-treated T cells are hypo-proliferative;

10 **[0024]** Figure 5B is a graph showing that ATRA-treated T cells are suppressive in function;

**[0025]** Figure 6A is a FACS dot plot showing granzyme expression in ATRA-treated FoxP3<sup>+</sup> T cells;

**[0026]** Figure 6B is a bar graph showing granzyme expression in ATRA-treated FoxP3<sup>+</sup> T cells;

15 **[0027]** Figure 7 is a FACS dot plot showing that ATRA-treated FoxP3<sup>+</sup> T cells efficiently kill immune cells;

**[0028]** Figure 8A is a bar graph showing the expression of mucosal tissue homing receptors in vitamin A-induced FoxP3<sup>+</sup> T cells;

**[0029]** Figure 8B is a bar graph showing the expression of mucosal tissue

homing receptors in vitamin A-induced FoxP3<sup>+</sup> T cells;

**[0030]** Figure 9A is a FACS dot plot showing ATRA induction of mouse FoxP3 T cells;

**[0031]** Figure 9B is a FACS dot plot showing expression of homing receptors  
5 in ATRA-induced mouse FoxP3 T cells;

**[0032]** Figure 9C is a FACS dot plot showing AM-580 induction of mouse FoxP3 T cells;

**[0033]** Figure 10 is a bar graph showing the tissue-specific migration of retinoid-induced FoxP3<sup>+</sup> T cells;

10 **[0034]** Figure 11A is a FACS dot plot showing the regulation of mucosal FoxP3<sup>+</sup> T cell numbers by vitamin A *in vivo*;

**[0035]** Figure 11B is a FACS dot plot showing the regulation of mucosal FoxP3<sup>+</sup> T cell numbers by vitamin A *in vitro*;

**[0036]** Figure 12 is a graph showing suppression of the induction of  
15 inflammation in mice treated with retinoid-induced FoxP3<sup>+</sup> regulatory T cells; and

**[0037]** Figure 13 is a bar graph showing the regulation of anti-inflammatory T cell numbers *in vivo* by controlling vitamin A consumption in mice.

## DETAILED DESCRIPTION OF THE INVENTION

**[0038]** The following detailed description is of the best currently contemplated modes of carrying out the invention. The description is not to be taken in a limiting sense, but is made merely for the purpose of illustrating the general principles of the invention, since the scope of the invention is best  
5 defined by the appended claims.

**[0039]** Broadly, the present invention provides a method for generating mucosal tissue homing immunosuppressive T-cells comprising treating naïve T-cells with a retinoid or a retinoid receptor agonist. By utilizing the FoxP3 induction activity of retinoid receptor agonists, FoxP3<sup>+</sup> regulatory T cells with  
10 specialized functions and migratory behavior can be generated. The selectivity of the homing capacity of retinoid-induced FoxP3<sup>+</sup> regulatory T cells for mucosal tissue and their unique biological function are the advantageous for treating inflammatory and immunological diseases, particularly in mucosal tissue.

**[0040]** In one embodiment of the present invention mucosal tissue homing  
15 immunosuppressive T-cells may be generated by treating naïve T-cells with retinoids and/or retinoid agonists. Immunosuppressive T-cells may be generated *ex vivo* by isolating naïve T-cells and culturing them with retinoids or retinoid agonists. The concentration of the retinoid and/or retinoid agonist may be from about 100 pM to about 1 mM. In an exemplary embodiment, the concentration  
20 may be from about 100 pM to about 100 nM. Conventional naïve T-cells may be

isolated from cord blood, peripheral blood or secondary lymphoid tissues. Non-limiting examples of secondary lymphoid tissues may be tonsils, spleen or appendix. Alternatively, immunosuppressive T-cells may be generated *in vivo* by administering retinoids and/or retinoid agonists to a mammal.

5   **[0041]**    In another embodiment of the present invention retinoids may be Vitamin A or derivatives of Vitamin A such as, but not limited to, retinol palmitate, retinyl palmitate, retinol acetate, beta-carotene or combinations thereof. In an exemplary embodiment, the retinoids may be all-trans-, 9-cis-, 11-cis retinoic acid or combinations thereof. Natural and non-natural derivative of retinoids are also  
10   contemplated. The skilled artisan may determine what derivatives of retinoids have similar activity to naturally occurring compounds. In an alternate exemplary embodiment, retinoid agonists may be AM-580, BMS641, BMS961, CD666, TTNPB, ATRA, Ro25-7386, methoprene acid or combinations of these. Other agonists that may activate the receptors RAR/RXR $\alpha$ ,  $\beta$  or  $\gamma$  are also  
15   contemplated by the present invention.

**[0042]**    In another embodiment of the present invention, mammals having an inflammatory and/or immunological disease may be treated by administering a therapeutically effective dose of a retinoid and/or a retinoid agonist. A therapeutically effective amount may be the amount necessary to prevent or treat  
20   the inflammation and/or immunological disease. It is to be understood that the

total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically-effective dose level for any particular patient will depend upon a variety of factors, including the disorder being treated  
5 and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, gender and diet of the patient: the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidentally with the specific compound  
10 employed; and like factors well known in the medical arts. Non-limiting examples of inflammatory or immunological diseases are Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, diabetes mellitus, Alzheimer's disease, Johne's disease or lupus.

**[0043]** The mammal may be treated by directly administering the retinoid  
15 and/or retinoid agonist. In an exemplary embodiment, the retinoid and/or retinoid agonist may be administered subcutaneously, intradermally, intraperitoneally, intramuscularly, orally, intravenously, topically or a combination thereof. The medical practitioner will be able to determine the therapeutically effective dose based on the severity of the disease and patient parameters. The retinoid and/or  
20 retinoid agonist may be administered alone or may be formulated with other

compounds. Non-limiting examples of compounds that may also be administered are TGF- $\beta$ 1, vitamin D, estrogen, progesterone or IL-10.

**[0044]** The retinoid and/or retinoid agonist may be part of a composition comprising a pharmaceutically acceptable carrier. The term “pharmaceutically acceptable carriers” includes nontoxic, inert solid, semi-solid or liquid filler, 5 diluent, encapsulating material or formulation auxiliary of any type. Illustrative examples of the materials that can serve as pharmaceutically-acceptable carriers are sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl 10 cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; 15 agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringers solution; ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations.

**[0045]** In one embodiment, the retinoid and/or retinoid agonist is 20 administered directly to the mammal. By way of non-limiting example, a

mammals diet may be supplemented with a retinoid or retinoid agonist. In an exemplary embodiment, a mammals diet is supplemented with from about 10,000 to about 30,000 U/kg. It will be appreciated that care may be taken in administering retinoids such as Vitamin A as they can be toxic in high doses.

5 Alternatively, a mammal may be administered a therapeutically effective dose of a retinoid or retinoic agonist orally.

**[0046]** In an alternate embodiment, naïve T-cells may be isolated and cultured with the retinoid and/or retinoid agonist to generate immunosuppressive T-cells. The immunosuppressive T-cells are then administered to the mammal to  
10 treat the inflammatory and/or immunological diseases. The naïve T-cells may be autologous, allogenic or xenogenic.

**[0047]** In a further embodiment, the immune system of a mammal may be boosted by suppressing immunosuppressive T-cells. Immunosuppressive T-cells may be suppressed by administering a therapeutically effective dose of a retinoid  
15 antagonist to the mammal. A retinoid antagonist may be any compound that blocks the function of RAR and/or RXR receptors. Non-limiting examples of retinoid antagonists may be Ro41-5253, Ro13-7410, AGN193109, LG100754 or combinations of these agonists. Other antagonists that can block the receptors RAR/RXR $\alpha$ ,  $\beta$  or  $\gamma$  are also contemplated by the present invention. Boosting a  
20 mammal's immune system may be used as a way to treat diseases caused by

pathogens such as, but not limited to, viruses, bacteria or fungi. Alternatively, the method of the present invention may be used to treat cancers such as, but not limited to, cancers of the intestine, respiratory tracts, pancreas, oral cavity, nasal cavity, lungs, mammary gland or cervix.

