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(54) Title: METHODS AND COMPOSITIONS FOR DETERMINING A GRAFT TOLERANT PHENOTYPE IN A SUBJECT

(57) Abstract: Methods are provided for determining whether a subject has a graft tolerant phenotype. In practicing the subject methods, the expression of at least one gene in a sample from the subject, e.g., a blood sample, is assayed to obtain an expression evaluation for the at least one gene. The obtained expression evaluation is then employed to determine whether the subject has a graft tolerant phenotype. Also provided are compositions, systems and kits that find use in practicing the subject methods. The methods and compositions find use in a variety of applications, including the determination of an immunosuppressive therapy regimen.



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## METHODS AND COMPOSITIONS FOR DETERMINING A GRAFT TOLERANT PHENOTYPE IN A SUBJECT

### CROSS-REFERENCE TO RELATED APPLICATIONS

5 Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing date of the United States Provisional Patent Application Serial No. 60/571,471 filed May 14, 2004 and to the filing date of United States Provisional Patent Application Serial No. 60/538,439 filed on January 21, 2004; the disclosures of which are herein incorporated by reference.

### INTRODUCTION

#### Background of the Invention

10 Transplantation of a graft organ or tissue from a donor to a host patient is a feature of certain medical procedures and treatment protocols. Despite efforts  
15 to avoid graft rejection through host-donor tissue type matching, in transplantation procedures where a donor organ is introduced into a host, immunosuppressive therapy is generally required to maintain viability of the donor organ in the host.

A variety of immunosuppressive agents have been employed in  
20 transplantation procedures, including azathioprine, methotrexate, cyclophosphamide, FK-506, rapamycin and corticosteroids. Agents finding increased use in immunosuppressive therapy due to their preferential effect on T-cell mediated reactions are the cyclosporins.

Following transplantation, administration of the immunosuppressive agent  
25 must be continued indefinitely since the benefits of immunosuppressive therapy are reversible and graft rejection may occur once administration of the immunosuppressive agent is discontinued. While use of immunosuppressive agents, such as Cyclosporin A, has been reported to prolong the survival of allogeneic transplants involving skin, heart, kidney, pancreas, bone marrow,  
30 small intestine and lung, use of such agents is not without undesirable side effects. Examples of undesirable side effects include increased risk of development of neoplastic disease conditions, e.g., skin cancer, lymphoma, etc.

While most recipients who discontinue their immunosuppressive treatment following a graft go on to suffer rejection, not all subjects suffer graft rejection. In a few cases, individuals tolerate their graft without immunosuppression, suggesting that immune non-responsiveness can be achieved in clinical practice.

5 The mechanisms of this process are not well understood, but may involve a combination of clonal deletion, clonal anergy and the generation of active regulatory T cells.

Because of the undesirable sides effects and risks of long term immunosuppressive therapy, it would be desirable to be able identify those  
10 individuals who are tolerant to their graft, i.e., graft tolerant, so that immunosuppression could be reduced or even discontinued in those individuals. Of particular interest would be the development of a way to identify graft tolerant individuals without first discontinuing immunosuppressive therapy, thereby avoiding the risk of graft rejection and damage to the graft associated therewith.  
15 The present invention meets this need.

#### Relevant Literature

Publications of interest include: United States Patent Application No. 2003/0104371.

#### 20 SUMMARY OF THE INVENTION

Methods are provided for determining whether a subject has a graft tolerant phenotype. In practicing the subject methods, the expression of at least one gene in a sample from the subject, e.g., a blood sample, is assayed to obtain an expression evaluation for the at least one gene. The obtained expression  
25 evaluation is then employed to determine whether the subject has a graft tolerant phenotype. Also provided are compositions, systems and kits that find use in practicing the subject methods. The subject methods and compositions find use in a variety of applications, including the determination of an immunosuppressive therapy regimen.

30 The subject invention provides methods of determining whether a subject has a graft tolerant phenotype, where the methods include: evaluating expression of at least one gene in a sample from the subject to determine whether the subject has a graft tolerant phenotype. In certain embodiments, the expression of at least one gene is evaluated by assaying the sample for a nucleic acid

transcript of the gene. In certain embodiments, the expression of at least one gene is evaluated by assaying the sample for an expression product of the gene. In certain embodiments, the sample is a blood sample. In certain embodiments, the blood sample is a peripheral blood sample. In certain embodiments, the at least one gene is a gene listed on Tables 1, 2, 3 and/or 4. In certain  
5      embodiments, expression of at least 5 genes from Tables 1, 2, 3 and/or 4 is evaluated. In certain embodiments, expression of at least 50 genes from Tables 1, 2, 3 and/or 4 is evaluated.

Also provided are methods of determining whether a subject has a graft  
10     tolerant phenotype by: (a) obtaining an expression profile for a sample from the subject; and (b) employing the obtained expression profile to determine whether the subject has a graft tolerant phenotype. In certain embodiments, the expression profile is compared to a reference expression profile in the employing step (b). In certain embodiments, the reference expression profile is a  
15     graft tolerant phenotype expression profile. In certain embodiments, the reference expression profile is a graft intolerant phenotype expression profile. In certain embodiments, the expression profile comprises expression measurements for at least 5 different genes. In certain embodiments, the at least 5 different genes are listed in Tables 1, 2, 3 and/or 4. In certain embodiments, the expression profile is  
20     determined using a microarray. In certain embodiments, the microarray is a genomic array. In certain embodiments, the microarray is a proteomic array.

Also provided is a method of managing immunosuppressive therapy in a subject having a graft, where the method includes: (a) evaluating whether the subject has an graft tolerant phenotype; and (b) determining a future  
25     immunosuppressive therapy protocol based on the the evaluation of step (a) to manage immunosuppressive therapy in said subject. In certain embodiments, the evaluating step (a) is by a method as described above. In certain embodiments, the method comprises at least reducing immunosuppression in the subject if said subject is found to have a graft tolerant phenotype. In certain  
30     embodiments, the method comprises discontinuing immunosuppression in the subject if the subject is found to have a graft tolerant phenotype.

Also provided are systems for determining whether a subject has an graft tolerant phenotype, where the systems include: (a) a gene expression evaluation element for evaluating expression of at least one gene in a sample to obtain a

gene expression result; and (b) a phenotype determination element for employing the gene expression result to determine whether a subject has a graft tolerant phenotype. In certain embodiments, the gene expression evaluation element comprises at least one reagent for assaying a sample for a nucleic acid transcript of said gene. In certain embodiments, the gene expression evaluation element  
5 comprises at least one reagent for assaying a sample for an expression product of said gene. In certain embodiments, the gene expression evaluation element comprises an array. In certain embodiments, the at least one gene is a gene listed in Tables 1, 2, 3 and/or 4. In certain embodiments, the phenotype  
10 determination element comprises a reference expression value for the at least one gene. In certain embodiments, the phenotype determination element comprises a reference expression profile that includes a reference expression value for at least one additional gene. In certain embodiments, the reference expression profile is a graft tolerant phenotype expression profile. In certain  
15 embodiments, the reference expression profile is a graft intolerant phenotype expression profile.

Also provided are kits for determining whether a subject has a graft tolerant phenotype, where the kits include: (a) a gene expression evaluation element for evaluating expression of at least one gene in a sample to obtain a  
20 gene expression result; (b) a phenotype determination element for employing the gene expression result to determine whether a subject has a graft tolerant phenotype; and (c) instructions for using said gene expression evaluation and phenotype determination elements in a method, e.g., as described above. In certain embodiments, the gene expression evaluation element comprises at least  
25 one reagent for assaying a sample for a nucleic acid transcript of said gene. In certain embodiments, the gene expression evaluation element comprises at least one reagent for assaying a sample for an expression product of said gene. In certain embodiments, the gene expression evaluation element comprises an array. In certain embodiments, the at least one gene is a gene listed in Tables 1,  
30 2, 3 and/or 4. In certain embodiments, the phenotype determination element comprises a reference expression value for the at least one gene. In certain embodiments, the phenotype determination element comprises a reference expression profile that includes a reference expression value for at least one additional gene. In certain embodiments, the reference expression profile is a

graft tolerant phenotype expression profile. In certain embodiments, the reference expression profile is a graft intolerant phenotype expression profile.

Also provided are reference expression profiles for a phenotype that is one of: (a) graft tolerant; or (b) graft intolerant; wherein the expression profile is recorded on a computer readable medium. In certain embodiments, the expression profile includes at least one of the genes from Tables 1, 2, 3 and/or 4. In certain embodiments, the expression profile is a profile for a phenotype that is allograft tolerant. In certain embodiments, the expression profile is a profile for a phenotype that is graft intolerant.

Also provide by the invention is a collection of reagents for evaluating gene expression, where the collection includes: reagents specific for at least two of the genes of Tables 1, 2, 3 and/or 4. In certain embodiments, the reagents are gene specific primers. In certain embodiments, the collection comprises at least 10 gene specific primers.

Also provided are arrays of probe nucleic acids immobilized on a solid support, where the arrays include: a plurality of probe nucleic acid compositions, wherein each probe nucleic acid composition is specific for a gene whose expression profile is indicative of a graft tolerance, wherein at least two of the probe nucleic acid compositions correspond to genes listed in Tables 1, 2, 3 and/or 4.

Also provided are kits that include at least one of: (a) an array according as described above; and (b) a collection of gene specific primers as described above, where in certain embodiments the kits include both of these components.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides Table 1, which lists 200 representative genes whose expression differs in OT patients relative to those with chronic graft injury.

Figure 2 provides Table 2, which is an analysis of 591 genes differentially expressed in PBL samples associated with tolerance in renal transplantation.

Figure 3 provides Table 3, which shows the gene expression differences between OT and CR samples by KEGG families.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for determining whether a subject has a graft tolerant phenotype. In practicing the subject methods, the expression of at least one gene in a sample from the subject, e.g., a blood sample, is assayed to obtain an expression evaluation for the at least one gene. The obtained expression evaluation is then employed to determine whether the subject has a graft tolerant phenotype. Also provided are compositions, systems and kits that find use in practicing the subject methods. The methods and compositions find use in a variety of applications, including the determination of an immunosuppressive therapy regimen.

Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Methods recited herein may be carried out in any order of the recited events which is logically possible, as well as the recited order of events.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar

or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described.

5 All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

10 It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

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The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication 20 provided may be different from the actual publication dates which may need to be independently confirmed.

25 As summarized above, the subject invention is directed to methods of determining whether a subject has a graft tolerant phenotype, as well as reagents and kits for use in practicing the subject methods. In further describing the invention, the subject methods are described first, followed by a review of the reagents and kits for use in practicing the subject methods.

#### METHODS OF DETERMINING WHETHER A SUBJECT HAS A GRAFT TOLERANT PHENOTYPE

30

The subject invention provides methods of determining whether a patient or subject has a graft tolerant phenotype. By graft tolerant phenotype is meant that the subject does not reject a graft organ, tissue or cell(s) that has been introduced into/onto the subject. In other words, the subject tolerates or maintains



the organ, tissue or cell(s) that has been transplanted to it. As is known in the transplantation field, the graft organ, tissue or cell(s) may be allogeneic or xenogeneic, such that the grafts may be allografts or xenografts. A feature of the graft tolerant phenotype detected or identified by the subject methods is that it is a phenotype which occurs without immunosuppressive therapy, i.e., it is present in a host that is not undergoing immunosuppressive therapy such that immunosuppressive agents are not being administered to the host.

