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(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY OF UTAH RESEARCH FOUNDATION** [US/US]; 615 Arapeen Drive, Suite 310, Salt Lake City, Utah 84108 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CHEN, Xinjian** [US/US]; 2679 Grand Vista Way, Salt Lake City, Utah 84121 (US). **GUO, Xiaohua** [CN/US]; 15 N. Medical Drive, 1520 EEJ, Department of Pathology, Salt Lake City, Utah 84112 (US). **JENSEN, Peter, E.** [US/US]; 15 N. Medical Drive., 1520 EEJ, Department of Pathology, Salt Lake City, Utah 84112 (US).

(74) Agents: **MEARA, Joseph, P.** et al.; Foley & Lardner LLP, 150 East Gilman Street, Post Office Box 1497, Madison, US 53701-1497 (US).

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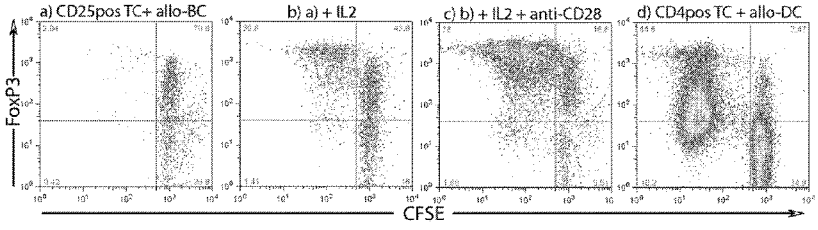
(54) **Title**: METHODS FOR EXPANDING HUMAN T REGULATORY CELLS AND USES OF SAME



(57) **Abstract**: This disclosure describes methods for expanding regulatory T cells. Human regulatory T cells are expanded by culturing a human regulatory T cell with a B cell to produce a population of allospecific human regulatory T cells. The methods include culturing a T cell and B cell in the presence of a cytokine and a stimulatory antibody. Regulatory T cells may also be expanded using weak stimulation of the T cell receptor or treatment with the phorbol ester phorbol 12-myristate 13-acetate. The disclosure further includes methods for treating immune-related disorders by administering the expanded human regulatory T cells to a subject in need thereof.

WO 2010/129770 A1

METHODS FOR EXPANDING HUMAN T REGULATORY CELLS AND USES OF SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/215,746, filed May, 7, 2009, and U.S. Provisional Application No. 61/236,619, filed August 25, 2009, the entire contents of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] This disclosure relates generally to compositions and methods for the production of regulatory T cells. More particularly, the disclosure relates to the production of human T regulatory cells using allogeneic B cells and to the production of regulatory T cells using weak stimulation of the T cell receptor (TCR).

BACKGROUND

[0003] The following description is provided to assist the understanding of the reader. None of the information provided or references cited is admitted to be prior art to the present invention.

[0004] The naturally arising regulatory FoxP3$^+$ CD4$^+$ T cells (Treg) play an important role in the induction and maintenance of immunological tolerance to self-antigens. Mouse Treg cells prevent allograft rejection and graft versus host disease (GVHD) when adoptively transferred into recipient hosts, unveiling the attractive prospect of using Tregs as therapeutic tools for treatment of transplant rejection and GVHD in humans. In contrast to conventional drug-based immunosuppressive therapy, Treg cell-based therapy has the potential to provide long lasting, nontoxic and antigen-specific suppression of graft rejection or GVHD without compromising protective immunity in the host. Since the number of Treg cells that can be obtained from a donor is limited, freshly isolated Treg cells need to be expanded *ex vivo* to generate a sufficient number of cells for therapeutic applications. In addition, expanded Treg cells have been reported to be more therapeutically effective than primary Treg cells.

SUMMARY

[0005] The present technology provides methods for expanding Treg cells. In one aspect, the disclosure provides a method for expanding human regulatory T cells comprising co-

culturing a human regulatory T cell and a B cell in the presence of a cytokine and a co-stimulatory agent to produce a population of allospecific human regulatory T cells.

[0006] In one embodiment, the cytokine is an interleukin. In one embodiment, the interleukin is IL-2. In one embodiment, in the co-stimulatory agent activates CD28. In one embodiment, the co-stimulatory agent is a CD28 ligand (*e.g.,* CD80 or CD86). In one embodiment, the co-stimulatory agent is an anti-CD28 antibody, and in a more specific embodiment, the anti-CD28 antibody is CD28.2.

[0007] In one embodiment, the B cell has been transformed to express the cytokine, the co-stimulatory agent, or both the cytokine and co-stimulatory agent. In one embodiment, the co-stimulatory agent is a B cell transformed to express the co-stimulatory agent or membranes derived from these cells. In one embodiment, the B cell has been transformed to express CD80 and/or CD86.

[0008] The technology provides for isolating T cells and B cells from different sources. Accordingly, some embodiments provide for isolating the regulatory T cell from a host, a donor, or a third party who is unrelated to the host and the donor. One specific source of regulatory T cells can be leukopheresis-derived leukocytes. In additional embodiments, the B cell is isolated from a host, a donor, or a third party who is unrelated to the host and the donor. In some embodiments, the B cell expresses a common alloantigen toward which regulatory T cell activity is desired. A similar embodiment provides that the B cell expresses a target HLA haplotype toward which regulatory T cell activity is desired. In one embodiment, the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a host. In one embodiment, the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a donor. In one embodiment, the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a third party who is unrelated to the host and the donor. In one embodiment, the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a host.

[0009] The technology provides that the regulatory T cell is a $CD25^+$ $CD4^+$ T cell or that the regulatory T cell is a $CD25^-$ $CD4^+$ T cell. In accordance with the methods described herein, the expanded human regulatory T cells express FoxP3 at a high level, remain anergic, and are potent suppressors of allospecific effector T cells.

[0010] In another aspect, the disclosure provides a method for expanding regulatory T cells, the method comprising: culturing a population of T cells with a T cell receptor agonist (TCR) under conditions wherein the T cell receptor is weakly stimulated, wherein Treg cells in the population of T cells are preferentially expanded compared to non-Treg cells.

[0011] In one embodiment, the preferential expansion includes proliferation of existing Treg cells and conversion of non-Treg cells to Treg cells. In one embodiment, the Treg cells have a $CD25^+$ $FoxP3^+$ $CD4^+$ phenotype.

[0012] In one embodiment, the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with a T cell receptor agonist. In one embodiment, the T cell receptor agonist is selected from the group consisting of: an anti-CD3 antibody, an antigen, and an allogenic antigen presenting cell. In one embodiment, the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with an anti-CD3 antibody. In one embodiment, the concentration of the anti-CD3 antibody in the culture is from about 0.0025 μg/mL to about 0.01 μg/mL.

[0013] In one embodiment, the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with an antigen. In one embodiment, the antigen is an OVA peptide. In one embodiment, the concentration of the OVA peptide in the culture is from about 0.01 μM to about 0.02 μM.

[0014] In another aspect, the disclosure provides a method for expanding regulatory T cells, the method comprising: culturing a population of T cells with a phorbol ester, wherein Treg cells in the population of T cells are preferentially expanded compared to non-Treg cells. In one embodiment, the phorbol ester is phorbol 12-myristate 13-acetate (PMA). In one embodiment, the concentration of the PMA in the culture is from about 10 nM to about 20 nM. In one embodiment, the culture does not include ionomycin.

[0015] The technology also provides allospecific regulatory T cell compositions produced by the present methods. These compositions may be used in therapeutic applications to treat a patient. In some embodiments, allospecific regulatory T cell products are used to treat a patient who suffers from an autoimmune disease, graft versus host disease, transplant rejection, or an immune-related inflammatory disease. In another aspect, the disclosure provides a method for treating an immunological disorder comprising administering to a transplant recipient an effective amount of the allospecific human regulatory T cells prepared

in accordance with the disclosed methods. In one embodiment, the immunological disorder is an autoimmune disease. In other embodiments, allospecific regulatory T cell products are used to treat a patient who suffers from multiple sclerosis, type 1 diabetes, inflammatory bowel disease, cardiovascular disease, Parkinson's disease, Alzheimer's disease, arthritis, IPEX, allergy, gastritis, lung inflammation, or from the destructive effects of an immune response to an infectious agent. Some embodiments provide for using allospecific regulatory T cell products to promote transplant tolerance in a host. In specific embodiments, the transplant is bone marrow or a solid organ.

BRIEF DESCRIPTION OF THE FIGURES

[0016] FIGs. 1A-1H show the results of a flow cytometry experiment to demonstrate that Treg cells were directly expanded with allogeneic B cells.

[0017] FIGs. 2A-2L show the results of a flow cytometry experiment to demonstrate that B-cell expanded Treg cells are anergic and potent suppressors.

[0018] FIGs. 3A-3J show the results of a flow cytometry experiment to demonstrate that B-cell expanded Treg cells have specificity for the alloantigens of the B-cells with which the Treg cells were expanded.

[0019] FIGs. 4A-4D show the results of a flow cytometry experiment to demonstrate that B-cell expanded Treg cells potently inhibit alloproliferation of third-party responder T cells in various Treg and responder cell combinations in an alloantigen-specific manner.

[0020] FIGs. 5A-5G show the results of a flow cytometry experiment in which Treg cells were expanded using the OVA peptide.

[0021] FIGs. 6A-6D show the results of a flow cytometry experiment in which Treg cells were expanded using an anti-CD3 antibody.

[0022] FIGs. 7A-C is a series of bar graphs showing that at high concentration, the anti-CD3 antibody 2c11 induced vigorous T cell proliferation dominated by $FoxP3^-$ non-Treg cells. At low concentrations, the antibody only induced modest T cell proliferation which is, however, dominated by $FoxP3^+$ Treg cells.

[0023] FIG. 8A is a graph showing the number of cells cultured with the anti-CD3 antibody 2c11 in the presence or absence of a TGF_β neutralizing antibody. FIG. 8B is a series of

graphs of flow cytometry data showing that when TGF_{β} neutralizing antibody was supplemented in the culture, the Treg cell conversion was diminished.

[0024] FIG. 9A-9C show a series of Western blots of $CD25^-$ non-Treg (CD25n) and $CD25^+$ Treg (CD25p) cells in whole cell lysate (A), cytoplasm (B) and nuclear extract (C). FIG. 9D shows analysis of these cells by immunofluorescent microscopy.

[0025] FIG. 10A-10F show the results of a flow cytometry experiment in which CFSE-labeled splenocytes were cultured with PMA alone or PMA plus ionomycin for five days.

[0026] FIGs. 11A-11D shows the results of differential DNA targeting of NFAT in Treg and activated non-Treg cells.

[0027] FIGs. 12A-D show the results of a flow cytometry experiment to demonstrate that third-party Treg cells prevent rejection of allogeneic bone marrow grafts in MHC-unrelated hosts.

DETAILED DESCRIPTION

[0028] The present technology generally provides methods for the *ex vivo* expansion of Treg cells. In one aspect, the methods involve *ex vivo* expansion of allospecific human Treg cells. In another aspect, the methods involve preferential expansion of Treg cells in a mixed population of T cells using weak stimulation of the TCR. The methods produce human regulatory T cells in sufficient amounts for therapeutic use in treating diseases and in reducing complications associated with tissue grafts and transplants. Accordingly, various aspects of the technology provide culture components and conditions that allow one to culture large amounts of Treg cells. Cultured human Treg cells are useful, alone or in combination with other therapies, for treating patients suffering from an autoimmune disease, graft versus host disease, transplant rejection, an immune-related inflammatory disease, or for promoting transplant tolerance in a host who is to receive a bone marrow, solid organ, or other transplant.

[0029] In accordance with one aspect, large amounts of allospecific human regulatory T cells may be produced by culturing a human regulatory T cell with a B cell in the presence of a cytokine and a stimulatory molecule. In certain embodiments, Treg cells were produced that expressed FoxP3 at a high level that was three-fold higher than that of the nondividing input $CD25^+$ $CD4^+$ T cells. In addition, while activated human non-Treg cells typically

express a low level of FoxP3, B-cell expanded Treg cells expressed FoxP3 at a level greater than 20-fold that of activated non-Treg cells.

[0030] Mouse primary B cells have the capacity to preferentially expand Treg cells in the absence of co-stimulation (Chen and Jensen, *Cutting Edge, J Immunol*, 2007). Moreover, addition of anti-CD28 stimulatory antibody to mouse B cells and mixtures of $FoxP3^+$ and $FoxP3^-$ cells resulted in T cell proliferation dominated by expansion of $FoxP3^-$ T cells (not Tregs). Attempts to reproduce preferential expansion of human Tregs using human primary B cells did not produce the same result. The present inventors found that exogenous stimulation of CD-28 is required for expansion of human Tregs. In some embodiments, this stimulation can be provided using anti-CD28 exposure or exposure to CD-28 ligands.

[0031] Besides high levels of FoxP3 expression, B-cell expanded Treg cells produced according to the methods described herein display other properties that are characteristic of Treg cells. The B-cell expanded Treg cells remain anergic, while activated non-Treg cells do not. As shown in Example 2, after resting in IL-2 for two days and re-stimulation with phorbol 12-myristate 13-acetate plus ionomycin, activated Treg cells produce levels of IL-2 and IFN-γ associated with an immune response, while the B-cell expanded Treg cells do not. Furthermore, the B-cell expanded Treg cells suppress responder T cells. For example, B-cell expanded Treg cells suppress the response of $CD25^-$ responder T cells to allogeneic monocyte-derived dendritic cells. As nonlimiting examples, the proliferation of responder T cells is abrogated at Treg to responder T cell ratios of from about 1:5 to about 1:20, and partially inhibited at a Treg to responder T cell ratio of from about 1:50 to about 1:100. The stable anergic phenotype and high suppressive activity of B-cell expanded human Treg cells is related to their high expression of FoxP3.

[0032] The B-cell expanded, allospecific Treg cells are much more potent than polyclonal Treg cells expanded with anti-CD3 and anti-CD28 antibodies plus IL-2. The high inhibitory potency of B-cell expanded Treg cells is the result of a high frequency of alloantigen-specific Treg cells in the expanded product and the high inhibitory capacity of the individual Treg cells.

[0033] In accordance with another aspect, weak TCR stimulation preferentially activates Tregs over Fox $P3^- CD4^+$ T cells in the culture. The weak TCR stimulation can be induced by low amounts of a TCR agonist, including antigen-specific or anti-CD3-mediated TCR

stimuli. In some embodiments, the TCR agonist is selected from the group consisting of: ovalbumin, anti-CD3 antibody, and allogenic APCs. A weak versus strong stimulation is provided by low or low high concentration of antigen or anti-CD3 antibody, respectively.

[0034] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. For example, reference to "a cell" includes a combination of two or more cells, and the like.

[0035] As used herein, "about" will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art, given the context in which it is used, "about" will mean up to plus or minus 10% of the enumerated value.

[0036] The term "alloantigen" refers to an antigen that differs from an antigen expressed by the recipient. Specifically, an alloantigen is a MHC polymorphism between a host individual and a donor individual of the same species, or between two populations of cells. In the context of a tissue graft or transplant, alloantigens are the nonself MHC expressed by the cells of allografted tissue that can induce an intense immune response in the recipient host and which is aimed at eliminating the transplanted cells. The immune reaction is the result of the host immune cells recognizing the alloantigenic cells or tissue as originating from a nonself source. If an alloantigen is presented to a member of the same species that does not have the alloantigen, it will be recognized as foreign and induce an immune response.

[0037] The term "allogeneic" refers to two or more individuals, cells, tissues, or other biological materials that differ at the MHC. Host rejection of grafted tissues from unrelated donors usually results from T-cell responses to allogeneic MHC molecules expressed by the grafted tissues. As used herein, a B cell and a T cell are allogeneic when they differ at the MHC as a result of originating from different individuals. In some contexts, these individuals are a transplant host and donor.

[0038] The term "allograft" refers to a graft of cells or tissue from a donor transplanted to a genetically dissimilar recipient, or host, of the same species.

[0039] The term "allospecific" refers to being reactive to, identifying, or binding cells or other biological components from genetically disparate individuals within the same species. Allospecific T cells can have effector or regulatory functions, and the relative proportions of the two populations activated following alloantigen presentation is one of the factors that determine the clinical outcome of a tissue graft or transplant, namely, graft rejection or persistence.

[0040] The term "anergic" refers to a state of being nonresponsive to an antigen. T cells and B cells are said to be anergic when they cannot respond to their specific antigen under optimal conditions of stimulation. Anergic Treg cells do not mount an immune response, but suppress the response of other effector T cells.

[0041] The term "antibody" refers to an immunoglobulin protein that binds specifically to a particular substance, which is called an antigen. Each antibody molecule has a unique structure that enables it to bind specifically to its corresponding antigen, but all antibodies have the same general gross structure.

[0042] The term "antigen" refers to any molecule that can bind specifically to an antibody. Antigens typically provoke an immune response in an individual, and this immune response may involve either antibody production or the activation of specific immunologically competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen.

[0043] The term "antigen presenting cell" (APC) refers to a cell that can process antigens and display antigen peptide fragments on the cell surface together with molecules required for T-cell activation. The main antigen-presenting cells for T cells are dendritic cells, macrophages, and B cells.

[0044] The term "autoimmune disease" refers to a condition that results from an adaptive immune response directed at an individual's own cells and tissues expressing self antigens. Autoimmunity can also be described as a loss of self-tolerance. The resulting immune response against self tissues and cells can lead to various acute and chronic disease states as a result of injury to vital organs and tissues. Examples of autoimmune diseases include, but are not limited to, Addison's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, type I diabetes, dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves's disease, Guillain-Barr syndrome,

Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

[0045] The term "autologous" refers to any material derived from the same individual to which it is later to be re-introduced into the same individual.

[0046] The term "B cell," or "B lymphocyte," refers to one of the two major types of lymphocytes. Each B cell expresses a particular antigen receptor on its cell surface. On activation by an antigen, B cells differentiate into cells producing antibody molecules of the same antigen specificity as this receptor.

[0047] The term "co-stimulatory agent" refers to an agent (*e.g.,* an antibody or ligand) that can bind to the cell surface to promote proliferation and activation of the cells in culture.

[0048] The term "cytokine" refers to a protein made by cells that affects the behavior of other cells. Cytokines made by lymphocytes are often called lymphokines or interleukins (abbreviated IL). Cytokines act *via* specific cytokine receptors on the cells that they affect.

[0049] The term "dendritic cell" (DC) refers to any member of a diverse population of morphologically similar cell types found in lymphoid or non-lymphoid tissues. These cells are characterized by their distinctive morphology and high levels of MHC expression. DCs can be isolated from a number of tissue sources. DCs have a high capacity for sensitizing MHC-restricted T cells and are very effective at presenting antigens to T cells. The antigens may be self-antigens that are expressed during T cell development of tolerance or foreign antigens.

[0050] The term "effective amount" of a composition refers to a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, *e.g.*, an amount which results in the prevention of, or a decrease in, the symptoms associated with a disease that is being treated. The amount administered to a subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight, and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

The Treg cells can also be administered in combination with one or more additional therapeutic compounds.

[0051] The term "effector cell" refers to a cell which mediates an immune response against an antigen. An example of an effector cell includes, but is not limited to, a T cell or a B cell.

[0052] The term "expansion" refers to growing cells in culture to achieve a larger homogenous population of the cells. Cells can be expanded in the presence of antigen presenting cells to produce a population of cells that is allospecific for the antigen presented by the antigen presenting cells. Regulatory T cells represent a small fraction of all T cells, so expansion in culture is required to produce enough regulatory T cells for an effective therapy.

[0053] The term "expression" refers to, but is not limited to, one or more of the following: transcription of a gene into precursor mRNA; splicing and other processing of a precursor mRNA to produce mature mRNA; mRNA stability; translating a mature mRNA into protein (including codon usage and tRNA availability); and glycosylation and/or other modifications of a translation product, if required for proper expression and function.

[0054] The term "graft versus host disease" (GVHD) refers to a condition that occurs when T cells present in donor tissue attack the host, or recipient, of the grafted cells or tissue.

