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(54) **Title:** METHODS FOR EXPANSION OR DEPLETION OF T-REGULATORY CELLS

(57) **Abstract:** The invention features methods of producing compositions enriched in Tregs and methods for treating immunologic-al disorders using these compositions. The invention also features methods for producing compositions enriched in lymphocytes and depleted of Tregs and the use of these compositions in the treatment of proliferative disorders.

METHODS FOR EXPANSION OR DEPLETION OF T-REGULATORY CELLS

BACKGROUND OF THE INVENTION

T-regulatory cells (Tregs) are a small subset of T-lymphocytes with diverse clinical applications in
5   transplantation, allergy, asthma, infectious diseases, graft versus host disease (GVHD), and
autoimmunity. Tregs are also involved in immunotolerance in conditions such as cancer. The use of
Tregs in clinical applications has been challenging because of their rarity in blood and the difficulty of
expanding them *ex vivo* into homogeneous populations. Naturally occurring Tregs constitute only 1-5%
of total CD4+ T cells in blood and they remain largely dormant until activated. Therefore, the harvesting
10   of sufficient quantities of Tregs in order to investigate their role in basic biology and for clinical medical
applications relies on the ability to expand Tregs *ex vivo*. More than a dozen protocols have been
developed worldwide to expand Tregs *ex vivo* for reinfusion into patients, but all of these protocols
produce heterogeneous progeny consisting of phenotypically and functionally mixed populations of CD4+
T cells. Heterogeneous CD4+ T cell populations hold risk because they are capable of releasing pro-
15   inflammatory cytokines and they possess cells with diverse, sometimes antagonistic functions.
Heterogeneous populations of CD4+ T cells are deemed by regulatory agencies to be impure and
irreproducible, so no clinical trials have proceeded beyond Phase I studies. Thus, a key research and
clinical goal has been to find methods to selectively expand Tregs without stimulating expansion of other
CD4+ T cell populations. A parallel goal in this field has been to find methods to selectively deplete Tregs
20   and to expand lymphocyte populations. Such lymphocyte populations would be useful to upregulate the
immune response in therapies for proliferative disorders, such as cancers.

SUMMARY OF THE INVENTION

The invention features a composition enriched in CD4+CD25^{hi} T regulatory cells (Tregs) in which
at least 60% (e.g., 70%, 80%, 90%, or 100%) of the cells in the composition are Tregs. Preferably, the
25   composition includes a homogeneous population of Tregs with desirable immune modulating properties,
e.g., expression of forkhead box P3 (FOXP3) protein. The composition also includes at least 5 X 10^6
(e.g., 5 x 10^7, 5 x 10^8, 5 x 10^9, 5 x 10^{10}, 5 x 10^{11}, or 5 x 10^{12}) Tregs. The Tregs in the composition can be
characterized as positive for the expression of one or more proteins selected from the group consisting of
CTLA4, TNFR2, FOXP3, CD62L, Fas, HLA-DR, and CD45RO, and as low or negative for the expression
30   of one or more proteins selected from the group consisting of CD127, CCR5, CCR6, CCR7, CXCR3, IFN-
gamma, IL10, and ICOS.

The invention also features a method for producing a composition enriched in CD4+CD25^{hi} Tregs,
such as the composition described above. This method generally includes contacting *in vitro* a population
of human cells that include T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) with a
35   tumor necrosis factor receptor 2 (TNFR2) agonist and/or an NF-κB activator (e.g., during one or more
culturing steps), thereby producing a composition that is enriched in the CD4+CD25^{hi} Tregs. The
population of human cells can be obtained from a human blood sample or a human bone marrow sample
from a patient. The population of human cells from the sample are, or can include, CD4+ cells, CD25+

cells, or CD4+CD25+ cells, which can be isolated or enriched from the blood or bone marrow sample prior to contacting with the TNFR2 agonist and/or the NF-κB activator. The TNFR2 agonist and/or the NF-κB activator promote enrichment of the CD4+CD25^{hi} Tregs, according to the method, by promoting an increase in the proliferation of CD4+CD25^{hi} Tregs present in the population of human cells and/or by

5   increasing the development of CD4+CD25^{hi} Tregs from T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) present in the population of human cells (e.g., by differentiation or activation). The method described above preferably produces a homogenous population of Tregs, e.g., where at least 60% (e.g., 70%, 80%, 90%, or substantially 100%) of the cells in the composition are Tregs.

        The TNFR2 agonist that can be used in the methods of the invention can be an agent selected

10   from the group consisting of an antibody (e.g., a monoclonal anti-TNFR2 antibody), a peptide, a small molecule, and a protein. Because TNFR2 signaling can proceed via the downstream NF-κB pathway, an NF-κB activator can be used to contact the population of human cells in order to produce the composition enriched in Tregs. The NF-κB activator can be selected from the group consisting of a small molecule (e.g., betulinic acid, topoisomerase poison VP16, and doxorubicin), a peptide, a protein, a virus, and a

15   small non-coding RNA.

        In addition to a TNFR2 agonist and/or a NF-κB activator, the method of producing a composition enriched in Tregs can include contacting the population of human cells (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) with one or more of interleukin-2 (IL2), rapamycin, anti-CD3 (e.g., an anti-CD3 antibody), and/or anti-CD28 (e.g., an anti-CD28 antibody). After *in vitro* proliferation, the above described

20   methods of the invention can produce at least 5 X 10^6 (e.g., 5 x 10^6, 5 x 10^7, 5 x 10^8, 5 x 10^9, 5 x 10^{10}, 5 x 10^{11}, or 5 x 10^{12}) Tregs in which at least 60% (e.g., 70%, 80%, 90%, or substantially 100%) of the cells in the composition are Tregs.

        The invention also features methods for treating an immunological disorder (e.g., an allergy, asthma, an autoimmune disorder, GVHD, or transplantation graft rejection) or an infectious disease (e.g.,

25   a bacterial infection, a viral infection, a fungal infection, and/or a parasitic infection) in a patient (e.g., a human patient) by administering to the patient any one or more of a composition enriched in Tregs, a TNFR2 agonist (e.g., a monoclonal anti-TNFR2 antibody), and a NF-κB activator. For example, the method of treatment can include administering the composition enriched in Tregs by itself or in combination with a NF-κB activator. The composition enriched in Tregs can be produced by any method

30   known in the art. One method of producing a composition enriched in Tregs is by using the methods of the invention described above. The TNFR2 agonist and the NF-κB activator for use in the method of treating an immunological disorder can be any one or more of those described above.

        Allergies that can be treated by the methods of the invention can be selected from the group consisting of food allergy, seasonal allergy, pet allergy, hives, hay fever, allergic conjunctivitis, poison ivy

35   allergy oak allergy, mold allergy, drug allergy, dust allergy, cosmetic allergy, and chemical allergy. Autoimmune disorders that can be treated by the methods of the invention can be selected from the group consisting of type I diabetes, Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Immune

Dysfunction Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Churg-Strauss Syndrome, Cicatricial Pemphigoid, CREST Syndrome, Cold Agglutinin Disease, Crohn's Disease, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Graves' Disease, Guillain-Barré, Hashimoto's Thyroiditis, Hypothyroidism, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia
5   Purpura (ITP), IgA Nephropathy, Juvenile Arthritis, Lichen Planus, Lupus, Ménière's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pemphigus Vulgaris, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis,
10   Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis.

The above described methods of treatment can include administering a composition enriched in Tregs that includes at least 5×10^6 (e.g., 5×10^6, 5×10^7, 5×10^8, 5×10^9, 5×10^{10}, 5×10^{11}, or 5×10^{12}) Tregs. Tregs having desirable immune-modulating properties include those expressing, e.g., FOXP3.

15   The invention also features an isolated antibody or antigen-binding fragment thereof that selectively binds to a first epitope of TNFR2, the first epitope includes positions 48-67 of SEQ ID NO: 1. The antibody or antigen-binding fragment thereof has an antagonistic effect on TNFR2 upon binding. The antibody or antigen-binding fragment thereof can further bind to a second epitope of TNFR2. The second epitope includes position 135 of SEQ ID NO: 1. The second epitope can include positions 135-147 of
20   SEQ ID NO: 1 (e.g., positions 130-149 of SEQ ID NO: 1, positions 128-147 of SEQ ID NO: 1, or positions 135-153 of SEQ ID NO: 1). The antibody or antigen-binding fragment thereof can be a monoclonal antibody or antigen-binding fragment thereof, a polyclonal antibody or antigen-binding fragment thereof, an Fab, a humanized antibody or antigen-binding fragment thereof, a bispecific antibody or antigen-binding fragment thereof, a monovalent antibody or antigen-binding fragment thereof, a chimeric antibody
25   or antigen-binding fragment thereof, a single-chain Fv molecule, a bispecific single chain Fv (($scFv'$)$_2$) molecule, a domain antibody, a diabody, a triabody, an affibody, a domain antibody, a SMIP, a nanobody, a Fv fragment, a Fab fragment, a F(ab')$_2$ molecule, or a tandem scFv (taFv) fragment. The equilibrium dissociation constant ("K_D") for binding of the antibody or antigen-binding fragment thereof to TNFR2 can be less than about 50 nM (e.g., less than about 30 nM, less than about 20 nM, less than about 10 nM,
30   less than about 5 nM, less than about 2 nM, less than about 1 nM, less than about 900 pM, less than about 800 pM, or less than about 700 pM). The equilibrium dissociation constant ("K_D") for binding of the antibody or antigen-binding fragment thereof to TNFR2 can be in the range of about 10 pM to about 50 nM (e.g., about 20 pM to about 30 nM, about 50 pM to about 20 nM, about 100 pM to about 5 nM, about 150 pM to about 1 nM, or about 200 pM to about 800 pM).

35   The invention also features a composition enriched in lymphocytes and depleted of Tregs, in which less than 10% (e.g., less than 9%, 8%, 7%, 5%, or 2% or substantially none) of the cells in the composition are Tregs. This composition can be produced by any method known in the art.

Furthermore, the invention also features methods for producing a composition enriched in lymphocytes and depleted of Tregs. This method generally includes contacting *in vitro* a population of

human cells that include Tregs with a tumor necrosis factor receptor 2 (TNFR2) antagonist and/or an NF-κB inhibitor. The TNFR2 antagonist and/or the NF-κB inhibitor is used to suppress the proliferation of Tregs, thereby producing a composition that is substantially depleted of Tregs. The population of human cells can be obtained from a human blood sample or a human bone marrow sample from a patient. The

5   population of human cells can include, e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells, which can be isolated or enriched from the blood or bone marrow sample prior to contacting with a TNFR2 antagonist and/or an NF-κB inhibitor. The method described above can be used to produce a composition enriched in lymphocytes (e.g., in which substantially 100% of the cells in the composition are lymphocytes) and in which less than 10% (e.g., less than 9%, 8%, 7%, 5%, or 2% or substantially none) of the cells in the

10   composition are Tregs.

The TNFR2 antagonist that can be used in the above method for producing a composition enriched in lymphocytes and depleted of Tregs can be an agent that is selected from the group consisting of an antibody (e.g., a monoclonal anti-TNFR2 antibody), a peptide, a small molecule, and a protein. The NF-κB inhibitor that can be used in the above method can be an agent selected from the group consisting

15   of a small molecule, a peptide (e.g., a cell penetrating inhibitory peptide), a protein, a virus, and a small non-coding RNA. For example, the NF-κB inhibitor can be a small molecule selected from the group consisting of 2-(1,8-naphthyridin-2-yl)-Phenol, 5-Aminosalicylic acid, BAY 11-7082, BAY 11-7085, CAPE (Caffeic Acid Phenethylester), Diethylmaleate, Ethyl 3,4-Dihydroxycinnamate, Helenalin, Gliotoxin, NF-κB Activation Inhibitor II JSH-23, NFκB Activation Inhibitor III, Glucocorticoid Receptor Modulator, CpdA,

20   PPM-18, Pyrrolidinedithiocarbamic acid ammonium salt, (R)-MG-132, Rocaglamide, Sodium Salicylate, QNZ, MG-132 [Z-Leu-Leu-Leu-CHO], Astaxanthin, (E)-2-Fluoro-4'-methoxystilbene, CHS-828, disulfiram, olmesartan, triptolide, withaferin, celastrol, tanshinone IIA, Ro 106-9920, cardamonin, BAY 11-7821, PSI, HU 211, ML130, PR 39, honokiol, CDI 2858522, andrographolide, and dithiocarbamates.

The TNFR2 antagonist can be a TNFR2 antagonist antibody that binds to a first epitope of

25   TNFR2. The first epitope includes the positions 48-67 of SEQ ID NO: 1. The antibody or antigen-binding fragment thereof can bind to a second epitope of TNFR2. The second epitope includes the position 135 of SEQ ID NO: 1 (e.g., positions 135-147 of SEQ ID NO: 1). The antibody or antigen-binding fragment thereof can be a monoclonal antibody or antigen-binding fragment thereof, a polyclonal antibody or antigen-binding fragment thereof, an Fab, a humanized antibody or antigen-binding fragment thereof, a

30   bispecific antibody or antigen-binding fragment thereof, a monovalent antibody or antigen-binding fragment thereof, a chimeric antibody or antigen-binding fragment thereof, a single-chain Fv molecule, a bispecific single chain Fv ((scFv')$_2$) molecule, a domain antibody, a diabody, a triabody, an affibody, a domain antibody, a SMIP, a nanobody, a Fv fragment, a Fab fragment, a F(ab')$_2$ molecule, or a tandem scFv (taFv) fragment. The equilibrium dissociation constant ("K_D") for binding of the antibody or antigen-

35   binding fragment thereof to TNFR2 can be less than about 50 nM (e.g., less than about 30 nM, less than about 20 nM, less than about 10 nM, less than about 5 nM, less than about 2 nM, less than about 1 nM, less than about 900 pM, less than about 800 pM, or less than about 700 pM). The equilibrium dissociation constant ("K_D") for binding of the antibody or antigen-binding fragment thereof to TNFR2 can be in the range of about 10 pM to about 50 nM (e.g., about 20 pM to about 30 nM, about 50 pM to about

40   20 nM, about 100 pM to about 5 nM, about 150 pM to about 1 nM, or about 200 pM to about 800 pM).

The invention features a method of treating a proliferative disorder (e.g., a cancer or a solid tumor) in a patient (e.g., a human patient) by administering to the patient any one or more of a composition enriched in lymphocytes and depleted of Tregs, a TNFR2 antagonist (e.g., a monoclonal anti-TNFR2 antibody), or an NF-κB inhibitor. For example, the method of treating proliferative disorders

5   can include administering the composition enriched in lymphocytes (and depleted of Tregs) by itself or in combination with a NF-κB inhibitor. The composition enriched in lymphocytes can be produced by any method known in the art. Preferably, the composition enriched in lymphocytes can be produced by the methods of the invention as described above. The TNFR2 antagonist and the NF-κB inhibitor for use in the method of treating a proliferative disorder can be any one or more of those described above.

10   The invention features a method of treating an infectious disease (e.g., a bacterial infection, a viral infection, a fungal infection, or a parasitic infection) in a patient by administering to the patient the composition enriched in lymphocytes and depleted of Tregs, a TNFR2 antagonist (e.g., a monoclonal anti-TNFR2 antibody), or an NF-κB inhibitor. For example, the method of treating an infectious disease can include administering the composition enriched in lymphocytes (and depleted of Tregs) by itself or in

15   combination with a NF-κB inhibitor. The composition enriched in lymphocytes can be produced by any method known in the art. Preferably, the composition enriched in lymphocytes can be produced by the methods of the invention as described above. The TNFR2 antagonist and the NF-κB inhibitor for use in the method of treating a proliferative disorder can be any one or more of those described above.

The invention features a method of treating an infectious disease in a patient by administering to

20   the patient an effective amount of the antibody or antigen-binding fragment thereof as described herein. The invention also features a method of treating a proliferative disease (e.g., a cancer) in a patient by administering to the patient an effective amount of the antibody or antigen-binding fragment thereof as described herein.

Cancers that can be treated according to the methods of the invention (e.g., by administering any

25   one or more of a composition enriched in lymphocytes (and depleted of Tregs), a TNFR2 antagonist and/or an NF-κB inhibitor) can be selected from the group consisting of Acute Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma; AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer; Bone Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma, Brain Stem Glioma, Visual Pathway and

30   Hypothalamic Glioma, Breast Cancer, Bronchial Adenomas/Carcinoids, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Clear Cell Sarcoma of Tendon Sheaths, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Epithelial Cancer, Esophageal Cancer,Ewing's Family of Tumors, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Intraocular Melanoma, Retinoblastoma,

35   Gallbladder Cancer, Gastric (Stomach) Cancer, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin's Lymphoma, Hypopharyngeal Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Pituitary Cancer, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer , Testicular Cancer, Thyroid Cancer, Urethral Cancer, Uterine Sarcoma, and

Vaginal Cancer. The solid tumors that can be treated with the methods of the invention can include solid tumors of the brain, lung, breast, lymphoid, gastrointestinal tract, genitourinary tract, pharynx, prostate, or ovary.

Definitions

5   The term "about" is used herein to mean a value that is ±10% of the recited value.

           The term "antibody," as used herein, includes whole antibodies or immunoglobulins and any antigen-binding fragment or single chains thereof. Antibodies, as used herein, can be mammalian (e.g., human or mouse), humanized, chimeric, recombinant, synthetically produced, or naturally isolated. In most mammals, including humans, whole antibodies have at least two heavy (H) chains and two light (L) chains connected by

10   disulfide bonds. Each heavy chain consists of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region consists of three domains, C_H1, C_H2, and C_H3 and a hinge region between C_H1 and C_H2. Each light chain consists of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region consists of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed

15   complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including

20   various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. Antibodies of the present invention include all known forms of antibodies and other protein scaffolds with antibody-like properties. For example, the antibody can be a monoclonal antibody, a polyclonal antibody, human antibody, a humanized antibody, a bispecific antibody, a monovalent antibody, a chimeric antibody, or a protein scaffold with antibody-like properties, such as fibronectin or ankyrin repeats.

25   The antibody can have any of the following isotypes: IgG (e.g., IgG1, IgG2, IgG3, and IgG4), IgM, IgA (e.g., IgA1, IgA2, and IgAsec), IgD, or IgE.

           The term "antigen-binding fragment," as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to a specific antigen (e.g., CD21 receptor). The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. The antibody

30   fragments can be a Fab, Fab'2, scFv, SMIP, diabody, a triabody, an affibody, a nanobody, an aptamer, or a domain antibody. Examples of binding fragments encompassed of the term "antigen-binding fragment" of an antibody include, but are not limited to: (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L, and C_H1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_H1 domains;

35   (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb including V_H and V_L domains; (vi) a dAb fragment (Ward et al., *Nature* 341:544-546, 1989), which consists of a V_H domain; (vii) a dAb which consists of a V_H or a V_L domain; (viii) an isolated complementarity determining region (CDR); and (ix) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by

separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see, e.g., Bird et al., *Science* 242: 423-426, 1988, and Huston et al., *Proc. Natl. Acad. Sci. USA* 85: 5879-5883, 1988). These antibody fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for utility in the same manner as intact antibodies. Antigen-binding fragments can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact immunoglobulins.

The term "chimeric antibody" refers to an immunoglobulin or antibody whose variable regions derive from a first species and whose constant regions derive from a second species. Chimeric antibodies can be constructed, for example, by genetic engineering, from immunoglobulin gene segments belonging to different species (e.g., from a mouse and a human).

The term "human antibody," as used herein, is intended to include antibodies, or fragments thereof, having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat et al (*Sequences of proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, 1991). Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (i.e., a humanized antibody or antibody fragment).

The term "humanized antibody" refers to any antibody or antibody fragment that includes at least one immunoglobulin domain having a variable region that includes a variable framework region substantially derived from a human immunoglobulin or antibody and complementarity determining regions (e.g., at least one CDR) substantially derived from a non-human immunoglobulin or antibody.

The term "TNF-α mutein," as used herein, refers to a polypeptide having an amino acid sequence that differs from the amino acid sequence of TNF-α by one or more amino acids, while retaining the ability to activate or inhibit TNFR2. For example, a TNF-α mutein may have an amino acid sequence with greater than 90% but less than 100% sequence identity relative to the amino acid sequence of a reference polypeptide (TNF-α).

The term "substantially 100%" or "substantially homogeneous" as used herein with respect to a Treg enriched composition of the invention means at least 90%, 95%, 96%, 97%, 98%, or 99% or more (e.g., all) of the cells in the composition are Tregs.

The term "treating" as used herein means stabilizing or reducing an adverse symptom associated with a condition; reducing the severity of a disease symptom; slowing the rate of the progression of a disease; inhibiting or stabilizing the progression of a disease condition; or changing a metric that is associated with the disease state in a desirable way.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a set of graphs showing that in a small double-blinded, placebo-controlled trial of human subjects, BCG treatment induces TNF-α (top left graph) and shortly thereafter Tregs appear in the treated subject (bottom left graph) versus placebo (right hand side graphs).

Figure 1B is a set of graphs showing that in freshly isolated CD4+ cells from fresh human blood, TNF-α alone does not induce FOXP3 in culture (left graph), but does induce it to higher levels when co-incubated with IL-2, compared to IL-2 alone (right graph). The data are from 14 subjects (left panel) and 10 subjects (right panel).

Figure 1C is a set of representative flow cytometry histograms that confirm greater intracellular induction of FOXP3 in CD4+CD25^{hi} Tregs after co-incubation with TNF-α and IL-2 than with IL-2 alone. Figures in flow diagrams are %. [*P < 0.05 or **P < 0.01, by paired t-test].

Figure 2A is a set of graphs showing that TNFR2 is preferentially expressed on CD4+CD25^{hi} T cells.

Figure 2B is a graph showing that one TNFR2 antibody induced FOXP3, acting as an agonist, and the other TNFR2 antibody suppressed FOXP3+ expression, acting as antagonist.

Figure 2C is a graph showing that in a signaling pathway assay, purified CD4+ cells, incubated with IL-2, the TNFR2 agonist and antagonist trigger differences in relative downstream expression of mRNA, especially in signaling proteins TRAF2, TRAF3 and apoptosis inhibitor cIAP2 that were preferentially induced by TNFR2 agonism. Data represented are means ± SEM from 4 subjects.

Figure 2D is a set of graphs showing that TNFR2 agonist triggers greater % increase in proliferation in samples from 6 subjects measured by flow cytometry (left panel) and with carboxyfluorescein diacetate succinimidyl ester (CFSE) measurements (right panels) and representative results from a typical experiment is presented with CFSE measurements (right panels). The numbers in a bar represent the percentage of cells that went into division. The TNFR2 antagonist suppressed CD4+ proliferation (left panel) and inhibited expansion AS measured by CFSE dilution (right panel). *P< 0.05 or ***P < 0.01, by paired t-test.

Figure 3A is a schematic showing the protocol for purifying CD4+CD25^{hi} cells from CD4+ cells from fresh blood and expanding for 16 days by incubation in 96 well round-bottom plate (2 ×10^4 cells/well) with anti-CD3 and anti-CD28 antibodies, human IL-2, and rapamycin.

Figure 3B is a set of graphs showing representative CD25 and FOXP3 flow diagrams of CD4+ cells before versus after CD25^{hi} purification and expansion, indicating purity of populations.

Figure 3C is a graph showing cell counts of purified Tregs, by treatment group, reveal that TNFR2 agonist induced more expansion than any other group. *p < 0.05, **p < 0.01, by paired t-test. The TNFR2 antagonist suppressed expansion versus no treatment. Data in Figure 3C are samples from 10 subjects.

Figure 4A is a set of graphs showing that all treatment groups are highly positive for Treg markers such as CD25, FOXP3, CTLA4, TNFR2, CD62L, and Fas and negative for CD127.

Figure 4B is a set of graphs showing that TNFR2 agonist-treated Tregs almost uniformly express HLA-DR and CD45RO and almost uniformly lack markers such as ICOS, CXCR3, CCR5, CCR6, CCR7, and CXCR3.

Figure 4C is a set of representative flow diagrams showing that TNFR2 agonist-treated Tregs have greater uniformity of Treg markers than do other groups (* p < 0.05, ** p < 0.01 by t-test).

Figure 5A is a set of graphs showing that in a representative case, TNFR2 agonist-treated Tregs exerted stronger and dose-dependent suppression of CD8+ cell numbers, compared to other groups, at all dilutions or suppression ratios (left panel, third column). Using a suppression index of 2:1 (CD8+ Responders to Tregs), TNFR2 agonist suppression of CD8+ cells is greater than no treatment and TNFR2 antagonist treatment (right panel). Data in Figure 5A (right panel) are samples from 5 subjects and data in Figure 5B (lower panel) are from 8 subjects.

Figure 5B is a set of graphs showing that TNFR2 agonist-treated Tregs produce a lower percentage of IFNγ+ cells.

Figure 5C is a graph showing lower numbers of T-bet+ cells after stimulation with PMA and ionomycin for 24 hours.

Figure 6 is a schematic showing the summary of findings with TNFR2 agonist versus antagonist. After purification and expansion, the TNFR2 agonist is better than TNFR2 antagonist at proliferating and yielding more phenotypically homogeneous Tregs (CD4+ CD25^{hi} FOXP3+ CTLA4+ TNFR2+ CD45RO+ CD62L+ CD127-, HLA-DR^{hi} CCR5- CCR7- CXCR3- ICOS-), with higher suppression capacity for CD8+ cells, and lower cytokine-producing capability.

Figure 7 is a graph showing the induction of FOXP3 expression by different TNFR agonists and antagonist antibodies. Screening anti-TNFR1 and anti-TNFR2 mAbs reveals that not all of the antibodies tested induce or inhibit FOXP3+ expression.

Figure 8 is a graph showing the proportion of CD25+FOXP3- cells after IL-2 overnight incubation with and without the TNFR2 agonist or antagonist. Significant percentage increases were observed if the treatment group was incubated in the presence of TNF or TNFR2 agonist. (**; p < 0.001). Data are of samples from 10 subjects.

Figure 9A is a schematic showing the expansion protocol. After 16 days of expansion, Tregs Expander Beads were removed and rested overnight for cell counting.

Figure 9B is a graph showing the magnitude of expansion by each treatment group. (*; p < 0.05, by paired t-test). Data in Figure 9B are of samples from 10 subjects.

Figure 10A is a set of graphs showing that all cells were positive for Fas.

Figure 10B is a set of graphs showing that some surface markers showed diverse patterns of expression according to the way the cells were expanded with a similar trend compared to cells expanded using rapamycin. (*; p < 0.05, **; p <0.01) determined by paired t test). Data are from 6 subjects.

Figure 11A is a graph showing the phenotypes of freshly separated Tregs before expansion (N=3, samples from 3 subjects).

Figure 11B is a set of graphs showing representative flow diagrams of Tregs markers before expansion.

Figure 12 is a set of graphs showing the density of cell surface markers measured by Mean Fluorescence Intensity (MFI). MFI of Tregs demonstrates clear differences between TNFR2 agonist expanded cells and TNFR2 antagonist expanded cells. (* p < 0.05, ** p < 0.01 determined by paired t test).

Figure 13A is a graph showing that the suppression capacity of expanded CD4+CD25+ cells was determined by CFSE dilution of CD8+ T responder cells. Flow cytometric figures of a typical result and summary of suppression index calculated based upon Responder:Treg of 2 : 1 from four independent experiments is also shown in Figure 13A.

Figure 13B is a set of graphs showing that CD4+CD25+ cells expanded with TNFR2 agonist exhibited significant enhanced suppression capacity (N=5). Those cells showed lowest cytokine producing capacity. (IFN, IL-10 and TNF after stimulation with PMA and ionomycin for 24 hours (*; p < 0.05, **; p < 0.01, by paired t test).

DETAILED DESCRIPTION OF THE INVENTION

The invention features methods for the production of a composition enriched in Tregs (e.g., CD4+,CD25^{hi} Tregs). The invention also features methods for treating immunological disorders and infectious diseases using a composition enriched in Tregs, e.g., a composition enriched in Tregs prepared by the methods described below. The invention also features methods for producing a composition enriched in lymphocytes (and depleted of Tregs) and methods of treating proliferative disorders using this composition.

Tregs and TNFR2

T regulatory cells (Tregs) are a small subset of T-lymphocytes with diverse clinical applications in transplantation, allergy, asthma, infectious diseases, GVHD, and autoimmunity. The Tregs can be used to suppress the abnormal immune response in patients in need thereof. Tregs are also known to be involved in immunotolerance in conditions such as cancer. Naturally occurring Tregs constitute only 1-5% of total CD4+ T cells in blood, and remain largely dormant until activated. In humans, Tregs are defined by co-expression of CD4+ and high expression of the interleukin-2 (IL-2) receptor alpha chain CD25^{hi}. Tregs also feature inducible levels of intracellular transcription factor FOXP3 and the expression of FOXP3 can be used to identify Tregs. TNF-α has two receptors, TNFR1 and TNFR2, each of which controls different signaling pathways. Unlike TNFR1, which has ubiquitous cellular expression, TNFR2 is expressed in a more limited manner, restricted primarily to subpopulations of T cells (in particular, Tregs),

endothelial cells, and neurons. Research in primates suggests that TNFR2-specific ligands are likely to have minimal systemic toxicity because of the restricted cellular distribution of TNFR2. Naturally occurring Tregs appear to express TNFR2 at a higher density than TNFR1. These features make TNFR2 an advantageous molecular target on Tregs.

5   *Methods for Producing an Enriched Treg Composition*

The invention features methods for expanding Tregs in a sample isolated from a patient (e.g., a human), such as a peripheral blood sample or a bone marrow sample, to produce a composition enriched in Tregs that are characterized as CD4+ and CD25^{hi}. Methods for promoting the proliferation of Tregs are known in the art, e.g., as described in Brunstein, C.G. et al., Infusion of *ex vivo* expanded T regulatory
10   cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics, *Blood* **117**, 1061-1070 (2011); Saas, P. & Perruche, S., (F1000 Immunology, 2012), Tresoldi, E. et al., Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells, *Haematologica* **96**, 1357-1365 (2011); Nadig, S.N. et al., *In vivo* prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* **16**, 809-813 (2010); Battaglia, M., Stabilini, A. & Tresoldi, E., Expanding human T
15   regulatory cells with the mTOR-inhibitor rapamycin, *Methods Mol Biol* **821**, 279-293 (2012); Pahwa, R. et al., Isolation and expansion of human natural T regulatory cells for cellular therapy, *Journal of immunological methods* **363**, 67-79 (2010); Hoffmann, P., Eder, R., Kunz-Schughart, L.A., Andreesen, R. & Edinger, M. ,Large-scale *in vitro* expansion of polyclonal human CD4(+)CD25high regulatory T cells, *Blood* **104**, 895-903 (2004); Lin, C.H. & Hunig, T., Efficient expansion of regulatory T cells *in vitro* and *in*
20   *vivo* with a CD28 superagonist, *European journal of immunology* **33**, 626-638 (2003); Lan, Q. et al., Induced FOXP3(+) regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? *Journal of molecular cell biology* **4**, 22-28 (2012); Sagoo, P. et al., Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells, *Science Translational Medicine* **83**, 1-10 (2011); Edinger, M. & Hoffmann, P.,
25   Regulatory T cells in stem cell transplantation: strategies and first clinical experiences, *Current opinion in immunology* **23**, 679-684 (2011); Trzonkowski, P. et al., First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127-T regulatory cells, *Clinical Immunology* **133**, 22-26 (2009); Di Ianni, M. et al., Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation, *Blood* **117**, 3921-3928 (2011); Hippen, K.L.
30   et al, Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of *in vivo* functional activity. *Sci Transl Med* **3**, 83ra41 (2011); Kim, Y.C. et al., Oligodeoxynucleotides stabilize Helios-expressing Foxp3+ human T regulatory cells during *in vitro* expansion, *Blood* **119**, 2810-2818 (2012); Bacchetta, R. et al., Interleukin-10 Anergized Donor T Cell Infusion Improves Immune Reconstitution without Severe Graft-Versus-Host-Disease After Haploidentical Hematopoietic Stem Cell
35   Transplantation. *ASH Annual Meeting Abstracts* **114**, 45- (2009); Desreumaux, P. et al., Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease, *Gastroenterology* **143**, 1207-1217 e1202 (2012); Clerget-Chossat, N. et al., in International Society for Cell Therapy Seattle, Washington, (2012); and Cardenas, P.A., Huang, Y. & Ildstad, S.T., The role of pDC, recipient T(reg) and donor T(reg) in HSC engraftment: Mechanisms of facilitation, *Chimerism* **2**, 65-
40   70 (2011). Each of these publications, and their methods for expanding Tregs, is incorporated herein by reference.

The protocols for promoting the proliferation of Tregs that are described in the above publications generally include obtaining fresh sample (e.g., a blood sample) from a patient (e.g., a human patient) that includes a population of CD4+ cells. The CD4+ cells can be further purified or enriched in one or more steps prior to the expansion of Tregs. The CD4+ cells can be separated from the sample using

5   techniques known in the art (e.g., using magnetic beads conjugated to anti-CD4+ antibodies such as Dynabeads® CD4 Positive Isolation kit (Invitrogen)). During culturing, the CD4+ cells can be contacted with one or more reagents to stimulate their proliferation. For example, one or more of anti-CD3 antibody, anti-CD28 antibody, human IL-2, and rapamycin can be added. However, this method alone produces a heterogeneous population of cells, some of which are capable of releasing pro-inflammatory cytokines

10   that can be detrimental to the patient. This heterogeneous population is not useful for treatment of immunological disorders or infectious diseases and Tregs cannot be easily isolated from this heterogeneous population without damaging them. The present invention improves upon these protocols by using a TNFR2 agonist that preferentially promotes proliferation of Tregs and produces a homogeneous population of Tregs with desirable traits, e.g., a sub-population of Tregs that express

15   FOXP3. The TNFR2 agonist and/or the NF-κB activator promote enrichment of the CD4+CD25^{hi} Tregs, according to the method, by promoting an increase in the proliferation of CD4+CD25^{hi} Tregs present in the population of human cells and/or by increasing the development of CD4+CD25^{hi} Tregs from T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) present in the population of human cells (e.g., by differentiation or activation). The invention produces a population of cells enriched in Tregs

20   that can be used for treatment of immunological disorders or infectious diseases as described herein.

In vitro Expansion of Tregs Using a TNFR2 Agonist

In general, the present method includes obtaining a starting population of T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) from a human sample, e.g., blood or bone marrow sample. When blood sample is used, typically 3-4 tubes of blood (~2-10 mL in each tube) can be used in

25   the protocol described below. One skilled in the art can adjust the amount of the blood sample that is used depending on the scale of the cell-culture protocol.

In general, anti-CD4 and/or anti-CD25 antibodies attached to a bead matrix, e.g., a magnetic bead (such as Dynabeads®), can be used for isolating the CD4+ cells, CD25+ cells, or CD4+CD25+ cells. For example, the CD4+ T cells can be isolated by using commercially available reagents, e.g.,

30   Dynabeads® CD4 Positive Isolation kit, and the CD25+ cells can be isolated using commercially available reagents, e.g., Dynabeads CD25 and/or DETACHaBEAD CD4/CD8 (Invitrogen). When CD4+CD25+ cells are used as the starting population, the cells can be isolated using both anti-CD4 and anti-CD25 beads in a single step or in a two step method by isolating the CD4+ cells first followed by isolation of the CD25+ cells, or vice versa.

35   The isolated CD4+ cells, CD25+ cells, or CD4+CD25+ cells can then be expanded in cell culture for about 16 days (e.g., about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 or more days) in a suitable cell culture vessel, e.g., a 96 well round-bottom plate (2×10^4 cells/well), in the presence of one or more of anti-CD3 and/or anti-CD28 antibodies. The amount of cells to be used in the starting culture will depend on the volume of the cell culture vessel. The anti-CD3 and anti-CD28 antibodies can be present

throughout the course of cell culture, e.g., for each days of culture or only during a portion of the cell culture (one or several days during culturing). Typically the anti-CD3 and anti-CD28 antibodies can be added in the form of commercially available Dynabead Human Treg Expander (Invitrogen) at a bead to cell ratio of 2:1.

5   Human IL-2 and/or rapamycin can also be added to the cell culture media, e.g., one or more times during the course of cell culture. For example, human IL-2 can be added every two days, e.g., at day 2, 4, 7, 9, 11, and 14 of a 16 day cell culture period. Rapamycin can be added at day 0, 2, 4, and 7 of a 16 day-cell culture period. Human IL-2 and rapamycin can be added together or on alternating days. Typically, human IL-2 is added two days after the start of cell culture. Human IL-2 and /or rapamycin can
10   also remain in the cell culture for the entire period of the expansion protocol. The cell culture media can be changed every 2-3 days by changing half of the media with fresh media; the fresh media may also contain human IL-2 and/or rapamycin. For example, half of the media can be changed every 2-3 days containing rapamycin (until day 7) and IL-2. Typically rapamycin can be used at a concentration of 0.5 nM to 100 µM (e.g., 0.5 nM, 1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 0.5 µM, 0.75 µM , 1 µM, 1.2 µM, or 2
15   µM). and human IL-2 can be used at a concentration of 0.05 to 6,000 U/ml (e.g., 0.05 U/ml, 1 U/ml, 2 U/ml, 10 U/ml, 20 U/ml, 50 U/ml, 100 U/ml, 150 U/ml, 200 U/ml, 250 U/ml, or 300 U/ml). During the course of the cell culture, as described above, the cells can be passaged as necessary. Protocols for passaging of cells are known in the art.

        A TNFR2 agonist can be added at the start of culture at day 0 or at later time points after initiation
20   of the culture (e.g., on any one of the days after initiation of culturing, so long as at least one or more days (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16, or more days) of culturing includes contacting the cells with a TNFR2 agonist). Additional TNFR2 agonist can be added at several points during the course of cell culture, e.g., at one or more of days 7, 8, 9, 10, 11, or 12. Typically a TNFR2 agonist is added on day 0 and additional TNFR2 agonist can be added on day 9 of a 16 day-cell culture
25   period (see, e.g., Figure 3A). The TNFR2 agonist that can be typically used is an anti-TNFR2 monoclonal antibody. Additional TNFR2 agonists that can be used in this method are described below. In general, the anti-TNFR2 antibody can be used at a concentration in the range of 0.05 µg/ml to 500 µg/ml, or more if necessary (e.g., 0.05 µg/ml, 0.1 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, 2.5 µg/ml, 3 µg/ml, 3.5 µg/ml, 4 µg/ml, 4.5 µg/ml, or 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400
30   µg/ml, or 500 µg/ml). The anti-TNFR2 antibody can be attached to a matrix, e.g., a bead, such as a magnetic bead, for removal at the end of the cell culture period.

        The cells can be harvested and the anti-CD3 and anti-CD28 regents can be removed, e.g., by removal of the Treg Expander beads by the Detach-a-bead reagent or by multiple rounds of proliferation that permits bead detachment. Cells can then be washed in an appropriate medium and rested. Cells
35   can then be analyzed for expression of various protein markers, stored appropriately, and/or used in methods for treating various disorders as described below.

        After *in vitro* proliferation, the Tregs in the enriched composition comprise at least 60% (e.g., 70%, 80%, 90%, or 100%) of the cells in the composition. The method described above preferably

produces a homogenous population of Tregs, e.g., where substantially 100% (e.g., at least 90%, 95%, 96%, 97%, 98%, or 99% or more (e.g., all)) of the cells in the composition are Tregs.

The method described above can result in an approximately 2 fold (e.g., 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, or 4-fold or more) expansion of Tregs. The Tregs produced by this expansion protocol

5   are characterized as expressing FOXP3, e.g., preferably at least 80% (e.g., 85%, 90%, 95%, 98%, 99%, or substantially 100%) of the cells in the expanded population of Tregs express FOXP3. In addition, it is preferable that the Tregs expanded by the methods of the invention express high levels of FOXP3. The Treg population also includes a low percentage of cells expressing IFNγ. The Treg population also exhibits increased capacity for suppressing activation of CD8+ cells.

10   In previously described Treg expansion protocols, a disadvantage was that the identification of the expanded Tregs in a heterogeneous population of cells required post-expansion sorting, often multiple rounds of sorting, of the Tregs. These multi-sorting procedures severely and adversely affected the viability, function, and yield of the Tregs and therefore limited the subsequent use of the sorted cells in therapeutic applications. Furthermore, this sorting could only enrich for Tregs expressing cell surface

15   markers and not for Tregs expressing FOXP3, which is an intracellular marker. The present invention features the proliferation of Tregs by contacting a population of human cells that is, or that includes, T lymphocytes (e.g., human CD4+ cells, CD25+ cells, or CD4+CD25+ cells) with a TNFR2 agonist to produce a substantially homogenous population of Tregs that express FOXP3, CD4+, and CD25^{hi}. The Tregs produced by the present method require no post-expansion sorting prior to use in therapeutic

20   applications. This is a significant advantage over previously described Treg expansion protocols. The Tregs produced by the present invention are also more potent than previously described Treg cell populations, which may be a result of their homogeneity or the subset of Tregs produced by the present method, or both. The present enriched Treg populations exhibit highly desirable qualities similar to those of immune-modulating Tregs.

25   *TNFR2 agonists*

The TNFR2 agonist that can be used in the methods of the invention include agents, such as an antibody, a peptide, a small molecule, and a protein. The TNFR2 agonist is an agent that can bind to TNFR2 and activate TNFR2 signaling. The TNFR2 agonist can be any agent that, when contacted with CD4+ T cells, can stimulate the expression of any one or more proteins selected from the group

30   consisting of FOXP3, TNF, TRAF2, TRAF3, and cIAP2.

In particular, the TNFR2 agonist can be a monoclonal antibody that binds TNFR2, such as Clone MR2-1 (Cell Sciences) or Clone MAB2261 (R&D Systems, Inc.). The TNFR2 agonist can also be a TNF-α mutein that binds only to TNFR2 as an agonist. TNF-α muteins that can be used as TNFR2 agonists include those described in, e.g., U.S. Patent Application Publication No. 2008/0176796 A1; U.S. Patent

35   Nos. 5,486,463 and 5,422,104; PCT Publication Nos. WO 86/02381; WO 86/04606; and WO 88/06625; and European Patent Nos. 155,549; 168,214; 251,037; 340,333; and 486,908. Each of these publications is incorporated herein by reference.

In addition, anti-TNFR2 antibodies that are capable of acting as TNFR2 agonists are described in Galloway et al. (*Eur. J. Immunol.* 22:3045-3048, 1992), Tartaglia et al. (*J. Biol. Chem.* 268:18542-18548, 1993), Tartaglia et al. (*J. Immunol.* 151:4637-4641, 1993), Smith et al. (*J. Biol. Chem.* 269:9898-9905, 1994), and Amrani et al. (*Am. J. Respir. Cell. Mol. Biol.* 15:55-63, 1996); each of which is incorporated

5   herein by reference.

Peptides that are capable of acting as a TNFR2 agonist can include an 11 amino acid TNF receptor agonist peptide (TNF$_{70-80}$) described in Laichalk et al. (Infection & Immunity 66:2822-2826, 1998), incorporated herein by reference.

Since activation of the NF-κB pathway is a downstream effect of TNFR2 agonism, the Treg

10   expansion method can instead, or in addition, include contacting the T lymphocyte population (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) with one or more activators of the NF-κB pathway in place of a TNFR2 agonist. The NF-κB activator can be a small molecule, a peptide, a protein, a virus, or a small non-coding RNA. For example, the NF-κB activator any one of the small molecules described in Manuvakhova et al., *J. Neurosci. Res.* 89: 58-72, 2011 (incorporated herein by reference). Alternatively,

15   the NF-κB activator can be betulinic acid. The NF-κB activator can also be the topoisomerase poison VP16. Additionally the NF-κB activator can be doxorubicin.

Characterization of Tregs

The Tregs in the enriched composition produced by the above described methods are CD4+ and CD25^{hi} and can be characterized by the presence or absence of one or more additional molecular

20   markers. For example, the Tregs produced by the methods of the invention may express one or more proteins selected from the group consisting of FOXP3, CTLA4, TNFR2, CD62L, Fas, HLA-DR, and CD45RO and are considered to be "positive" for these markers. Alternatively, the Tregs may not express, or may express in low to near undetectable amounts, one or more proteins selected from the group consisting of CD127, CCR5, CCR6, CCR7, CXCR3, IFN-gamma, IL10, and ICOS and may be considered

25   to be considered "negative" for these markers. Preferably, the method produces a composition enriched in Tregs, in which at least 90% of Tregs express HLA-DR and less than 5% of Tregs express ICOS.

Treatment Using an Enriched Treg Composition of the Invention

The invention features methods for treating a variety of diseases, e.g., immunological disorders and conditions, such as allergies, asthma, autoimmune diseases, GVHD, transplantation graft rejection,

30   and infectious diseases by administering a composition enriched in Tregs to a patient (e.g., a human) in need thereof. The composition enriched in Tregs can be produced by the methods described above, for example, by contacting a human sample, e.g., a blood or bone marrow sample, containing T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) with a TNFR2 agonist (e.g., a TNFR2 agonist antibody) and/or an NF-κB activator to produce the composition enriched in Tregs (e.g., a substantially

35   homogenous population of Tregs). The TNFR2 agonist and/or the NF-κB activator promote enrichment of the CD4+CD25^{hi} Tregs, according to the method, by promoting an increase in the proliferation of CD4+CD25^{hi} Tregs present in the population of human cells and/or by increasing the development of

CD4+CD25^{hi} Tregs from T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) present in the population of human cells (e.g., by differentiation or activation).

A patient in need of treatment for an immunological disorder or condition, such as an allergy, asthma, an autoimmune disease, GVHD, or a transplantation graft rejection, or for an infectious disease
5   can be administered an enriched composition of Tregs (e.g., CD4+CD25^{hi} Tregs or CD4+CD25^{hi}FOXP3+ Tregs) produced from their own blood or bone marrow using the following steps: i) obtaining a cell sample, e.g., a blood or bone marrow sample, from the human patient; ii) isolating T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) from the sample as described in the method above; iii) subjecting these cells to the Tregs expansion method described above (e.g., contact and/or culturing of
10   the CD4+ cells, CD25+ cells, or CD4+CD25+ cells with anti-CD3 antibody, anti-CD28 antibody, and a TNFR2 agonist in combination with IL-2 and/or rapamycin, and/or contact or culturing of the CD4+ cells, CD25+ cells, or CD4+CD25+ cells with an NF-κB activator) to produce a composition enriched in Tregs (e.g., a substantially homogenous population of Tregs in which the Tregs comprise, e.g., >90% of cells in the composition); and iv) introducing the composition enriched in Tregs into the patient without any (or
15   with limited) post-expansion sorting of the enriched Tregs in order to treat the disease or disorder. The above steps of the method of treatment can be performed in iterative cycles, where the number of cycles of treatment provided to the patient can be determined by the disorder being treated, the severity of the disorder, and/or the outcome of each treatment cycle (i.e., a change in the disease state). For example, changes in efficacy markers and/or in clinical outcomes can be used for determining the frequency with
20   which blood or bone marrow should be obtained from a patient, Tregs enriched from that blood or bone marrow, and/or the enriched Tregs administered to the patient. The enriched Treg composition can also be stored (e.g., frozen) for future administration.

Preferably, the Tregs are obtained from a patient's own blood or bone marrow (i.e., autologous cells), although the following methods of treatment may also include the use of allogeneic Tregs with
25   possible best fit HLA matching or from unrelated donors that have been expanded according to the above methods. Allogeneic Tregs preferentially share at least 4/6 HLA markers in common with the patient receiving the enriched Treg composition.

Treatment of an Immunological Disorder or Condition Using an Enriched Treg Composition of the Invention
30   The enriched Treg composition produced by the methods described above can be administered to a patient suffering from an immunological disorder or condition, such as an allergy, asthma, an autoimmune disorder, GVHD, or a transplantation graft rejection, to treat the immunological disorder or condition.

1) Allergies:

35   An enriched Treg composition of the invention can be used to treat one or more allergic conditions in a patient, such as an allergy selected from the group consisting of food allergy, seasonal allergy, pet allergy, hives, hay fever, allergic conjunctivitis, poison ivy allergy oak allergy, mold allergy, drug allergy, dust allergy, cosmetic allergy, and chemical allergy. Administration and dosage of the Treg composition are discussed herein below.

2) Asthma:

An enriched Treg composition of the invention can be used to treat asthma by administering the composition to a patient in need thereof.

3) Autoimmune disorders:

An enriched Treg composition of the invention can be used to treat one or more autoimmune disorder selected from the group consisting of type I diabetes, Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Churg-Strauss Syndrome, Cicatricial Pemphigoid, CREST Syndrome, Cold Agglutinin Disease, Crohn's Disease, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Graves' Disease, Guillain-Barré, Hashimoto's Thyroiditis, Hypothyroidism, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, Juvenile Arthritis, Lichen Planus, Lupus, Ménière's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pemphigus Vulgaris, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis, Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis. Additional autoimmune diseases that can be treated with the methods of the invention are disclosed in U.S. Patent No. 8,173,129, incorporated herein by reference. Administration and dosage of the Treg composition are discussed herein below.

4) Transplantation graft rejection or GVHD:

An enriched Treg composition of the invention can be used to reduce or inhibit transplantation graft rejection or GVHD that occurs when transplanted tissue is rejected by the recipient's immune system. The transplantation graft rejection can be a chronic rejection, an acute rejection, or a hyperacute rejection. Administration and dosage of the Treg composition are discussed herein below.

In addition to the composition enriched in Tregs, other treatments that can be administered to the patient can include, e.g., steroid treatment, antibody-based treatment, immosuppressive drugs, blood transfer, and marrow transplant, according to techniques known in the art.

Treatment of an Infectious Disease Using an Enriched Treg Composition of the Invention

The invention also features methods of treating infectious diseases caused any one or more of a virus, a bacteria, a fungus, or a parasite by administering a composition enriched in Tregs. The composition enriched in Tregs can be produced by the methods described above. The methods of the invention can be used for treating viral infections caused by, e.g., a member of the *Flaviviridae* family (e.g., a member of the *Flavivirus, Pestivirus,*and *Hepacivirus* genera), which includes the hepatitis C virus, Yellow fever virus; Tick-borne viruses, such as the Gadgets Gully virus, Kadam virus, Kyasanur

Forest disease virus, Langat virus, Omsk hemorrhagic fever virus, Powassan virus, Royal Farm virus, Karshi virus, tick-borne encephalitis virus, Neudoerfl virus, Sofjin virus, Louping ill virus and the Negishi virus; seabird tick-borne viruses, such as the Meaban virus, Saumarez Reef virus, and the Tyuleniy virus; mosquito-borne viruses, such as the Aroa virus, dengue virus, Kedougou virus, Cacipacore virus, Koutango virus, Japanese encephalitis virus, Murray Valley encephalitis virus, St. Louis encephalitis virus, Usutu virus, West Nile virus, Yaounde virus, Kokobera virus, Bagaza virus, Ilheus virus, Israel turkey meningoencephalo-myelitis virus, Ntaya virus, Tembusu virus, Zika virus, Banzi virus, Bouboui virus, Edge Hill virus, Jugra virus, Saboya virus, Sepik virus, Uganda S virus, Wesselsbron virus, yellow fever virus; and viruses with no known arthropod vector, such as the Entebbe bat virus, Yokose virus, Apoi virus, Cowbone Ridge virus, Jutiapa virus, Modoc virus, Sal Vieja virus, San Perlita virus, Bukalasa bat virus, Carey Island virus, Dakar bat virus, Montana myotis leukoencephalitis virus, Phnom Penh bat virus, Rio Bravo virus, Tamana bat virus, and the Cell fusing agent virus; a member of the Arenaviridae family, which includes the Ippy virus, Lassa virus (e.g., the Josiah, LP, or GA391 strain), lymphocytic choriomeningitis virus (LCMV), Mobala virus, Mopeia virus, Amapari virus, Flexal virus, Guanarito virus, Junin virus, Latino virus, Machupo virus, Oliveros virus, Paraná virus, Pichinde virus, Pirital virus, Sabiá virus, Tacaribe virus, Tamiami virus, Whitewater Arroyo virus, Chapare virus, and Lujo virus; a member of the *Bunyaviridae* family (e.g., a member of the *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, and *Phlebovirus* genera), which includes the Hantaan virus, Sin Nombre virus, Dugbe virus, Bunyamwera virus, Rift Valley fever virus, La Crosse virus, California encephalitis virus, and Crimean-Congo hemorrhagic fever (CCHF) virus; a member of the *Filoviridae* family, which includes the Ebola virus (e.g., the Zaire, Sudan, Ivory Coast, Reston, and Uganda strains) and the Marburg virus (e.g., the Angola, Ci67, Musoke, Popp, Ravn and Lake Victoria strains); a member of the *Togaviridae* family (e.g., a member of the *Alphavirus genus*), which includes the Venezuelan equine encephalitis virus (VEE), Eastern equine encephalitis virus (EEE), Western equine encephalitis virus (WEE), Sindbis virus, rubella virus, Semliki Forest virus, Ross River virus, Barmah Forest virus, O'nyong'nyong virus, and the chikungunya virus; a member of the *Poxviridae* family (e.g., a member of the *Orthopoxvirus* genus), which includes the smallpox virus, monkeypox virus, and vaccinia virus; a member of the *Herpesviridae* family, which includes the herpes simplex virus (HSV; types 1, 2, and 6), human herpes virus (e.g., types 7 and 8), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella-Zoster virus, and Kaposi's sarcoma associated-herpesvirus (KSHV); a member of the *Orthomyxoviridae* family, which includes the influenza virus (A, B, and C), such as the H5N1 avian influenza virus or H1N1 swine flu; a member of the *Coronaviridae* family, which includes the severe acute respiratory syndrome (SARS) virus; a member of the *Rhabdoviridae* family, which includes the rabies virus and vesicular stomatitis virus (VSV); a member of the *Paramyxoviridae* family, which includes the human respiratory syncytial virus (RSV), Newcastle disease virus, hendravirus, nipahvirus, measles virus, rinderpest virus, canine distemper virus, Sendai virus, human parainfluenza virus (e.g., 1, 2, 3, and 4), rhinovirus, and mumps virus; a member of the *Picornaviridae* family, which includes the poliovirus, human enterovirus (A, B, C, and D), hepatitis A virus, and the coxsackievirus; a member of the *Hepadnaviridae* family, which includes the hepatitis B virus; a member of the *Papillamoviridae* family, which includes the human papilloma virus; a member of the *Parvoviridae* family, which includes the adeno-associated virus; a member of the *Astroviridae* family, which includes the astrovirus; a member of the *Polyomaviridae* family, which includes the JC virus, BK virus, and SV40 virus; a member of the *Calciviridae* family, which includes the Norwalk virus; a member of the *Reoviridae* family, which includes

the rotavirus; and a member of the *Retroviridae* family, which includes the human immunodeficiency virus (HIV; e.g., types 1 and 2), and human T-lymphotropic virus Types I and II (HTLV-1 and HTLV-2, respectively)).

The methods of the invention can also be used for treating bacterial infections. Examples of bacterial infections that may be treated include, but are not limited to, those caused by bacteria within the genera *Salmonella, Streptococcus, Bacillus, Listeria, Corynebacterium, Nocardia, Neisseria, Actinobacter, Moraxella, Enterobacteriacece, Pseudomonas, Escherichia, Klebsiella, Serratia, Enterobacter, Proteus, Salmonella, Shigella, Yersinia, Haemophilus, Bordatella, Legionella, Pasturella, Francisella, Brucella, Bartonella, Clostridium, Vibrio, Campylobacter,* and *Staphylococcus.*

The methods of the invention can also be used for treating parasitic infections caused by a protozoan parasite (e.g., an intestinal protozoa, a tissue protozoa, or a blood protozoa) or a helminthic parasite (e.g., a nematode, a helminth, an adenophorea, a secementea, a trematode, a fluke (blood flukes, liver flukes, intestinal flukes, and lung flukes), or a cestode). Exemplary protozoan parasites include *Entamoeba hystolytica, Giardia lamblia, Cryptosporidium muris, Trypanosomatida gambiense, Trypanosomatida rhodesiense, Trypanosomatida crusi, Leishmania mexicana, Leishmania braziliensis, Leishmania tropica, Leishmania donovani, Toxoplasma gondii, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium falciparum, Trichomonas vaginalis,* and *Histomonas meleagridis.* Exemplary helminthic parasites include *richuris trichiura, Ascaris lumbricoides, Enterobius vermicularis, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Wuchereria bancrofti,* and *Dracunculus medinensis, , Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Fasciola hepatica, Fasciola gigantica, Heterophyes heterophyes,* and *Paragonimus westermani, Taenia solium, Taenia saginata, Hymenolepis nana,* and *Echinococcus granulosus.*

The methods of the invention can also be used for treating fungal infections. Examples of fungal infections that may be treated include, but are not limited to, those caused by, e.g., *Aspergillus, Candida, Malassezia, Trichosporon, Fusarium, Acremonium, Rhizopus, Mucor, Pneumocystis,* and *Absidia.*

Administration and dosage of the Treg composition in methods for treating an infectious disease are discussed herein below.

Treatment Using an NF-κB Activator

Because TNFR2 signaling is transduced via activation of the NF-κB pathway, activators of NF-κB signaling can also be used in place of, or in combination with, administration of an enriched Treg composition for treating immunological disorders or conditions and infectious diseases according to the methods described above. For example, any one of the NF-κB activators described above can be used in the methods of treatment described above. The NF-κB activator can be used by itself or in combination with the composition enriched in Tregs.

Methods for Producing an Enriched Lymphocyte Composition Depleted of Tregs

The invention also features methods for producing a composition enriched in lymphocytes (and depleted of Tregs) *in vitro.* Preferably the method produces a composition in which less than 10% of the

cells (e.g., less than 10%, 5%, 3%, 1%, or 0.5%, or none of the cells) in the composition are Tregs. The method is similar to the method for producing a composition enriched in Tregs except a TNFR2 antagonist, such as an anti-TNFR2 monoclonal antibody, is used in place of a TNFR2 agonist. Additional TNFR2 antagonists that can be used in this method are described below.

The method generally involves the separation of T lymphocytes (e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) from human samples, e.g., human blood or bone marrow sample, followed by expansion of the cells during culturing by incubation of the cells with anti-CD3 and anti-CD28 antibodies. During the expansion step, the cells are contacted with a TNFR2 antagonist. The TNFR2 antagonist suppresses the proliferation of Tregs in the culture, thereby producing a composition that is enriched in lymphocytes and depleted of Tregs. Human-IL-2 and/or rapamycin may optionally be added to the cell culture during the expansion of cells.

After *in vitro* enrichment of lymphocytes (and depletion of Tregs), less than 10% (e.g., less than 10%, 9%, 8%, 7%, 5%, or 2% or substantially none) of the cells in this composition are Tregs. The method described above can result in approximately 2 fold (e.g., 2.5-fold, 3-fold, 3.5-fold, or 4-fold or more) enrichment of non-Treg lymphocytes (e.g., CD4+ T cells, CD8+ T cells, CD4+ CD8+ T cells, B cells, natural killer cells, etc.). The enriched lymphocyte population may also include dendritic cells, monocytes, macrophages, and neutrophils.

TNFR2 antagonists

The TNFR2 antagonist that can be used in this method of the invention can include agents, such as an antibody, a peptide, a small molecule, and a protein that can bind to TNFR2 and suppress TNFR2 signaling. The TNFR2 antagonist can be an agent that, when contacted with CD4+ T cells, can stimulate the expression of cIAP but not the expression of TRAF2, TRAF3, or FOXP3.

The TNFR2 antagonist can be a monoclonal antibody that binds TNFR2. There are two epitopes of TNFR2 that the TNFR2 antagonist antibody can bind. The first epitope includes positions 48-67 (QTAQMCCSKCSPGQHAKVFC) of SEQ ID NO: 1 (amino acid sequence of human TNFR2). The second epitope includes position 135 (R) of SEQ ID NO: 1 (e.g., positions 135-153 (RLCAPLRKCRPGF) of SEQ ID NO: 1). For example, the TNFR2 antagonist antibody can be any one of Clone MAB726 (R&D Systems, Inc.) or Clone M1 (BD Biosciences). While each MAB726 and M1 binds the second epitope, an antibody of the invention may bind the first epitope or both epitopes. The TNFR2 antagonist antibody or antigen-binding fragment thereof can bind TNFR2 with a K_D of less than about 50 nM (e.g., less than about 30 nM, less than about 20 nM, less than about 10 nM, less than about 5 nM, less than about 2 nM, less than about 1 nM, less than about 900 pM, less than about 800 pM, or less than about 700 pM). The TNFR2 antagonist antibody or antigen-binding fragment thereof can bind TNFR2 with a K_D in the range of about 10 pM to about 50 nM (e.g., about 20 pM to about 30 nM, about 50 pM to about 20 nM, about 100 pM to about 5 nM, about 150 pM to about 1 nM, or about 200 pM to about 800 pM). The TNFR2 antagonist antibody avidity can be determined using methods known in the art (e.g., surface plasmon resonance. For example, MAB 726 binds TNFR2 with a K_D of 621 pM (determined by surface plasmon resonance (Pioneer SensiQ®, Oklahoma City, OK)). The TNFR2 antagonist can also be a TNF-α mutein that is capable of binding to TNFR2 and suppressing downstream signaling.

The TNFR2 antagonist can function via downstream signaling by inhibition of the NF-κB pathway. Thus, the method of the invention can also include contacting the human sample e.g., a blood or bone marrow sample, with one or more inhibitors of the NF-κB pathway in order to achieve the same effect as that of using a TNFR2 antagonist. The NF-κB inhibitor can be a small molecule, a peptide, a protein, a

5   virus, or a small non-coding RNA. In one embodiment, the NF-κB inhibitor that can be used in the methods to produce a composition enriched in lymphocytes can be any one or more of 2-(1,8-naphthyridin-2-yl)-Phenol, 5-Aminosalicylic acid, BAY 11-7082, BAY 11-7085, CAPE (Caffeic Acid Phenethylester), Diethylmaleate, Ethyl 3,4-Dihydroxycinnamate, Helenalin, Gliotoxin, NF-κB Activation Inhibitor II JSH-23, NFκB Activation Inhibitor III, Glucocorticoid Receptor Modulator, CpdA, PPM-18,

10   Pyrrolidinedithiocarbamic acid ammonium salt, (R)-MG-132, Rocaglamide, Sodium Salicylate, QNZ, MG-132 [Z-Leu-Leu-Leu-CHO], Astaxanthin, (E)-2-Fluoro-4'-methoxystilbene, CHS-828, disulfiram, olmesartan, triptolide, withaferin, celastrol, tanshinone IIA, Ro 106-9920, cardamonin, BAY 11-7821, PSI, HU 211, ML130, PR 39, honokiol, CDI 2858522, andrographolide, and dithiocarbamates. The NF-κB inhibitor can also be a peptide inhibitor, e.g., a cell penetrating inhibitory peptide as described in May et

15   al., *Science*, 2000 Sep 1;289(5484):1550-4 and in Orange and May, *Cell Mol. Life Sci.,* 2008 Nov;65(22):3564-91, each of which is incorporated herein by reference. Additional NF-κB inhibitors are also described in Gilmore and Herscovitch, *Oncogene* (2006) **25,** 6887–6899; Nam, *Mini Rev. Med. Chem.,* 2006 Aug;6(8):945-51; and in U.S. Patent No. 6,410,516, each of which is incorporated herein by reference.

20   *Methods of Treatment Using a Treg-Depleted, Lymphocyte-Enriched Composition and/or Antagonist of the TNFR2 Signaling Pathway*

The invention features methods for treating proliferative disorders, e.g., cancers, by administering a composition enriched in lymphocytes and depleted of Tregs to a patient in need thereof. The composition enriched in lymphocytes can be produced by the methods described above, for example, by

25   contacting cells obtained from a human sample, e.g., blood or bone marrow sample, with a TNFR2 antagonist, e.g., a TNFR2 antagonist antibody, and/or an NF-κB inhibitor to produce a composition enriched in lymphocytes and depleted of Tregs. An NF-κB inhibitor may be used in methods of treating proliferative disorders, e.g., cancers, instead of the composition enriched in lymphocytes and depleted of Tregs or in combination with this composition. The invention also features methods for treating

30   proliferative disorders, e.g., cancers, by administering a composition containing a TNFR2 antagonist (e.g., an anti-TNFR2 antagonist antibody) to a patient in need thereof.

The invention features methods for treating infectious diseases, by administering a composition enriched in lymphocytes and depleted of Tregs to a patient in need thereof. The composition enriched in lymphocytes can be produced by the methods described above, for example, by contacting cells obtained

35   from a human sample, e.g., blood or bone marrow sample, with a TNFR2 antagonist, e.g., a TNFR2 antagonist antibody, and/or an NF-κB inhibitor to produce a composition enriched in lymphocytes and depleted of Tregs. An NF-κB inhibitor may be used in methods of treating infectious diseases, instead of the composition enriched in lymphocytes and depleted of Tregs or in combination with this composition. The invention also features methods for treating infectious diseases, by administering a composition

containing a TNFR2 antagonist (e.g., an anti-TNFR2 antagonist antibody) alone to a patient in need thereof.

Treatment of Proliferative Disorders Using an Enriched Lymphocyte Composition of the Invention

5   Non-Treg lymphocytes in a patient's blood can be expanded and administered back to the patient in order to treat a proliferative disorder (e.g., a cancer). The enriched lymphocyte composition can be administered alone or in combination with one or more anti-cancer agents known in the art.

The enriched lymphocyte composition can be prepared as follows: i) obtaining a sample e.g., a blood or bone marrow sample, from a human patient and isolating nucleated cells (e.g., lymphocytes,
10   such as T lymphocytes, e.g., CD4+ cells, CD25+ cells, or CD4+CD25+ cells) present therein; ii) subjecting these cells to the lymphocyte expansion method described above to produce a composition enriched in lymphocytes and depleted of Tregs (e.g., a substantially homogenous population of lymphocytes in which Tregs comprise less than 10% of cells in the composition, preferably less than 5% of the cells in the composition or are absent in the expanded composition); and iii) introducing the
15   composition enriched in lymphocytes into the patient without any post-expansion sorting of the lymphocytes. The above steps of the method of treatment can be performed in iterative cycles, where the number of cycles of treatment provided to a patient can be determined by the proliferative disorder being treated, the severity of the disorder, and/or the outcome of each treatment cycle, i.e., a change in the disease state. For example, changes in efficacy markers and/or in clinical outcomes can be used for
20   determining the frequency with which blood or bone marrow should be drawn from a patient, the lymphocytes enriched (and Tregs depleted) from that blood, and the enriched lymphocytes administered to the patient. The enriched lymphocyte composition may also be prepared and stored for later use (e.g., frozen).

Preferably, the lymphocytes are obtained from a patient's own blood or bone marrow (i.e.,
25   autologous cells), although the following methods of treatment may also include the use of allogeneic lymphocytes with possible best fit HLA matching or from unrelated donors that have been expanded according to the above methods. Allogeneic lymphocytes preferentially share at least 4/6 HLA markers in common with the patient receiving the enriched lymphocyte composition.

Treatments for proliferative disorders according to the present invention may include inhibiting the
30   NF-κB signaling pathway in combination with the administration of an enriched lymphocyte composition depleted of Tregs. Because TNFR2 signaling is transduced via the NF-κB pathway, the treatment of proliferative disorders according to the present invention can also include administering to a patient an inhibitor of the NF-κB pathway. For example, any one of the NF-κB inhibitors described above can be administered for treating a proliferative disorder. The NF-κB inhibitor can be administered by itself or in
35   combination with the composition enriched in lymphocytes. The NF-κB inhibitor can also function independently of the TNFR2 signaling pathway.

Proliferative disorders that can be treated by administering the enriched lymphocyte/Treg depleted composition include one or more cancers selected from the group consisting of Acute

Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma; AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer; Bone Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma, Brain Stem Glioma, Visual Pathway and Hypothalamic Glioma, Breast Cancer, Bronchial Adenomas/Carcinoids,

5   Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Clear Cell Sarcoma of Tendon Sheaths, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Epithelial Cancer, Esophageal Cancer,Ewing's Family of Tumors, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Intraocular Melanoma, Retinoblastoma, Gallbladder Cancer, Gastric (Stomach) Cancer, Hairy Cell Leukemia, Head

10   and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin's Lymphoma, Hypopharyngeal Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Pituitary Cancer, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer, Testicular Cancer, Thyroid Cancer, Urethral Cancer, Uterine Sarcoma, and Vaginal Cancer. The proliferative disorders can also include solid tumors including

15   malignancies (e.g., sarcomas, adenocarcinomas, and carcinomas) of the various organ systems, such as those of brain, lung, breast, lymphoid, gastrointestinal (e.g., colon), and genitourinary (e.g., renal, urothelial, or testicular tumors) tracts, pharynx, prostate, and ovary. Exemplary adenocarcinomas include colorectal cancers, renal-cell carcinoma, liver cancer, non- small cell carcinoma of the lung, and cancer of the small intestine.

20   Administration and dosage of the enriched lymphocyte composition in the method for treating a proliferative disease are discussed herein below.

Treatment of an Infectious Disease Using an Enriched Lymphocyte Composition of the Invention

          The invention also features methods of treating infectious diseases caused by any one or more of
25   a virus, bacteria, a fungus, or a parasite. The methods involve administering a composition enriched in lymphocytes (e.g., CD8+ T cells, B cells, or natural killer cells) and depleted of Tregs. This composition can be produced by the methods described above. The methods of the invention can be used for treating viral infections caused by, e.g., a member of the *Flaviviridae* family (e.g., a member of the *Flavivirus*, *Pestivirus,*and *Hepacivirus* genera), which includes the hepatitis C virus, Yellow fever virus; Tick-borne

30   viruses, such as the Gadgets Gully virus, Kadam virus, Kyasanur Forest disease virus, Langat virus, Omsk hemorrhagic fever virus, Powassan virus, Royal Farm virus, Karshi virus, tick-borne encephalitis virus, Neudoerfl virus, Sofjin virus, Louping ill virus and the Negishi virus; seabird tick-borne viruses, such as the Meaban virus, Saumarez Reef virus, and the Tyuleniy virus; mosquito-borne viruses, such as the Aroa virus, dengue virus, Kedougou virus, Cacipacore virus, Koutango virus, Japanese encephalitis virus,

35   Murray Valley encephalitis virus, St. Louis encephalitis virus, Usutu virus, West Nile virus, Yaounde virus, Kokobera virus, Bagaza virus, Ilheus virus, Israel turkey meningoencephalo-myelitis virus, Ntaya virus, Tembusu virus, Zika virus, Banzi virus, Bouboui virus, Edge Hill virus, Jugra virus, Saboya virus, Sepik virus, Uganda S virus, Wesselsbron virus, yellow fever virus; and viruses with no known arthropod vector, such as the Entebbe bat virus, Yokose virus, Apoi virus, Cowbone Ridge virus, Jutiapa virus, Modoc

40   virus, Sal Vieja virus, San Perlita virus, Bukalasa bat virus, Carey Island virus, Dakar bat virus, Montana

myotis leukoencephalitis virus, Phnom Penh bat virus, Rio Bravo virus, Tamana bat virus, and the Cell fusing agent virus; a member of the Arenaviridae family, which includes the Ippy virus, Lassa virus (e.g., the Josiah, LP, or GA391 strain), lymphocytic choriomeningitis virus (LCMV), Mobala virus, Mopeia virus, Amapari virus, Flexal virus, Guanarito virus, Junin virus, Latino virus, Machupo virus, Oliveros virus,

5   Paraná virus, Pichinde virus, Pirital virus, Sabiá virus, Tacaribe virus, Tamiami virus, Whitewater Arroyo virus, Chapare virus, and Lujo virus; a member of the *Bunyaviridae* family (e.g., a member of the *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, and *Phlebovirus* genera), which includes the Hantaan virus, Sin Nombre virus, Dugbe virus, Bunyamwera virus, Rift Valley fever virus, La Crosse virus, California encephalitis virus, and Crimean-Congo hemorrhagic fever (CCHF) virus; a member of the *Filoviridae*

10   family, which includes the Ebola virus (e.g., the Zaire, Sudan, Ivory Coast, Reston, and Uganda strains) and the Marburg virus (e.g., the Angola, Ci67, Musoke, Popp, Ravn and Lake Victoria strains); a member of the *Togaviridae* family (e.g., a member of the *Alphavirus genus*), which includes the Venezuelan equine encephalitis virus (VEE), Eastern equine encephalitis virus (EEE), Western equine encephalitis virus (WEE), Sindbis virus, rubella virus, Semliki Forest virus, Ross River virus, Barmah Forest virus,

15   O'nyong'nyong virus, and the chikungunya virus; a member of the *Poxviridae* family (e.g., a member of the *Orthopoxvirus* genus), which includes the smallpox virus, monkeypox virus, and vaccinia virus; a member of the *Herpesviridae* family, which includes the herpes simplex virus (HSV; types 1, 2, and 6), human herpes virus (e.g., types 7 and 8), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella-Zoster virus, and Kaposi's sarcoma associated-herpesvirus (KSHV); a member of the *Orthomyxoviridae*

20   family, which includes the influenza virus (A, B, and C), such as the H5N1 avian influenza virus or H1N1 swine flu; a member of the *Coronaviridae* family, which includes the severe acute respiratory syndrome (SARS) virus; a member of the *Rhabdoviridae* family, which includes the rabies virus and vesicular stomatitis virus (VSV); a member of the *Paramyxoviridae* family, which includes the human respiratory syncytial virus (RSV), Newcastle disease virus, hendravirus, nipahvirus, measles virus, rinderpest virus,

25   canine distemper virus, Sendai virus, human parainfluenza virus (e.g., 1, 2, 3, and 4), rhinovirus, and mumps virus; a member of the *Picornaviridae* family, which includes the poliovirus, human enterovirus (A, B, C, and D), hepatitis A virus, and the coxsackievirus; a member of the *Hepadnaviridae* family, which includes the hepatitis B virus; a member of the *Papillamoviridae* family, which includes the human papilloma virus; a member of the *Parvoviridae* family, which includes the adeno-associated virus; a

30   member of the *Astroviridae* family, which includes the astrovirus; a member of the *Polyomaviridae* family, which includes the JC virus, BK virus, and SV40 virus; a member of the *Calciviridae* family, which includes the Norwalk virus; a member of the *Reoviridae* family, which includes the rotavirus; and a member of the *Retroviridae* family, which includes the human immunodeficiency virus (HIV; e.g., types 1 and 2), and human T-lymphotropic virus Types I and II (HTLV-1 and HTLV-2, respectively)).

35   The methods of the invention can also be used for treating bacterial infections. Examples of bacterial infections that may be treated include, but are not limited to, those caused by bacteria within the genera *Salmonella, Streptococcus, Bacillus, Listeria, Corynebacterium, Nocardia, Neisseria, Actinobacter, Moraxella, Enterobacteriacece, Pseudomonas, Escherichia, Klebsiella, Serratia, Enterobacter, Proteus, Salmonella, Shigella, Yersinia, Haemophilus, Bordatella, Legionella, Pasturella,*

40   *Francisella, Brucella, Bartonella, Clostridium, Vibrio, Campylobacter,* and *Staphylococcus.*

The methods of the invention can also be used for treating parasitic infections caused by a protozoan parasite (e.g., an intestinal protozoa, a tissue protozoa, or a blood protozoa) or a helminthic parasite (e.g., a nematode, a helminth, an adenophorea, a secementea, a trematode, a fluke (blood flukes, liver flukes, intestinal flukes, and lung flukes), or a cestode). Exemplary protozoan parasites include *Entamoeba hystolytica, Giardia lamblia, Cryptosporidium muris, Trypanosomatida gambiense, Trypanosomatida rhodesiense, Trypanosomatida crusi, Leishmania mexicana, Leishmania braziliensis, Leishmania tropica, Leishmania donovani, Toxoplasma gondii, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium falciparum, Trichomonas vaginalis,* and *Histomonas meleagridis.* Exemplary helminthic parasites include *richuris trichiura, Ascaris lumbricoides, Enterobius vermicularis, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Wuchereria bancrofti, and Dracunculus medinensis, , Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Fasciola hepatica, Fasciola gigantica, Heterophyes heterophyes, and Paragonimus westermani, Taenia solium, Taenia saginata, Hymenolepis nana,* and *Echinococcus granulosus.*

The methods of the invention can also be used for treating fungal infections. Examples of fungal infections that may be treated include, but are not limited to, those caused by, e.g., *Aspergillus, Candida, Malassezia, Trichosporon, Fusarium, Acremonium, Rhizopus, Mucor, Pneumocystis,* and *Absidia.*

Administration and dosage of the lymphocyte-enriched composition in methods for treating an infectious disease are discussed herein below.

Dosage

The composition enriched in Tregs can be administered to a patient in need thereof one or more times per day, week, month (e.g., one or more times every 2 weeks), or year depending on the severity of the disease and change in disease state of the patient during the treatment. Generally it is expected that a typical dosage would include 5×10^5 to 5×10^{12} (e.g., 5×10^5, 5×10^6, 5×10^7, 5×10^8, 5×10^9, 5×10^{10}, 5×10^{11}, or 5×10^{12}) Tregs. Disease metrics, such as severity of symptoms, change in symptoms, patient response to treatment, any adverse effects of treatment, and/or effect of any additional treatment(s), can be used to determine the frequency of treatment and the dosage, i.e., the number of Tregs to be administered to a patient.

The composition enriched in lymphocytes (and depleted of Tregs) can be administered one or more times per day, week, month (e.g., one or more times every 2 weeks), or year depending on the severity of the disease and change in disease state of the patient during the treatment. Preferably, less than 10 % of the cells (e.g., less than 9%, 8%, 7%, 5%, %, 1% or none of the cells) in this composition enriched in lymphocytes are Tregs. Generally it is expected that a typical dosage would include 5×10^5 to 5×10^{12} (e.g., 5×10^5, 5×10^6, 5×10^7, 5×10^8, 5×10^9, 5×10^{10}, 5×10^{11}, or 5×10^{12}) cells in the enriched lymphocyte composition. Disease metrics, such as severity of symptoms, change in symptoms, patient response to treatment, any adverse effects of treatment, and/or effect of any additional treatment(s), can be used to determine the frequency of treatment and the dosage, i.e., the number of lymphocytes to be administered to a patient.

Administration

Generally, the compositions of the invention (e.g., the composition enriched in Tregs or the composition enriched in lymphocytes and depleted of Tregs) can be administered in any medically useful form. For example, such compositions may include the addition of compounds, e.g., adjuvants,

5   preservatives, carriers, excipients, diluents, anti-bacterial or anti-mycotic agents, anti-inflammatory agents, and/or anti-cancer agents, where appropriate. The compositions of the invention can be administered intravenously, intramuscularly, orally, by inhalation, parenterally, intraperitoneally, intraarterially, transdermally, sublingually, nasally, transbuccally, liposomally, adiposally, ophthalmically, intraocularly, subcutaneously, intrathecally, orally, or locally, and they are formulated, as appropriate,

10   depending on the chosen route of administration.

Administration of Antibodies of the Invention

Pharmaceutical compositions containing an anti-TNFR2 antibody of the invention (e.g., an anti-TNFR2 antagonist antibody) are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers in the form of aqueous

15   solutions, lyophilized or other dried formulations. The acceptable excipient or carrier is selected on the basis of the mode and route of administration. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in *Remington: The Science and Practice of Pharmacy*, 21st edition, Ed. Gennaro; Lippincott, Williams & Wilkins (2005), a well-known reference text in this field, and in the USP/NF (United States Pharmacopeia and the National

20   Formulary). Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben;

25   catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or

30   sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Other exemplary excipients are described in *Handbook of Pharmaceutical Excipients*, 6th Edition, Rowe et al., Eds., Pharmaceutical Press (2009).

The compositions of the invention may be prepared in a pharmaceutically acceptable carrier or

35   excipient. Such suitable carriers or excipients may be selected from, for example, water, saline (e.g., phosphate-buffered saline (PBS) or acetate-buffered saline (ABS), or Ringer's solution), dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, a composition for administration to a mammal can contain minor amounts of auxiliary substances, such as wetting or emulsifying agents, or pH buffering agents that enhance the effectiveness of the composition. The

compositions of the invention may also be prepared in any acceptable salt formulation. Other agents that may be used in preparation of the compositions of the invention include, e.g., adjuvants, preservatives, diluents, anti-bacterial or anti-mycotic agents, anti-inflammatory agents, and/or anti-cancer agents, where appropriate.

5   The compositions may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

          The active ingredients may also be entrapped in microcapsule prepared, for example, by
10   coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington: The Science and Practice of Pharmacy*, 21st edition, Ed. Gennaro; Lippincott, Williams & Wilkins (2005).

15   The compositions to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

          Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin of the invention, which matrices are in the form of shaped articles, *e.g.,* films, or
20   microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric
25   acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

          The compositions containing one or more anti-TNFR2 antibodies (e.g., anti-TNFR2 antagonist antibody) may be administered to a patient prior to the development of symptoms of a proliferative disease or an infectious disease or the compositions may be administered to the patient after diagnosis
30   with a proliferative disease or an infectious disease after presentation with one or more (e.g., 1, 2, 3, 4, or 5) symptoms of the disease. The dosage of the anti-TNFR2 antibodies depends on the patient's state of health, but generally ranges from about 0.1 mg to about 400 mg of an antibody per dose (e.g., 1 mg, 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 300 mg, or 400 mg or more per dose).

          The compositions may be administered to a patient in one or more doses (e.g., 2, 3, 4, 5, 6, 7, 8,
35   9, or 10 or more doses). If more than one dose is administered, the doses may be administered via the same mode of administration (e.g., intravenous administration) or by different modes of administration (e.g., intravenous and intramuscular administration). The patient may also be administered different

doses at different times. For example, the patient may be administered a higher initial dose and lower subsequent doses over the course of treatment or vice versa.

The compositions may be administered daily, weekly, monthly, or yearly. For example, a dose of the composition may be administered twice daily, biweekly, bi-annually, tri-annually, or quarterly. The

5   dose of the composition may be determined by a skilled physician upon consideration of a subject's clinical symptoms and/or physical condition (e.g., weight, sex, height, and severity of the proliferative or infectious disease). The composition may be administered by intravenous, intradermal, parenteral, intra-arterial, subcutaneous, intramuscular, intraorbital, topical, intraventricular, intraspinal, intraperitoneal, intranasal, intracranial, or oral administration.

10   *Kits of the invention*

The invention features a kit for the production of a composition enriched in Tregs. The kit can include a TNFR2 agonist (e.g., a TNFR2 agonist antibody) or an NF-κB activator (e.g., one or more of the NF-κB activators described above), reagents and/or devices for isolating a human sample, e.g., a blood or bone marrow sample, reagents and/or devices for isolating blood or bone marrow cells (e.g.,

15   CD4+CD25+ cells) from the sample, and reagents for culturing the blood or bone marrow cells (e.g., anti-CD3 antibody, anti-CD28 antibody, interleukin-2, and/or rapamycin). Additionally the kit of the invention can also include instructions for performing the method of the invention, e.g., instructions for isolating a blood or bone marrow sample and blood or bone marrow cells therefrom, instructions for contacting blood or bone marrow cells with a TNFR2 agonist, and/or instructions for culturing, harvesting and/or storing the

20   enriched Tregs. The kit of the invention can also include reagents and instructions for assaying the expression of various marker genes that can be used to characterize the Tregs. For example, these can include reagents and instructions for detecting the mRNA or protein levels of one or more of FOXP3, CTLA4, TNFR2, CD62L, Fas, HLA-DR, CD45RO, CD127, CCR5, CCR6, CCR7, CXCR3, IFN-gamma, IL10, and ICOS.

25   The invention features a kit for the production of a composition enriched in lymphocytes and depleted of Tregs. The kit can include a TNFR2 antagonist (e.g., a TNFR2 antagonist antibody) or an NF-κB inhibitor (e.g., one or more of the NF-κB inhibitors described above), reagents and/or devices for isolating a human blood or bone marrow sample, reagents and/or devices for isolating blood or bone marrow cells (e.g., T lymphocytes, such as CD4+ cells, CD25+ cells, or CD4+CD25+ cells) from the

30   sample, and reagents for culturing the blood cells (e.g., anti-CD3 antibody, anti-CD28 antibody, interleukin-2, and/or rapamycin). Additionally the kit of the invention can also include instructions for performing the method of the invention, e.g., instructions for isolating a blood or bone marrow sample and blood or bone marrow cells therefrom, instructions for contacting blood cells with a TNFR2 antagonist, and/or instructions for culturing, harvesting and/or storing the enriched lymphocytes. The kit of the

35   invention can also include reagents and instructions for assaying the expression of various marker genes that can be used to characterize the lymphocytes. For example, these can include reagents and instructions for detecting the mRNA or protein levels of one or more of FOXP3, TRAF2, TRAF3, and cIAP.

The invention also features kits that include a composition containing an anti-TNFR2 antibody (e.g., an anti-TNFR2 antagonist antibody), a pharmaceutically-acceptable carrier or excipient, and, optionally, other agents as described herein; the composition contains an effective amount of the anti-TNFR2 antibody for treating a proliferative disease or an infectious disease. The kits may include instructions explaining how a practitioner (e.g., a physician, nurse, or patient) may administer the composition contained therein. Furthermore, the kits may also include additional components, such as one or more additional components described above, instructions or administration schedules for a patient suffering from a proliferative disease or an infectious disease, and, optionally, a device(s) for administering the composition (e.g., a syringe).

The following examples are meant to illustrate the invention. They are not meant to limit the invention in any way.

EXAMPLES

Materials and Methods

Human subjects and TNF-α induction with BCG vaccine

Two BCG vaccinations were used to induce TNF-α. Administration of BCG was approved by the Human Studies Committee at Massachusetts General Hospital and by the FDA (NCT00607230).

For the double-blinded placebo-controlled trial, one subject was injected with BCG at a dose of $1.6\text{-}3.2\text{x}10^6$ cfu and the placebo subject was injected with saline. The BCG or saline injection was administered intradermally on two occasions four-weeks apart. All blood samples were blinded and simultaneously sent to the laboratory for monitoring TNF-α and Treg levels.

Reagents and Flow Cytometry

Recombinant human TNF-α was purchased from Leinco Technologies (St. Louis, MO), and recombinant human IL-2 was purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against TNFR1 and TNFR2 used for screening purposes were from internal sources and external vendors (Table 1). External vendors included R&D Systems, Inc., Hycult-Biotechnology, BD-Pharmingen, Accurate, Abcam and Sigma. All other antibodies were purchased from BD-Biosciences. Intracellular staining of FOXP3 and CD152 were performed using either FOXP3 Fix/Perm Buffer set (Biolegend) or Human FOXP3 Buffer set (BD Biosciences). The avidities of MAB726 and M1 antibodies for TNFR2 were determined using surface plasmon resonance on Pioneer SensiQ (SensiQ Technologies, Oklahoma City, OK).

Table 1

Clone	MR2-1	80M2	MAB726	M1	MAB2261
Isotype	Mouse IgG1	Mouse IgG1	Mouse IgG1	Rat IgG2b	Mouse IgG2A
Properties	Agonist	Neutral	Antagonist	Antagonist	Agonist
Vendor	Cell Sciences	Cell Sciences	R&D	BD Biosciences	R&D

CD4+ Cell isolation, induction of FOXP3, and expansion of CD4+CD25+ cells

CD4+ T cells were isolated using Dynal CD4 Positive Isolation Kit (Invitrogen). Extraction of CD25 positive cells was subsequently performed after CD4+ isolation using Dynabeads CD25 and DETACHaBEAD CD4/CD8 (Invitrogen). After isolation, 2×10^4 cells were cultured in 96-round-bottom well plate. Dynabeads for human Treg Expander (Invitrogen) (Dynabeads coupled with anti-CD3 and anti-CD28 monoclonal antibodies) was added at a beads-to-cell ratio of 2:1. In selected wells, TNF-α (20ng/ml), TNFR2 mAbs (2.5 μg/ml), rapamycin (1μM, EMD Biosciences, San Diego, CA) were added. After two days, IL-2 (200U/ml) was added to the culture. Half of the media was changed every 2 to 3 days containing rapamycin (until day 7) and 100 U/ml of IL-2. On day 9, additional TNF-α or TNFR2 mAbs were supplied into the media. On day 16, cells were harvested, Dynabeads Human Treg Expander was removed, washed and rested. On the following day, cells were analyzed.

Intracellular staining

Expanded CD4+CD25+ cells were stimulated with phorbol myristate acetate (PMA) (2 ng/ml) and ionomycin (500 ng/ml) (Sigma) for 24 hours. Monensin (GolgiStop, BD Biosciences) was added for the last 4 hours of incubation. Cells were fixed and permeabilized using Human FOXP3 Buffer Set, followed by staining with fluorochrome-conjugated IFNγ and IL-10 mAbs.

mRNA isolation

Isolated CD4+ cells were incubated in presence of IL-2 (50U/ml) with or without TNFR2 mAbs (2.5μg/ml). After 3 hours, cells were collected and total RNA was isolated using RNAqueous-4PCR kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied-Biosystems, Foster City, CA).

Cell proliferation and suppression assays

For CD4 proliferation experiments, CD4+ cells were stained with 1μM carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were plated at the density of 2×10^5 cells / well in 96-well plate with anti-CD3mAbs . Four days later, cells were collected and analyzed.

For Treg suppression assay, autologous PBMCs were used as responders. PBMC were collected using Ficoll-Paque, cryopreserved at -80 ℃, and thawed the day before mixing with Tregs and rested

overnight in RPMI 1640 and 10U/ml IL-2. The next day, responder cells were stained with CFSE (1μM). Responder cells (5×10^4 cells) and expanded Tregs were mixed at various ratios, and stimulated with anti-CD3mAb and IL-2. After 4 days, cells were collected and analyzed.

Statistical analysis

5   All data analyses were performed by the paired Student *t* test using GraphPad Prism-5 software (GraphPad Software, La Jolla, CA). I considered two-sided p value 0.05 as significant.

Example 1: Clinical trial induction of human Tregs

Unexpanded, naturally occurring human Tregs are heterogeneous and rare in blood. Homogeneous populations of Tregs are difficult to expand *in vitro* even with multiple ligand mixtures.
10   With the goal of expanding sufficient numbers of homogeneous populations of human Treg cells TNF-α, I first sought to confirm or refute an increase in Treg concentrations by induction with native TNF-α. Because an FDA-approved version of TNF-α does not exist and manufacturing of a stable form is difficult, I administered a well-known, strong inducer of TNF-α, *Bacillus Calmette Guérin* (BCG), a generic vaccine already on the market for decades for tuberculosis and bladder cancer. This method of inducing
15   endogenous TNF-α eliminated the manufacturing problems of TNF-α that forms unnatural monomers, dimers and trimers with differential cellular effects.

The small, double-blinded placebo-controlled clinical trial enrolled two subjects. One human subject received BCG injections ($1.6-3.2 \times 10^6$ cfu/injection) and the placebo received saline, twice, 4-weeks apart. Both were monitored weekly for 20-weeks to study the pharmacokinetics of TNF-α and
20   Treg induction. After each injection TNF-α induced Tregs in a bi-modal fashion with slightly delayed kinetics (Figure 1A, left panels). After 20 weeks of observation, saline injections induced neither TNF-α nor Tregs. The total CD4+ cell counts did not change in BCG and placebo patients other than in the percentages of CD4+CD25^{hi}FOXP3+ depicted in Figure 1A. This *in vivo* evidence confirms endogenous TNF-α *in vivo* increases the numbers of Tregs but since TNF-α binds to both the TNFR1 and TNFR2
25   receptors, it does not clarify which receptor was more central for the Tregs effect.

Example 2: Functional effects of TNFR monoclonal antibodies and signaling pathways

I cultured freshly isolated human CD4+ cells from 14 human subjects only with TNF-α for 16 hours (Figure 1B). While finding no induction of Tregs, assessed by inducible FOXP3, I observed a significant increase in Tregs after adding IL-2. Co-incubation of TNF-α and IL-2 produced a significant
30   increase in Tregs over IL-2 alone (Figure 1B). IL-2 is important for Tregs induction and maintenance in mice. Findings were confirmed by flow cytometry, in which co-incubation increased the percentage of CD4+CD25^{hi} FOXP3 cells in human cultured cells from blood (Figure 1C). Thus, the data in Figures 1B and 1C confirm that TNF-α can induce a homogeneous population of Tregs *in vitro*.

In freshly isolated CD4+ cells, I examined expression levels of each TNFR in relation to CD25+
35   expression. TNFR1 expression on CD4+ cells, regardless of CD25+ expression levels, was unchanged using flow cytometry (Figure 2A, middle panel), whereas TNFR2 preferentially expressed CD4$^+$CD25^{hi}

Tregs by nearly a factor of 10 (Figure 2A, right panel). This confirms earlier studies that TNFR2 is more densely expressed on Tregs.

Screening several TNFR1 and TNFR2 monoclonal antibodies (mAbs) on isolated CD4+ cells sampled from fresh human blood enabled selective study of each TNF receptor, unlike studying TNF-α,
5   which acts through both receptors and can have manufacturing problems from the use of *E. coli* and yeast systems. Although most of the screened TNFR1 or TNFR2 mAbs failed to induce or suppress FOXP3+ Tregs after stimulation by presence of IL-2 for 16 hours (Figures 7 and 8), I found two types of TNFR2 mAbs with significant, and opposing, effects on FOXP3 induction (Figure 2b). Studying freshly cultured cells from 10 subjects, one TNFR2 antibody significantly induced FOXP3 expression in CD4+
10   human T cells (which I designated the "TNFR2 agonist"), whereas the other TNFR2 monoclonal suppressed intracellular FOXP3 expression (which I designated the "TNFR2 antagonist") (Figure 2B). Thus, I have identified M1 and MAB726 as TNFR2 antagonists.

Having identified two functionally-opposing types of TNFR2 mAbs, I measured their effects on isolated CD4+ T cells by examining downstream mRNA expression in signaling proteins specific to
15   TNFR2 activation. After 24h stimulation by the TNFR2 agonist or antagonist, relative mRNA expression was significantly different. The TNFR2 agonist stimulated expression of TNF, TRAF2, TRAF3, cIAP2 and FOXP3. In contrast, the TNFR2 antagonist stimulated expression of cIAP1, but not TRAF2, TRAF3 or FOXP3 (Figure 2C).

The effects of the TNFR2 agonist and antagonist were studied on purified human CD4+ T cells
20   co-cultured with anti-CD3 or IL-2. When CD4+ proliferation was studied with anti-CD3 combined with the TNFR2 agonist, the highest degree of proliferation was shown by the agonist. In contrast, the TNFR2 antagonist (e.g., M1 or MAB726) suppressed CD4+ proliferation even relative to the control, anti-CD3 alone (Figure 2D, left-most panel). The same findings were observed after 4 days by directly measuring CD4+ proliferation by flow cytometry and measuring CD4+ proliferation by CFSE dilution (Figure 2D,
25   three right-most panels). Thus, the opposing effects of TNFR2 agonist treatment versus TNFR2 antagonist treatment on Tregs have been demonstrated, as shown in Figures 2B-2D.

Despite high expression of TNFR2 on Tregs, some TNFR2 expression is also observable on CD4+ T cells that are not true Tregs because they only express intermediate levels of CD25, i.e., CD4$^+$CD25^{mid} cells. I therefore studied the impact of overnight incubation on CD25^{mid} cell subpopulations
30   of IL-2 alone, IL-2 and TNF-α, or IL-2 and TNFR2 agonist, or IL-2 and TNFR2 antagonist. I found a rise in the proportion of CD25^{hi}FOXP3$^-$ cells similar to effector cells with IL-2 and TNF-α stimulation alone, or IL-2 and TNFR2 agonist alone (Figure 8). However, I observed suppression with IL-2 and TNFR2 antagonist relative to the other three groups. Therefore, the TNFR2 agonist and antagonist, studied by the same assay, showed opposing trends on the same CD25$^+$FOXP3- cell population. Thus, the addition
35   of a TNFR2 antagonist (e.g., M1 or MAB726) inhibited expression of Foxp3 on CD4+CD25+ T cells.

I separated fresh human blood to obtain pure CD4+ and CD25^{hi} co-expressing Tregs (Figure 3). I purified and expanded these Tregs *in vitro* using standard protocols of anti-CD3 anti-CD28 plus IL-2 for 16 days (Figure 3A), then rested them overnight before counting. I added rapamycin (until day 7) because it selectively expands the highest number of Tregs with greatest capacity for suppressing CD8+

cells. This process successfully produced CD4+CD25^{hi} Tregs (Figure 3B). I assessed T$_{reg}$ expansion by treatment group: no treatment, treatment with TNF-α, TNFR2 agonist, or TNFR2 antagonist. The TNFR2 agonist outperformed every other group, expanding Tregs at least twofold higher than no treatment or antagonist treatment. The latter suppressed expansion because its effect was less than that of no treatment. Because rapamycin is known to inhibit proliferation, I examined the effects of treatment without rapamycin, yet found similarly opposing effects between agonist versus antagonist treatment, albeit at smaller mean absolute values (Figure 9B). The yields of expanded cells tended to be less without rapamycin, but the agonist still expanded Tregs.

Example 3: TNFR2 agonist expansion and homogeneity of Tregs

I next investigated whether *in vitro* Tregs, treated by TNFR2 agonist, possessed more homogeneous Treg cell surface markers than those treated by the antagonist. Comparing phenotypes for 14 cell surface markers, all treatment groups highly expressed Treg signature markers FOXP3 and CD25 (Figure 4A). The expression levels of FOXP3 were similar to levels before treatment. However, CD25+ expression was much higher after agonist treatment, which can be considered an expansion effect rather than an antagonist effect (data not shown). Nearly 100% of expanded CD25^{hi} Tregs in each group were positive for CTLA4, TNFR2, CD62L, Fas, and negative for CD127 (Figure 4A). Tregs treated with TNFR2 antagonist also maintained expression for these markers. In contrast, several other surface markers, such as HLA-DR, ICOS, CD45RO and chemokine receptors, were differentially expressed between the agonist vs. antagonist treatment (Figures 4B and 4C and Figure 10). Similar results were observed in Tregs expanded without rapamycin (Figure 10). Tregs expanded by TNFR2 agonist—relative to most other comparator groups, especially the TNFR2 antagonist—yielded a surprisingly homogeneous population of cells with this phenotype: CD4^{+}CD25^{hi}FOXP3^{+}CTLA4^{+}TNFR2^{+}CD127^{-}CD62L^{+}Fas^{+}HLA-DR^{+}CD45RO^{+}CCR5^{-}CCR6^{-}CCR7^{-}CXCR3^{-}ICOS^{-}. The mean fluorescence intensity (MFI), a direct measure of the average density of the protein per cell, similarly revealed that for most surface markers, the TNFR2-agonist treated cells showed opposing expression levels compared to TNFR2-antagonist treated cells (Figure 12). Further investigation should define whether these expanded cells maintain phenotypic homogeneity over time but the evidence from this conventional expansion protocol shows that they were more homogeneous than other groups. Before treatment, Treg markers were more heterogeneous (Figure 11). Unexpanded, naturally occurring human Tregs are heterogeneous populations. *In vitro* studies of mixed Treg populations, which include CD45RO^{+}FOXP3^{low} T cells, produce pro-inflammatory cytokines. This particular phenotype is found in up to 50% of FOXP3+ T cells.

One of the most upregulated markers by TNFR2 agonist-treated Tregs was HLA-DR, which is reported to have higher suppressive activity against CD8 T+ cells, suggestive of an effector Treg. In contrast to HLA-DR, all four chemokine receptors were strongly down-regulated. Although the lack of chemokine receptors might result in failure to migrate to the site of inflammation, another homing receptor, CD62L, which was highly expressed in all treatment groups (Figure 4), is crucial for entering the site of pathogenic T cell presence in acute GVHD. The fact that agonist-treated Tregs were CD45RO+ and CCR7- and displayed significantly higher expression levels of Fas, measured by MFI (Figure 12), contributes to the view that they are activated effector Tregs.

Example 4: TNFR2 agonist-treated Tregs and CD8+ suppression

One key function of Tregs, especially in autoimmunity, is to suppress the function of autoreactive cytotoxic CD8+ T cells. I assessed this capacity by mixing Tregs from each treatment group with CFSE-stained autologous PBMC, after having stimulated them with anti-CD3mAb and IL-2 for 4 days.

5   Autologous CD8+ T cells, the responder cells, were tested for suppression by observing the ratios of responders to Tregs. Ratios of dilution enable study of dose-dependence. All groups of Tregs displayed suppressive function on CD8+ T cells, but the degree varied by treatment group (Figure. 5A, left panel). TNFR2 agonist-treated Tregs, for example, showed the strongest suppressive capacity at 1:1 ratio (by leaving the fewest number of CD8+ cells) and then became progressively weaker at higher ratios.

10   However, the antagonist-treated cells displayed weaker suppressive capacity that was essentially no different from that of no treatment. With a suppression index of 2:1, the TNFR2 agonist-treated group showed greater suppression than did the antagonist and no treatment groups (Figure 5A, right panel). Similar results were observed with Tregs treated without rapamycin (Figure 13A). The results are consistent with known phenotypes and expansion capacity of functional Tregs.

15   *Example 5: TNFR2 agonist-treated Tregs and cytokine production*

I found that all treatment groups had relatively limited ability to produce intracellular IFNγ and IL-10 after PMA and ionomycin stimulation. But TNFR2 agonist and the TNF only-treated Tregs produced the lowest percentages of IFNγ+ cells (Figure 5B, lowest left panel). The antagonist-treated Tregs showed significantly higher IFNγ production than the agonist (Fig. 5b, lower left panel). In similar

20   experiments without rapamycin, the TNFR2 agonist-treated group not only produced lower IFNγ, but also lower IL-10 and TNF production relative to TNF or no treatment, respectively (Figure 13B). Agonist-treated Tregs also showed the fewest number of TH1 Transcription Factor (T-bet)+ Tregs (Figure 5C), which is consistent with lower IFNγ production (Figure 5C). One of the reasons for these Tregs showing high suppression capacity over CD8+ T cells may be due to Tregs lacking the ability to produce IFNγ.

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Other Embodiments

The disclosures of U.S. Provisional Patent Application No. 61/762,136, filed on February 7, 2013, and U.S. Provisional Patent Application No. 61/763,217, filed on February 11, 2013, are hereby incorporated by reference in their entirety.

5   While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

10   All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated as being incorporated by reference in their entirety.

           Other embodiments are in the claims.

CLAIMS

1.   A method for producing a composition enriched in CD4+CD25^{hi} T regulatory cells (Tregs) comprising contacting *in vitro* a population of human cells comprising T-lymphocytes obtained from a human blood or bone marrow sample from a patient with a tumor necrosis factor receptor 2 (TNFR2) agonist or an NF-κB activator, thereby producing said composition enriched in Tregs, wherein said Tregs comprise at least 60% of the cells in said composition.

2.   The method of claim 1, wherein said population comprises CD4+ cells, CD25+ cells, or CD4+CD25+ cells and said method further comprises isolating said CD4+ cells, CD25+ cells, or CD4+CD25+ cells, respectively, from said sample prior to said contacting.

3.   The method of claim 1, wherein said Tregs comprise at least 70% of the cells in said composition.

4.   The method of claim 1, wherein said Tregs comprise at least 80% of the cells in said composition.

5.   The method of claim 1, wherein said Tregs comprise at least 90% of the cells in said composition.

6.   The method of claim 1, wherein said Tregs comprise substantially 100% of the cells in said composition.

7.   The method of any one of claims 1 to 6, wherein said Tregs are further characterized as positive for the expression of one or more proteins selected from the group consisting of FOXP3, CTLA4, TNFR2, CD62L, Fas, HLA-DR, and CD45RO.

8.   The method of any one of claims 1 to 7, wherein said Tregs are further characterized as low or negative for the expression of one or more proteins selected from the group consisting of CD127, CCR5, CCR6, CCR7, CXCR3, IFN-gamma, IL10, and ICOS.

9.   The method of any one of claims 1 to 8, wherein said TNFR2 agonist is selected from the group consisting of an antibody, a peptide, a small molecule, and a protein.

10.   The method of claim 9, wherein said agonist is an antibody.

11.   The method of claim 10, wherein said antibody is a monoclonal anti-TNFR2 agonist antibody.

12.   The method of any one of claims 1 to 8, wherein said NF-κB activator is selected from the group consisting of a small molecule, a peptide, a protein, a virus, and a small non-coding RNA.

13.   The method of claim 12, wherein said NF-κB activator is a small molecule.

14.   The method of claim 13, wherein said NF-κB activator is selected from the group consisting of betulinic acid, topoisomerase poison VP16, and doxorubicin.

15.   The method of any one of claims 1 to 14, wherein said method further comprises contacting said population of human cells with any one or more of interleukin-2 (IL2), rapamycin, anti-CD3, and/or anti-CD28.

16.   The method of any one of claims 1 to 15, wherein said method produces at least 5×10^6 Tregs.

17.   A composition enriched in CD4+CD25^{hi} Tregs, wherein said Tregs comprise at least 60% of the cells in said composition, and wherein said composition comprises at least 5×10^6 Tregs.

18.   The composition of claim 17, wherein said Tregs comprise at least 70% of the cells in said composition.

19.   The composition of claim 18, wherein said Tregs comprise at least 80% of the cells in said composition.

20.   The composition of claim 19, wherein said Tregs comprise at least 90% of the cells in said composition.

21.   The composition of claim 20, wherein said Tregs comprise substantially 100% of the cells in said composition.

22.   The composition of any one of claims 17 to 21, wherein said Tregs are further characterized as positive for the expression of one or more proteins selected from the group consisting of FOXP3, CTLA4, TNFR2, CD62L, Fas, HLA-DR, and CD45RO.

23.   The composition of any one of claims 17 to 22, wherein said Tregs are further characterized as negative for the expression of one or more proteins selected from the group consisting of CD127, CCR5, CCR6, CCR7, CXCR3, IFN-gamma, IL10, and ICOS.

24.   A composition enriched in CD4+ and CD25^{hi} Tregs produced by the method of any one of claims 1 to 16, wherein said composition comprises at least 5×10^6 Tregs.

25.   The composition of claim 24, wherein said Tregs comprise at least 70% of the cells in said composition.

26.   The composition of claim 25, wherein said Tregs comprise at least 80% of the cells in said composition.

27.   The composition of claim 26, wherein said Tregs comprise at least 90% of the cells in said composition.

28.   The composition of claim 27, wherein said Tregs comprise substantially 100% of the cells in said composition.

29.   The composition of any one of claims 24 to 28, wherein said Tregs are further characterized as positive for the expression of one or more proteins selected from the group consisting of FOXP3, CTLA4, TNFR2, CD62L, Fas, HLA-DR, and CD45RO.

30.   The composition of any one of claims 24 to 29, wherein said Tregs are further characterized as low or negative for expression of one or more proteins selected from the group consisting of CD127, CCR5, CCR6, CCR7, CXCR3, IFN-gamma, IL10, and ICOS.

31.   A method of treating an immunological disorder in a patient comprising administering to said patient the composition of any one of claims 17 to 30, wherein said composition treats said immunological disorder.

32.   The method of claim 31, wherein said immunological disorder is selected from the group consisting of an allergy, asthma, an autoimmune disorder, graft versus host disease (GVHD), and transplantation graft rejection.

33.   The method of claim 32, wherein said allergy is selected from the group consisting of food allergy, seasonal allergy, pet allergy, hives, hay fever, allergic conjunctivitis, poison ivy allergy oak allergy, mold allergy, drug allergy, dust allergy, cosmetic allergy, and chemical allergy.

34.   The method of claim 32, wherein said autoimmune disorder is selected from the group consisting of type I diabetes, Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Churg-Strauss Syndrome, Cicatricial Pemphigoid, CREST Syndrome, Cold Agglutinin Disease, Crohn's Disease, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Graves' Disease, Guillain-Barré, Hashimoto's Thyroiditis, Hypothyroidism, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, Juvenile Arthritis, Lichen Planus, Lupus, Ménière's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pemphigus Vulgaris, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis, Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis.

35.   A method of treating an infectious disease in a patient comprising administering to said patient the composition of any one of claims 17 to 30, wherein said composition treats said infectious disease.

36.   The method of claim 35, wherein said infectious disease is selected from the group consisting of a bacterial infection, a viral infection, a fungal infection, and a parasitic infection.

37.   The method of any one of claims 31 to 36, wherein said composition comprises at least 5 X 10^6 Tregs.

38.   The method of claim 37, wherein said composition comprises at least 5 X 10^7 Tregs.

39.   The method of claim 38, wherein said composition comprises at least 5 X 10^8 Tregs.

40.   The method of claim 39, wherein said composition comprises at least 5 X 10^9 Tregs.

41.   A method of treating an immunological disorder in a patient comprising administering to said patient the composition produced by the method of any one of claims 1 to 16, wherein said composition treats said immunological disorder.

42.   The method of claim 41, wherein said immunological disorder is selected from the group consisting of an allergy, asthma, an autoimmune disorder, GVHD, and transplantation graft rejection.

43.   The method of claim 42, wherein said allergy is selected from the group consisting of food allergy, seasonal allergy, pet allergy, hives, hay fever, allergic conjunctivitis, poison ivy allergy oak allergy, mold allergy, drug allergy, dust allergy, cosmetic allergy, and chemical allergy.

44.   The method of claim 42, wherein said autoimmune disorder is selected from the group consisting of type I diabetes, Alopecia Areata, Ankylosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Churg-Strauss Syndrome, Cicatricial Pemphigoid, CREST Syndrome, Cold Agglutinin Disease, Crohn's Disease, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Graves' Disease, Guillain-Barré, Hashimoto's Thyroiditis, Hypothyroidism, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, Juvenile Arthritis, Lichen Planus, Lupus, Ménière's Disease, Mixed Connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, Pemphigus Vulgaris, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis, Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis.

45.   A method of treating an infectious disease in a patient comprising administering to said patient the composition produced by the method of any one of claims 1 to 16, wherein said composition treats said infectious disease.

46.   The method of claim 45, wherein said infectious disease is selected from the group consisting of a bacterial infection, a viral infection, a fungal infection, and a parasitic infection.

47.   The method of any one of claims 41 to 46, wherein said composition comprises at least 5×10^6 Tregs.

48.   The method of claim 47, wherein said composition comprises at least 5×10^7 Tregs.

49.   The method of claim 48, wherein said composition comprises at least 5×10^8 Tregs.

50.   The method of claim 49, wherein said composition comprises at least 5×10^9 Tregs.

51.   An isolated antibody or antigen-binding fragment thereof that selectively binds to a first epitope of TNFR2, said first epitope comprising positions 48-67 of SEQ ID NO: 1, said antibody or antigen-binding fragment thereof having an antagonistic effect on TNFR2 upon binding.

52.   The antibody or antigen-binding fragment thereof of claim 51 further binding to a second epitope of TNFR2, said second epitope comprising position 135 of SEQ ID NO: 1

53.   The antibody or antigen-binding fragment thereof of claim 52, wherein said second epitope comprises positions 135-147 of SEQ ID NO: 1.

54.   The antibody or antigen-binding fragment thereof of claim 53, wherein said second epitope comprises positions 130-149 of SEQ ID NO: 1.

55.   The antibody or antigen-binding fragment thereof of claim 53, wherein said second epitope comprises positions 128-147 of SEQ ID NO: 1.

56.   The antibody or antigen-binding fragment thereof of claim 53, wherein said second epitope comprises positions 135-142 of SEQ ID NO: 1.

57.   The antibody or antigen-binding fragment thereof of claim 53, wherein said second epitope comprises positions 135-153 of SEQ ID NO: 1.

58.   The antibody or antigen-binding fragment thereof of any one of claims 51 to 57, wherein said antibody or antigen-binding fragment thereof is selected from the group consisting of a monoclonal antibody or antigen-binding fragment thereof, a polyclonal antibody or antigen-binding fragment thereof, an Fab, a humanized antibody or antigen-binding fragment thereof, a bispecific antibody or antigen-binding fragment thereof, a monovalent antibody or antigen-binding fragment thereof, a chimeric antibody or antigen-binding fragment thereof, a single-chain Fv molecule, a bispecific single chain Fv ((scFv')$_2$) molecule, a domain antibody, a diabody, a triabody, an affibody, a domain antibody, a SMIP, a nanobody, a Fv fragment, a Fab fragment, a F(ab')$_2$ molecule, and a tandem scFv (taFv) fragment.

59.   The antibody or antigen-binding fragment thereof of any one of claims 51 to 58, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 50 nM.

60.   The antibody or antigen-binding fragment thereof of claim 59, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 10 nM.

61   The antibody or antigen-binding fragment thereof of claim 60, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 1 nM.

62.   A method for producing a composition enriched in lymphocytes comprising contacting *in vitro* a population of human cells comprising said lymphocytes obtained from a human blood or bone marrow sample from a patient with a tumor necrosis factor receptor 2 (TNFR2) antagonist or an NF-κB inhibitor that suppresses proliferation of T regulatory cells (Tregs), thereby producing said composition enriched in lymphocytes, wherein said Tregs comprise less than 10% of the cells in said composition.

63.   The method of claim 62 further comprising isolating said human cells from a human blood sample or a human bone marrow sample prior to said contacting.

64.   The method of claim 62, wherein said Tregs comprise less than 5% of cells in said composition.

65.   The method of any one of claims 62 to 64, wherein said TNFR2 antagonist is selected from the group consisting of an antibody or antigen-binding fragment thereof, a peptide, a small molecule, and a protein.

66.   The method of claim 65, wherein said antagonist is an antibody or antigen-binding fragment thereof.

67.   The method of claim 66, wherein said antibody or antigen-binding fragment thereof is a monoclonal anti-TNFR2 antagonist antibody or antigen-binding fragment thereof.

68.   The method of claim 65 or 66, wherein said antibody or antigen-binding fragment thereof binds to a first epitope of TNFR2, said first epitope comprising positions 48-67 of SEQ ID NO: 1.

69.   The method of claim 68, wherein said antibody or antigen-binding fragment thereof binds to a second epitope of TNFR2, said second epitope comprising position 135 of SEQ ID NO: 1.

70.   The method of claim 69, wherein said second epitope comprises positions 135-147 of SEQ ID NO: 1.

71.   The method of any one of claims 66 to 70, wherein said antibody or antigen-binding fragment thereof is selected from the group consisting of a monoclonal antibody or antigen-binding fragment thereof, a polyclonal antibody or antigen-binding fragment thereof, an Fab, a humanized antibody or antigen-binding fragment thereof, a bispecific antibody or antigen-binding fragment thereof, a monovalent antibody or antigen-binding fragment thereof, a chimeric antibody or antigen-binding fragment thereof, a single-chain Fv molecule, a bispecific single chain Fv ((scFv')$_2$) molecule, a domain antibody, a diabody, a triabody, an affibody, a domain antibody, a SMIP, a nanobody, a Fv fragment, a Fab fragment, a F(ab')$_2$ molecule, and a tandem scFv (taFv) fragment.

72.   The method of any one of claims 66 to 71, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 50 nM.

73.   The method of claim 72, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 10 nM.

74.   The method of claim 73, wherein an equilibrium dissociation constant ("K_D") for binding of said antibody or antigen-binding fragment thereof to TNFR2 is less than about 1 nM.

75.   The method of any one of claims 62 to 64, wherein said NF-κB inhibitor is selected from the group consisting of a small molecule, a peptide, a protein, a virus, and a small non-coding RNA.

76.   The method of claim 75, wherein said NF-κB inhibitor is a small molecule.

77.   The method of claim 76, wherein said NF-κB inhibitor is selected from the group consisting of 2-(1,8-naphthyridin-2-yl)-Phenol, 5-Aminosalicylic acid, BAY 11-7082, BAY 11-7085, CAPE (Caffeic Acid Phenethylester), Diethylmaleate, Ethyl 3,4-Dihydroxycinnamate, Helenalin, Gliotoxin, NF-κB Activation Inhibitor II JSH-23, NFκB Activation Inhibitor III, Glucocorticoid Receptor Modulator, CpdA, PPM-18, Pyrrolidinedithiocarbamic acid ammonium salt, (R)-MG-132, Rocaglamide, Sodium Salicylate, QNZ, MG-132 [Z-Leu-Leu-Leu-CHO], Astaxanthin, (E)-2-Fluoro-4'-methoxystilbene, CHS-828, disulfiram, olmesartan, triptolide, withaferin, celastrol, tanshinone IIA, Ro 106-9920, cardamonin, BAY 11-7821, PSI, HU 211, ML130, PR 39, honokiol, CDI 2858522, andrographolide, and dithiocarbamates..

78.   The method of claim 77, wherein said NF-κB inhibitor is a peptide.

79.   The method of claim 78, wherein said peptide is a cell penetrating inhibitory peptide.

80.   A composition produced by the method of any one of claims 62 to 79, wherein said composition is enriched in lymphocytes, and wherein Tregs in said composition comprise less than 10% of the cells in said composition.

81.   The composition of claim 80, wherein said Tregs comprise less than 5% of cells in said composition.

82.   A method of treating a proliferative disorder in a patient comprising administering to said patient the composition of claim 80 or 81.

83.   The method of claim 82, wherein said proliferative disorder is a cancer selected from the group consisting of Acute Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma; AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer; Bone Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma, Brain Stem Glioma, Visual Pathway and Hypothalamic Glioma, Breast Cancer, Bronchial Adenomas/Carcinoids, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Clear Cell Sarcoma of Tendon Sheaths, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Epithelial Cancer, Esophageal Cancer,Ewing's Family of Tumors, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Intraocular Melanoma, Retinoblastoma, Gallbladder Cancer, Gastric (Stomach) Cancer, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin's Lymphoma, Hypopharyngeal Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Pituitary Cancer, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer , Testicular Cancer, Thyroid Cancer, Urethral Cancer, Uterine Sarcoma, and Vaginal Cancer.

84.   The method of claim 82, wherein said proliferative disorder is a solid tumor of the brain, lung, breast, lymphoid, gastrointestinal tract, genitourinary tract, pharynx, prostate, or ovary.

85.   A method of treating an infectious disease in a patient comprising administering to said patient the composition of claim 80 or 81.

86.   The method of claim 85, wherein said infectious disease is selected from the group consisting of a bacterial infection, a viral infection, a fungal infection, and a parasitic infection.

87.   The composition of any one of claims 17 to 30 for use in a method of treating an immunological disorder or an infectious disease.

88.   The composition of claim 80 or 81 for use in a method of treating a proliferative disorder.

89.   A method of treating an infectious disease in a patient comprising administering to said patient an effective amount of the antibody or antigen-binding fragment thereof of any one of claims 51-61.

90.   A method of treating a proliferative disease in a patient comprising administering to said patient an effective amount of the antibody or antigen-binding fragment thereof of any one of claims 51-61.

Figure 1

A

B

C

Figure 2

A

B

C

D
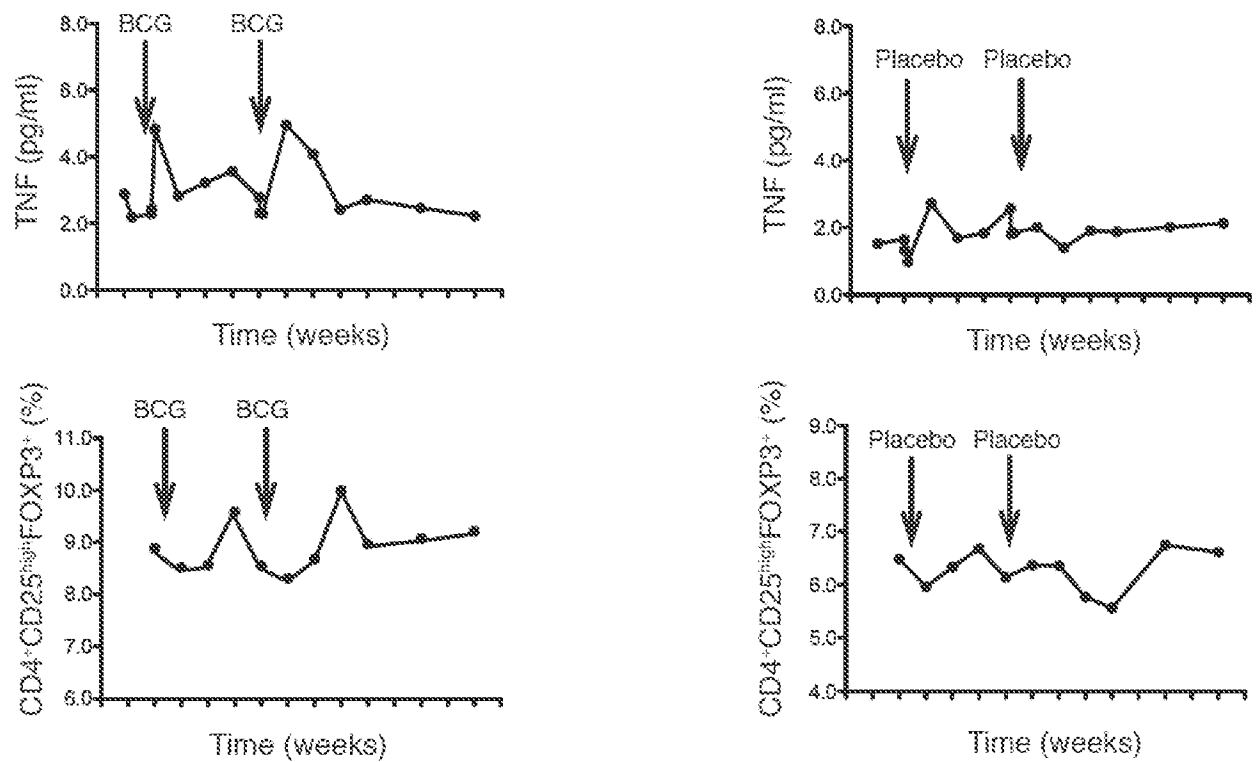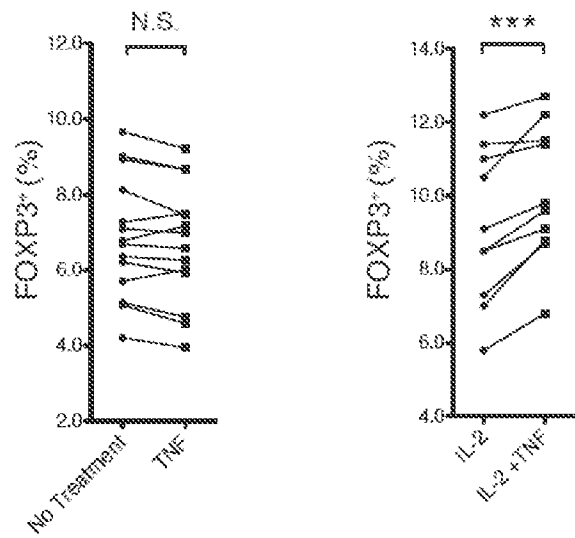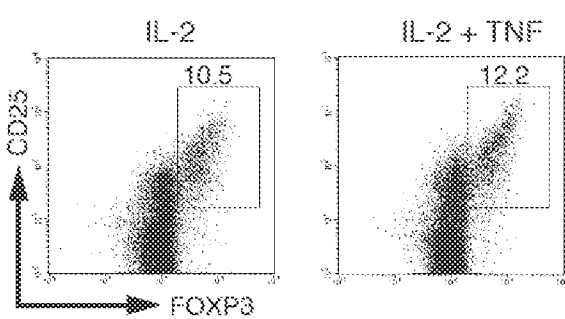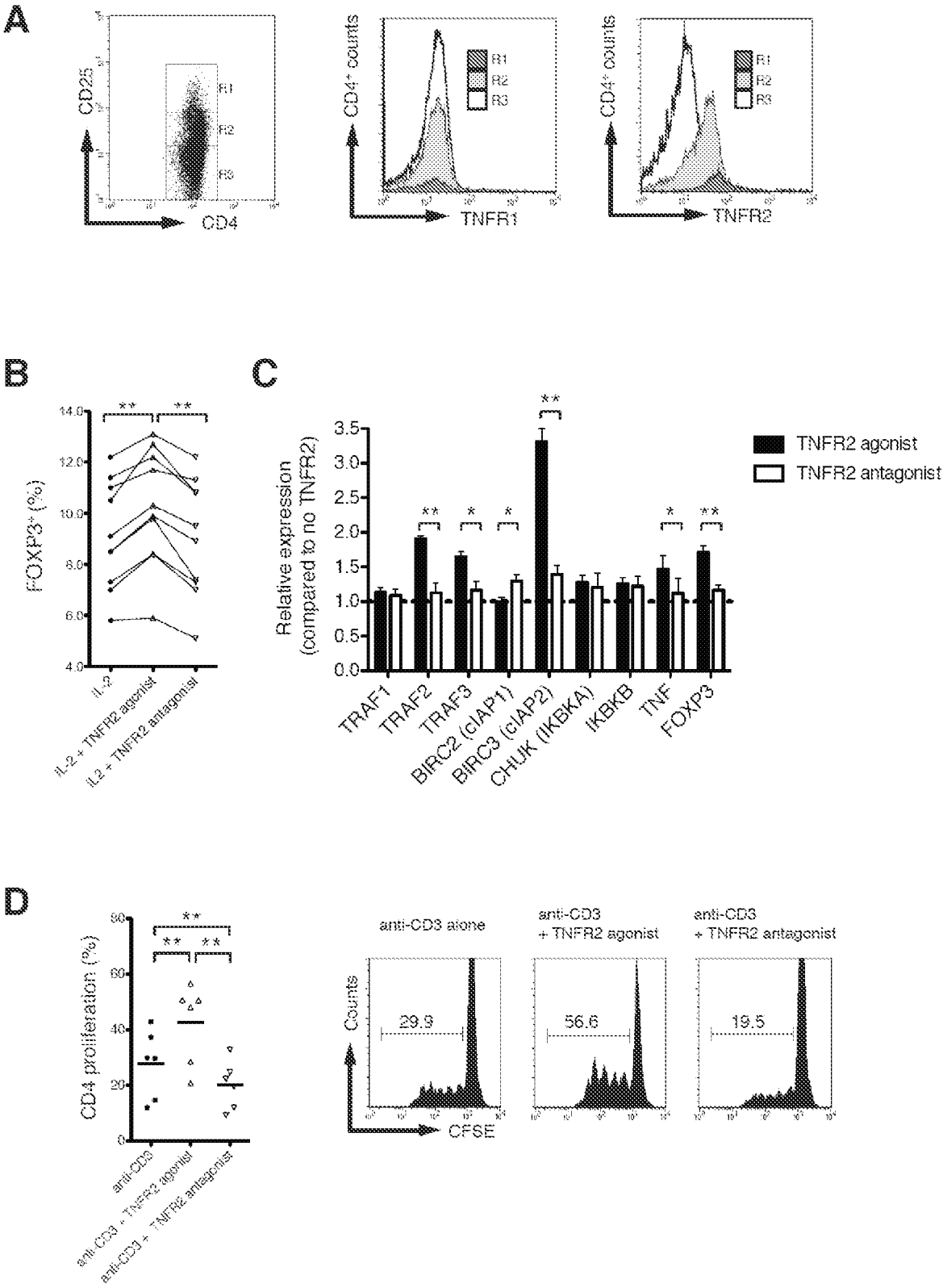
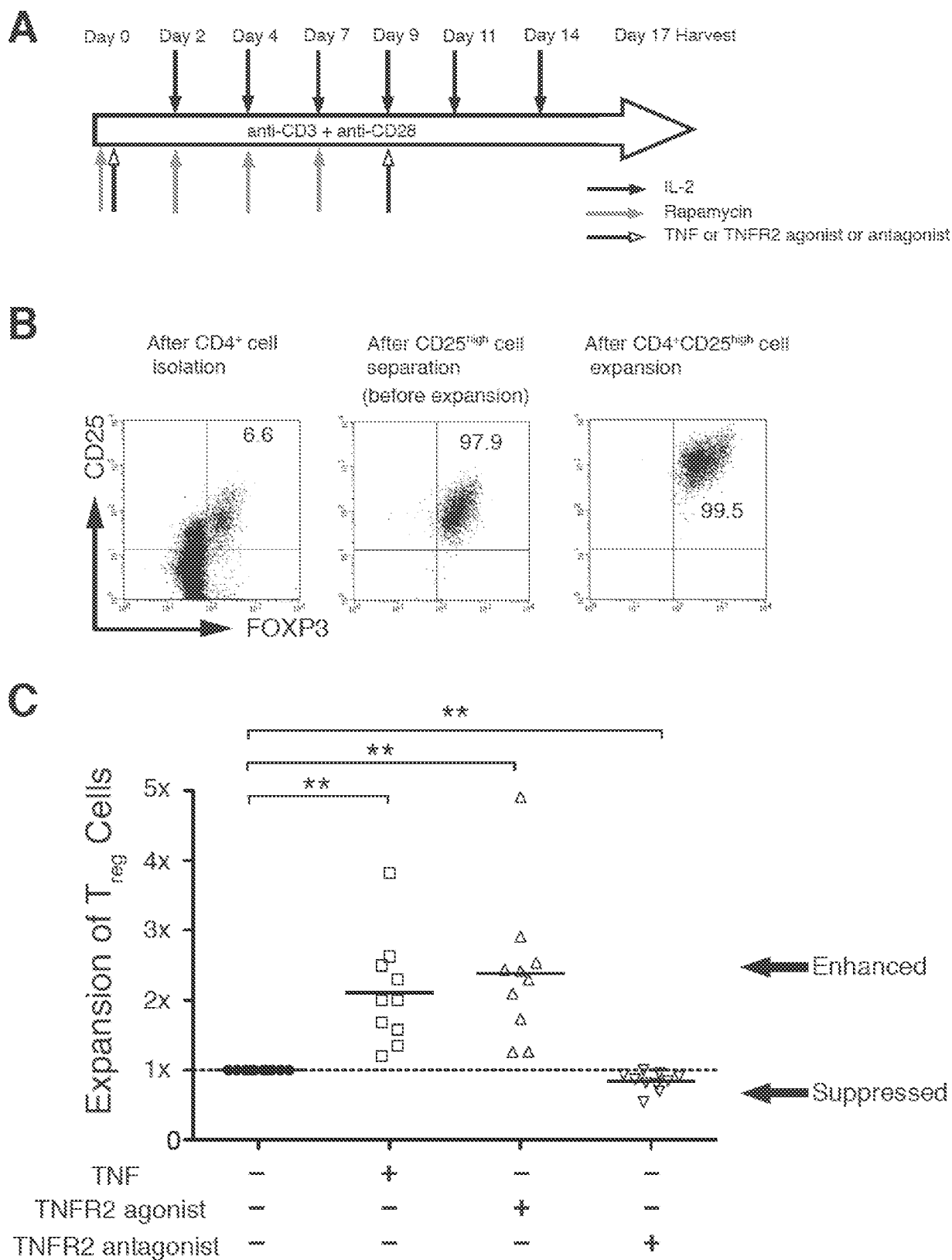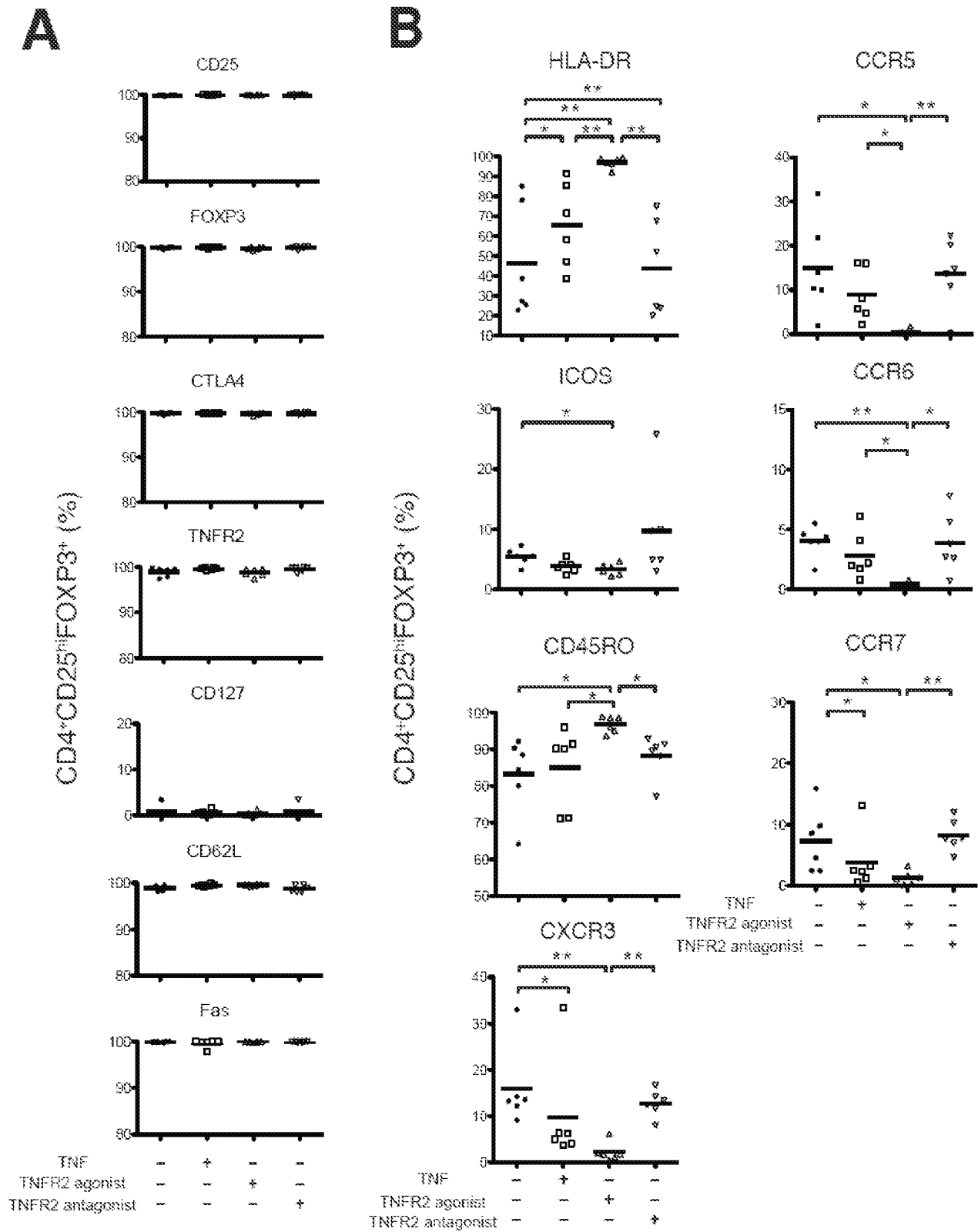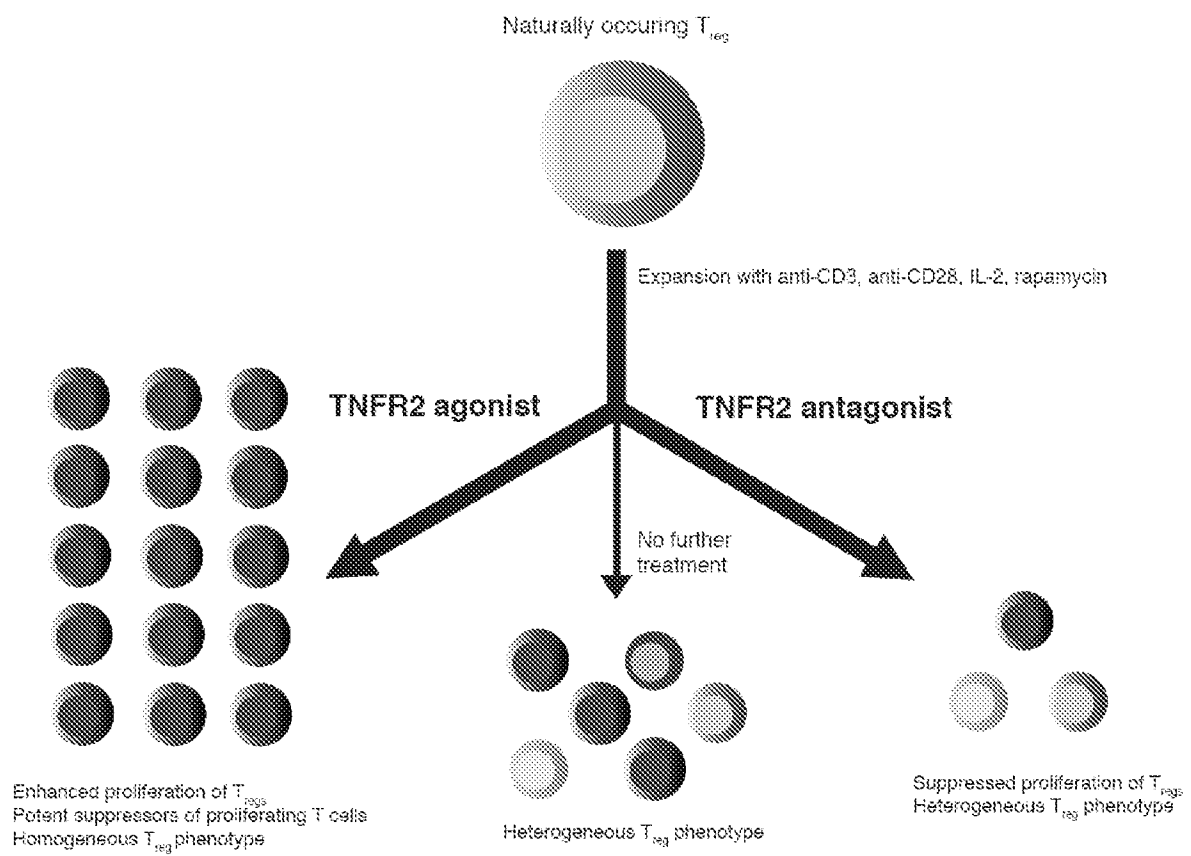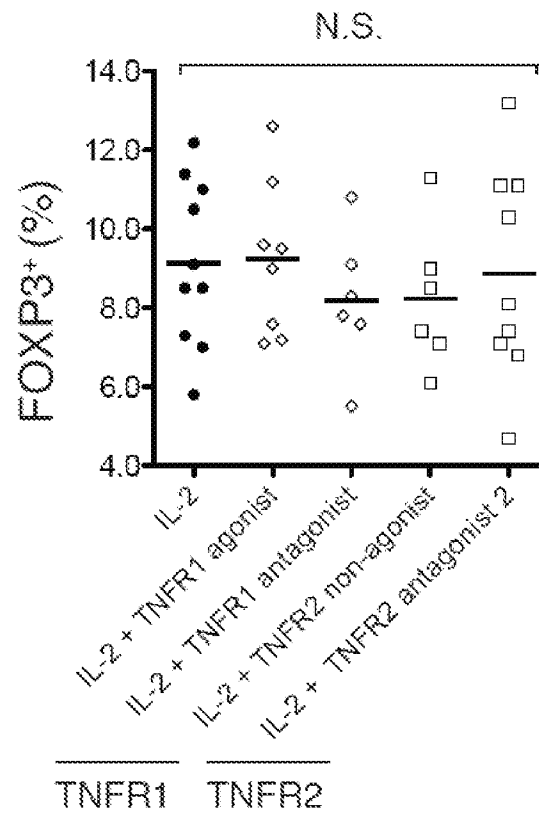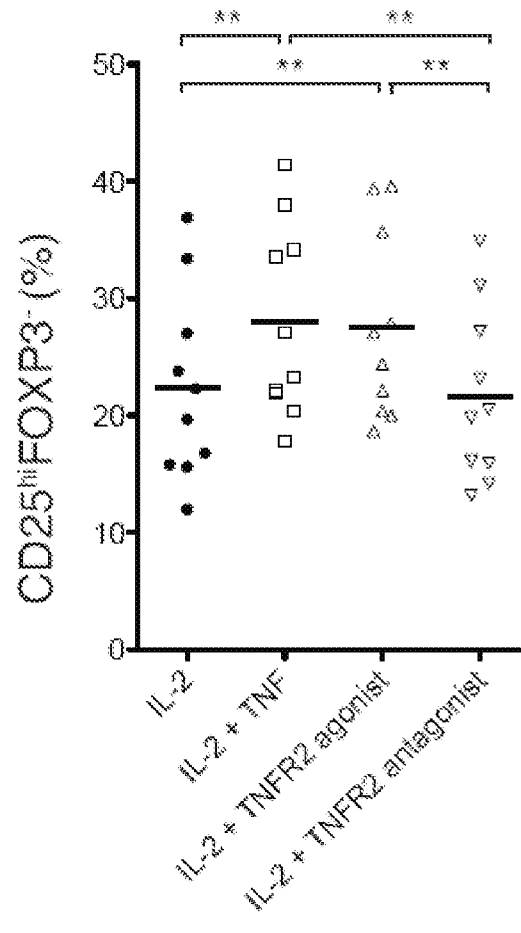# Figure 3

Figure 4

Figure 4
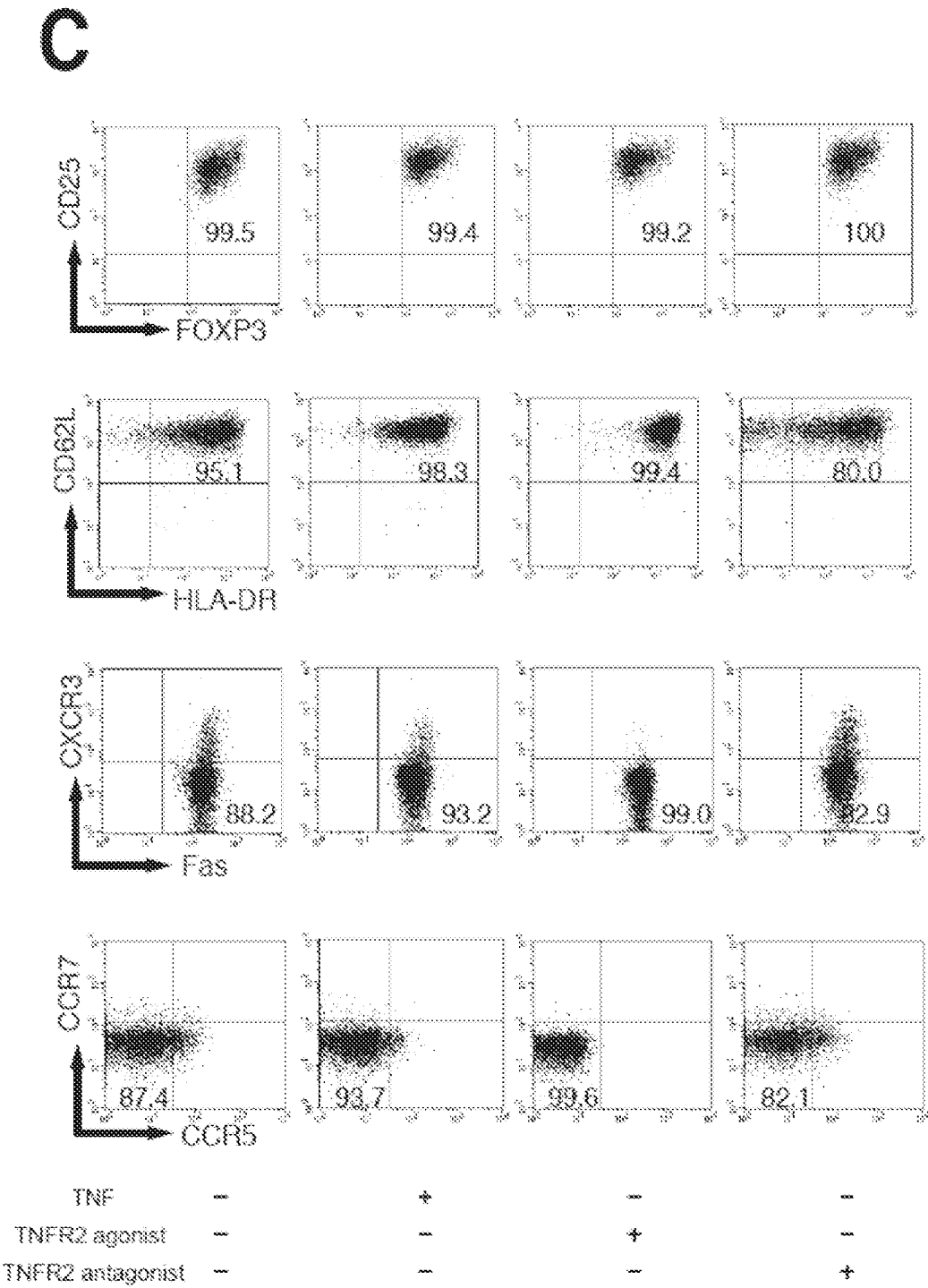
Figure 5
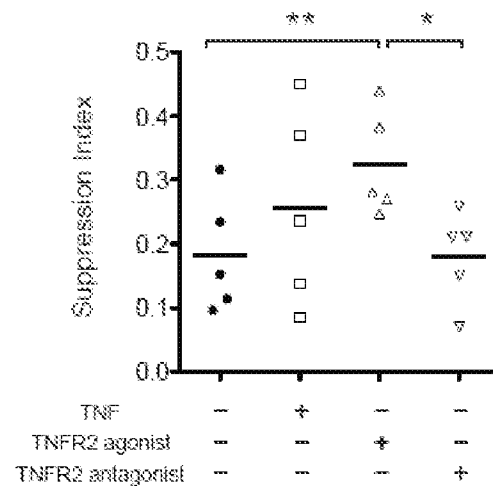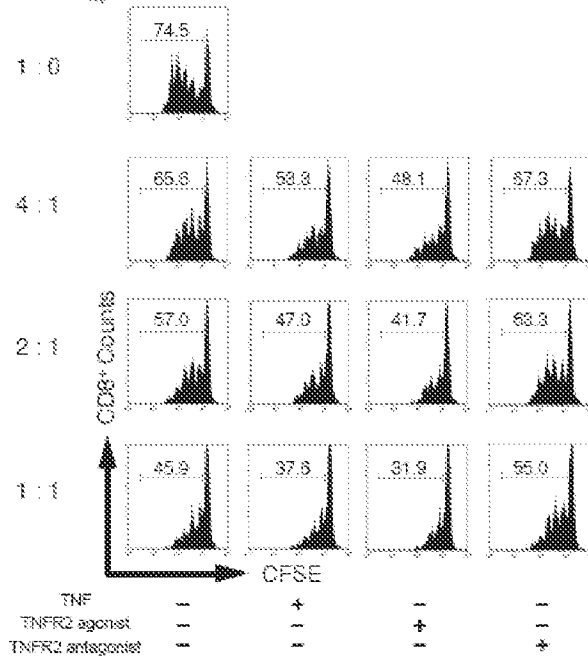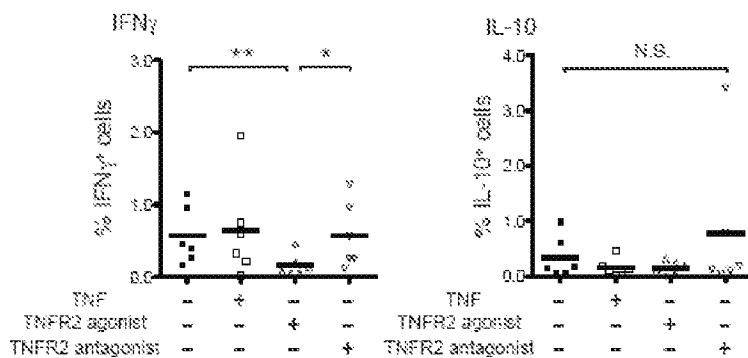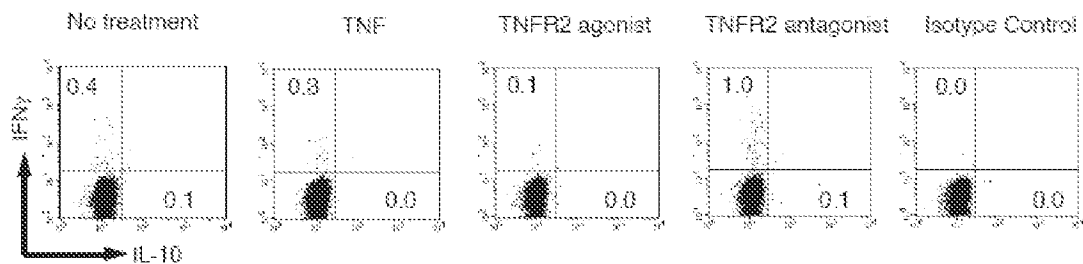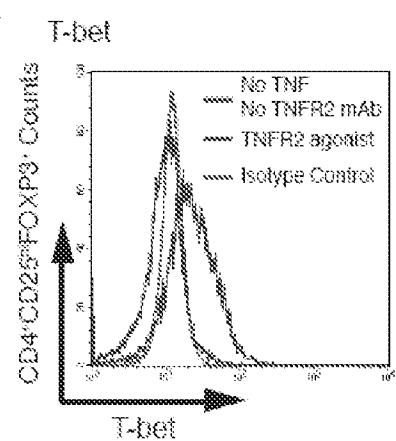
Figure 6

Figure 7

Figure 8

Figure 9

A

B
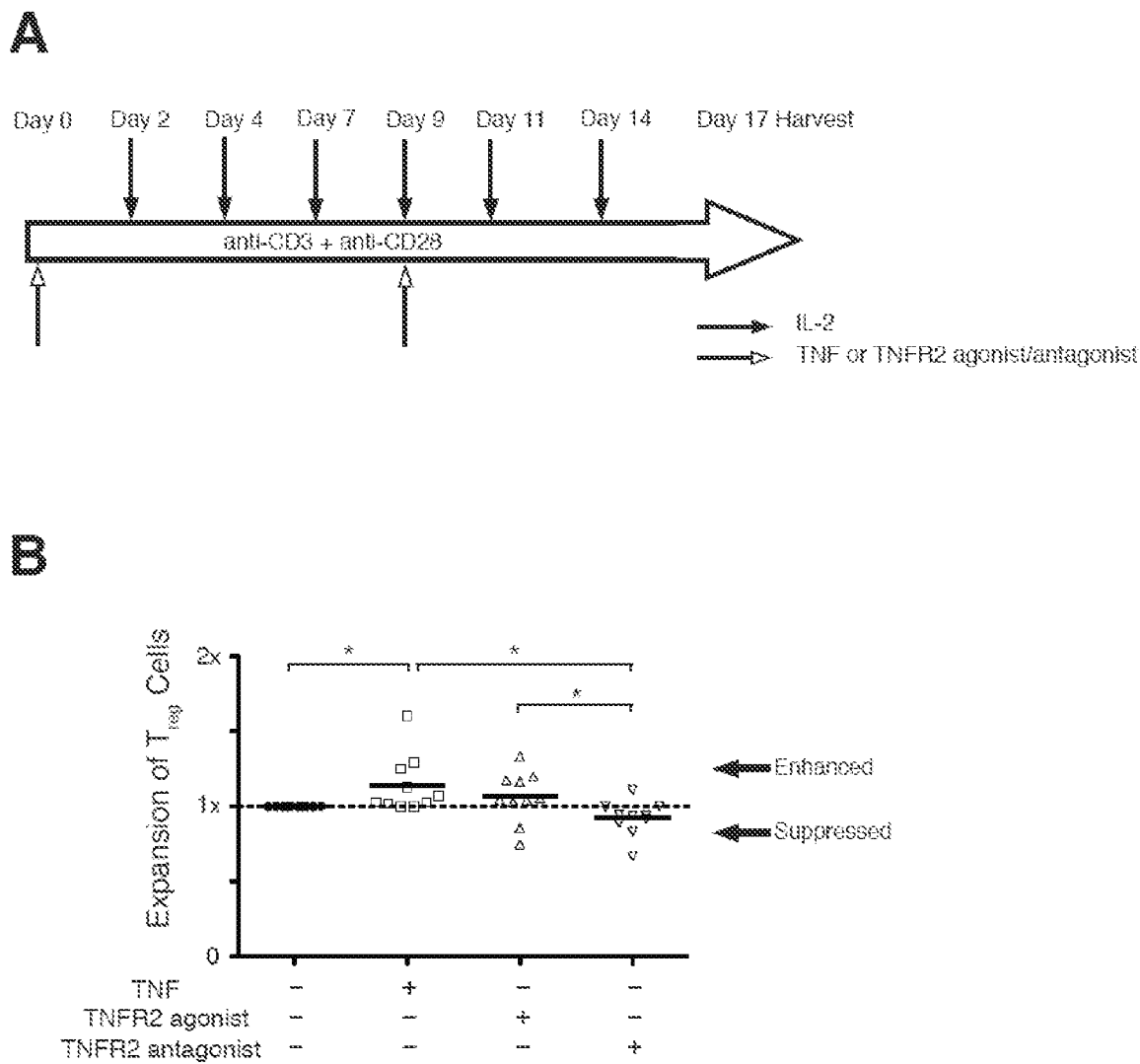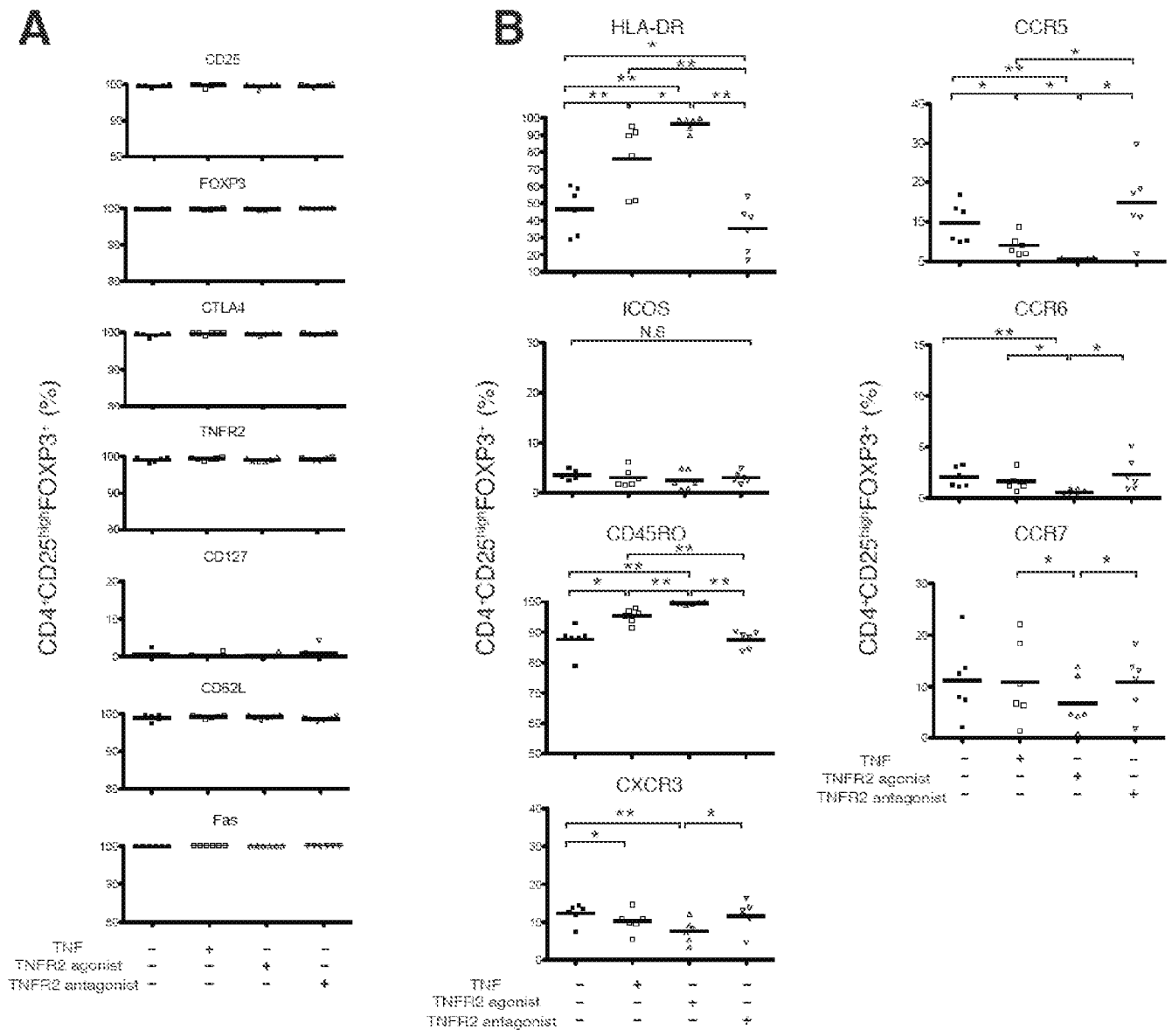
Figure 10

Figure 11
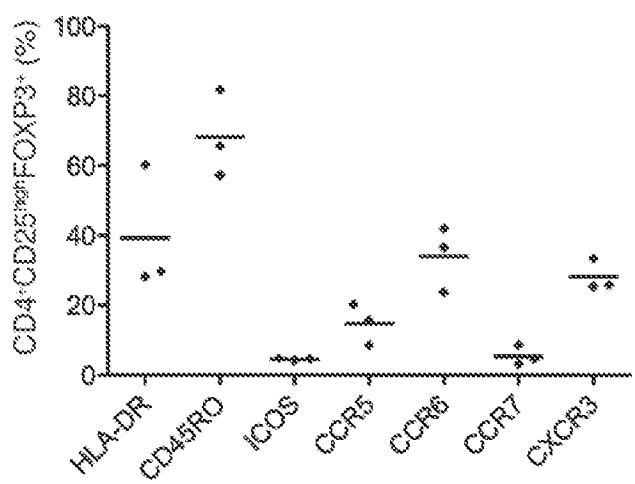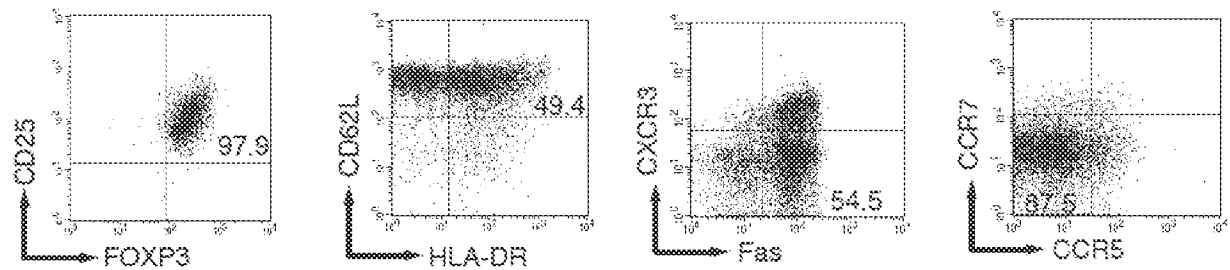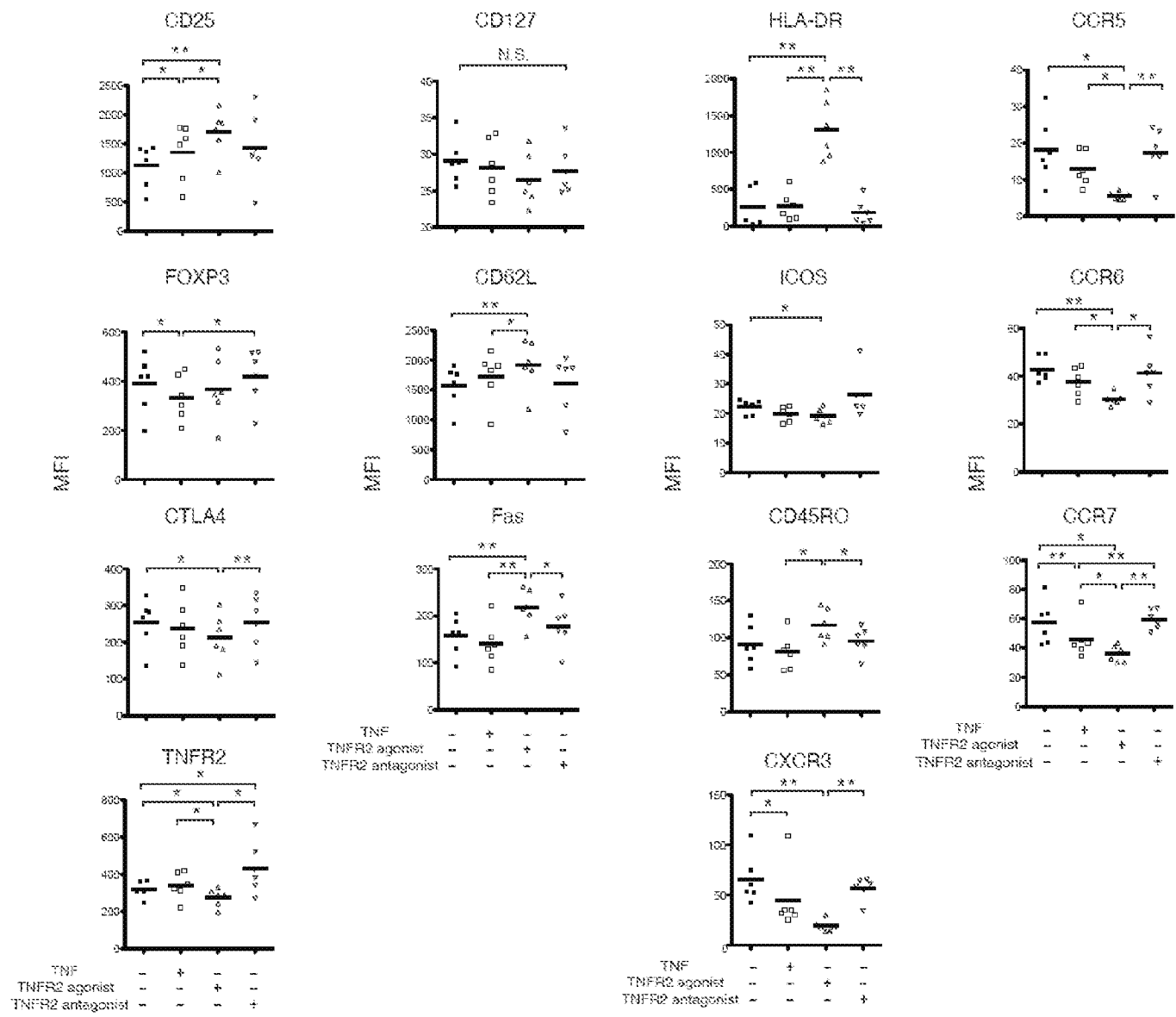
Figure 12

Figure 13

A
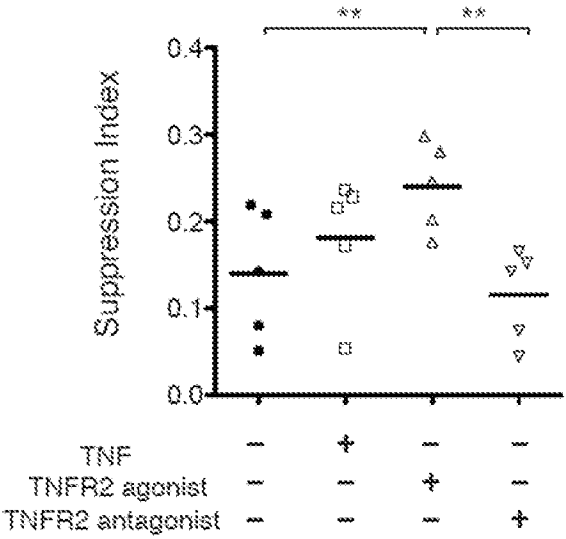
B
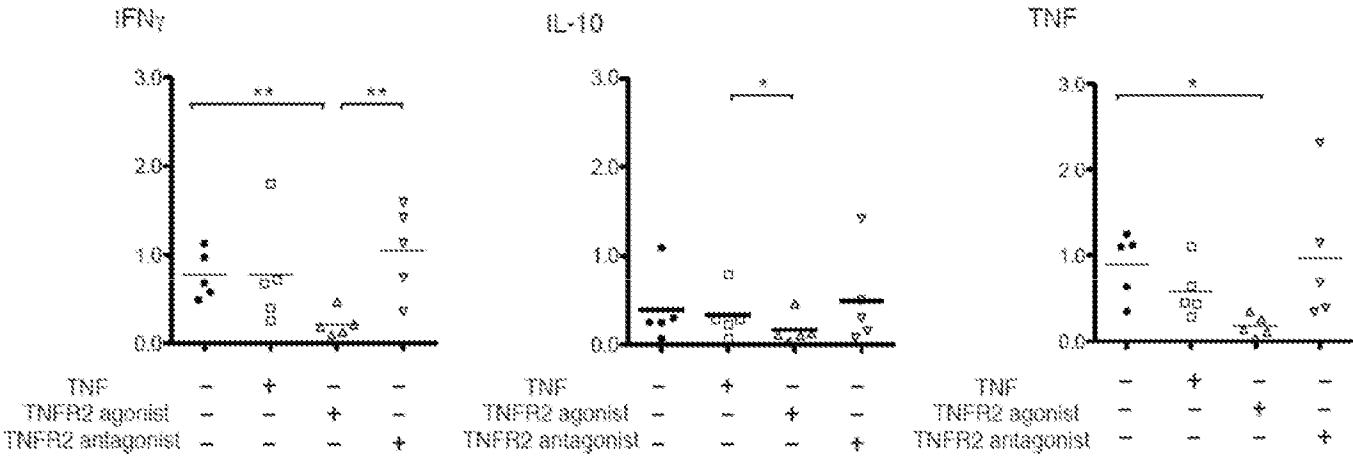
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/12, 39/00; C12N 5/10 (2014.01)
USPC - 424/93.71, 184.1; 435/375
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)

IPC(8): A61K 35/12, 39/00; C12N 5/00, 5/10; A61P 31/18, 35/00, 37/02 (2014.01)
USPC: 424/93.71, 184.1; 435/39, 325, 372, 375

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)

MicroPatent (US-Granted, US-Applications, EP-A, EP-B, WO, JP, DE-G, DE-A, DE-T, DE-U, GB-A, FR-A); ProQuest; IP.com; Google; Google Scholar; 'enriched CD4+CD25hi T regulatory cells,' 'Tregs,' 'T-lymphocytes,' 'TNFR2 agonist,' 'NF-KB activator'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0175830 A1 (STEINMAN, RM et al.) July 24, 2008; abstract; paragraphs [0009], [0021], [0031], [0114], [0124]	1-7, 17-22
Y	HOFFMANN, P et al. Large-Scale In Vitro Expansion Of Polyclonal Human CD4+CD25(sup)(hi) Regulatory T Cells. Blood. 01 August 2004, Vol. 104, No. 3; abstract; pages 895-903. DOI: 10.1182/blood-2004-01-0086.	1-7, 17-22
A	EP 2295588 A1 (RILEY, JL et al.) March 16, 2011; entire document	1-7, 17-22
A	US 2010/0298232 A1 (LIU, C) November 25, 2010; entire document	1-7, 17-22
A	US 2012/0196919 A1 (BROWN, M et al.) August 02, 2012; entire document	1-7, 17-22

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 June 2014 (05.06.2014)	2 4 JUN 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450	Shane Thomas
Facsimile No. 571-273-3201	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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.: 8-16, 23-50, 59-61, 68-74, 80-90
   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:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   Group I: Claims 1-7, 17-22

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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

Box No. IV: Lack of Unity of Invention:

Group I: Claims 1-7 and 17-22 are directed toward a composition enriched in CD4+ CD25hi Tregs, wherein said Tregs comprise at least 60% of the cells in said composition.

Group II: Claims 51-58 are directed toward an isolated antibody or antigen-binding fragment thereof that selectively binds to a first epitope of TNFR2, said first epitope comprising positions 48-67 of SEQ ID NO: 1, said antibody or antigen-binding fragment thereof having an antagonistic effect on TNFR2 upon binding.

Group III: Claims 62-67 and 75-79 are directed toward a method for a composition enriched in lymphocytes, wherein Tregs comprise less than 10% of the cells in said composition.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a composition enriched in CD4+ CD25hi T regulatory cells (Tregs), and contacting in vitro a population of human cells comprising T-lymphocytes with a tumor necrosis factor receptor 2 (TNFR2) agonist or an NF-KB activator, which are not present in Groups II or III; the special technical features of Group II including an antagonistic antibody that binds to an epitope in TNFR2, which are not present in either of Groups I or III; the special technical features of Group III including contacting in vitro a population of human cells comprising lymphocytes obtained from a human blood or bone marrow sample from a patient with a tumor necrosis factor receptor 2 (TNFR2) antagonist or an NF-KB inhibitor.

Groups I-II share the technical features including a composition enriched in lymphocytes in vitro, said lymphocytes obtained from a human blood or bone marrow sample from a patient; a TNFR2 modulator or NF-kB modulator; and T regulatory (Treg) cells. Groups II-III share the technical features including a TNFR2 antagonist.

However, these shared technical features are previously disclosed by EP 2,295,588 A1 to Riley, et al. (hereinafter 'Riley') in view of US 2010/0298232 A1 (LIU), as further evidenced by the publication entitled 'The Role Of Tumor Necrosis Factor (TNF)-alpha And TNF Receptor Polymorphisms In SUsceptibility To Ankylosing Spondylitis' by Chatzikyriakidou, et al. (hereinafter 'Chatzikyriakiou'). Riley discloses a composition enriched in lymphocytes (a population of expanded T cells in vitro (a composition enriched in lymphocytes in vitro); paragraphs [0160], [0161]), said lymphocytes obtained from a sample (lymphocytes obtained from a sample; paragraph [0160]); a TNFR2 modulator or NF-kB modulator (aAPC comprising a lentiviral vector encoding TNFa; paragraphs [0009], [0017], [0018]; wherein TNF-alpha is a TNFR2 modulator (see the abstract of the Chatzikyriakidou Evidentiary reference); and T regulatory (Treg) cells (T regulatory (Treg) cells; paragraph [0161]); the administration of the composition to a human patient in need thereof (administration of the composition to a human patient in need thereof; paragraph [0153]); and the inhibition of unwanted T cell responses by inhibiting T cell activation (inhibition of unwanted T cell responses by inhibiting T cell activation, paragraph [0128]; including antibodies that bind to costimulatory molecules or cytokines, antibodies that bind to costimulatory molecules or cytokines; paragraph [0138]).

Riley does not disclose wherein the sample is of lymphocytes obtained from a human blood or bone marrow sample from a patient; and a TNFR2 antagonist or antibody.

Liu discloses antagonists of TNFR2 (antagonists of TNFR2; paragraphs [0182], [0191], [0192]), including peptides (including peptides; paragraphs [0177], [0179]) and antibodies (antibodies; paragraph [0182]), and the modulation of diseases or disorders mediated by TNFR activity, including immune disorders (modulation of diseases or disorders mediated by TNFR activity, including immune disorders; paragraph [0169]).

Since Riley discloses both the use of a sample for inducing proliferation of T cells, as well as treatment including the administration of said composition including aAPC cells and T cells to a human in need thereof, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have utilized a sample of lymphocytes obtained from a human blood or bone marrow from a patient, for the induction process disclosed by Riley, for enabling autologous cell stimulation and expansion before reintroducing the cells to the subject as a form of treatment, for minimizing the potential to introduce foreign antigens and/or pathogens to a subject in need of treatment. Additionally, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Riley, for including the specific use of modulators of TNFR2, such as antagonistic antibodies or peptides, as previously disclosed by Liu, in order to specifically modulate the response of a set of T cells to the compositions disclosed by Riley, for effectively treating immune disorders, as previously disclosed by Liu.

Since none of the special technical features of the Groups I-III inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Riley and Liu references, unity of invention is lacking.