In practicing the subject methods, a subject or patient sample, e.g., cells or collections thereof, e.g., tissues, is assayed to determine whether the host from which the assayed sample was obtained is graft tolerant, i.e., has a graft tolerant phenotype. Accordingly, the first step of the subject methods is to obtain a suitable sample from the subject or patient of interest, i.e., a patient on immunosuppressive therapy and having at least one graft, e.g., allograft. The sample is derived from any initial suitable source, where sample sources of interest include, but are not limited to, many different physiological sources, e.g., CSF, urine, saliva, tears, tissue derived samples, e.g., homogenates, and blood or derivatives thereof.

In certain embodiments, a suitable initial source for the patient sample is blood. As such, the sample employed in the subject assays of these embodiments is generally a blood-derived sample. The blood derived sample may be derived from whole blood or a fraction thereof, e.g., serum, plasma, etc., where in many embodiments the sample is derived from blood cells harvested from whole blood. Of particular interest as a sample source are peripheral blood lymphocytes (PBL). Any convenient protocol for obtaining such samples may be employed, where suitable protocols are well known in the art.

In practicing the subject methods, the sample is assayed to obtain an expression evaluation, e.g., expression profile, for one or more genes, where the term expression profile is used broadly to include a genomic expression profile, e.g., an expression profile of nucleic acid transcripts, e.g., mRNAs, of the one or more genes of interest, or a proteomic expression profile, e.g., an expression profile of one or more different proteins, where the proteins/polypeptides are expression products of the one or more genes of interest. As such, in certain embodiments the expression of only one gene is evaluated. In yet other embodiments, the expression of two or more, e.g., about 5 or more, about 10 or

more, about 15 or more, about 25 or more, about 50 or more, about 100 or more, about 200 or more, etc., genes is evaluated. Accordingly, in the subject methods, the expression of at least one gene in a sample is evaluated. In certain  
5 transcriptosome, as that term is employed in the art. See e.g., Gomes et al., Blood (2001 Jul 1) 98(1):93-9.

In generating the expression profile, in many embodiments a sample is assayed to generate an expression profile that includes expression data for at least one gene/protein, usually a plurality of genes/proteins, where by plurality is  
10 meant at least two different genes/proteins, and often at least about 5, typically at least about 10 and more usually at least about 20 different genes/proteins or more, such as 50 or more, 100 or more, etc.

In the broadest sense, the expression evaluation may be qualitative or quantitative. As such, where detection is qualitative, the methods provide a  
15 reading or evaluation, e.g., assessment, of whether or not the target analyte, e.g., nucleic acid or expression product, is present in the sample being assayed. In yet other embodiments, the methods provide a quantitative detection of whether the target analyte is present in the sample being assayed, i.e., an evaluation or assessment of the actual amount or relative abundance of the target analyte,  
20 e.g., nucleic acid in the sample being assayed. In such embodiments, the quantitative detection may be absolute or, if the method is a method of detecting two or more different analytes, e.g., target nucleic acids, in a sample, relative. As such, the term "quantifying" when used in the context of quantifying a target analyte, e.g., nucleic acid(s), in a sample can refer to absolute or to relative  
25 quantification. Absolute quantification may be accomplished by inclusion of known concentration(s) of one or more control analytes and referencing the detected level of the target analyte with the known control analytes (e.g., through generation of a standard curve). Alternatively, relative quantification can be accomplished by comparison of detected levels or amounts between two or more  
30 different target analytes to provide a relative quantification of each of the two or more different analytes, e.g., relative to each other.

Genes/proteins of interest are genes/proteins that are differentially expressed or present at different levels in graft tolerant and graft intolerant individuals. Representative genes/proteins of interest in certain embodiments

include, but are not limited to, the genes/proteins provided in Table 1. (Note that for Table 1, the exact sequence of the clone identified in the table can be determined through the NCBI Entrez nucleotide database at located at the website produced by placing "http://www." before:

- 5 "ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&db=nucleotide"; the sequence for a specific clone is then obtained by entering the clone ID in quotes as the search term).

In certain embodiments, at least one of the genes/proteins in the prepared expression profile is from Tables 1, 2, 3 and/or 4, where the expression profile  
10 may include expression data for 5, 10, 20, 50, 75 or more of, including all of, the genes/proteins listed in Tables 1, 2, 3 and/or 4. The number of different genes/proteins whose expression and/or quantity data, i.e., presence or absence of expression, as well as expression/quantity level, that are included in the expression profile that is generated may vary, but may be at least 2, and in many  
15 embodiments ranges from 2 to about 100 or more, sometimes from 3 to about 75 or more, including from about 4 to about 70 or more.

In certain embodiments, the expression profile obtained is a genomic or nucleic acid expression profile, where the amount or level of one or more nucleic acids in the sample is determined, e.g., the nucleic acid transcript of the gene of  
20 interest. In these embodiments, the sample that is assayed to generate the expression profile employed in the diagnostic methods is one that is a nucleic acid sample. The nucleic acid sample includes a plurality or population of distinct nucleic acids that includes the expression information of the phenotype determinative genes of interest of the cell or tissue being diagnosed. The nucleic  
25 acid may include RNA or DNA nucleic acids, e.g., mRNA, cRNA, cDNA etc., so long as the sample retains the expression information of the host cell or tissue from which it is obtained. The sample may be prepared in a number of different ways, as is known in the art, e.g., by mRNA isolation from a cell, where the isolated mRNA is used as is, amplified, employed to prepare cDNA, cRNA, etc.,  
30 as is known in the differential expression art. The sample is typically prepared from a cell or tissue harvested from a subject to be diagnosed, e.g., via biopsy of tissue, using standard protocols, where cell types or tissues from which such nucleic acids may be generated include any tissue in which the expression

pattern of the to be determined phenotype exists, including, but not limited to, peripheral blood lymphocyte cells, etc, as reviewed above.

The expression profile may be generated from the initial nucleic acid sample using any convenient protocol. While a variety of different manners of generating expression profiles are known, such as those employed in the field of differential gene expression analysis, one representative and convenient type of protocol for generating expression profiles is array-based gene expression profile generation protocols. Such applications are hybridization assays in which a nucleic acid that displays "probe" nucleic acids for each of the genes to be assayed/profiled in the profile to be generated is employed. In these assays, a sample of target nucleic acids is first prepared from the initial nucleic acid sample being assayed, where preparation may include labeling of the target nucleic acids with a label, e.g., a member of signal producing system. Following target nucleic acid sample preparation, the sample is contacted with the array under hybridization conditions, whereby complexes are formed between target nucleic acids that are complementary to probe sequences attached to the array surface. The presence of hybridized complexes is then detected, either qualitatively or quantitatively. Specific hybridization technology which may be practiced to generate the expression profiles employed in the subject methods includes the technology described in U.S. Patent Nos.: 5,143,854; 5,288,644; 5,324,633; 5,432,049; 5,470,710; 5,492,806; 5,503,980; 5,510,270; 5,525,464; 5,547,839; 5,580,732; 5,661,028; 5,800,992; the disclosures of which are herein incorporated by reference; as well as WO 95/21265; WO 96/31622; WO 97/10365; WO 97/27317; EP 373 203; and EP 785 280. In these methods, an array of "probe" nucleic acids that includes a probe for each of the phenotype determinative genes whose expression is being assayed is contacted with target nucleic acids as described above. Contact is carried out under hybridization conditions, e.g., stringent hybridization conditions, and unbound nucleic acid is then removed.

30

The term "stringent assay conditions" as used herein refers to conditions that are compatible to produce binding pairs of nucleic acids, e.g., surface bound and solution phase nucleic acids, of sufficient complementarity to provide for the desired level of specificity in the assay while being less compatible to the

formation of binding pairs between binding members of insufficient complementarity to provide for the desired specificity. Stringent assay conditions are the summation or combination (totality) of both hybridization and wash conditions.

5       The term "stringent assay conditions" as used herein refers to conditions that are compatible to produce binding pairs of nucleic acids, e.g., surface bound and solution phase nucleic acids, of sufficient complementarity to provide for the desired level of specificity in the assay while being less compatible to the formation of binding pairs between binding members of insufficient  
10 complementarity to provide for the desired specificity. Stringent assay conditions are the summation or combination (totality) of both hybridization and wash conditions.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization (e.g., as in array, Southern  
15 or Northern hybridizations) are sequence dependent, and are different under different experimental parameters. Stringent hybridization conditions that can be used to identify nucleic acids within the scope of the invention can include, e.g., hybridization in a buffer comprising 50% formamide, 5×SSC, and 1% SDS at 42°C, or hybridization in a buffer comprising 5×SSC and 1% SDS at 65°C, both  
20 with a wash of 0.2×SSC and 0.1% SDS at 65°C. Exemplary stringent hybridization conditions can also include a hybridization in a buffer of 40% formamide, 1 M NaCl, and 1% SDS at 37°C, and a wash in 1×SSC at 45°C. Alternatively, hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1×SSC/0.1% SDS  
25 at 68°C can be employed. Yet additional stringent hybridization conditions include hybridization at 60°C or higher and 3×SSC (450 mM sodium chloride/45 mM sodium citrate) or incubation at 42°C in a solution containing 30% formamide, 1M NaCl, 0.5% sodium sarcosine, 50 mM MES, pH 6.5. Those of ordinary skill will readily recognize that alternative but comparable hybridization and wash  
30 conditions can be utilized to provide conditions of similar stringency. In certain embodiments, the stringency of the wash conditions that set forth the conditions which determine whether a nucleic acid is specifically hybridized to a surface bound nucleic acid. Wash conditions used to identify nucleic acids may

include, e.g.: a salt concentration of about 0.02 molar at pH 7 and a temperature of at least about 50°C or about 55°C to about 60°C; or, a salt concentration of about 0.15 M NaCl at 72°C for about 15 minutes; or, a salt concentration of about 0.2×SSC at a temperature of at least about 50°C or about 55°C to about 60°C for about 15 to about 20 minutes; or, the hybridization complex is washed twice with a solution with a salt concentration of about 2×SSC containing 0.1% SDS at room temperature for 15 minutes and then washed twice by 0.1×SSC containing 0.1% SDS at 68°C for 15 minutes; or, equivalent conditions. Stringent conditions for washing can also be, e.g., 0.2×SSC/0.1% SDS at 42°C.

10 A specific example of stringent assay conditions is rotating hybridization at 65°C in a salt based hybridization buffer with a total monovalent cation concentration of 1.5 M (e.g., as described in U.S. Patent Application No. 09/655,482 filed on September 5, 2000, the disclosure of which is herein incorporated by reference) followed by washes of 0.5×SSC and 0.1×SSC at room temperature.

Stringent assay conditions are hybridization conditions that are at least as stringent as the above representative conditions, where a given set of conditions are considered to be at least as stringent if substantially no additional binding complexes that lack sufficient complementarity to provide for the desired specificity are produced in the given set of conditions as compared to the above specific conditions, where by "substantially no more" is meant less than about 5-fold more, typically less than about 3-fold more. Other stringent hybridization conditions are known in the art and may also be employed, as appropriate.