[0055] The term "HLA" is an acronym for "human leukocyte antigen" and refers to the human MHC.

[0056] The term "HLA haplotype" refers to a linked set of genes associated with one haploid genome, which determines the HLA of cells from an individual. The linked genes of the HLA are usually inherited as one haplotype from each parent. This set of genes resides on chromosome 6, and encodes cell-surface antigen-presenting proteins and many other genes.

[0057] The term "host" refers to an individual to whom transplanted cells, tissues, organs, or other biological material is transplanted. "Recipient" and "host" are used interchangeably with an equivalent meaning.

[0058] The term "immune response" refers to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by these cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from an individual's body of cells that

originate from a source other than that individual's body. In cases of autoimmunity or pathological inflammation, the immune response is directed to the normal cells or tissues of the same individual rather than to nonself cells.

[0059] The term "leukocyte" refers generally to a white blood cell. Leukocytes include lymphocytes, polymorphonuclear leukocytes, and monocytes. The term "lymphocyte" refers a class of white blood cells that bear variable cell-surface receptors for antigens. The two main classes of lymphocytes are B lymphocytes (B cells) and T lymphocytes (T cells), which mediate humoral and cell-mediated immunity, respectively.

[0060] The term "preferential expansion" refers to conditions that favor the growth or proliferation of one cell type versus another in a mixed population of cells. In one embodiment, a preferential expansion of Treg cells refers to conditions where the number of Treg cells in a culture increase (on a percentage basis) to a greater extent than non-Treg cells in the culture. For example, a preferential expansion of Treg cells may be an increase in the cell number that is at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, or at least 100% greater than the increase in the number of non-Treg cells. In one embodiment, only Treg cells proliferate (and non-Treg cells do not proliferate) in response to the culture conditions.

[0061] The term "regulatory T cell" or "Treg cell" refers to a naturally occurring subtype of T cell that can inhibit T-cell immune responses to an antigen. Treg cells represent a distinct T-cell lineage that has a key role in an individual's tolerance of self-antigens and the prevention of autoimmune disease and inappropriate immune responses. When activated, they are anergic and suppress the proliferation and cytokine production of conventional T cells. Like all T cells, Treg cells require T cell receptor activation and costimulation to become fully active.

[0062] The term "self-antigen" refers to an antigen that is expressed by a host cell or tissue.

[0063] The term "T cell", or "T lymphocyte", refers to an immune cell defined by its development in the thymus and having heterodimeric receptors associated with the proteins of the CD3 complex.

Overview

[0064] The development of regulatory T cell-based immunotherapy in humans provides an important advance for promoting tissue graft and transplant tolerance, and for treating autoimmune and other immune-related disorders. Accordingly, in one aspect, the present technology provides methods for expanding T cells *ex vivo*. In particular, the methods involve the preferential expansion *ex vivo* of Treg cells in a mixed population of Treg cells and non-Treg cells. The methods also involve expanding Treg *ex vivo* with allogenic B cells. In some embodiments, the regulatory T cells express very high levels of FoxP3, maintain an anergic phenotype, and potently suppress responder T cell alloproliferation in an alloantigen-specific manner.

[0065] The naturally arising $CD25^+$ $FoxP3^+$ $CD4^+$ T cells (Tregs) represent a distinct lineage of CD4 T cells that have suppressive function. Although constituting approximately only 5 to 10% of total CD4 T cells, the Tregs are required for the maintenance of immunological tolerance to self-antigens. Transfer of $CD25^+$ cell-depleted T cell or thymocyte suspensions from normal mice into syngeneic T cell-deficient nude mice results in various autoimmune diseases in the recipient mice, and transfer of $CD25^+CD4^+$ T cells or thymocytes together with the $CD25^+$ cell-depleted population prevents those diseases. In scurfy mice or patients with the X-linked immunodeficiency syndrome, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), developmental lack of Tregs due to loss-of-function mutation of FoxP3 gene is associated fatal, fulminate autoimmune disease affecting multi-organ systems. Even in adult animals where a normal immune repertoire is fully developed, acute *in vivo* depletion of Treg cells results in similar catastrophic fatal autoimmunity. These observations indicate that a normal immune repertoire contains a sufficient number of auto-reactive lymphocytes capable of causing fatal autoimmunity and the auto-reactive lymphocytes are constantly suppressed by the Tregs. Since Treg cells are present in a relatively low frequency, their marked efficacy in suppressing the autoimmunity suggests that Treg cells are highly potent suppressors. Despite their remarkable potency in suppressing autoimmunity, Tregs appear not to compromise protective immunity against common microbial infections, as indicated by the fact that a Treg-intact host is fully immunocompetent. Only with certain chronic viral, fungal, special bacterial or parasitic infections, do Tregs appear to play a role in attenuating protective immunity.

Techniques for isolating T cells and B cells

[0066] T cells and B cells for use in the methods described may be isolated from a biological sample taken from a mammalian subject, such as a human subject, originating from a number of sources, including for example, peripheral blood mononuclear cells, bone marrow, thymus, tissue biopsy, tumor, lymph node tissue, gut associated lymphoid tissue, mucosal-associated lymph node tissue, spleen tissue, or any other lymphoid tissue. T cells and B cells can be isolated as peripheral blood mononuclear cells (PBMC) from a blood sample obtained from the peripheral blood of a subject. PBMCs are prepared from peripheral blood by centrifugation on a gradient of Ficoll and Hypaque (metrizamide). One exemplary gradient centrifugation method is described in Walker *et al.*, *J. Clin. Invest.* 112: 1437–43 (2003). Alternatively, T cells and B cells may be isolated from leukocytes derived from blood using an apheresis or leukopheresis procedure. Leukocytes derived from leukopheresis filters can be enriched for lymphocytes using density gradient centrifugation, *e.g.*, through a Ficoll-metrizamide gradient.

[0067] $CD25^+$ CD4 T cells and $CD25^-$ CD4 T cells may be isolated from a lymphocyte-enriched sample comprising human T cells through the use of gradient centrifugation and positive/negative selection techniques well known to those of skill in the art. Total CD4 T cells can be isolated by panning, affinity separation, cell sorting using antibodies specific for CD4, and other techniques that provide enrichment of CD4 T cells. CD4 T cells may be prepared by magnetic depletion of other lineage-positive cells. For example, using a magnetic depletion kit, CD4 T cells may be prepared by depleting cells expressing CD8, CD11b, CD16, CD19, CD36, and CD56. The $CD4^+$ No-Touch T cell isolation kit is one example of such a kit manufactured by Miltenyi Biotec (Auburn, CA). $CD25^+$ CD4 T cells and $CD25^-$ CD4 T cells can be prepared by positive and negative selection, respectively, of CD4 T cells using anti-CD25 microbeads. Using similar methods, B cells and monocytes may be prepared from Ficoll-enriched lymphocytes by positive selection with anti-CD19 and anti-CD14 microbeads, respectively. Anti-CD25, anti-CD19, and anti-CD14 microbeads are available from Miltenyi Biotec. If desired, the purity of cell preparations can be determined by flow cytometric analyses.

[0068] *Sources of B cells*. For therapeutic administration, the Treg cells administered to a transplant patient should selectively inhibit pathological immunity against allografts, or the host tissue in the case of allogeneic bone marrow transplantation, without compromising

protective immunity. The B-cell expanded Treg cells have a high alloantigen specificity that is not determined by the HLA haplotypes of the Treg cells, but that is induced and determined by the haplotype of the B cells used to expand them. Consequently, using the methods described herein, Treg cells from a given individual can be made specific for a particular alloantigen by expanding them with allogeneic B cells presenting that particular alloantigen. Thus, the B cells can be isolated from a transplant or tissue graft donor to produce Treg cells that are allospecific for the transplant or tissue graft donor, the B cells can be isolated from a transplant or tissue graft recipient to produce Treg cells that are allospecific for the transplant or tissue graft recipient, or B cells from an individual having a target HLA haplotype can be used to produce Treg cells that are allospecific for that HLA haplotype.

[0069] Moreover, a B cell bearing a synthetic genetic construct could be used to present an antigen with a defined amino acid sequence toward which Treg cell activity is desired. Methods for expressing antigen constructs by B cells are known in the art. *See, e.g.*, Melo, *et al.*, Gene Transfer of Ig-Fusion Proteins into B cells Prevents and Treats Autoimmune Diseases, *The Journal of Immunology*, 168:4788–95 (2002); Lei & Scott, Induction of Tolerance to Factor VIII Inhibitors by Gene Therapy with Immunodominant A2 and C2 Domains Presented by B cells as Ig-Fusion Proteins, *Blood*, 105:4865–70 (2005).

[0070] Antigenic peptides useful in the methods may be identified by eluting peptides from MHC molecules known to be associated with autoimmunity, for example the HLA-DQ and DR molecules that confer susceptibility to several common autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, and multiple sclerosis. Antigenic peptides useful in the present methods also include synthesized peptides predicted to bind to MHC molecules associated with autoimmune diseases. Suitable antigenic peptides for use in the methods herein and for producing the pharmaceutical compositions are known in the art.

[0071] *Sources of T cells*. In addition, the Treg cells may be isolated from a transplant or tissue graft recipient, or from a transplant or tissue graft donor. Furthermore, Treg cells can be isolated from a "third-party" individual other than the donor or the recipient. B-cell expanded Treg cells can potently inhibit the antigen-specific alloproliferation of HLA-unrelated responder T cells, even when the HLA haplotypes of the Treg and responder cells differ at both the class I and class II HLA alleles. Effective inhibition is obtained with either antigen-specific or nonspecific Treg cells at a high Treg cell to responder T cell ratio. At a

low Treg to responder cell ratio, potent inhibition is associated with antigen-specific Treg cells.

[0072] In some embodiments, B-cell expanded Treg cells inhibit alloproliferation of responder T cells at a Treg to responder cell ratio of at least or about 1:5, regardless of the HLA haplotypes of the Treg and responder cells. In other embodiments, when the B-cell expanded Treg cells are stimulated with the same alloantigens against which the Treg cells were expanded, the B-cell expanded Treg cells can inhibit alloproliferation of responder T cells at a Treg to responder cell ratio of at least or about 1:50, regardless of the HLA haplotypes of the Treg and responder cells. Also, when B-cell expanded Treg cells are stimulated with dendritic cells expressing different alloantigens from which the Treg cells were expanded, the Treg cells inhibit proliferation of responder cells at a Treg to responder cell ratio of at least or about 1:5. Under the same conditions, but at a Treg to responder cell ratio of at least or about 1:50, only partial or marginal inhibition occurs. Thus, Treg cells from any individual unrelated to the donor or recipient can be directed or enriched toward a desired specificity and unwanted reactivity can be attenuated through B-cell expansion of alloantigen specific T cell clones.

[0073] Isolating and expanding "third-party" Treg cells that are allospecific to a particular alloantigen may provide "off the shelf" Treg cell pharmaceutical compositions that can be rapidly employed in therapy. Such a Treg cell product overcomes several limitations of producing allospecific Treg cells from a transplant or tissue graft host or donor. For example, because at least two weeks are needed to expand Treg cells on a large scale, the expansion of allospecific Treg cells from unexpected hosts, such as cadaver donors, is not possible. Also, isolating and expanding allospecific Treg cells from a donor or host for immediate use in an emergency transplantation or tissue graft is not possible because of this time requirement. The limited availability of Treg cells from a single donor or cord blood source might also constitute a barrier to providing enough Treg cells for therapy. Thus, the availability of an "off the shelf" Treg cell product is useful to treat, for example, acute graft-versus-host disease or acute allograft rejection under conditions where the availability of donor or recipient T cells is limiting, or when an insufficient time period is available to expand the Treg cells.

[0074] In some embodiments, the pharmaceutical compositions comprise an enriched antigen specific Treg cell population in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise

buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans; mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA; adjuvants and preservatives. Compositions are suitably formulated for intravenous administration. In some embodiments, the composition contains a therapeutically effective amount of the $CD4^+CD25^+$ Treg cells in combination with an effective amount of another bioactive material.

Culturing techniques

[0075] For expansion of allospecific regulatory T cells, isolated $CD25^+$ CD4 T cells are cultured in a suitable culture medium with isolated allogeneic B cells in the presence of a cytokine and a co-stimulatory agent. One example of a suitable culture medium is MEM-α plus GlutaMAX MEM supplemented with 50 μM 2-ME, HEPES, pyruvate, nonessential amino acids, penicillin/streptavidin, and 10% fetal calf serum.

[0076] In an illustrative embodiment, the co-stimulatory agent activates CD28. Accordingly, any agent capable of stimulating or cross-linking CD28 can be used to stimulate T cells, such as an anti-CD28 antibody or a ligand for CD28 (*e.g.,* CD80 and/or CD86). Exemplary anti-CD28 antibodies or fragments thereof include monoclonal antibody 93 (IgG2; Bristol Myers Squibb, Princeton, NJ), monoclonal antibody KOLT-2 (IgG1), CD28.2 (Pharmigen, San Diego, CA). Exemplary ligands include the B7 family of proteins such as B7-1 (CD80) and B7-2 (CD86) (Freedman *et al.*, *J. Immunol.* 137:3260-3267 (1987)). In one embodiment, the method uses a CD28.2 antibody concentration of about 1 μg/ml. In an illustrative embodiment, the cytokine is an interleukin. In a particular embodiment, the interleukin is IL-2. In another embodiment, the interleukin is IL-15.

[0077] For preferential expansion of Treg cells in a mixed population of T cells, isolated T cells are cultured in a suitable culture medium under conditions for the weak stimulation of the TCR. One example of a suitable culture medium is MEM-α plus GlutaMAX MEM supplemented with 50 μM 2-ME, HEPES, pyruvate, nonessential amino acids, penicillin/streptavidin, and 10% fetal calf serum. In some embodiments, weak stimulation of the TCR is achieved using a low concentration of a TCR agonist. In one embodiment, weak stimulation of the TCR is achieved using an anti-CD3 antibody. In one embodiment, weak stimulation of the TCR is achieved using an antigen.

[0078] For preferential expansion of Treg cells in a mixed population of T cells, isolated T cells are cultured in a suitable culture medium containing a phorbol ester, including, but not limited to, phorbol 12-myristate 13-acetate (PMA). One example of a suitable culture medium is MEM-α plus GlutaMAX MEM supplemented with 50 μM 2-ME, HEPES, pyruvate, nonessential amino acids, penicillin/streptavidin, and 10% fetal calf serum.

[0079] $CD25^+$ CD4 T cells are cultured in standard laboratory culture plates, dishes, bottles, or other containers at an appropriate cell density. As an example of culture conditions useful for expanding $CD25^+$ CD4 T cells, the $CD25^+$ CD4 T cells can be cultured in 96-well round-bottom plates at a cell density from 2×10^3 to 1×10^4 cells per well. In these cultures, while other B cell to T cell ratios and cytokine concentrations may be used, $CD25^+$ CD4 T cells can be expanded at a ratio of B cells to T cells ranging from about 1 to about 4, from about 1 to about 1, or from about 4 to about 1.

[0080] In one embodiment, the cytokine (*e.g.,* IL-2) concentration is from 4 to 100 units/ml. As an example of the degree of regulatory T cell expansion that can be achieved using conditions within the ranges provided, a 20- to 40-fold expansion of the Treg cells may be accomplished within 14 days without further addition of B cells to the cultures.

Assay and characterization of expanded regulatory T cells

[0081] The alloantigen-specific Treg cells suitably possess the following characteristics. First, the alloantigen-specific Treg cells may express the cell surface markers $CD4^+$ and $CD25^+$. These markers may be measured, for example, using anti-CD4 and anti-CD25 antibody reagents. Second, the alloantigen-specific Treg cells express FoxP3. Fox P3 may be measured, for example, as a function of protein expression as measured by a Western blot; as a function of FoxP3 mRNA transcription measured using a Northern blot, RT-PCR, or another method of mRNA quantification; or by flow cytometry. Third, the alloantigen-specific Treg cells may maintain an anergic state, which may be measured, for example, by quantifying IL-2 and IFN-γ production as in Example 2. Finally, the alloantigen-specific Treg cells may suppress proliferation of autologous responder T cells stimulated in culture by exposure to the antigen used for expansion. The suppression may be measured, for example, using a proliferation assay as in Example 2.

[0082] *Quantifying FoxP3 expression.* FoxP3 is a forkhead family transcription factor that is necessary, but not sufficient, for human Treg cell suppressive activity. Accordingly, FoxP3

expression is a useful marker for verifying the presence of and quantifying the number of regulatory T cells present in the expanded population, for example, by quantifying FoxP3 protein, FoxP3 mRNA, or FoxP3-expressing cells. FoxP3 protein expression can be quantified by western blot analysis. An example of an exemplary protocol consists of the following steps. T cell populations are washed in PBS and lysed and sonicated in lysis buffer comprising 25 mM Tris pH 8.5, 2% lithium dodeccyl sulfate, 1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1× Roche Complete protease inhibitors and protein levels are quantified (or example, by using a BCA assay; Pierce). Lysates are separated on 4–12% gradient bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes are blocked for 3 hours in TBS/0.1% Tween-20 with 5% nonfat dry milk, probed with polyclonal rabbit-anti-FoxP3 antiserum (1:2000) overnight at 4°C in the same buffer and developed using standard protocols. Western blots are stripped and re-probed with TFIIB (Santa Cruz) for a loading control. For a positive control, 293T cells are transfected with a human FoxP3 cDNA clone.

[0083] Alternatively, FoxP3 levels can be assayed by a quantitative PCR assay. An example of an exemplary protocol consists of the following steps. RNA is extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and cDNA is prepared with 2.5 μM random hexamers (Applied Biosystems Inc., Foster City, CA). Message levels are quantified by real-time PCR using the ABI 7000 Sequence Detection System (Applied Biosystems Inc.). Amplification is carried out in a total volume of 25 μl for 40 to 50 cycles of 15 seconds at 95°C, 1 minute at 60°C, and product can be detected using SYBR Green I dye (Molecular Probes Inc., Eugene, OR). The relative expression can be determined by normalizing expression of each target to GAPDH, and then comparing this normalized value to the normalized expression in a reference sample to calculate a fold-change value. Primers can be designed so that amplicons spanned intron/exon boundaries to minimize amplification of genomic DNA.

[0084] FoxP3-expressing cells can also be quantified using, for example, flow cytometry and an anti-FoxP3 antibody. One specific protocol is described in Roncador *et al.*, *Eur. J. Immunol.* 35:1681-1691, 2005. Generally, cells are washed and suspended in phosphate-buffered saline containing 2% FCS and 0.02% sodium azide, then incubated with an anti-FoxP3 antibody using standard protocols and manufacturers' instructions. The anti-FoxP3 antibody can be fluorescently labeled, or a second fluorescently-labeled antibody that

recognizes the anti-FoxP3 antibody can be used. The anti-FoxP3 antibody 236A/E7 from eBioscience is one such antibody that can be used to label cells expressing FoxP3. Stained cells can be analyzed using a flow cytometry analysis machine or a fluorescence-activated cell sorter such as a FACSaria (Becton Dickinson, Franklin Lakes, NJ) or FACScalibur and the data can be analyzed with appropriate software such as FloJo.

Pharmaceutical Compositions

[0085] In another aspect, the present technology provides a pharmaceutical composition comprising a T cell population containing antigen-specific $CD4^+CD25^+$ Treg cells in a formulation which is suitable for administration to a patient in need thereof. In some embodiments, the antigen-specific $CD4^+CD25^+$ Treg cells are specific for a self-antigen associated with an autoimmune or inflammatory disease. In some embodiments, the Treg cells are useful for promoting transplant tolerance. The methods of generating antigen-specific $CD4^+CD25^+$ Treg cells described herein are useful for generating the T cell population for use in the composition according to this embodiment.