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## EXAMPLE 1

**[0048]** *All-trans-retinoic acid (ATRA), a major vitamin A metabolite, induces FoxP3 expression in T cells.* FoxP3 is the master transcription factor for CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. The majority of CD4<sup>+</sup>CD25<sup>+</sup> cells are FoxP3<sup>+</sup>,  
10 whereas most CD4<sup>+</sup>CD25<sup>-</sup> cells are FoxP3<sup>-</sup>. Neonatal human cord blood CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells were cultured in a T cell activation condition with IL-2 (25 U/ml) and phytohemagglutinin (PHA, 5 µg/ml) in the presence or absence of all-trans retinoic acid (ATRA, 2 nM) for 6 days. Naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells were activated in the absence and presence of ATRA, and examined for expression of  
15 FoxP3 mRNA and protein by RT-PCR analysis (Figure 1A), flow cytometry analysis (Figure 1B) and dose-dependent induction of FoxP3 by ATRA (Figure 1C). Representative data from at least three independent experiments are shown. The T cells, activated in the presence of IL-2, expressed the FoxP3 mRNA (Figure 1A). When the T cells were cultured with both ATRA and IL-2, the  
20 levels of FoxP3 mRNA expression increased compared to IL-2 alone (Figure 1A).

Also, at the protein level, significantly more of the T cells cultured in the presence of ATRA than in its absence were FoxP3<sup>+</sup> cells (Figure 1B). These results suggest that ATRA is a novel inducer of FoxP3<sup>+</sup> cells. The optimal ATRA concentration for induction of FoxP3 was 2-10 nM. The culture condition  
5 included only T cells and not other cell types, and thus, the effect of ATRA on induction of FoxP3 is thought to be direct on T cells.

## EXAMPLE 2

**[0049]** *RAR $\alpha$  is induced in ATRA-treated T cells.* RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$   
10 are the major receptors that are likely to mediate the function of ATRA. The mRNA expression of RARs and RXRs in freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells was determined, as well as in CD4<sup>+</sup>CD25<sup>-</sup> T cells activated in ATRA. Neonatal human cord blood CD4<sup>+</sup> CD25<sup>-</sup> naïve T cells were cultured in a T cell activation condition with IL-2 (25 U/ml) and PHA (5  $\mu$ g/ml) in the presence  
15 or absence of ATRA (2 nM) for 6 days. Freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells are shown for comparison. RT-PCR was performed, and a representative set of results out of 4 independent experiments are shown.

**[0050]** The most highly expressed receptor in response to ATRA was RAR $\alpha$  among the 6 RAR and RXR family receptors. Expression of RAR $\alpha$  & RAR $\beta$  and  
20 RXR $\gamma$  at low levels was detected in freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup>

CD4 T cells (Figure 2).

### EXAMPLE 3

**[0051]** *Retinoids induce FoxP3 expression in T cells through RAR $\alpha$ .* As

5 RAR $\alpha$  can be induced in T cells in response to ATRA, the function of this receptor in induction of FoxP3 was examined using an RAR $\alpha$  antagonist, Ro41-5253. The RAR $\alpha$  antagonist, Ro41-5253 completely suppressed the FoxP3<sup>+</sup> cell induction effect of ATRA. An RAR $\alpha$ , but not the RXR, agonist (methoprene acid), was able to induce FoxP3. FoxP3 protein expression in CD4 T cells, determined  
10 by intracellular staining by PE-labeled anti-FoxP3 antibody, is shown as FACS dot plots (Figure 3A) and graphs (Figure 3B). Neonatal human cord blood CD4<sup>+</sup> CD25<sup>-</sup> T cells were cultured in a T cell activation condition with IL-2 (25 U/ml) and PHA (5  $\mu$ g/ml) in the presence and absence of indicated agonists or antagonists for 6 days. The concentrations used to obtain the data were 2 nM (ATRA), 1  $\mu$ M  
15 (Ro41-5253), 5 nM (AM-580), and 10  $\mu$ M (methoprene acid). Data from four independent experiments were combined (Figure 3B).

**[0052]** The RAR $\alpha$  antagonist completely suppressed the induction of FoxP3 by ATRA (Figures 3A and B), suggesting that RAR $\alpha$  is a dominant receptor that mediates the effect of ATRA. Although RARs are major receptors for ATRA, it is

possible that metabolites of ATRA would bind RXRs to induce FoxP3. Therefore, a RAR $\alpha$ -specific agonist, AM-580, was also employed to induce FoxP3. In a manner similar to ATRA, AM-580 was able to induce FoxP3 (Figures 3A and B). The optimal concentration of AM580 for induction of FoxP3 was 5 nM (not shown). A pan-RXR agonist, methoprene acid, had only marginal effects on induction of FoxP3 even at a very high concentration (20  $\mu$ M, Figure 3A). The two agonists when combined did not induce FoxP3 at higher than the levels induced by the RAR $\alpha$  agonist alone. Taken together these data support the role of RAR $\alpha$  in ATRA-induced FoxP3 expression.

10

#### EXAMPLE 4

**[0053]** *Retinoids enhance the effect of TGF- $\beta$  in induction of FoxP3.* TGF- $\beta$  is a potent inducer of FoxP3 (Chen et al., 2003, J Exp Med). Therefore, the interaction between ATRA and TGF- $\beta$  in their effects on FoxP3 expression was examined. Figures 4A and 4B show that ATRA and TGF- $\beta$  cooperate in induction of FoxP3<sup>+</sup> T cells (Figure 4A), and FoxP3 induction by TGF- $\beta$ 1 (2 ng/ml) is not dependent on RAR $\alpha$  (Figure 4B). Neonatal human cord blood CD4<sup>+</sup> CD25<sup>-</sup> T cells were cultured in a T cell activation condition with IL-2 (100 U/ml) and PHA (5  $\mu$ g/ml) in the presence and absence of indicated agonists or

15

antagonists for 6 days. Ro41-5253 is a RAR $\alpha$  antagonist. A representative data set out of four independent experiments is shown. ATRA was used at 2 nM.

**[0054]** Both ATRA and TGF- $\beta$  had clear effects on induction of FoxP3<sup>+</sup> T cells, and ATRA, when added to the culture with TGF- $\beta$ , enhanced the effect of  
5 TGF- $\beta$  (Figure 4A). The RAR $\alpha$  antagonist, Ro41-5253, blocked only ATRA-induced but not TGF- $\beta$ -induced FoxP3 induction (Figure 4B).

#### EXAMPLE 5

**[0055]** *ATRA-treated T cells have the phenotype of anti-inflammatory or  
10 suppressor T cells.* Generally, regulatory T cells are hypo-proliferative and can suppress the proliferation of target T cells (CD4<sup>+</sup>CD25<sup>-</sup> T cells) in co-culture. It was therefore determined if the ATRA-induced FoxP3<sup>+</sup> cells had these features. Figures 5A and 5B show that ATRA-treated T cells are hypo-proliferative (A) and suppressive in function (B). Freshly isolated CD4<sup>+</sup> CD25<sup>-</sup> T cells were cultured  
15 with ATRA (2 nM), TGF- $\beta$ 1 (2 ng/ml) or ATRA+TGF- $\beta$ 1 in a T cell activation condition (IL-2 +PHA) for 6-7 days. Then, the T cells were co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> cells for 5 additional days. A 3H-thymidine incorporation assay was performed to assess the proliferation potentials of the cultures.

