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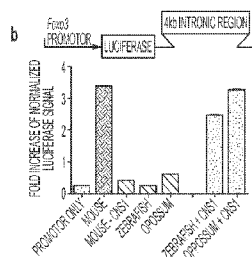
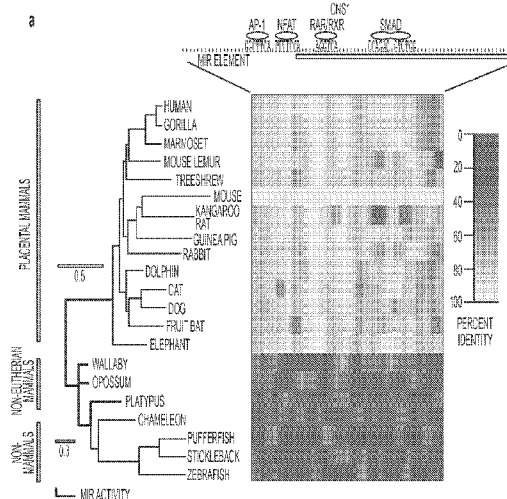
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(54) Title: METHOD OF REDUCING THE RISK OF PREGNANCY COMPLICATIONS



(57) Abstract: The present invention relates to compositions and methods for reducing the risk of pregnancy complications, including miscarriage and preeclampsia. In particular, the invention provides compositions and methods for use in pregnancy complication risk assessment and prevention based on the use of pTregs. The present invention permits identification and/or characterization of pTreg levels, and also provides systems for using such pTreg levels, for example to assess and/or reduce risk of pregnancy complications.



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## METHOD OF REDUCING THE RISK OF PREGNANCY COMPLICATIONS

### Government Support

[0001] This invention was made with government support under AI21609, DK091968, and GM07739 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

### Related Applications

[0002] This application claims priority to United States Provisional Patent Application serial number 61/664,898, filed June 27, 2012; the entirety of which is hereby incorporated by reference.

### Background

[0003] Major complications of pregnancy include miscarriage and preeclampsia. A miscarriage is the cessation of a pregnancy before the fetus can survive and is defined as occurring before the twentieth week of gestation. The etiology is typically due to multiple factors and quite complex. Statistically speaking, one in four pregnancies ends in miscarriage. In large part this high number is the result of the efficiency of the human reproductive system in screening out chromosomal abnormalities. Other reasons for miscarriages include: uterine or cervical defects; systemic maternal disorders; infectious diseases; hormonal deficiencies; blood group incompatibility; maternal age; injury; and environmental or industrial toxins.

[0004] A miscarriage is a traumatic event. The sight of blood, the intensity of physical pain, and the knowledge that one's baby has died can result in clinical shock. Not only is a miscarriage frightening, it can also be emotionally overwhelming. Grief and mourning are a normal response to pregnancy loss. The incidence of clinical depression in women who have experienced a miscarriage is reported to be 85%.

[0005] Preeclampsia is a hypertensive disorder that complicates up to 8-10% of pregnancies and remains the leading cause of maternal and perinatal morbidity and mortality.

### Summary

[0006] The present invention encompasses the recognition that reproducible and detectable changes in pTreg levels are associated with incidence and/or risk of pregnancy

complications, including miscarriage and preeclampsia. The present invention permits identification and/or characterization of pTreg levels, and also provides systems for using such pTreg levels, for example to assess and/or reduce risk of pregnancy complications. The present invention also permits identification and/or characterization of novel agents to assess and/or reduce risk of pregnancy complications by virtue of their effect on pTreg levels.

**[0007]** In certain embodiments, the present disclosure provides methods of identifying an elevated risk for pregnancy complications comprising providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized, processing the sample to determine a CNS1 region sequence, and classifying the woman as having an elevated risk of pregnancy complications if the determined sequence includes one or more alterations relative to a reference sequence.

**[0008]** In certain embodiments, the present disclosure provides methods of identifying an elevated risk for pregnancy complications comprising providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized, processing the sample to determine a level of pTreg cells, and classifying the woman as having an elevated risk of pregnancy complications if the determined level is lower than a reference low threshold or higher than a reference high threshold.

**[0009]** In certain embodiments, the present disclosure provides methods of reducing risk for pregnancy complications comprising a step of administering to a subject a composition comprising pTregs. In some embodiments, the subject is a pregnant female. In some embodiments, the pTregs are autologous. In some embodiments, the pTregs are generated and/or expanded ex-vivo in the presence of a paternal alloantigen.

**[0010]** In certain embodiments, the present disclosure provides a population of maternal pTregs from a pregnant female, wherein at least 25% of the pTregs in the population recognize paternal alloantigens.

**[0011]** In certain embodiments, the present disclosure provides methods of identifying agents that promote pTreg generation comprising determining an activity level of CNS1 exposed to an agent and identifying the agent as promoting pTreg generation if the CNS1 activity level is elevated relative to a CNS1 activity level in an untreated control.

**[0012]** In certain embodiments, the present disclosure provides methods of reducing the risk for pregnancy complications comprising administering to a subject one or more

agents that promote pTreg generation. In some embodiments, the methods further comprise administering to a subject a composition comprising pTregs.

[0013] In some embodiments, the present disclosure provides methods as described herein wherein pregnancy complications are selected from miscarriage and preeclampsia.

### Brief Description of the Drawing

[0014] **Figures 1A-B** show *Foxp3* CNS1 element essential for extrathymic induction of Treg cells is present only in placental mammals. Figure 1A shows a schematic of a *Foxp3* CNS1 element and binding sites of transcription factors implicated in pTreg cell differentiation (not to scale). Overlap with annotated MIR retrotransposon is indicated. Phylogenetic tree of vertebrates with spatial conservation of CNS1 is shown as percent identity smoothened across a 15 bp window. Scale bars are noted. Figure 1B is a graphical representation of a luciferase assay of enhancer activity performed in EL-4 cells using *Foxp3*-4 kB fragments including the first intron of the *Foxp3* locus from indicated species inserted downstream of a mouse *Foxp3* promoter and luciferase (*Luc*) gene (schematic above). Error bars represent standard error (n=3).

[0015] **Figures 2A-B** show CNS1-dependent generation of pTreg cells specific for fetal alloantigen during pregnancy. Figure 2A shows a schematic diagram of experimental design with representative flow cytometric analysis of Foxp3 expression in CNS1-sufficient T cells in deciduas of pregnant TCR  $\beta\delta^{-/-}$  recipient females. Figure 2B is a graphical representation showing percent of CD4<sup>+</sup> cells that express Foxp3 in indicated tissues on day E13.5-E14.5 of pregnancy in TCR  $\beta\delta^{-/-}$  mice transferred with CNS1-sufficient (WT) or -deficient (KO) TEa Foxp3-negative CD4<sup>+</sup> T cells and mated with B6 or BALB/c males. Error bars indicate standard error.

[0016] **Figures 3A-G** show CNS1 deficiency results in increased resorption of embryos. Figure 3A shows macroscopic evaluation of resorption of allogeneic embryos in uteri of CNS1-sufficient (WT) and -deficient (KO) mice on day E14.5. Arrows indicate resorptions. Images representative of 20-30 females are shown. Figure 3A shows a graph of percent of resorbed embryos in all CNS1-sufficient and -deficient pregnancies with BALB/c males. Two-sided Fisher's exact test was used to assess significance. Figure 3C shows a graph of incidence of pregnancies with at least one resorption. Two-sided Fisher's exact test was used to assess significance. Figure 3D shows a graph of percent resorption observed in

individual mothers with indicated genotype. Error bars indicate standard error. Figure 3E shows a graph of percent of embryos resorbed in pregnant CNS1-sufficient and -deficient B6 mice mated with B6 males. Figure 3F shows a graph of incidence of pregnancies with at least one resorption. Figure 3G shows a graph of percent resorption observed in individual mothers. Error bars indicate standard error. See also Figure 4.

**[0017]**        **Figure 4** show that CNS1-deficiency results in decreased numbers of viable embryos. Figure 4 is a graph of total number of nonresorbed embryos observed in CNS1-sufficient (WT) and -deficient (KO) pregnant mice sired by BALB/c males at day E13-14.5.

**[0018]**        **Figures 5A-B** show experimental design for acute DT-mediated ablation of CNS1-sufficient Treg cells in heterozygous  $\text{Foxp3}^{\text{CNS1KO/DTR}}$  females. Figure 5A shows a timeline of DT treatments (arrowheads) and mating. Mice were treated with diphtheria toxin (DT) 3 days before mating and continuously throughout mating and pregnancy. Figure 5B shows heterozygous  $\text{Foxp3}^{\text{CNS1KO/DTR}}$  and  $\text{Foxp3}^{\text{GFP/DTR}}$  females containing one copy of  $\text{Foxp3}^{\text{DTR}}$  and CNS1 KO ( $\text{Foxp3}^{\text{CNS1KO}}$ ) or  $\text{Foxp3}^{\text{GFP}}$  (control) allele each, respectively. Due to random X chromosome inactivation Treg cells express Foxp3 from either the  $\text{Foxp3}^{\text{DTR}}$  allele or the  $\text{Foxp3}^{\text{CNS1KO}}$  allele. Administration of DT to these mice results in ablation of 50% of thymic Treg cells that contain the DTR allele and all pTreg cells.

**[0019]**        **Figures 6A-B** show acute depletion of pTreg and all Treg cells results in comparable increase in embryo resorption. Figure 6A shows a graph of percent of embryos resorbed in pregnant  $\text{Foxp3}^{\text{GFP/DTR}}$  or  $\text{Foxp3}^{\text{CNS1KO/DTR}}$  females treated with DT continuously starting 3 days prior to mating with BALB/c males; incidence of pregnancies with at least one resorption; percent resorption observed in individual mothers. Error bars indicate standard error. Figure 6B shows a graph of percent of resorbed embryos, incidence of resorption, and percent resorption per mother for wild-type or  $\text{Foxp3}^{\text{DTR}}$  B6 mice mated with BALB/c males and treated with DT on day E5.5 and E7.5. See also Figure 5.

**[0020]**        **Figures 7A-E** show decreased Treg cell numbers and histological features of immune-mediated resorption in deciduas of CNS1-deficient female mice. Figure 7A shows a representative flow cytometric analysis of  $\text{Foxp3}^+$  Treg cells in decidua and analysis of decidua and lymph nodes (LN) of CNS1-sufficient (WT) and -deficient (KO) mice mated with BALB/c males and analyzed on day E13.5-E14.5. Error bars indicate standard error (n=8-12). Figure 7B shows a representative flow cytometric analysis of activated  $\text{CD62L}^{\text{lo}}$   $\text{Foxp3}^-$   $\text{CD4}^+$  T cells within the decidua and analysis of decidua and LN. Figure 7C

shows a histopathological evaluation of placentas from WT (left) and CNS1 KO (right) females mated with BALB/c males; low power magnification survey of representative sections of hematoxylin and eosin (H&E) stained placenta. Maternal spiral arteries (SA) are more frequently clustered and prominent in KO placenta at day E12.5 (arrows, upper right panel) (representative of 6-8 mice analyzed per group with 4-10 placental sites each). Figure 7D shows analysis of H&E stained sections of KO placentas at day E13.5; early necrosis of SA (arrow, left) in the decidua (DB) and edema (arrowhead, left) at the chorionic plate. Resorption sites (lower right) shown in the same animal were characterized by loss of embryo and necrotic labyrinths (L) with variable necrosis in the trophoblast (T) layer; embryo (E) and yolk sac (YS) as indicated. Scale bars=500µm. Figure 7E shows immunohistochemical staining for CD3 in day E12.5 placentas from CNS1-sufficient (WT) and -deficient (KO) females. CD3<sup>+</sup> T cells (darker staining) are more numerous in KO placentas in proximity to maternal spiral arteries (SA). Scale bars=100µm. See also Figures 8-10.

**[0021]** **Figure 8** shows lack of compensatory proliferation of thymic Treg cells in pregnant CNS1-deficient females. Proliferative activity in Treg cell subsets in the draining lymph node (DLN) and decidua during allogeneic pregnancy of CNS1-sufficient (WT) and -deficient (KO) mice was assessed by intracellular Ki67 staining. Data are shown as percent of Foxp3<sup>+</sup> Treg cells staining positive for Ki67.

**[0022]** **Figures 9A-D** show graphical analysis of effector cytokine production during allogeneic pregnancy in CNS1-sufficient (WT) and -deficient (KO) mice. Figure 9A shows percent of Foxp3-negative CD4<sup>+</sup> cells in the draining lymph node of pregnant CNS1 WT and CNS1 KO mice expressing the indicated cytokines as measured by intracellular cytokine staining after ex vivo stimulation with PMA/Ionomycin for 5 hours. Figure 9B shows percent of Foxp3-negative CD4<sup>+</sup> cells in the decidua of pregnant CNS1 WT and CNS1 KO mice expressing indicated cytokines as measured by intracellular cytokine staining after ex vivo stimulation with PMA/Ionomycin for 5 hours. Figure 9C shows fold expression of indicated cytokine and transcription factor mRNAs normalized to GAPDH in CNS1 WT and CNS1 KO mice measured by quantitative RT-PCR using total RNA isolated from decidua of allogeneic mated mice. Figure 9D shows serum levels of indicated cytokines measured in Foxp3<sup>WT/CNS1KO</sup> (Het) and Foxp3<sup>CNS1KO/CNS1KO</sup> (KO) pregnant females using Milliplex multi-analyte assay.

**[0023]**        **Figures 10A-C** shows histopathology of placentas and embryos in CNS1-deficient females mated with BALB/c males. Figure 10A shows representative HE-stained sections of CNS1KO placentas. In ED12.5 KO decidua, maternal spiral arteries (SA) with thickened walls were more frequently noted in clusters with occasional early regions of necrosis (arrows, top row). At ED13.5 days, in viable placenta there were occasional degenerative to necrotic spiral arteries (arrow, middle left). In resorption sites (see also Figures 3a and 7d) there is extensive thrombosis and necrosis of vasculature (arrow, middle right). Edema (lower row) is occasionally noted in ED13.5 placenta with early degeneration within the decidua basils surrounding SA (lower left) and in one instance at the chorionic plate (CP) and labyrinth (L) (arrow, lower right). Scale bars=100µm. In Figure 10B, representative HE-stained sections of ED13.5 CNS1KO embryo demonstrate early degeneration along with placental changes. There is edema (arrow, left; E inset) in connective tissues adjacent to an optic cup where there is focal hemorrhage (arrow). Scale bar= 500µm. Inset: higher magnification of affected optic cup. Scale bar=50µm. Within the same embryo, there is edema and mild hemorrhage within the thorax; heart (H) and liver (L) are indicated. Scale bar =50µm. Figure 10C shows representative images of histological staining using B220 (CD45R) and F4/80 antibodies in CNS1 WT and CNS1 KO deciduas demonstrating no major increase in numbers of B cells and macrophages respectively. Scale bars=100µm.

### Definitions

**[0024]**        *Allogeneic:* As used herein, the term “allogeneic” means from a different organism of the same species. In the context of transplantation, the term is used to mean that the cells, tissues and/or organs referred to as “allogeneic” are from a different individual than a recipient into which said cells, tissues and/or organs are being transplanted. Typically, allogeneic cells, tissues or organs have a different genotype than the recipient.

**[0025]**        *Amplification:* The term “amplification” is used herein to refer to any in vitro process for increasing copy number of one or more nucleic acids. In some embodiments, amplification involves exponentially increasing copy number of nucleic acids. In some embodiments, amplification increases copy number of specific nucleic acids of known (i.e., predetermined) sequence. In some embodiments, amplification involves addition of nucleotides (e.g., ribonucleotides or deoxyribonucleotides) to ends of primers, so that polynucleotides complementary to a template nucleic acid molecule to which the primer is

hybridized are formed. As used herein, a single amplification reaction may consist of many rounds of primer extension. For example, a single amplification reaction may consist of several cycles of denaturation and extension ranging from about 5 cycles to 1000 cycles, or more. In some embodiments, amplification involves exponential amplification of a target nucleic acid sequence with limited exponential amplification undesired nucleic acid sequences. In some embodiments, linear amplification of undesired nucleic acids may still occur. Linear amplification does not usually represent a substantial problem in amplification procedures. For example, a single product molecule that contaminates a reaction through 20 cycles of PCR will result in only about 20 molecules if amplified linearly. However, this molecule could result in up to about a million molecules if amplified exponentially at maximal theoretical efficiency. Thus, linear amplification is generally inconsequential. Presence of amplified nucleic acid can be detected by any number of methods known in the art. One method of detection is to differentiate reaction products of a specific size by means of molecular weight. Methods for molecular weight differentiation may include gel filtration, sedimentation velocity, osmotic pressure, or gel electrophoresis, etc. Amplified nucleic acid can also be sequenced using other methods also known in the art. Alternatively or additionally, methods of detecting presence of amplified nucleic acid may include, but are not limited to, labeling nucleotides with a physical label capable of generating a detectable signal. Various “signal generating compounds” (labels) contemplated include radiolabels, chromogens, catalysts such as enzymes, luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds, radioactive elements, and direct visual labels. Useful labels include, but are not limited to,  $^{32}\text{P}$ , fluorescent dyes, colored or fluorescent proteins, electron-dense reagents, enzymes (as commonly used in ELISAs), biotin, digoxigenin, and haptens and proteins for which antisera or monoclonal antibodies are available. Examples of enzymes include, but are not limited to, alkaline phosphatase, horseradish peroxidase, luciferase, beta-galactosidase, etc. Selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances.

**[0026]**        *Amplification Reaction Reagents:* As used herein, the term “amplification reaction reagents”, refers to reagents used in nucleic acid amplification reactions and may include, but are not limited to, buffers, reagents, enzymes having reverse transcriptase and/or polymerase activity or exonuclease activity, enzyme cofactors such as magnesium or manganese, salts, nicotinamide adenine dinuclease (NAD) and deoxynucleoside

triphosphates (dNTPs), such as deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and thymidine triphosphate. Amplification reaction reagents may readily be selected by one skilled in the art depending on the amplification method used.