[0086] The pharmaceutical composition comprising $CD4^+CD25^+$ antigen-specific regulatory T cells is administered to a subject in need thereof in a manner appropriate to the disease to be treated and/or prevented. The quantity and frequency of administration will be determined by such factors as the condition of the patient and the type and/or severity of the patient's disease. Appropriate dosages may also be determined by clinical trials. An effective amount of the composition can be determined by a physician with consideration of individual differences in age, weight, disease severity, condition of the patient, route of administration and any other factors relevant to treatment of the patient. In general, a pharmaceutical composition comprising Treg cells may be administered at a dosage of about 10^5 to 10^8 cells/kg body weight, suitably 10^5 to 10^6 cells/kg body weight, including all integer values within these ranges. The compositions may also be administered multiple times at these dosages. The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

[0087] The cells can be administered by using infusion techniques that are commonly used in immunotherapy, and may be administered to a patient subcutaneously, intradermally, intramuscularly, or by intravenous injection.

Methods of Treating and/or Preventing Autoimmune Diseases and Inflammatory Conditions

[0088] The methods described herein may be useful in a therapeutic application to treat a patient. In particular, the methods are useful for treating immunological disorders, such as various autoimmune diseases and immune-related inflammatory disease, and in promoting transplant tolerance in a transplant recipient. For example, the expanded Treg cells are useful in promoting tolerance of bone marrow or a solid organ transplanted from a donor to a recipient. The expanded Treg cells are also useful in preventing or treating graft-versus-host disease associated with a transplant or tissue graft. Other particular, but non-limiting, examples of patient conditions that can be treated with the expanded Treg cells are multiple sclerosis, type 1 diabetes, inflammatory bowel disease, cardiovascular disease, Parkinson's disease, Alzheimer's disease, arthritis, IPEX, allergy, gastritis, lung inflammation, or the destructive effects of an immune response to an infectious agent. The use of HLA haplotype-shared third-party B cells for expansion of alloantigen-specific Treg cells are also useful for bone marrow transplantation situations where the recipients cannot be a source of the B cells because of ongoing hematological malignancies such as leukemia or multiple myeloma.

[0089] In one aspect, the present technology provides methods for treating and/or preventing an autoimmune disease or inflammatory condition. The pathogenesis of a number of autoimmune diseases is believed to be caused by autoimmune T cell responses to self-antigens present in the organism. For example, autoreactive T cells have been implicated in the pathogenesis of multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes (T1D), and Pemphigus. The importance of Treg in the protection from autoimmunity has been demonstrated in various animal models and in humans. In humans, the ability of Treg to regulate T cells in an antigen-specific manner has been demonstrated in the context of various diseases, including regulation of T cells specific to tumor antigens (Viguier *et al.*, *J. Immunol.*, 173:1444–53 (2004)); alloantigens in the setting of bone marrow transplantation (Ng *et al.*, *Blood*, 98:2736–44 (2001)); and the nonself alloantigens as described herein and in Walker *et al.*, *PNAS*, 102:4103–08 (2005)). Therefore, immunotherapy with Treg cells obtained from T cells of a human subject is useful in the context of a cellular therapy for regulating the immune response in the subject. For example, the Treg cells may be used for preventing and/or treating a disease or condition such as an autoimmune disease, inflammatory disease, or in the treatment and/or prevention of transplant rejection and also to prevent graft-versus-host reactions.

[0090] *Methods of Treating and/or Preventing Graft Versus Host Disease.* One problem in hematopoietic stem cell transplantation is graft-versus-host disease (GVHD), which is caused by alloreactive T cells present in the infused hematopoietic stem cell preparation. Studies in mice have demonstrated that adoptive transfer of Treg can block graft-versus-host disease without affecting the graft-versus-leukemia response (Edinger *et al.*, *Nat. Med.*, 9:1144–50 (2003)). Accordingly, in one aspect, the technology provides a method for reducing the risk of, or the severity of, an adverse GVHD effect in a patient who is undergoing a hematopoietic stem cell transplant, comprising administering to the patient an amount of regulatory T cells specific for mismatched antigens between the recipient and donor according to the methods described herein effective to reduce the risk or severity of an adverse GVHD effect in the patient.

[0091] *Methods of Treating and/or Preventing an Transplanted Organ Rejection.* Graft rejection mediated by alloreactive host T cells is a problem that is treated by long-term immunosuppression of the transplant recipient. Accordingly, in one embodiment, the technology provides methods for reducing the risk of, or the severity of, an adverse immune response in a patient that has undergone, is undergoing, or will undergo, an organ transplant, comprising administering to the patient an amount of a population of transplant-specific Treg cells according to the methods described herein effective to reduce the risk or severity of an adverse immune response in the patient. The transplant-specific Treg cells may be generated using the methods described herein. For example, in one embodiment, the method comprises obtaining a sample containing T cells from the patient. A population of $CD4^+CD25^+$ T cells is isolated from the sample and transplant-specific regulatory T cells are produced by contacting the isolated T cells in a culture vessel with B cells isolated from the transplant donor and using the culture conditions disclosed herein. In some embodiments, B cells can be used that comprise at least one antigenic peptide specific to the transplant organ or tissue. The cells are administered in an amount effective to reduce the risk and/or the severity of an adverse immune response in the patient.

[0092] Cyclosporin (CsA) is the mainstay drug used in treating GVHD and solid organ rejection. From time to time, the drug is ineffective for reasons not yet understood, and its prolonged use often causes multi-organ damage or toxicity. The present inventors discovered that CsA when used in conjunction with IL-2 selectively inhibited activation of effector cells while promoting expansion of Treg cells (FIG. 4). This finding suggests CsA used alone

could worsen GVHD by damaging Treg cells through inhibiting IL-2 secretion from effector T cells. Adding IL-2 to the regimen will promote Treg cell propagation in the patients while the inhibition of effector T cells is maintained. In one embodiment, low doses of IL-2 with CsA are adiminstered to prevent GVHD in allogeneic BMT patients. If CsA is used with IL-2 for treatment of solid organ rejection, it is predicted that patients may no longer need to take CsA for a lifetime but do well without taking the drug because of generation of a stable population of Treg cells in the immune system.

[0093] The use of 3rd party Treg cells to establish mixed bone marrow chimerism opens a new avenue to achieving transplant tolerance. To achieve mixed bone marrow chimerism can not only cure a variety of hematological disorders but it can establish permanent tolerance to allogeneic solid organ transplant. Currently no effective feasible method has been established to achieve allogeneic mixed bone chimerism.

[0094] The methods described in this aspect are useful for reducing the risk of, or the severity of, any adverse immune response in a transplant recipient, such as graft-versus-host disease. The methods may be applied to solid organ (*e.g.*, kidney(s), heart, lung(s), liver, and pancreas) transplant recipients, to allogeneic bone marrow, or to autoimmune patients with autologous or allogeneic bone marrow. A reduction of severity of an adverse immune response may be measured by any suitable method. Nonlimiting examples include the reduction or elimination of acute graft rejection, the reduction or elimination of chronic rejection, the reduction or elimination of graft-versus-host disease, and/or the reduction or elimination of the need for high doses of immunosuppressive drugs.

[0095] These methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to cells and compositions known to exert the desired effect.

EXAMPLES

[0096] The present methods, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present methods.

Example 1 –Procedures for Isolating and Expanding T cells.

[0097] The following is a description of the materials and experimental procedures used throughout Examples 2-5 below.

[0098] *Isolation of $CD25^+$ and $CD25^-$ CD4 T cells, B cells and monocytes*. Leukocytes were back flushed out of leukophoresis filters with cold Ca^{2+}- and Mg^{2+}-free PBS. Lymphocytes were enriched with Ficoll gradients and labeled with 10 μM carboxyfluorescein succinimidyl ester (CFSE). Total CD4 T cells were isolated by magnetic depletion of other lineage-positive cells using a CD4 T cell isolation kit (Miltenyi Biotec). $CD25^+$ cells were isolated from CD4 T cells by positive selection with anti-CD25 microbeads (Miltenyi Biotec). B cells were isolated from Ficoll-enriched lymphocytes by positive selection with anti-CD19 beads (Miltenyi Biotec). Monocytes were positively selected with CD14 beads (Miltenyi Biotec), and cultured with 50 ng/ml each of GM-CFS and IL-4 for 6 days with addition 500 ng of LPS on day 5 to generate mature DCs.

[0099] *Cell cultures and flow cytometry.* The culture medium was made of MEM-α plus GlutaMAX MEM (Invitrogen Life Technologies) supplemented with 50 μM 2-ME, HEPES, pyruvate, nonessential amino acids, penicillin/streptavidin, and 10% fetal calf serum. For expansion of allospecific Treg cells, $CD25^+$ cells were cultured for various time periods in 96-well round-button plates at 2×10^3 to 1×10^4 cells per well with allogeneic B cells at the indicated B to T cell ratios in the presence of IL-2 and stimulatory anti-CD28 antibody, CD28.2 (Biolegend) at the indicated concentrations. For expansion of polyclonal Treg cells, $CD25^+$ cells were cultured with anti-CD3 antibody (OKT3) (1 μg/ml) plus CD28.2 (0.5 μg/ml). For re-stimulation, the cells were cultured for 4 hours with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) plus ionomycin (300 ng/ml). Inhibition assays were performed by culturing $CD25^-$ CD4 responder cells with allogeneic DCs at a 1 to 5 DC to T cell ratio in the presence of various numbers of Treg cells expanded with B cells of the same or different donor origin as the DCs. The Treg cells in the cultures were enumerated by combining cell counts and flow cytometry. B cells constituted only a small fraction (<10%) of the cells in the expanded product at the end of culture, and were not removed from expanded Treg cells used in suppressor assays. OKT3 plus CD28.2 was also used to stimulate the cultures as indicated. Antibodies for flow cytometry were purchased from BD Bioscience, except for anti-FoxP3 (236A/E7), which was from eBioscience. Intracellular cellular staining was performed using

Perm/Fix kits (eBioscience). The data were collected on FACS Calibur and analyzed with FlowJo software.

[0100] *Molecular HLA Typing*. Genomic deoxyribonucleic acid (DNA) was prepared from PBMC using a standard phenol-chloroform extraction procedure. HLA-I (HLA-A, -B) and HLA-II (HLA-DR) alleles were identified in PCR-amplified products of exon 2 and 3 of the HLA-A and B loci and exon 2 of the HLA-DR locus by sequence-specific oligonucleotide probe hybridization using a Luminex platform (Tepnel, Stamford, CT).

Example 2. Primary Human B Cells Can Efficiently Expand Allogeneic Treg Cells

[0101] Experiments have shown that freshly isolated mouse B cells can expand isolated allogeneic $CD25^+$ $CD4^+$ T cells at a B to T cell ratio of 4 to 1 in the absence of exogenous IL-2, we set up similar cultures with human B cells and magnetic beads-sorted allogeneic $CD25^+$ $CD4^+$ T cells at various B to T ratios in the presence or absence of exogenous IL-2. CFSE-labeled $CD25^+$ CD4 T cells were cultured for 7 days with allogeneic B cells at a B to T cell ratio of 4 to 1 either alone (FIG. 1Aa), in the presence of IL-2 (100 U/ml) (FIG. 1B) or IL-2 plus stimulatory anti-CD28 antibody CD28.2 (0.5 μg/ml) (FIG. 1C). CFSE-labeled $CD25^-$ CD4 non-Treg cells were cultured for 3 days with allogeneic DCs (FIG. 1D). To examine expression of costimulatory molecules (FIG. 1E), primary B cells (solid thick lines) were stained with anti-CD80, CD86, or isotype-control antibodies (dotted line); EBV-transformed B cells were used as positive controls. $CD25^+$ CD4 T cells were cultured for 7 days with allogeneic B cells at a B to T cell ratio of 1 to 1 in the presence of 0.5 μg/ml CD28.2 plus various concentrations of IL-2 (FIG. 1F), or cultured with allogeneic B cells at different B to T cell ratios in fixed concentrations of IL-2 (120 U/ml) and CD28.1 (1 μg/ml) (FIG. 1G), and enumerated by flow cytometry. $CD25^+$ CD4 T cells were cultured with allogeneic B cells at B to T cell ratio of 1 to 1 in the presence of 200 u/ml of IL-2 and 1 μg/ml of CD28.1, and enumerated at various days of culture (FIG. 1H). The results are representative of more than three experiments.

[0102] No significant Treg cell proliferation was obtained when the Treg cells were cultured with allogeneic B cells alone (FIG. 1A), and supplementation of IL-2 resulted in only minimal Treg cell proliferation (FIG. 1B) in multiple attempts. The lack of significant Treg cell expansion under these culture conditions raised the possibility that human Treg cells might require costimulation, which was not required by mouse Treg cells, for activation and proliferation. Freshly isolated human B cells were analyzed for expression of CD80 and

CD86, and neither of these costimulatory molecules was detected (FIG. 1E). To provide costimulation, stimulatory anti-CD28 antibody CD28.2 was supplemented in the cultures along with exogenous IL-2, and this resulted in robust proliferation of $FoxP3^+$ cells (FIG. 1C). The concentration of IL-2 and the B cell to Treg cell ratio influenced the degree of Treg cell expansion, but the impact was modest (FIGs. 1F & 1G), since a 25-fold change in IL-2 concentration and 16-fold change in B to T cell ratio resulted in less than 2-fold change in expansion of Treg cells. The kinetics of allogeneic B-cell induced Treg cell proliferation was slow (FIG. 1H). Very little cell division was detected at day 3, with minimal division observed on day five. Robust cell proliferation was observed at later time points. A 20- to 40-fold expansion of the cells could be obtained within 14 days without further addition of B cells to the cultures. The expanded Treg cells expressed high levels of FoxP3 (with a mean fluorescence intensity, MFI, 1630) (FIG. 1C), which was three-fold higher than that of the non-dividing input $CD25^+$ CD4 cells (MFI, 550).

[0103] Several studies have reported that activated human non-Treg cells also express Foxp3 and that TGF-β can convert $CD45RA^+$ $CD25^-$ naïve CD4 T cells to FoxP3-expressing cells, but neither activated non-Treg cells nor the TGF-β-converted human FoxP3-expressing CD4 T cells are suppressive, complicating the previously proposed defining role of FoxP3 in determining the Treg-cell phenotype. To understand the reason for the dissociation of FoxP3 expression from the Treg-cell phenotype, we examined activated non-Treg CD4 cells for FoxP3 expression. $CD25^-$ CD4 T cells were cultured with allogeneic DCs for 3 days, which resulted in a clear increase in FoxP3 expression (MFI 80) in proliferating CD4 T cells (FIG. 1d) as compared to the non-dividing CD4 T cells (MFI 9). This level, however, was much lower (>20 fold) than that detected in the B-cell expanded Treg cells or in the non-dividing input Treg cells. Therefore, the expression of FoxP3 in B-cell expanded Treg cells was quantitatively different from that of activated non-Treg cells.

Example 3. B cell-Expanded $CD25^+$ Cells Remain Anergic and are Potent Suppressors

[0104] The high level of expression of FoxP3 in the B-cell expanded Treg cells suggested that these cells maintained the properties of Treg cells. To confirm this possibility, B-cell expanded Treg and activated non-Treg cells were rested in IL-2 containing media for 2 days and then re-stimulated with PMA plus ionomycin. Expanded $CD25^+$ CD4 Treg cells (FIGs. 2A & 2B) or $CD25^-$ CD4 non-Treg cells (FIGs. 2C & 2D) were rested and then re-stimulated with PMA plus ionomycin, and analyzed for IL-2 and IFN-γ production by intracellular flow

cytometry. CFSE-labeled $CD25^-$ CD4 responder T cells were cultured with allogeneic DCs alone (FIG. 2E) or in the presence of B-cell expanded Treg cells at a Treg to responder cell ratio of 1 to 5 (FIG. 2F), 1 to 20 (FIG. 2g) or 1 to 80 (FIG. 2H), or in the presence of OKT3/CD28.2-expanded Treg cells at Treg to responder ratio of 1 to 5 (FIG. 2I), 1 to 20 (FIG. 2J) or 1 to 80 (FIG. 2K). To provide polyclonal stimulation (FIG. 2L), OKT3 plus CD28.2 instead of allogeneic DCs was used to stimulate the cultures consisting of $CD25^-$ CD4 responder T cells with various numbers of either B cell-expanded (open square) or OKT3/CD28.1-expanded (open circle) Treg cells for 6 days. The total number of proliferating responder cells was enumerated by flow cytometry, and the number presented in each histogram represents the average of duplicates or triplicates. The results are representative of at least two experiments.

[0105] The re-stimulation induced production of high levels of IL-2 and IFN-γ in the non-Treg but not in the Treg cells (FIG. 2A–D), indicating the expanded Treg cells remained anergic, one of the key distinguishing features of Treg cells. To determine whether the B cell-expanded Treg cells were suppressive, $CD25^-$ responder CD4 T cells were cultured with allogeneic monocyte-derived, LPS-matured DCs in mixed leukocyte cultures (MLCs) in the presence or absence of Treg cells. In the absence of Treg cells, the responder cells underwent vigorous proliferation, generating a large number of dividing T cells in the cultures (FIG. 2E). This intensive proliferation, however, was abrogated by the presence of the B-cell expanded Treg cells at a Treg to responder cell ratio of 1 to 5 (FIG. 2F) or 1 to 20 (FIG. 2G). Even at a very low Treg to responder cell ratio of 1 to 80, the proliferation of responder cells was partially inhibited (FIG. 2H). The inhibitory potency of B-cell expanded Treg cells was compared to that of the Treg cells expanded with anti-CD3/CD28 antibodies (OKT3 and CD28.2) plus IL-2. At a Treg to responder cell ratio of 1 to 5, the OKT3-expanded Tregs only partially inhibited proliferation of the responder cells (FIG. 2I), and the inhibition was no longer detectable at a Treg to responder ratio of 1 to 20 (FIG. 2J) or 1 to 80 (FIG. 2K). Therefore, the B-cell expanded Treg cells were much more potent than OKT3-expanded polyclonal Treg cells in suppressing alloreactivity of responder T cells. Since the low inhibitory capacity of OKT3-expanded Treg cells could be due to a low frequency of alloantigen-specific Treg cells in the expanded cells, OKT3 plus CD28.2 instead of allogeneic DCs were used as a global stimulus to provide polyclonal activation of both Treg and responder T cells in the cultures. Stronger inhibition was observed with OKT3-expanded Treg cells (FIG. 2L), as compared with alloantigenic stimulation (FIG. 2I–2K). Nevertheless,

the B-cell expanded Treg cells still demonstrated stronger inhibition than OKT-expanded Treg cells (FIG. 2L). These results taken together suggest that the high inhibitory potency of B-cell expanded Treg cells is the result of high frequency of alloantigen-specific Treg cells in the expanded products and high inhibitory capacity of individual cells.

Example 4. Allogeneic B cell-expanded Tregs are enriched for alloantigen specificity

[0106] Ideally, for therapeutic applications, the Treg cells administered to a transplant patient should selectively inhibit pathological immunity against allografts or the host tissue in the case of allogeneic bone marrow transplantation (BMT) without compromising protective immunity. This might be accomplished by generating Treg cells that are specific for donor or host alloantigens. To determine whether allogeneic B cell-expanded Tregs have specificity for the stimulating alloantigens, $CD25^+$ CD4 T cells from a given donor #32 (DRB1 *0701/*1322) were divided and expanded separately with B cells from two unrelated donors designated Y and Z, whose HLA-DR haplotypes were DRB1 *0701/*0301 and DRB1 *1201/*0101, respectively (Table 1). CFSE-labeled $CD25^-$ CD4 responder T cells were cultured for 5 days with allogeneic DCs from donor Y either alone (FIG. 3A) or in the presence of Treg cells expanded by B cells from either donor Y at Treg to responder cell ratio of 1 to 5 (FIG. 3B) or 1 to 50 (FIG. 3C), or of Treg cells expanded by B cells from donor Z at Treg to responder cell ratio of 1 to 5 (FIG. 3E) or 1 to 50 (FIG. 3F). In a parallel experiment, the same CFSE-labeled $CD25^-$ CD4 responder T cells were cultured for 5 days with allogeneic DCs from donor Z either alone (FIG. 3G) or in the presence of Treg cells expanded by B cells from donor Z at Treg to responder cell ratio of 1 to 5 (FIG. 3H) or 1 to 50 (FIG. 3I), or of Treg cells expanded by B cells from donor Y at Treg to responder cell ratio of 1 to 5 (FIG. 3J) or 1 to 50 (FIG. 3K). The results are representative of two sets of experiments involving B cells and DCs of from different donors, Treg cells from two different donors, and responder T cells of three different donor origins.