25 The resultant pattern of hybridized nucleic acid provides information regarding expression for each of the genes that have been probed, where the expression information is in terms of whether or not the gene is expressed and, typically, at what level, where the expression data, i.e., expression profile (e.g., in the form of a transcriptosome), may be both qualitative and quantitative.

Alternatively, non-array based methods for quantitating the levels of one or more nucleic acids in a sample may be employed, including quantitative PCR, and the like.

Where the expression profile is a protein expression profile, any convenient protein quantitation protocol may be employed, where the levels of

one or more proteins in the assayed sample are determined. Representative methods include, but are not limited to: proteomic arrays, flow cytometry, standard immunoassays (e.g., ELISA assays), etc.

Following obtainment of the expression profile from the sample being  
5 assayed, the expression profile is compared with a reference or control profile to determine the particular graft tolerant/intolerant phenotype of the cell or tissue, and therefore host, from which the sample was obtained/derived. The terms "reference" and "control" as used herein mean a standardized pattern of gene expression or levels of expression of certain genes to be used to interpret the  
10 expression signature of a given patient and assign a graft tolerant/intolerant phenotype thereto. The reference or control profile may be a profile that is obtained from a cell/tissue known to have the desired phenotype, e.g., tolerant phenotype, and therefore may be a positive reference or control profile. In addition, the reference/control profile may be from a cell/tissue known to not have  
15 the desired phenotype, e.g., an intolerant phenotype, and therefore be a negative reference/control profile.

In certain embodiments, the obtained expression profile is compared to a single reference/control profile to obtain information regarding the phenotype of the cell/tissue being assayed. In yet other embodiments, the obtained expression  
20 profile is compared to two or more different reference/control profiles to obtain more in depth information regarding the phenotype of the assayed cell/tissue. For example, the obtained expression profile may be compared to a positive and negative reference profile to obtain confirmed information regarding whether the cell/tissue has the phenotype of interest.

25 The comparison of the obtained expression profile and the one or more reference/control profiles may be performed using any convenient methodology, where a variety of methodologies are known to those of skill in the array art, e.g., by comparing digital images of the expression profiles, by comparing databases of expression data, etc. Patents describing ways of comparing expression profiles  
30 include, but are not limited to, U.S. Patent Nos. 6,308,170 and 6,228,575, the disclosures of which are herein incorporated by reference. Methods of comparing expression profiles are also described above.

The comparison step results in information regarding how similar or dissimilar the obtained expression profile is to the control/reference profile(s),

which similarity/dissimilarity information is employed to determine the phenotype of the cell/tissue being assayed. For example, similarity with a positive control indicates that the assayed cell/tissue has a tolerant phenotype. Likewise, similarity with a negative control indicates that the assayed cell/tissue has an intolerant phenotype.

Depending on the type and nature of the reference/control profile(s) to which the obtained expression profile is compared, the above comparison step yields a variety of different types of information regarding the cell/tissue that is assayed. As such, the above comparison step can yield a positive/negative determination of a tolerant phenotype of an assayed cell/tissue. In many embodiments, the above-obtained information about the cell/tissue being assayed is employed to diagnose a host, subject or patient with respect to that host's graft tolerance, as described above.

The subject methods further find use in pharmacogenomic applications. In these applications, a subject/host/patient is first diagnosed for the presence of absence of the graft tolerant phenotype using a protocol such as the diagnostic protocol described in the preceding section. The subject is then treated using a protocol whose suitability is determined using the results of the diagnosis step. More specifically, where the identified phenotype is tolerant, a protocol that may include a reduced level of immunosuppression (i.e., immunosuppression at a level less than that which is indicated for patients not known to be graft tolerant), or no immunosuppression, may be employed to manage/treat the subject. Alternatively, where a patient is identified as having an intolerant phenotype, full immunosuppressive protocols are then employed/continued.

In many embodiments, a host is screened for the presence of a graft tolerant phenotype following receipt of a graft or transplant. The host may be screened once or serially following transplant receipt, e.g., weekly, monthly, bimonthly, half-yearly, yearly, etc., as long as the host is on immunosuppressive therapy. In certain embodiments, monitoring of the host expression profile even after immunosuppressive therapy has been reduced or discontinued is conducted to determine whether the host has maintained the tolerogenic expression profile and may continue for the lifetime of the host.



## DATABASES OF EXPRESSION PROFILES OF PHENOTYPE DETERMINATIVE GENES

Also provided are databases of expression profiles of graft tolerant phenotype determinative genes. Such databases will typically comprise  
5 expression profiles of various cells/tissues having graft tolerant phenotypes, negative expression profiles, etc., where such profiles are further described below.

The expression profiles and databases thereof may be provided in a variety of media to facilitate their use. "Media" refers to a manufacture that  
10 contains the expression profile information of the present invention. The databases of the present invention can be recorded on computer readable media, e.g. any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such  
15 as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. One of skill in the art can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising a recording of the present database information. "Recorded" refers to a process for storing  
20 information on computer readable medium, using any such methods as known in the art. Any convenient data storage structure may be chosen, based on the means used to access the stored information. A variety of data processor programs and formats can be used for storage, e.g. word processing text file, database format, etc.

25 As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the information of the present invention. The minimum hardware of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any  
30 one of the currently available computer-based system are suitable for use in the present invention. The data storage means may comprise any manufacture comprising a recording of the present information as described above, or a memory access means that can access such a manufacture.

A variety of structural formats for the input and output means can be used to input and output the information in the computer-based systems of the present invention. One format for an output means ranks expression profiles possessing varying degrees of similarity to a reference expression profile. Such presentation  
5 provides a skilled artisan with a ranking of similarities and identifies the degree of similarity contained in the test expression profile.

#### REAGENTS AND KITS

10 Also provided are reagents and kits thereof for practicing one or more of the above-described methods. The subject reagents and kits thereof may vary greatly. Reagents of interest include reagents specifically designed for use in production of the above-described expression profiles of phenotype determinative genes, i.e., a gene expression evaluation element made up of one or more  
15 reagents.

One type of such reagent is an array of probe nucleic acids in which the phenotype determinative genes of interest are represented. A variety of different array formats are known in the art, with a wide variety of different probe structures, substrate compositions and attachment technologies. Representative  
20 array structures of interest include those described in U.S. Patent Nos.: 5,143,854; 5,288,644; 5,324,633; 5,432,049; 5,470,710; 5,492,806; 5,503,980; 5,510,270; 5,525,464; 5,547,839; 5,580,732; 5,661,028; 5,800,992; the disclosures of which are herein incorporated by reference; as well as WO 95/21265; WO 96/31622; WO 97/10365; WO 97/27317; EP 373 203; and EP 785  
25 280.

In many embodiments, the arrays include probes for at least 1 of the genes listed in Tables 1, 2, 3 and/or 4. In certain embodiments, the number of genes that are from Tables 1, 2, 3 and/or 4 that is represented on the array is at least 5, at least 10, at least 25, at least 50, at least 75 or more, including all of the  
30 genes listed in Tables 1, 2, 3 and/or 4. The subject arrays may include only those genes that are listed in Tables 1, 2, 3 and/or 4, or they may include additional genes that are not listed in Tables 1, 2, 3 and/or 4. Where the subject arrays include probes for such additional genes, in certain embodiments the number % of additional genes that are represented does not exceed about 50%, usually

does not exceed about 25 %. In many embodiments where additional "non-Table 1" genes are included, a great majority of genes in the collection are phenotype determinative genes, where by great majority is meant at least about 75%, usually at least about 80 % and sometimes at least about 85, 90, 95 % or higher, including embodiments where 100% of the genes in the collection are phenotype determinative genes.

Another type of reagent that is specifically tailored for generating expression profiles of phenotype determinative genes is a collection of gene specific primers that is designed to selectively amplify such genes. Gene specific primers and methods for using the same are described in U.S. Patent No. 5,994,076, the disclosure of which is herein incorporated by reference. Of particular interest are collections of gene specific primers that have primers for at least 1 of the genes listed in one Tables 1, 2, 3 and/or 4, often a plurality of these genes, e.g., at least 2, 5, 10, 15 or more. In certain embodiments, the number of genes that are from Tables 1, 2, 3 and/or 4 that have primers in the collection is at least 5, at least 10, at least 25, at least 50, at least 75 or more, including all of the genes listed in Tables 1, 2, 3 and/or 4. The subject gene specific primer collections may include only those genes that are listed in Tables 1, 2, 3 and/or 4, or they may include primers for additional genes that are not listed in Tables 1, 2, 3 and/or 4. Where the subject gene specific primer collections include primers for such additional genes, in certain embodiments the number % of additional genes that are represented does not exceed about 50%, usually does not exceed about 25 %. In many embodiments where additional "non-Table 1", "non-Table 2", "non-Table 3" or "non-Table 4" genes are included, a great majority of genes in the collection are phenotype determinative genes, where by great majority is meant at least about 75%, usually at least about 80 % and sometimes at least about 85, 90, 95 % or higher, including embodiments where 100% of the genes in the collection are phenotype determinative genes.

The kits of the subject invention may include the above-described arrays and/or gene specific primer collections. The kits may further include one or more additional reagents employed in the various methods, such as primers for generating target nucleic acids, dNTPs and/or rNTPs, which may be either premixed or separate, one or more uniquely labeled dNTPs and/or rNTPs, such as biotinylated or Cy3 or Cy5 tagged dNTPs, gold or silver particles with different

scattering spectra, or other post synthesis labeling reagent, such as chemically active derivatives of fluorescent dyes, enzymes, such as reverse transcriptases, DNA polymerases, RNA polymerases, and the like, various buffer mediums, e.g. hybridization and washing buffers, prefabricated probe arrays, labeled probe  
5 purification reagents and components, like spin columns, etc., signal generation and detection reagents, e.g. streptavidin-alkaline phosphatase conjugate, chemifluorescent or chemiluminescent substrate, and the like.

The subject kits may also include a phenotype determination element, which element is, in many embodiments, a reference or control expression profile  
10 that can be employed, e.g., by a suitable computing means, to make a phenotype determination based on an "input" expression profile, e.g., that has been determined with the above described gene expression evaluation element. Representative phenotype determination elements include databases of expression profiles, e.g., reference or control profiles, as described above.

15 In addition to the above components, the subject kits will further include instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of  
20 paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g., diskette, CD, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a removed site. Any convenient means may  
25 be present in the kits.

## SYSTEMS

Also provided are systems for practicing one or more of the above-  
30 described methods. The subject systems may vary greatly, but typically include at least a gene expression evaluation element, e.g., one or more reagents, and a phenotype determination element.

Reagents of interest include reagents specifically designed for use in production of the above-described expression profiles of phenotype determinative

genes, i.e., a gene expression evaluation element made up of one or more reagents. One type of such reagent is an array of probe nucleic acids in which the phenotype determinative genes of interest are represented. A variety of different array formats are known in the art, with a wide variety of different probe structures, substrate compositions and attachment technologies. Representative  
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In many embodiments, the arrays include probes for at least 1 of the genes listed in Tables 1, 2, 3 and/or 4. In certain embodiments, the number of genes that are from Tables 1, 2, 3 and/or 4 that is represented on the array is at  
15 least 5, at least 10, at least 25, at least 50, at least 75 or more, including all of the genes listed in Tables 1, 2, 3 and/or 4. The subject arrays may include only those genes that are listed in Tables 1, 2, 3 and/or 4, or they may include additional genes that are not listed in Tables 1, 2, 3 and/or 4. Where the subject arrays include probes for such additional genes, in certain embodiments the number %  
20 of additional genes that are represented does not exceed about 50%, usually does not exceed about 25 %. In many embodiments where additional "non-Table 1", "non-Table 2", "non-Table 3" or "non-Table 4" genes are included, a great majority of genes in the collection are phenotype determinative genes, where by great majority is meant at least about 75%, usually at least about 80 % and  
25 sometimes at least about 85, 90, 95 % or higher, including embodiments where 100% of the genes in the collection are phenotype determinative genes.

Another type of reagent that is specifically tailored for generating expression profiles of phenotype determinative genes is a collection of gene specific primers that is designed to selectively amplify such genes. Gene specific  
30 primers and methods for using the same are described in U.S. Patent No. 5,994,076, the disclosure of which is herein incorporated by reference. Of particular interest are collections of gene specific primers that have primers for at least 1 of the genes listed in one Tables 1, 2, 3 and/or 4, often a plurality of these genes, e.g., at least 2, 5, 10, 15 or more. In certain embodiments, the number of

genes that are from Tables 1, 2, 3 and/or 4 that have primers in the collection is at least 5, at least 10, at least 25, at least 50, at least 75 or more, including all of the genes listed in Tables 1, 2, 3 and/or 4. The subject gene specific primer collections may include only those genes that are listed in Tables 1, 2, 3 and/or 4, or they may include primers for additional genes that are not listed in Tables 1, 2, 3 and/or 4. Where the subject gene specific primer collections include primers for such additional genes, in certain embodiments the number % of additional genes that are represented does not exceed about 50%, usually does not exceed about 25 %. In many embodiments where additional "non-Table 1", "non-Table 2", "non-Table 3" or "non-Table 4" genes are included, a great majority of genes in the collection are phenotype determinative genes, where by great majority is meant at least about 75%, usually at least about 80 % and sometimes at least about 85, 90, 95 % or higher, including embodiments where 100% of the genes in the collection are phenotype determinative genes.

The systems of the subject invention may include the above-described arrays and/or gene specific primer collections. The systems may further include one or more additional reagents employed in the various methods, such as primers for generating target nucleic acids, dNTPs and/or rNTPs, which may be either premixed or separate, one or more uniquely labeled dNTPs and/or rNTPs, such as biotinylated or Cy3 or Cy5 tagged dNTPs, gold or silver particles with different scattering spectra, or other post synthesis labeling reagent, such as chemically active derivatives of fluorescent dyes, enzymes, such as reverse transcriptases, DNA polymerases, RNA polymerases, and the like, various buffer mediums, e.g. hybridization and washing buffers, prefabricated probe arrays, labeled probe purification reagents and components, like spin columns, etc., signal generation and detection reagents, e.g. streptavidin-alkaline phosphatase conjugate, chemifluorescent or chemiluminescent substrate, and the like.

The systems may also include a phenotype determination element, which element is, in many embodiments, a reference or control expression profile that can be employed, e.g., by a suitable computing means, to make a phenotype determination based on an "input" expression profile, e.g., that has been determined with the above described gene expression evaluation element. Representative phenotype determination elements include databases of expression profiles, e.g., reference or control profiles, as described above.

The following examples are offered by way of illustration and not by way of limitation.

5

## EXPERIMENTAL

### ***I. Methods and Materials***

#### *Patient selection*

10 The protocol was accepted by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks. All patients and normal individuals were informed of the protocol and signed an informed consent. IRB approval was also obtained to perform DNA microarrays on all patient samples at Stanford University. The 42 adult participants in this  
15 study were classified into four groups for comparison: operationally tolerant (OT), chronic rejection (CR), stable, or normal.

Operationally tolerant patients were kidney graft recipients with stable graft function who had not taken any immunosuppressive drugs for at least three years (N=5) with a mean drug free time period was  $8 \pm 3.3$  years [range 3-12]. Eleven  
20 additional patients who received low doses ( $\leq 10$ mg/day) of Prednisone® or Cortancyl® as the only immunosuppressive drug for at least 3 years before analysis were also classified Op-Tol (or OT). The mean time period of immunosuppression was  $9.8 \pm 4.4$  years [range 4-15]. The renal function of drug-free tolerant patients and minimally immunosuppressed patients remained stable  
25 and blood creatinemia was not significantly different before and after immunosuppression interruption or modification.

Patients with chronic rejection, patients with stable function under immunosuppressive maintenance regimen and normal healthy individuals are used as control groups. The chronic rejection group included patients with  
30 histological chronic rejection lesions (CR group) associated or not to an allograft glomerulopathy and with a degradation of their renal function. Inclusion was evidenced by clinical indices indicative of end-stage renal disease requiring hemodialysis (N=3) or serum creatine and proteinuria  $> 1.5$  g/day and

creatinemia  $>60\mu\text{mol}$  and by evidence of rejection on kidney biopsies. The stable function group included patients with stable graft function and under a bitherapy immunosuppressive regimen including a calcineurin inhibitor (StaCNI+) or not (StaCNI-). The inclusion of patients with stable function under standard IS

5 required all following criteria: a) Patients over 18 years old; b) received kidney transplant between 5 to 8 years; c) patients without acute rejection history; d) patients with good immunosuppressive treatment compliance (as judged by medication intake and medical survey); e) patients under standard dual immunosuppressive maintenance treatment including: CsA or FK506 and

10 mycophenolate mofetil, azathioprine or corticosteroids; f) stable kidney function (i.e.  $\pm 25\%$  of the mean value of the year that preceded the test) and with a Nankivell calculated clearance (\*please provide reference) above 40 ml/min after at least five year of transplantation and at one and three months after the inclusion in the study; g) proteinuria  $< 1.5\text{g /day}$  and creatinemia  $< 160\mu\text{mol}$  after

15 at least five year of transplantation and at one and three months after the inclusion in the study; h) blood trough-levels within the International standard range (75-250 ng/ml) for Neoral and 5-10 ng/ml for Prograf). Normal individuals included healthy individuals were 25 to 60 years old (N=3) with a normal blood formula and no known infectious pathology for at least 6 months prior to the

20 study.

#### *RNA extraction from whole samples*

Blood, harvested in EDTA tubes, was obtained from a peripheral vein or arterio-venous fistula. Ionogramm, WBC, formula and CNI blood levels (RIA) were performed on each sample. Peripheral blood leukocytes (PBL) were

25 separated on a Ficoll layer (Eurobio, Les Ulis, France) and frozen in Trizol reagent (Invitrogen, Life Technologies, San Diego, CA) for RNA extraction.

#### *Microarray hybridization and data analysis*

The cDNA microarrays were processed using protocols as previously described by our laboratory using 2  $\mu\text{g}$  RNA in each channel (Sarwal, et al., N. Engl. J. Med. (2003) 349: 125-138). A "common reference" RNA pool (Perou, et

30 al., Nature (2000) 406:747-752) was used as an internal standard with each hybridization. Sample or reference RNA was subjected to two successive rounds of amplification before hybridization to the cDNA microarrays using a protocol



essentially as described by Wang et al. (Wang, et al., Nat. Biotechn. (2000) 18:457-459). Hybridized microarrays were scanned using GenePix 4000 (Axon Instruments, Union City, CA) and fluorescent images were analyzed with the GenePix Pro software package. Each microarray contains approximately 30,000  
5 cDNAs representing over 12,400 unique genes.

Array data for the 42 samples were stored in the Stanford Microarray database (Sherlock, et al., Nuc. Acids Res. (2001)29:152-155) and gene lists filtered at retrieval. The hybridized raw data of 30,000 cDNA spots was manually examined for single spots or blemishes (Alizadeh et al., Cold Spring Harbor  
10 Symp. Quant. Biol. (1999) 64:71-78). These spots or blemishes were flagged and removed, generating a data file with 22,090 clones under low stringency (70% representative data and signal/noise ratio of expression measurements > 1.5). Further high-stringency data filtering, choosing only clones with 90% representative data and a 4-fold expression level difference at >2 arrays in the  
15 study set generated a file of 2,083 cDNA clones representing 1,846 unique genes. This higher-stringency gene set was further analyzed for potential biomarkers in this study and in a study of the T-cell expression profiles from the same patient groups. Cluster (V3.0) generated hierarchical clusters of the samples measuring similarity of expression of the genes and similarity across  
20 arrays which are visualized with the TreeView program (Eisen et al., Proc. Nat'l Acad. Sci USA (1998) 95:14863-8); (See also the website found by positioning "http://www." in front of "microarrays.org/software." Significance of gene expression differences among the groups of samples was performed using Statistical Analysis of Microarray (SAM) (Tusher et al., Proc. Nat'l Acad. Sci USA  
25 (2001) 98:5116-21) using a false discovery rate threshold of <5%.

#### *Taqman-based Quantitative RT-PCR*

One hundred nanograms of amplified RNA were subjected to real-time PCR analysis to assess quantization of marker genes identified in the microarray  
30 screen. Assay-on-demand primer sets and Taqman EZ RT-PCR core reagents (Applied Biosystems, Foster City, CA) were used according to manufacturer's directions in 20  $\mu$ L reaction volumes using the 7900HT Sequence Detection System. Transcript levels were calculated according to the  $2^{-\Delta\Delta C_t}$  method of

the manufacturer (1997). The labeled TaqMan® probes (Assays-on-demand, Applied Biosystems) used for these assays include:

- β-Actin: 5'FAM- TCGCCTTTGCCGATCCGCCGCCCGT-NFQ3' (SEQ ID NO:01);  
 5 TK1: 5'FAM-ACACATGACCGGAACACCATGGAGG-NFQ3' (SEQ ID NO:02);  
 C1S: 5'FAM- TTGCCACAGACATAAATGAATGCAC-NFQ3' (SEQ ID NO:03);  
 EV12A: 5'FAM- AAGCATTTTGTAAAGATTGCCAAGTA-NFQ3' (SEQ ID NO:04);  
 MMP2: 5'FAM- GCAGGGCGGCGGTACAGCTACTTC-NFQ3' (SEQ ID NO:05);  
 MAPK9: 5'FAM-GATTGTTTGTGCTGCATTTGATACA-NFQ3' (SEQ ID NO:06);  
 10 TCEB3: 5'FAM-AGACATTCTTGCGGAGACTGGGGTT-NFQ3' (SEQ ID NO:07);  
 KIFC3: 5'FAM-CAAGGCCGAGATAGGCCAGGCCATC-NFQ3' (SEQ ID NO:08);  
 PRAME: 5'FAM-CGTTTGTGGGGTTCCATTCAGAGCC-NFQ3' (SEQ ID NO:09);  
 DLK1: 5'FAM-GATCAACGGCTCCCCCTGCCAGCAC-NFQ3' (SEQ ID NO:10);  
 CYR61: 5'FAM-CTGCAGAGCTCAGTCAGAGGGCAGA-NFQ3' (SEQ ID NO:11);  
 15 HPRT: 5'FAM-GGTCAAGGTCGCAAGCTTGCTGGTG-NFQ3' (SEQ ID NO:12);  
 GAPDH: 5'FAM-GGGCGCCTGGTCACCAGGGCTGCTT-NFQ3' (SEQ ID NO:13).

## II. Results

- 20 We compared gene expression profiles from RNA extracted using unfractionated peripheral blood samples from 16 operationally tolerant (OT) patients (4 without any treatment, 12 with low dose steroid monotherapy) with that from 11 patients with signs of chronic rejection (CR), 12 transplant patients with stable function (S), and from three normal controls (N; healthy un-grafted  
 25 individuals). Unsupervised analysis revealed that the majority of the CR and OT samples segregate into separate groups. In addition, all of the normal samples and most of the stable samples were observed to cluster with the CR patient groups. A recent reference study of whole gene expression profiling using the same cDNA array platform employed in this study, Whitney et al. (Whitney et  
 30 al., Proc. Nat'l Acad. Sci. USA (2003) 100:1896-901) made at least two key discoveries that assisted in the interpretation of our expression results. First, blood-based gene expression profiles from the same individual were quite stable over time; second, genes from a common cell origin in PBL samples tend to

tightly cluster as evidenced by correlation analysis between cell counts and expression measurements.

Separation of the sample groups was primarily driven by three gene clusters identified as A, B and C with a large percentage of the genes expressed at higher levels in the blood from OT patients than others. By identifying cell signaling genes tightly clustered to ones of limited tissue expression, one can then infer the putative cell origin, and immune activation status of the primary clusters. In addition, genes might be highly differentially expressed between the two phenotypes of interest, OT and CR in this study.

Because the OT and CR patients largely group into separate branches in the unsupervised expression analysis, close examination of the observed gene clusters provided insight into potential functional roles for the major genes involved in the tolerance signature. Both pro- and anti-apoptotic genes were identified as highly differentially expressed between OT and CR patients.

Expression patterns of the anti-apoptotic genes TOSO, CASP7 and FIAM2 in Cluster A parallel both T- and B-cell specific genes including the T-cell receptor (TCR), HLA class II genes, several immunoglobulin genes (IGL, IG1L1, IGJ3, IGHM). This cluster also contained a number of key cytokines that play a role in the immune signaling: (CCL20, CCL19, CCL8 and CCR7). This cluster also contained the T-cell activation antigen CD27, which serves as a regulator of B-cell activation is the receptor for the pro-apoptotic CD27-binding protein (SIVA). In contrast, the pro-apoptosis genes RGN and RIS1 co-clustered with platelet and T-suppressor cell specific genes CD9 and GFBB2 respectively. It is this cluster, Cluster C, which contained a number of immune response genes (C1s, IER3, and CD59) and several cell cycle regulated genes (TK1, CCNA2, CDC20, CCND2, CCNA2, CCND1, MCM2 and CDC6) and GAGE4, an antigen of unknown function that is recognized by cytotoxic T-cells. IER3 (intermediate early response 3) functions to protect cells from Fas- or tumor necrosis factor (TNF $\alpha$ )-induced apoptosis. CD59, also known as protectin (MIC1), functions to restrict lysis of erythrocytes and leukocytes by homologous complement and is involved in signal transduction for T-cell activation. CD59 interacts with T-cell surface antigen CD2, a gene also highly differentially expressed in this dataset and found to co-cluster in Cluster B with NK4, CD5, CD6, CD81, CD8A, granulysin, and granzyme C and B. Additional NK-specific genes segregated with

this gene group include NKG7 and ICAM3. Thus, both cytotoxic T-cell antigens and NK-cell specific genes are expressed in parallel and characterized this cluster. The NK-cell specific cluster also had elevated expression of IL-2 regulated genes including CTSW. Together, the expression data shows that at least 3 populations of circulating leukocytes are involved in the tolerance signature identified: increased expression of T- and B-cell genes and anti-apoptotic genes serving a protective role (Cluster A); T-cells expression NK antigens with elevated expression of NK4, NKG7 and cytotoxic signaling chemokines (Cluster B); and a rapidly proliferating platelet or suppressor T-cell population modulated by the pro-apoptotic genes RGN and RIS1 (Cluster C).

#### *Supervised Statistical Analysis of the Expression Profiles*

Of the 2,083 highly differentially expressed genes in this study, 550 were found to show significant differences in expression between the OT and CR patient groups using the SAM statistical analysis tool (Tusher, et al. supra) ( $q < 5\%$ ). In contrast, no significant differences were observed within the 4 tolerant patients comparing those without any immunosuppressive drug vs. 12 tolerant patients under minimal immunosuppression (steroid monotherapy) (minimum false discovery rate  $> 16\%$ ). This observation showed that the expression patterns were sufficiently similar to group all the tolerant patients together in class prediction analysis and collectively consider them "operationally tolerant". Far more up-regulated genes are evident in the genes highly correlated with the OT phenotype: 523 genes were up-regulated and only 27 down-regulated and these genes clustered the patient groups in a similar manner as the unsupervised analysis. This observation showed that the state of operational tolerance in these patients is not a passive process but is actively regulated. Further, the expression profiles showed that the state of operational tolerance involves active transcription across several hundred genes in concert in peripheral blood cells. This mechanism is consistent with the observation that a much larger number of differentially expressed genes were initially identified using the SAM analysis tool when 22,897 clones were retrieved from the SMD database with stringency filtering (70% representative data and signal/noise ratio  $> 1.5$ ). From this larger gene set, a total of 3,125 cDNA clones representing ~1,820 genes were identified in the full gene list with SAM significance score  $< 5\%$  (data not shown).

To identify genes whose expression differed in OT and CR patients from healthy control individuals, each gene was zero transformed to the average expression level in the 3 normal PBL samples and supervised analysis with SAM repeated. In this normalized dataset, 591 significant genes were identified, all of which are at higher levels in the OT samples than CR (clustered data not shown).

The differentially expressed genes that segregate the OT and CR patient groups include several interesting candidate genes involved in immune signaling cascades including specific transcription factors, cell adhesion molecules, cell cycle-specific genes, inhibitors of inflammation/alloresponse, growth factors, and genes with important roles in vascular biology. This subset is provided in Table 2. Each of these gene families are represented by multiple group members in the identified group of 591 significantly differentially expressed cDNAs as are B- and T-cell specific genes, apoptotic gene family members, protein kinases, several cell matrix/structural proteins as well as other functional categories. Expression fold differences between OT and CR patients of representative members of these gene families are summarized in Table 3. For the remainder of the study, we analyzed both the normalized data (zero transformed) and clustered data to identify putative biomarkers predictive of tolerance.

#### 20 *Class Prediction Analysis*

PAM or Predictive Analysis of Microarray data (Tibshirani et al., Proc. Nat'l Acad. Sci. USA (2002) 99:6567-72) was used to identify those genes with highest predictive value in identifying a tolerogenic state in the blood samples. This program identifies a minimum gene set characteristic of user defined sample groups (the learning set) and then scores both known and unknown samples based on similarity to identified expression profile differences. Normalized expression ratios for OT and CR groups were used as a learning set and classification scores for the 12 stable patients determined by PAM analysis using threshold log-ratio difference of  $>1.5$  (3-fold expression difference between OT and CR (Table 3). Although there was substantial overlap in the identified genes, the 30 top ranked genes from PAM class prediction analysis using zero-transformed data (and raw expression values) differ in how they cluster the samples. The latter dataset segregate OT and CR samples into separate

branches that are in agreement with computed phenotype goodness-of-fit scores reported by the PAM class prediction program.

With the objective of further refining the gene set into a manageable number of genes for PCR-based analysis, the array data was analyzed by logistic regression. Class prediction using logistic regression modeling requires that markers independently correlate with the test phenotype which initially proved challenging with the array data due to the extremely tight clustering of many of the highest ranked genes. In all analysis performed, the expression of thymidine kinase 1 (TK1) showed the greatest difference comparing OT vs. CR (T-test  $p < .00001$ ) and highest expression in the tolerant patients. More importantly, the expression of several hundred genes positively correlated with TK1. For example, expression of 102 genes correlated with TK1 with  $R^2 > 0.7$  and 398 genes with  $R^2 > 0.5$  and, at least on the basis of the array data, these tightly correlating genes consistently had the highest significance scores. We therefore selected four candidate genes from the PAM class prediction set, two of which positively correlate with the tolerant phenotype (TK1 and C1s) and two of which negatively correlated (EV12A and MAPK9). Classification based on array data for these four genes give 95.8% concordant classification and 4.2% discordant classification within the test groups (Table 3). Remarkably, array-based expression ratios of TK1 alone yield 94.5% concordant classification and 4.2% discordant classification (see the web supplement for full output on the logistic regression and expression ratios for the test groups). In addition, the expression of TK1 also correlates with prediction scores generated by both PAM and logistic regression;  $R^2 > 0.85$  for logistic regression scores and  $R^2 > 0.80$  for PAM scores. Only 2 of the 39 samples classify discordantly between the two methods (5% discordance): Chronic patient #88 that PAM/microarray data profiles as consistent with the tolerant phenotype and Stable patient #89 which classifies as CR by PAM and OT by logistic regression. The overall expression profile of Stable patient #89 is sufficiently distinct from the tolerant patients that it failed to co-cluster with this group in all three sets of genes analyzed.

#### *Confirmation of gene signature with Taqman PCR*

Real-time quantitative RT-PCR was used to confirm expression difference between OT and CR patient groups using  $\beta$ -actin as a reference gene. RNA was

available for this analysis from 8 of the OT patients, 11 CR patients and all 12 stable post-transplant patients and residual amplified cDNA prepared for the array expression study was used as the template source. In the small sample group analyzed, the expression of EV12A was the only gene tested to reach statistical significance. However, a significant correlation between array and PCR results were seen for EV12A, TK1 and C1s and average expression of MAPK9 was higher in the CR patients than OT, also in agreement with the array-based data. To determine whether all four markers combined would provide greater statistical power, logistic regression modeling was used to analyze the PCR data. The overall best the differentiation between OT and CR groups is obtained a cut-off of 0.02 using a curve described by the following algorithm ( $p < 0.00005$ ):

$$1 / (1 + \exp -[10.2373 + 17.2638(\text{MPK9}) + 2.7098 (\text{EV12A}) - 4.8491 (\text{C1S}) - 10.1332(\text{TK1})])$$

where gene names are the fold-difference measurements at each locus. The logistic regression model from these data had correct prediction of phenotype of 72.2% (sensitivity=75%, specificity=66.7%).

TK1 expression by quantitative RT-PCR was still the most informative of the genes in this analysis, although more inter-individual variation was observed in the Taqman results and hence this marker alone failed to reach the predictive power of the array data. However, we did find that the combined expression profiles of TK1, MAPK9, EV12A, and C1s, when analyzed by logistic regression, were in complete agreement with PAM prediction scoring of the OT and CR patients tested. One of the patients originally classified as tolerant at the time of the study (Patient #T/C04) was found to have evidence of chronic injury as evidenced by the detection of proteinuria. By including the data from this patient in the CR group of the learning set and repeating the analysis only using expression measurements on the TK1 locus, cutoffs at 0.10 and at 0.59 the logistic regression model have correct prediction probability of 83.3% (sensitivity 100%, specificity 50%) and 83.3% (sensitivity 91.7% and specificity 66.7%), respectively. Thus, a cutoff of 0.59 would maximize both the sensitivity and specificity in the classification by TK1 alone by logistic regression and this model also gave better fit than the 4-gene profile. However, agreement among the stable patients between the two test measures (PAM class prediction of global

gene expression profiles vs. logistic regression analysis of RT-PCR data) was not as conclusive in that we found consistent classification between the two test methods in 9 of 12 stable patients (75%). Higher concordance within the OT and CR patients (100%) might be expected because these are the learning set in the analyses and the stable patients the test set (75% concordance). Together these data indicated that the blood-based expression signature of the tolerogenic state is subtle and a larger panel of genes, such as the 30 gene sets identified by PAM class prediction, may be beneficial in certain embodiments for accurate identification of the tolerance in a diagnostic setting.

Additional genes of interest are found in Table 4.

Table 4			
	Accession	Gene	Description
	NM_001003927	EVI2A	ecotropic viral integration site 2A
	NM_005601	NKG7	NATURAL KILLER CELL GROUP 7 SEQUENCE
	M38690	CD9	LEUKOCYTE ANTIGEN MIC3
	U25931	CD27	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY
	NM_014009	FOXP3	forkhead box P3
	NM_000953	DP1	prostaglandin D2 receptor

### III. Discussion and Conclusions

Identification of blood-based biomarkers with predictive power in directing clinical outcomes is an active area of clinical research. We used global gene expression monitoring to test whether blood-based markers diagnostic of spontaneously achieved tolerance could be identified in a cohort of long-term renal transplant patients. By normalizing to the average expression levels in normal control blood samples, several functional groups of genes were identified to be at significantly higher levels in the tolerant patients, where the identified genes included genes characteristic of rapid cell proliferation, anti-apoptotic activity and senescence, as well as genes involved in immune signaling cascades including specific transcription factors, cell adhesion molecules.

Overall the data shows that the spontaneously achieved state of immune tolerance is an active regulatory process as evidenced by the large numbers of up-regulated genes relative to normal blood and by the highly synchronized expression signature that is observed. Further, this signature was observed in the



blood from ~25% of long-term stable graft recipients showing that expression monitoring could become an invaluable method in realizing the goal of predictably reducing long-term immunosuppressive therapy when merited. The gene expression signatures were sufficiently strong that two independent classification methods, logistic regression and PAM analysis, gave similar predictive power (~90% overall) with differences in predicted phenotype for only 3 of 39 participants in this pilot study. Hierarchical clustering of the top 30 genes scored by PAM separated all patients classified tolerant and from those with chronic renal injury or graft rejection with 100% concordance to the logistic regression scoring. Identification of a large number of differentially expressed genes reported in this study of spontaneously tolerant adult renal transplant patients provides several biomarkers for spontaneously achieved immune tolerance. The gene expression study reported here shows that expression differences characteristic of spontaneously achieved tolerance can be detected in whole blood lysates obviating the need for more invasive methods of sampling.

It is evident that subject invention provides a convenient and effective way of determining whether a subject has a graft tolerant phenotype, without first removing the subject from immunosuppressive therapy. As such, the subject invention provides a number of distinct benefits, including the ability to easily identify subjects undergoing immunosuppressive therapy that have a graft tolerant phenotype, and therefore may be removed from immunosuppressive therapy, so that these individuals can avoid the adverse conditions, as well as costs, associated with such therapy. As such, the subject invention represents a significant contribution to the art.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without  
5 departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of determining whether a subject has a graft tolerant phenotype,  
5 said method comprising:  
evaluating expression of at least one gene in a sample from said subject to  
determine whether said subject has a graft tolerant phenotype.
2. The method according to Claim 1, wherein said expression of at least one  
10 gene is evaluated by assaying said sample for a nucleic acid transcript of said  
gene.
3. The method according to Claim 1, wherein said expression of at least one  
15 gene is evaluated by assaying said sample for an expression product of said  
gene.
4. The method according to any of Claims 1 to 3, wherein said sample is a  
blood sample.
- 20 5. The method according to Claim 4, wherein said blood sample is a  
peripheral blood sample.
6. The method according to Claim 1, wherein said at least one gene is a  
gene listed on Tables 1, 2, 3 and/or 4.  
25
7. A method according to any of the preceding claims, wherein the method  
comprises:  
    (a) obtaining an expression profile for a sample from said subject; and  
    (b) employing said obtained expression profile to determine whether  
30 said subject has a graft tolerant phenotype.
8. The method according to Claim 7, wherein said expression profile is  
compared to a reference expression profile in said employing step (b).

9. The method according to Claims 7 or 8, wherein said expression profile is determined using a microarray.
- 5 10. A method of managing immunosuppressive therapy in a subject having a graft, said method comprising:
- (a) evaluating whether said subject has an graft tolerant phenotype according to any of Claims 1 to 9; and
  - (b) determining a future immunosuppressive therapy protocol based on
- 10 said the evaluation of step (a) to manage immunosuppressive therapy in said subject.
11. A system for determining whether a subject has a graft tolerant phenotype comprising:
- 15 (a) a gene expression evaluation element for evaluating expression of at least one gene in a sample to obtain a gene expression result; and
  - (b) a phenotype determination element for employing said gene expression result to determine whether a subject has a graft tolerant phenotype.
- 20 12. A reference expression profile for a phenotype that is one of:
- (a) graft tolerant; or
  - (b) graft intolerant;
- wherein said expression profile is recorded on a computer readable medium.
- 25 13. A collection of gene specific primers for evaluating gene expression, said collection comprising:
- primers specific for at least two of the genes of Tables 1, 2, 3 and/or 4.
- 30 14. A array of probe nucleic acids immobilized on a solid support, said array comprising:
- a plurality of probe nucleic acid compositions, wherein each probe nucleic acid composition is specific for a gene whose expression profile is indicative of a

graft tolerance, wherein at least two of said probe nucleic acid compositions correspond to genes listed in Tables 1, 2, 3 and/or 4.

15. A kit comprising:
- 5 (a) an array according to Claim 14; and
- (b) a collection of gene specific primers according to Claim 13.

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Figure 1  
Table 1.

Table 1				
Clone	Accession	Gene	Description	Fold-Diff
IMAGE:379920	AA778098	TK1	thymidine kinase 1, soluble	4.60
IMAGE:436094	AA700832	RBP1	retinol binding protein 1, cellular	3.47
IMAGE:2108366	AI392840	EPHA2	EphA2	3.75
IMAGE:210717	H64346	SDC2	syndecan 2 (heparan sulfate proteoglycan 1, fibroglycan)	4.45
IMAGE:2557762	AW050510	PYCR1	pyrroline-5-carboxylate reductase 1	3.17
IMAGE:196992	R93124	AKR1C1	aldo-keto reductase family 1, member C1	3.97
IMAGE:783936	AA443405	SLC7A7	solute carrier family 7 (cationic amino acid transporter)	4.07
IMAGE:1577805	AA954999	CDH2	cadherin 2, type 1, N-cadherin (neuronal)	4.26
IMAGE:753038	AA436460	KIFC3	kinesin family member C3	2.83
IMAGE:436121	AA701996	SLC7A7	solute carrier family 7 (cationic amino acid transporter)	4.33
IMAGE:122159	T98611	COL3A1	collagen, type III, alpha 1	4.83
IMAGE:2108490	AI394016	C20orf42	chromosome 20 open reading frame 42	2.94
IMAGE:526657	AA128607	TCEB3	transcription elongation factor B (SIII)	6.45
IMAGE:486110	AA040703	PFN2	profilin 2	4.47
IMAGE:1283012	AA745260	CCNB2	cyclin B2	5.98
IMAGE:1474174	AA936799	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase)	3.39
IMAGE:2545705	AI969128	CNN3	calponin 3, acidic	4.41
IMAGE:2457449	AI932735	CCNB2	cyclin B2	4.26
IMAGE:897956	AA598817	PRAME	preferentially expressed antigen in melanoma	2.80
IMAGE:451907	AA706968	ZWINT	ZW10 interactor	3.47
IMAGE:307471	N93505	TM4SF2	transmembrane 4 superfamily member 2	3.04
IMAGE:139009	R62612	FN1	fibronectin 1	7.65
IMAGE:2109103	AI380951	COL6A3	collagen, type VI, alpha 3	3.73
IMAGE:897910	AA598653	OSF-2	osteoblast specific factor 2 (fasciclin I-like)	7.88
IMAGE:377384	AA055076	NR2F2	nuclear receptor subfamily 2, group F, member 2	4.32
IMAGE:785933	AA448569	SRPX	sushi-repeat-containing protein, X chromosome	4.15
IMAGE:241029	H81023	STK12	serine/threonine kinase 12	2.99
IMAGE:199367	R95691	SERPINE2	serine (or cysteine) proteinase inhibitor, clade E	6.36
IMAGE:74537	T59043	AFP	alpha-fetoprotein	4.77
IMAGE:2449395	AI924357	AKR1C1	aldo-keto reductase family 1, member C1	3.46
IMAGE:815526	AA456878	MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	3.18
IMAGE:784593	AA443302	PDE1A	phosphodiesterase 1A, calmodulin-dependent	2.84
IMAGE:360254	AA012892	CYR61	cysteine-rich, angiogenic inducer, 61	9.01
IMAGE:1517595	AA903137	MELK	maternal embryonic leucine zipper kinase	3.59
IMAGE:2577230	AW075585	TNC	tenascin C (hexabrachion)	3.29
IMAGE:758088	AA426421	CALD1	caldesmon 1	2.98
IMAGE:490995	AA136707	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase	3.09
IMAGE:250654	H95959	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	2.89
IMAGE:344430	W73473	BMP7	bone morphogenetic protein 7 (osteogenic protein 1)	2.95
IMAGE:1316861	AA768291	MELK	maternal embryonic leucine zipper kinase	3.54
IMAGE:898092	AA598794	CTGF	connective tissue growth factor	6.49
IMAGE:1307938	AA767775	TAL2	T-cell acute lymphocytic leukemia 2	2.17
IMAGE:133273	R26732	PMP22	peripheral myelin protein 22	3.58
IMAGE:1845885	AI218117	TYR	tyrosinase (oculocutaneous albinism IA)	2.22
IMAGE:1250917	AA731503	FLJ20311	hypothetical protein FLJ20311	2.81

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Table 1

Clone	Accession	Gene	Description	Fold-Diff
IMAGE:346696	W72159	TEAD4	TEA domain family member 4	3.31
IMAGE:878564	AA775872	GPC3	glypican 3	3.56
IMAGE:744647	AA621315	CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	2.63
IMAGE:359412	AA010487	CCND2	cyclin D2	6.77
IMAGE:2569190	AW073393	FSTL1	folliculin-like 1	4.03
IMAGE:124345	R02117	CENPF	centromere protein F, 350/400ka (mitosin)	3.02
IMAGE:378461	AA775616	SPP1	secreted phosphoprotein 1 (osteopontin, T-cell activation 1)	4.90
IMAGE:460470	AA677687	C4BPB	complement component 4 binding protein, beta	2.25
IMAGE:116743	T89453	FADS1	fatty acid desaturase 1	7.70
IMAGE:358168	W95586	MGC9850	hypothetical protein MGC9850	4.46
IMAGE:809473	AA443119	PTRF	polymerase I and transcript release factor	2.75
IMAGE:786673	AA451903	RBM9	RNA binding motif protein 9	2.60
IMAGE:68950	T54121	CCNE1	cyclin E1	2.61
IMAGE:2057931	AI340883	TACSTD1	tumor-associated calcium signal transducer 1	3.68
IMAGE:526184	AA076645	RAB21	RAB21, member RAS oncogene family	5.23
IMAGE:781047	AA430092	BUB1	BUB1 budding uninhibited by benzimidazoles 1	3.61
IMAGE:788566	AA452826	PCP4	Purkinje cell protein 4	3.75
IMAGE:2391979	AI738499	DHRS2	dehydrogenase/reductase (SDR family) member 2	10.27
IMAGE:2019101	AI359985	AGT	angiotensinogen (serine (or cysteine) proteinase inhibitor	3.85
IMAGE:753184	AA400464	SOX9	SRY (sex determining region Y)	2.64
IMAGE:450533	AA704242	SERPINA3	serine proteinase inhibitor(antitrypsin)	3.87
IMAGE:142788	R71093	SERPINH2	serine proteinase inhibitor (heat shock protein 47)	3.64
IMAGE:1269639	AA714672	M96	metal response element binding transcription factor 2	3.58
IMAGE:1871508	AI262459	TM4SF5	transmembrane 4 superfamily member 5	2.45
IMAGE:378488	AA777187	CYR61	cysteine-rich, angiogenic inducer, 61	4.13
IMAGE:625016	AA181040	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	2.72
IMAGE:2019116	AI359981	SOX3	SRY (sex determining region Y)-box 3	2.97
IMAGE:487878	AA045463	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	2.92
IMAGE:772437	AA405486	KIAA0638	KIAA0638 protein	2.43
IMAGE:212188	H68848	APOH	apolipoprotein H (beta-2-glycoprotein I)	4.84
IMAGE:665356	AA194983	TNFRSF11B	tumor necrosis factor receptor 11b (osteoprotegerin)	2.26
IMAGE:774471	AA429989	LAMB1	laminin, beta 1	3.04
IMAGE:2108629	AI492226	C20orf42	chromosome 20 open reading frame 42	2.12
IMAGE:725680	AA394236	TFAP2C	transcription factor AP-2 gamma (activating enhancer)	5.04
IMAGE:624627	AA187351	RRM2	ribonucleotide reductase M2 polypeptide	2.88
IMAGE:2569884	AW084720	C1S	complement component 1, s subcomponent	2.38
IMAGE:855563	AA664212	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	2.31
IMAGE:1584142	AA972716	GAGEB1	G antigen, family B, 1 (prostate associated)	2.59
IMAGE:209066	H63492	STK6	serine/threonine kinase 6	3.62
IMAGE:342640	W68219	KIAA0101	KIAA0101 gene product	3.37
IMAGE:897788	AA598513	PTPRF	protein tyrosine phosphatase, receptor type, F	3.39
IMAGE:796694	AA460685	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	2.33
IMAGE:796606	AA460520	CDV-1	carnitine deficiency-associated gene	2.10
IMAGE:1323591	AA858026	SERPINA5	serine (or cysteine) proteinase inhibitor	4.79
IMAGE:50562	H16793	C8orf4	chromosome 8 open reading frame 4	2.80
IMAGE:1475476	AA857809	MAGEA4	melanoma antigen, family A, 4	2.72
IMAGE:121551	T97710	LAD1	ladinin 1	2.39
IMAGE:683100	AA213393	CCNA2	cyclin A2	2.61
IMAGE:2063390	AI375135	AMBP	alpha-1-microglobulin/bikunin precursor	5.72
IMAGE:839991	AA490172	COL1A2	collagen, type I, alpha 2	2.49
IMAGE:1948085	AI352345	CBR3	carbonyl reductase 3	2.28

Table 1				
Clone	Accession	Gene	Description	Fold-Diff
IMAGE:377461	AA055368	CAV1	caveolin 1, caveolae protein, 22kDa	4.94
IMAGE:1302907	AA732416	PARVG	parvin, gamma	3.64
IMAGE:626001	AA187933	TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)	2.26
IMAGE:263716	H99676	COL6A1	collagen, type VI, alpha 1	3.19
IMAGE:884418	AA773070	MYO5B	myosin VB	2.04
IMAGE:1472743	AA872397	LGALS2	lectin, galactoside-binding, soluble, 2 (galectin 2)	2.86
IMAGE:344139	W69790	DOC1	downregulated in ovarian cancer 1	2.34
IMAGE:686172	AA262211	KIAA0008	Drosophila discs large-1 tumor suppressor-like	2.09
IMAGE:290378	N64508	PODXL	podocalyxin-like	2.59
IMAGE:2092597	AI381509	GAGE8	G antigen 8	3.81
IMAGE:898286	AA598974	CDC2	cell division cycle 2, G1 to S and G2 to M	2.33
IMAGE:773495	AA427924	MGC10724	hypothetical protein MGC10724	3.64
IMAGE:212429	H69531	TF	transferrin	2.99
IMAGE:2543464	AW029498	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A	5.04
IMAGE:825470	AA504348	TOP2A	topoisomerase (DNA) II alpha 170kDa	2.45
IMAGE:300411	N80273	CCL19	chemokine (C-C motif) ligand 19	2.04
IMAGE:2489436	AI972269	MYLK	myosin, light polypeptide kinase	1.91
IMAGE:489519	AA099153	TIMP3	tissue inhibitor of metalloproteinase 3	2.41
IMAGE:2568350	AW070825	KRT19	keratin 19	5.68
IMAGE:234237	H69334	PIR	Pirin	2.72
IMAGE:810131	AA464250	KRT19	keratin 19	2.83
IMAGE:2139448	AI470584	TNRC9	trinucleotide repeat containing 9	1.98
IMAGE:2223790	AI571948	TFF1	trefoil factor 1 (breast cancer, estrogen-inducible)	2.41
IMAGE:33603	R19521	GPRC5B	G protein-coupled receptor, family C5B	2.40
IMAGE:782575	AA447522	HSJ001348	cDNA for differentially expressed CO16 gene	2.03
IMAGE:49569	H15115	KIAA1026	KIAA1026 protein	1.96
IMAGE:1335954	AA810530	POLQ	polymerase (DNA directed), theta	2.41
IMAGE:148800	H13424	N33	Putative prostate cancer tumor suppressor	3.08
IMAGE:343821	W69496	TYRO3	TYRO3 protein tyrosine kinase	2.32
IMAGE:2021931	AI361763	B4GALT2	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase	2.88
IMAGE:1493107	AA876375	LTB4DH	leukotriene B4 12-hydroxydehydrogenase	2.61
IMAGE:45099	H05140	RGN	regucalcin (senescence marker protein-30)	1.92
IMAGE:29205	R14517	MIG2	mitogen inducible 2	2.68
IMAGE:487297	AA040613	CAP2	adenylyl cyclase-associated protein 2	2.73
IMAGE:141815	R70684	JAG1	jagged 1 (Alagille syndrome)	2.29
IMAGE:1272470	AA743293	FOXM1	forkhead box M1	2.52
IMAGE:782730	AA447978	ALDH1A2	aldehyde dehydrogenase 1 family, member A2	2.59
IMAGE:1241341	AA714513	HLA-DRB3	major histocompatibility complex, class II, DR beta 3	2.26
IMAGE:898062	AA598776	CDC20	CDC20 cell division cycle 20 homolog (S. cerevisiae)	2.06
IMAGE:66406	T66935	DKFZp762E1312	hypothetical protein DKFZp762E1312	2.48
IMAGE:41648	R52795	IL13RA2	interleukin 13 receptor, alpha 2	3.28
IMAGE:2308409	AI653116	KIAA1077	sulfatase FP	3.22
IMAGE:1235082	AA713690	BRN1	barren homolog (Drosophila)	2.32
IMAGE:827384	AA669106	UHRF1	ubiquitin-like, containing PHD/RING finger domains, 1	2.33
IMAGE:1913366	AI308916	PRSS3	protease, serine, 3 (mesotrypsin)	2.81
IMAGE:714106	AA284668	PLAU	plasminogen activator, urokinase	2.08
IMAGE:32983	R18944	SIN3A	SIN3 homolog A, transcriptional regulator (yeast)	4.99
IMAGE:1284490	AA744546	TTK	TTK protein kinase	2.40
IMAGE:344854	W72972	ANKRD3	ankyrin repeat domain 3	2.22
IMAGE:1056107	AA628360	C20orf16	chromosome 20 open reading frame 16	2.18
IMAGE:760299	AA425947	DKK3	dickkopf homolog 3 (Xenopus laevis)	3.01



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Table 1

Clone	Accession	Gene	Description	Fold-Diff
IMAGE:809910	AA464416	IFITM3	interferon induced transmembrane protein 3 (1-8U)	3.06
IMAGE:2488862	AI971229	FKBP9	FK506 binding protein 9, 63 kDa	2.65
IMAGE:141314	R63811	LMCD1	LIM and cysteine-rich domains 1	2.32
IMAGE:1597856	AA961402	APOA2	apolipoprotein A-II	2.67
IMAGE:2488265	AI970855	SEMA3F	sema domain, immunoglobulin domain (Ig)	2.45
IMAGE:2012757	AI356709	D2S448	Melanoma associated gene	2.25
IMAGE:684238	AA251325	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	2.50
IMAGE:259291	N29501	ITGB5	integrin, beta 5	2.26
IMAGE:1550909	AA913206	GAGE8	G antigen 8	2.26
IMAGE:135221	R32848	S100P	S100 calcium binding protein P	2.23
IMAGE:1241524	AA729003	TCL1A	T-cell leukemia/lymphoma 1A	2.33
IMAGE:795730	AA460282	EFS2	signal transduction protein (SH3 containing)	1.87
IMAGE:40580	R55130	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	1.79
IMAGE:897865	AA598640	MID1	midline 1 (Opitz/BBB syndrome)	1.89
IMAGE:2018976	AI362866	PTTG1	pituitary tumor-transforming 1	2.22
IMAGE:50182	H17882	KAL1	Kallmann syndrome 1 sequence	1.84
IMAGE:47142	H10964	PEX12	peroxisomal biogenesis factor 12	2.20
IMAGE:549383	AA081414	PLAB	prostate differentiation factor	2.37
IMAGE:815709	AA485172	HLA-DOA	major histocompatibility complex, class II, DO alpha	1.92
IMAGE:682989	AA210804	PAICS	phosphoribosylaminoimidazole carboxylase	2.29
IMAGE:773322	AA425419	SHOX2	short stature homeobox 2	1.96
IMAGE:293339	N64741	SNAI2	snail 2	2.94
IMAGE:512337	AA057826	RPS5	ribosomal protein S5	2.44
IMAGE:927027	AA535146	CCL20	chemokine (C-C motif) ligand 20	2.22
IMAGE:375682	AA032221	STEAP	six transmembrane epithelial antigen of the prostate	3.12
IMAGE:753248	AA406231	DAAM2	dishevelled associated activator of morphogenesis 2	1.96
IMAGE:739155	AA421819	CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)	2.92
IMAGE:2017415	AI369629	CENPA	centromere protein A, 17kDa	4.10
IMAGE:1558642	AA976544	MLPH	melanophilin	2.91
IMAGE:1357100	AA832260	SLC2A5	solute carrier f(facilitated glucose/fructose transporter)	1.86
IMAGE:1407750	AA861203	IGFBP3	insulin-like growth factor binding protein 3	3.40
IMAGE:813387	AA455538	NQO1	NAD(P)H dehydrogenase, quinone 1	2.44
IMAGE:2428020	AI870548	HP	haptoglobin	2.51
IMAGE:1356393	AA831559	DDR1	discoidin domain receptor family, member 1	2.49
IMAGE:2018154	AI364521	ETFB	electron-transfer-flavoprotein, beta polypeptide	1.87
IMAGE:1234404	AA687354	CD22	CD22 antigen	2.24
IMAGE:796398	AA459941	ZIM2	zinc finger, imprinted 2	2.82
IMAGE:1928791	AI313387	F3	coagulation factor III (thromboplastin, tissue factor)	2.24
IMAGE:246524	N53057	CHEK1	CHK1 checkpoint homolog (S. pombe)	1.91
IMAGE:2494168	AI986336	FLJ20151	hypothetical protein FLJ20151	2.23
IMAGE:434768	AA701860	FST	follistatin	2.38
IMAGE:2016775	AI356028	GPRC5B	G protein-coupled receptor, family C, group 5, member B	2.14
IMAGE:811740	AA463257	ITGA2	integrin, alpha 2 (CD49B, subunit of VLA-2 receptor)	1.94
IMAGE:2569769	AW084638	CA2	carbonic anhydrase II	1.97
IMAGE:878438	AA670362	TEAD2	TEA domain family member 2	2.23
IMAGE:773330	AA425450	GPNMB	glycoprotein (transmembrane) nmb	2.25
IMAGE:347560	W81317	H1FX	H1 histone family, member X	1.79
IMAGE:1470446	AA864299	FLJ32987	proapoptotic caspase adaptor protein	2.10
IMAGE:753969	AA478949	DLG5	discs, large (Drosophila) homolog 5	1.87
IMAGE:2014382	AI362029	NR2F1	nuclear receptor subfamily 2, group F, member 1	2.02
IMAGE:770859	AA434397	ITGB5	integrin, beta 5	2.27

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Table 1				
Clone	Accession	Gene	Description	Fold-Diff
IMAGE:2409751	AI816969	ESPL1	extra spindle poles like 1 (S. cerevisiae)	1.83
IMAGE:840493	AA485893	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	3.00

Figure 2  
Table 2.

Gene category/class	# cDNA	Most highly-differentially expressed gene in the category			Fold*
		Accession	Clone ID	Gene	
Apoptosis	5	AA864299	IMAGE:1470446	PACAP proapoptotic caspase adaptor protein	2.22
B-Cell related	27	AI186799	IMAGE:1745349	MYOM2 myomesin (M-protein) 2, 165kDa	3.6
Cell adhesion/calcium channel	8	AA954999	IMAGE:1577805	CDH2 cadherin 2, type 1, N-cadherin (neuronal)	3.61
Cell cycle genes	33	AA010487	IMAGE:359412	CCND2 cyclin D2	8.19
Cell matrix	67	AA598653	IMAGE:897910	OSF-2 osteoblast specific factor 2	9.71
Cell signaling	41	AI394733	IMAGE:2109760	SLC38A6 solute carrier family 38, member 6	6.37
Chemokines	17	AI950929	IMAGE:2548121	C1S complement component 1s	4.55
Detoxification	28	R93124	IMAGE:196992	AKR1C-pseudo-chlordecone reductase	4.63
DNA replication	32	AI369629	IMAGE:2017415	CENPA centromere protein A, Core histone	4.94
ESTs or unknown cDNAs	181	AI738499	IMAGE:2391979	cDNA FLJ20338 fis, clone HEP12179	12.60
Growth factors	8	AA012892	IMAGE:360254	CYR61 cysteine-rich, angiogenic inducer 61	10.83
Lipid/cholesterol	8	T89453	IMAGE:116743	FADS1 fatty acid desaturase 1	8.44
Oncogenes	17	AA076645	IMAGE:526184	RAB21, member RAS oncogene family	6.35
Other genes of known function	22	AA452826	IMAGE:788566	PCP4 Purkinje cell protein 4	4.41
Protease inhibitors	18	R95691	IMAGE:199367	SERPINE2 Serine/cysteine proteinase inhibitor, E2	6.12
Protein kinases	25	AA832386	IMAGE:1357548	STK6 serine/threonine kinase 6 IPL1-related kinase	4.54
Ribosomal genes	3	AA057826	IMAGE:512337	RPS5 ribosomal protein S5	2.71
T-Cell related	17	AA775616	IMAGE:378461	SPPI secreted T-lymphocyte activation 1	5.79
Transcription factors	26	AA128607	IMAGE:526657	TCEB3 transcription elongation factor B (SIII)	7.37

\*1. Fold reported is the average expression level in PBL from OT patient group relative to CR.

7/8  
Figure 3  
Table 3.

Table 3				
Accession	Clone ID	Gene	Fold-change	Cellular role
<b>Apoptosis</b>				
AA460685	IMAGE:796694	BIRC5	2.65	Inhibitor of Apoptosis domain
AA864299	IMAGE:1470446	PACAP	2.22	Proapoptotic caspase adaptor
T50675	IMAGE:72778	CASP7	2.06	Apoptosis-related cysteine protease
AA125792	IMAGE:502664	RIS1	2.28	Ras-induced senescence 1
H05140	IMAGE:45099	RGN	2.36	Senescence marker protein-30
AI886854	IMAGE:2413733	FAIM2	1.56	Fas apoptotic inhibitory molecule
AA291229	IMAGE:700619	BNIP3	1.88	Pro-apoptotic 19K/Bcl-2-interacting protein
<b>Complement</b>				
AI950929	IMAGE:2548121	C1S	4.55	Complement component 1s
T69603	IMAGE:83549	C1R	1.91	Complement component 1r
T68274	IMAGE:83210	C8B	1.5	Complement component 8
AA746481	IMAGE:1251210	CD59	2.61	Complement membrane attack regulator
<b>Serine/cysteine protease inhibitors</b>				
R95691	IMAGE:199367	SERPINE2	6.11	Plasminogen activator inhibitor
AA858026	IMAGE:1323591	SERPINA5	5.77	a-1 antiproteinase, antitrypsin
AA704242	IMAGE:450533	SERPINA3	4.52	a-1 antiproteinase, antitrypsin
R71093	IMAGE:142788	SERPINH2	4.36	Heat shock protein 47
AI084671	IMAGE:1681489	SERPINE1	1.82	Plasminogen activator inhibitor
T62086	IMAGE:85690	SERPIND1	1.45	Heparin cofactor/ proteinase inhibitor
<b>Growth factors/receptors</b>				
AA012892	IMAGE:360254	CYR61	10.83	Angiogenic inducer
AA463257	IMAGE:811740	ITGA2	1.93	Integrin, alpha 2/VLA-2 receptor
W73473	IMAGE:344430	BMP7	3.19	TGF-beta family osteogenic protein
AA598794	IMAGE:898092	CTGF	7.86	Connective tissue growth factor
<b>Cyclins</b>				
AA010487	IMAGE:359412	CCND2	8.19	Cyclin D2
AA745260	IMAGE:1283012	CCNB2	7.27	Cyclin B2
AA213393	IMAGE:683100	CCNA2	3.04	Cyclin A2
T54121	IMAGE:68950	CCNE1	2.68	Cyclin E1
<b>Lipid metabolism/cholesterol transport</b>				
T89453	IMAGE:116743	FADS1	8.44	Fatty acid desaturase 1
H68848	IMAGE:212188	APOH	5.83	Apolipoprotein H
AA961402	IMAGE:1597856	APOA2	3.06	Apolipoprotein A-II
R00706	IMAGE:123474	SCD	1.6	Stearoyl-CoA desaturase
T53121	IMAGE:68504	APOB	2.14	Apolipoprotein B
NM_000237	LCP:7381	LPL	1.96	Lipoprotein lipase
<b>Calcium dependent T-cell-cell adhesion</b>				
AA954999	IMAGE:1577805	CDH2	3.61	Cadherin 2, type 1, N-cadherin
AA421819	IMAGE:739155	CDH6	3.58	Cadherin 6, type 2, K-cadherin
AW007700	IMAGE:2506265	CACNA2D2	3.2	Calcium channel, voltage-dependent
R32848	IMAGE:135221	S100P	2.64	S100 calcium binding protein
<b>Cell surface stimulation</b>				
H64346	IMAGE:210717	SDC2	5.25	Syndecan 2 (fibroglycan)
AI969128	IMAGE:2545705	CNN3	5.21	Calponin 3, acidic
AA936799	IMAGE:1474174	MMP2	4.45	Matrix metalloproteinase 2
AA775447	IMAGE:878182	A2M	4.08	Alpha-2-macroglobulin
AA478662	IMAGE:754106	TIMP3	3.33	Tissue inhibitor of metalloproteinase 3
R14517	IMAGE:29205	MIG2	3.21	Mitogen inducible chemokine

Table 3				
Accession	Clone ID	Gene	Fold-change	Cellular role
<b>Cell matrix/ structural proteins</b>				
AA598653	IMAGE:897910	OSF-2	9.71	Osteoblast-specific factor/adhesion
R62612	IMAGE:139009	FN1	9.27	Fibronectin 1
AW070825	IMAGE:2568350	KRT19	6.88	Keratin 19
AA055368	IMAGE:377461	CAV1	5.92	Caveolin 1, caveolae protein
T59043	IMAGE:74537	AFP	5.49	Alpha-fetoprotein
T98611	IMAGE:122159	COL3A1	5.45	Collagen, type III, alpha 1
AA040703	IMAGE:486110	PFN2	5.23	Profilin 2
AA701996	IMAGE:436121	DLK1	5.01	Delta-like 1 homolog (Drosophila)
AI380951	IMAGE:2109103	COL6A3	4.47	Collagen, type VI, alpha 3
AA427924	IMAGE:773495	SPON1	4.42	Spondin 1, extracellular matrix
R26732	IMAGE:133273	PMP22	3.97	Peripheral myelin protein 22
AW075585	IMAGE:2577230	TNC	3.83	Tenascin C (hexabrachion)
H99676	IMAGE:263716	COL6A1	3.54	Collagen, type VI, alpha 1
AA436460	IMAGE:753038	KIFC3	3.38	Kinesin family member C3
H69531	IMAGE:212429	TF	3.23	Transferrin
W73473	IMAGE:344430	BMP7	3.19	Osteogenic protein - TGF-beta family
AA464250	IMAGE:810131	KRT19	2.91	Keratin 19

## SEQUENCE LISTING

<110> Sarwal, Minnie  
Mansfield, Elaine  
Brouard, Sophie  
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<120> METHODS AND COMPOSITIONS FOR DETERMINING  
A GRAFT TOLERANT PHENOTYPE IN A SUBJECT

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