**[0027]** *Autologous:* As used herein, the term “autologous” means from the same organism. In the context of transplantation, the term is used to mean that cells, tissues and/or organs referred to as “autologous” are derived from the recipient itself. Typically, autologous cells, tissues and/or organs do not contain any substantial amount of material which could be regarded as allogeneic or xenogeneic, that is to say derived from a “foreign” cellular source.

**[0028]** *Carrier or diluent:* As used herein, the terms “carrier” and “diluent” refers to a pharmaceutically acceptable (e.g., safe and non-toxic for administration to a human) carrier or diluting substance useful for preparation of a pharmaceutical formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

**[0029]** *Comparable:* The term “comparable” as used herein refers to a system, set of conditions, effects, or results that is/are sufficiently similar to a test system, set of conditions, effects, or results, to permit scientifically legitimate comparison. Those of ordinary skill in the art will appreciate and understand which systems, sets of conditions, effect, or results are sufficiently similar to be “comparable” to any particular test system, set of conditions, effects, or results as described herein.

**[0030]** *Consensus sequence:* As used herein, the term “consensus sequence” is used to refer to a nucleotide or peptide sequence, wherein at each position in the sequence, the residue or amino acid is that found most commonly in a population. In some embodiments, a consensus sequence is based on sequence alignment data. In some embodiments, the population is a random sample. In some embodiments, the population is a population of individuals known to have a low or no risk for a disease, disorder, or condition (i.e. pregnancy complications).

**[0031]** *Detectable label:* In general, a detectable label may be directly detectable or indirectly detectable, e.g., through combined action with one or more additional members of a signal producing system. Examples of directly detectable labels include radioactive,

paramagnetic, fluorescent, light scattering, absorptive and colorimetric labels. Examples of indirectly detectable include chemiluminescent labels, e.g., enzymes that are capable of converting a substrate to a chromogenic product such as alkaline phosphatase, horseradish peroxidase and the like

**[0032]**        *Dosage form:* As used herein, the terms “dosage form” and “unit dosage form” refer to a physically discrete unit of a therapeutic agent for a patient to be treated. Each unit contains a predetermined quantity of active material calculated to produce a desired therapeutic effect. It will be understood, however, that a total dosage of the composition will be decided by an attending physician within the scope of sound medical judgment.

**[0033]**        *Dosing regimen:* A “dosing regimen” (or “therapeutic regimen”), as that term is used herein, is a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regime comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, the therapeutic agent is administered continuously over a predetermined period. In some embodiments, the therapeutic agent is administered once a day (QD) or twice a day (BID).

**[0034]**        *Improve, increase, or reduce:* As used herein, the terms “improve,” “increase” or “reduce,” or grammatical equivalents, indicate values that are relative to a comparable baseline or reference measurement. In some embodiments, a comparable baseline or reference measurement is a measurement in the same individual prior to initiation of treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of treatment described herein. In some embodiments “control individual” is an individual afflicted with the same form of disease as an individual being treated, who is about the same age as an individual being treated (to ensure that stages of disease in the treated individual and the control individual(s) are comparable).

[0035] *In vitro*: As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, *etc.*, rather than within a multi-cellular organism.

[0036] *In vivo*: As used herein, the term “*in vivo*” refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

[0037] *Nucleic Acid*: The terms “nucleic acid”, “nucleic acid molecule”, and “polynucleotide” each is used herein to refer to a polymers of nucleotide monomers or analogs thereof, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Unless otherwise stated, the terms encompass nucleic acid-like structures with synthetic backbones, as well as amplification products. In some embodiments, nucleic acids involved in the present invention are linear nucleic acids.

[0038] *Oligonucleotide*: The term “oligonucleotide”, as used herein, refers to a string of nucleotides or analogs thereof. These stretches of nucleic acid sequences may be obtained by a number of methods including, for example, chemical synthesis, restriction enzyme digestion or an amplification reaction (*e.g.*, polymerase chain reaction, “PCR”). As will be appreciated by one skilled in the art, the length of an oligonucleotide (*i.e.*, the number of nucleotides it contains) can vary widely, often depending on its intended function or use. Generally, oligonucleotides comprise between about 5 and about 150 nucleotides, usually between about 10 and about 100 nucleotides, and more usually between about 15 and about 75 nucleotides, or between about 15 and about 50 nucleotides. Throughout the specification, whenever an oligonucleotide is represented by a sequence of letters (chosen, for example, from the four base letters: A, C, G, and T, which denote adenosine, cytidine, guanosine, and thymidine, respectively), nucleotides are presented in the 5'→3' order from left to right.

[0039] *Pregnancy Complications*: The term “pregnancy complications”, as used herein, refers to any disease, disorder, or condition associated with pregnancy. In some embodiments, pregnancy complications are or comprise anemia. In some embodiments, pregnancy complications are or comprise ectopic pregnancy. In some embodiments, pregnancy complications are or comprise gestational diabetes. In some embodiments, pregnancy complications are or comprise hyperemesis gravidarum. In some embodiments,

pregnancy complications are or comprise infection. In some embodiments, pregnancy complications are or comprise miscarriage. In some embodiments, pregnancy complications are or comprise placenta previa. In some embodiments, pregnancy complications are or comprise placental abruption. In some embodiments, pregnancy complications are or comprise preeclampsia. In some embodiments, pregnancy complications are or comprise preterm labor.

**[0040]**        *Primer:* The term “primer”, as used herein, refers to an oligonucleotide that hybridizes in a sequence specific manner to a complementary nucleic acid molecule (*e.g.*, a nucleic acid molecule comprising a target sequence) and can be extended. In many embodiments, a primer is a probe that has at least one extendable terminus. The term “primer”, in particular, generally refers to an oligonucleotide that acts as a point of initiation of a template-directed synthesis using methods such as PCR (polymerase chain reaction) or LCR (ligase chain reaction) under appropriate conditions (*e.g.*, in the presence of four different nucleotide triphosphates and a polymerization agent, such as DNA polymerase, RNA polymerase or reverse-transcriptase, DNA ligase, etc, in an appropriate buffer solution containing any necessary co-factors and at suitable temperature(s)). Such a template directed synthesis is also called “primer extension”. For example, a primer pair may be designed to amplify a region of DNA using PCR. Such a pair will include a “forward primer” and a “reverse primer” that hybridize to complementary strands of a DNA molecule and that delimit a region to be synthesized/amplified.

**[0041]**        *Probe:* The term “probe”, as used herein, refers to an oligonucleotide that hybridizes to a target sequence. In many embodiments, a probe hybridizes in a sequence specific manner, in that it favors its target sequence in the presence of competing other sequences. Typically, a probe has a nucleotide sequence including at least a portion that hybridizes to at least about 8, more preferably at least about 10 or at least about 15, typically about 20 to about 40 consecutive nucleotides of a target nucleic acid (*i.e.*, will hybridize to a contiguous sequence of a target nucleic acid). Oligonucleotides that exhibit differential or selected binding to a polymorphic site may readily be designed by one of ordinary skill in the art. For example, an oligonucleotide that is perfectly complementary to a sequence that encompasses a polymorphic site will hybridize to a nucleic acid comprising that sequence as opposed to a nucleic acid comprising an alternate polymorphic variant. In many embodiments, a probe is an oligonucleotide whose hybridization to its target is directly or

indirectly detectable. For example, in some embodiments, a probe comprises an oligonucleotide and a detectable label or tag.

**[0042]**       *Risk:* As will be understood from context, a “risk” of a disease, disorder condition, or event (cardiac defect) comprises a likelihood that a particular individual will develop a disease, disorder, or condition, and/or will suffer an undesirable cardiac event (together, that a person will suffer a cardiac defect). In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 100%. In some embodiments risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference samples have a known risk of a disease, disorder, condition and/or event (cardiac defect). In some embodiments a reference sample or group of reference samples are from individuals comparable to a particular individual. In some embodiments, relative risk is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more.

**[0043]**       *Reference:* As will be understood from context, a reference sample, population, or individual is one that is sufficiently similar to a particular sample, population, or individual of interest to permit a relevant comparison (i.e., to be comparable). In some embodiments, information about a reference sample is obtained simultaneously with information about a particular sample. In some embodiments, information about a reference sample is historical. In some embodiments, information about a reference sample is stored for example in a computer-readable medium. In some embodiments, comparison of a particular sample of interest with a reference sample establishes identity with, similarity to, or difference of a particular sample of interest relative to a reference.

**[0044]**       *Sample:* As used herein, the term “sample” typically refers to a biological sample obtained or derived from a source of interest, as described herein. In some embodiments, a source of interest comprises an organism, such as an animal or human. In some embodiments, a biological sample comprises biological tissue or fluid. In some embodiments, a biological sample may be or comprise bone marrow; blood; blood cells; ascites; tissue or fine needle biopsy samples; cell-containing body fluids; free floating nucleic acids; sputum; saliva; urine; cerebrospinal fluid, peritoneal fluid; pleural fluid; feces; lymph; gynecological fluids; skin swabs; vaginal swabs; oral swabs; nasal swabs; washings or lavages such as a ductal lavages or bronchoalveolar lavages; aspirates; scrapings; bone marrow specimens; tissue biopsy specimens; surgical specimens; feces, other body fluids, secretions, and/or excretions; and/or cells therefrom, *etc.* In some embodiments, a

biological sample is or comprises cells obtained from an individual. In some embodiments, obtained cells are or include cells from an individual from whom the sample is obtained. In some embodiments, obtained cells are or include microbial cells of an individual's microbiome. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. For example, in some embodiments, a primary biological sample is obtained by methods selected from the group consisting of biopsy (*e.g.*, fine needle aspiration or tissue biopsy), surgery, collection of body fluid (*e.g.*, blood, lymph, feces *etc.*), *etc.* In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (*e.g.*, by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to techniques such as amplification or reverse transcription of mRNA, isolation and/or purification of certain components, *etc.*

**[0045]**        *Therapeutically effective amount:* As used herein, the term "therapeutically effective amount" refers to an amount of a microbiome altering agent which confers a therapeutic effect on a treated subject, at a reasonable benefit/risk ratio applicable to any medical treatment. A therapeutic effect may be objective (*i.e.*, measurable by some test or marker) or subjective (*i.e.*, subject gives an indication of or feels an effect). In particular, a "therapeutically effective amount" refers to an amount of a therapeutic agent effective to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect, such as by ameliorating symptoms associated with a disease, preventing or delaying onset of a disease, and/or also lessening severity or frequency of symptoms of a disease. A therapeutically effective amount is commonly administered in a dosing regimen that may comprise multiple unit doses. For any particular therapeutic agent, a therapeutically effective amount (and/or an appropriate unit dose within an effective dosing regimen) may vary, for example, depending on route of administration, on combination with other agents. Also, a specific therapeutically effective amount (and/or unit dose) for any particular patient may depend upon a variety of factors including what disorder is being treated; disorder severity; activity of specific agents employed; specific composition employed; age, body weight, general health, sex and diet of a patient; time of administration, route of administration; treatment duration; and like factors as is well known in the medical arts.

### Detailed Description of Certain Embodiments

#### Maternal fetal conflict

[0046] Adaptive immune systems of vertebrates allow for highly efficient protection against invading pathogens. However, these benefits have come with a substantial trade-off due to overzealous or “unwanted” immune responses and associated inflammation caused by infectious agents, commensal microbiota, autoantigens, and fetal alloantigens during pregnancy in placental animals. Numerous mechanisms operating within mammalian immune systems cooperatively limit deleterious immune responses.

[0047] Mammalian mothers are faced with a problem: within their womb they carry fetus having a genome that is half maternal and half paternal. Fetuses express paternal antigens early in development. Thus, fetal antigens that are paternal in origin would be considered foreign by maternal immune systems (Billingham & Medawar, 1953, *Nature* 172:603-606). Maternal immune systems may be prevented from recognizing foreign fetal tissue and/or maternal immune systems may be prevented from developing immune responses in successful pregnancies (Raghupathy, 2001, *Immunol* 13:219-227; Vince & Johnson, 1995, *Human Reproduction* 10: 107-113).

[0048] While not intending to be bound by any particular theory of operation, it is believed that pregnancy complications, in particular miscarriages and preeclampsia, are caused or associated with inappropriate immune responses in a pregnant subject. In particular, it is believed that subjects at risk for pregnancy complications present inappropriate immune cytokines associated with a T-helper 1 (Th1) immune response known to those of skill in the art. (See, Kwak-Kim et al, 2003, *Hum. Reprod.* 18(4): 676-773.) In contrast, subjects that have healthy pregnancies typically present immune cytokines associated with a T-helper 2 (Th2) immune response.

#### Miscarriage

[0049] Miscarriage is generally defined as spontaneous abortion of pregnancy a stage where an embryo or fetus is incapable of surviving independently. In humans, this stage is generally defined as prior to 20 weeks of gestation. Miscarriage is the most common complication of early pregnancy.

[0050] Miscarriage occurs in 15% of diagnosed pregnancies in women between fifteen and forty-five years of age. Recurrent miscarriage is defined as loss of three or more

consecutive pregnancies and occurs in about 3-4% of these women. Risk of pregnancy loss increases from 15-20% in a first pregnancy to 40% after one miscarriage.

**[0051]** Miscarriage is largely a disorder of unknown etiology. It has been theorized that miscarriage are a natural rejection of a fetus with abnormalities incompatible with life, however, this theory has yet to be substantiated. Approximately 80% of unexplained miscarriages are thought to be caused by an immune mechanism.

**[0052]** Risk factors for miscarriage include age, weight and a woman's overall health. Prevalence of miscarriage increases with increasing maternal age, although not with gravidity. Risk begins to increase rapidly at age 35 years. Risk of miscarriage at age 40 is approximately twice that at age 20. As families are planned later and later in life, frequency of miscarriage will only increase without effective methods of prevention.

**[0053]** Accompanying rising age of hopeful parents is an increasing use of assisted reproductive techniques such as in vitro fertilization, gamete intrafallopian tube transfer (GIFT), ect. These techniques have their own attending risks, especially to women during ovarian hyperstimulation. Moreover, assisted reproduction and in vitro fertilization are costly, time consuming and have a high failure rate, resulting in pregnancy in only about 25% of cases. (see Merck Manual 17th edition, 1999, Merck Research Laboratories, Whitehouse Station, New Jersey, p. 1995).

**[0054]** New methods and compositions are needed to reduce risks associated with pregnancy to maternal and fetal health. Effective prevention of miscarriage can allow women, especially women at risk, to have successful pregnancies and reduce or eliminate one common health risk of pregnancy.

### **Preeclampsia**

**[0055]** Preeclampsia is a life-threatening condition affecting pregnant women in the late second or third trimesters, and postnatal women in the first six weeks after delivery. It is characterized by proteinuria and high blood pressure. Preeclampsia may develop gradually or suddenly, and may remain mild throughout the pregnancy or become severe. Preeclampsia occurs in approximately eight to ten percent of pregnancies.

**[0056]** Risk factors for preeclampsia in pregnant women include age over 35 years or under 18 years, familial history of preeclampsia, individual history of preeclampsia, preexisting hypertension, diabetes, autoimmune disease, inherited thrombophilias, renal

disease, obesity, and/or multiple gestations (twins, triplets, ect.) or combinations thereof. The single most significant risk for developing preeclampsia is having had preeclampsia in a previous pregnancy.

**[0057]** Common symptoms in addition to high blood pressure and proteinuria include elevated uric acid, vision problems such as blinking lights or blurry vision, persistent headaches, extreme swelling of hands and/or feet, fluid retention, and/or pain in the upper right abdomen, or combinations thereof. If untreated, preeclampsia can cause maternal liver and/or kidney damage, deprive the fetus of oxygen, and cause eclampsia (seizures).

**[0058]** A diagnosis of preeclampsia is made when a pregnant woman presents with high blood pressure (two separate readings taken at least four hours apart of 140/90 mm Hg or more) and 300 mg of protein in a 24-hour urine sample.

**[0059]** Treatment for preeclampsia includes close monitoring by a physician. Moderate to severe preeclampsia is often treated in the hospital with bed rest, magnesium sulfate, and/or medication for high blood pressure. When a woman has severe preeclampsia or is near term with mild to moderate preeclampsia, delivery by induction of labor or cesarean section is the remedy of choice. Within the first few days following delivery, the mother's blood pressure usually returns to normal. However, with severe preeclampsia, it may take several weeks for blood pressure to return to normal.

**[0060]** While not intending to be bound by any particular theory of operation, it is believed that at least some instances of preeclampsia are caused or triggered by aberrant immune responses (Ahn, H., et al., "Immunologic Characteristics of Preeclampsia, a Comprehensive Review", *Am J Reprod Immunol* 65: 377–394, 2011). Decreases in Treg cells and increases in effector cells expressing cytokines have been observed in preeclampsia (Darmochwal-Kolar, D., et al., "The predominance of Th17 lymphocytes and decreased number and function of Treg cells in preeclampsia" *Journal of Reproductive Immunology* 93: 75– 81, 2012).

## **Tregs**

**[0061]** Immune responses are a complex phenomenon that is heavily modulated by T cells. Antigen presenting cells, including macrophages, B lymphocytes, and dendritic cells are able to activate T-cells. Different types of T-cells are able to be activated by different antigen presenting cells. Any antigen presenting cells are able to activate helper T-cells, which are activated in response to a specific antigen and are responsible for a variety of

functions including production of cytokines, and memory T-cells, which retain long term memory of antigens. Only dendritic cells can activate naive T cells, which are capable of responding to pathogens that have not yet been encountered.

**[0062]** In some embodiments, T cells are T lymphocytes. T lymphocytes (T-cells) are critical in development of all cell-mediated immune reactions. In some embodiments, T cells are Helper T-cells. Helper T-cells control and modulate development of immune responses. In some embodiments, T cells are Cytotoxic T-cells. Cytotoxic T-cells (killer T-cells) are effector cells which play an important role in immune reactions against intracellular parasites and viruses by means of lysing infected target cells. Cytotoxic T-cells have also been implicated in protecting bodies from developing cancers through an immune surveillance mechanism. In some embodiments, T cells are Regulatory T cells. Regulatory T cells block induction and/or activity of T helper cells. T-cells do not generally recognize free antigen, but recognize it on the surface of other cells. These other cells may be specialized antigen-presenting cells capable of stimulating T cell division or may be virally-infected cells within bodies that become a target for cytotoxic T-cells.