[0107] Each expanded Treg population was used to inhibit alloproliferation of the $CD25^-$ CD4 responder T cells from an unrelated donor #40 (DRB *04AMAD/*0407) elicited by mature DCs derived from either donor Y or Z. In the absence of Treg cells, the DCs of both donors Y and Z elicited vigorous proliferation of the responder T cells in mixed leukocyte cultures (MLCs) (FIG. 3A & F). When the Treg cells expanded by B cells of donor Y were added to the MLCs elicited by DCs of donor Y at a Treg to responder cell ratio of 1 to 5 (FIG. 3B) or 1 to 50 (FIG. 3C), proliferation of responder T cells was nearly abrogated.

However, when the Treg cells of the same donor origin, which had been expanded by B cells of donor Z, were added to the culture, they imposed significant inhibition at a Treg to responder ratio of 1 to 5 (FIG. 3D), but at the ratio of 1 to 50, only marginal inhibition was observed (FIG. 3E). The lack of substantial inhibition by the Z-BC expanded Treg cells at this low Treg to responder ratio of 1 to 50 was not due to an intrinsic low inhibitory capacity of the Treg cells, since, when added to MLCs elicited by Z-DCs at similar Treg to responder ratios, the Treg cells imposed strong inhibition to the alloproliferation (FIG. 3G & H). By contrast, the Treg cells expanded by B cells of donor Y imposed only minimal inhibition to the MLCs elicited by DCs of donor Z at a Treg to responder ratio of 1 to 50 (FIG. 3J), in contrast to strong inhibition in MLCs elicited by DCs from donor Y (FIG. 3C). Similar results were obtained in multiple cultures involving different allogeneic BC/DC and Treg cell combinations (FIG. 4). The results demonstrate that allogeneic B cell-expanded Treg cells are highly suppressive and, at a relatively high Treg to responder cell ratio (1 to 5), they are capable of imposing potent inhibition on alloreactive, third-party responder T cells in a haplotype nonspecific manner. At a very low Treg to responder cell ratio (1 to 50), however, the Treg cells lose their capacity to potently inhibit alloreactivity elicited by non-specific alloantigens while remaining highly potent in inhibiting alloproliferation of responder cells elicited by specific alloantigens. The alloantigen specificity demonstrated by B-cell expanded Treg cells is not determined by the HLA-haplotypes of the Treg cells but induced and determined by the haplotype of the B cells used to expand them.

Example 5. B Cell-Expanded Tregs Can Inhibit HLA-Unrelated "Third-Party" Responder T Cells

[0108] We used third-party responder T cells in the above inhibition experiments based on previous reports that mouse Treg cells, when activated, could suppress responder T cells of different Ag specificities or MHC allotypes. If B cell-expanded human Treg cells can inhibit HLA-unrelated responder T cells in an alloantigen-specific manner, it then opens the possibility of using alloantigen-specific third-party Treg cells for therapeutic applications. The frequency of $FoxP3^+$ CD4 T cells in human peripheral blood is very low (about 1% of CD4 T cells), and this could pose a formidable challenge if a large number of Treg cells needs to be harvested from a single donor for ex vivo expansion for therapeutic utilization. While the information regarding the availability of Treg cells from a single donor is scant in the literature, in our experience, with magnetic beads sorting, only a very small number (1 to 3×10^5) of $CD25^+$ cells of purity greater than 90% $FoxP3^+$ cells could be obtained from a

leuko-filter of 1 unit (500 ml) of blood. Although the number of Treg cells could be expanded ex vivo substantially, excessive expansion has been shown to increase the frequency of FoxP3$^-$ T cells in the expanded product. Since transfer of activated effector T cells is potentially harmful to the recipients, the requirement for highest quality of expanded Treg cells might pose a limit to the extent which Treg cells could be expanded *ex vivo*, and thus make it necessary to have a sufficient number of freshly isolated Treg cells as starting input cells for expansion. Another potential barrier to the use of Treg cells in transplantation could be related to the time needed to expand Treg cells. According to all current protocols, at least two weeks are needed to expand Treg cells on a large scale, which makes expansion of Treg cells specific for alloantigens of unexpected donors, such as cadaver donors, impossible. The use of third-party Treg cells expanded for specificity for various major target HLA haplotypes opens the potential for "off the shelf" cell products that could be rapidly employed in therapy.

[0109] To include a variety of combinations of HLA-unrelated Treg and responder cells in inhibition experiments, CD25$^-$ CD4 T cells from three HLA-unrelated blood donors, #38, #39 and #40 (Table 1) were used in cultures with CD25$^+$ CD4 T cells from each of two HLA-unrelated donors, #31 and #32, which had been expanded separately with B cells from donors Y and Z (Table 1). Treg cells from donor #31 or #32, each of which had been expanded separately with B cells from either donor Z (FIG. 4A & D) or Y (FIG. 4B & C), were used to inhibit alloproliferation of responder T cells derived from three HLA-unrelated donors, #38, #39 and # 40, elicited by DCs from either donor Z (FIG. 4A & C) or Y (FIG. 4B & D).

[0110] Although the HLA haplotypes of responder T cells were disparate from that of Treg cells at both class I and II alleles (Table 1), the B cell-expanded Treg cells potently inhibited alloproliferation of the responder T cells in all Treg and responder cell combinations at a Treg to responder T cell ratio of 1 to 5, or at 1 to 50 when the Treg cells were stimulated with the same alloantigens against which the Treg cells were expanded (FIG. 4A & 4B). When the B cell-expanded Treg cells were stimulated with DCs expressing different alloantigens from which they were expanded (FIG. 4C & 4D), the Treg cells still substantially inhibited alloproliferation of the responder cells at Treg to responder cell ratio of 1 to 5. At Treg to responder cell ratio of 1 to 50, however, only partial or marginal inhibition was observed. These results demonstrate that when used as third-party suppressor cells, the B cell-expanded Treg cells can potently inhibit alloproliferation of HLA-unrelated responder T cells. Effective

inhibition was obtained with either antigen-specific or nonspecific Treg cells at relatively high Treg to responder cell ratio. At a low Treg to responder cell ratio, however, potent inhibition was only associated with antigen-specific Treg cells.

Table 1. HLA allele typing of HLA-DR, A and B loci of the blood donors

	DRB1	A	B
Donor Y	*0701/0301	0101/0201	0801/1302
Donor Z	1201/0101	0101/2301	5101/5501
Donor 31	#0404/1301	0101/0201	1301/3501
Donor 32	*0701/1322	0301/2402L	4001/5501
Donor 38	0409/0101	0201/2402	0702/3906
Donor 39	#0404/0301	0101/3101	0801/4001
Donor 40	0401/0407	0101/6801	0702/5701

* DRB1 *0701 is a shared allele between donor Y and #32; # DRB1 *0404 is a shared allele between donor #31 and #39.

Example 6 – Preferential Expansion of Allospecific Tregs by Weak TCR Stimulation.

[0111] In this Example, we show that all weak TCR stimulation including Ag-specific or anti-CD3-mediated TCR stimuli preferentially activate Tregs. To examine how the strength of specific antigen stimulation impact activation of Treg cells, CD4 T cells of OT-II TCR transgenic mice were stimulated with different concentrations of OVA peptide, $OVA_{323-339}$, presented by splenic APCs, and proliferation of Treg and non-Tregs cells were determined by flow cytometry (FIG. 5). Similar to allostimulation, high doses of the peptide antigen (0.5 μM) induced an enormous expansion of OT-II T cells with expansion of non-Treg T cells exceeding that of Treg cells, resulting in a decrease in Treg to non-Treg cell ratio from 1 to 10 (of the non-stimulated cells) (FIG. 5A) to 1 to 100 (FIG. 5B). As the concentration of the antigen was lowered down, the magnitude of T cell proliferation decreased. Notably, however, reduction in proliferation was confined only to non-Treg cells (FIG. 5C to 5E), since the number of proliferating Treg cells was not decreased but in stead increased (FIG. 5F). It was only as the antigen concentration reached a very low level that the expansion of Treg cells began to attenuate (FIG. 5E). The reduction in proliferation of non-Treg cells, along with an increase in expansion of Treg cells as a function of decrease in antigen concentration, resulted in about 50 fold increase in the ratio of proliferating Treg to non-Treg cells (FIG. 5G).

[0112] To further determine whether the weak polyclonal TCR-stimulation also preferentially induced Treg cell proliferation, total spleen cells were cultured in the presence of various concentrations of mitogenic anti-CD3 antibody, 2C11, from 1 to 0.001 μg/ml.

Similar to $OVA_{323-339}$-derived stimulation, high concentration of 2C11 primary induced expansion of non-Treg cells (FIG. 6A), while at low antibody concentration, proliferation of non-Treg cells diminished but that of Treg cells enhanced, with the number of proliferating Treg cells exceeding that the non-Treg cells the number of non-Treg cells in most experiments (FIG. 6D). These results demonstrate that the weak TCR stimulation induced preferential Treg cell expansion is associated not only with alloantigen, but also specific antigen and polyclonal T cell stimuli, such as mitogenic anti-CD3 antibody.

[0113] Previous studies have reported that low doses of peptide delivered by means of osmotic pumps transforms mature T cells into CD4+25+ suppressor cells *in vivo* and targeting low doses of DEC-205-conjugated antigen to dendritic cells convert non-Treg cells to FoxP3+ Treg cells. We asked whether the observed the weak TCR stimulation-induced preferential expansion of Treg cells *in vitro* resulted from conversion of non-Treg cells. Responder cells composed of $CD45.1^+CD25^+$ and $CD45.2^+CD25^-$ CD4 T cells were cultured with various concentration of 2C11 mAb, and expanded $FoxP3^+$ $CD4^+$ T cells were gated out and assessed for CD45 expression to define their origin. As shown in FIG. 7, at high antibody concentrations, the $CD45.1^+$ Treg cells underwent only mild proliferation, with a small number of $CD45.1^-$ converted Treg cells contributing to the $FoxP3^+CD4^+$ T cell pool. At low antibody concentration, however, the $CD45.1^+$ Treg cells under more vigorous expansion, and at the same time, more $FoxP3^+$ $CD4^+$ T cells are converted from $CD45.1^-$ non-Treg cells.

[0114] To determine whether weak anti-CD3 stimulation induced production of TGF_β in the culture, which in turn promoted Treg cells conversion, we examined TGF_β concentration for each culture condition. More TGF_β was detected in low 2C11 stimulated cultures (FIG. 8A). When TGF_β neutralizing antibody was supplemented in the culture, the Treg cell conversion was diminished (FIG. 8B). These results demonstrate that weak-TCR stimulation promote Treg cell expansion through promoting preferential proliferation of pre-existing Treg cells as well as conversion of non-Treg cells to Treg cells.

[0115] The finding of preferential expansion of Treg cells in response to weak-TCR stimulation was intriguing since it raised a question of how a subset of phenotypically anergic cells were more sensitive to weak TCR stimulation than the phenotypically competent non-Treg cells. In an attempt to understand the mechanism, we examined the status of three major transcription factors, NFAT, NF-kB and AP-1 in freshly isolated Treg cells. It is known that in resting T cells, these three (families) of transcription factors are primarily located in the

cytoplasm. During T cell activation, they become translocated into the nucleus in order to activate transcription of a large number of genes including the *il-2* gene whose products are involved in T cell activation. The total amount of NFATc1, NFATc2 and c-Fos (AP-1) was found to be similar in the total cell lysates of freshly isolated Treg and non-Treg $CD4^+$ cells as detected by Western blots (FIG. 9), but two to three folds more c-Rel (NF-κB) was identified in Treg than in non-Treg cells in all (five) experiments. In non-Treg cells, all these factors were detected only in the cytoplasm but not in the nucleus, consistent with previous findings. Similarly, in Treg cells, all the c-Fos and most of c-Rel proteins were also identified in the cytoplasm, with only a minute fraction of c-Rel was detected in the nucleus. In contrast to the non-Tregs, however, a major fraction (40 -60%) of NFATc1 and NFATc2 were found in the nucleus Treg cell, suggesting their nuclear translocation prior to the isolation of the cells.

[0116] Since nuclear translocation of NFAT requires dephosphorylation of cytoplasmic NFAT proteins by calcineurin, and treatment of T cells with the calcineurin inhibitor cyclosporin A (CsA) results in the immediate export of NFAT from nucleus by NFAT kinases, we asked whether the constitutive nuclear translocation of NFAT in Treg cells was the result of continuous activation of calcineurin. Freshly isolated Treg cells were treated in culture with CsA and the examined for translocation of NFATc2. As a control, freshly isolated non-Treg CD4 T cells were treated with Ca^{2+} ionophor ionomycin, washed, and then treated with CsA. Treatment of the non-Treg cells with ionomycin resulted in rapid nuclear translocation of NFATc2, which then swiftly returned back to the cytoplasm as ionomycin was washed away or the cells were treated with CsA. The same treatment of Treg cells with CsA, however, did not alter the nuclear location of NFATc2. These results demonstrate that in Treg cells, a major fraction of NFATc1 and NFATc2 is constitutively located in the nucleus. The constitutive nuclear localization of NFATc2 does not require continuous activation of calcineurin, suggesting that its nuclear localization of NFAT is the result of retention in rather than continuous import of the protein into the nucleus. These observations are consistent with previous prediction that NFAT can complexes with FoxP2 and the interaction of NFATc2 with FoxP3 is necessary for FoxP3 to function. Using luciferase report assay, studies have also reported that Smad3 and NFAT cooperate to induce expression FoxP3 expression its enhancer. Therefore, in order for Treg cells to maintain their phenotype, NFATs have to be constitutively located in the nucleus.

[0117] It has been known since the early studies that the combination ionomycin and the phorbol ester PMA can activate T cells and induce them to undergo robust proliferation, bypassing the requirement for TCR signaling at early onset of T cell activation, but neither alone can do so. The ability for PMA to complement ionomycin to induce T cell proliferation indicates that PMA can activate all other transcription factors required for T cell activation, except NFAT. Therefore, if the nuclear translocated NFAT in Treg cells is functional, PMA alone should induce proliferation of Treg but not non-Treg cells. To test this possibility, total spleen cells were culture with PMA or PMA plus ionomycin. As expected, PMA plus ionomycin induced T cells to undergo blast transformation as early as 24 hrs, and vigorous T cell proliferation at culture day 3. As shown in FIG. 10, CFSE-labeled splenocytes (FIG. 10A) were cultured with PMA plus ionomycin for four days (FIG. 10B) or PMA alone for 6 days (FIG. 10C). While PMA plus ionomycin induced vigorous non-Treg cell proliferation, PMA alone selectively induced proliferation of Treg cells with the non-Treg cells undergoing apoptosis (FIG. 10F). When $CD45.1^+$ Treg cells were added to $CD45.1^-$ non-Treg cells (FIG. 10D) and cultured with PMA for 5 days (FIG. 10E), the expanded FoxP3+ were composed primarily of $CD45.1^+FoxP3^+$ cells, suggesting conversion of non-Treg to Treg cells did not significantly contribute to the PMA-induced expansion of Treg cells.

[0118] To study the differential DNA targeting of NFAT in Treg and activated non-Treg cells, Treg and non-Treg cells were isolated and rested. Some non-Treg cells were stimulated with PMA plus ionomycin for 30 minutes. Chromatin Immunoprecipitation (ChIP) was performed as described previously. Antibodies against NFATc1 and NFATc2 (Santa Cruz) were mixed and used to precipitate NFAT-bound DNA. PCR were performed with primer sets for *Il2*, *Pde3b-20*, *Irf4*, *miR-155*, *FasL*, *Il4* promoter and enhancer regions. Of the DNA regions tested, NFAT bound to *IL-2*, *Pde-20* and *IrF4* in both Treg and activated non-Treg cells, but *IL-4* promoter and enhancer and *Pde3b* only in activated non-Treg cells (FIG. 11). The binding of miR-155 was detected only in Treg but not in activated non-Treg cells. NFAT did not bind to *FasL* in either Treg or activated non-Treg cells (FIG. 11).

Example 7 – Third-Party Treg Cells Prevent Rejection of Allogenic Bone Marrow Grafts in a MHC-related Host.

[0119] As described above, we reported that B-cell expanded allospecific third-party human Treg cells are capable of inhibiting allo-reactivity of effector T cells in vitro. An immediate important question relevant to clinical application of this discovery is that whether

the 3rd-party Treg cells would be functional or rejected when transplanted to HLA-unrelated recipient hosts. To address this question, we isolated Treg cells from C3H mice (MHC of k allotype) and expanded the Treg cells in vitro with a mixture of B cells from three different strains of mice, C3H, BALB/c (d allotype) and B6 (b allotype). The expanded Treg cells were tested for their ability to suppress rejection of allogeneic bone marrow grafts as follows.

[0120] B6 mice were sub-lethally irradiated (with 500 rads of X-ray), then transplanted with bone marrow cells from BALB/c mice (2 x10*7 cells/mouse) with or without the expanded Treg cells described above). The BM-transplanted mice were followed of their peripheral blood cells by flow cytometry for evidence of BM engrafment. As shown in FIG. 12, show that the that mice received B6 bone marrow alone rejected the bone marrow cells (FIG .12C) while the mice that received the bone marrow plus the Treg cells did not reject (FIG 12D) (as indicated by the co-presence of H2-Kd+ and H2-Kb+ cells in the blood. These findings should have tremendous implication to transplant medicine and treatment for hematological disorders. As such, the present methods may be used to produce third-party Treg cells that may be used in allogenic bone marrow grafts for mammalian hosts.

* * * *

[0121] While certain embodiments have been illustrated and described, it should be understood that changes and modifications can be made therein in accordance with ordinary skill in the art without departing from the technology in its broader aspects as defined in the following claims.

[0122] The present disclosure is not to be limited in terms of the particular embodiments described in this application. Many modifications and variations can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and compositions within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this disclosure is not limited to particular methods, reagents, compounds compositions or biological systems, which can of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0123] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0124] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

[0125] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

[0126] Other embodiments are set forth within the following claims.

CLAIMS

What is claimed is:

1. A method for expanding human regulatory T cells comprising co-culturing a human regulatory T cell and a B cell in the presence of a cytokine and a co-stimulatory agent to produce a population of allospecific human regulatory T cells.

2. The method of claim 1, wherein the cytokine is an interleukin.

3. The method of claim 2, wherein the interleukin is IL-2.

4. The method of claim 1, where in the co-stimulatory agent activates CD28.

5. The method of claim 4, wherein the co-stimulatory agent is an anti-CD28 antibody.

6. The method of claim 1, wherein the B cell has been transformed to express the cytokine, the co-stimulatory agent, or both the cytokine and co-stimulatory agent.

7. The method of claim 1, wherein the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a host.

8. The method of claim 1, wherein the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a donor.

9. The method of claim 1, wherein the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a third party who is unrelated to the host and the donor.

10. The method of claim 1 wherein the regulatory T cell, the B cell, or both the regulatory T cell and the B cell are isolated from a host.

11. A method for expanding regulatory T cells, the method comprising: culturing a population of T cells with a T cell receptor agonist (TCR) under conditions wherein the T cell receptor is weakly stimulated, wherein Treg cells in the population of T cells are preferentially expanded compared to non-Treg cells.

12. The method of claim 11, wherein the preferential expansion includes proliferation of existing Treg cells and conversion of non-Treg cells to Treg cells.

13. The method of claim 11, wherein the Treg cells have a $CD25^+$ $FoxP3^+CD4^+$ phenotype.

14. The method of claim 11, wherein the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with a T cell receptor agonist.