**[0056]** Similar to TGF- $\beta$ 1-treated cells, ATRA-treated T cells had a low

proliferation potential compared to fresh CD4<sup>+</sup>CD25<sup>-</sup> cells and cultured control cells. ATRA+TGF- $\beta$ 1-treated cells even had lower proliferative potentials. ATRA-treated T cells had suppressive activity on CD4<sup>+</sup>CD25<sup>-</sup> T cells at levels similar to that of TGF- $\beta$ 1-treated cells. TGF- $\beta$ 1+ATRA-treated cells were most  
5 suppressive among the three (Figures 5A-5B). Overall, the suppressive activities of the cells were closely correlated with their FoxP3<sup>+</sup> cell frequencies or the FoxP3 induction activities of the agonists.

#### EXAMPLE 6

10 **[0057]** *Retinoid-induced FoxP3<sup>+</sup> cells have cell-killing activities.* Regulatory T cells may use a number of different effector mechanisms to suppress target cells. Granzymes are a class of such effector molecules implicated in regulatory function. Figures 6A and 6B show that ATRA-treated FoxP3<sup>+</sup> T cells uniquely express granzymes as potential effector molecules. FoxP3<sup>+</sup> T cells, generated  
15 from cord blood CD4<sup>+</sup> CD25<sup>-</sup> T cells with ATRA, were examined for their expression of granzyme A, granzyme B and perforin. Also included were FoxP3<sup>+</sup> T cells, generated with IL-2 (control) or IL-2 (25 U/ml) and TGF- $\beta$  (2 ng/ml).

**[0058]** To determine the cell killing activity, FoxP3<sup>+</sup> T cells, generated from cord blood CD4<sup>+</sup> CD25<sup>-</sup> T cells with IL-2, ATRA, TGF  $\beta$ 1 or ATRA+TGF  $\beta$ 1, were  
20 examined for cell killing activity (Figure 7). PHA-activated B cells were used as

target cells at a target:effector ratio of 1:10. EGTA was added to suppress perforin-dependent cell killing activity. The retinoid-induced FoxP3<sup>+</sup> cells had efficient cell killing activity towards B cells as shown in Figures 8A and 8B. This cell killing activity was inhibited by EGTA (an inhibitor of perforin pathway),  
5 suggesting that the cells kill target cells in a perforin-dependent manner.

#### EXAMPLE 7

**[0059]** *Retinoids induce FoxP3<sup>+</sup> cells with homing receptors for mucosal tissues.* The migration ability of T cells is regulated by the trafficking receptors  
10 expressed by the T cells and determines the tissue sites of T cell effector function. Therefore the homing receptor phenotype of retinoid-induced FoxP3<sup>+</sup> cells was examined and the results shown in Figure 8. Neonatal human cord blood CD4<sup>+</sup> CD25<sup>-</sup> T cells were cultured in a T cell activation condition (IL-2 and PHA; 100 U/ml and 5 mg/ml respectively) in the presence and absence of ATRA  
15 (2 nM) for 6 days. Cells were stained with monoclonal antibodies to chemokine receptors for flow cytometry analysis (Figure 8A). Chemotaxis assay to the mucosal tissue chemokine CCL25 (5 µg/ml) was performed using a Transwell migration assay (Figure 8B). Three independent experiments were combined and averages and SEM are shown. Retinoid-induced FoxP3<sup>+</sup> cells expressed a  
20 number of trafficking receptors: homeostatic secondary lymphoid (CCR7 and L-

selectin), antigen-induced inflammatory (CCR4 and CXCR3), and mucosal tissue homing receptors (CCR9 and integrin  $\beta 7$  chain). Only the mucosal tissue homing receptors (CCR9 and integrin  $\beta 7$  chain) were uniquely expressed by the retinoid-induced FoxP3<sup>+</sup> cells compared with control (IL-2-induced) FoxP3<sup>+</sup> cells (Figure 5 8).

#### EXAMPLE 8

**[0060]** *The FoxP3 induction effect of retinoids is conserved in both humans and mice.* It was determined if ATRA could induce FoxP3 in mouse T cells.

10 DO11.10<sup>+</sup> (Rag2<sup>-/-</sup>) mice do not have FoxP3<sup>+</sup> T cells, because the antigen (ovalbumin) specific for DO11.10 T cells is not expressed in the mice (Walker et al., 2003, J Exp Med). These CD4<sup>+</sup> CD25<sup>-</sup> T cells were cultured in a T cell activation condition (irradiated splenocytes with 2 mg/ml of OVA<sub>323-339</sub> antigen peptide and 100 U/ml of IL-2) in the presence or absence of ATRA. Similar to 15 human T cells, ATRA induced FoxP3 expression in T cells (Figure 9A). Mouse FoxP3<sup>+</sup> T cells, primed in retinoic acid, expressed the mucosal tissue homing receptors CCR9 and  $\alpha 4\beta 7$  as unique trafficking receptors (Figure 9B). Other retinoids such as AM-580 (a RAR $\alpha$  agonist) and methoprene acid (a RXR agonist) were also tested. AM-580 was a good inducer of FoxP3 at low 20 concentrations (Figure 9C). Methoprene acid was able to induce FOXP3 only at

very high concentrations (optimal dose =  $\sim 100 \mu\text{M}$ ). Figures 9A-9C show a representative data set out of four independent experiments is shown.

#### EXAMPLE 9

5   **[0061]**     *Retinoid-induced FoxP3<sup>+</sup> cells selectively migrate to several mucosal tissues.* The in vivo homing behavior of the retinoid-induced FoxP3<sup>+</sup> T cells was determined. For this, retinoid-induced FoxP3<sup>+</sup> cells were prepared from CD4<sup>+</sup>CD25<sup>-</sup> T cells as described in Example 8, and injected intravenously into host mice. Mice were sacrificed 20 hours later, and migration of injected FoxP3<sup>+</sup> cells and FoxP3<sup>-</sup> T cells to 10 different tissue sites was determined. Control T cells were labeled with the red fluorescent dye tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC), while test T cells were labeled with the green dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). The two T cell subsets ( $10^7$  cells per mouse) were co-injected into BALB/c mice via a tail vein.

10   The lamina propria lymphocytes (LPLs) were isolated after removing the epithelial cells with 5 mM EDTA (5 times), digestion (3 times, 45 min each) with 300 U/ml collagenase (type 3, Worthington, Lakewood, NJ) and 100  $\mu\text{g/ml}$  DNase I (Worthington), filtration through a nylon mesh, and centrifuge in a 40/75% Percoll-gradient. The cells were stained with antibodies to CD4 and FoxP3.

15   Normalized homing indexes were calculated by a formula ( $\text{HI} = a/b$ ), where

20

a=[test cells migrated into the tissue site]÷[control cells migrated into the tissue site], and b=[test cells in input]÷[control cells in input]. LP (SI) and LP (LI) are lamina propria of small and large intestine respectively. "P. cavity" is peritoneal cavity, and "Peyer's p." is Peyer's patches. Three independent experiments were  
5 combined and averages and SEM are shown.

**[0062]** As shown in Figure 10, the retinoid-induced FoxP3<sup>+</sup> T cells preferentially migrate to the small intestinal lamina propria and Peyer's patches among the tissue sites examined. Migration to peritoneal cavity and lymph nodes (MLN) was also slightly elevated for retinoid-induced compared to control FoxP3<sup>+</sup>  
10 T cells. Interestingly, FoxP3<sup>+</sup> CD4 T cells were more efficient in migration to the mucosal tissue sites than FoxP3<sup>-</sup> cells.

#### EXAMPLE 10

**[0063]** *Regulation of mucosal FoxP3<sup>+</sup> cell numbers by vitamin A in vivo and*  
15 *in vitro.* The *in vivo* effect of vitamin A on numbers of intestinal FoxP3<sup>+</sup> T cells was determined. Adult mice were fed with food with (control mice) or without vitamin A for 6 weeks, and then the numbers of FoxP3<sup>+</sup> T cells in small and large intestine, and mesenteric lymph nodes were determined by flow analysis (Figure 11A). The mice fed with vitamin A-deficient food had significantly smaller  
20 numbers of  $\alpha 4\beta 7^{+}$  mucosal tissue homing FoxP3<sup>+</sup> T cells in the large and small

intestines compared to the mice fed with control food having vitamin A. However, there was no reduction in lymph nodes (MLN), demonstrating a tissue specificity. These data demonstrate that the induction of FoxP3<sup>+</sup> cells by vitamin A and retinoids takes place *in vivo*.