**[0063]** Regulatory T cells are a subset of CD4<sup>+</sup> T cells. Regulatory T cells express an X-chromosome encoded transcription factor Foxp3 and suppress inflammatory immune responses against “self” and foreign antigens in a variety of physiological and pathological settings (Littman, D.R., et al., “Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140, 845–858, 2010). Loss-of-function mutations in Foxp3 result in congenital Treg cell deficiency and severe systemic immunopathology in both mice and humans, which reveal a vital role these cells play in immune homeostasis (Chatila, T.A., et al., “JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome.” *J Clin Invest* 106, R75–81, 2000; Brunkow, M.E., et al., “Disruption of a new forkhead/wingedhelix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse.” *Nat Genet* 27, 68–73, 2001; Wildin, R., et al., “X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy.” *Nat Genet* 27, 18–20, 2001; Fontenot, J.D., et al., “Foxp3 programs the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.” *Nat Immunol* 4, 330–336, 2003). Depletion of Treg cells in normal mice also results in a fatal lympho- and myeloproliferative disorder with widespread inflammatory lesions (Kim, J.M. et al., “Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice.” *Nat Immunol* 8, 191–197, 2007). Analysis of CD4<sup>+</sup> T cells expressing a functional

Foxp3 reporter allele and a Foxp3 reporter null allele showed that Foxp3 is essential for suppressor function of Treg cells (Gavin, M.A. et al., “Foxp3-dependent programme of regulatory T-cell differentiation.” *Nature* 445, 771–775, 2007; Lin, W. et al., “Regulatory T cell development in the absence of functional Foxp3.” *Nat Immunol* 8, 359–368, 2007). Recent studies implicated Treg cells in suppression of different types of inflammatory responses during infection, autoimmunity, metabolic inflammation, tissue injury, autoinflammatory responses at barrier sites, and tumor immunity (reviewed in Josefowicz, S.Z. et al., “Regulatory T cells: mechanisms of differentiation and function,” *Annu Rev Immunol.* 2012;30:531-64. Epub 2012 Jan 6).

**[0064]** In some embodiments, regulatory T cells are tTreg cells. tTreg cells are generated in the thymus. Some thymocytes expressing TCR with a heightened reactivity for “self” antigens up-regulate Foxp3 and differentiate into tTreg cells.

**[0065]** In some embodiments, regulatory T cells are pTreg cells. Peripheral pTreg cell generation occurs upon stimulation of naïve CD4<sup>+</sup> T cells with high affinity cognate TCR ligands in the presence of TGF $\beta$  and retinoic acid (Chen, W. et al., “Conversion of peripheral CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells to CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells by TGF- $\beta$  induction of transcription factor Foxp3.” *J Exp Med* 198, 1875–1886, 2003; Zheng, S.G. et al., “Natural and induced CD4<sup>+</sup>CD25<sup>+</sup> cells educate CD4<sup>+</sup>CD25<sup>-</sup> cells to develop suppressive activity: the role of IL-2, TGF- $\beta$ , and IL-10.” *J Immunol* 172, 5213–5221, 2004; Kretschmer, K. et al., “Inducing and expanding regulatory T cell populations by foreign antigen.” *Nat Immunol* 6, 1219–1227, 2005; Hall, J.A. et al., “The role of retinoic acid in tolerance and immunity.” *Immunity* 35, 13–22, 2011).

**[0066]** A recent observation that an intronic Foxp3 enhancer CNS1, that contains Smad3 and RAR (retinoic acid receptor) binding sites, facilitates TGF- $\beta$ -dependent Foxp3 induction and pTreg cell differentiation, but is dispensable for tTreg generation suggests that biological functions of these two Treg cell subsets are distinct (Zheng, Y. et al, “Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate.” *Nature* 463, 808–812, 2010). Indeed, in contrast to fatal early onset inflammatory lesions resulting from congenital Treg cell deficiency, selective pTreg cell paucity leads to a rather late onset allergic and asthma-like inflammation in gut and lung tissue (Josefowicz et al., 2012). Since a principal difference between these two Treg cell subsets is location and type of antigen that facilitate their differentiation, tTreg cells are likely responsible for tolerance to self-

antigens, whereas pTreg cells restrain immune responses to non-self antigens such as allergens, commensal microbiota, and food.

**[0067]** Pregnancy represents a physiological situation where tolerance to paternal alloantigens is critical for successful reproduction of placental mammals. Treg cells have been suggested to play a role in pregnancy based on their increased numbers in pregnant mice and humans (Somerset, D.A., et al, “Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset.” *Immunology* 112, 38–43, 2004). Antibody-mediated depletion of CD25+ Treg cells results in increased resorption of embryos in allogeneic matings in mice (Aluvihare, V.R. et al., “Regulatory T cells mediate maternal tolerance to the fetus.” *Nat Immunol* 5, 266–271, 2004; Shima, T. et al., “Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice.” *J. Reprod. Immunol.* 85, 121–129, 2010) and women with repeated spontaneous abortions and preeclampsia display decreased numbers of CD25+CD4+ Treg cells (Munoz-Suano, A. et al., “Gimme shelter: the immune system during pregnancy.” *Immunol Rev* 241, 20–38, 2011; Winger, E.E., et al. “Low circulating CD4(+) CD25(+) Foxp3(+) T regulatory cell levels predict miscarriage risk in newly pregnant women with a history of failure.” *Am. J. Reprod. Immunol.* 66, 320–328, 2011). These studies left open a question as to whether a role for Treg cells during pregnancy is largely due to their general “house-keeping” role in immune homeostasis and observed modulation in their numbers is secondary to altered immune balance, or if there is an evolutionary selected mechanism of Treg cell-mediated maternal-fetal tolerance.

**[0068]** The present invention encompasses the recognition that the pTreg subset of Tregs is responsible for maternal-fetal tolerance and that these cells are therapeutically useful for reducing risk of pregnancy complications, including miscarriage and preeclampsia.

**[0069]** In some embodiments, the present invention comprises a population of Tregs. In some embodiments, a provided population of Tregs comprises pTregs. In some embodiments, a population of pTregs comprises pTregs from a pregnant female. In some embodiments, a population of pTregs from a pregnant female comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% of pTregs having specificity for paternal alloantigens. Thus, in some embodiments, the present invention provides compositions comprising Tregs wherein substantially all of the Tregs in the composition are pTregs. Alternatively or additionally, in some embodiments, the present

invention provides compositions comprising Tregs wherein substantially all of the pTregs are from a pregnant female. Alternatively or additionally, in some embodiments, the present invention comprises compositions comprising Tregs wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% of the Tregs in the compositions have specificity for paternal alloantigens

**[0070]** In some embodiments, a population of pTregs comprises pTregs generated from pTreg precursors. In some embodiments, pTreg precursors comprise T cells. In some embodiments, pTreg precursors comprise CD4<sup>+</sup> T cells. In some embodiments, pTreg precursors comprise CD4<sup>+</sup> CD25<sup>-</sup> T cells.

**[0071]** In some embodiments, pTregs or pTreg precursors are isolated from tissue or tissue samples of a subject. In some embodiments, tissue or tissue samples comprise spleen tissue. In some embodiments, tissue or tissue samples comprise lymph node tissue. In some embodiments, tissue or tissue samples are chemically or mechanically disrupted. In some embodiments, tissue or tissue samples are homogenized. In some embodiments, tissue or tissue samples are sonicated.

**[0072]** In some embodiments, isolation of pTregs or pTreg precursors thereof comprises obtaining bone marrow of a subject. In addition to dendritic cells, bone marrow includes hematopoietic cells, such as osteoclasts, monocytes, macrophages, lymphoid cells and their precursors (for review, see, for example, Dexter et al., in Long-Term Bone Marrow Culture: 57-96, Alan R. Liss, 1984).

**[0073]** In some embodiments, isolation of pTregs or pTreg precursors thereof comprises isolating peripheral blood. In some embodiments, isolating peripheral blood comprises isolating blood by venipuncture. In some embodiments, isolation of pTregs or pTreg precursors thereof comprises isolating white blood cells by leukapheresis. Methods for isolating peripheral blood are well known to practitioners in the art. In some embodiments, isolation of pTregs or pTreg precursors thereof comprises isolating peripheral blood mononuclear cells (PBMCs) from peripheral blood. In some embodiments, isolation of pTregs or pTreg precursors thereof comprises isolating peripheral blood mononuclear cells (PBMCs) from peripheral blood comprises density gradient centrifugation, in which PBMCs are separated from other components of peripheral blood based on density by centrifugation. After centrifugation, blood cells will separate into fractions based on density. Methods of density gradient centrifugation to isolate PBMCs from peripheral blood are

well known in the art and include, for example, Ficoll-Paque density gradient centrifugation.

**[0074]** In some embodiments, isolation of pTreg precursors thereof further comprises exposing a population of cells to at least one antibody selected from the group comprising anti-CD8 antibody, anti-CD10 antibody, anti-CD14 antibody, anti-CD15 antibody, anti-CD16 antibody, anti-CD19 antibody, anti-CD25 antibody, anti-CD35 antibody, anti-CD36 antibody, anti-CD49b antibody, anti-CD56 antibody, anti-CD66a antibody, anti-CD66b antibody, anti-CD66c antibody, anti-CD66d antibody, anti-CD89 antibody, anti-CDw92 antibody, anti-CD93 antibody, anti-CD111 antibody, anti-CD112 antibody, anti-CD123 antibody, anti-CD141 antibody, anti-CD156a antibody, anti-CD170 antibody, anti-TCRg/d antibody, anti-CD235a antibody, anti-CD282 antibody, and anti-CDw329 antibody, anti-137-Integrin antibody or mixtures, and depleting antibody bound cells to enrich for CD4<sup>+</sup> T cells.

**[0075]** In some embodiments, isolation of pTregs further comprises exposing a population of cells to anti-CD49d and depleting antibody bound cells to enrich for pTregs. In some embodiments, isolation of pTregs further comprises exposing a population of cells to anti-CD127 and depleting antibody bound cells to enrich for pTregs. In some embodiments, isolation of pTregs further comprises exposing a population of cells to anti-CD62L and depleting antibody bound cells to enrich for pTregs. In some embodiments, isolation of pTregs further comprises exposing a population of cells to an anti-CD25 antibody and enriching for antibody bound cells. In some embodiments, isolation of pTregs further comprises exposing a population of cells to an anti-CD4 antibody and enriching for antibody bound cells. In some embodiments, isolation of pTregs further comprises exposing a population of cells to an anti-Fox3 antibody and enriching for antibody bound cells.

**[0076]** In some embodiments, steps of enriching and/or depleting comprise centrifugation. In some embodiments, steps of enriching and/or depleting comprise cell elutriation. In some embodiments, steps of enriching and/or depleting comprise magnetic separation. In some embodiments, steps of enriching and/or depleting comprise fluorescence activated cell sorting. In some embodiments, steps of enriching and/or depleting comprise immunological separation. In some embodiments, immunological separation comprises an antibody column. In some embodiments, steps of enriching and/or depleting comprise complement lysis. In some embodiments, steps of enriching and/or depleting comprise flow cytometry.

***Ex vivo* generation of pTregs**

[0077] The present invention encompasses the recognition that pTregs are therapeutically useful for reducing the risk of pregnancy complications, and that such pTregs can be generated *ex vivo*.

[0078] Techniques for generating pTregs *ex vivo* are known in the art (Chen, W. et al., "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med 198, 1875–1886, 2003; Zheng, S.G. et al., "Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10." J Immunol 172, 5213–5221, 2004; Hall, J.A. et al., "The role of retinoic acid in tolerance and immunity." Immunity 35, 13–22, 2011).

[0079] In some embodiments, *ex vivo* generation of a cell type comprises isolating a population of pTregs or precursors thereof, as described herein, and culturing pTreg precursors in the presence of agents for promoting maturation and/or expansion of pTregs.

[0080] In certain embodiments, pTregs or precursors thereof are cultured in cell culture media. Cell culture media utilized in accordance with the present invention is or comprises serum-free cell culture media. In certain embodiments, utilized cell culture media is fully defined synthetic cell culture media. In certain embodiments, utilized cell culture media is Dulbecco's Modified Eagle Medium (DMEM). In certain embodiments, utilized cell culture media is RPMI, Ham's F-12, or Mammary Epithelial Cell Growth Media (MEGM). In some embodiments, cell culture media comprises additional components including Fetal Bovine Serum (FBS), Bovine Serum (BS), and/or Glutamine or combinations thereof. In some embodiments, utilized media are supplemented with an antibiotic to prevent contamination. Useful antibiotics in such circumstances include, for example, penicillin, streptomycin, and/or gentamicin and combinations thereof. Those of skill in the art are familiar with parameters relevant to selection of appropriate cell culture media.

[0081] In some embodiments, agents comprise agents for promoting maturation of pTregs from for pTreg precursors. In some embodiments, agents for promoting maturation of pTregs from for pTreg precursors comprise Antigen Presenting Cells, allogeneic cells, IL-2, IL-10, pTregs, retinoic acid, TGF- $\beta$ , T Cell Receptors (TCRs) and combinations thereof. In some embodiments, IL-2 is provided at a concentration of 0.02, 0.2, 2, 20, or 200

or 2,000 ng/ml. In some embodiments, IL-10 is provided at a concentration of 0.001, 0.01, 0.1, 1, 10, or 100 ng/ml. In some embodiments, TGF- $\beta$  is provided at a concentration of 0.002, 0.02, 0.2, 2, 20, or 200 ng/ml TGF- $\beta$ .

**[0082]** In some embodiments, TCRs comprise anti-CD3. In some embodiments, TCRs comprise anti-CD28. In some embodiments, TCRs comprises TCRs specific for OVA. In some embodiments, TCRs comprises cells form an alloTCRs specific for paternal antigens.

## Uses

### *Assessing Risk of Pregnancy Complications*

**[0083]** The present invention encompasses the recognition that pTreg levels, or factors affecting pTreg levels, can be relied upon as a diagnostic tool to identify and characterize risk of pregnancy complications generally, and miscarriage and preeclampsia specifically. As described herein, immune intolerance a major cause of miscarriage and preeclampsia worldwide. As such, there is a constant need for more accurate tests for assessing risk of pregnancy complications.

**[0084]** In some embodiments, the current invention provides methods of identifying an elevated risk for pregnancy complications comprising, providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized, processing the sample to determine a level of pTreg cells; and classifying the woman as having an elevated risk of pregnancy complications if the determined level is lower than a reference low threshold or higher than a reference high threshold.

**[0085]** In some embodiments, an elevated risk for pregnancy complications comprises a risk from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 1000% or more relative to a reference. In some embodiments, a reference comprises an average occurrence of pregnancy complications in a population. In some embodiments, a reference comprises a statistical occurrence of pregnancy complications deemed to be acceptable or unavoidable in a population by medical professionals. In some embodiments a reference comprises a subject who is otherwise comparable to the woman whose risk of pregnancy complications is to be identified or characterized and whose level of pTregs is higher than the reference low threshold and lower than the reference high threshold. In some embodiments a reference comprises a subject who is otherwise comparable to the woman whose risk of pregnancy complications

is to be identified or characterized and whose CNS1 sequence is identical to a reference sequence.

**[0086]** In some embodiments, processing the sample comprises contacting the sample with one or more antibodies that are specific to pTregs to identify pTregs using the techniques described herein. In some embodiments, antibodies comprise anti-CD4 antibodies. In some embodiments, antibodies comprise anti-CD-25 antibodies. In some embodiments, antibodies comprise anti-Fox3 antibodies.

**[0087]** In some embodiments, processing the sample comprises contacting the sample with one or more antibodies that are specific to activated pTregs to identify activated pTregs using the techniques described herein. In some embodiments, antibodies comprise anti- CD45RA antibodies.

**[0088]** In some embodiments, processing the sample comprises contacting the sample with one or more antibodies that do not have affinity for pTregs to identify cells that are not pTregs using the techniques described herein. In some embodiments, antibodies comprise anti-CD127 antibodies.

**[0089]** In some embodiments, processing a sample comprises any means for determining a level of pTregs in the sample. Techniques for determining a level of a given cell type in a population of cells are well known in the art. For example, as shown in Zheng et al. ( “Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10.”), a number of pTregs in a sample can be determined by performing flow cytometry analysis on cells exposed to antibodies specific to cell surface markers for a cell type of interest (pTregs). Other techniques for determining a level of pTregs include but are not limited to magnetic cell sorting (MACS), fluorescence-activated cell sorting (FACS), ELISA, PCR, including real time and quantitative PCR and/or fluorescence microscopy.

**[0090]** In some embodiments, a reference low threshold is a threshold below which a subject is at elevated risk for pregnancy complications relative to a reference. In some embodiments, a reference low threshold comprises a proportion of CD4+ T-cells that are pTregs. In some embodiments, a reference low threshold comprises less than 4%, less than 3%, less than 2% or less than 1% of CD4+ T-cells.

**[0091]** In some embodiments, a reference high threshold is a threshold above which a subject is at elevated risk for pregnancy complications relative to a reference. In some

embodiments, a reference high threshold comprises a proportion of CD4+ T-cells that are pTregs. In some embodiments, a reference high threshold comprises more than 11%, more than 12%, more than 15% or more than 20% of CD4+ T-cells.

**[0092]** In some embodiments, reference high thresholds and reference low thresholds comprise historical levels of pTregs of the woman whose risk of pregnancy complications is to be identified or characterized. In some embodiments, historical levels of pTregs comprise levels of pTregs at points in the menstrual cycle of the woman whose risk of pregnancy complications is to be identified or characterized.

**[0093]** In some embodiments, the current invention provides methods of identifying an elevated risk for pregnancy complications comprising providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized; processing the sample to determine a CNS1 region sequence; and classifying the woman as having an elevated risk of pregnancy complications if the determined sequence includes one or more alterations relative to a reference sequence.

**[0094]** The CNS1 enhancer is located between the promoter and the first exon at the Foxp3 locus. In some embodiments a reference sequence comprises a full length CNS1 sequence. In some embodiments a reference sequence comprises a portion of a CNS1 sequence. In some embodiments, a reference sequence is a CNS1 consensus sequence, or fraction thereof. In some embodiments, a reference sequence is a CNS1 sequence or fraction thereof from a woman at low risk for pregnancy complications.