15. The method of claim 14, wherein the T cell receptor agonist is selected from the group consisting of: an anti-CD3 antibody, an antigen, and an allogenic antigen presenting cell.

16. The method of claim 11, wherein the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with an anti-CD3 antibody.

17. The method of claim 11, wherein the conditions wherein the T cell receptor is weakly stimulated include contacting the population of T cells with an antigen.

18. The method of claim 17, wherein the antigen is an OVA peptide.

19. A method for expanding regulatory T cells, the method comprising: culturing a population of T cells with a phorbol ester, wherein Treg cells in the population of T cells are preferentially expanded compared to non-Treg cells.

20. The method of claim 19, wherein the phorbol ester is phorbol 12-myristate 13-acetate (PMA).

FIG. 1

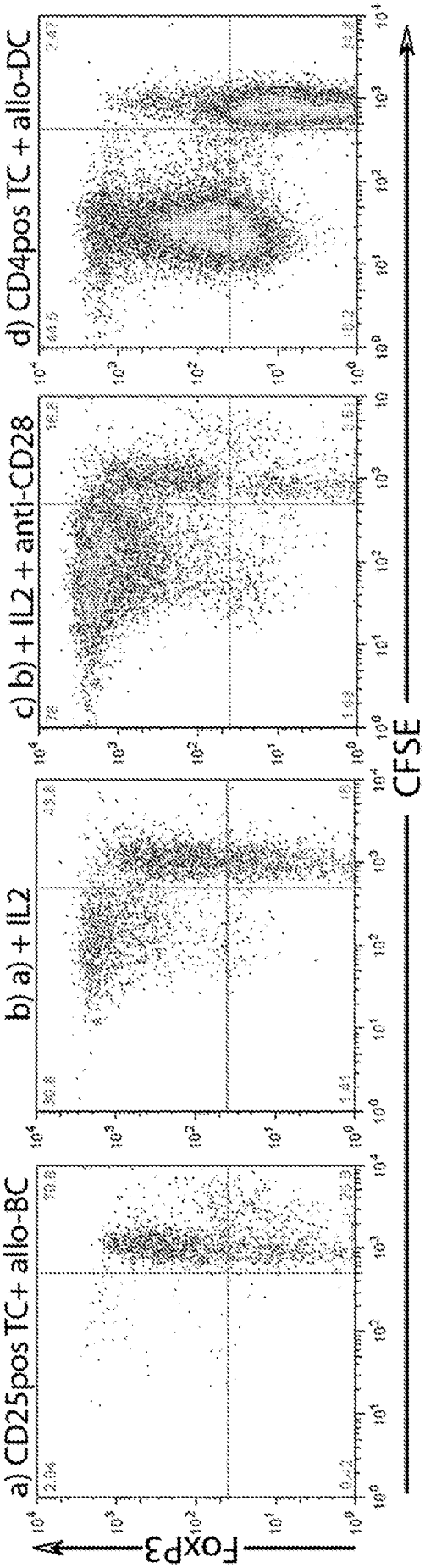

a) CD25pos TC+ allo-BC
b) a) + IL2
c) b) + IL2 + anti-CD28
d) CD4pos TC + allo-DC
FoxP3
CFSE

FIG. 1 (CONT.)

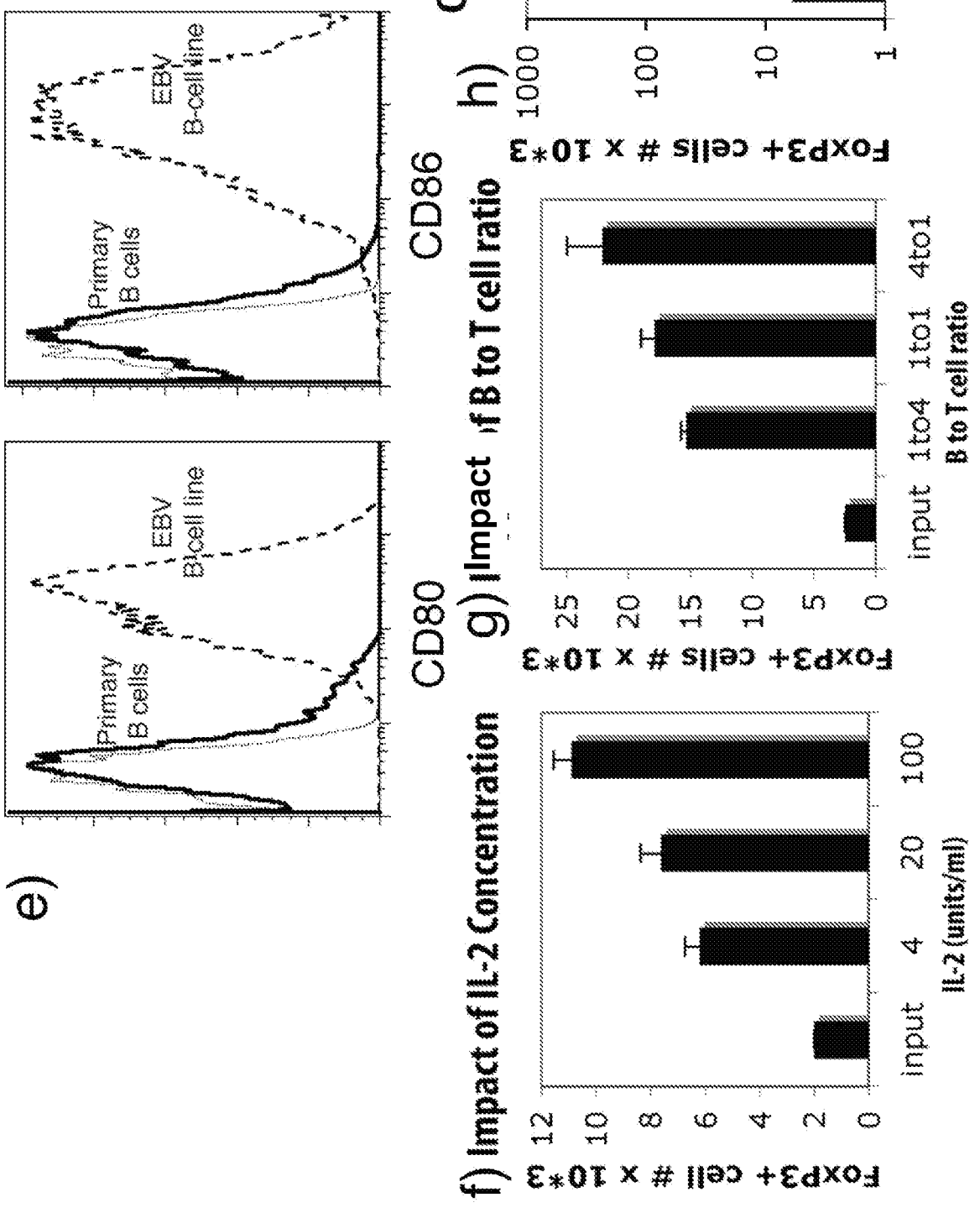

e)
Primary B cells
EBV B-cell line
CD80
CD86
f) Impact of IL-2 Concentration
FoxP3+ cell # x 10*3
IL-2 (units/ml)
input
4
20
100
g) Impact of B to T cell ratio
FoxP3+ cells # x 10*3
B to T cell ratio
input
1to4
1to1
4to1
h)
Cell expansion as function of time
FoxP3+ cells # x 10*3
Culture days

FIG. 2

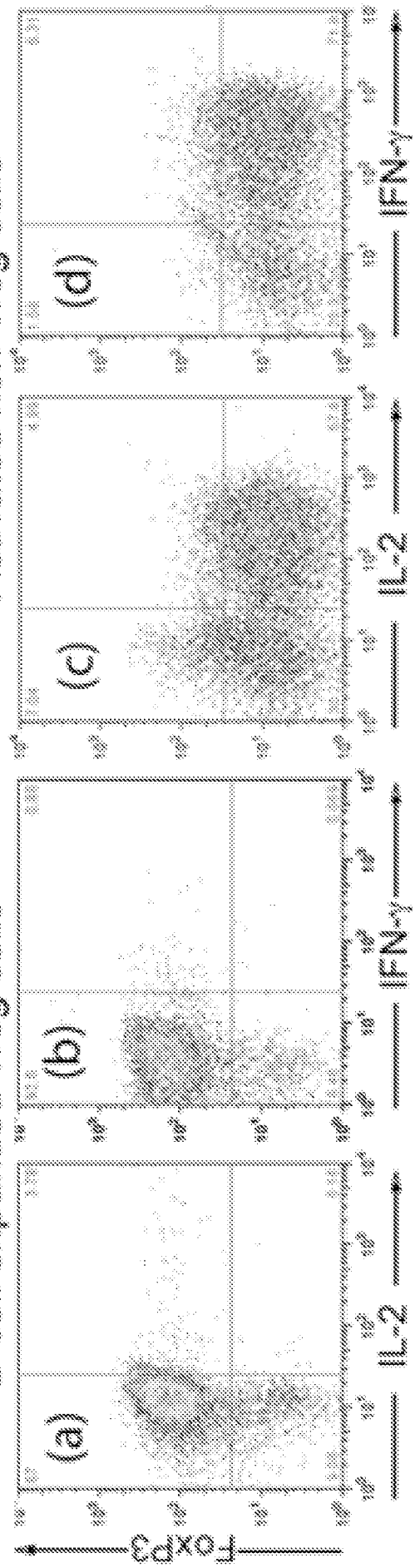

B-cell expanded Treg cells
Activated non-Treg cells
(a)
(b)
(c)
(d)
FoxP3
IL-2
IFN-γ
IL-2
IFN-γ

FIG. 2 (CONT.)

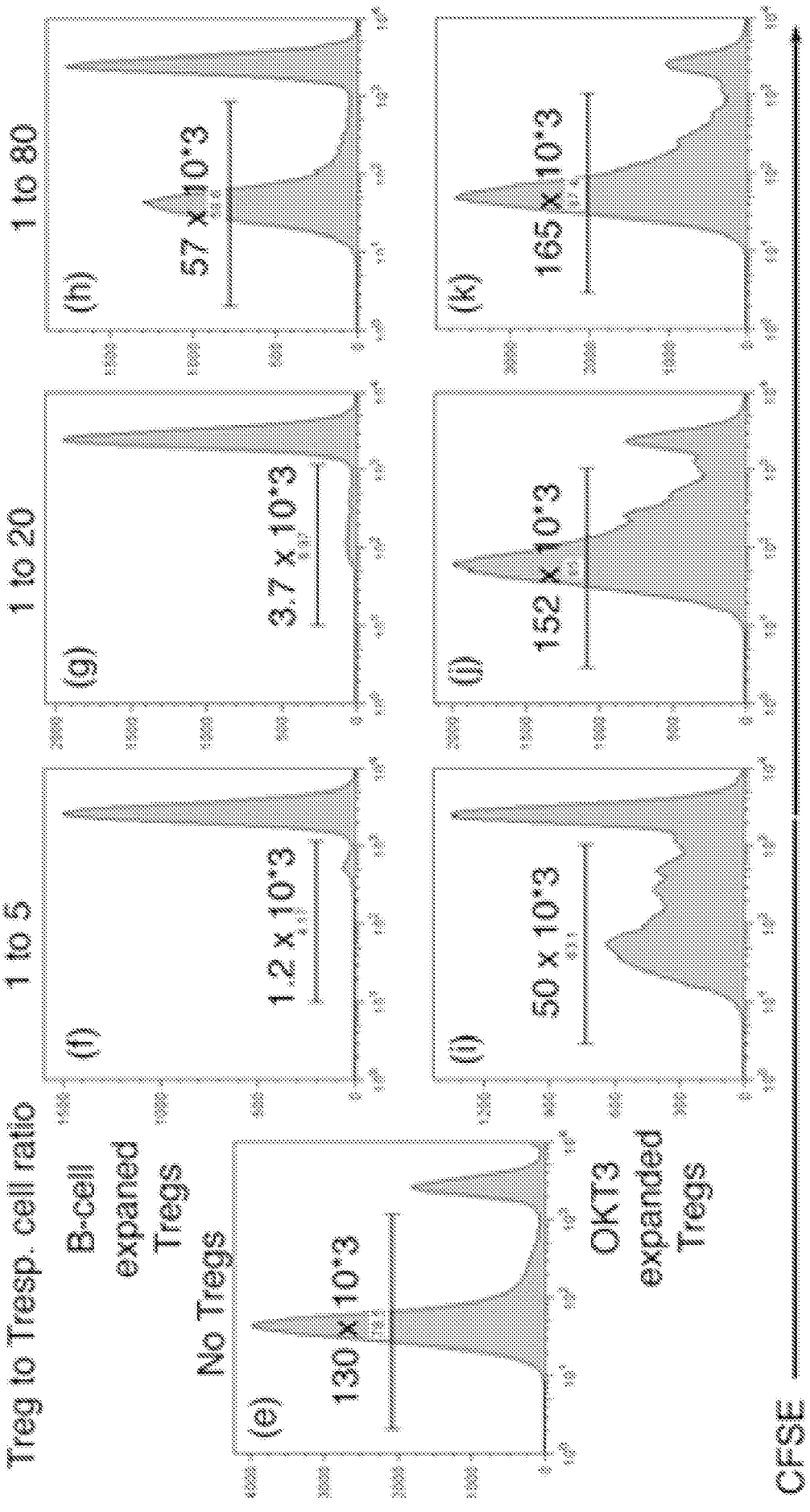

Treg to Tresp. cell ratio
1 to 5
1 to 20
1 to 80
B-cell expaned Tregs
No Tregs
OKT3 expanded Tregs
(e)
130 x 10^3
(f)
1.2 x 10^3
(g)
3.7 x 10^3
(h)
57 x 10^3
(i)
50 x 10^3
(j)
152 x 10^3
(k)
165 x 10^3
CFSE

FIG. 2 (CONT.)

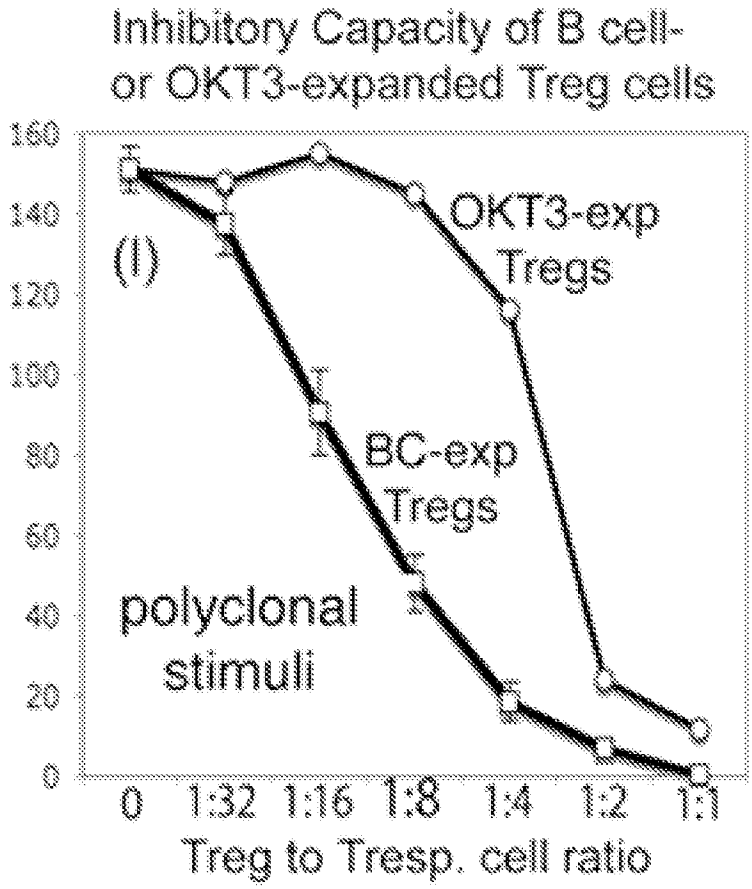

Inhibitory Capacity of B cell- or OKT3-expanded Treg cells
160
140
120
100
80
60
40
20
0
(I)
OKT3-exp Tregs
BC-exp Tregs
polyclonal stimuli
0
1:32
1:16
1:8
1:4
1:2
1:1
Treg to Tresp. cell ratio

FIG. 3

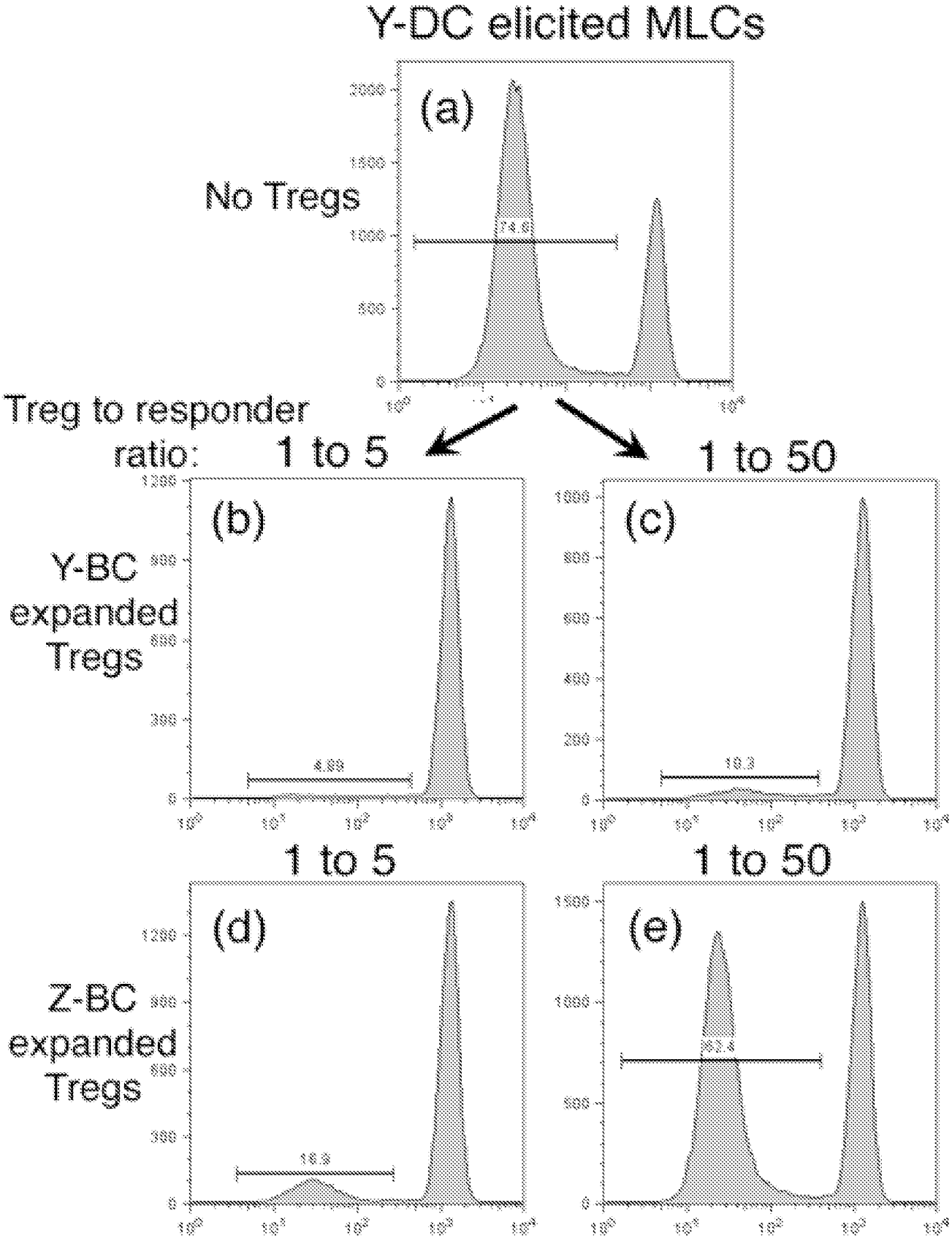

Y-DC elicited MLCs
(a)
No Tregs
Treg to responder ratio:
1 to 5
1 to 50
(b)
(c)
Y-BC expanded Tregs
1 to 5
1 to 50
(d)
(e)
Z-BC expanded Tregs

FIG. 3 (CONT.)

