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(54) Title: TREATMENT OF ORGAN TRANSPLANT PATIENTS

(57) Abstract: Methods for treating an organ transplant recipient receiving an immunosuppressive therapy by administering CD4+/
CD25+ T-reg cells.

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Treatment of Organ Transplant Patients

FIELD

[0001] The disclosure relates generally to the treatment of organ transplant patients with regulatory T cells to ameliorate or prevent the negative consequences of the transplant.

BACKGROUND

[0002] Transplantation is the treatment of choice for most causes of end stage renal disease. However, without some modification of the recipient's immune system, all donor organs succumb to rejection after transplant to a host patient. To prevent this, patients must take immunosuppressive drugs for life, generally a combination of steroids, a calcineurin inhibitor (CNI), such as cyclosporine or tacrolimus, and an antiproliferative agent (azathioprine, mycophenolate mofetil or sirolimus). Induction with a brief course of an anti-T lymphocyte antibody preparation (daclizumab, basiliximab, muromonab, alemtuzumab, polyclonal anti-thymocyte globulin) is also used in approximately 70% of U.S. transplant centers.

[0003] Dependence on immunosuppression tempers the substantial benefit obtained from transplantation. The typical regimens are relatively complex, expensive, and they increase the risk of opportunistic infection and malignancy and have many non-immune side effects that hamper their tolerability. Specifically, CNIs are nephrotoxic, a side effect of significant concern in renal transplantation. Steroids exacerbate osteoporosis and hyperlipidemia and cause avascular osteonecrosis. Both classes of agents worsen glucose tolerance and hypertension and are associated with cosmetic effects causing non-compliance. As such, methods of transplantation that lessen the dependence on chronic immunosuppression stand to reduce the risk and expense of transplantation. They must, however, also prevent rejection. Accordingly, there is a need for alternate therapies that help to minimize the need for lifelong immunosuppression or eliminate entirely the need for drugs through the induction of tolerance, are therefore of great interest.

[0004] Regulatory CD4⁺CD25⁺ T cells (T-regs) derived from the thymus and/or peripheral tissues have been demonstrated to broadly control T cell reactivity. T-regs have been shown to control immune responsiveness to alloantigens and significantly contribute to operational tolerance in transplantation models. However, there have been limited efforts to

harness the therapeutic potential of directly isolated CD4+CD25+ T-reg cells for controlling graft rejection and inducing transplantation tolerance, such as for kidney transplants.

SUMMARY

[0005] In one aspect, the disclosure is directed to a method of treating an organ transplant recipient receiving an immunosuppressive therapy that includes at least two immunosuppressants. The method includes administering to the recipient between about 500,000,000 and 5,000,000,000 CD4+/CD25+ T-reg cells and decreasing the dosage amount of one or both of the immunosuppressants. The immunosuppressants may be selected from cyclosporine, tacrolimus, everolimus, azathioprine, mycophenolate mofetil, sirolimus, alemtuzumab, azathioprine, belatacept, and corticosteroids.

[0006] In various aspects of the disclosure, T-reg cells may be administered on about day 50 to about day 70 following an organ transplant, the decreasing the dosage may begin on about day 80 to about day 120 following the organ transplant, and immunosuppressant dosage amount may be incrementally decreased over the course of three to 15 months following the transplant. The dose of one of the immunosuppressants may be reduced by at least 25%, at least 50%, at least 75%, at least 90%, or may be completely eliminated. In addition, the dosage of both of the immunosuppressants may be decreased.

[0007] In further aspects of the disclosure, the T-reg cells may be selected and expanded from a frozen apheresis product.

[0008] Still further, aspects of the disclosure include methods for reducing, stopping, and/or preventing a cellular immune response that causes cellular, organ, or tissue rejection in the recipient. In addition, the method may include monitoring a patient for adverse events and stopping the decreasing the dosage amount one or both immunosuppressants, which may include returning the patient to full immune suppressant therapy.

DESCRIPTION

[0009] All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

[0010] Before describing the present invention in detail, a number of terms will be defined. As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. For example, reference to a “nucleic acid” means one or more nucleic acids.

[0011] It is noted that terms like “commonly,” and “typically” are not utilized herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that can or cannot be utilized in a particular embodiment of the present invention.

[0012] As used herein, the term “about” refers to $\pm 10\%$ of any particular value unless otherwise noted.

[0013] As used herein, the terms “or” and “and/or” are utilized to describe multiple components in combination or exclusive of one another. For example, “x, y, and/or z” can refer to “x” alone, “y” alone, “z” alone, “x, y, and z,” “(x and y) or z,” “x or (y and z),” or “x or y or z.”

[0014] As used herein, the term “viability” when used to describe a cell population, refers to the percentage of viable cells within the population.

[0015] The term "autoimmune disease" as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include, but are not limited to, Addison's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease,

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

[0016] In one aspect, the disclosure is directed to the use of a T-reg cellular product to provide donor specific immune regulation and control in an organ transplant recipient. This aspect of the disclosure includes a method of treating an organ transplant recipient that is receiving an immunosuppressive therapy of at least two immunosuppressants. The method includes administering to the recipient between about 500,000,000 and 5,000,000,000 CD4+/CD25+ T-reg cells followed by a period of weaning the patient, either partially or completely, from one of the immunosuppressants.

[0017] A T-reg cellular product according to the disclosure includes peripheral blood mononuclear cells (PBMCs) collected from eligible subjects by apheresis that are selected and expanded prior to infusion into a subject. The cells may be obtained approximately one year to about one week prior to the transplant. Briefly, a T-reg cellular product for selection and expansion can be collected and manufactured from a patient that undergoes one or more courses of leukapheresis (20 - 25L collection) that should yield at least 1×10^{10} Total Nucleated Cells (TNC). The leukapheresis product may be from a fresh leukapheresis sample or the sample (or multiple samples) may be cryopreserved until used for T-reg cellular expansion. One such product is described in U.S. Patent Publication No. WO2017/132446 and US Provisional Patent Application Serial No. 63/391,209 filed July 21, 2022, which are incorporated herein by reference in their entirety, which describe the preparation of a T-reg cellular product that includes between about 500,000,000 and 5,000,000,000 CD4+/CD25+ T-reg cells in a solution comprising 5% Human Serum Albumin (HSA) and Plasmalyte A Infusion (Baxter, Inc). Plasmalyte A is a solution of the following substances in water: sodium chloride, potassium chloride, magnesium chloride hexahydrate, sodium acetate trihydrate and sodium gluconate.

[0018] An example release criteria for the cellular product is: > 70% viable; gram stain negative; endotoxin < 5 EU/mL; CD4+/CD25+ > 90%; CD8+ or CD20+ < 5%; CD25+/FOXP3+ > 70%, aerobic, anaerobic and fungal sterility negative, mycoplasma not detected; residual bead

count < 10,000 beads per 10^8 cells (if beads are used); and able to elicit >50% suppression at a 1:2 T-reg:Teffector cell ratio in a mixed lymphocyte reaction. Preparation of an example T-reg cellular product is shown in the Examples below.

[0019] Infusion of T-regs

[0020] In various embodiments of the disclosure, the T-reg cellular product may be infused about 45 to about 75 days post-transplant, for example, about 50 to 70 days post-transplant, about 53 to 67 post-transplant, about 55 to 65 days post-transplant, or about 56, 57, 58, 59, 60, 61, 62, 63, 64, or 65 days post-transplant. A dose range for the T-reg cellular product is 0.5 to 5.0×10^9 T-reg cells, for example about 0.5×10^9 , 0.7×10^9 , 0.9×10^9 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , or 5×10^9 T-reg cells.

[0021] One aspect of the disclosure includes a pre-medication plan to be given approximately prior to infusion (e.g., 15-, 30- or 45-minutes prior), which includes, for example, hydrocortisone (about 100 mg IV), diphenhydramine (about 50 mg PO) and acetaminophen (about 650 mg PO).

[0022] In one aspect, the T-reg cellular product is administered only once as an infusion of approximately 150 mL solution containing 0.5 to 5.0×10^9 T-reg cells. Infusion may include gravity feed through a large bore (18 gauge or larger) peripheral IV at a flow rate of no greater than 10cc/minute. Two rinse backs of the infusion bag may be performed immediately following the infusion using up to approximately 30 mL of normal saline followed by a flush of the access line with approximately 100 mL normal saline.