**[0095]** In some embodiments, processing the sample to determine a CNS1 region sequence comprises amplifying the CNS1 region sequence and sequencing the CNS1 region sequence.

**[0096]** In some embodiments, amplifying the CNS1 region sequence comprises contacting a sample containing genomic DNA from an individual with amplification reagents and primers for amplifying the CNS1 region sequence, and performing PCR.

**[0097]** PCR (or polymerase chain reaction) technique is well-known in the art and has been disclosed, for example, in K.B. Mullis and F.A. Faloona, *Methods Enzymol.*, 1987, 155: 350-355 and U.S. Pat. Nos. 4,683,202; 4,683,195; and 4,800,159 (each of which is incorporated herein by reference in its entirety). In its simplest form, PCR is an *in vitro* method for enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank a region of interest in a target DNA. A

plurality of reaction cycles, each cycle comprising: a denaturation step, an annealing step, and a polymerization step, results in exponential accumulation of a specific DNA fragment (“*PCR Protocols: A Guide to Methods and Applications*”, M.A. Innis (Ed.), 1990, Academic Press: New York; “*PCR Strategies*”, M.A. Innis (Ed.), 1995, Academic Press: New York; “*Polymerase chain reaction: basic principles and automation in PCR: A Practical Approach*”, McPherson et al. (Eds.), 1991, IRL Press: Oxford; R.K. Saiki et al., *Nature*, 1986, 324: 163-166). Termini of amplified fragments are defined as 5' ends of primers. Examples of DNA polymerases capable of producing amplification products in PCR reactions include, but are not limited to: *E. coli* DNA polymerase I, Klenow fragment of DNA polymerase I, T4 DNA polymerase, thermostable DNA polymerases isolated from *Thermus aquaticus* (Taq), available from a variety of sources (for example, Perkin Elmer), *Thermus thermophilus* (United States Biochemicals), *Bacillus stearothermophilus* (Bio-Rad), or *Thermococcus litoralis* (“Vent” polymerase, New England Biolabs).

**[0098]** In some embodiments, primers for amplifying a CNS1 region sequence are primers such that an amplification product generated from an amplification reaction comprises an entire CNS1 region. In some embodiments, primers for amplifying a CNS1 region sequence are primers such that an amplification product generated from an amplification reaction comprises a portion of a CNS1 region.

**[0099]** In some embodiments, any of a variety of sequencing reactions known in the art can be used to directly sequence at least a portion of amplified DNA. The sequence can be compared with sequences of known reference sequence to detect alterations relative to reference sequence. Exemplary sequencing reactions include those based on techniques developed by Maxam and Gilbert, *Proc. Natl. Acad. Sci. USA*, 74:560, 1977 or Sanger, *Proc. Nat. Acad. Sci.* 74:5463, 1977. It is also contemplated that any of a variety of automated sequencing procedures may be utilized when performing subject assays (*Biotechniques* 19:448, 1995; Venter, et al., *Science*, 291:1304-1351, 2001; Lander, et al., *Nature*, 409:860-921, 2001), including sequencing by mass spectrometry (see, for example, U.S. Pat. No. 5,547,835 and international patent application Publication Number WO 94/16101, entitled DNA Sequencing by Mass Spectrometry by H. Koster; U.S. Pat. No. 5,547,835 and international patent application Publication Number WO 94/21822 entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Koster), and U.S. Pat. No. 5,605,798 and International Patent Application No. PCT/US96/03651 entitled DNA Diagnostics Based on Mass Spectrometry by H. Koster; Cohen et al. (1996) Adv

Chromatogr 36:127-162; and Griffin et al. (1993) Appl Biochem Biotechnol 38:147-159). It will be evident to one skilled in the art that, for certain embodiments, occurrence of only one, two or three nucleic acid bases need be determined in a sequencing reaction. Yet other sequencing methods are disclosed, e.g., in U.S. Pat. No. 5,580,732 entitled "Method of DNA sequencing employing a mixed DNA-polymer chain probe" and U.S. Pat. No. 5,571,676 entitled "Method for mismatch-directed in vitro DNA sequencing", and in Melamede, U.S. Pat. No. 4,863,849; Cheeseman, U.S. Pat. No. 5,302,509, Tsien et al, International application WO 91/06678; Rosenthal et al, International application WO 93/21340; Canard et al, Gene, 148: 1-6 (1994); Metzker et al, Nucleic Acids Research, 22: 4259-4267 (1994) and U.S. Pat. Nos. 5,740,341 and 6,306,597.

#### *Identification of Therapeutics*

**[0100]** The present invention encompasses the recognition that the level of CNS1 activation corresponds to pTreg levels and that the level of CNS1 activation can be relied upon to identify new agents for promoting pTreg activation. As described herein, immune intolerance a major cause of pregnancy complications generally, and specifically miscarriage and preeclampsia, worldwide. As such, there is a constant need for agents for reducing risk of pregnancy complications.

**[0101]** In some embodiments, the current invention provides methods of identifying agents that promote pTreg generation comprising determining an activity level of CNS1 exposed to an agent and identifying the agent as promoting pTreg generation if the CNS1 activity level is elevated relative to a CNS1 activity level in a comparable reference (e.g., an untreated control).

**[0102]** According to methods of the present invention, an enhancer is operably linked to a reporter on a reporter gene construct. A reporter gene construct is a nucleic acid molecule that includes a nucleic acid encoding a reporter operatively linked to a transcriptional control sequences. Transcription of the reporter gene is controlled by these sequences. Activity of at least one or more of these control sequences is directly or indirectly regulated by transcription factors and other proteins or biomolecules. Transcriptional control sequences include a promoter and other regulatory regions, such as enhancer sequences, that modulate activity of the promoter, or control sequences that modulate activity or efficiency of RNA polymerase that recognizes the promoter, or control

sequences are recognized by effector molecules. Such sequences are herein collectively referred to as transcriptional regulatory elements or sequences.

**[0103]** A reporter refers to any moiety that allows for detection of a molecule of interest, such as a protein expressed by a cell, or a biological particle. Typical reporter moieties include, include, for example, light emitting proteins such as luciferase, fluorescent proteins, such as red, blue and green fluorescent proteins (see, e.g., U.S. Pat. No. 6,232,107, which provides GFPs from *Renilla* species and other species), lacZ gene from *E. coli*, alkaline phosphatase, secreted embryonic alkaline phosphatase (SEAP), chloramphenicol acetyl transferase (CAT), hormones and cytokines and other such well-known genes. For expression in cells, nucleic acid encoding a reporter moiety can be expressed as a fusion protein with a protein of interest or under control of a promoter of interest. Expression of these reporter genes can also be monitored by measuring levels of mRNA transcribed from these genes. Techniques for assessing activity level of enhancers using reporter genes are well known in the art. In some embodiments, reporter gene protein levels are assayed through ELISA, western blot, FACS, MACS, flow cytometry,  $\beta$ -galactosidase assays and/or immunohistochemistry.

**[0104]** In some embodiments, CNS1 is upstream of a reporter gene. In some embodiments, a promoter is or comprises a sequence located upstream of a reporter gene. In some embodiments, CNS1 is upstream of a promoter. In some embodiments, a promoter is upstream of a reporter gene and CNS1 is downstream of a reporter gene. In some embodiments, a promoter is a FOX3 promoter.

**[0105]** Reporter gene constructs may be or include any vector that facilitates expression of a reporter sequence in a construct in a host cell. Any suitable vector can be used. There are many known in the art. Examples of vectors that can be used include, for example, plasmids or modified viruses. Vectors are typically compatible with a given host cell into which they are introduced to facilitate vector replication and expression of an encoded reporter. Examples of specific vectors that may be useful in the practice of the present invention include, but are not limited to, *E. coli* bacteriophages, for example, lambda derivatives, or plasmids, for example, pBR322 derivatives or pUC plasmid derivatives; phage DNAs, e.g., numerous derivatives of phage 1, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast vectors such as 2 $\mu$  plasmids or derivatives thereof; vectors useful in eukaryotic cells, for example, vectors useful in insect cells, such as baculovirus vectors, vectors useful in mammalian cells such as retroviral

vectors, adenoviral vectors, adenovirus viral vectors, adeno-associated viral vectors, SV40 viral vectors, herpes simplex viral vectors and vaccinia viral vectors; vectors derived from combinations of plasmids and phage DNAs, plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

[0106] Useful cell types include yeast and bacteria cells, insect cell culture and primary and transformed mammalian cell lines to which exogenous DNA may be introduced by lipofection, electroporation, or infection.

[0107] Techniques for culturing a wide variety of cell types are well known in the art. See, for example, Current Protocols in Molecular Biology (N.Y., John Wiley & Sons; Davis et al. 1986).

[0108] Methods for cloning amplified sequences into reporter gene constructs are well known in the art. For general references describing methods of molecular biology which are mentioned in this application, e.g., isolating, cloning, modifying, labeling, manipulating, sequencing and otherwise treating or analyzing nucleic acids and/or proteins, see, e.g., Sambrook, J. et al. (1989). Molecular Cloning, a Laboratory Manual. Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel, F. M. et al. (1995). Current Protocols in Molecular Biology, N.Y., John Wiley & Sons; Davis et al. (1986), Basic Methods in Molecular Biology, Elsevier Sciences Publishing, Inc., New York; Hames et al. (1985), Nucleic Acid Hybridization, IL Press; Dracopoli, N.C. et al. Current Protocols in Human Genetics, John Wiley & Sons, Inc.; and Coligan, J. E., et al. Current Protocols in Protein Science, John Wiley & Sons, Inc.

#### *Treatment*

[0109] The present invention encompasses the recognition that pTregs represent an effective preventative therapy for pregnancy complications caused by immune intolerance. In some embodiments, the current invention provides methods of reducing risk for pregnancy complications comprising a step of administering to a subject a composition comprising pTregs.

[0110] In some embodiments, pTregs are autologous. In some embodiments, autologous pTregs are expanded *ex vivo* as described herein from pTreg precursors obtained as described herein. In some embodiments, autologous pTregs are expanded *ex vivo* in the presence of paternal antigens as described herein from pTreg precursors obtained as described herein.

**[0111]** In some embodiments, pTregs are allogeneic. In some embodiments, allogeneic pTregs are paternal origin. In some embodiments, allogeneic pTregs are isolated from a subject as described herein. In some embodiments, allogeneic pTregs are expanded ex vivo as described herein from pTreg precursors obtained as described herein.

**[0112]** In some embodiments, a subject is any mammalian subject at risk for a pregnancy complications. In some embodiments, the subject is a human female. In certain embodiments, the subject is identified as having an elevated risk for pregnancy complications using methods described herein. In certain embodiments, the subject has previously had one or more miscarriages. In further embodiments, the subject has previously had two or more miscarriage. In other embodiments, the subject has had recurrent miscarriage, i.e., three or more miscarriage. In certain embodiments, the subject has previously had preeclampsia.

**[0113]** In some embodiments, the subject can be any subject in a population at risk for pregnancy complications. For instance, the subject can be a human female in an age group at risk for pregnancy complications. In some embodiments, the subject can be a human female greater than 35 years of age, greater than 40 years of age or greater than 45 years of age. In some embodiments, the subject can be a human female less than 20 years of age or less than 15 years of age. However, essentially a woman of any age that presents with a reproductive infirmity, such as miscarriage, preeclampsia and preterm labor, is a candidate for obtaining the materials and methods of the instant invention.

**[0114]** In some embodiments, the subject is in any other population at risk for pregnancy complications as determined by a practitioner of skill in the art. In certain embodiments, the subject is threatening abortion. In some embodiments, the subject is obese, morbidly obese, has overall poor health or comorbid conditions that indicate a risk of miscarriage to the skilled practitioner. In certain embodiments, these conditions can be incompetent cervix, uterine anomalies, hypothyroidism, diabetes mellitus, chronic nephritis, acute infection, use of illicit drugs (such as cocaine or crack), immunologic problems, severe emotional shock and viral infection (especially cytomegalovirus, herpes virus and rubella) (see Merck Manual 17th edition, 1999, Merck Research Laboratories, Whitehouse Station, New Jersey, p. 2053). In certain embodiments, the subject has had an implantation failure during a previous assisted reproduction procedure. Other subjects at risk include those with unusually high Th1 immune responses or unusually low Th2 immune responses. In some

embodiments, the subject can also be in any other population at risk for pregnancy complications as determined by a practitioner of skill in the art.

[0115] In accordance with the methods of the invention, pTregs of the present invention are typically administered a subject alone, or in compositions or medicaments comprising the pTregs (*e.g.*, in the manufacture of a medicament for the treatment of the disease), as described herein. The compositions can be formulated with a physiologically acceptable carrier or excipient to prepare a pharmaceutical composition. The carrier and composition can be sterile. The formulation should suit the mode of administration.

[0116] Suitable pharmaceutically acceptable carriers are capable of maintaining a pTregs in a form that, upon arrival of pTregs at a target cell, tissue, or site in the body, pTregs are capable of suppressing an immune response at the target site (noting that the target site can be systemic). Suitable carriers of the present invention include carriers or formularies that transport, but do not specifically target the vaccine to a site (also referred to herein as non-targeting carriers). Examples of pharmaceutically acceptable carriers include, but are not limited to water, saline, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

[0117] Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal,—or o-cresol, formalin and benzol alcohol.

[0118] According to another aspect of the invention, pTregs can be preserved by cryopreservation. Methods of cellular cryopreservation are well known in the art and generally involves freezing cells in liquid nitrogen.

[0119] pTregs (or a composition or medicament containing pTregs) are administered by any appropriate route. In some embodiments, pTregs are administered intravenously. In other embodiments, pTregs are administered by direct administration to a target tissue, such as heart or muscle (*e.g.*, intramuscular), tumor (intratumorally), nervous system (*e.g.*, direct injection into the brain; intraventricularly; intrathecally). Alternatively, pTregs can be administered by inhalation, parenterally, subcutaneously, intradermally, transdermally, or

transmucosally (*e.g.*, orally or nasally). More than one route can be used concurrently, if desired.

**[0120]** pTregs (or a composition or medicament containing pTregs) are administered in a therapeutically effective amount (*i.e.*, a dosage amount that, when administered at regular intervals, is sufficient to treat a disease, disorder, or condition (*i.e.* pregnancy complications including miscarriage and/or preeclampsia) such as by ameliorating symptoms associated with the disease, disorder, or condition, preventing or delaying onset of the disease, disorder, or condition, and/or also lessening severity or frequency of symptoms of the disease, disorder, or condition). The dose which will be therapeutically effective for treatment of the disease, disorder, or condition will depend on the nature and extent of the disease, disorder, or condition's effects, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges using methods known in the art. The precise dose to be employed will also depend on route of administration, and should be decided according to judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. The therapeutically effective dosage amount can be, for example, about 100 to  $10^{15}$  cells, about 1000 to  $10^{14}$  cells, about  $10^4$  to  $10^{13}$  cells, about  $10^5$  to  $10^{12}$  cells or about  $10^7$  to  $10^{11}$  cells. The effective dose for a particular individual can be varied (*e.g.*, increased or decreased) over time, depending on the needs of the individual.

**[0121]** In some embodiments pTregs (or a composition or medicament containing pTregs) are administered as a single dose. In some embodiments, pTregs (or a composition or medicament containing pTregs) are administered at regular intervals, depending on the nature and extent of the disease, disorder, or condition's effects, and on an ongoing basis. Administration at an "interval," as used herein, indicates that the therapeutically effective amount is administered periodically (as distinguished from a one-time dose). The interval can be determined by standard clinical techniques. In some embodiments, pTregs are administered bimonthly, monthly, twice monthly, triweekly, biweekly, weekly, twice weekly, thrice weekly, or daily. The administration interval for a single individual need not be a fixed interval, but can be varied over time, depending on the needs of the individual.

**[0122]** Following treatment, levels of pTreg cells can be detected to determine whether treatment was effective and a sufficient level of pTregs was transferred. If levels of pTregs are still low, additional pTregs can be transferred. In some embodiments, methods

further comprise detecting an amount of pTreg cells as described herein. In some embodiments, methods further comprise classifying the woman as having an elevated risk of pregnancy complications if the determined level is lower than a reference low threshold or higher than a reference high threshold as described herein. In some embodiments, the method further comprises administering to a subject a composition comprising pTregs as described herein.

**[0123]** Agents of the present invention may be used to induce proliferation and/or activation of a patient's pTregs *in vivo*. In some embodiments, the present invention comprises methods of *in vivo* stimulation of pTregs, comprising administering to a subject an effective amount of one or more agents that promote pTreg generation.

**[0124]** In some embodiments, administering to a subject an effective amount of an agents that promote pTreg generation comprises a pre-treating step performed before methods for *ex vivo* treatment of pTregs or precursors thereof described above. Without wishing to be held to a specific theory, pre-treatment of a subject with one or more agents of the present invention may result in an increase in number of pTregs and/or enhance activity of pTregs harvested after such administration. Enhanced pTregs may then be further treated, including with the same or additional agents of the present invention, and then reintroduced to a patient's body where they may produce an improved immunogenic effect.

**[0125]** In some embodiments, the agents that promote pTreg generation are administered before isolation of pTregs or precursors thereof, as described in methods for *ex vivo* treatment of pTregs or precursors thereof. In some embodiments, the agents that promote pTreg generation are administered immediately before isolation of pTregs or precursors thereof. In some embodiments, the last administration of agents that promote pTreg generation is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more days of isolation of pTregs or precursors thereof.

**[0126]** In some embodiments, agents that promote pTreg generation are administered concurrently with pTregs. In some embodiments, agents that promote pTreg generation are administered after administration of pTregs. In some embodiments, the last administration of pTregs is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more days of administration of agents that promote pTreg generation.

**[0127]** In some embodiments, administering to a subject an effective amount of an agents that promote pTreg generation comprises a stand-alone immunostimulatory

treatment. Again, without wishing to be held to a specific theory, treatment of a subject with one or more compositions of the present invention may result in an increase in number of pTregs and/or enhance activity of pTregs in a subject.

**[0128]** In accordance with methods of the invention, agents that promote pTreg generation of the invention are typically administered a subject alone, or in compositions or medicaments comprising agents (*e.g.*, in manufacture of a medicament for treatment of a disease), as described herein. Compositions can be formulated with a physiologically acceptable carrier or excipient to prepare a pharmaceutical composition. The carrier and composition can be sterile. The formulation should suit the mode of administration.

**[0129]** Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (*e.g.*, NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, sugars such as mannitol, sucrose, or others, dextrose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, *etc.*, as well as combinations thereof. Pharmaceutical preparations can, if desired, be mixed with auxiliary agents (*e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like) which do not deleteriously react with active compounds or interference with their activity. In a preferred embodiment, a water-soluble carrier suitable for intravenous administration is used.

**[0130]** Composition or medicament, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can also be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrrolidone, sodium saccharine, cellulose, magnesium carbonate, *etc.*

**[0131]** The composition or medicament can be formulated in accordance with routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, in a preferred embodiment, a composition for intravenous administration typically is a solution in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at a

site of injection. Generally, ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that ingredients may be mixed prior to administration.

**[0132]** In some embodiments, agents that promote pTreg generation can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

**[0133]** Agents that promote pTreg generation are administered by any appropriate route. In a preferred embodiment, agents that promote pTreg generation are administered intravenously. In other embodiments, agents that promote pTreg generation are administered by direct administration to a target tissue, such as heart or muscle (*e.g.*, intramuscular), tumor (intratumorally), nervous system (*e.g.*, direct injection into the brain; intraventricularly; intrathecally). Alternatively, agents that promote pTreg generation can be administered by inhalation, parenterally, subcutaneously, intradermally, transdermally, or transmucosally (*e.g.*, orally or nasally). More than one route can be used concurrently, if desired.

**[0134]** Agents that promote pTreg generation administered in a therapeutically effective amount (*i.e.*, a dosage amount that, when administered at regular intervals, is sufficient to achieve a desired effect, such as by promote pTreg generation, as described above). The dose which will be therapeutically effective will depend on nature and extent of desired effects, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges using methods known in the art. The precise dose to be employed will also depend on route of administration, and should be decided according to judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[0135] In some embodiments, agents that promote pTreg generation are administered as a single dose. In some embodiments, agents that promote pTreg generation are administered at regular intervals. Administration at an “interval,” as used herein, indicates that a therapeutically effective amount is administered periodically (as distinguished from a one-time dose). The interval can be determined by standard clinical techniques. In some embodiments, agents that promote pTreg generation are administered bimonthly, monthly, twice monthly, triweekly, biweekly, weekly, twice weekly, thrice weekly, daily, twice daily, or every six hours. In some embodiments, agents that promote pTreg generation are administered daily for 1, 3, or 7 days. The administration interval for a single individual need not be a fixed interval, but can be varied over time, depending on the needs of the individual.

[0136] As used herein, the term “bimonthly” means administration once per two months (*i.e.*, once every two months); the term “monthly” means administration once per month; the term “triweekly” means administration once per three weeks (*i.e.*, once every three weeks); the term “biweekly” means administration once per two weeks (*i.e.*, once every two weeks); the term “weekly” means administration once per week; and the term “daily” means administration once per day.

[0137] In some embodiments, agents that promote pTreg generation are administered at regular intervals indefinitely. In some embodiments, agents that promote pTreg generation are administered at regular intervals for a defined period. In some embodiments, agents that promote pTreg generation are administered at regular intervals for a year, 11 months, 10 months, 9 months, 8 months, 7 months, 6 months, 5 months, 4 months, 3 months, 2 months, a month, 3 weeks, 2 weeks, a week, 6 days, 5 days, 4 days, 3 days, 2 days or a day.

### Exemplification

#### **Example 1: Effect of extrathymic regulatory T cells in placental mammals on maternal-fetal conflict**

[0138] The following example investigates the hypothesis that, given the capacity of pTreg cells to mediate tolerance against non-self antigens, mechanisms supporting their generation arose to mitigate maternal-fetal conflict caused by immune responses to paternal

alloantigens in placental mammals. The following example further investigates the hypothesis that pTreg cell mediated suppression represents such a mechanism.

[0139] In support of this hypothesis, the results presented herein demonstrate that CNS1 enhancer is present only in eutherian mammals and that CNS1-dependent generation of pTreg cells during allogeneic pregnancy in mice plays an important role by preventing embryo resorption and associated defective spiral artery remodeling with accumulation of placental activated T cells. The results presented herein suggest that extrathymic generation of regulatory T cells emerged during evolution as a means of mitigation of maternal-fetal allogeneic conflict.

#### *Experimental Procedures*

[0140] Mouse strains and timed matings

[0141] *Foxp3<sup>GFP</sup>*, *Foxp3<sup>DTR</sup>*, TEa, and CNS1-deficient mice on a B6 background were previously described (Grubin, C.E. et al, “Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides.” *Immunity* 7, 197–208, 1997; Fontenot, J.D. et al., “Regulatory T cell lineage specification by the forkhead transcription factor *foxp3*.” *Immunity* 22, 329–341, 2005; Kim et al., 2007; Zheng et al., 2010). TCRβδ-deficient B6 mice were purchased from Jackson Laboratory and maintained as a homozygous colony. All mice were bred and housed in a specific pathogen-free animal facility at the Memorial Sloan-Kettering Cancer Center and used in accordance with institutional guidelines. Diphtheria toxin (DT) (Sigma) was administered twice i.p. as indicated.

[0142] Timed matings

[0143] 1 or 2 female mice were setup in the afternoon with individual males. Females were checked daily for presence of a vaginal plug in the mornings and plugged females were separated from males; the day of plug detection was considered day E0.5. Plugged females were analyzed for resorbed fetuses at E14.5 and resorption was always analyzed in 3 different ways to confirm significance (see Statistical analysis).

[0144] Adoptive cell transfers

[0145] TEa CD4+Foxp3-negative (GFP-) cells were purified using an Aria2 cell sorter (BD Biosciences) after enrichment of CD4 cells using Dynal CD4 magnetic beads according to manufacturer instructions (Invitrogen).  $4 \times 10^6$  cells were injected i.v. into

TCR $\beta\delta$ -deficient B6 females and recipient mice were time mated with B6 or BALB/c males. Pregnant mice were analyzed on day E13.5.

[0146] CNS1 sequence analysis

[0147] The *Foxp3* locus, including the 100kb flanking sequence 5' and 3' of the *Foxp3* gene, was found in each species by ENSEMBL annotations. The most CNS1-like sequence in all species was determined by scanning for mouse CNS1 across the *Foxp3* locus using global-local alignment. For scoring alignments, there was no penalty for opening gaps, -1 for extending gaps or mismatched nucleotides, and +1 for matched nucleotides. All of CNS1 was aligned to a moving window twice the size of CNS1 and the window was moved 50% of the size of CNS1. Genome-wide phylogenies were downloaded from <http://hgdownload.cse.ucsc.edu/goldenPath/hg19/phyloP46way/vertebrate.mod> and branch lengths were scaled to number of substitutions per site (Nikolaev, S. et al., “Early history of mammals is elucidated with ENCODE multiple species sequencing data.” PLoS Genet. 3, e2, 2007; Pollard, K.S. et al., “Detection of nonneutral substitution rates on mammalian phylogenies.” Genome Res. 20, 110–121, 2010). Phylogenetic tree was unrooted and the root was arbitrarily selected. The tree had two scales since the distance between non-eutherians is much larger than non-eutherians.

[0148] Luciferase assays

[0149] Foxp3 luciferase expression constructs were generated using Infusion cloning system (Clontech) and verified by restriction digests and sequencing.  $5 \times 10^6$  EL4-LAF cells were mixed with 5  $\mu$ g of indicated vector and 0.8  $\mu$ g of pRL-TK control vector in complete RPMI with 20% fetal bovine serum (FBS) and electroporation was performed using a Biorad electroporator (300V, 1000  $\mu$ F). Cells were rested for 15 minutes and then incubated in complete RPMI supplemented with 10% FBS for 1 hour before addition of PMA, ionomycin, and TGF $\beta$  (250 ng/ml, 25 ng/ml, and 4 ng/ml, respectively). After 18-24 hours cells were lysed and dual luciferase activity was measured according to manufacturer instructions (Promega). Firefly luciferase activity levels were normalized to Renilla luciferase and resulting normalized values with stimulation were divided by those without to determine stimulation dependent enhancement of promoter activity.

[0150] Flow cytometry

[0151] Single-cell suspensions from lymph nodes and spleen were prepared by mechanical disruption after dissection. Deciduas were isolated by careful dissection from the

uterus and separation of the yolk sac containing the fetus followed by mechanical disruption. Fluorophore-conjugated antibodies were purchased from BD-Biosciences and eBioscience. Intracellular Foxp3 staining was performed using Foxp3 mouse Treg cell staining kit (eBioscience). Stained cells were analyzed using an LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Treestar).

**[0152]**           Histological analyses

**[0153]**           Hematoxylin-, eosin- and periodic acid Schiff-stained placental-fetal units from day E12.5 to 13.5 were examined histologically for qualitative changes to morphologically characterize early resorptions. Changes evaluated included clustered and thickened decidual spiral arteries, necrosis, thrombosis, edema, and inflammation in any placental layer and necrotic placenta sites that lacked embryos (resorptions). For each uterus, all intact placental sites were evaluated individually and scored for viability (resorptions) and prominence of clustered spiral arteries, necrosis in the metrial gland and necrosis and inflammation in the trophoblast layer. A total of 6 WT uteri with 60 gestational sites and 7 KO uteri with 51 sites were examined.

**[0154]**           Immunohistochemical methods

**[0155]**           Placental-embryo units were stained with antibodies against CD3 (T cells; Clone CD3-12, AbD Serotec), F4/80 (macrophages; CALTAG), and CD31 (blood vessels; Abcam). Appropriate isotype controls and Bond Polymer Refine (DAB) detection kit including peroxide block, polymer and hematoxylin (Leica) were used. Slides were qualitatively examined for signal detected by DAB staining (brown). Images were captured with Nikon Eclipse 80i with CFI plan apo-objectives and Nikon Digital Sight DS-Fi1 12 mega pixel camera and Nikon Basic Elements Software (Nikon). Raw images were edited for brightness in Adobe Photoshop Elements.

**[0156]**           Statistical analysis

**[0157]**           Unless otherwise noted statistical analysis was performed using the unpaired two-tailed Student's *t*-test with Welch's correction for unequal variances for individual biological replicates in Prism (GraphPad). The Fisher's exact test was used to assess significance of ratios of healthy and resorbed fetuses. This method assumes that each individual fetus represents an independent event due to physical separation of individual embryos from mother's alloreactive T cells and likely probabilistic factors affecting alloreactive T cell activation in the placenta. In support of the choice of the Fisher's exact

test as an adequate way to analyze data in question, large numbers of resorbed embryos in a given female or strings of resorbing embryos, i.e. frequent resorptions of neighboring embryos, were rarely observed. These observations suggested that resorption of each fetus is an independent event with a considerable stochastic component. Nevertheless, to exclude the potential concern of non-independence, Fisher's exact test on incidence of mother's having any resorption event was also performed. Lastly, the Student's *t*-test was used to compare the percent resorption of fetuses in individual females. Nonparametric tests were also significant but *p* values are not shown.

### *Results*

**[0158]** CNS1 emerges in placental mammals

**[0159]** Extrathymic induction of Foxp3 and pTreg differentiation is facilitated by *Foxp3* CNS1 enhancer, that contains binding sites for transcription factors activated downstream of three major signaling pathways that have been implicated in this process (Tone, Y. et al., "Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 9, 194–202, 2008; Xu, L. et al., "Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I." *Immunity* 33, 313–325, 2010) (Figure 1a). Thus, to test the hypothesis that pTreg differentiation may have been gained during evolution to assist fetal tolerance, conservation of the CNS1 element in a variety of vertebrates for which annotated genome sequences are available was examined. While CNS1 is highly conserved throughout placental mammals, no evidence of a CNS1 sequence homologue was found within 100kb of the transcription start site of any forkhead family member in the monotreme platypus, in marsupials wallaby and opossum, or in non-mammals such as zebrafish (Figure 1a)(Margulies, E.H. et al., "Analyses of deep mammalian sequence alignments and constraint predictions for 1% of the human genome." *Genome Res.* 17, 760–774, 2007). Importantly, a Foxp3 homologue was previously identified in zebrafish and its forced expression in mouse Foxp3- CD4+ T cells conferred suppressive capacity (Quintana, F.J. et al., "Adaptive autoimmunity and Foxp3-based immunoregulation in zebrafish." *PLoS ONE* 5, e9478, 2010). The results presented herein suggested that species without identifiable *Foxp3* CNS1 sequence homologues lack a proximal regulatory element conferring efficient TGF- $\beta$ -mediated Foxp3 induction, a key factor in pTreg differentiation. To test this notion, 4kb regions downstream of the promoter of zebrafish, opossum, and mouse *Foxp3* genes

(*Foxp3*-4kb) were cloned and their enhancer activities were evaluated upon TCR (PMA/ionomycin) and TGF- $\beta$  induced activation of the *Foxp3* promoter in EL-4 cells in a luciferase assay previously used for assessment of CNS1 enhancer activity (Figure 1b)(Tone et al., 2008). Indeed, only mouse, but not zebrafish or opossum *Foxp3*-4kb sequence markedly augmented *Foxp3* promoter activity. Furthermore, mouse *Foxp3*-4kb sequence devoid of CNS1 was lacking enhancer activity whereas incorporation of CNS1 into zebrafish or opossum *Foxp3*-4kb sequence reconstituted enhancer activity. While the remote possibility remains that in non-eutherians TGF- $\beta$  can facilitate Foxp3 induction in a species-specific manner, these results support the idea that CNS1-like enhancer activity, a prerequisite for pTreg differentiation, arose in placental mammals.

[0160] Interestingly, the majority of the CNS1 element sequence was contained within an annotated SINE retrotransposon of the mammalian-wide interspersed repeat (MIR) family (Figure 1a) suggesting a mechanism for the abrupt emergence of CNS1 in the *Foxp3* locus since the MIR family is thought to have been amplified during the Mesozoic era in the course of the radiation of placental mammals, marsupials, and monotremes (Jurka, J. et al., "Ubiquitous mammalian-wide interspersed repeats (MIRs) are molecular fossils from the mesozoic era." Nucleic Acids Res 23, 170–175, 1995; Smit, A.F. et al., "MIRs are classic, tRNA-derived SINEs that amplified before the mammalian radiation." Nucleic Acids Res 23, 98–102, 1995).

[0161] Extrathymic generation of fetal alloantigen-specific Treg cells in pregnancy

[0162] To test whether pTreg cells specific for paternal alloantigen are generated during pregnancy in a CNS1-dependent manner, CNS1-sufficient and -deficient *Foxp3*<sup>GFP</sup> mice expressing transgenic (tg) TEa TCR that recognizes Ea52-68 peptide derived from I-E<sup>d</sup> molecule bound to MHC class II molecule I-A<sup>b</sup> were used (Grubin et al., 1997). This complex is highly expressed in H-2<sup>bxd</sup> (B6 x BALB/c) F1 mice, yet is absent in either parental strains because B6 do not express peptide donor Ea chain, while BALB/c mice lack the appropriate presenting molecule, I-A<sup>b</sup>. FACS purified Foxp3<sup>+</sup> CD4<sup>+</sup> T cells from CNS1-deficient and -sufficient TEa tg *Foxp3*<sup>GFP</sup> B6 mice were transferred into T-cell-deficient B6 recipients, which were subsequently mated with BALB/c (H-2<sup>d</sup>) or B6 (H-2<sup>b</sup>) male. In this experimental setup TEa cells were able to recognize endogenous Ea52-68:I-A<sup>b</sup> complex presented by antigen-presenting cells (APC) of the embryo (H-2<sup>dx</sup>) or processed by maternal APC only when recipient B6 females were mated with a BALB/c male (Figure 2a).

Induction of Foxp3 in transferred CNS1-sufficient (WT) TEa T cells was observed primarily in the draining lymph node (DLN) and decidua of females mated with BALB/c, but not B6 males. In contrast, no significant induction of Foxp3 was observed upon transfer of CNS1-deficient (KO) TEa T cells (Figure 2b). Although the possibility that under a particular condition extrathymic Treg cells can be generated in the absence of CNS1 cannot be formally excluded, the results presented herein demonstrate that pTreg cells specific for fetal alloantigens are generated during pregnancy in a CNS1-dependent manner.

[0163] pTreg cell paucity results in increased resorption of fetuses

[0164] A role for CNS1 and extrathymic generation of Treg cells in maternal-fetal tolerance and fertility was directly assessed. CNS1-deficient (KO) and -sufficient (WT) B6 females were mated with allogeneic BALB/c males and embryo resorption was assessed on day E13.5-E14.5 (Figure 3a). Increased resorption was observed in CNS1-deficient females compared with wild-type littermate controls (Figure 3b). Incidence of resorption and its rate per pregnant female was also increased (Figures 3c-d). Consistent with an effect that can account for evolutionary pressure for emergence of CNS1-dependent pTreg cell differentiation, nonresorbed fetuses was also significantly decreased in CNS1-deficient females (Figure 4). Females impaired in pTreg cell generation did not show increased resorption of embryos sired by B6 males expressing syngeneic MHC allele (Figure 3e-g). The latter observation indicates that CNS1-deficient females are not generally predisposed to spontaneous abortion, but reject embryos expressing mismatched MHC alleles. CNS1-deficient females were always compared to their WT counterparts in identical breedings with syngeneic or allogeneic males to ensure the genetic make-up of embryos is the same in the two groups compared as different strains of mice can vary in rates of embryo loss due to early fetal death unrelated to immunologic conflicts and since F1 embryos are frequently more robust and survive better.

[0165] To exclude potential effects of congenital pTreg cell deficiency, heterozygous *Foxp3*<sup>CNS1KO/DTR</sup> females containing one CNS1 KO allele and one *Foxp3*<sup>DTR</sup> allele were generated. Due to random X chromosome activation half of the cells in a *Foxp3*<sup>CNS1KO/DTR</sup> mouse express each allele and are either susceptible to DT-mediated ablation (*Foxp3*<sup>DTR</sup>) or unable to induce Foxp3 in the periphery (*Foxp3*<sup>CNS1</sup>). Administration of DT allows for

depletion of all CNS1-dependent pTreg cells while sparing a pool of CNS1-deficient tTreg cells (Figure 5). Treatment with DT before and during pregnancy in these mice resulted in increased resorption compared to control heterozygous *Foxp3*<sup>GFP/DTR</sup> females where DT ablation leaves CNS1-sufficient Foxp3 (Figure 6a). Observed increases were similar to those observed in intact CNS1-deficient females. Thus, acute pTreg ablation during allogeneic pregnancy results in increased embryo resorption comparable to that observed in CNS1-deficient females.

**[0166]** Extrathymically generated Treg cells play a predominant role in maternal-fetal tolerance

**[0167]** Although analysis of allogeneic pregnancy in CNS1-deficient and *Foxp3*<sup>CNS1KO/DTR</sup> females suggested that pTreg cells generated in a CNS1-dependent manner prevent “rejection” of MHC-mismatched fetuses, it was possible that their contribution to overall Treg cell-mediated suppression of maternal-fetal allogeneic conflict was relatively minor. To address this question the effect of essentially complete ablation of Treg cells in pregnant *Foxp3*<sup>DTR</sup> B6 females expressing diphtheria toxin receptor under control of the endogenous *Foxp3* locus was addressed (Kim et al., 2007). Increased resorption of MHC-mismatched embryos was observed in *Foxp3*<sup>DTR</sup> B6 females, compared with control B6 mice, when mated with BALB/c males and treated with diphtheria toxin (DT) at day E5.5 and E7.5 (Figure 6b). In contrast to selective pTreg deficiency, DT-mediated combined ablation of pTreg and tTreg cells also increased resorption of syngeneic embryos most likely due to a loss of restraint of T cell reactivity against self-antigens. Despite widespread immune mediated inflammation and lympho- and myeloproliferative syndrome in pregnant *Foxp3*<sup>DTR</sup> females subjected to “wholesale” Treg ablation, rates of resorption observed in these mice were similar to those in CNS1-deficient females and ablation of pTreg cells. The results presented herein suggest that pTreg cells play a predominant role in maternal-fetal tolerance.

**[0168]** CNS1-deficient females exhibit signs of inflammation and abnormal spiral artery remodeling

**[0169]** Consistent with impaired pTreg induction during allogeneic pregnancy in CNS1-deficient females, markedly reduced Treg cell numbers in the decidua in these mice mated with BALB/c males were observed (Figure 7a). Proliferative activity assessed by Ki67 expression was not increased in Treg cell subsets in the decidua and DLN in CNS1-

deficient mice in comparison to CNS1-sufficient controls (Figure 8). The results presented herein suggest that paucity of pTreg cells is not associated with compensatory expansion of tTreg cells as suggested by Josefowicz et al., 2012. The observed decrease in the Treg cell population inversely correlated with the increased presence of activated effector CD62L<sup>lo</sup>CD4<sup>+</sup> T cells (Figure 7b), however, no significant changes in effector cytokine production was detected in the DLN or decidua (Figure 9). Fluid composition and activation state of immune cells in a rapidly changing placental environment could mask potential differences. Histologic examination of placentas of CNS1-deficient and -sufficient females performed in a blinded fashion showed that the genotype of the dams was accurately predicted by the morphological status of the decidual spiral arteries; although there was some variability between individual placentas in any one uterus, CNS1-deficient placentas exhibited more prominent clusters of thickened blood vessels as compared to CNS1-sufficient littermate controls (Figure 7c). At day E13.5, placentas of surviving embryos of CNS1-deficient females exhibited early necrosis of spiral arteries and edema, while resorptions were characterized by embryo loss with necrotic labyrinths (Figure 6d). Presumptive early resorption sites exhibited necrosis or thrombosis of decidual vessels and edema in placentas and embryos (Figure 10). Consistent with immune-mediated pathology, more prominent T cell presence was noted in CNS1-deficient placentas, where single T cells were scattered within all layers of the placenta with clusters prominent in the decidua near spiral arteries (Figure 7e). In contrast, only rare single T cells were observed in CNS1-sufficient placentas and no major changes in numbers of B cells and macrophages were detected (Figure 10). The results presented herein are indicative of an inflammatory pathology associated with allogeneic pregnancy in CNS1-deficient females resembling that of human pregnancy-associated disorders such as preeclampsia (Redman, C.W. et al., "Latest advances in understanding preeclampsia." *Science* 308, 1592–1594, 2005; Renaud, S.J. et al., "Spontaneous pregnancy loss mediated by abnormal maternal inflammation in rats is linked to deficient uteroplacental perfusion." *J Immunol* 186, 1799–1808, 2011).

### *Discussion*

**[0170]** Over the last decade numerous studies have led to the realization that suppressive function of Treg cells extends far beyond autoimmunity, the originally suggested sphere of their activity. Treg cells have been implicated in control of acute and chronic infections, tissue homeostasis at barrier sites populated by commensal microbiota, allergy, injury response and tissue repair, metabolic syndrome, and cancer (Josefowicz et al.,

2012). The results presented herein suggest that the *Foxp3* intronic enhancer CNS1, essential for extrathymic differentiation of Treg cells, is present only in eutherian mammals, but not in marsupials or monotremes and that pTreg cell paucity in CNS1-deficient females mated to MHC mismatched males results in increased spontaneous abortion of embryos. The histological features observed in pTreg cell-deficient females described in the results presented herein are similar to what is reported in preeclampsia patients, suggesting that there may also be a direct link between preeclampsia and impaired pTreg generation or function (Avagliano, L., et al., “Abnormal spiral artery remodelling in the decidual segment during pregnancy: from histology to clinical correlation” *J Clin Pathol* 64:1064-1068, 2011; Redman, C.W., et al., “Latest Advances in Understanding Preeclampsia”, *Science* 308, 1592, 2005).

**[0171]** An implication of these observations is that generation of Treg cells in the thymus does not afford adequate protection of the fetus expressing allogeneic MHC alleles from immune mediated attack by maternal T cells. This latter notion is also supported by the comparable extent of embryo resorption associated with acute depletion of pTreg and pan-Treg ablation, i.e. elimination of both pTreg and tTreg cells. This result suggests that extrathymically generated Treg cells serve as a predominant subset mitigating maternal-fetal allogeneic conflict, suggesting that once in place, extrathymic generation of Treg cells, primarily driven by pressure to enforce maternal fetal tolerance, likely assumed additional functions including control of responses to non-self antigens leading to allergy and asthma and to commensal organisms in the gut (Lathrop, S.K. et al. “Peripheral education of the immune system by colonic commensal microbiota.” *Nature* 478, 250–254, 2011; Josefowicz et al., 2012).

**[0172]** The results presented herein demonstrate that, although pTreg cell deficiency in CNS1-deficient mice resulted in significantly increased resorption of fetuses during allogeneic pregnancy, its penetrance was incomplete, suggesting that pTreg cell mediated suppression enforces maternal-fetal tolerance not single-handedly, but jointly with other numerous immunomodulatory mechanisms. This result was likely due to multiple mechanisms that operate during pregnancy to limit encounter of maternal alloreactive T cells with, or their response to, fetal alloantigens. These mechanisms include, but are not limited to, inactivation of immune cells by tryptophan deprivation by indoleamine 2,3-dioxygenases (Munn, D.H. et al, “Prevention of allogeneic fetal rejection by tryptophan catabolism.” *Science* 281, 1191–1193, 1998), Fas-Fas ligand mediated apoptosis of

activated alloreactive T cells (Hunt, J.S. et al., “Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus.” *J Immunol* 158, 4122–4128, 1997), expression of immunosuppressive mediators such as TGF- $\beta$  and galectin-1 (Simpson, H. et al., “Transforming growth factor beta expression in human placenta and placental bed during early pregnancy.” *Placenta* 23, 44–58, 2002; Blois, S.M. et al., “A pivotal role for galectin-1 in fetomaternal tolerance.” *Nat Med* 13, 1450–1457, 2007), entrapment of endometrial dendritic cells (Collins, M.K. et al., “Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice.” *J Clin Invest* 119, 2062–2073, 2009), limited expression of MHC molecules on trophoblasts (Erlebacher, A. et al., “Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus.” *J Clin Invest* 117, 1399–1411, 2007), and increased expression of inhibitory B7 family members (PD-L1, B7H3, B7H4) (reviewed in Petroff, M.G. et al., “B7 family molecules as regulators of the maternal immune system in pregnancy.” *Am. J. Reprod. Immunol.* 63, 506–519, 2010).

**[0173]** Additional factors, which may influence degree of immune mediated resorption associated with pTreg cell or pan-Treg cell deficiency include genetic background, microbial status, and stress exposure. It is likely that in the absence of pTreg cells, infection may result in a more severe pregnancy disruption. It must be also noted that the three week-long gestation period in mice is relatively short; extrathymic generation of Treg cells may play a more pronounced role in maternal-fetal tolerance in mammals with longer gestation times where there would be higher probability of the encounter of alloreactive T cells of the mother with paternally encoded alloantigens and for the immune response to develop.

**[0174]** The aforementioned possible influences affecting severity of pregnancy disruption and differences in experimental design might account for a varying degree of embryo resorption observed in the results presented herein and in previous reports employing adoptive T cell transfers and CD25 antibody mediated Treg cell depletion in lymphopenic or lymphoreplete mice (Aluvihare et al., 2004; Shima et al., 2010). However, pervasive fetal death observed previously upon continuous DT-mediated ablation of Treg cells starting at mid-gestation daily which was likely due to secondary effects of poor health condition of the mother has not been observed in the results presented herein (Rowe, J.H. et al., “Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens.” *Cell Host Microbe* 10, 54–64, 2011).

**[0175]** The results presented herein demonstrate a key role of extrathymic generation of Treg cells in maternal fetal tolerance that substantially adds to previous studies demonstrating general importance of Treg cells in control of maternal immune responses to the allogeneic fetus in mice. In humans, Treg cells are present in increased numbers during pregnancy in the blood and the decidua (Heikkinen, J. et al, "Phenotypic characterization of regulatory T cells in the human decidua." Clin. Exp. Immunol. 136, 373–378, 2004; Somerset et al., 2004). Decreases in Treg cells have been associated with frequent human pregnancy disorders including preeclampsia and repeated spontaneous abortions (Arruvito, L. et al., "IL-6 transsignaling and the frequency of CD4+FOXP3+ cells in women with reproductive failure." J. Reprod. Immunol. 82, 158–165, 2009; Darmochwal-Kolarz, D. et al., "The predominance of Th17 lymphocytes and decreased number and function of Treg cells in preeclampsia." J. Reprod. Immunol. 93, 75–81, 2012). Histological features of allogeneic pregnancy in pTreg cell-deficient females were redolent of abnormal spiral artery remodeling associated with pre-eclampsia and other complications of pregnancy in humans and accompanying increased local inflammation (Redman and Sargent, 2005; Avagliano, L. et al., "Abnormal spiral artery remodelling in the decidual segment during pregnancy: from histology to clinical correlation." J. Clin. Pathol. 64, 1064–1068, 2011; Renaud et al., 2011). These observations raise an intriguing possibility of a link between these conditions and impaired pTreg generation or function. The results presented herein suggest that the reduced Treg accumulation and resulting pathology may be partially due to defective peripheral induction of Treg cells to paternal antigens and that potential therapies could be developed to address this defect.

**[0176]** The analysis of CNS1 sequence conservation suggests that this enhancer was gained during evolution of eutherian mammals. CNS1 contains binding sites for transcription factors downstream of three major signaling pathway required for pTreg generation and its deletion results in a selective impairment of this differentiation process (Zheng et al., 2010). Thus, introduction of this several hundred base pair-long DNA sequence into the *Foxp3* locus could have been sufficient to enable differentiation of pTreg cells. This line of reasoning implies that increased interaction between mother and fetus during gestation necessitated a mechanism of acquired active tolerance afforded by peripheral generation of regulatory T cells. Consistent with this idea, the data presented herein demonstrates diminished litter size observed in allogeneic pregnancy in CNS1-

deficient vs. –sufficient females, suggested that pTreg generation afforded a reproductive advantage.

[0177] The process of insertion of CNS1 sequence into the first intron of the *Foxp3* locus appears to have occurred via a MIR family retrotransposon activity during the Mesozoic era at a time overlapping with evolution of placental mammals. The results presented herein suggest that in the mouse genome MIR elements resembling CNS1 are enriched for SMAD and RXR binding sites suggesting that these elements may have endowed TGF- $\beta$  and retinoic acid response capacity to *Foxp3* and other genes in agreement with the idea of exaptation where portions of transposable elements acquire a function that serves their host (Brosius, J. et al., “On “genomenclature”: a comprehensive (and respectful) taxonomy for pseudogenes and other “junk DNA”.” *Proc Natl Acad Sci U S A* 89, 10706–10710, 1992). These elements can then confer novel signaling pathway responsiveness to existing genes, thereby augmenting their function. A mechanism of retrotransposon-mediated exaptation affecting structure or regulation of pre-existing genes upon introduction of novel exons or enhancers has been previously reported (Bejerano, G. et al. “A distal enhancer and an ultraconserved exon are derived from a novel retroposon.” *Nature* 441, 87–90, 2006; Mikkelsen, T.S. et al., “Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences.” *Nature* 447, 167–177, 2007). The results presented herein suggest that acquisition of CNS1 supporting extrathymic Treg cell generation in eutherian mammals represents a novel example of retrotransposition-mediated innovation in regulation of gene expression where a distinct biological purpose and novel functionality associated with the CNS1 enhancer is implicit of the potential evolutionary pressure underlying its conservation.

[0178] It is noteworthy in this regard that emergence of chorioallantoic placenta, which allowed for viviparity in therian mammals, was assisted by appropriation of several retroviral genes including retrotransposon-derived *Peg10* and *Peg11/Rtl1* and syncytin-A and –B originating from the envelope protein of a defective retrovirus. These genes are essential for normal function of placenta and trophoblast fusion, respectively (Mi, S. et al., “Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis.” *Nature* 403, 785–789, 2000; Ono, R. et al., “Deletion of *Peg10*, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality.” *Nat Genet* 38, 101–106, 2006; Sekita, Y. et al., “Role of retrotransposon-derived imprinted gene, *Rtl1*, in the feto-maternal interface of mouse placenta.” *Nat Genet* 40, 243–248, 2008; Dupressoir, A. et al., “A pair of co-opted

retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast.” Proc Natl Acad Sci U S A 108, E1164–73, 2011). Taken together, these results and the results presented herein suggest that, in addition to facilitating placentation during mammalian evolution, retrotransposon-mediated innovation helped to alleviate immune conflict associated with this acquisition.

**[0179]** The results presented herein suggest that the mechanism of extrathymic differentiation of Treg cells may have been gained during evolution to reinforce tolerance to paternal alloantigens presented by the fetus during the increasingly long gestation period in placental mammals. This adaptation was realized with the aid of the *Foxp3* CNS1 enhancer responsible for induction of Foxp3 in peripheral CD4<sup>+</sup> Foxp3<sup>+</sup> T cells, which likely emerged upon capture of a MIR retrotransposon containing TGF- $\beta$  and retinoic acid receptor response elements. A role of extrathymically generated Treg cells in maternal-fetal tolerance may provide an important insight into potential clinical complications of human pregnancies.

### **Equivalents**

**[0180]** Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the following claims:

### Claims

What is claimed is:

1. Methods of identifying an elevated risk for pregnancy complications comprising:  
providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized;  
processing the sample to determine a CNS1 region sequence; and  
classifying the woman as having an elevated risk of pregnancy complications if the determined sequence includes one or more alterations relative to a reference sequence.
2. Methods of identifying an elevated risk for pregnancy complications comprising:  
providing a sample from a woman whose risk of pregnancy complications is to be identified or characterized;  
processing the sample to determine a level of pTreg cells; and  
classifying the woman as having an elevated risk of pregnancy complications if the determined level is lower than a reference low threshold or higher than a reference high threshold.
3. Methods of reducing risk for pregnancy complications comprising a step of administering to a subject a composition comprising pTregs.
4. The method of claim 3, wherein the subject is a pregnant female.
5. The method of claims 3-4, wherein the pTregs are allogeneic.
6. The method of claims 3-4, wherein the pTregs are autologous.
7. The method of claims 4-6, wherein the pTregs are paternal cells.
8. The method of claims 3-7 wherein the pTregs are generated and/or expanded ex-vivo.

9. The method of claims 4-8 wherein the pTregs are generated and/or expanded ex-vivo in the presence of a paternal alloantigen.
10. The method of claims 3-9 wherein the subject has a reduced amount and/or type of pTreg cells relative to a reference.
11. The method of claims 3-10 further comprising detecting an amount and/or type of pTreg cells.
12. A population of maternal pTregs from a pregnant female, wherein at least 25%% of the pTregs in the population recognize paternal alloantigens.
13. Methods of identifying agents that promote pTreg generation comprising:  
determining an activity level of CNS1 exposed to an agent; and  
identifying the agent as promoting pTreg generation if the CNS1 activity level is elevated relative to a CNS1 activity level in an untreated control.
14. Methods of reducing the risk for pregnancy complications comprising administering to a subject one or more agents that promote pTreg generation.
15. The method of claim 14 further comprising administering to a subject a composition comprising pTregs.
16. The method of claims 1-11, 14, or 15 wherein pregnancy complications are selected from miscarriage and preeclampsia.

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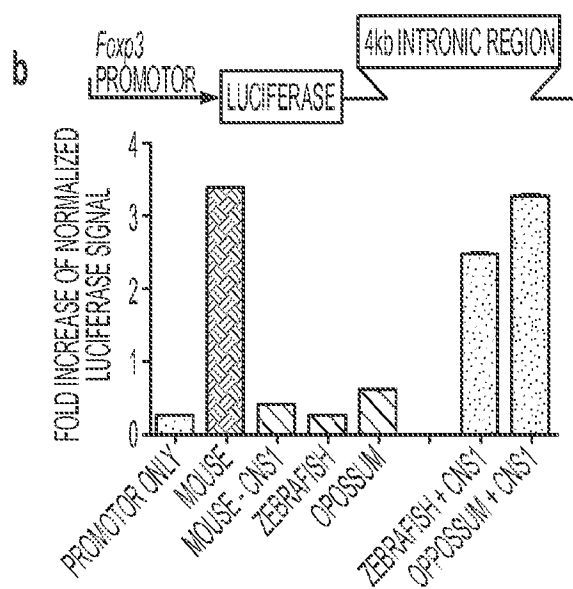
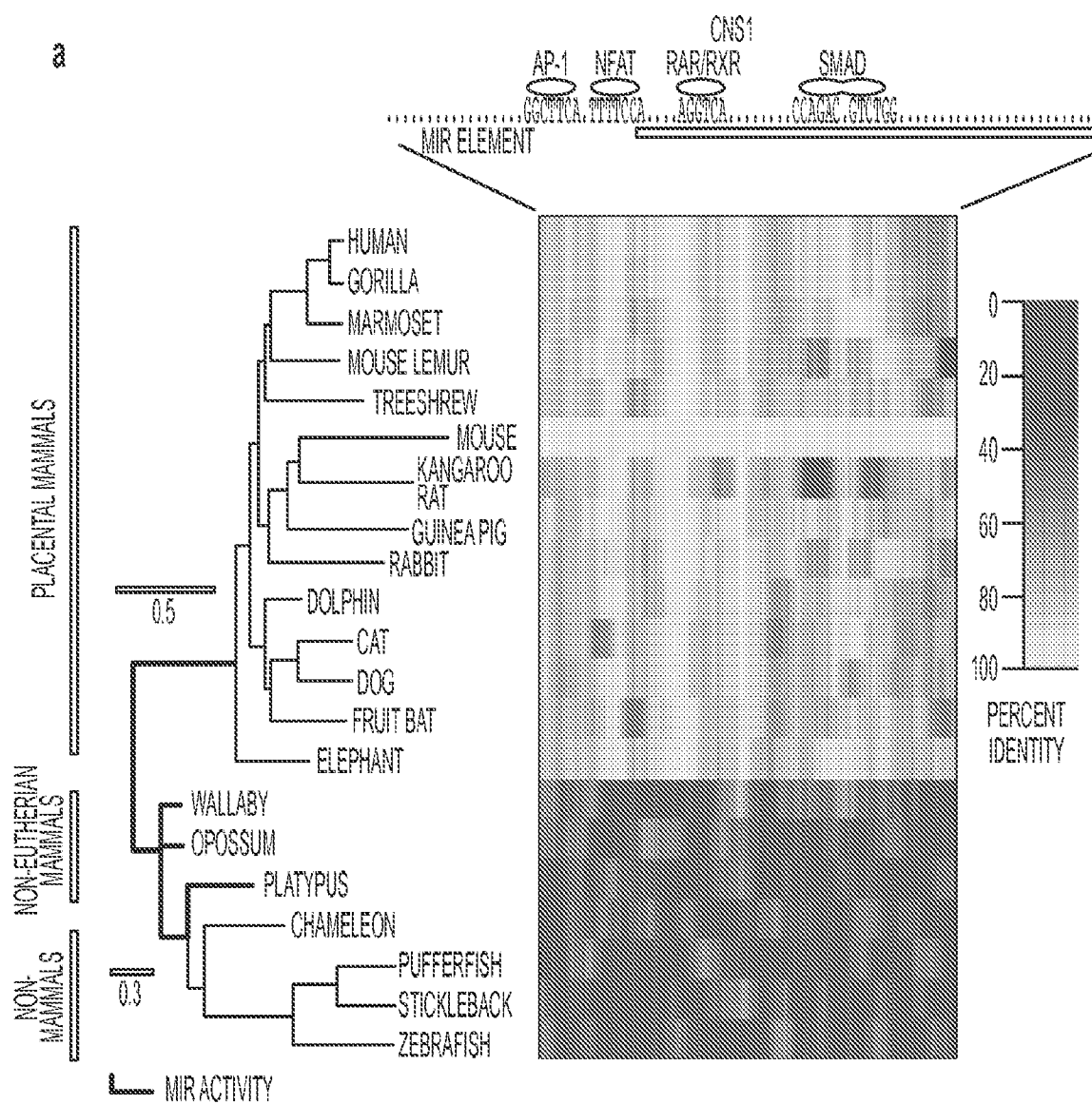


FIG. 1

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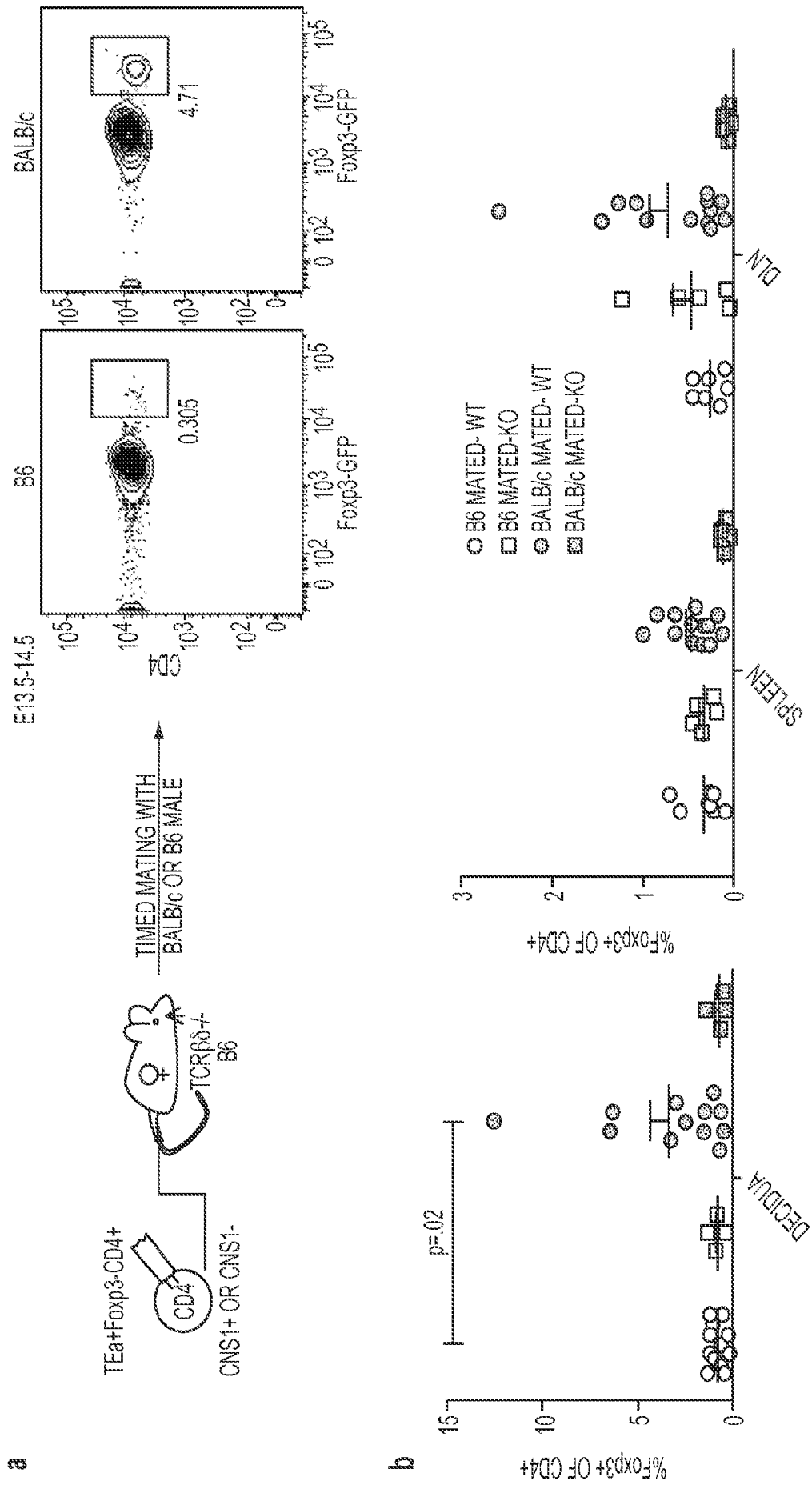


FIG. 2

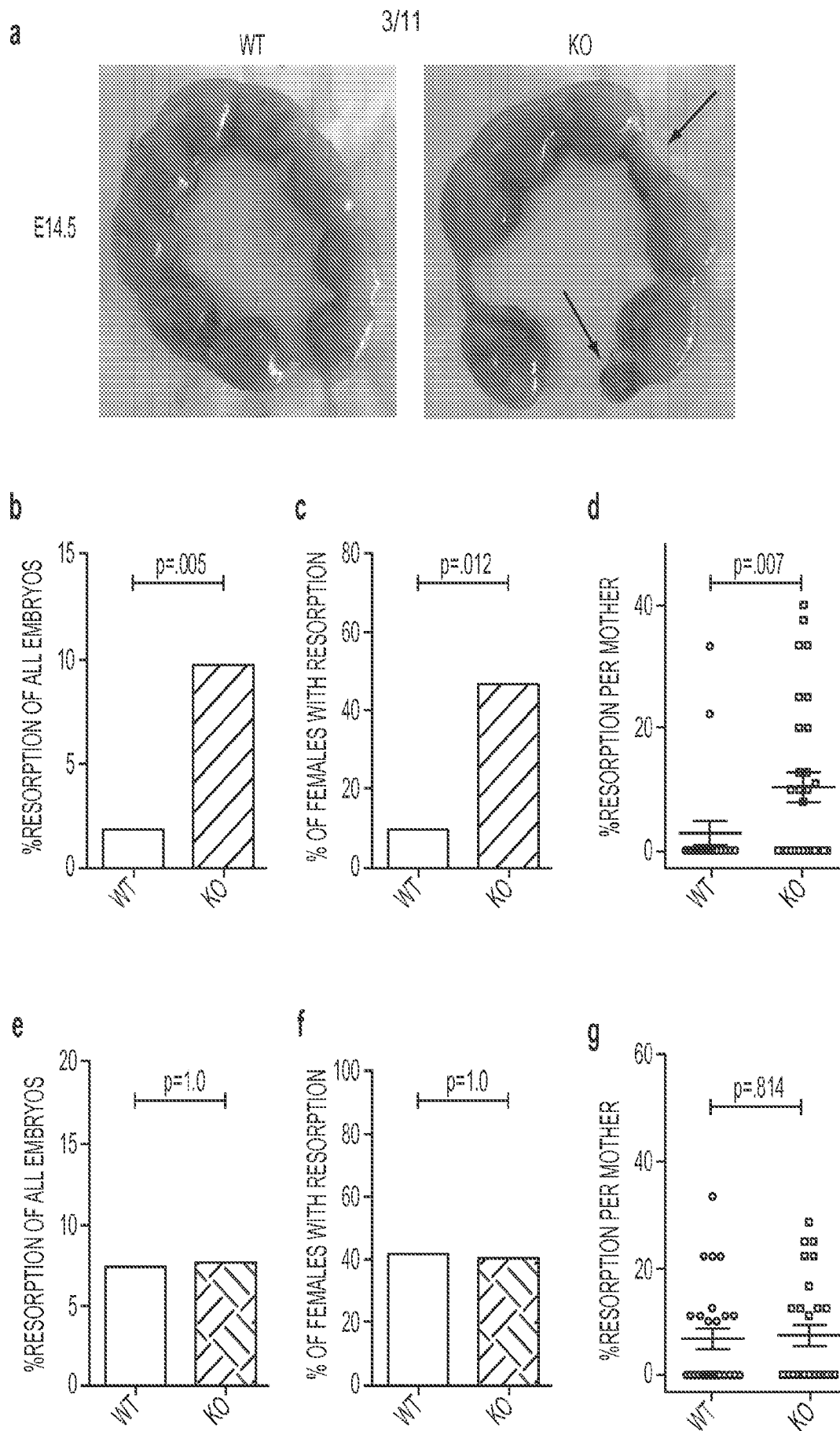


FIG. 3

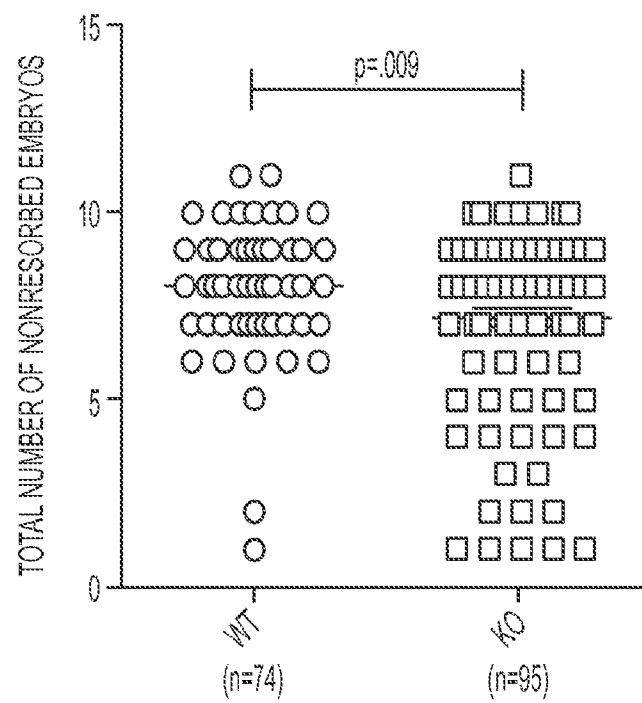


FIG. 4

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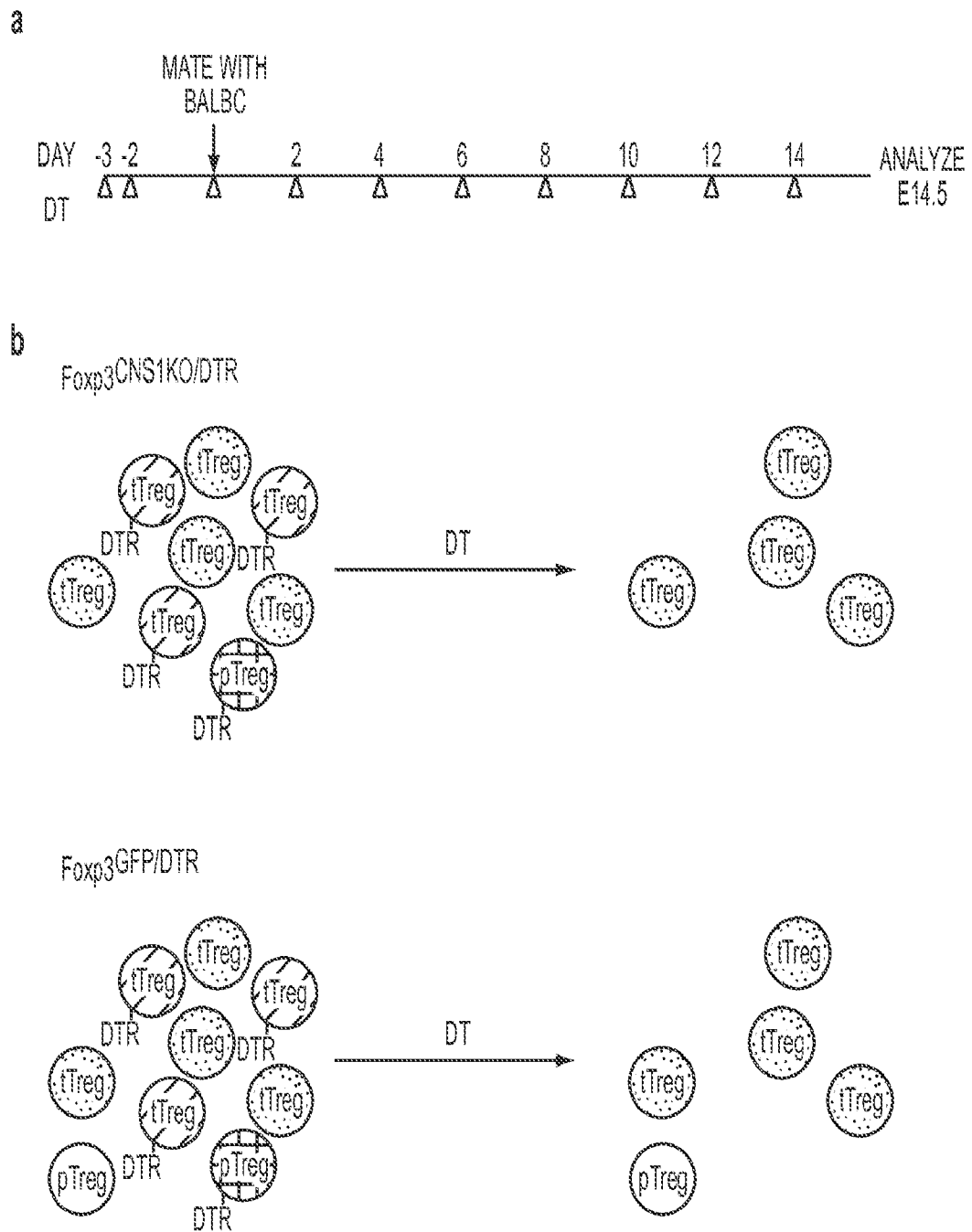


FIG. 5

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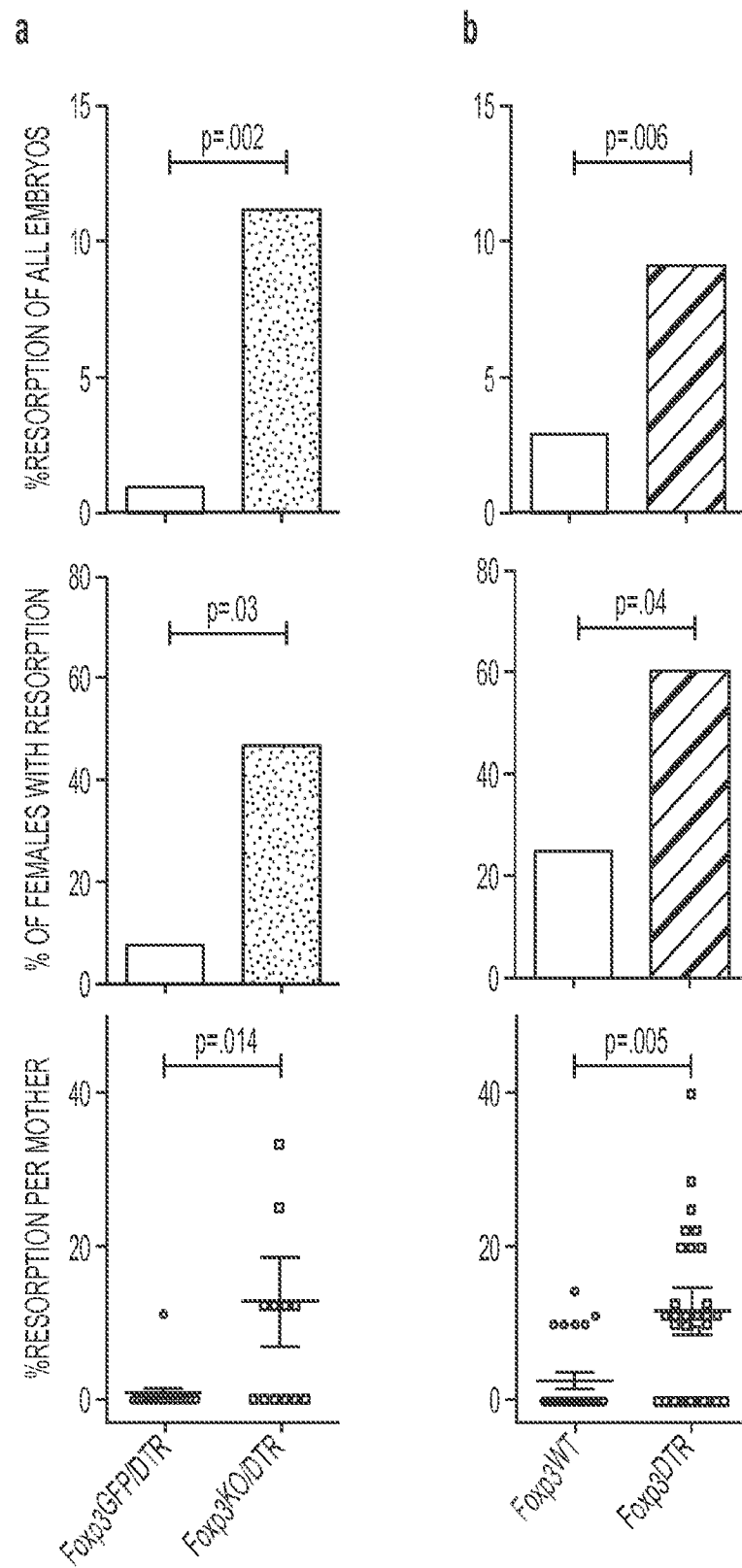


FIG. 6

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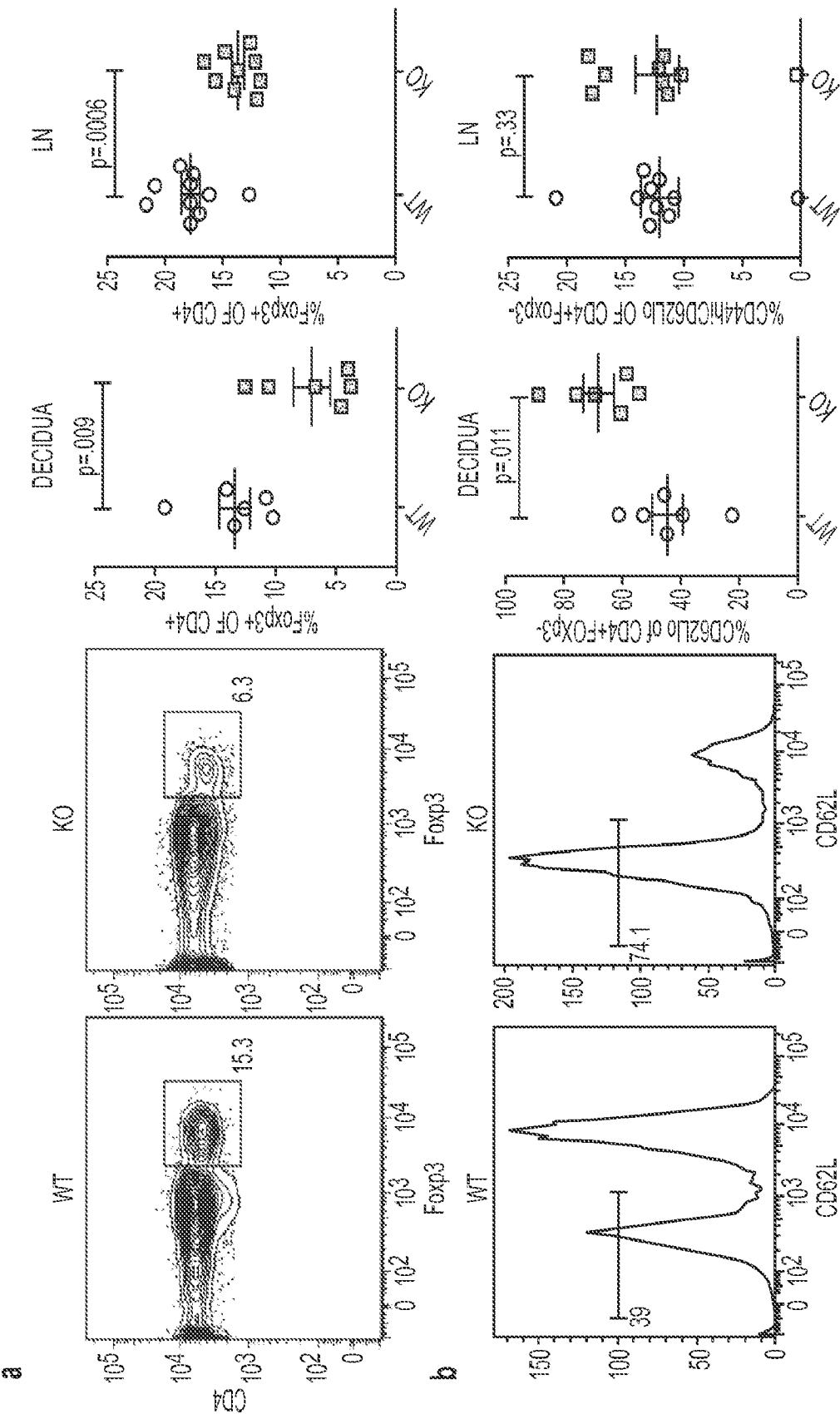


FIG. 7

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FIG. 7  
CONTINUED

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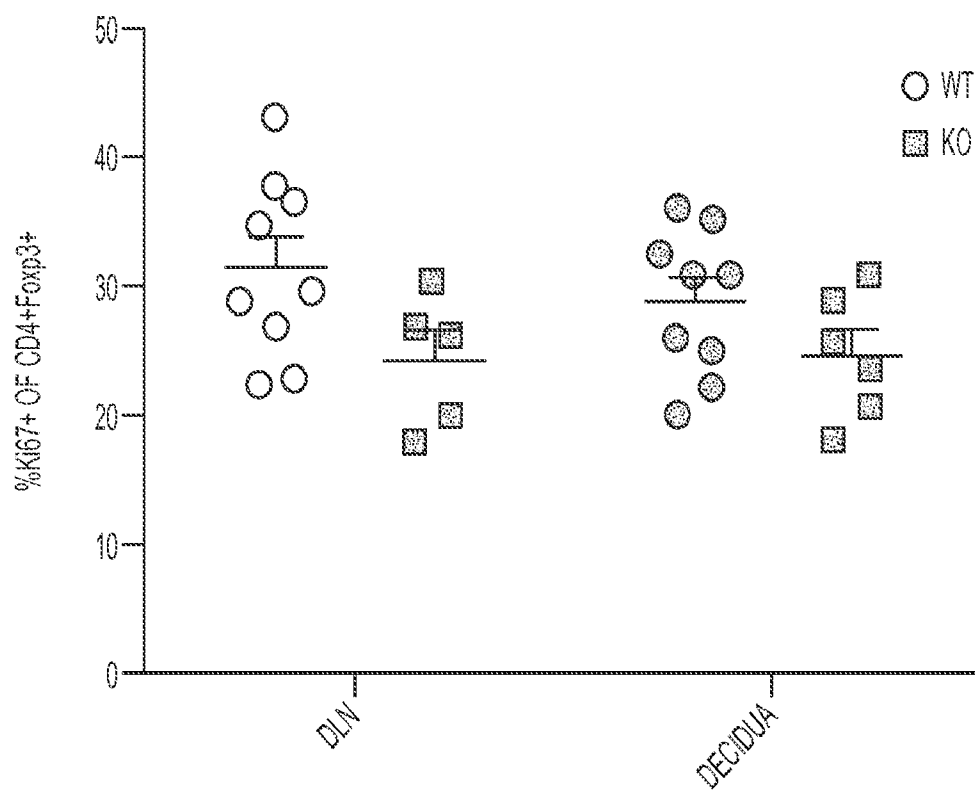


FIG. 8

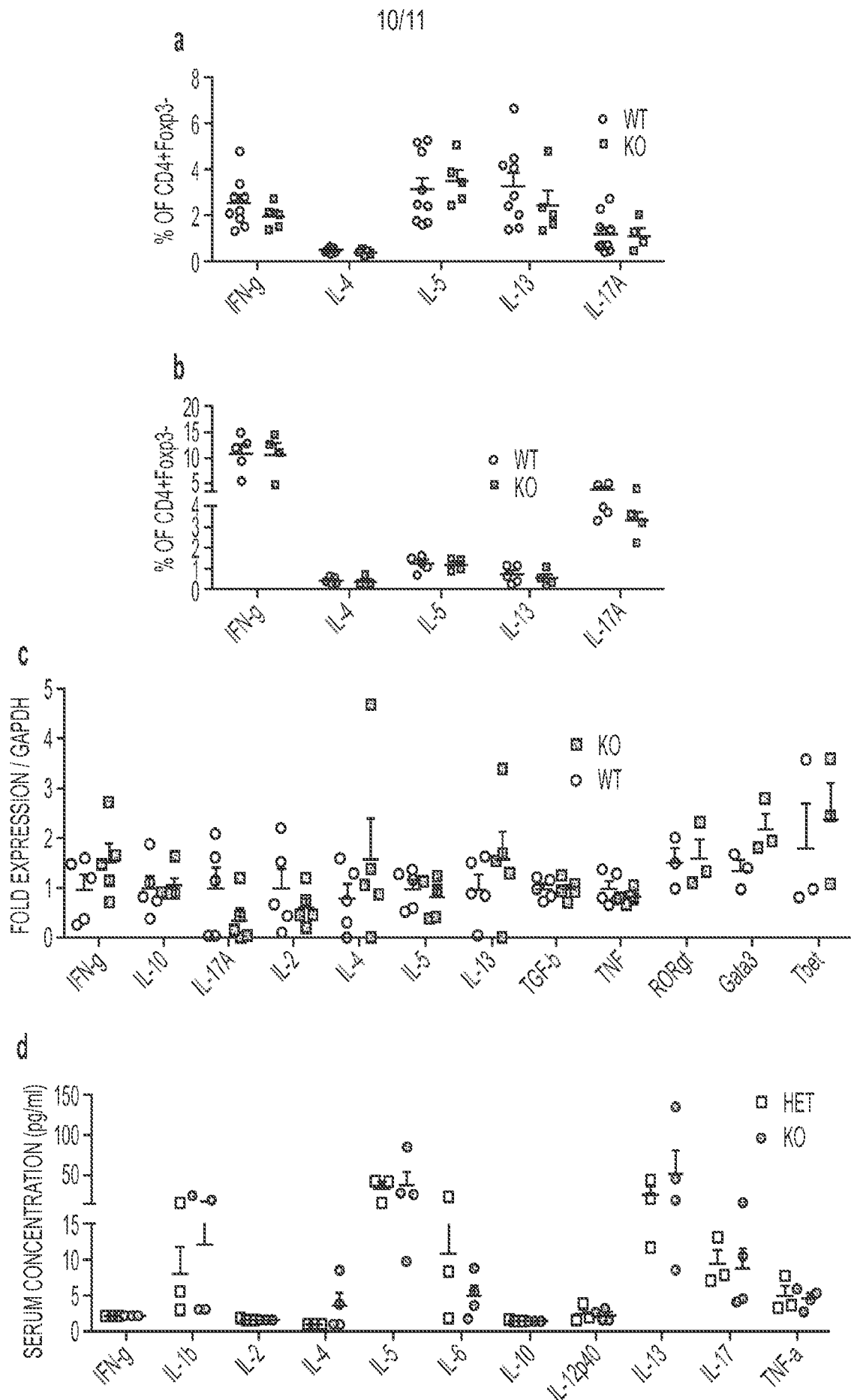


FIG. 9

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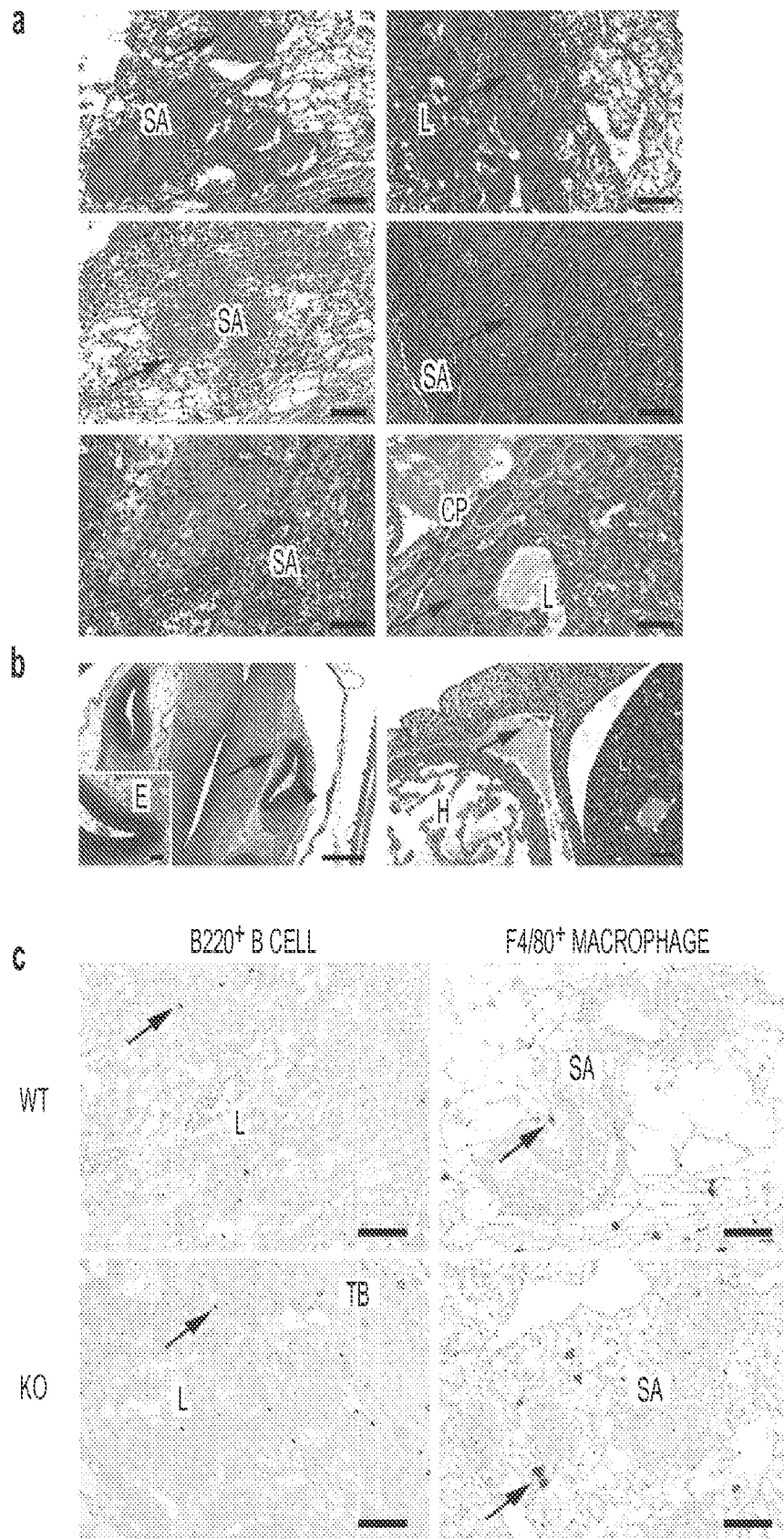


FIG. 10

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US2013/047896

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/56 (2013.01)

USPC - 424/93.71

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) - A01N 63/00; A61K 35/56, 45/00; C12N 1/68 (2013.01)

USPC - 424/93.1, 93.7, 93.71, 184.1; 435/6.1

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

CPC - A01N 63/00; A61K 35/56, 45/00; C12N 1/68 (2013.01)

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

PatBase, Google Patents, Google, PubMed

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                   | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | US 2012/0076805 A1 (SHARPE et al) 29 March 2012 (29.03.2012) entire document                                                                                                                         | 3, 4                  |
| X         |                                                                                                                                                                                                      | 14, 15                |
| --        |                                                                                                                                                                                                      | -----                 |
| Y         | US 2009/0297539 A1 (KOSHIBA et al) 03 December 2009 (03.12.2009) entire document                                                                                                                     | 12, 13                |
| Y         | US 2012/0107825 A1 (WINGER et al) 03 May 2012 (03.05.2012) entire document                                                                                                                           | 1, 2                  |
| Y         | ZHENG et al., "Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate," Nature, Vol. 463, No. 7282, Pgs. 808-812. 11 February 2010 (11.02.2010) entire document       | 1, 2, 13.             |
| Y         | TAFURI et al., "T Cell Awareness of Paternal Alloantigens During Pregnancy," Science, Vol. 270, Pgs. 630-633, 27 October 1995 (27.10.1995) entire document                                           | 12                    |
| P, X      | SAMSTEIN et al., "Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict," Cell, Vol. 150, No. 1, Pgs. 29-38, 06 July 2012 (06.07.2012) entire document | 1-4, 12-15            |

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


## \* Special categories of cited documents:

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

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

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

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

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

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

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

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

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

Date of the actual completion of the international search

04 November 2013

Date of mailing of the international search report

22 NOV 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

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PCT Helpdesk: 571-272-4300  
PCT OSP: 571-272-7774

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/047896

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☒ Claims Nos.: 5-11, 16  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

### Remark on Protest

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