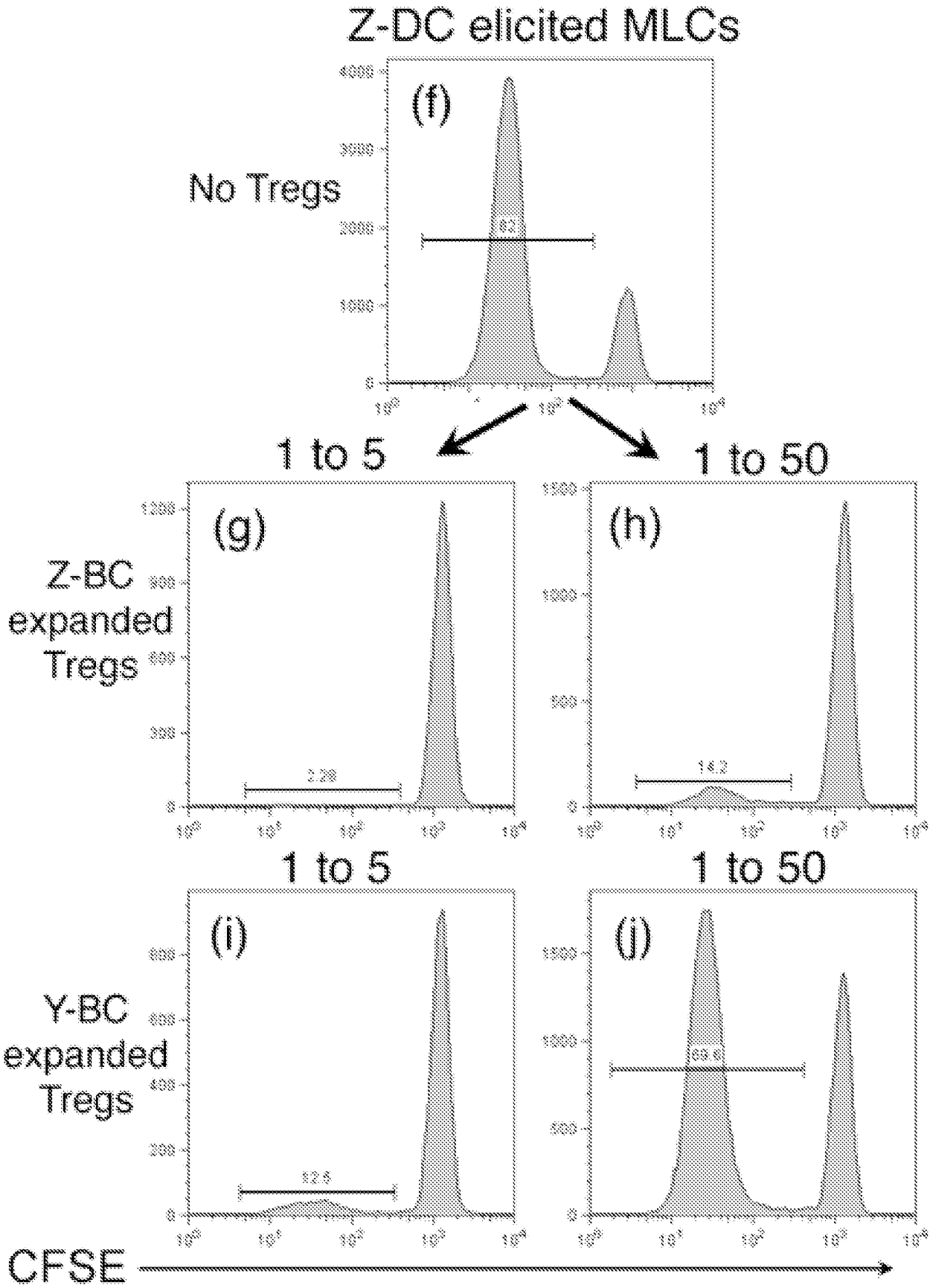

Z-DC elicited MLCs
(f)
No Tregs
1 to 5
1 to 50
(g)
(h)
Z-BC expanded Tregs
1 to 5
1 to 50
(i)
(j)
Y-BC expanded Tregs
CFSE

FIG. 4

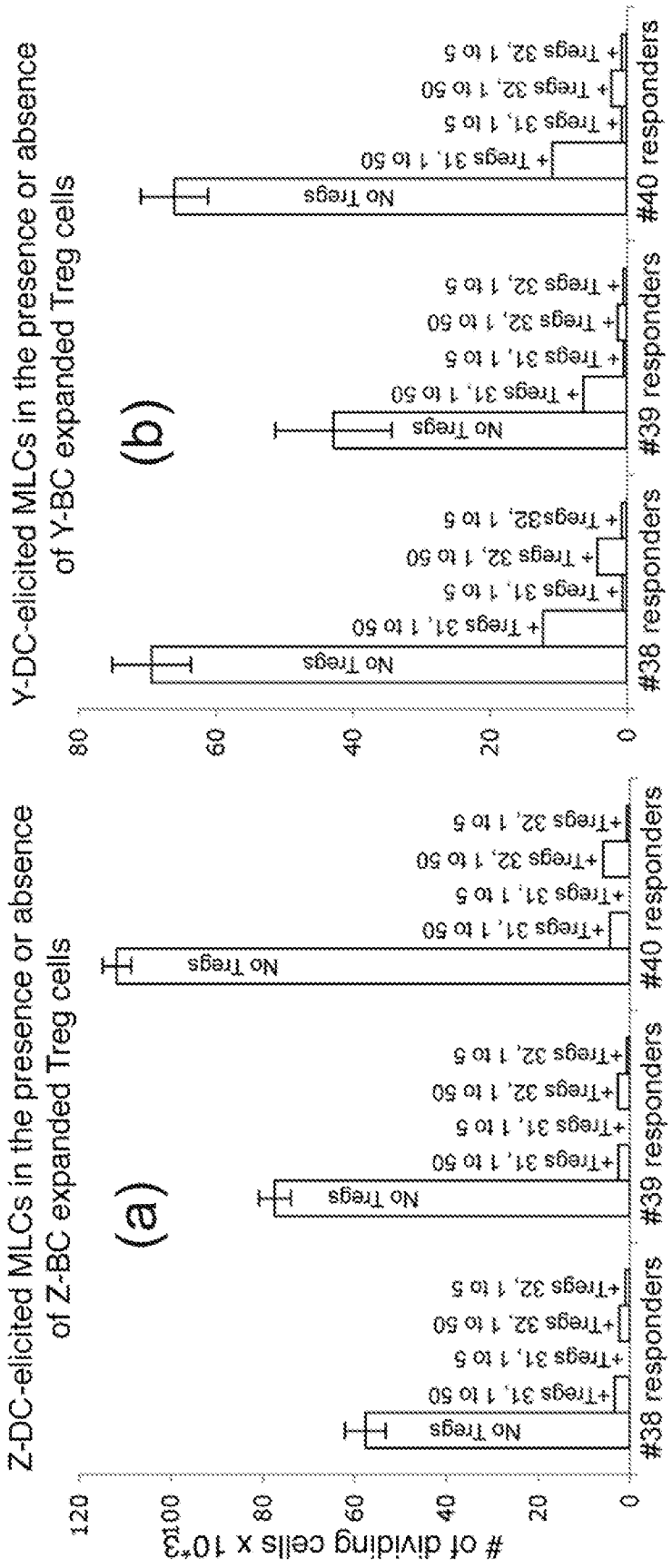

Z-DC-elicited MLCs in the presence or absence of Z-BC expanded Treg cells
(a)
# of dividing cells x 10^3
0
20
40
60
80
100
120
No Tregs
+Tregs 31, 1 to 50
+Tregs 31, 1 to 5
+Tregs 32, 1 to 50
+Tregs 32, 1 to 5
#38 responders
#39 responders
#40 responders
Y-DC-elicited MLCs in the presence or absence of Y-BC expanded Treg cells
(b)
0
20
40
60
80
No Tregs
+ Tregs 31, 1 to 50
+ Tregs 31, 1 to 5
+ Tregs 32, 1 to 50
+ Tregs 32, 1 to 5
#38 responders
#39 responders
#40 responders

FIG. 4 (CONT.)

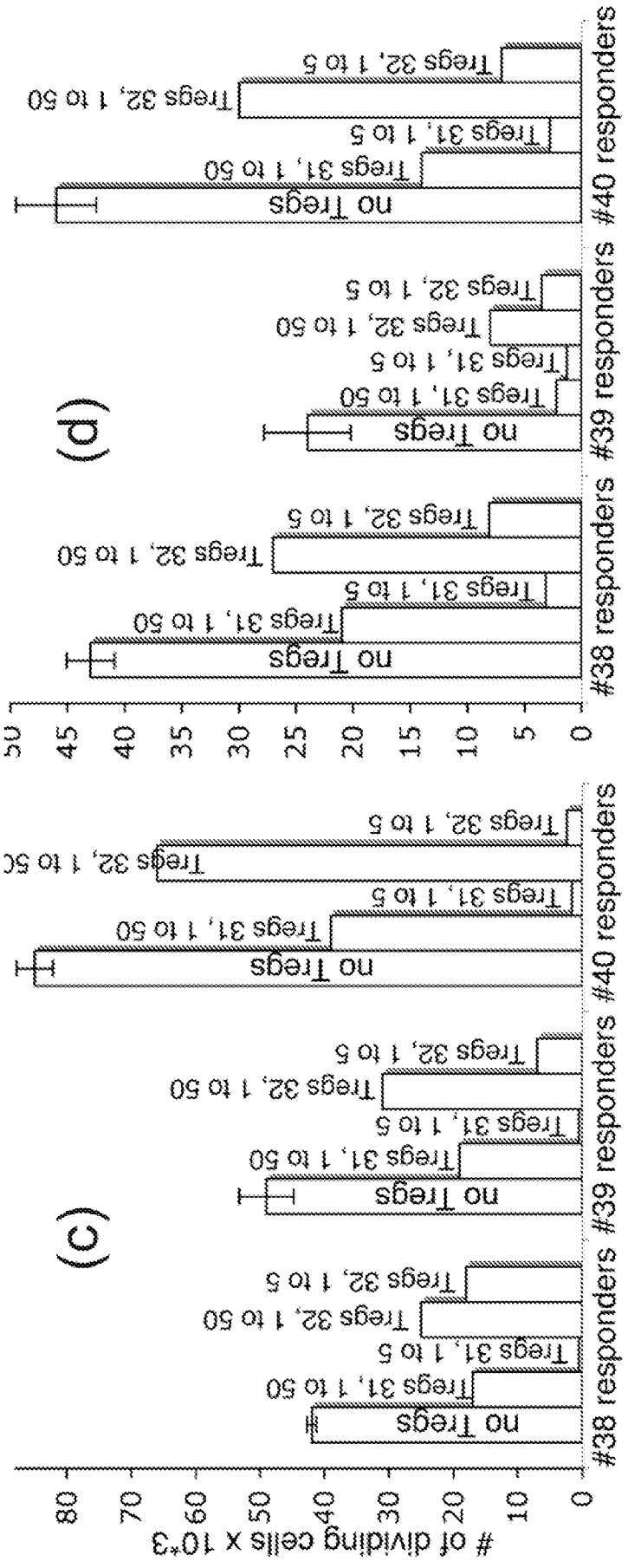

(c)
(d)
# of dividing cells x 10^3
0
10
20
30
40
50
60
70
80
no Tregs
Tregs 31, 1 to 50
Tregs 31, 1 to 5
Tregs 32, 1 to 50
Tregs 32, 1 to 5
#38 responders
#39 responders
#40 responders

FIG. 5

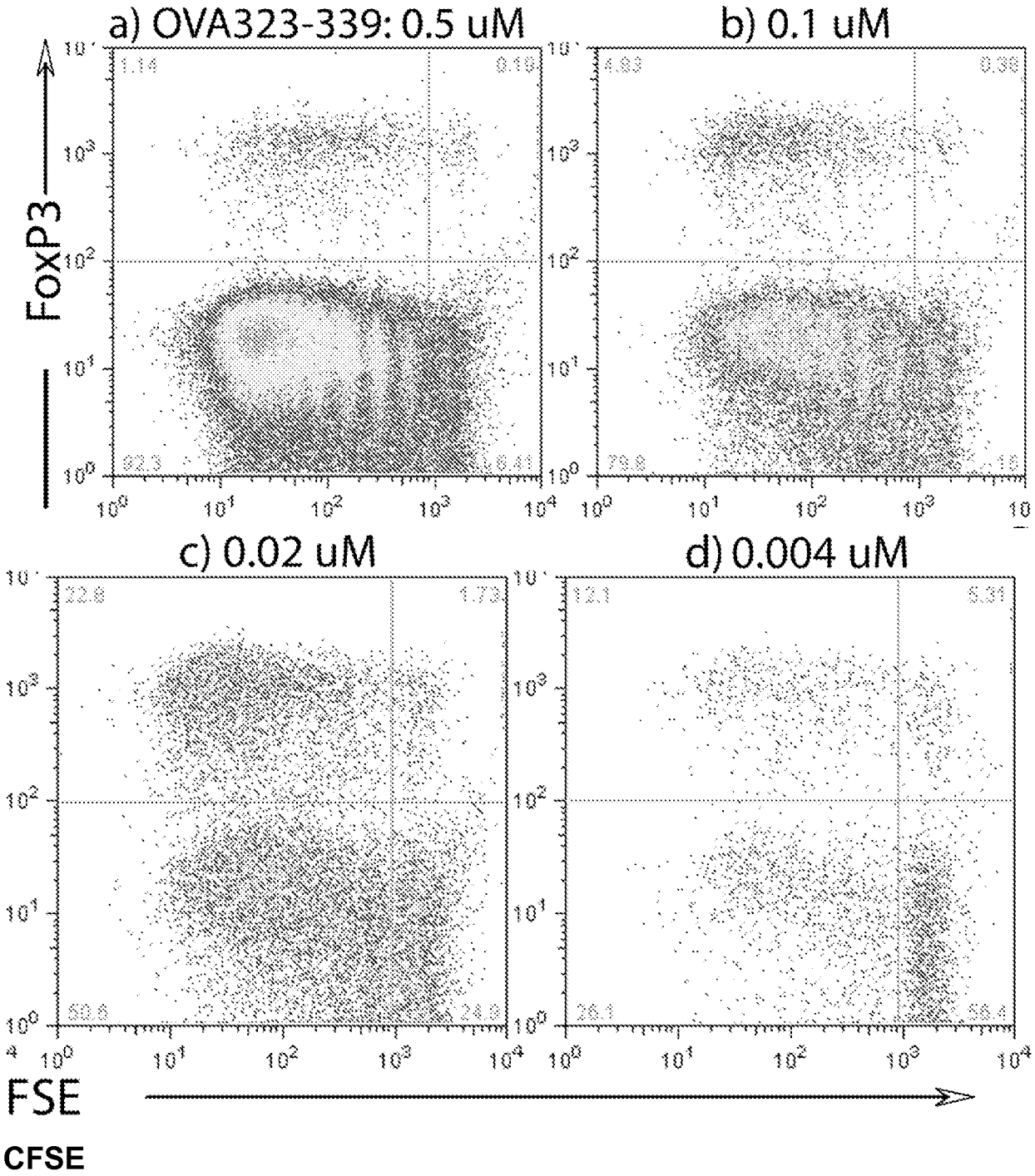


FIG. 5 (CONT.)

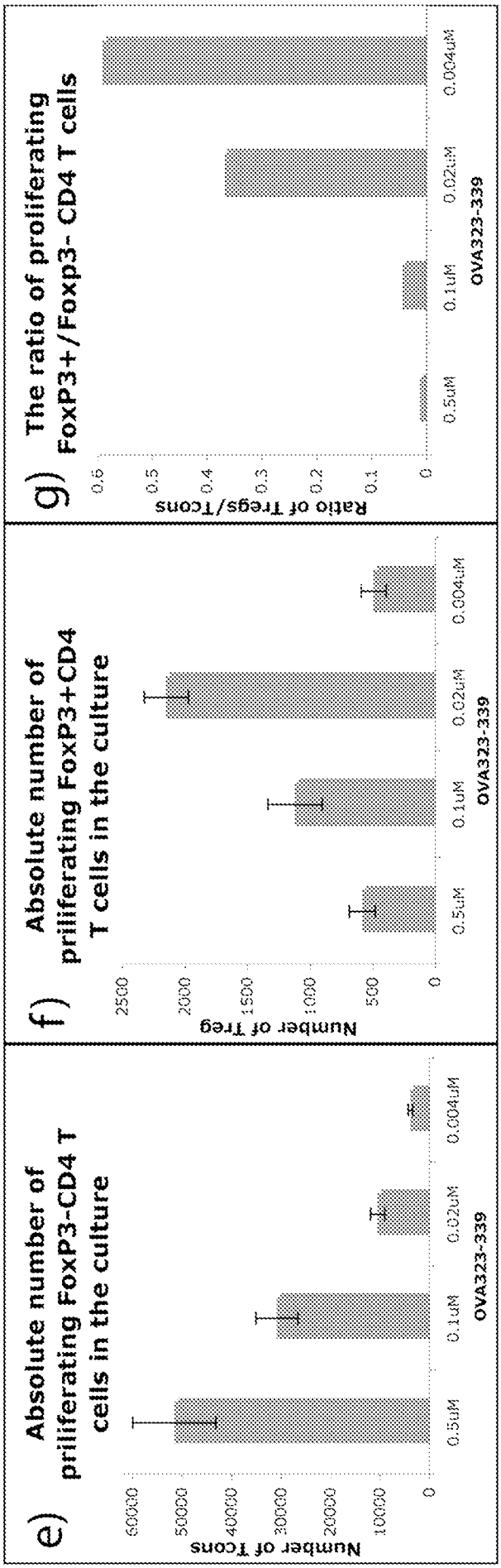

e)
Absolute number of prilliferating FoxP3-CD4 T cells in the culture
Number of Tcons
60000
50000
40000
30000
20000
10000
0
0.5uM
0.1uM
0.02uM
0.004uM
OVA323-339
f)
Absolute number of prilliferating FoxP3+CD4 T cells in the culture
Number of Treg
2500
2000
1500
1000
500
0
0.5uM
0.1uM
0.02uM
0.004uM
OVA323-339
g)
The ratio of proliferating FoxP3+/Foxp3- CD4 T cells
Ratio of Tregs/Tcons
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5uM
0.1uM
0.02uM
0.004uM
OVA323-339