5   **[0064]**     Also provided is a method with which almost all of naïve T cells can be converted into mucosal tissue homing FoxP3<sup>+</sup> T cells by utilizing ATRA and cytokines. Mouse naïve T cells were cultured with ATRA (1-100 nM), IL-2 (10-1000 U/ml) and TGF-β1 (1-100 ng/ml), together with TCR activators (concanavalin A or PHA at 1-10 μg/ml) for 6-7 days. Figure 11B shows that  
10   ~95% of T cells after culture were FoxP3<sup>+</sup> regulatory T cells expressing the mucosal tissue homing receptor α4β7. For comparison, the control culture generated fewer FoxP3<sup>+</sup> T cells and these cells did not express α4β7. This method reproducibly generates 92-99% pure mucosal tissue homing FoxP3<sup>+</sup> T cells.

15

#### EXAMPLE 11

**[0065]**     *Regulation of inflammation by retinoid-induced FoxP3<sup>+</sup> T cells in vivo.*

The immune regulatory function of retinoid-induced FoxP3<sup>+</sup> T cells was determined *in vivo*. Figure 12 shows that retinoid-induced FoxP3<sup>+</sup> regulatory T  
20   cells can suppress the induction of inflammation in mice. A mouse model of

inflammation (SCID mouse) was utilized, which is commonly used as a general animal model of inflammatory immune responses. These mice lose weight and eventually die when they are injected with inflammatory T cells ( $CD4^+CD62L^+CD45RB^+$ /mouse). The major cause of the weight loss is the inflammation in the intestine. SCID mice were injected with inflammatory T cells ( $0.5 \times 10^6$   $CD4^+CD62L^+CD45RB^+$ /mouse) to induce intestinal inflammation. At the same time, some mice were injected with retinoid-induced FoxP3<sup>+</sup> regulatory T cells ( $0.5 \times 10^6$ /mouse). Control SCID mice are the mice that were not treated. It should be noted that the weight loss due to the inflammation was effectively suppressed by the retinoid-induced FoxP3<sup>+</sup> regulatory T cells (vitamin A FoxP3<sup>+</sup> cells). Each group contained 3-5 mice. The data in Figure 12 show that the weight loss in these mice due to the inflammation was effectively suppressed by the retinoid-induced FoxP3<sup>+</sup> regulatory T cells. The effective dose was  $0.5 \times 10^6$  cells/mouse injected intravenously. This dose may be even lower and the same efficacy obtained.

## EXAMPLE 12

**[0066]** *Regulation of anti-inflammatory T cell numbers in vivo by controlling vitamin A consumption and retinoic acid concentration.* BALB/c mice were fed with rodent diets containing a normal level of retinol acetate (2500 U/kg; VAN),

excessive level (25,000 U/kg; VAS) or no retinol acetate (0 U/kg; VAD) for 12 weeks following birth. At 12 weeks, naïve CD4<sup>+</sup> T cells from DO11.10 rag2<sup>-/-</sup> mice were transferred into the VAN, VAS, and VAD mice and the mice were immunized intraperitoneally with ovalbumin (500 µg/mouse) in incomplete  
5 adjuvant. All-trans retinoic acid (1 mg/ kg) was injected intraperitoneally on day 0, 2, 4, and 6. The frequencies of mucosal FoxP3<sup>+</sup> T cells (% of CD4<sup>+</sup> T cells) were determined in various organs 7 days post immunization. This experiment provided a method to control the numbers of suppressor T cells (i.e. anti-inflammatory T cells) *in vivo* by controlling the consumption of vitamin A and  
10 concentration of retinoic acid. Abbreviations of organ names: MLN (mesenteric lymph node), Per. Cavity (peritoneal cavity), PLN (peripheral lymph node), PP (Peyer's patches), and Col. Patches (colonic patches).

**[0067]** Figure 13 is an example that the numbers of anti-inflammatory T cells can be increased or decreased in the body by controlling the consumption of  
15 vitamin A. More specifically, by lowering the vitamin A/retinoic acid concentrations, the numbers of anti-inflammatory T cells in mucosal tissues can be decreased. Lowering the vitamin A/retinoic acid concentrations in the body can be achieved through special diet with reduced vitamin A or through administering retinoid receptor antagonists. By increasing the concentrations,  
20 the numbers of the anti-inflammatory T cells can be increased. Increasing the

retinoid concentrations in the body can be achieved through use of fortified vitamin A supplements or through administering retinoid receptor agonists. It has been shown that these anti-inflammatory T cells induced by retinoic acid are highly effective in suppression of tissue inflammation (Figure 12).

- 5   **[0068]**   It should be understood, of course, that the foregoing relates to exemplary embodiments of the invention and that modifications may be made without departing from the spirit and scope of the invention as set forth in the following claims.

## WE CLAIM:

1. A method for generating mucosal tissue homing immunosuppressive T-cells comprising treating naive T-cells with a retinoid or a retinoid receptor agonist.

2. The method of claim 1 wherein the retinoid is retinol palmitate, retinyl palmitate, retinol acetate, beta-carotene or combinations thereof.

3. The method of claim 1 wherein the retinoid is all-trans-, 9-cis-, 11-cis retinoic acid or combinations thereof.

4. The method of claim 1 wherein the retinoid receptor agonist is AM-  
5 580, BMS641, BMS961, CD666, TTNPB, ATRA, Ro25-7386, methoprene acid or combinations thereof.

5. The method of any one or more of claims 1-4 wherein the retinoid or retinoid receptor agonist has a concentration of from about 100 pM to about 1 mM.

6. The method of any one or more of claims 1-4 wherein the retinoid or retinoid receptor agonist has a concentration of from about 100 pM to about 100 nM.

7. The method of any one or more of claims 1-6 wherein the mucosal tissue is alimentary tract, respiratory tract, lung, stomach, intestines or colon.

8. The method of any one of claims 1-7 wherein the naïve T-cells are obtained from cord blood, peripheral blood, secondary lymphoid tissue or combinations thereof.

9. The method of any one or more of claims 1-8 wherein the naïve T-cells are treated *ex vivo*.

10. The method of any one or more of claims 1-9 wherein the naïve T-cells are treated *in vivo*.

11. The method of any one or more of claims 1-10 further comprising  
5 the reduction or prevention of inflammation in a mucosal tissue by the generated immunosuppressive T-cells.

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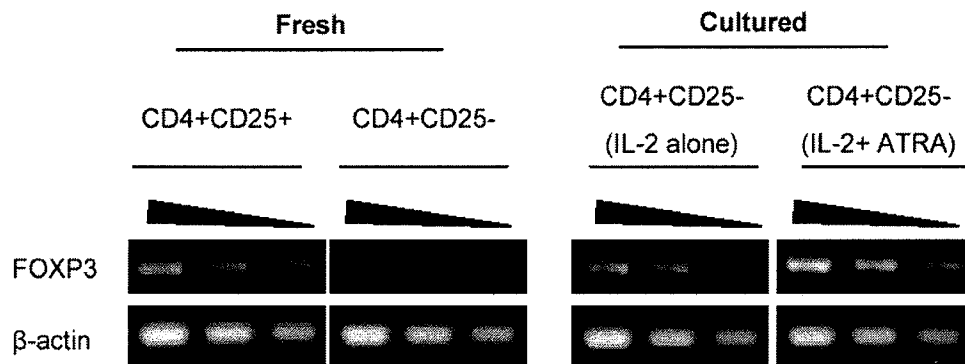


**FIGURE 1A**

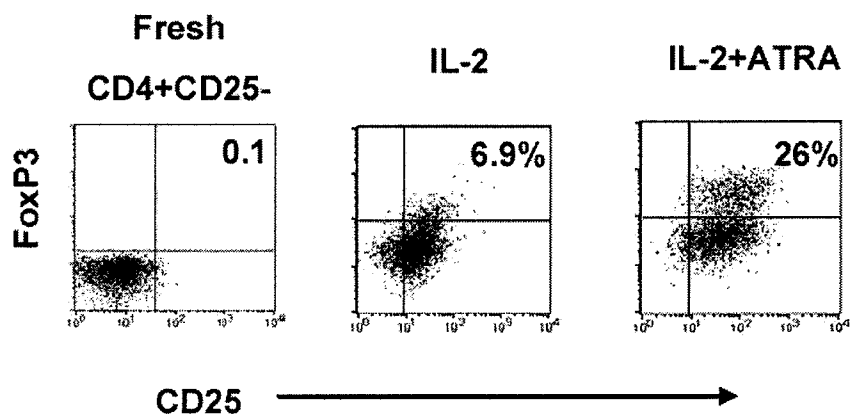


**FIGURE 1B**

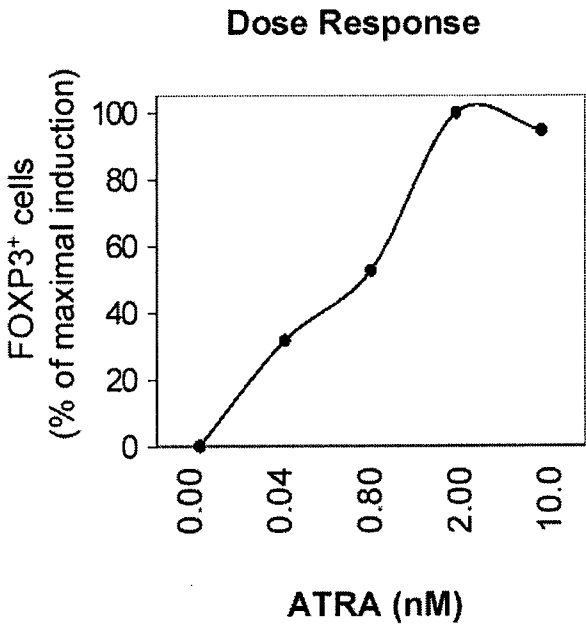


**FIGURE 1C**

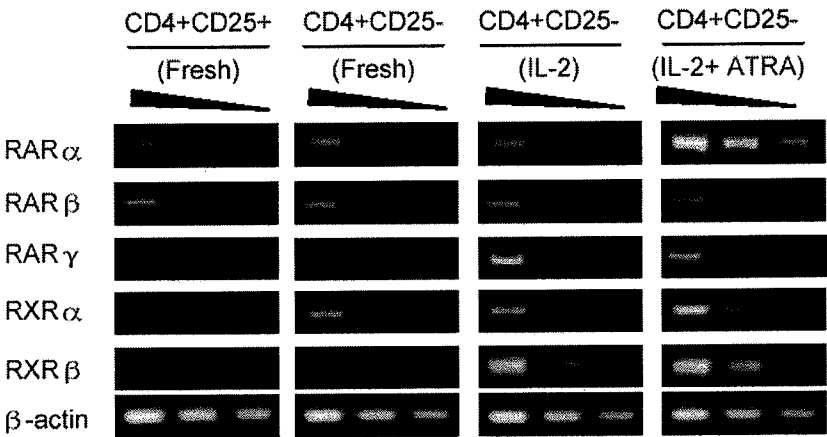


**FIGURE 2**

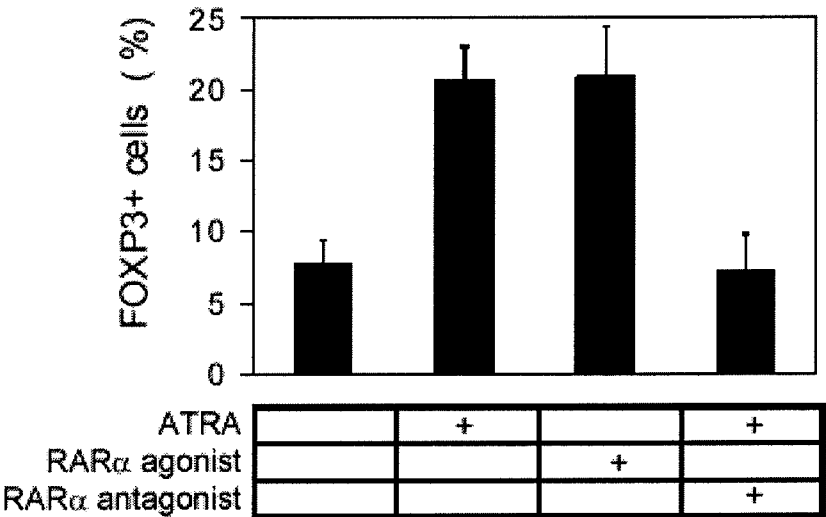


FIGURE 3A

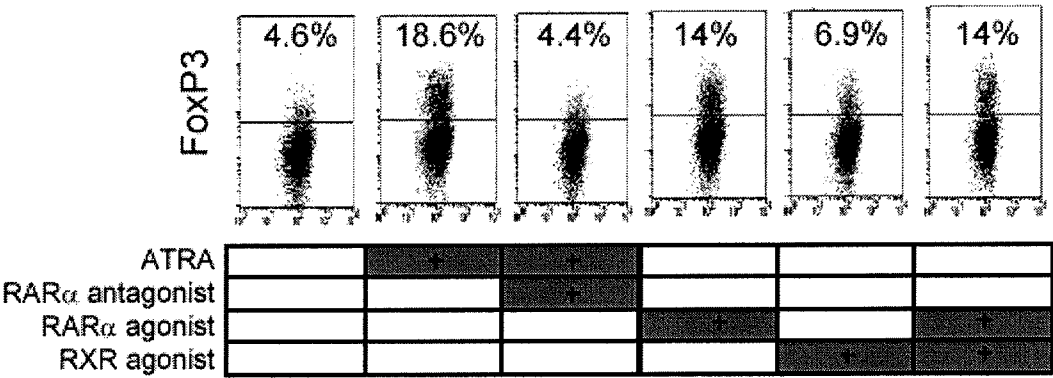


FIGURE 3B

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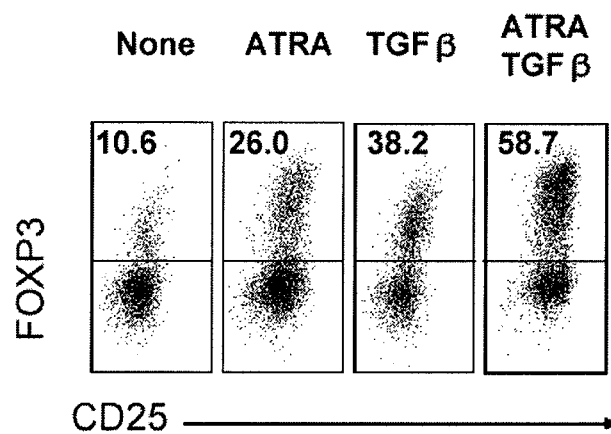


FIGURE 4A

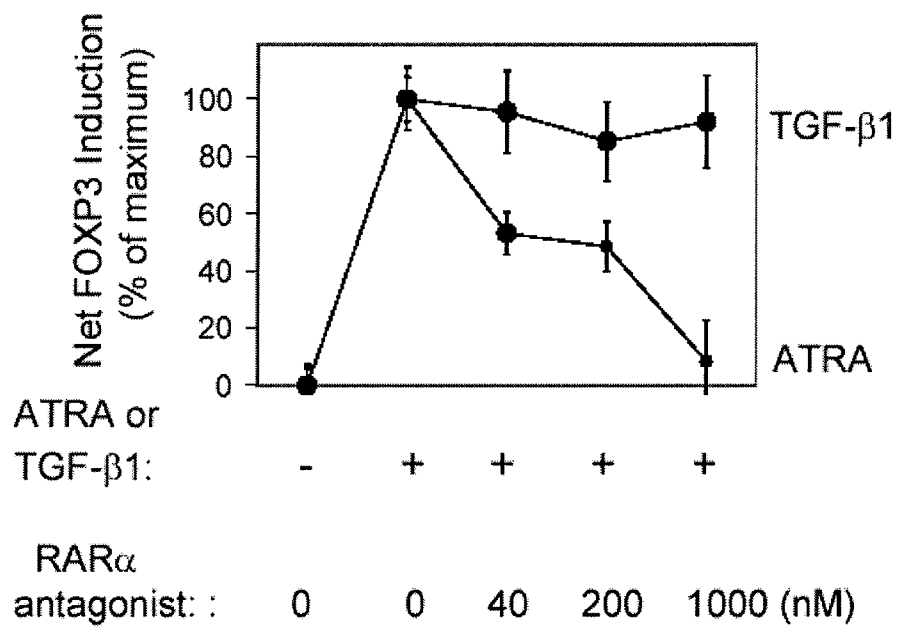
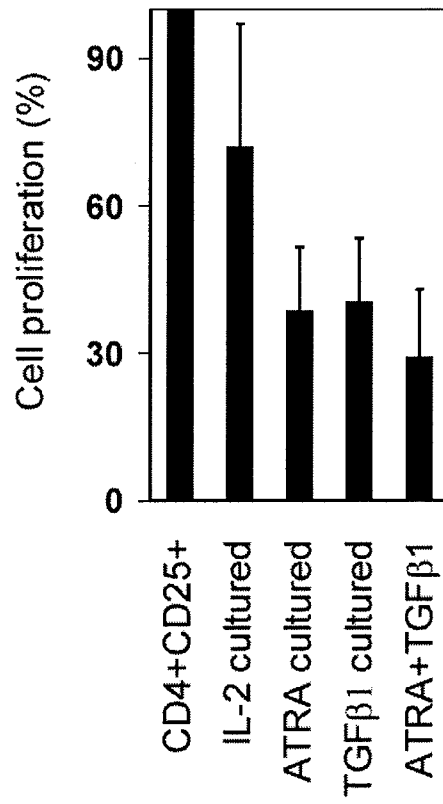


FIGURE 4B

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**FIGURE 5A**

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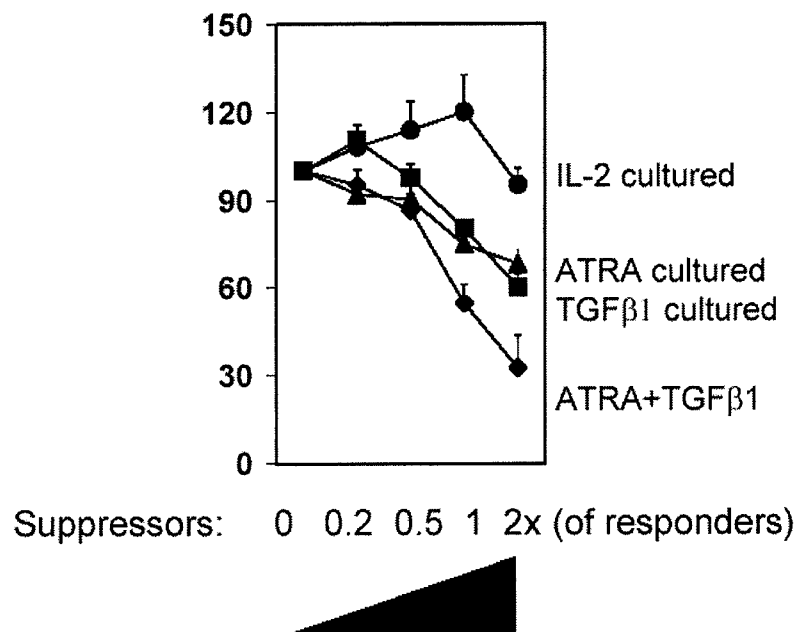
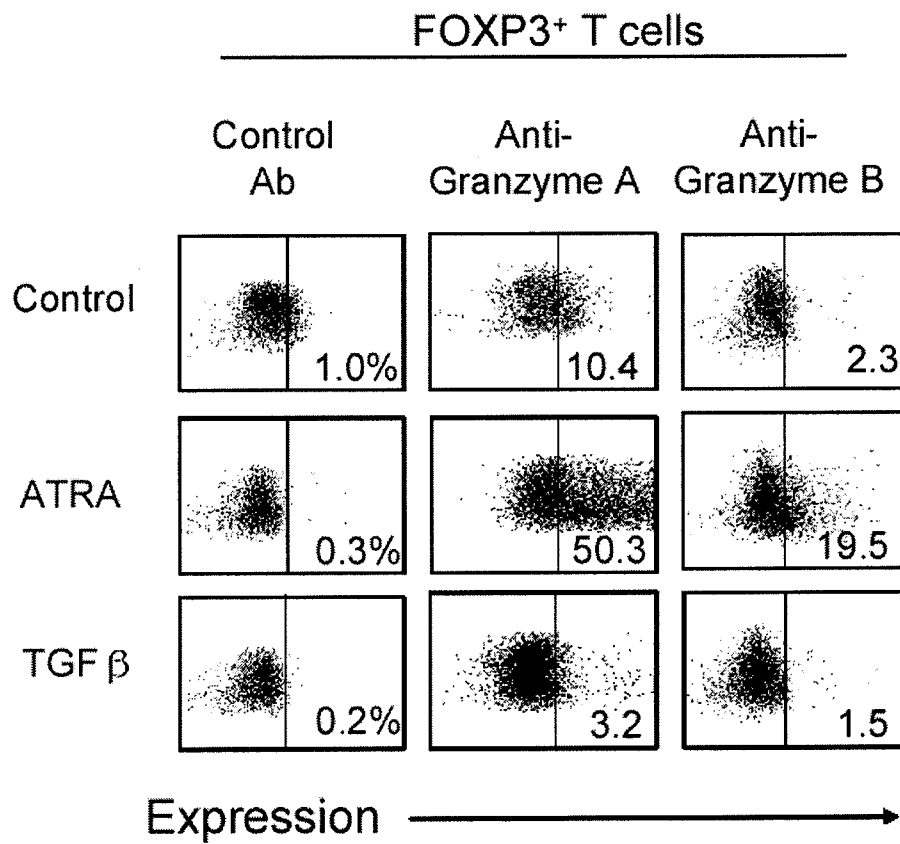


FIGURE 5B

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**FIGURE 6A**

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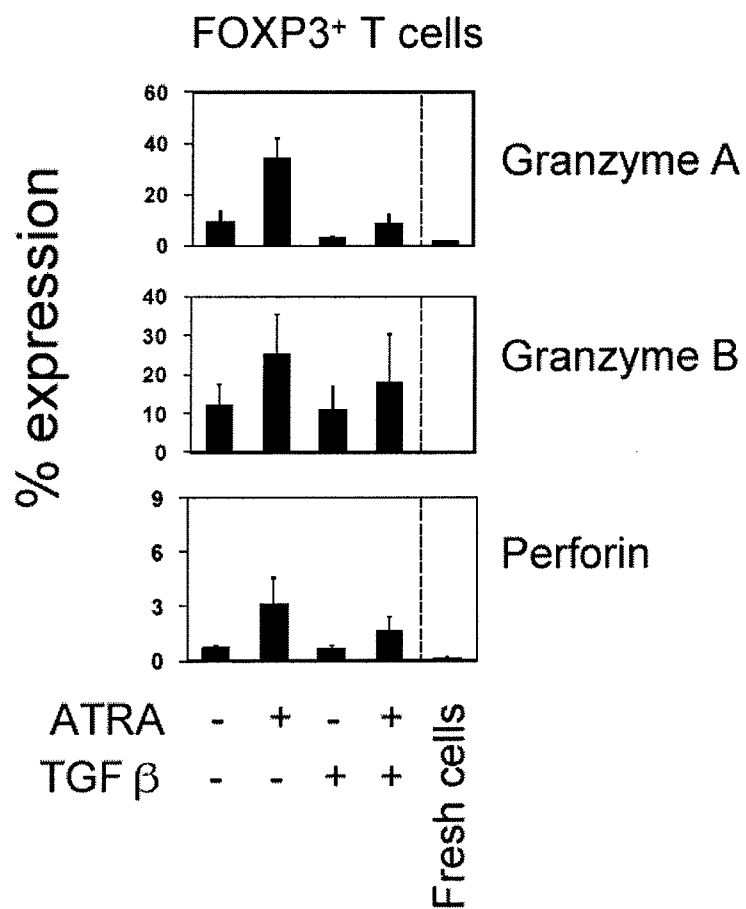


**FIGURE 6B**

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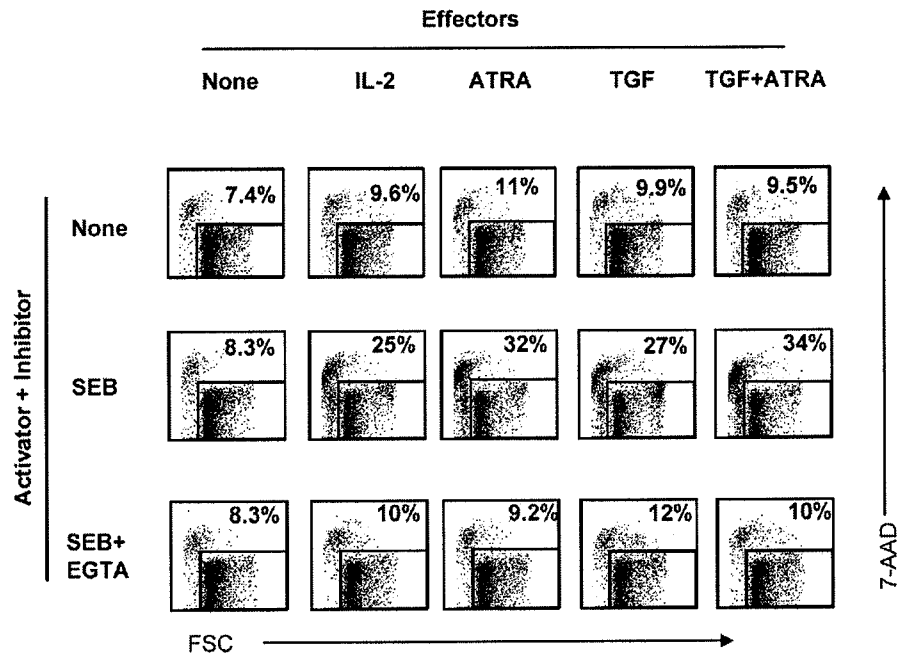


FIGURE 7

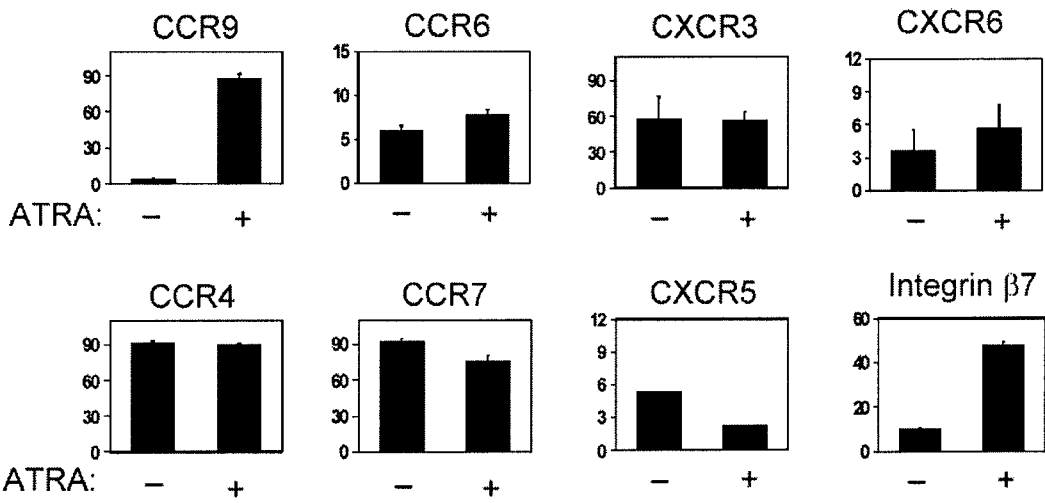


FIGURE 8A

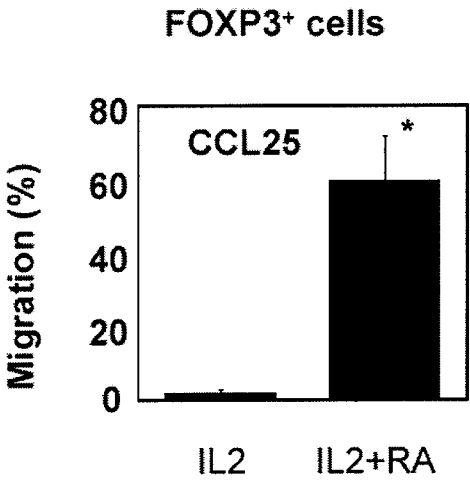


FIGURE 8B

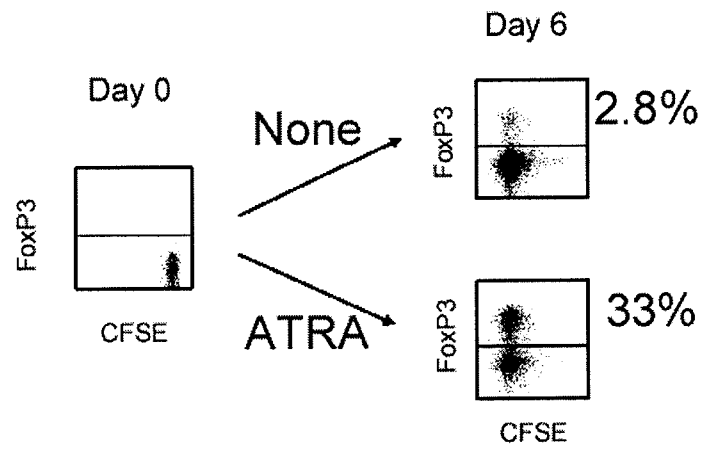


FIGURE 9A

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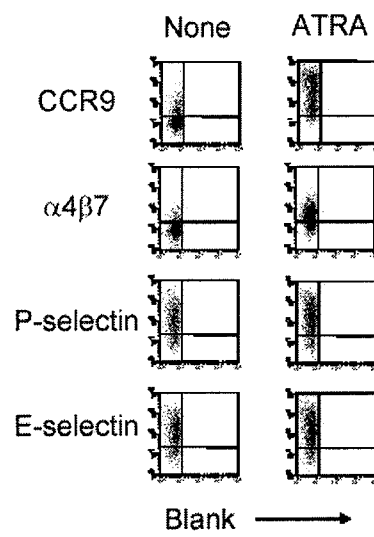


**FIGURE 9B**

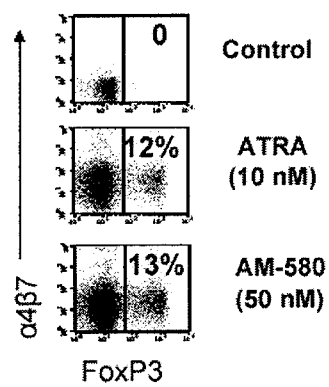


**FIGURE 9C**

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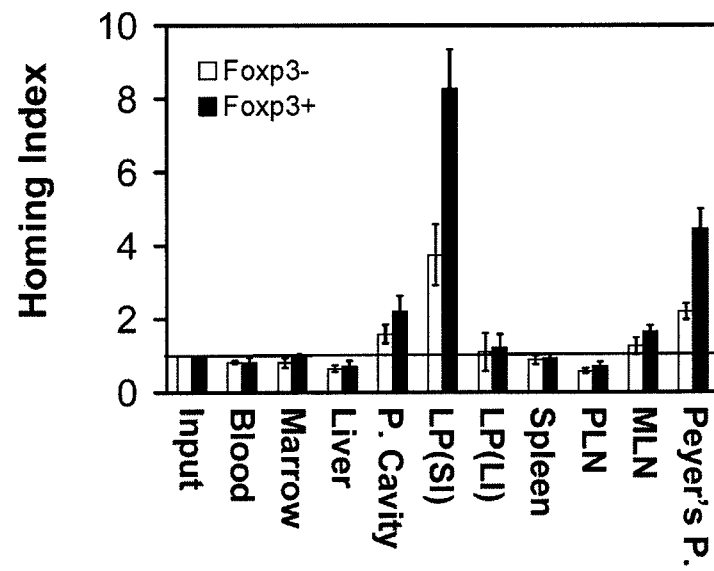


FIGURE 10

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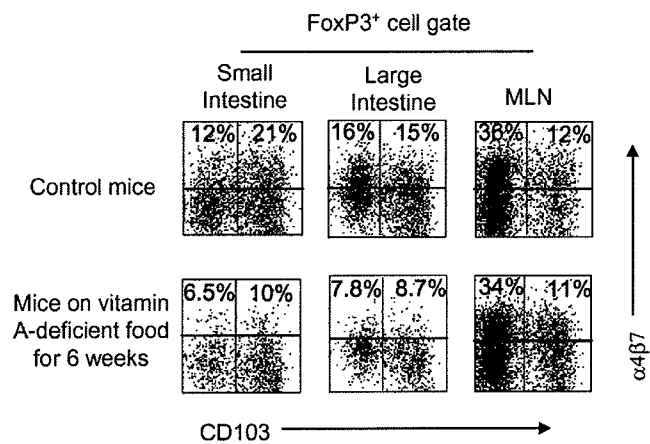


FIGURE 11A

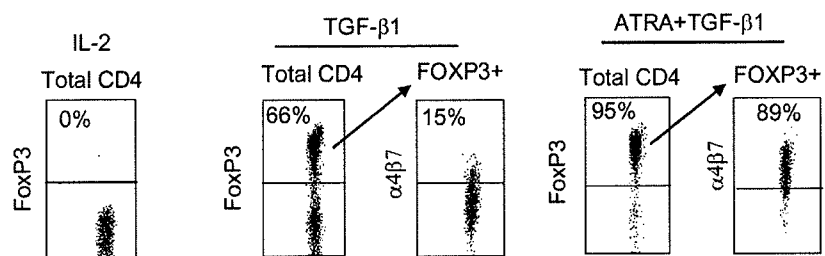
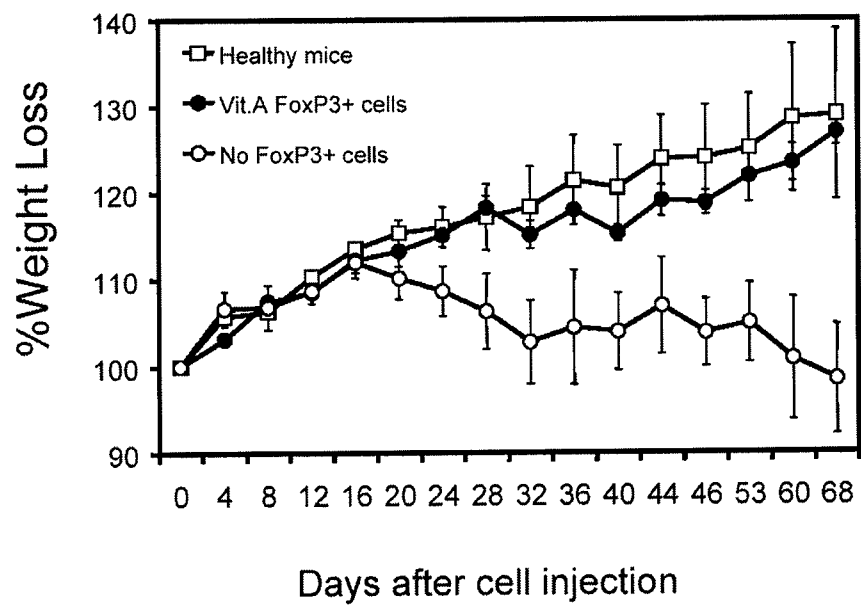


FIGURE 11B

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**FIGURE 12**

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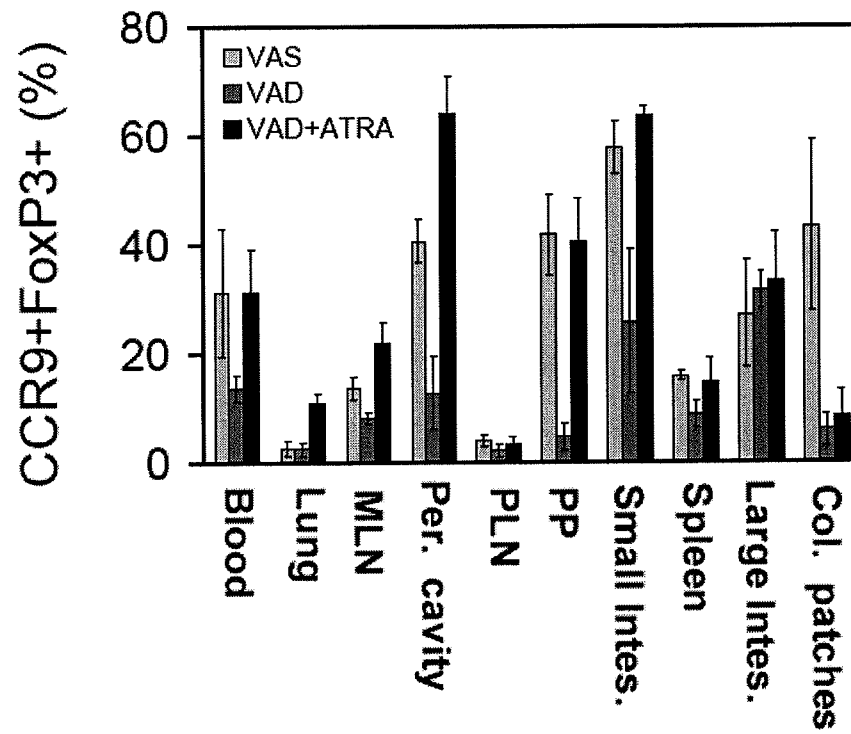


FIGURE 13

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/62125

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/07 (2008.04)

USPC - 514/725

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC- 514/725

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
USPC- 514/529, 703, 885, 886 (text search-see search terms below)

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

PubWEST (USPT, PGPB, EPAB, JPAB) and Google Patent/Scholar

Search terms: retinoid, retinoic acid, naive, T cell, homing, retinyl palmitate, foxp3

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*       | Citation of document, with indication, where appropriate, of the relevant passages                                                       | Relevant to claim No. |
|-----------------|------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X<br>-----<br>Y | IWATA et al. Retinoic acid imprints gut-homing specificity on T cells IN Immunity 2004, 21:527-538; abstract, p528, para 1, p529, para 2 | 1, 3, 4<br>-----<br>2 |
| Y               | US 5,571,692 A (EVANS et al.) 05 November 1996 (05.11.1996) col 14, ln 17-28                                                             | 2                     |

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

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

23 July 2008 (23.07.2008)

Date of mailing of the international search report

30 JUL 2008

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/62125

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-11  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

### Remark on Protest

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