[0023] Post-transplant Immunosuppression

[0024] Immunosuppressive therapy following organ transplant is well-known. Agents that are commonly used include a calcineurin inhibitor (CNI), such as cyclosporine or tacrolimus, and an antiproliferative agent (azathioprine, mycophenolate mofetil or sirolimus) or low-dose CNI and a mammalian target of rapamycin (mTor) agent such as low dose tacrolimus with everolimus. Other immunosuppressants include alemtuzumab, azathioprine, belatacept, and corticosteroids (e.g., Methylprednisolone). In addition, one or more induction agents, which are intended to prevent acute rejection and typically used only for first thirty days after transplant.

Induction agents include daclizumab, basiliximab, muromonab, alemtuzumab, polyclonal anti-thymocyte globulin, ethylprednisolone, Atgam, Thymoglobulin, or OKT3.

[0025] One example of a post-transplant immunosuppressant regimen according to the disclosure includes THYMOGLOBIN[®] (anti-thymocyte globulin [rabbit]) (Genzyme Corporation) as an induction agent. THYMOGLOBIN is available in single-use 10 mL vials containing 25 mg of anti-thymocyte globulin (rabbit) lyophilized, sterile powder. An example dose of thymoglobulin for induction is 1.5 mg/kg of body weight administered intravenously once per day for five days so that a total dose of 7.5 mg/kg may be given. The first dose must be given prior to reperfusion of the donor kidney on Day 0 and the final dose is given on Day +4 post-transplant.

[0026] An immunosuppressant regimen may also include everolimus (40-O-(2-hydroxyethyl) derivative of sirolimus) (Novartis, under the trade names ZORTRESS[®] in the USA). Zortress is an oral medication and available as 0.25 mg, 0.5 mg, 0.75 mg, and 1 mg tablets. A starting dose of everolimus is 1.5 mg BID. Beginning on Day -2, subjects may receive oral daily dosing targeted to trough concentration of 3-8 ng/mL.

[0027] The immunosuppressant regimen may also include tacrolimus (FK506), which is a macrolide immunosuppressant produced by *Streptomyces tsukubaensis* and marketed with the tradename PROGRAF[®] (Astellas Pharmaceuticals). Tacrolimus is an oral medication and is available in 0.5 mg, 1.0 mg, and 5 mg capsules (also available as a liquid) and has an extended-release formulation. A starting dose of tacrolimus may be 1-2 mg BID. Beginning on Day -2, a patient may receive oral daily dosing targeted to trough concentration of 4-6 ng/mL.

[0028] The therapy may include the following prophylaxis regimens and other appropriate therapy and/or regimens that are allowed at the discretion of a treating physician.

[0029] Antiviral prophylaxis may include (a) oral valganciclovir (e.g., for 3-6 months in the following CMV serostatus combination: D+R-, D-R+, D+R+); and/or (b) CMV serostatus combination D-R-: Oral prophylaxis with valganciclovir. Antibacterial prophylaxis may include Cefazolin[®] (e.g., 2gm q 8hr x 3 doses). Anti-pneumocystis prophylaxis may include

Trimethoprim/sulfamethoxazole (e.g., single strength daily for 12 months) or patients with sulfa allergies may receive oral atovaquone or alternative therapy.

[0030] Weaning of a patient from an immunosuppressant following administration of T-regs includes decreasing the dosage amount of at least one immunosuppressant incrementally over the course of three to 15 months, for example over three to 12 months, three to 9 months, three to six months or over three months. The dose of at least one immunosuppressant therapy may be decreased partially or completely. For example, the dose may be reduced by at least 25%, at least 50%, at least 75%, at least 90%, or 100%. Weaning may begin at about day 80 to about day 120 following the organ transplant, for example about 80, 90, 100, 110 or 120 days post-transplant, or about one week to about 7 weeks days post T-reg infusion, for example about 1, 2, 3, 4, 5, 6, or 7 weeks post infusion. Weaning may begin by an initial reduction in a dose of about 5%, about 10%, or about 25% per week or per month, as may be determined by a clinician until a target dose is achieved or the dose of at least one immunosuppressant has been eliminated. Weaning may be continued as tolerated unless an immunologic event occurs that requires resuming a multi-immunosuppression regimen. For some patients, the dose of at least one immunosuppressant may be eliminated completely. One of more immunosuppressant may be subject to weaning.

[0031] An example of protocol for weaning a post-transplant, post-T-reg infusion patient on an immunosuppressant therapy of everolimus and tacrolimus is shown in Table 1.

Table 1

	Tacrolimus + Everolimus (no weaning)	T-reg + Everolimus + Tacrolimus (weaning of Tacrolimus)	T-reg + Everolimus + Tacrolimus (weaning of Everolimus)
Thymoglobulin			
Dose	1.5 mg/kg per day x 5 days Total dose of 7.5 mg/kg	1.5 mg/kg per day x 5 days Total dose of 7.5 mg/kg	1.5 mg/kg per day x 5 days Total dose of 7.5 mg/kg
Start	Day 0	Day 0	Day 0
End	Day +4	Day +4	Day +4

Everolimus (mTOR)			
Dose	Target dose to 3-8 ng/ml Starting dose 1.5 mg BID No weaning	Target dose to 3-8 ng/ml Starting dose 1.5 mg BID No weaning	Target dose to 3-8 ng/ml Starting dose 1.5 mg BID Weaning begins after 3 Month normal biopsy/no de novo DSA
Start	Day -2	Day -2	Day -2
Tacrolimus (CNI)			
Dose	Target dose to 4-6 ng/ml Starting dose 1-2 mg BID No weaning	Target dose to 4-6 ng/ml Starting dose 1-2 mg BID Weaning begins after 3 Month normal biopsy/no de novo DSA	Target dose to 4-6 ng/ml Starting dose 1-2 mg BID No weaning
Start	Day -2	Day -2	Day -2

[0032] In this example, a patient may begin weaning of either everolimus or tacrolimus at month three post-transplant if the month 3 biopsy is normal and no de novo donor specific antibodies (DSA) are identified. Weaning of everolimus or tacrolimus may be completed by 12 months post-transplant if subjects do not develop evidence of allograft dysfunction. The dose may be reduced by at least 50% every 3 months beginning at month 3 post-transplant and fully withdrawn by 12 months post-transplant (e.g., a daily dose of 3.0 mg everolimus taken at month 3 post-transplant is reduced to 1.5 mg by month 6 post-transplant, 0.75 mg by month 9 post-transplant, and 0 mg by month 12 post-transplant).

[0033] In one example where the organ transplant is a kidney transplant, for-cause renal allograft biopsies may be performed for subjects who have: (1) an unexplained increase in serum creatinine of > 0.3 mg/dL from baseline after fluid resuscitation and exclusion of obstructive uropathy; (2) proteinuria of 1000 mg /day; and/or (3) the development of de novo donor specific antibodies (DSA). The serum creatinine result obtained at one month post-transplant may be used as the baseline reference point for this assessment. In addition, a for-cause biopsy may be performed for all subjects with a positive renal transplant biomarker result (e.g., TRUGRAF® test result indicating “not-TX”) and/or at the discretion of a clinician. Successful weaning -- to a

reduced or eliminated dose of at least one immunosuppressant and maintenance of the decreased or eliminated dose -- is defined as the ability to taper to/maintain a reduced or eliminated dose without the need to re-instate a standard dual immunosuppression regimen (e.g., mTOR and CNI) because of an immunologic event.

[0034] Another aspect of the disclosure is directed to a method for returning a patient to complete (e.g., dual) immunosuppressant therapy if the patient develops signs of allograft dysfunction or an immunologic event

[0035] In this aspect of the disclosure, patients subject to immunosuppressant weaning as described above may be returned to full immunosuppressant therapy in the event of an immunologic event such as development of de novo donor-specific antibodies, biopsy-proven acute rejection, biopsy-proven sub-clinical rejection, and development of significant (2+) interstitial fibrosis/tubular atrophy.

EXAMPLES

[0036] **Example 1: Selection of CD4+ and CD25+ T regulatory Cells**

[0037] Overview: This example establishes the selection protocols for successful enrichment of CD4+/CD25+ Treg cells.

[0038] Declumping Buffers

[0039] Three 1 L CLINIMACS® PBS/EDTA buffer bags were labeled (Bags #1, #2, and #3) and prepared by adding 20 mL of 25% human serum albumin (HSA) and 1.8 mL of 200 mg/mL MgCl₂. To Bag #1, 54 mL of 1 mg/mL PULMOZYME® (dornase alpha) was added. To each of Bags #2 and #3, 10.5 mL of 1 mg/mL PULMOZYME® was added. From Bag #1, 200 mL of solution was transferred to a sterile culture bag, which was labeled as Bag #1A, and supplemented with 45 mL 25% HSA. The approximate volumes, percentages, and/or final concentrations of each component in the Bags are shown in Table 2.

Table 2

Declumping Buffer Bag Components

Bag (total volume)	Bag #1A (245 mL)	Bag #1 (876 mL)	Bag #2 (1032 mL)	Bag #3 (1032 mL)
HSA	5%	0.5%	0.5%	0.5%
MgCl ₂	2.9 mM	3.5 mM	3.5 mM	3.5 mM
PULMOZYME [®]	41 U/mL	50 U/mL	50 U/mL	50 U/mL

[0040] Leukapheresis Product Cryopreservation

[0041] A collected leukapheresis sample was centrifuged at 2800 rpm for 11 minutes. Using a plasma extractor and an electronic scale, the supernatant was removed (*i.e.*, plasma was expressed). Plasma was added back to the centrifuged sample to reach a volume of 60 mL. The leukapheresis sample was then transferred to a cryopreservation bag. A freezing solution of 50% DMSO (dimethyl sulfoxide) in Normal Saline (0.9% sodium chloride) was prepared, then placed in a 2-8°C refrigerator for at least 10 minutes. The 50% DMSO freezing solution was added to the cryopreservation bag to reach a final DMSO concentration of 10%.

[0042] The cryopreservation bag was frozen in a controlled-rate freezer at an average cooling rate of 1°C/min, to an endpoint of -100°C. Once frozen, the bag was transferred to a monitored liquid nitrogen freezer and stored in the vapor phase.

[0043] Leukapheresis Product Thawing

[0044] Thawing of apheresis products generally results in some cellular clumping with associated decreased viability. Column separation of thawed apheresis products can be problematic due to the clumping of the product and the potential loss of cellular starting material. Therefore, a “thawing buffer” consisting of a PBS/EDTA buffer (phosphate-buffered saline, pH 7.2, supplemented with 1 mM EDTA), such as CliniMACS[®] PBS/EDTA buffer (Miltenyi Biotec), 5% human serum albumin, 3.5 mM MgCl₂ and 50 U Pulmozyme[®]/mL was used for the initial washing of the thawed apheresis product as well as for the selection process.

[0045] The cassette holding the frozen leukapheresis bag was removed from the dry shipper and immersed completely in a $37 \pm 2^{\circ}\text{C}$ water bath. Once the cells thawed, but were still cold, the leukapheresis bag was removed from the cassette.

[0046] CD8/CD19 Negative Selection

[0047] Thawed leukapheresis cells were transferred to a 600 mL TRANSFER PACK™ container (FENWAL™, Lake Zurich, IL). The entire contents of Bag #1A were added to the container. The container was incubated for 30 minutes, and then centrifuged at 1800 rpm for 15 minutes, all at room temperature. Using a plasma extractor and an electronic scale, the supernatant was removed and discarded. An aliquot of 200 mL of buffer from Bag #1B was added to the container. The container was centrifuged at 1800 rpm for 15 minutes at room temperature. Using a plasma extractor and an electronic scale, the supernatant was removed and discarded. The final product volume was adjusted to 85 ± 5 mL using buffer solution from Bag #1B. The cells were resuspended by gently agitating the container.

[0048] The entire contents of the CLINIMACS® CD8 microbead kit and the CD19 microbead kit were added to the resuspended cells. The microbead kits include a colloid of magnetic antibodies specific to the cells of interest (e.g., CD8+ CD19+ cells) and Iron-Dextran. The container was mixed gently before incubating for 30 minutes at room temperature on a rotator at 25 rpm. The incubated cells were diluted to 450 mL with buffer solution from Bag #1B. The container was centrifuged at 1800 rpm for 15 minutes. Using a plasma extractor and an electronic scale, the supernatant containing excess microbeads was removed and discarded. The product volume was adjusted to 100 ± 2 mL with buffer solution from Bag #1B. The cells were resuspended by gently agitating the container.

[0049] The container and Buffer Bag #2 were connected to a CLINIMACS® Plus instrument, and a CD8/CD19 depletion program was executed, which separates CD8+/CD19+ cells using a high-gradient magnetic separation column (Miltenyi). The magnetically-labeled CD8+CD19+ cells are retained in the magnetized column and separated from the unlabeled cells. The unlabeled cells are eluted out of the column and consist of a cell population depleted of CD8+/CD19+ cells. The CD8/CD19 depleted cells were collected in a “CD8/19 Depleted Product Preparation” Bag.

[0050] CD4/CD25 Enrichment

[0051] The CD8/CD19 depleted cells were diluted with cold (4-8°C) buffer (Buffer Bag #3) to a total volume of 380 mL. The cells were resuspended by gently agitating the CD8/19 Depleted Product Preparation Bag. The diluted product was cooled to 4-8°C in a refrigerator for 20 minutes.

[0052] The entire contents of a CLINIMACS® CD25 microbead kit were combined with the diluted, cooled CD8/CD19 depleted cells in the CD8/19 Depleted Product Preparation Bag and the combination was mixed gently before incubating for 15 minutes at 4-8°C on a rotator at 25 rpm. The incubated cells were transferred to a 600 mL TRANSFER PACK™ container, and then diluted with buffer solution from Bag #3. The container was centrifuged at 1800 rpm for 15 minutes at room temperature. Using a plasma extractor and an electronic scale, the supernatant containing excess Microbead reagent was removed and discarded. The product volume was adjusted to 100 ± 2 mL with buffer solution from Bag #3. The cells were resuspended by gently agitating the container.

[0053] The container and Buffer Bag #3 were connected to a CLINIMACS® Plus instrument, and a CD4/CD25 enrichment program was executed. This protocol is similar to that described above, with the exception that the desired CD4+/CD25+ cells are selected for and undesired cells are washed out. CLINIMACS® Magnetic Cell Separation Systems separate mixed cell populations in a magnetic field using an immunomagnetic label specific for the cells of interest (e.g., CD4+/CD25+ (bright), referred to as regulatory T-cells, or Treg). Thus, once the unlabeled cells were removed from the column, the retained cells (CD4+/CD25+ cells) were eluted by removing the magnetic field from the column, washing the cells out and collecting them. The resulting CD4+/CD25+ enriched cells were collected in a “CD25 Enriched Cell” Bag.

[0054] Flow cytometry was used to determine the phenotypic characteristics of the selected population. The selection protocol yielded 98.7% CD4+ cells, 86.8% CD25+ cells, 0.0% CD8+ cells, and 0.1% CD20+ cells (see, Table 2 “Day 0,” which is a representative data set and Figure 1, which includes the data shown in Table No. 2). FoxP3+ was used as a separate marker for Treg cells.

Table 3

CD4+/CD25+ Enriched Cells

	Day 0
CD4+	98.7%
CD25+	86.8%
FoxP3+	44.0%
CD8+	0.0%
CD20+	0.1%
Viability	94.9%
Cell #	1 x 10⁷
Sterility	negative

[0055] The data in Table No. 3 demonstrate that the combined selection processes used for selecting Treg cells were successful.

[0056] Example 2: T regulatory Cell Expansion

[0057] Overview: Enriched populations of Treg cells obtained in Example 1 are expanded to provide therapeutically relevant numbers of Treg cells.

[0058] Growth media (GM) was TexMACS medium (Miltenyi) supplemented with 10% heat-inactivated AB serum, 1,000 IU/ml IL-2 (Clinigen), and 1 µg/ml TGF-β (Invitrogen). G-Rex bioreactor (Wilson Wolf,) cultures were initiated with 5x10⁶ (G-Rex 10M) or 50x10⁶ (G-Rex 100M) cells and TRANSACT™ simulation reagent (Miltenyi) at 60 µl/ml. GM is added every two to three days to maintain a cell density of 1x10⁶/ml. During days 0-9 the GM is also added with Everolimus (EVR, Novartis) at 100 ng/ml. On Day 11-12, the cells are restimulated with TRANSACT™ reagent (Miltenyi) at 60 µl/ml. On Day 14, in-process testing is performed for sterility, endotoxin detection, cell counts, viability, phenotyping, and the Treg suppression assay. EVR is not added after Day 9 and cells are harvested on Day 21. On Day 21, additional quality testing is performed, again for sterility, endotoxin detection, cell counts, viability, phenotyping, and the Treg suppression assay. The target Treg numbers at the end of the culture is 4-5x10⁹ and if this number is not obtained, the culture is extended to another week to 28 days in total. This

would ensure that sufficient cell numbers are obtained for Treg therapy. An example expansion protocol is shown in Tables 4 and 5.

Table 4

Protocol of Large-scale Bead-free Treg Expansion in G-Rex 100M				
Growth Medium (GM) contains: TexMACS GMP Medium with 10% Normal AB serum.				
Everolimus (Evr): (no more addition after day 7)				
Whenever cell count reaches 2×10^9 , split into 2 G-Rex 10M. Make up volume to 1000ml in each				
Day		Activity	Volume (ml) on the day	Cells
0	Wednesday	Stimulate with TRANSACT™ (3000 µl), 50ml GM +IL2 (50,000U) +TGF-B (50ng) + Evr (5µg)	50	50×10^6
2	Friday	Add 50ml GM +IL2 (100,000U) +TGF-B (100ng) +Evr (10ug)	100	Do not disturb
5	Monday	Add 50ml GM +IL2 (150,000U) +TGF-B (150ng) +Evr (15ug)	150	Do not disturb
7	Wednesday	Add 50ml GM +IL2 (200,000U) +TGF-B (200ng) +Evr (20ug)	200	Count
9	Friday	Add 100ml GM +IL2 (300,000U) +TGF-B (300ng)	300	Do not disturb
12	Monday	Restimulate with TRANSACT™ (24 ml). Add 100ml GM +IL2 (400,000U) +TGF-B (400ng)	400	Count
13	Tuesday	Add 200ml GM +IL2 (600,000U) +TGF-B (600ng)	600	Do not disturb
14	Wednesday	In Process Testing.	600	Count
16	Friday	Add 200ml GM +IL2 (800,000U) +TGF-B (800ng)	800	Do not disturb
19	Monday	Add 200ml GM +IL2 (1,000,000U) +TGF-B (1,000ng)	1000	Count
21	Wednesday	Harvest if Tregs grow to 4 billion total cells (Continue culture if Tregs do not grow to 4 billion total cells)	1000	Count
23	Friday	Add IL2 (1,000,000U) +1,000ng TGF-B	1000	Do not disturb
26	Monday	Add IL2 (1,000,000U) +1,000ng TGF-B	1000	Count
27	Tuesday	Add IL2 (1,000,000U) +1,000ng TGF-B	1000	Do not disturb
28	Wednesday	Harvest		Count

Table 10

Protocol of Large-scale Bead-free Treg Expansion in G-Rex 10M				
Growth Medium (GM) contains: TexMACS GMP Medium with 10% Normal AB serum.				
Everolimus (Evr): (no more addition after day 7)				
Whenever cell count reaches 2×10^8 , split into 2 G-Rex 10M. Make up volume to 100ml in each				
Day		Activity	Volume (ml) on the day	Cells
0	Wednesday	Stimulate with TRANSACT™ (300 µl), 5ml GM +IL2 (5,000U) +TGF-B (5ng) + Evr (500ng)	5	50×10^6
2	Friday	Add 5ml GM +IL2 (10,000U) +TGF-B (10ng) +Evr (1ug)	10	Do not disturb
5	Monday	Add 5ml GM +IL2 (15,000U) +TGF-B (15ng) +Evr (1.5ug)	15	Do not disturb
7	Wednesday	Add 5ml GM +IL2 (20,000U) +TGF-B (20ng) +Evr (2ug)	20	Count
9	Friday	Add 10ml GM +IL2 (30,000U) +TGF-B (30ng)	30	Do not disturb
12	Monday	Restimulate with TRANSACT™ (2.4 ml). Add 10ml GM +IL2 (40,000U) +TGF-B (40ng)	40	Count
13	Tuesday	Add 20ml GM +IL2 (60,000U) +TGF-B (60ng)	60	Do not disturb
14	Wednesday	In Process Testing	60	Count
16	Friday	Add 20ml GM +IL2 (80,000U) +TGF-B (80ng)	80	Do not disturb
19	Monday	Add 20ml GM +IL2 (100,000U) +TGF-B (100ng)	100	Count
21	Wednesday	Harvest if Tregs grow to 4 billion total cells (Continue culture if Tregs do not grow to 4 billion total cells)	100	Count
23	Friday	Add IL2 (100,000U) +100ng TGF-B	100	Do not disturb
26	Monday	Add IL2 (100,000U) +100ng TGF-B	100	Count
27	Tuesday	Add IL2 (100,000U) +100ng TGF-B	100	Do not disturb
28	Wednesday	Harvest		Count