FIG. 6

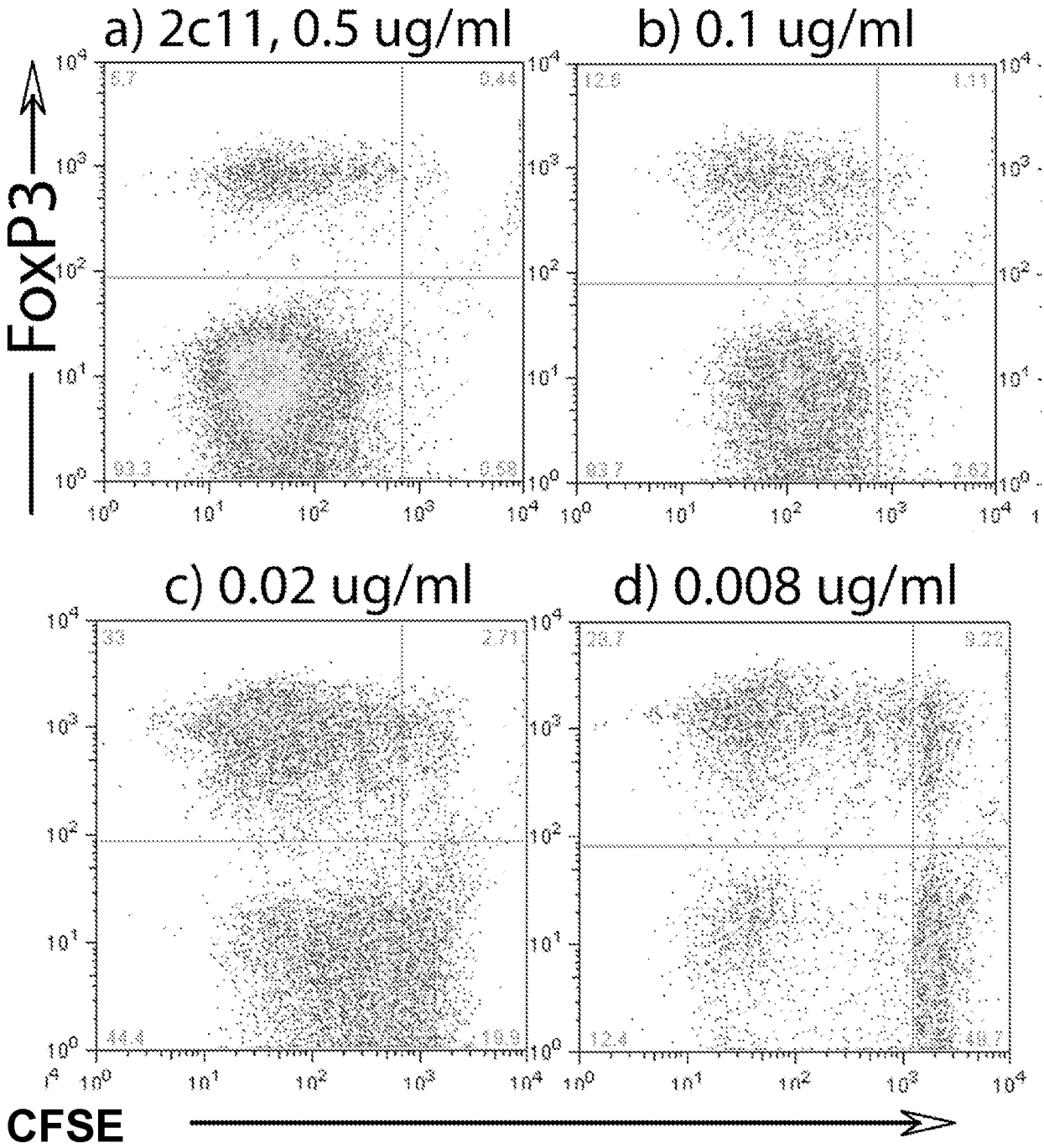


FIG. 7A

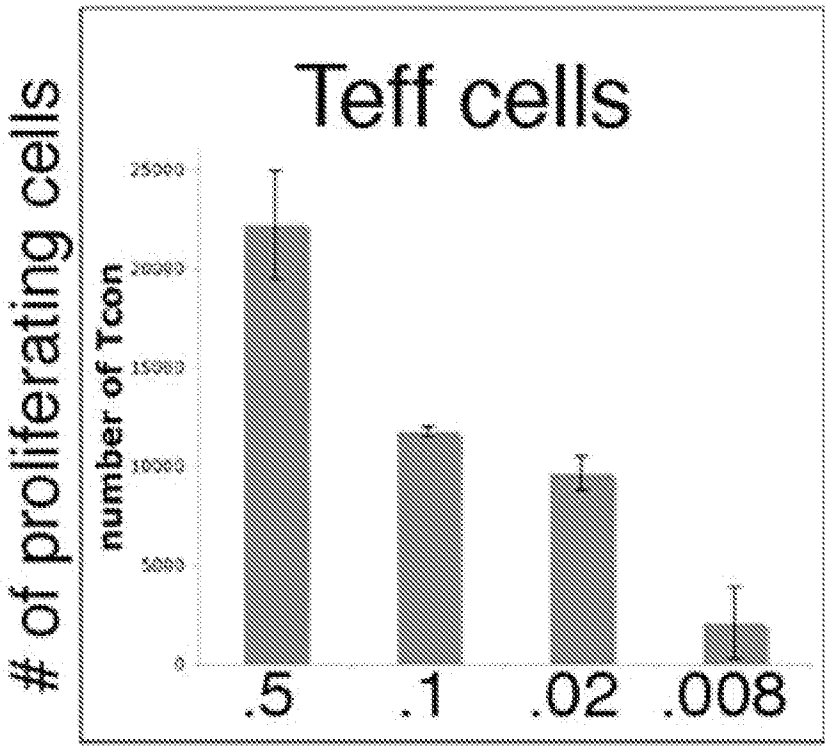

Teff cells
# of proliferating cells
number of Tcon
.5
.1
.02
.008

FIG. 7B

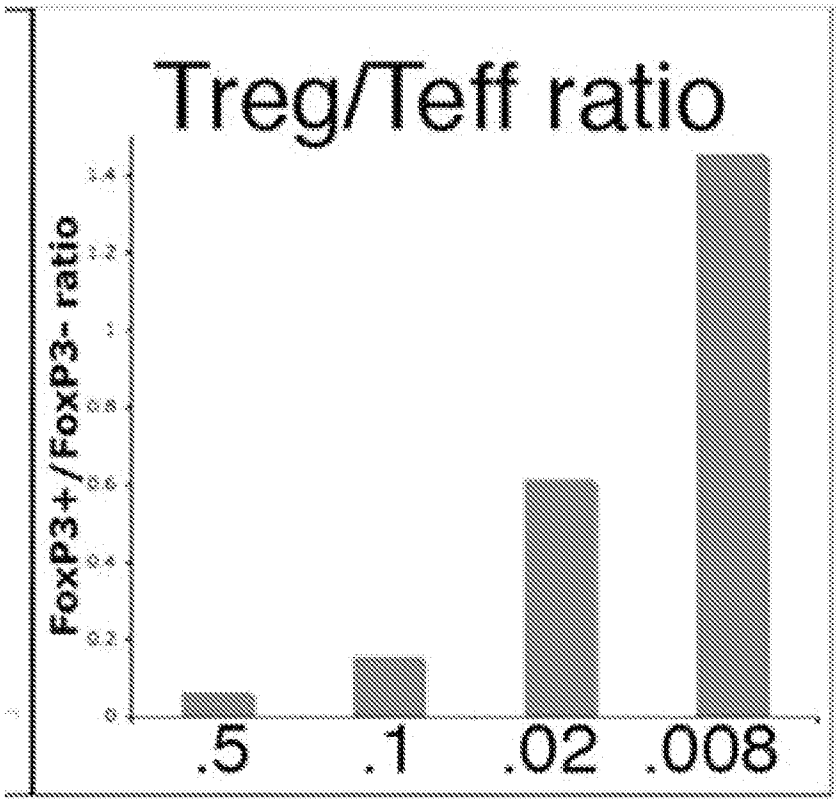

Treg/Teff ratio
FoxP3+/FoxP3- ratio
.5
.1
.02
.008

FIG. 7C

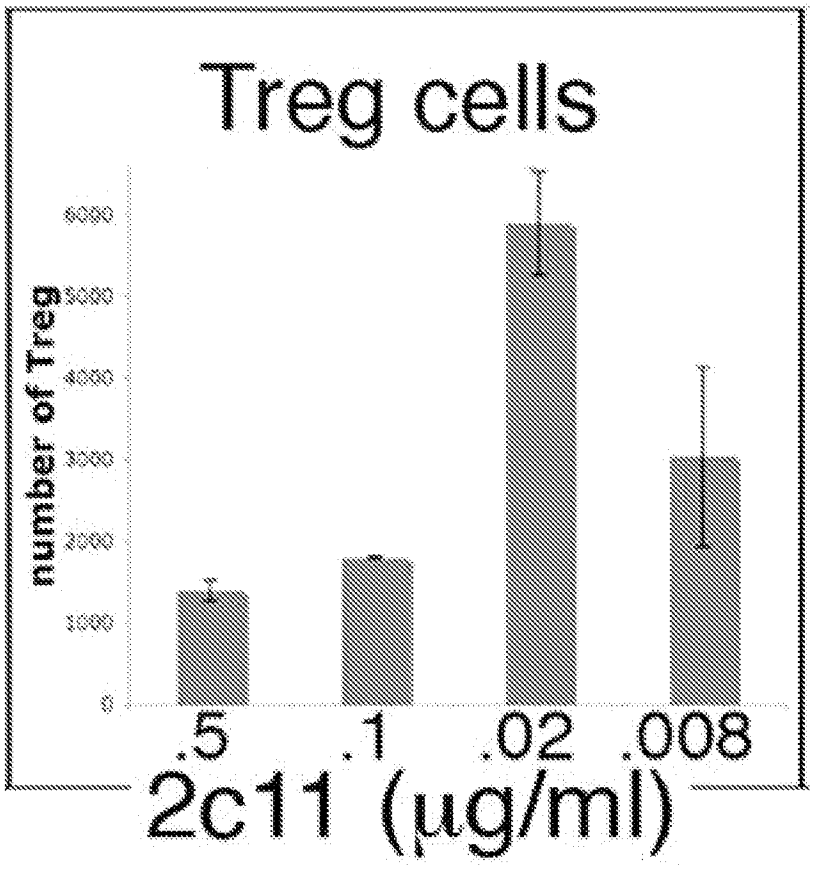

Treg cells
number of Treg
6000
5000
4000
3000
2000
1000
0
.5
.1
.02
.008
2c11 (µg/ml)

FIG. 8A

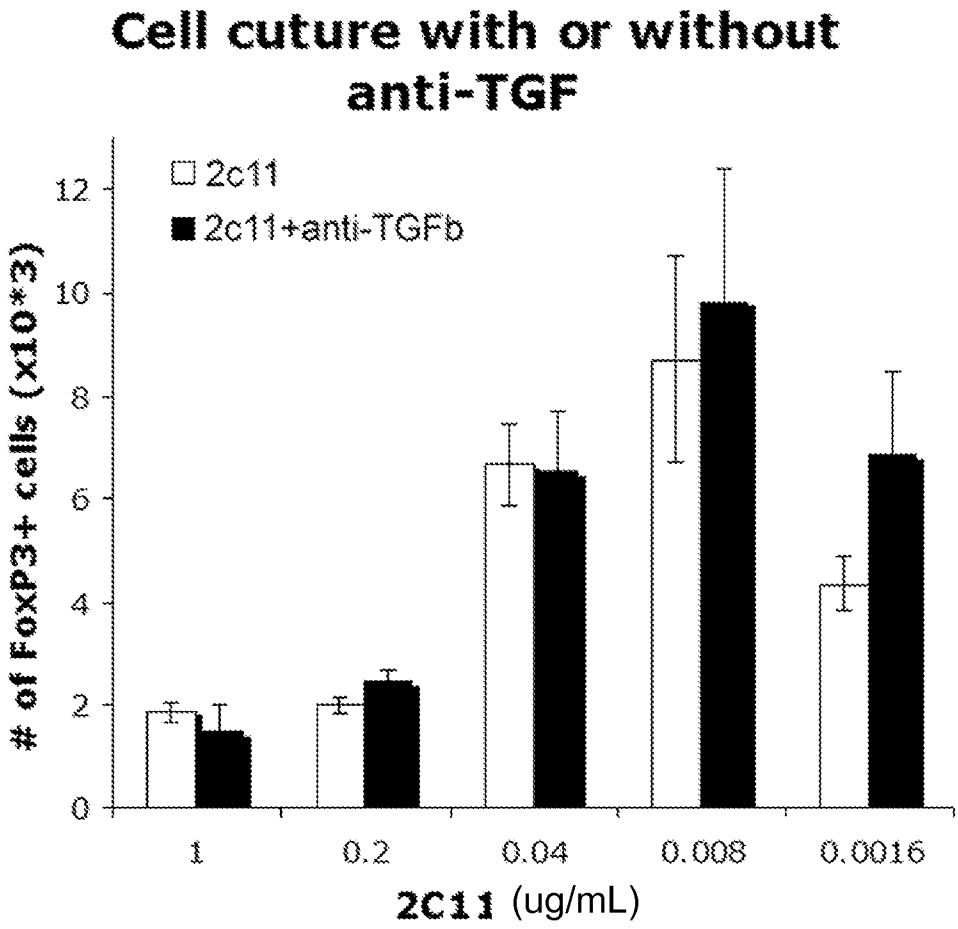

Cell cuture with or without anti-TGF
2c11
2c11+anti-TGFb
# of FoxP3+ cells (x10*3)
0
2
4
6
8
10
12
1
0.2
0.04
0.008
0.0016
2C11 (ug/mL)

FIG. 8B

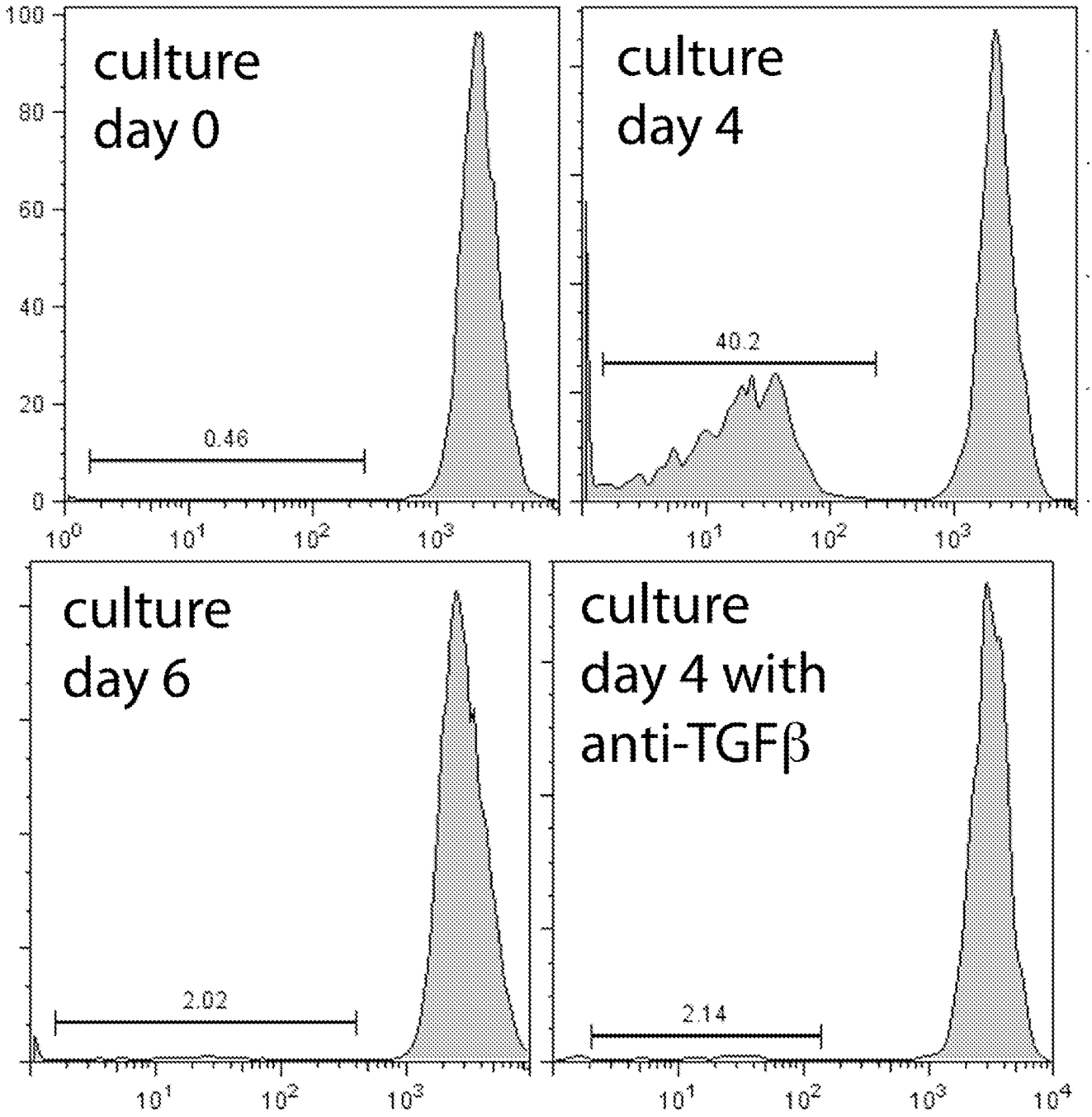

culture
day 0
0.46
culture
day 4
40.2
culture
day 6
2.02
culture
day 4 with
anti-TGFβ
2.14

FIG. 9

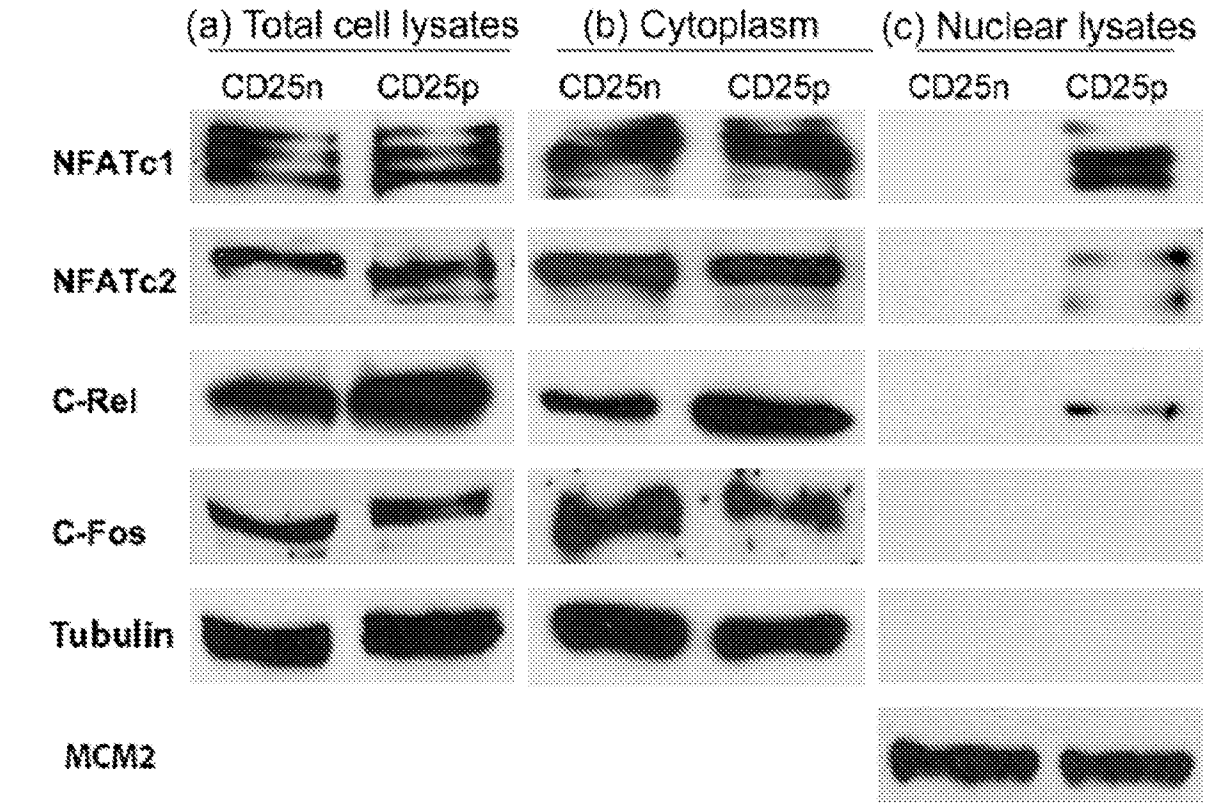

(a) Total cell lysates
(b) Cytoplasm
(c) Nuclear lysates
CD25n
CD25p
CD25n
CD25p
CD25n
CD25p
NFATc1
NFATc2
C-Rel
C-Fos
Tubulin
MCM2
(d)
CD25- Tc
NFATc1
CD25+ Tc
NFATc1
CD25- Tc
NFATc2
CD25+ Tc
NFATc2

