METHODS FOR IDENTIFYING PATIENTS AT RISK FOR COSTIMULATION BLOCKADE RESISTANT REJECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 61/806,206, filed March 28, 2013; the entire content of which is incorporated herein by reference.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

This invention relates to methods for determining the susceptibility of a transplant patient to costimulation blockade resistant rejection. These methods are useful for identifying effective drug regimens for the treatment of immune disorders associated with graft transplantation and/or maintenance of a transplant.

BACKGROUND OF THE INVENTION

Given the central role of T-cells in transplant rejection, a common goal among current immunosuppressive therapies is to block T-cell activation and function (Sayegh, M.H. et al., *N. Engl. J. Med.*, 338(25):1813-1821 (1998)). T-cells require both an antigen-specific (Signal 1) and co-stimulatory signal (Signal 2) for full activation (Lenschow, D.J. et al., *Annu. Rev. Immunol.*, 14:233-258 (1996)). One of the best-characterized co-stimulatory pathways involves the CD28-CD80/86 (B7-1/2) interaction (Linsley, P.S. et al., *Annu. Rev. Immunol.*, 11:191-212 (1993)). Cytotoxic T-lymphocyte antigen 4 (CTLA4) binds to CD80/86 with higher avidity than CD28, and is transiently expressed on T-cells following their activation, where it interrupts the interaction between CD28 and CD80/86 (Oosterwegel, M.A. et al., *Curr. Opin. Immunol.*, 11(3):294-300 (1999)). This creates a negative feedback signal for T-cell activation.

Functionally distinct T cell populations can be defined by the expression of specific cell surface antigens. Numerous studies describe associations between T cell surface phenotype and function (Appay, V. et al., *Nat. Med.*, 8:379-385 (2002)). CD57, a surface molecule expressed on T and NK cells, is a marker with functional associations. CD57 has been commonly described as an external marker of cell senescence, a state in which the cell is in persistent cell cycle arrest. Expansions of CD4+ T cells expressing CD57 have been associated with a number of chronic pathological conditions such as tuberculosis, malaria, rheumatoid arthritis, and HIV-1 infection (Maeda, T. et al., *Arthritis Rheum.*, 46:379-384 (2002); Imberti, L. et al., *Blood*, 89:2822-2832 (1997)).

Belatacept (L104EA29YIg) is the first biologic agent approved for primary maintenance immunosuppression, selectively blocking the CD28 co-stimulation pathway to prevent T-cell activation (Larsen, C.P. et al., *Am. J. Transplant.*, 5:443-453 (2005)). Belatacept combined with corticosteroids and a mycophenolic acid is indicated for prophylaxis of graft rejection in adults receiving a renal transplant. While Belatacept has been associated with improved long-term graft function as well as patient and graft survival, patients treated with this costimulation blockade molecule tended to experience early acute rejection.

Further, while the introduction of kidneys from extended criteria donors has been necessary to alleviate donor organ shortages, recipients are at greater risk of complications and graft loss than recipients of living or standard criteria deceased donor kidneys (Tullius, S.G. et al., *Eur. Renal Dis.*, 1:51-54 (2007)).

With the increasing use of costimulation blockade molecules and higher risk extended criteria donor organs, it is clear that there is a need to prospectively identify patients at risk for costimulation blockade resistant rejection. The Inventors have identified a CD4+ T cell phenotype that strongly correlates with risk for costimulation blockade resistant rejection.

At present, there are no predictive assays to identify patients at risk of costimulation blockade resistant rejection. Thus, there is a need in the art for a diagnostic assay that could determine whether a patient would be eligible to be treated with costimulation blockade molecules or at risk of acute rejection. The present invention provides such novel assay.

SUMMARY OF INVENTION

In one aspect, the invention provides a method of predicting if a transplant patent is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a blood sample from a person receiving costimulation blockade therapy, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

In another aspect, the invention provides a method of predicting if a patient in need of a transplant is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a peripheral blood sample from a person prior to transplantation, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a graph of PBMCs from belatacept treated patients examined for markers of senescence (CD57) and exhaustion (PD1). Patients who experienced ACR had a significantly higher percentage of CD57+PD1- cells in the CD4+, but not CD8+, compartment. These differences were significant in each memory subset of CD4+, but not CD8+, T cells

Figure 2 shows a graph of PBMCs from tacrolimus treated patients examined for markers of senescence (CD57) and exhaustion (PD1). No significant correlations with outcome were found in the CD57+PD1- compartment of either CD4+ or CD8+ T cells.

Figure 3 shows that CD28- T cells do not predict acute cellular rejection outcome. PBMCs from belatacept and tacrolimus treated patients were examined for surface expression of costimulation markers CD28 and CD27. A trend existed towards increased prevalence of CD28 negative CD4 T cells in the belatreated patients who rejected, but this did not reach statistical significance. No correlations between CD28 expression on CD8 cells and outcome were observed.

Figure 4 shows the phenoflow panels utilized in Example 1. *CD3 antibody was diluted 1:8 before use, while the CD45RA antibody was diluted 1:10 before use.

Figure 5 (SEQ ID NOS: 1 and 2) depicts a nucleotide and amino acid sequence of L104EA29YIg, a CTLA4 mutant molecule comprising a signal peptide; a mutated extracellular domain of CTLA4 starting at methionine at position +1 and ending at aspartic acid at position +124, or starting at alanine at position -1 and ending at aspartic acid at position +124; and an Ig region. SEQ ID NOS: 1 and 2 depict a nucleotide and amino acid sequence, respectively, of L104EA29YIg, a CTLA4 mutant molecule, comprising a signal peptide; a mutated extracellular domain of CTLA4 starting at methionine at position +27 and ending at aspartic acid at position +150, or starting at alanine at position +26 and ending at aspartic acid at position +150; and an Ig region.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

As used herein, "ligand" refers to a molecule that specifically recognizes and binds another molecule, for example, a ligand for CTLA4 is a B7 molecule.

"L104EA29YIg", "belatacept" and "LEA29YIg" are used interchangeable herein and refer to a fusion protein that is a soluble CTLA4 mutant molecule comprising an extracellular domain of wildtype CTLA4 with amino acid changes A29Y (a tyrosine amino acid residue substituting for an alanine at position 29) and L104E (a glutamic acid amino acid residue substituting for a leucine at position +104), joined to an Ig tail (included in Figure 4, SEQ ID NOS: 1 and 2; DNA encoding L104EA29YIg was deposited on June 20, 2000 with ATCC® number PTA-2104. Belatacept is commercially available as the IV formulation, NULOJIX® (Bristol-Myers Squibb Company). Belatacept is a fusion protein that acts as a selective costimulation blocker by binding surface ligands (CD80 and CD86) on antigen-presenting cells. In antigen recognition, the binding of the costimulation receptor on T cells (CD28) to CD80 and CD86 (signal 2) is

required for efficient T-cell activation when the T-cell receptor encounters MHC alloantigens (signal 1).

As used herein, "B7" refers to the B7 family of molecules including, but not limited to, B7-1 (CD80), B7-2 (CD86) and B7-3 that may recognize and bind CTLA4 and/or CD28.

As used herein, the term "CD4" refers to "cluster of differentiation 4" which is a glycoprotein expressed on the surface of T helper cells, monocytes, macrophages, and dendritic cells

As used herein, "CD57+ T cells" are T cells with CD57 molecules expressed on the cell surface.

As used herein, to "block" or "inhibit" a receptor, signal or molecule means to interfere with the activation of the receptor, signal or molecule, as detected by an art-recognized test. Blockage or inhibition may be partial or total. For example, blockage of a cell-mediated immune response can be detected by determining the functionality of the transplant, such as the serum creatinine concentrations following renal transplantation.

As used herein, "costimulation blockade resistant rejection" and "acute rejection" are used interchangeable herein and refer to a reaction against allograft or xenograft tissue that is incompatible with the recipient, which typically occurs 2 to 60 days after transplantation.

As used herein, "treat" or "treating" a disorder or disease means to manage a disease or disorder by medicinal or other therapies. Treatment of a disease or disorder may suppress immune-mediated events associated with a disease, ameliorate the symptoms of a disease or disorder, reduce the severity of a disease or disorder, alter the course of disease or disorder progression and/or ameliorate or cure the basic disease or disorder problem. For example, to treat an immune disorder associated with graft transplantation may be accomplished by regulating an immune response *e.g.*, by regulating functional CTLA4- and/or CD28- positive cell interactions with B7-positive cells. For example, treating renal transplant rejection includes inhibition of renal transplant rejection as measured by glomerular filtration rate (GFR). For example treating immune disorders associated with graft transplantation includes prophylaxis of organ rejection by administration of L104EA29YIg. Further, treating immune disorders

associated with graft transplantation may prolong the survival of the host and transplanted organ.

As used herein, "immune disorders associated with graft transplantation" means any transplant related disease mediated by T-cell interactions with B7-positive cells including, but not limited to, immune disorders associated with graft transplantation rejection, graft related disorders, graft versus host disease (GVHD) (*e.g.*, such as may result from bone marrow transplantation, or in the induction of tolerance), rejection of the graft or transplant including acute rejection of the graft or transplant and chronic rejection of the graft or transplant.

As used herein, the "graft" or "transplant" may be solid organ allografts or xenografts, tissue or cell allografts or xenografts or external anatomy allografts or xenografts, including but not limited to skin, islet cells (also known as islets), muscles, hepatocytes, neurons, heart, liver, kidney, lung, appendages, limbs, nose, ear or face.

Early costimulation blockade resistant rejection has been seen in some patients on costimulation blockade immunosuppression, such as belatacept. Hypothesizing that T cell phenotype might prospectively identify patients at risk for costimulation blockade resistant rejection, transplant patients receiving costimulation blockade, were studied by specifically quantifying terminally differentiated memory cells expressing CD57, a marker of T cell senescence, and relating this expression to clinical outcome (see Example 1).

As discussed in Example 1, peripheral blood samples were obtained from kidney transplant patients prior to drug administration and analyzed by multiparameter flow cytometry, specifically analyzing the samples for markers of memory, differentiation, activation, exhaustion and senescence. Patients who eventually experienced acute rejection had a markedly higher percentage of CD4+CD57+PD1- T cells ($p=0.0055$) at the time of transplantation compared to patients without rejection (Fig. 1). Although typically considered a marker of senescence, CD57 expression on CD4+ T cells was paradoxically correlated with expression of the proliferation marker Ki67 ($p=0.0087$), and was not associated with shorter telomere length ($p=0.7424$). These relationships were limited to the CD4 compartment. CD8+CD57+ T cells marginally trended higher in acute rejection patients ($p=0.2358$, Fig. 1), but CD57 expression on CD8+ T cells was

associated with more traditional indices of senescence including reduced Ki67 expression and shorter telomere length.

In one aspect, the invention provides a method of predicting if a transplant patent is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a blood sample from a person receiving costimulation blockade therapy, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

In another aspect, the invention provides a method of predicting if a patient in need of a transplant is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a peripheral blood sample from a person prior to transplantation, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

In one embodiment, the invention provides a method of predicting if a kidney transplant patent is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a blood sample from a person receiving costimulation blockade therapy, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

In one embodiment, the invention provides a method of predicting if a patient in need of a kidney transplant is at risk of costimulation blockade resistant rejection, the method comprising: (a) acquiring a peripheral blood sample from a person prior to transplantation, (b) quantitating the percent of CD+CD57+ T cells and (c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells in a reference, wherein the percentage of CD4+CD57+ T cells in the test sample is elevated compared to a reference.

In one embodiment, the reference may be blood from normal healthy controls or stable transplant patients; or a reference value determined from a representative number of normal healthy controls or stable transplant patients.

In another embodiment the percentage of CD4+CD57+ T cells is elevated at least 2 fold, from 2 to 25 fold, from 2 to 20 fold, from 2 to 15 fold, from 2 to 10 fold, from 2 to 5 fold compared to the percentage of CD4+CD57+ T cells in a reference.

In another embodiment the CD4+CD57+ T cell content of the blood sample is measured by flow cytometry. Flow cytometry has been used with increasing regularity in clinical laboratories for immunophenotyping. The advantages of flow cytometry include speed, sensitivity, precision and objectivity. The components and operation of flow cytometers is well known to those skilled in the art and will not be described in detail herein. For purposes of a description of such, applicants refer to U.S. Patent No. 5,567,627, issued Oct. 22, 1996 which is incorporated herein by reference in its entirety. It is sufficient to indicate that the components and methodology of flow cytometry can be used to provide specific information on a number of parameters of a sample. For example it is possible to provide information on components of different sizes within a sample, while simultaneously providing information on signals of different wavelengths received from different components received from the sample. Thus, when a sample includes components of varying sizes and also includes components with labels which emit different wavelengths of light the flow cytometry data obtained can provide multidimensional information to the user. The present invention utilizes this technology by providing different types of labeled antibodies and labeled and unlabelled cells expressing known antigens. By exposing a sample to such and thereafter analyzing such within a flow cytometer it is possible to obtain substantial amounts of information regarding the blood in a quick and efficient manner.

Multiparameter flow cytometry is the method of choice, when immunophenotyping is desired, because more information can be objectively obtained from fewer cells due to the use of antibodies to differentiation-associated antigens and antibodies to activation-associated antigens at the same time to define what functions specific populations of cells are performing. For example, multi-color fluorescence-activated cell sorter analysis can be performed by obtaining peripheral blood samples from patients and normal healthy controls by hemolysis using the Whole Blood Lysing

Reagent Kit (Beckman Coulter, Fullerton, CA). Cell surface antigens of peripheral blood leukocytes can be detected using a FACS®Calibur flow cytometer with the CELLQuest program (Becton Dickinson, Mountain View, CA). Cell surface antigens and intracellular cytokines can be detected using fluor-labeled monoclonal antibodies, typically available from Becton Dickinson.

Cell stimulation and staining for intracellular cytokine analysis can be performed according to the manufacturer's instructions (PharMingen, San Diego, CA). Briefly, peripheral blood leukocytes are stimulated with phorbol myristate acetate and calcium ionophore for 4 hours. For the last 3 hours of stimulation, the intracellular transport inhibitor, nomensin (GOLGISTOP®) is added for accumulation of intracellular cytokines. Afterward, cell surface antigens are stained with fluorescent-labeled monoclonal antibodies specific for the desired antigen. After CYTOFIX®/CYTOPERM® solution is added to fix and permeabilize the cells, intracellular cytokines are stained with fluorescent-labeled monoclonal antibodies and multi-color fluorescence-activated cell sorter (FACS) analysis is performed.

In a further embodiment, the costimulation blockage therapy comprises administering L104EA29YIg (belatacept), a mutant CTLA4Ig molecule, which is a fusion protein that acts as a selective costimulation blocker by binding surface ligands (CD80 and CD86) on antigen-presenting cells.

The typical belatacept pharmaceutical composition exemplified in the instant invention is an intravenous (i.v.) formulation listed below.

Composition of Lyophilized L104EA29YIg 100mg/Vial Drug Product

Component	Amount/Vial (mg)[a]
L104EA29YIg	110[a]
Sucrose	220
Sodium Phosphate Monobasic Monohydrate	15.18
Sodium Chloride	2.55
1N Sodium Hydroxide	Adjust to pH 7.5
1N Hydrochloric Acid	Adjust to pH 7.5

[a] Each vial contains 10% overfill for vial, needle and syringe holdup of the reconstituted solution.

The lyophilized drug is typically reconstituted to about 25mg/ml with 10 ml of either Sterile Water for Injection, USP (SWFI) or 0.9% Sodium Chloride Injection, USP. The reconstituted solution is further diluted to drug product concentrations between 1 and 10 mg/ml with 0.9% Sodium Chloride Injection, USP. The diluted drug product for injection is isotonic and suitable for administration by intravenous infusion.

L104EA29Y is typically administered at 10 mg/kg weight of the patient during the early phase, high risk period that follows transplantation and decreased to 5 mg/kg weight of the patient for a maintenance dosage.

Typical target trough serum concentration of L104EA29Y are between about 3µg/mL and about 30µg/mL over the first 3 to 6 months post-transplant which is sufficient to maintain function of the allograft, preferably between about 5µg/mL and about 20 µg/mL. Typically, target trough serum concentration of L104EA29Y during the maintenance phase are between about 0.2 µg/mL and about 3µg/mL, preferably between about 0.25µg/mL and about 2.5µg/mL.

A typical administration regimen includes an early phase, in which doses are higher and the frequency of administration is increased during the period of greatest immunologic risk, followed by a maintenance phase. The early phase regimen may range from the first 3 to 6 months post-transplantation and involves administration that initially is more frequent than monthly, preferably as frequently as daily, weekly or every two weeks depending on the immunologic risk and/or target trough serum concentration. The maintenance phase begins when the early phase ends and involves administration that is not more frequent than monthly, and lasts for as long as needed, typically for as long as the patient retains the transplant. As used herein, day 1 is defined as the day of the transplant or the first day of treatment with L104EA29Y or pharmaceutical compositions thereof.

The dosage of L104EA29Y in the early phase is about 8 to about 12 mg/kg weight of the patient, preferably about 10 mg/kg. The dosage of L104EA29YIg of the invention in the maintenance phase is about 3 to about 7 mg/kg weight of the patient, preferably about 5 mg/kg.

The early phase may range from the first 3 to 6 months post- transplantation. The administration regimen during early phase may vary depending on the status of the

recipient and/or graft. For example, a more intensive early phase regimen would administer a higher dose of the molecules or the pharmaceutical compositions of the invention on day 1, day 5, week 2 visit (*e.g.*, day 13-17), then every two weeks for the first 3 months (*e.g.*, on week 4 visit, week 6 visit, week 8 visit, week 10 visit, and week 12 visit), followed by monthly administration through month 6 visit (*e.g.*, on month 4 visit, month 5 visit, and month 6 visit). An example of a typical more intensive early phase regimen is administration of 10mg/kg weight of the patient of L104EA29YIg at days1, 5, 15, 29, 43, 57, 71, 85, 113, 141 and 169. A less intensive regimen, for example, would administer the molecules or the pharmaceutical compositions of the invention on day 1, week 2 visit, week 4 visit, then monthly through month 3 visit. An example of a typical less intensive early phase regimen is administration of 10mg/kg weight of the patient of L104EA29YIg on days1, 15, 29, 57 and 85.

Typically, an early phase is followed by a maintenance phase where lower doses of the molecules or pharmaceutical compositions of the invention are administered at one to two month intervals for as long as needed, typically for as long as the patient retains the transplant. An example of the maintenance phase for the more intensive regimen described above includes monthly administration of 5mg/kg weight of the patient of L104EA29YIg starting at month 7 visit. While an example of the maintenance phase for the less intensive regimen above would include monthly administration of 5mg/kg weight of the patient of L104EA29YIg starting at month 4 visit.

Alternatively, one knowledgeable in the art would be able to modify the administration regimen in response to the patients risk status and/or response to the therapy post transplantation. For example, the early phase of the less intensive regimen described above could be modified by adding administration day 5 to the regimen, thereby increasing the frequency of administration during the period of greatest immunologic risk.

As used herein, "four weeks", "month", "months" or "monthly" refers to a period of 28 ± 5 days. As used herein, "two weeks" refers to a period of 14 ± 3 days.

Flexibility in the administration regimens is required to facilitate administration scheduling in the lives of transplant recipients, while maintaining the target trough profile of L104EA29Y. Permitted windows for administering the doses may be as follows:

Visit	Visit window
Day 1 and day 5	96 hours apart ± 6 hours
Week 2	Target date ± 2 days
Week 4 - Month 6	Target date ± 3 days
from Month 7 thereafter	Target date ± 5 days

The target date is a result of adding the desired duration to the previous actual visit date. The desired duration for the week 2 visit is 10 days. The desired duration is 14 days for a visit planned for two weeks from the previous visit, *e.g.*, a week 6 visit following a week 4 visit. The desired duration is 28 days for a visit planned for a month or four week from the previous visit, *e.g.*, a month 4 visit following a month 3 visit. The desired duration is 56 days for a visit planned for two months from the previous visit, *e.g.*, a month 8 visit after a month 6 visit. For example, a day 15 actual visit date plus 14 days results in a week 4 target date of day 29. Based on the visit windows above, the administration may occur on day 29 ± 3 days. Should the administration occur on day 26, that day becomes the actual visit date utilized for the calculation of the next target date.

Low risk of acute rejection recipients typically include those who receive transplants from living related donors and well matched recipient/donors. High risk of acute rejection recipients typically include those who receive transplants from marginal donors or re-transplants, have high panel reactive antibodies or are African American.

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described above, are, therefore, to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

Example 1

Early costimulation blockade resistant rejection has been seen in some patients on belatacept-based immunosuppression. Hypothesizing that T cell phenotype might prospectively identify patients at risk for costimulation blockade resistant rejection,

patients receiving belatacept-based costimulation blockade, were studied by specifically quantifying terminally differentiated memory cells expressing CD57, a marker of T cell senescence, and relating this expression to clinical outcome.

Methods: Samples from renal allograft recipients receiving belatacept-based immunosuppression were selected from an IRB-approved tissue acquisition protocol.

Study Population:

Organ transplant recipients, candidates for organ transplantation, and their organ donors under evaluation at the Emory University Hospital/ Emory Transplant Center (Emory) or Children's Healthcare of Atlanta (CHOA) were considered for study.

Inclusion Criteria:

- Recipients of or candidates for organ transplantation or organ donors for recipients under evaluation at Emory or CHOA.
- Normal volunteers to include individuals without any known end-stage organ disease who are not on any immunosuppressive medication, and individuals with conditions requiring immunosuppression (such as dermatological diseases) that do not require transplant therapies.

Exclusion Criteria:

- Patients who fail to meet the criteria for transplantation or post transplant follow-up by Emory or CHOA physicians.
- Any condition that, in the opinion of the attending physician, would place the patient at undue risk by participating. Specific conditions include but are not limited to anemia prohibitive of phlebotomy, coagulopathy or technical considerations that would prevent acquisition of sufficient tissue on biopsy for clinical use, or medical urgency preventing timely administration of the consenting process.

Enrollment

Volunteers were recruited for each of the following scenarios:

- *Group 1:* Development of a clinically recognized complication with potential immune etiology or ramifications. Examples include opportunistic infection, rejection, malignancy, alloantibody formation, or immunosuppressive drug toxicity.

- *Group 2:* Maintenance of immune stability characterized by stable graft function without evident complication. These patients served as comparators for individuals with specific complications under scenario 1. These individuals were enrolled to establish demographically matched cohorts of up to 20 individuals for each identified complication identified in Group 1.

- *Group 3:* Pre-transplant status. Recipient candidates for kidney, pancreas, liver or lung transplantation were enrolled and they were followed through the waiting period, transplantation and post transplantation. These individuals served to prospectively capture immune complications prior to clinical presentation of an immune complication to facilitate the retrospective evaluation of assays with potential utility in the prospective, sub-clinical detection of burgeoning complications.

- *Group 4:* Donors for individuals meeting the criteria for one of the 3 above mentioned scenarios. As many of the immune related complications are triggered by or involve immune responses that are specific for donor antigens, donor tissue ease required for the assessment of donor specific immunity. As kidney transplant recipients received organs from live donors, their donors were enrolled to provide target peripheral blood lymphocytes for mechanistic analysis.

- *Group 5*: Individuals with liver, renal, or pulmonary diseases that may lead to the development of organ failure. Including these individuals allowed the study of mechanisms of diseases that may recur post transplantation, and better enable one to predict which patients are at increased risk for recurrence. Moreover, this group allowed the study of disease complications that may affect the post transplantation course.

- *Group 6:* Normal volunteers and people on immunosuppressive agents for non-transplanted conditions were recruited to serve as reference comparators for patient samples.

Patients were recruited in a manner that was sensitive to the inclusion of women and members of underserved minority groups. Enrollment targeted demographics that generated a collection of samples from individuals of age, gender, and ethnicity as appropriate for the disease process under evaluation.

Procedure

Following enrollment volunteers were able to donate samples. In general, the volume of blood donated for this study was 50 mL or 3 mL/kg, whichever was less, for each time point. Under circumstances where the needs of a specific assay require larger amounts (*e.g.,* in lymphocyte depleted patients, or multiple planned assays), a larger blood volume, up to a maximum of 150 mL for any single phlebotomy session was collected and did not exceed 3mL/kg. The total amount of blood drawn did not exceed 450 mL, or 7mL/kg whichever was less, over any 6-week period in keeping with current NIH guidelines. Biopsy tissue and BAL fluid was limited to tissue or fluid in excess of that required for clinical diagnostic needs, as determined by the physician overseeing the procedure. Urine was limited to that which can be donated without interfering with diagnostic testing, and was typically only collected from normal volunteers and kidney donors or recipients. The donation schedule varied by group. In general, patients with documented complications (Group 1) were enrolled and studied at the time the complication was diagnosed, 1 and 6 months following the resolution (or persistence in the case of chronic conditions) of the complication.

Samples were delivered to the Emory Transplant Biorepository where it was processed for study or storage. In general, blood samples were be separated and stored in the form of peripheral blood mononuclear cells (PBMC), serum, plasma, PBMC derived DNA, and PBMC derived RNA converted to cDNA. Biopsy tissue was snap frozen in liquid nitrogen and converted to DNA, RNA, and cDNA. BAL fluid was separated into a cellular fluid and mononuclear cells, and cells were stored or converted to DNA, RNA, and cDNA. Urine was spun and separated in to a cellular supernatants and cell pellets.

Pellets were stored and/or converted to RNA and cDNA. Samples may have been studied fresh or discarded as excess.

Demographic data including age, gender, and race was recorded to assure diversity of this population. Clinical information was collected at the time of each sample collection and included information such as primary disease requiring transplant, date of transplant, date of sample donation, medication dose at time of transplant, most recent level of immunosuppressants, creatinine at time of donation, and baseline creatinine. Each time a subject was asked to donate blood their health status will be reviewed. Clinically concerning information that becomes apparent as a result of study visits (*e.g.*, excessive bruising post phlebotomy, gross hematuria, etc.) was relayed to the patient's attending physician for disposition.

Assay

Peripheral blood samples obtained prior to drug administration and analyzed by multiparameter flow cytometry, specifically analyzing the samples for markers of memory, differentiation, activation, exhaustion and senescence.

The PBMC samples were thawed and resuspended at $5x10^5$ cells/50µl/tube. 1µl live/dead fixable aqua dead cell stain was added per tube. The tubes were then incubated in the dark at room temperature for 30mins and then washed 2 times with 1ml 1x PBS and resuspended in 50µl of PBS.

Antibodies: The antibodies conjugated to fluorescein isothiocyanate (FITC) included CD2 (BD Pharmingen #555326), CD11a (BioLegend #301206), CD39 (Miltenyi Biotec #130-093-502), CD57 (BD Pharmingen #555619), CD103 (Beckman Coulter #IM1856U), IgD (BD Pharmingen #555778), Ki67 (BD Pharmingen #556026), and Perforin (BD Pharmingen #556577).

The antibodies conjugated to phycoerythrin (PE) included CD25 (Miltenyi Biotec #130-091-024), CD28 (BD Pharmingen #555729), CD31 (Miltenyi Biotec #130-092-653), CD38 (BD Pharmingen #555460), BCL2 (BD Pharmingen #556535), IgM (BD Pharmingen #555783), PD1 (CD279) (BioLegend #329906) and Granzyme B (Invitrogen #MHGB04).

PE-Cy7 is a tandem fluorochrome composed of R-phycoerythrin (PE), which is excited by 488-nm light and serves as an energy donor, coupled to the cyanine dye

Cy7(Cy7), which acts as an energy acceptor and fluoresces maximally at 780 nm. The antibodies conjugated to PE-Cy7 tandem fluorochrome included CD19 (BD Pharmingen #341093), CD127 (eBioscience #25-1278-42), and CCR7(CD197) (BD Pharmingen #557648).

The antibodies conjugated to allophycocyanin (APC) included CD27 (BioLegend #302810), HLADR (BD Pharmingen #340549), CCR5(CD195) (R&D #FAB1802A), and IgG (BD Pharmingen #550931). CD127 (BD Pharmingen #558598) was conjugated to ALEXA FLUOR®647. ALEXA FLUOR®647 fluorochrome emission is collected at the same instrument settings as for APC.

The antibodies conjugated to ALEXA FLUOR®700 included CD3 (BD Pharmingen #557943), and CD27 (eBioscience #50-168-83).

The antibodies conjugated to APC-eFluor 780 CD8 (eBioscience #47-0088-42) and CD24 (eBioscience #47-0247-42).

The antibodies conjugated to V450 included CD4 (BD Horizon #560345) and CD5 (BD Horizon #644487).

The antibodies conjugated to Pacific Orange (PacOrange) included CD3 (Invitrogen #CD0330TR), CD14 (Invitrogen #MHCD1430) and CD20 (Invitrogen #MHCD2030).

The antibodies conjugated to QDOT®655 nanocrystal conjugate (QDOT®655) included CD45RA (Invitrogen #Q10069). CD38 (eBioscience #95-0388-42) was conjugated to eFluor650NC. eFluor650NC fluorochrome emission is collected at the same instrument settings as for QDOT®655.

The Extracellular stain: The required amount of antibodies listed in Figure 4 were added to the appropriate tubes and incubated in the dark on ice for 30mins, then washed 2 times with 1ml 1x PBS and resuspended in 200µl of PBS.

The Intracellular stain: 300µl BD CYTOFIX®/CYTOPERM® was added to tubes7 and 8 and incubated in the dark, on ice for 30mins and then washed 2 times with 1ml 1x BD Perm/Wash buffer. ICS antibodies shown in Figure 4 were added to the appropriate tubes and incubated in the dark at room temperature for 30mins, then washed 2 times with 1ml 1x BD Perm/Wash buffer and resuspended in 200µl of PBS.

The samples were run on a BD LSRFortessa (BD Biosciences, San Jose, California) according to the manufactures instructions.

PCR analysis of telomere length, a parameter relevant to senescence, also was performed with QuantStudio (Life Technologies) with iTaq SYBR® (Bio-Rad) and compared to reference samples from 5 normal healthy controls. Within 7 months of transplantation, 9 patients experienced acute cellular rejection and 5 were rejection-free.

Results: Patients who eventually experienced acute cellular rejection had a markedly higher percentage of CD4+CD57+PD1- T cells (p=0.0055) at the time of transplantation compared to patients without rejection (Fig. 1). Although typically considered a marker of senescence, CD57 expression on CD4+ T cells was paradoxically correlated with expression of the proliferation marker Ki67 (p=0.0087), and was not associated with shorter telomere length (p=0.7424). These relationships were limited to the CD4 compartment. CD8+CD57+ T cells marginally trended higher in acute cellular rejection patients (p=0.2358, Fig. 1), but CD57 expression on CD8+ T cells was associated with more traditional indices of senescence including reduced Ki67 expression and shorter telomere length.

Discussion: This study identified a CD4+ T cell phenotype that strongly correlates with risk for belatacept-resistant rejection. CD57 has been commonly described as an external marker of cell senescence, a state in which the cell is in persistent cell cycle arrest. However, the non-senescent (Ki67+, normal telomere length) phenotype associated with CD57, and its limitation to CD4+ T cells suggests that in this setting it is a FLAG® for activated T follicular helper (T_{FH}) cells. T_{FH} cells are antigen-experienced cells known to be critical for certain immune responses and driven by alternative costimulatory pathways including CD154 and the CD28 homologue ICOS.

WHAT IS CLAIMED IS:

1. A method of predicting if a transplant patent is at risk for costimulation blockade resistant rejection, the method comprising:

(a) acquiring a blood sample from a person receiving costimulation blockade therapy;

(b) quantitating the amount of CD4+CD57+ T cells and

(c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells of a reference,

wherein the percentage of CD4+CD57+ T cells in the test sample is higher than the reference.

2. A method of predicting if a patent is at risk for costimulation blockade resistant rejection, the method comprising:

(a) acquiring a blood sample from a person in need of a transplant;

(b) quantitating the amount of CD4+CD57+ T cells and

(c) comparing the percentage of CD4+CD57+ T cells in the test sample with the percentage of CD4+CD57+ T cells of a reference,

wherein the percentage of CD4+CD57+ T cells in the test sample is higher than the reference.

3. The method according to claim 1 or 2 wherein the percentage of CD4+CD57+ T cells is elevated at least 2 fold when compared to the percentage of CD4+CD57+ T cells in the reference.

4. The method according to claim 1 or 2 wherein the reference is blood from normal healthy controls or stable transplant patients; or a reference value determined from a representative number of normal healthy controls or stable transplant patients.

5. The method according to claim 1 or 2 wherein the costimulation blockade therapy is L104EA29YIg comprising:

(a) an amino acid sequence beginning with methionine at position 27 and ending with lysine at position +357 or glycine at position +356 of Figure 4, or

(b) an amino acid sequence beginning with alanine at position 26 and ending with lysine at position +357 or glycine at position +356 of Figure 4.

6. The method according to claim 5 wherein the L104EA29YIg molecule administration regimen comprises an early phase regimen, wherein the early phase regimen may range from the first 3 to 6 months post-transplantation and involves administration that initially is more frequent than monthly.

7. The method according to claim 6 wherein the early phase administration regimen comprises administration of the L104EA29YIg molecule on day 1, week 2 visit, week 4 visit, week 8 visit and week 12 visit.

8. The method according to claim 7 wherein the early phase administration regimen comprises administration of L104EA29YIg molecule on day 5.

9. The method according to claim 7 wherein the early phase administration regimen comprises administration of L104EA29YIg molecule on week 6 visit, week 10 visit, month 4 visit, month 5 visit and month 6 visit.

10. The method according to claim 6 wherein the administration regimen further comprises a maintenance phase regimen wherein the maintenance phase regimen begins after the early phase regimen ends and wherein administration of L104EA29YIg molecule is not more frequent than monthly.

11. The method according to claim 6 wherein the effective dose of L104EA29YIg molecule during the early phase is about 10 mg/kg weight of the subject.

12. The method according to claim 10 wherein the effective dose of L104EA29YIg during the maintenance phase is about 5 mg/kg weight.

13. The method according to claim 5 wherein the effective dose of the L104EA29YIg molecule is about 10mg/kg weight of the subject with an administration regimen comprising administration on days 1, 15, 29, 57, 85 and 5gm/kg monthly thereafter.

14. The method according to claim 5 wherein the effective dose of the L104EA29YIg molecule is about 10mg/kg weight of the subject with an administration regimen comprising administration on days 1, 5, 15, 29, 57, 85 and 5mg/kg monthly thereafter.

15. The method according to claim 5 wherein the effective dose of the L104EA29YIg molecule is about 10 mg/kg weight of the subject with an administration regimen comprising administration on days1, 5, 15, 29, 43, 57, 71, 85, 113, 141, 169 and 5 mg/kg monthly thereafter.

16. The method according to claim 1 or 2 wherein the transplant rejection comprises solid organ, tissue and/or cell transplant rejection.

17. The method according to claim 16 wherein the transplant rejection comprises skin, islet cells, muscles, hepatocytes, neurons, heart, liver, kidney, lung, appendages, limbs, nose, ear or face transplant rejection.

18. The method according to claim 16 wherein the transplant rejection is kidney transplant rejection.

19. The method according to claim 1 or 2 wherein the costimulation blockade therapy is L104EA29YIg administered so to maintain a target trough serum concentration between about 3µg/ml and about 30µg/ml.