[0059] In view of the foregoing disclosure and the description of the invention by reference to specific embodiments thereof, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims.

More specifically, although some aspects of the present invention are identified herein as particularly advantageous, it is contemplated that the present invention is not necessarily limited to these particular aspects of the invention.

CLAIMS

1. A method of treating an organ transplant recipient receiving an immunosuppressive therapy comprising at least two immunosuppressants, the method comprising:
 - (a) administering to the recipient between about 500,000,000 and 5,000,000,000 CD4+/CD25+ T-reg cells;
 - (b) decreasing the dosage amount of one of the at least two immunosuppressants.
2. The method of claim 1, wherein one of the at least two immunosuppressants is selected from cyclosporine, tacrolimus, everolimus, azathioprine, mycophenolate mofetil, sirolimus, alemtuzumab, azathioprine, belatacept, and corticosteroids.
3. The method of claim 1 or claim 2, wherein the at least two immunosuppressants comprise everolimus and tacrolimus.
4. The method of any preceding claim, wherein the dosage amount is incrementally decreased over the course of three to 15 months.
5. The method of claim 4, comprising administering the T-reg cells on about day 50 to about day 70 following an organ transplant.
6. The method of claim 5, wherein the decreasing the dosage begins on about day 80 to about day 120 following the organ transplant.
7. The method of any of claims 1-6, wherein the dose of one of the at least two immunosuppressants is eliminated.
8. The method of any of claims 1-6, wherein the dose of the one of the at least two immunosuppressants is reduced by at least 25%, at least 50%, at least 75%, at least 90%, or 100%.
9. The method of any of the preceding claims, wherein decreasing the dosage amount comprises decreasing the dosage amount of two immunosuppressants.

10. A method of any of the preceding claims, wherein the T-reg cells are selected and expanded from a frozen apheresis product.
11. The method of any of the preceding claims, wherein the method at least one of reduces, stops, and prevents a cellular immune response that causes cellular, organ, or tissue rejection in the recipient.
12. The method of any of the preceding claims, further comprising monitoring the patients for adverse events and stopping the decreasing the dosage amount of one of the at least two immunosuppressants.
13. The method of claim 12, further comprising returning the patient to full immune suppressant therapy.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/019975

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - INV. - A61K 35/17; A61K 31/365; A61K 38/13; A61P 37/06 (2023.01)

ADD. - C12N 5/0783 (2023.01)

CPC - INV. - A61K 39/4611; A61P 37/06; A61K 35/17; A61K 38/13; A61K 31/365 (2023.05)

ADD. - A61K 2035/122; C12N 5/0637 (2023.05)

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)
See Search History documentDocumentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History documentElectronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATHEW et al. A Phase I Clinical Trial with Ex Vivo Expanded Recipient Regulatory T cells in Living Donor Kidney Transplants. Sci Rep. 09 May 2018, Vol. 8, Pgs. 1-12. Entire Document	1-3
A	US 2006/0115899 A1 (BUCKNER et al.) 01 June 2006 (01.06.2006) entire document	1-3
A	US 2016/0152951 A1 (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERV) 02 June 2016 (02.06.2016) entire document	1-3
A	ZWANG et al. Cell Therapy in Kidney Transplantation: Focus on Regulatory T Cells. Journal of the American Society of Nephrology. 31 July 2017, Vol. 28, Pgs. 1960-1972. Entire Document	1-3
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

10 June 2023

Date of mailing of the international search report

AUG 11 2023

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/019975

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.: 4-13
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.