FIG. 10

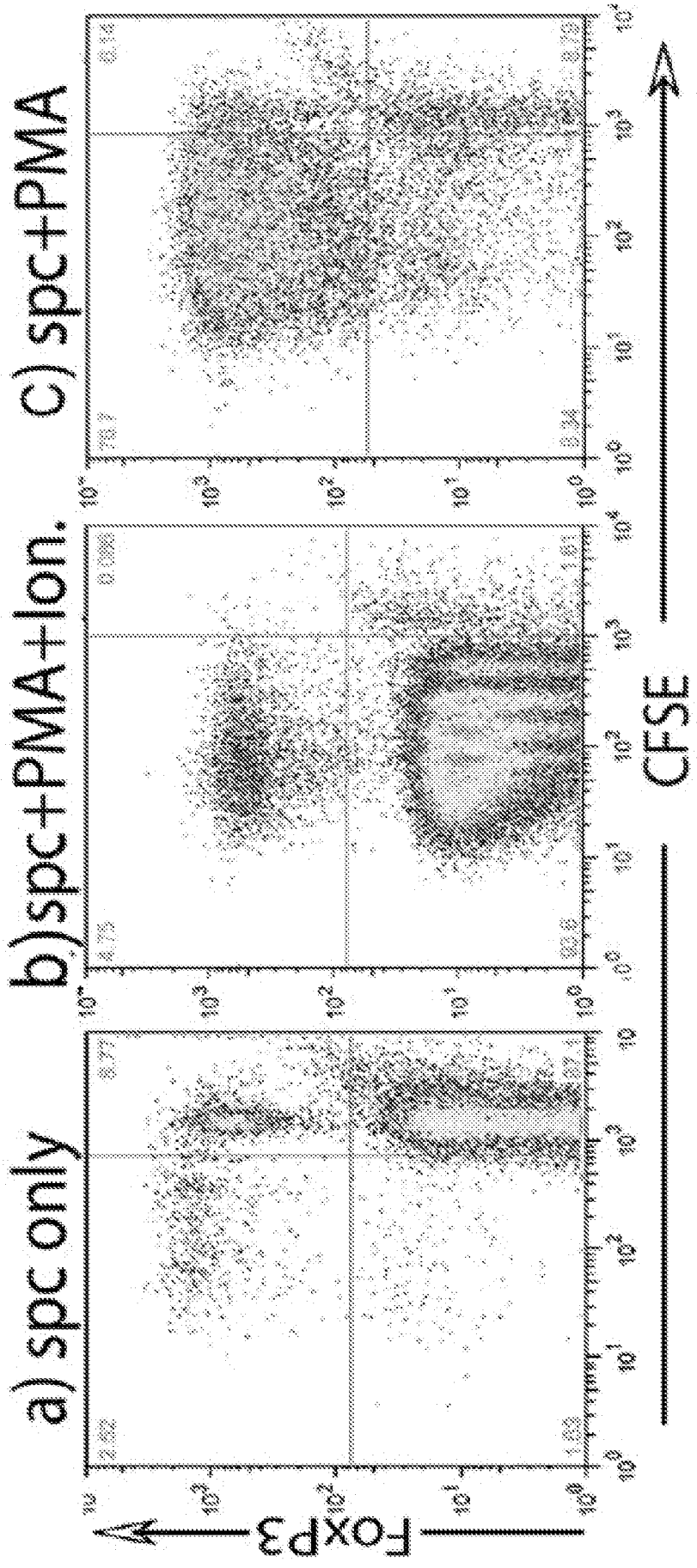


FIG. 10 (CONT.)

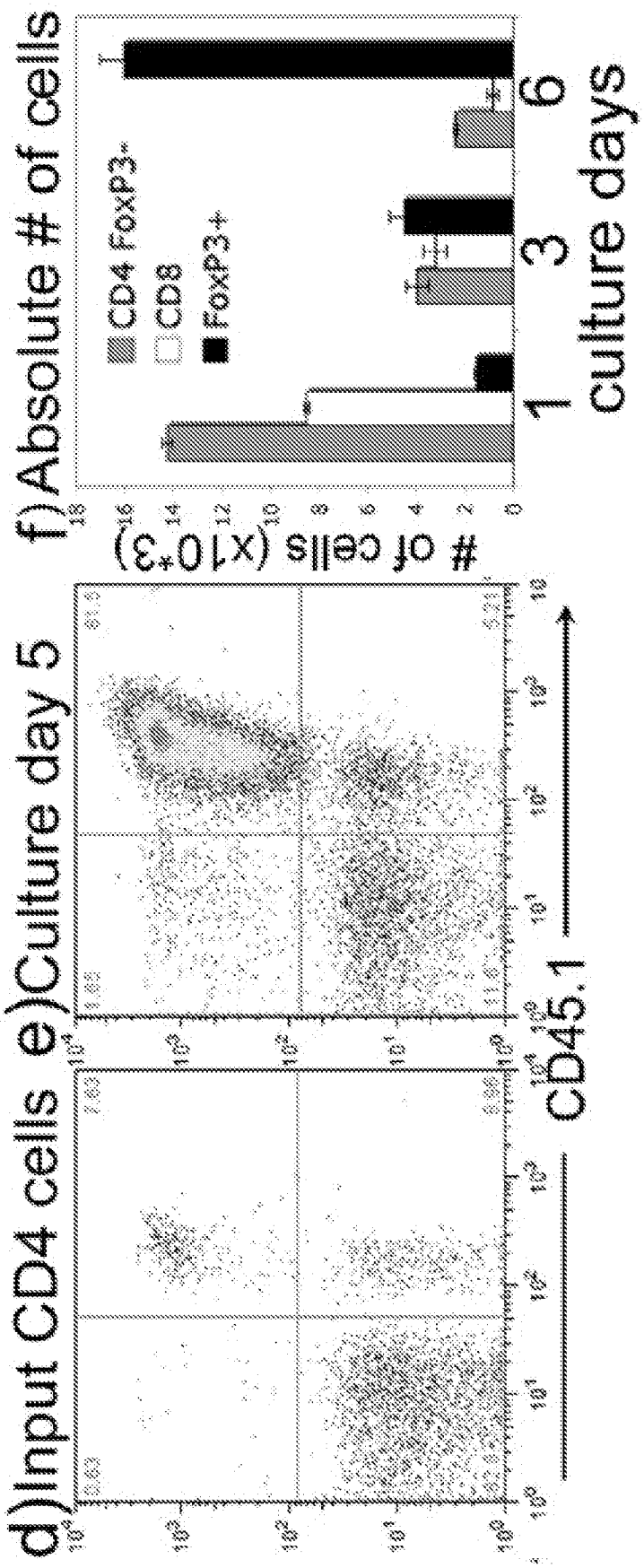

d)Input CD4 cells
e)Culture day 5
f)Absolute # of cells
CD45.1
CD4 FoxP3-
CD8
FoxP3+
# of cells (x10^3)
culture days
1
3
6

FIG. 11

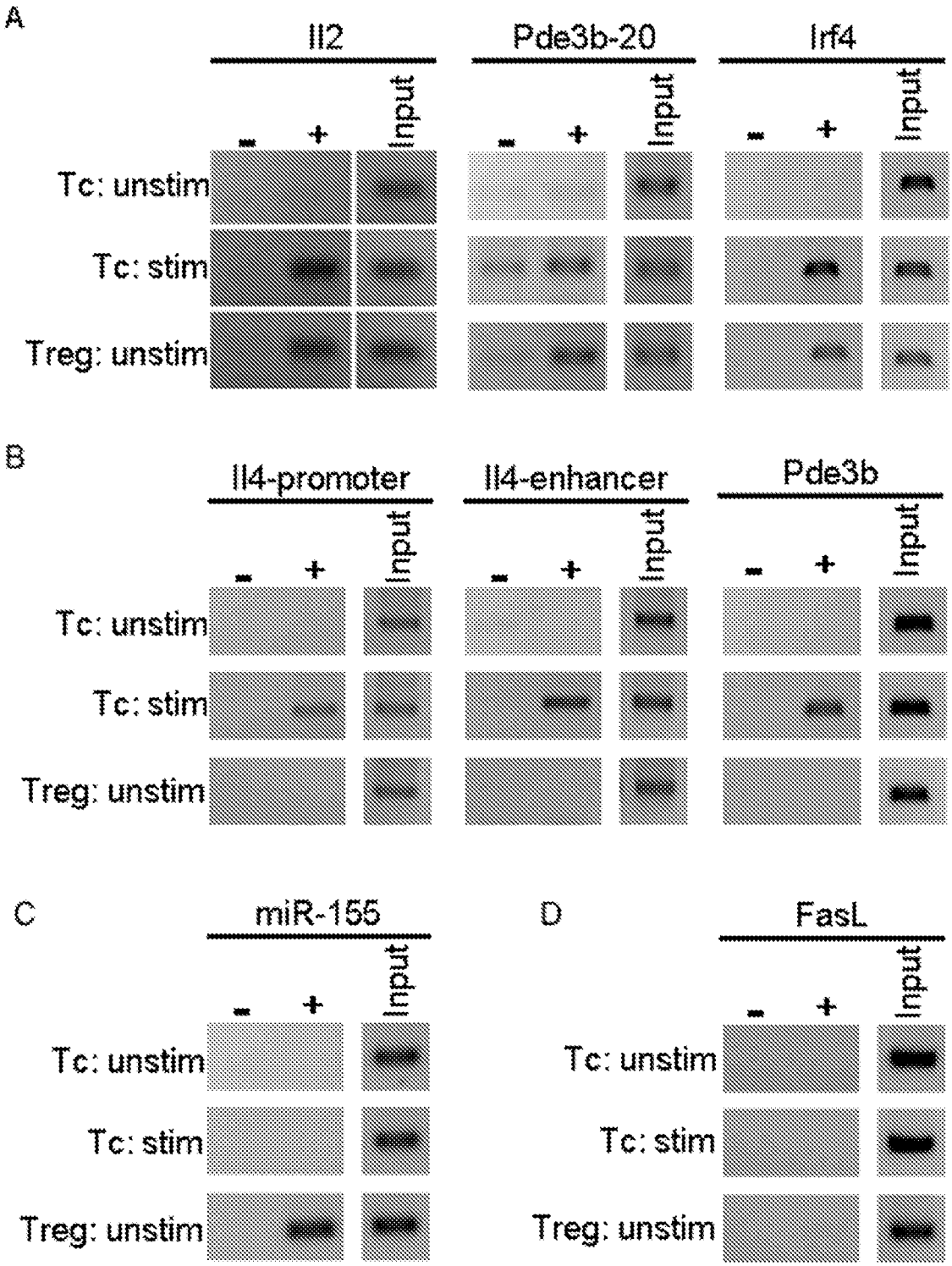
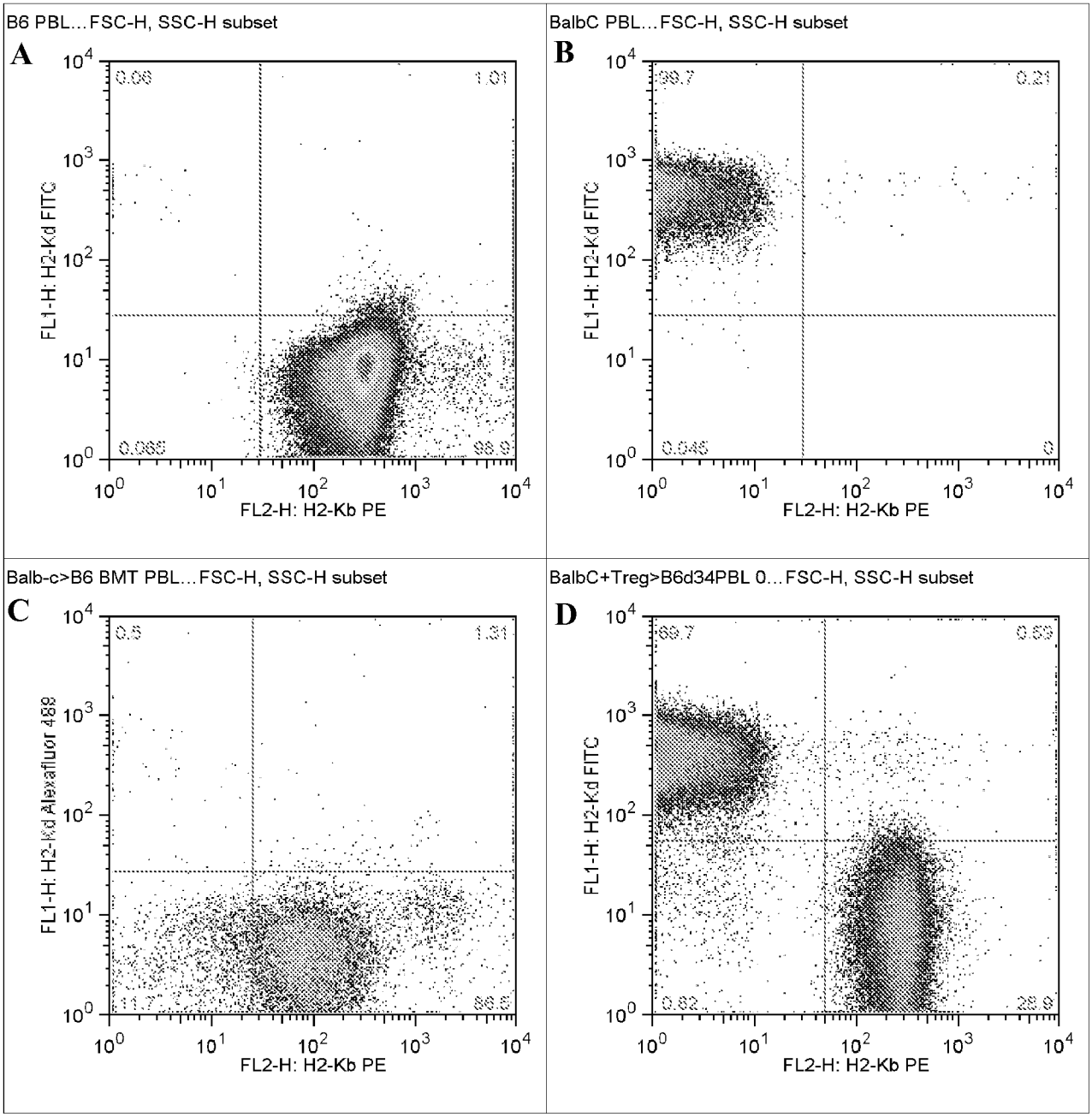

A
Il2
Pde3b-20
Irf4
-
+
Input
Tc: unstim
Tc: stim
Treg: unstim
B
Il4-promoter
Il4-enhancer
Pde3b
C
miR-155
D
FasL

FIG. 12


B6 PBL...FSC-H, SSC-H subset
A
0.06
1.01
0.065
98.9
FL1-H: H2-Kd FITC
FL2-H: H2-Kb PE
BalbC PBL...FSC-H, SSC-H subset
B
99.7
0.21
0.045
0
FL1-H: H2-Kd FITC
FL2-H: H2-Kb PE
Balb-c>B6 BMT PBL...FSC-H, SSC-H subset
C
0.5
1.31
11.7
86.6
FL1-H: H2-Kd Alexafluor 488
FL2-H: H2-Kb PE
BalbC+Treg>B6d34PBL 0...FSC-H, SSC-H subset
D
69.7
0.59
0.62
28.9
FL1-H: H2-Kd FITC
FL2-H: H2-Kb PE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/033869

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 5/0783 (2010.01) *C12N 5/02* (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases: EPODOC, WPI, CAPLUS, BIOSIS, MEDLINE; Keywords: Treg, B cell, co-stimulation, IL-2, CD28, allospecific, expansion, culture, weak stimulation and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHEN, L. C. *et al.*, "Direct expansion of Human allospecific FoxP3$^+$ CD4$^+$ regulatory T cells with allogeneic B cells for therapeutic application", The Journal of Immunology, 2009, Vol. 183, No. 6, pages 4094-4102, online publication date: 14 August 2009 See: page 4095, second column, first paragraph to page 4098, first paragraph	1-5, 7-10
P,X	ZHENG, J. *et al.*, "Efficient induction and expansion of human alloantigen-specific CD8 regulatory T cells from naïve precursors by CD40-activated B cells", The Journal of Immunology, 2009, Vol. 183, No. 6, pages 3742-3750, online publication date: 14 August 2009 See: page 3743, first column, last paragraph; page 3744, first to last paragraph; figures 1, 7	1-4, 7-10

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 September 2010	09 SEP 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **DANIEL SHEAHAN** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 7969

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/033869

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.:
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:

See Supplemental Box

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.:

1-18

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.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TU, W. *et al.*, "Efficient generation of human alloantigen-specific $CD4^+$ regulatory T cells from naïve precursors by CD40-activated B cells", Blood, 2008, Vol. 112, No. 6, pages 2554-2562 See: page 2555, second column, second paragraph; page 2556, first column, last paragraph to page 2569, first column, first paragraph; figure 1C	1-4, 7-10
X	ALBERT, M. H. *et al.*, "Ethylenecarbodiimide-coupled allogeneic antigen presenting cells induce human $CD4^+$ regulatory T cells", Clinical Immunology, 2008, Vol. 129, pages 381-393 See: page 382, second column, last paragraph; page 383, second column, third full paragraph; figure 3D	1-4, 7-10
X	WO 2007/033291 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 22 March 2007 See: [0006], [0043]-[0054]; example 1	11, 14-18
X	CHEN, T.-G. *et al.*, "Induction of dominant transplantation tolerance by an altered peptide ligand of the male antigen Dby", The Journal of Clinical Investigation, 2004, Vol. 113, No. 12, pages 1754-1762 See: page 1755, first column, first paragraph; page 1757, first paragraph to page 1758; second paragraph; page 1760, first two paragraphs	11-13, 17-18
X	WO 2006/052844 A2 (MEDICAL COLLEGE OF GEORGIA RESEARCH INSTITUTE) 18 May 2006 See: [00062]-[00079]	11, 13-16
P,X	WO 2010/000127 A1 (VERSITECH LIMITED *et al.*) 7 January 2010 See: examples 1-6	1-4, 7-10

Supplemental Box
(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

This international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, consideration has been given to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features they define different inventions.

The International Searching Authority has found that there are different inventions as follows:

- Invention 1: Claims 1-10 are directed to a method for expanding human regulatory T cells wherein human regulatory T cells are co-cultured with a B cell in the presence of a cytokine and a co-stimulatory agent.

- Invention 2: Claims 11-18 are directed to a method for expanding human regulatory T cells wherein a population of T cells is cultured with a T cell receptor (TCR) agonist so that the TCR is weakly stimulated.

- Invention 3: Claims 19-20 are directed to a method for expanding regulatory T cells wherein a population of T cells is cultured together with a phorbol ester.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

In the above groups of claims, the identified distinguishing features may have the potential to make a contribution over the prior art but are not common to all the claims and therefore cannot provide the required technical relationship. The only feature common to all of the claims and which provides a technical relationship among them is that they are all directed to methods of expanding regulatory T cells *in vitro*. However this feature does not make a contribution over the prior art because it is disclosed in a number of documents including:

WO 2007/033291 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 22 March 2007

TU, W. *et al.*, "Efficient generation of human alloantigen-specific $CD4^+$ regulatory T cells from naïve precursors by CD40-activated B cells", Blood, 2008, Vol. 112, No. 6, pages 2554-2562

Therefore in light of these documents there is no special technical feature present in the claims and the requirements for unity of invention are consequently not satisfied *a posteriori*.

The applicant elected to pay an additional fee for invention 2. Therefore this search has been carried out with respect to those claims which correspond to inventions 1 and 2, namely claims 1-18.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2010/033869

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2007033291	EP	1937309	US	2007190052
WO	2006052844	EP	1814393	US	2006093580
WO	2010000127	US	2009324557		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX