

Microarrays: Monitoring for Transplant Tolerance and Mechanistic Insights

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Transplantation is the preferred therapy for the end-stage kidney diseases and the only definitive treatment of various solid organ diseases. The success of transplantation has largely been attributable to improvements in surgical techniques and immunosuppressive medications that impair the adaptive immune system [1]. Transplantation results in a burden of lifelong immunosuppression, however, with its associated complications (infection, neoplasm, drug toxicity), while still failing to control chronic immune-mediated graft injury, resulting in the eventual decline in graft function and limited graft life expectancy [2–4]. Given the current deceased organ shortage, preserving graft function is a prime objective for organ transplantation. There is thus an acute need for molecular research to better understand the divergence of the immune response from graft acceptance to rejection, and the progression of molecular changes in the transplant to immune and nonimmune injury. The ultimate benefit of this research is the ability to avoid biopsies and individualize immunosuppression while avoiding rejection and supporting tolerance.

Operational tolerance: coining a new term for a rare clinical phenotype

Achieving long-term, drug-free graft acceptance is an unsolved problem in clinical transplantation, because dependency on immunosuppression is gated by the occurrence of acute rejection in most patients if immunosuppression withdrawal is attempted randomly. Strategies to understand,

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monitor for, and support the development of transplantation tolerance will be a great advance for the management of transplant patients and for the prevention and treatment of autoimmune diseases.

Tolerance, often called the “Holy Grail” of organ transplantation, is defined by the presence of donor-specific hyporesponsiveness in the presence of an intact immune system. Immune tolerance refers to an acquired modification of the host immune system, achieved without chronic drug therapy, whereby specific responses against the graft are suppressed but the immune system is otherwise fully competent. Clinically, this results in the maintenance of stable allograft function without clinically evident immunosuppression [2,5], including true tolerance (the absence of any detectable detrimental immune response and no immunocompromise) and operational tolerance (the gross phenotype of tolerance with an immune response or deficit that has no significant clinical impact). Although true tolerance avoids acute and chronic allograft injury, there is justifiable concern that operational tolerance will eventually give way to chronic allograft injury if there is even modest intra-graft inflammation [5].

Diverse mechanisms in transplantation tolerance

There are different mechanisms contributing to the development of transplantation tolerance. Tolerance can be associated with anergy [6–9], suppression [10,11], clonal deletion [12], costimulation blockade [13], and chimerism [14]. Anergy refers to a long-lasting state of partial or total unresponsiveness that develops after suboptimal or incomplete T cell activation [8,9]. T and B cell tolerance can be established (or lost) either centrally, at the site of primary lymphocyte development in the thymus or bone marrow, or peripherally, at locations of antigen recognition and processing in the secondary (eg, central lymph nodes and spleen) and tertiary (eg, intestinal Peyer patch) lymphoid tissues [15].

For this article we have divided the study of tolerance in the clinical arena into three broad areas, focusing only on the field of solid organ transplantation: (1) evaluation of existing tolerant transplant recipients as to their mechanism of tolerance, (2) evaluation of prospective tolerance trials, and (3) immunologic assays to assess the likelihood of rejection or tolerance [16].

Evaluation of existing tolerant transplant recipients as to the mechanism of metastable tolerance

Tolerance is a rare spontaneous event, more frequently seen after liver transplantation. The number of kidney transplant recipients reported as tolerant is less than 1% compared with the total number of kidney transplants performed. Studies of these rare individuals allow a few generalizations: (1) tolerance typically appears years after transplantation, suggesting that it was

achieved by a process rather than through a sudden induction, (2) there is no relationship to the recipient's underlying renal disease, and (3) donor age and mismatch tends to be lower in tolerant patients compared with the general population [5,6,17]. Most cases of operational tolerance in solid organ transplant recipients are the result of nonadherence, although the typical outcome of random drug discontinuation in a transplant recipient is graft loss within 8 months [18].

The progress and evaluation of prospective tolerance trials

Different protocols have been developed by different groups in an attempt to deliberately induce stable graft tolerance just before the time of organ engraftment. The main clinical approaches have been recently reviewed by Girlanda and Kirk [5], and St. Clair and colleagues [15], and have used hematopoietic cells as a tolerance-facilitating antigen [19], establishment of mixed chimerism [20–22], pretransplantation targeted irradiation to lymphoid tissue (TLI) [23,24], lymphocyte depletion with polyclonal or monoclonal antibodies [25], and costimulation blockade [26–29]. The implementation of these tolerance protocols is thus difficult in routine practice despite early success in animals, although there is the caveat that some of the animals in these trials have developed chronic graft rejection over time. The mechanisms involved in the clinical experience may be somewhat different from those in the experimental setting. So far, the most exciting data to date on renal transplant recipients were published by two groups simultaneously in the *New England Journal of Medicine*, the protocols of Scandling and colleagues at Stanford University [30] and Kawai and colleagues from the Massachusetts General Hospital [20]. These two protocols have different induction approaches and likely different mechanisms of stable tolerance that may or may not rely on donor chimerism and thus may require different strategies for immune monitoring.

Immunologic assays to assess the likelihood of rejection or tolerance

A major barrier to clinical tolerance is the absence of a method to detect it prospectively. Despite the pressing need to develop tolerance assays, to date no assays have been shown to predict accurately the development or presence of stable donor-specific tolerance after transplantation. Development of tolerance assays requires an in-depth understanding of the complex and rare phenomenon of tolerance. The rarity of spontaneous transplantation tolerance and the difficulty of identifying such individuals pose major challenges in studying tolerance. In addition, the mechanisms of tolerance probably are numerous, may change over time, and vary depending on the organs involved. It now has become increasingly clear that tolerance in experimental rodent transplant models may differ from clinical

transplantation in humans. Many promising candidate assays measure the presence of antidonor responses or proinflammatory responses *ex vivo*, usually in peripheral blood lymphocytes, but hyporesponsiveness as determined by these assays does not always indicate or correlate with long-term stable clinical tolerance. The extent to which the response in peripheral blood mirrors what happens in the tissue also is widely debated, and the peripheral compartment, although easier to monitor, may not reflect all the key processes critical for maintaining tissue tolerance. For all of these reasons, the development and applicability of a tolerance assay has eluded the transplant community.

In humans, many immunologic assays have been used as surrogate tests (Table 1) to monitor the immune response after transplantation [9,48–50]. An ideal monitoring assay for clinical use should be noninvasive, sensitive, specific, and relatively inexpensive. Immune monitoring assays that currently are in development can be divided broadly into two major categories: donor antigen specific and antigen nonspecific. Donor antigen-specific assays measure the response of T and B cells to specific donor antigens; these methods require that a significant number of donor cells will be available over an extended period of time. Tests of antigen-specific T cell responses (mixed lymphocyte reaction, limiting dilution) have not been shown to predict the development of tolerance and have not been helpful guides for immunosuppression withdrawal. More recent tests of precursor frequency (enzyme-linked immunosorbent spot and tetramer analysis [51,52]), although promising during immunosuppression, have not yet been applied in clinical practice for monitoring tolerant patients. Antigen-nonspecific assays for the most part determine the phenotype of surface markers or functional state of cells within a tissue of interest, with the goal of identifying a pattern that is associated with a particular clinical status [9]. Very sensitive molecular techniques have become available to quantify relevant gene expression patterns [41,44,45,53–56] and protein signatures [47,57] in biologic samples. These gene and protein markers, whose expression within the graft, fluids draining from the graft (eg, urine, bile, or lavage fluid), and the peripheral blood, if correlative with either long-term graft function or rejection, would provide at least one means to monitor for the success or failure of a tolerance induction therapy or immunosuppression minimization strategy. Investigators have attempted to identify markers of immune function to help diagnose acute rejection or the onset of chronic rejection and to distinguish them from other disorders [53,58]. The polymerase chain reaction (PCR)-based analysis of genes expressed by cytotoxic T cells, such as perforin, fas, granzyme B [59], and granulysin [60], are up-regulated within the graft during rejection episodes in tissue and blood, and in urine samples during acute and chronic rejection [46], but despite the high sensitivity and specificity for detection of graft rejection in these studies, their ability to differentiate rejection from different types of infection has not been fully studied, limiting their application in clinical practice.

More recently, researchers have applied microarray technology to identify genes whose expression is increased in the graft during rejection of human renal [44,53,61–69] and cardiac allografts [70,71], and to predict the development of chronic graft injury based on immunohistochemical examination of different cells infiltrating the rejecting graft [53].

Microarrays, history, and clinical application

Microarray, a high-throughput technology used in molecular biology and in medicine, is based on the principle of complementary, single-stranded, nucleic acid sequences forming double-stranded hybrids. The principle of the microarray technology is evolved from similar principles used for Southern blotting, wherein radioactive probe labeling has been replaced by fluorescent single or dual color sample labeling. Microarrays now consist of an arrayed series of thousands of microscopic spots of DNA cDNAs or oligonucleotides (called features), each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that is used as a probe to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine the relative abundance of nucleic acid sequences in the target.

Microarrays were first described in 1987, when a collection of distinct cDNAs was used in arrays, made by spotting cDNAs onto filter paper with a pin-spotting device, to identify genes mediating the antiproliferative action of interferon [72]. The use of miniaturized microarrays for gene expression profiling was first reported in 1995 [73], and a complete eukaryotic genome on a microarray was published in 1997 [74]. The gene probes on the array are either small (20–60 bp) single-stranded oligonucleotides synthesized in situ (provided by Affymetrix and Agilent), or cloned cDNA amplified by PCR and obtained by reverse transcription of mRNA. The evolution of microarray technology and differences and relative advantages and disadvantages of cDNA versus oligonucleotide-based arrays were recently summarized [75]. Today, microarrays are fabricated using various technologies, including printing with fine-pointed pins onto glass slides [73], photolithography using pre-made masks, photolithography using dynamic micromirror devices, ink-jet printing [76], or electrochemistry on microelectrode arrays. Illumina uses designed oligonucleotide probes attached to beads deposited randomly on a substrate.

Currently, the global expression profiling with microarrays has become a driving tool for discovering novel genes and co-regulated, functionally significant pathways in diseases of interest. Microarray technology can be used for many different purposes, most prominently to measure differential gene expression (determining biomarkers for clinical phenotypes of disease and for survival analysis when selected genes are combined with clinical classes

Table 1
Monitoring strategies for tolerance and their use in clinical tolerance studies

Assay	Technique/detection	Advantages	Disadvantages	Use in clinical trials
Antigen-specific assays				
<i>T cell reactivity</i>				
MLR	Assays of T cell reactivity that reflect antigen-specific responses	Measures complex Th1/Th2 function and kinetic or effector function	Low sensitivity and reproducibility	The results of these assays have not been shown consistently to be correlated with the development of tolerance or the ability to wean immunosuppression
ELISA		Sensitive and reproducible Measures Th1/Th2 pattern	Decrease in sensitivity with longer incubation	
Limiting dilution assay		Measures function and frequency; the function of regulatory cells can be assessed	Requires in vitro expansion and is labor intensive and mathematically complex	
CTL	Quantifies the frequency of previously activated or memory T cells that respond to donor antigens by producing a selected cytokine	May be useful for guiding decisions about immunosuppressive drug management and for the identification of tolerant transplant recipients	Assesses CD8 ⁺ T cell– mediated activity only and does not give a comprehensive picture of alloreactivity	May be useful for guiding decisions about immunosuppressive drug management and for the identification of tolerant transplant recipients [128]
ELISPOT		Donor specificity High sensitivity Measures frequency and phenotype of cells Short in vitro culture Measure direct and indirect alloreactivity Uncover regulation Use of frozen cells possible Relatively easy to perform	Frequency measured on the basis of the cytokine production of cells Snapshot of events Counting of cells/spots is subjective	Not yet used to study tolerant patients

Trans vivo DTH	Index of reactivity of T cells to antigens measured by quantification of resultant swelling in mouse footpad	Direct and indirect alloreactivity Uncover regulation	Requires mice Cumbersome Larger number of cells required Measurement of swelling is subjective	Used to study tolerant patients [31]
<i>Flow cytometry</i> Tetramers	Frequency of T cells measured by their binding to specific peptide-MHC complexes using flow cytometry	Direct visualization of T cells regardless of their function No activation or expansion needed Easy to perform Requirement for cell numbers low Could be combined with other flow cytometric assays to evaluate cytokines/functional status of cells	Need for large numbers of MHC/peptide tetramers to study multiple antigens Not well established for class II	Not yet studied in clinical transplantation
CFSE	Measurement of proliferation by dilution of the dye in dividing cells using flow cytometry	Avoidance of radioisotopes Ability to distinguish dividing/nondividing cells Proliferation of cells followed over time Characterization of the phenotype/activation/death of the dividing cells using multicolor flow cytometric techniques	Requires optimization for human cells Lack of sensitivity Toxicity of the dye	Studied only in a single clinical liver transplantation trial [32]

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Assay	Technique/detection	Advantages	Disadvantages	Use in clinical trials
Intracellular cytokine staining	Frequency of cytokine-producing T cells by flow cytometry	Measures frequency and phenotype of cells Easy to perform Requires few cells Multiparametric staining possible	Requires specific activation procedures/ inhibitors of intracellular transport Toxic to cells Limited sensitivity	Not yet studied in clinical transplantation
Non-antigen-specific assays				
<i>Immunophenotyping</i>				
Phenotyping of recipient immune cells	Flow cytometric analysis of peripheral blood cells	Donor cells and donor antigen are not requested	No systematic studies have been performed to date Does not provide direct mechanistic information	Several types of regulatory cells are associated with tolerance, such as CD4 ⁺ CD25 ^{high} , CD3 ⁺ CD4 ⁺ CD8 ⁺ , CD8 ⁺ CD28 ⁺ , NK1.1 ⁺ , CD4 ⁺ CD25 ^{high} [33], CD8 ⁺ CD28 ⁺ [34], pDC [35–38]
Characterization of the TCR repertoire: TcLandscape	Assay uses PCR to analyze the T cell repertoire with respect to Vβ chain use and CDR3 length	Identification of populations of T cells that are over- or underexpressed in tolerant transplant recipients Does not require a source of donor cells to stimulate a response	Lack of donor specificity, no difference from the normal repertoire Influence of immunosuppression on repertoire not well studied	Longitudinal studies are needed to determine the stability of the repertoire changes described and their ability to predict tolerance

T cell responses to polyclonal, non-antigen-specific stimulation	Commercial assay: ImmunKnow—the Cylex immune cell function assay This assay is performed by stimulating whole blood in the presence of circulating levels of immunosuppressive drugs with phytohemagglutinin	Easily available in labs Correlates well with severe infection	Poor correlation with rejection, where dynamic range varies with immunosuppression, recipient age, or potentially other comorbidities	A single report has described the clinical use of this assay for managing immunosuppression after transplantation [39]
<i>Gene expression and transcriptional profiling</i> Gene array	Quantification of gene expression footprint	Simultaneous assessment of several thousand genes, useful for unbiased discovery Can be applied for prediction on independent test sets	Challenges with respect to data analysis with small patient numbers	Patterns of gene expression displayed by tolerant transplant recipients can be applied for testing by PCR [40–45]
RT-PCR	Quantification of gene expression	Precise quantification over a larger dynamic range Highly reproducible and sensitive	Less well suited for large numbers of genes based on a biased selection of genes and will thus miss new gene markers	Quantification by real-time PCR of the genes expressed by cells shed in the urine after renal transplantation has also been shown to reflect the status of the immune system [46]

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Assay	Technique/detection	Advantages	Disadvantages	Use in clinical trials
<i>Proteomics</i>	Examination of the concentration of soluble proteins in the supernatants of centrifuged urine samples using mass spectrometry.	Accurate quantification of processes regulated at the translational level Useful for “discovery” in that the expression of proteins not known to affect immune responses or for which the encoding gene has not been identified or sequenced is determined	Applicable only to kidney transplant recipients(?), although this has not been proved and may also apply to other organs, for the study of filtered plasma proteins	Can define those proteins present in the urine of renal transplant recipients that are associated with acute cellular rejection [47]

Abbreviations: CFSE, assays based on flow cytometry—carboxy-fluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte assay; DSA, donor-specific antibody; DTH, trans vivo delayed-type hypersensitivity assay; ELISPOT, enzyme-linked immunospot assay; MLR, mixed leukocyte reaction; PCR, polymerase chain reaction.

and incorporated into regression models to detect variations in survival times), and to detect single nucleotide polymorphism (SNPs) of the human genome (Hap Map project) [77], aberrations in methylation patterns [78], alterations in gene copy number [79], alternative RNA splicing [80], pathogen detection [81,82], and micro-RNA [83]. The advent of microarray technology has greatly enhanced our potential to understand, classify, and prognosticate on human diseases [53,84–86], and to further increase our understanding of the molecular basis of various outcomes and causes of graft dysfunction [41,44,45,53,87].

Gene expression microarrays can estimate the prevalence of mRNA for thousands of genes in a small sample of cells or tissue. Organ transplant researchers are increasingly using microarrays to identify specific patterns of gene expression that predict and characterize acute and chronic rejection, or tolerance, and to improve our understanding of the mechanisms underlying organ allograft dysfunction. In organ transplantation, a prospective study of the gene-expression profile of graft injury usually involves sample collection from tissue biopsy, blood, and biofluids, such as urine, bile, or bronchoalveolar lavage, taken before, during, and after injury.

A recent report describes patterns of gene expression (signatures) and a data analysis schema that distinguished healthy kidney donors, recipients free from rejection who had good graft function, recipients who had acute rejection, and recipients who had impaired graft function because of causes other than rejection using samples from the allograft or peripheral blood [66]. These markers can be potentially used to monitor induction and maintenance of tolerance by assaying for a “lack” of this signature in developing tolerance. Nevertheless, based on the premise that antigen recognition is necessary for allorecognition and subsequent accommodation or tolerance, and given that a recently published study of array analysis of operationally tolerant patients showed that many of the operationally tolerant patients had prior episodes of acute rejection [41], it remains to be seen whether definition of tolerance at a given point in time will be gated by a prior gene signature for rejection or will subsequently predict one’s future risk for alloimmune activation.

In humans, the certain genes that classify acute [88–90] and chronic [91] lung rejections were recently identified in bronchoalveolar lavage cells. Gene microarray analysis of peripheral blood samples were reported in heart [92], liver [43], and kidney [40–42,44,45,61,93] transplantation. Urine is another noninvasive promising sample to analyze by microarray for the discovering of genes associated with renal graft rejection or tolerance [44,61]. The array-based published studies and key findings in transplantation and rejection were recently reviewed by our group [75]. Clearly, larger-scale prospective clinical validation studies are required to cross-validate the diagnostic and predictive potential of noninvasive biomarkers for graft rejection. It can be hypothesized that the most informative rejection-based biomarkers would obviously fail in the presence of immune quiescence and tolerance

development, providing a negative control for a microarray-based tolerance test. In Table 2 we have reviewed human studies that identify sets of genes associated with operational tolerance in the blood of kidney and liver recipients and that may have usefulness as a minimally invasive monitoring tool for guiding immunosuppression titration. Operational tolerance in transplantation tolerance refers to good and stable allograft function in the absence of any immunosuppression for an extended period of time. This state has rarely been reported in renal transplantation, in contrast to its more common occurrence in liver transplantation. The immune characterization of such patients has not been performed in detail because of the paucity of these patients and the difficulty in recruiting them. The discovery of a diagnostic biomarker signature would allow the identification of quasi-tolerant patients in whom immunosuppressive drugs could be safely decreased [96]. Based on this premise a study of long-term stable kidney transplant patients was conducted across 91 cDNA microarrays [41], comparing patients who had operational tolerance with patients who had chronic rejection, with and without drugs (the latter group was patients on dialysis after a failed transplant), patients on minimal single drug immunosuppression (steroid monotherapy in this study), patients on triple immunosuppression without any graft dysfunction or rejection (also called stable patients in the study), and healthy volunteers. Extensive clinical and hematologic indices were collected in these patients and correlated with gene expression changes (<http://www.pnas.org/content/104/39/15,448/suppl/DC1>). As seen in Fig. 1A, a small subset of genes in peripheral blood can identify and predict tolerant patients and additionally predict that 40% of patients on steroid monotherapy and 8% to 10% of patients on triple drug therapy may be quasi-tolerant; this latter group may thus be potentially able to significantly wean their immunosuppression in a safely monitored manner. These genes have the potential to be used as a minimally invasive monitoring biomarker panel in peripheral blood, useful for guiding immunosuppression titration and minimization in transplant patients on maintenance immunosuppression. The inferred molecular mechanisms and pathways that guide the expression of some of the most highly regulated genes in this study are shown in Fig. 1B. Which drug to wean, how slowly to wean and when to wean the drug—these all currently remain unanswered questions that will require careful clinical trial design and patient monitoring in the future. A survey of gene expression profiles of 75 healthy adult volunteers by Whitney and colleagues [97] shows that expression signatures, in the absence of disease, are stable with time and variability among healthy individuals is lower than within disease states (infection, lymphoma, and leukemia; Fig. 1C). The variance in gene expression signatures in tolerance from normal is lower than in bacterially infected peripheral blood mononuclear cells (PBMC) and almost 50% of the variance in cancer, suggesting that a study of gene expression pattern changes in peripheral blood during tolerance may be sufficient for accurate phenotype classification from the normal state.

Limited studies of long-term human renal allograft transplantation tolerance are consistent with a peripheral tolerance mechanism rather than central tolerance [24], with the emergence of regulatory T cells (Tregs). $CD4^+CD25^+$ Tregs decrease indirect T-cell allorecognition of donor HLA-DR peptides in renal allograft recipients [100]. Recent work suggests that surface proteins, such as FOXP3, GITR [101,102] and neuropilin [103], may help distinguish $CD4^+CD25^+$ T cells with regulatory activity from non-regulatory cells; some of these genes were also found to be differentially regulated in the microarray-based tolerance study mentioned above [41]. De novo peripheral generation of $CD4^+CD25^+FOXP3^+$ Treg cells from $CD4^+CD25^-FOXP3^+$ T cells, under the influence of TGF- β , has been reported [104]. The array studies on human renal transplant operationally tolerant patients discussed earlier and in Fig. 1, support that the peripheral generation of Treg from naïve precursors might be a consequence of sustained suboptimal antigenic stimulation that is facilitated by TGF- β , with altered costimulation by CD9 and CD28 down-modulating T-cell activation [105,106], and increased expression of ubiquitin pathway genes, promoting degradation of signaling molecules involved in the immune synapse [107]. It could be that the repertoire of natural $CD4^+CD25^+$ Treg in operationally tolerant patients may be skewed toward self-antigens that are shared with those derived from the grafted tissue.

As presented at the 8th International Conference on New Trends in Immunosuppression and Immunotherapy in 2008 (<http://www.kenes.com/immuno2008>), The European Union Indices of Tolerance Network [108], and the National Institutes of Health's Immune Tolerance Network (ITN) [109], are also collecting additional quasi-tolerant patients for similar analyses by microarray (reviewed by Sawitzki and colleagues [96]). These efforts are currently being conducted jointly among multiple groups because of the paucity of patients who have true operational tolerance. At the recent American Transplant Congress meeting in June, 2008 in Toronto, the European consortium [45], suggested that drug-free tolerant patients show a population expansion of peripheral blood B lymphocytes and natural killer (NK) lymphocytes in the absence of donor-specific antibodies when compared with patients on triple immunosuppression. A similar population expansion of B cells and selective expression of B cell-related genes in samples obtained from drug-free tolerant patients was also noted by the ITN group [44], when the comparative denominator again was patients on triple immunosuppression. Flow cytometry analysis on peripheral blood B cells in patients who had kidney transplants did not identify any differences in the percentage/distribution of naïve (Bm1), activated (Bm2/Bm2'), or memory B cells (EBm5/Bm5) [110], between tolerant, stable and chronic rejection patients. Although these data on potential differences in cell subsets in operational tolerance are intriguing, the impact of immunosuppression on B cell numbers and B cell subsets is difficult to separate from the presence of operational tolerance in a drug-free state.

Table 2
Key array-based human studies in transplantation tolerance

Author	Array type	Tissue	Transplanted organ	Phenotype (number of patients)	Key findings: mechanism or biomarkers
Published studies					
Braud, et al [40]	6864 genes were tested by Oligoarray	PBMC	Kidney	TOL (n = 8) HV (n = 8) CR (n = 18)	223 genes are associated with TOL (43 up-regulated and 180 down-regulated): TOL has increased genes of response to wounding (10), immune response (14), defense response (14), response to biotic stimulus (14), response to pest/pathogen/parasite (10), response to external stimulus (15), response to stress (11); TOL has reduced genes of immune response (49), defense response (51), response to biotic stimulus (52), response to pest/pathogen/parasite (34), response to external stimulus (60), response to stress (44), cell motility (18), response to wounding (21), inflammatory response (16), receptor activity (45), innate immune response (16), defense/immunity protein activity (13), transmembrane receptor activity (31), obsolete molecular function (15), carbohydrate metabolism (14).
Brouard, et al [41]	cDNA microarray	Peripheral blood	Kidney	Training group: TOL (n = 5) STA (n = 11) HV (n = 8) Validation group: TOL (n = 12) STA (n = 12) MIS (n = 10) AR (n = 14) CR (n = 11) HV (n = 8)	“Tolerant footprint” of 49 genes; 33 genes correctly segregated TOL and CAN phenotypes with 99% and 86% specificity: TOL has reduced costimulatory signaling, immune quiescence, apoptosis, and memory T cell responses.

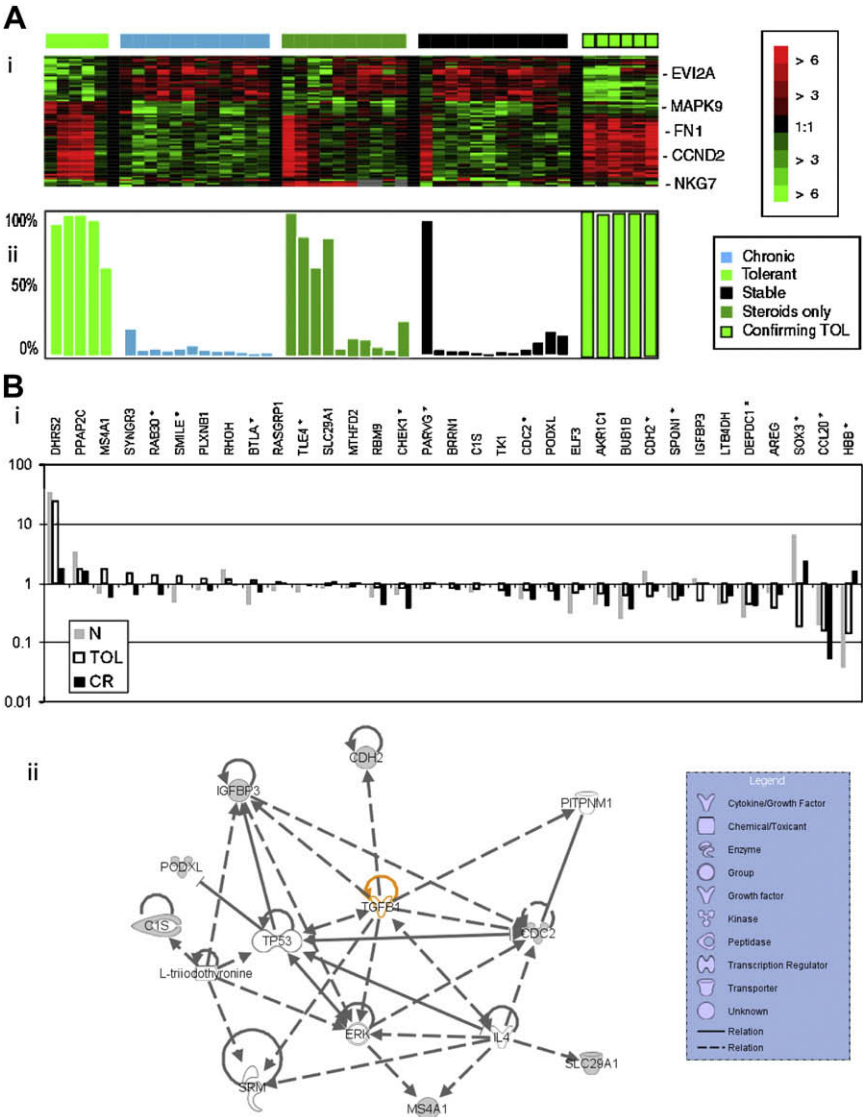
Kawasaki, et al [42]	cDNA (Agilent)	PBMC	Liver	TOL (n = 11) HV (n = 11)	717 genes are specific to TOL (627 up-regulated and 90 down-regulated): Significant three up- and down-regulated genes were categorized (numbers of genes HV < TOL/ TOL < HV): apoptosis (8/0); calcium (8/1); cell cycle (5/0); chemokine (2/1); cytokine (7/3); immunity (40/2); kinase (18/2); membrane (49/2); metabolism (9/0); phosphatase (11/3); receptor (55/10); signal transduction (11/1); transcription (20/3); and others (461/75)
Martinez-Llordella, et al [43]	cDNA (Affymetrix)	PBMC	Liver	TOL (n = 16) STA (n = 16) HV (n = 10)	628 genes are associated with TOL (462 up-regulated and 166 down-regulated): TOL exhibited increased $\gamma\delta$ T-cell and NK receptors, regulatory T-cell subsets (CD4 ⁺ CD25 ⁺ T cells and Vdelta1 ⁺ T cells) than STA or HV
ATC-2008 abstracts (http://www.atcmeeting.org/2008):					
Hernandez Fuentes, et al [45]	Microarray	Peripheral blood	Kidney	TOL STA (<10 mg/d prednisone) STA (CNI) STA (CNI free) CR HV	Selective expansion of peripheral blood B and NK lymphocytes in TOL, high ratio of foxp3/ α 1,2-mannosidase
Newell, et al [44]	Microarray	PBMC Whole blood Urine	Kidney	TOL (n = 22) STA (n = 34) CAN (n = 20) HV (n = 18)	Significantly higher expression of B cell differentiation genes in TOL, higher numbers of naïve B cells in peripheral blood; increased expression of CD20 in urine in STA and CAN

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Table 2
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Author	Array type	Tissue	Transplanted organ	Phenotype (number of patients)	Key findings: mechanism or biomarkers
Braud, et al [94]	Microarray	PBMC	Kidney	TOL CR	343 differentially expressed genes: key genes, such as BANK1 (a negative modulator of CD40-mediated AkT activation, thereby preventing hyperactive B cell response in blood from TOL patients), and IL7R (a specific marker absent on potentially regulatory CD4 ⁺ CD25 ^{high} T cells in blood from patients who have CR)
Martinez-Llordella, et al [95]	Affymetrix microarray	PBMC	Liver Kidney	TOL (n = 22) Non-TOL (n = 31) HV Previously published cDNA array in kidney TOL [41]	The most significantly enriched in non-tolerant liver recipients: hypertrophy pathway (eg, ILR1, IFNg, VEGF), CTLA4 pathway (eg, CTLA4, ICOS, CD28), hypoxia-inducible factor pathway (eg, HIF1A, JUN), NTHI pathway (eg, TNF, IL1B), LAIR pathway (eg, C5, ICAM1), and IL1R pathway (eg, ILR1, IL1B, TRAF).Significantly enriched in liver TOL: NK related genes (eg, NKG7, KLRD1, KIR2SD2); Comparison of the liver and kidney data sets yielded 61 identical genes (cell cycle pathway and carboxylic acid metabolism pathway).

Abbreviations: AR, acute rejection; CAN, chronic allograft nephropathy; CNI, calcineurin inhibitors; CR, chronic rejection; HV, healthy volunteers; MIS, steroid immunosuppression; NK, natural killers; PBMC, peripheral blood mononuclear cells; STA, stable on immunosuppression; TOL, operational tolerance.



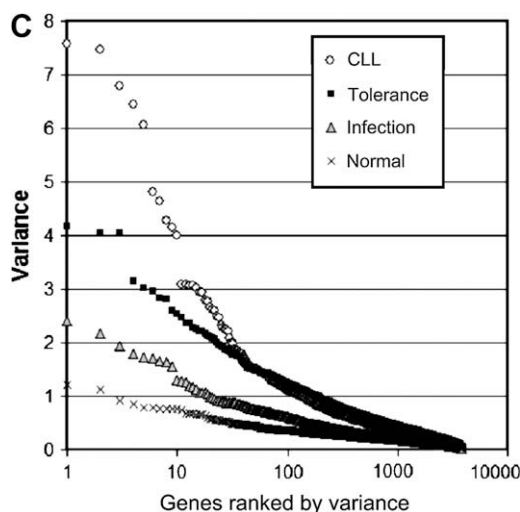


Fig. 1 (continued)

Fig. 1. (A) Microarray analysis and prediction scores for the operationally tolerant phenotype in renal transplantation. In panel i, the gene clustering is shown for the tolerance footprint of 49 genes, with the most regulated genes in operational tolerance marked on the far left by their gene symbols. In panel ii, the prediction score on the Y axis ranges from 0% to 100% for fit-to-class for operational tolerance; the X axis shows individual blood samples with different clinical categories. A small subset of genes in peripheral blood from renal transplant recipients can predict operational tolerance (TOL) in 5 of 6 patients studied in the learning set (green bars), in 6 of 6 patients in the prediction set (green bars with black outlines), in none of the patients who had chronic rejection (blue bars), in 4 of 10 patients on steroid monotherapy (dark green bars), and in 1 of 12 patients on triple immunosuppression and stable graft function (black bars). (Adapted from Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* 2007;104:15448–53; with permission.) (B) Potential peripheral mechanism supporting operational tolerance. In panel i, quantitative reverse transcriptase (RT)–PCR results on the 33 most abundant genes are shown. Triplicate measurements were averaged and median fold expression relative to HRPT (normalizing gene). Genes with statistically significant expression differences between TOL and chronic rejection (CR) patients are indicated with * following the gene symbol and $P < .001$ for CCL20, TLE4, CDH2, PARVG, and SPON1, whereas $P < .006$ for RAB30, BTLA, and SMILE; $P < .03$ for SOX3, CHEK1, HBB, and DEPDC1; and $P = .045$ for CDC2. Most genes in the tolerance signature are regulated by way of the TGF β axis (panel ii). The Network Pathway has been created using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA). (C) Global expression differences in peripheral blood samples in normal controls and disease. Variance in global gene expression in peripheral blood was examined across 24 training group samples composed of immunosuppressive drug-free tolerant transplant recipients (TOL), chronic rejection (CR), and normal (N) cohorts, and compared with global expression variance analysis conducted on published peripheral blood microarray studies on the lymphochip platform used in our study: (1) circadian variations in peripheral blood from 75 healthy volunteers [97], (2) infection of peripheral blood with different pathogenic bacteria [98] and (3) chronic lymphocytic leukemia (CLL) [99]. Global expression variance was calculated across 3826 randomly selected overlapping transcripts and plotted in rank order. Variance across the data from this study (“tolerance”) was intermediate between chronic lymphocytic leukemia and host response to different bacteria (“infection”), and variance in all three studies was greater than in resting whole blood from normal adult volunteers (“normal”).

Although array data do not exist on other cell types in human transplantation tolerance, published studies suggest that manipulation of other pathways in the allo- and innate immune response can promote graft accommodation. Data from several groups have reshaped the role of NK cells in allograft rejection and tolerance [111]. Recent publications have described a novel paradigm whereby NK cells can be protolerogenic and tolerance cannot be induced in their absence [112]. Earlier publications also demonstrated that NK cells are required for induction of tolerance to islet allografts [113], NK cells promote skin transplant tolerance by killing donor antigen-presenting cells [114], and NK cells play an essential role in augmentation of allograft survival mediated by donor spleen cells [115]. The decay-accelerating factor (Daf) axis may also be a critical pathway for the manipulation of tolerance and anergy [116] because Daf dissociates C3/C5 convertases and thereby prevents complement activation. Absence of MyD88, a key innate TLR signal adaptor, has been found to promote inducible allograft acceptance, implying that inhibiting innate immunity may be a potential, clinically relevant strategy to also facilitate transplantation tolerance [117]. Reducing inflammation (avoidance of infections) plus protocols to reduce inflammation will be important adjuvant therapy for future transplant tolerance induction. Daf dissociates C3/C5 convertases that assemble on host cells and thereby prevents complement activation on their surfaces. During primary T cell activation, the absence of Daf on antigen-presenting cells (APCs) and on T cells enhances T cell proliferation and augments the induced frequency of effector cells. Population expansion of CD4⁺CD25⁺CD45RO⁺IL-7R^{hi} cells has been demonstrated in patients who have chronic humoral rejection, in contrast to a lack of such expansion in stable transplant recipients [118]. Dendritic cells (DC) also have a potential tolerogenic role, and in the absence of danger signals, DCs exist in an “immature” state expressing few or no costimulatory molecules, and cognate engagement of antigen-specific T cells results in anergy or apoptosis [119,120]. Given this, the potential use of immature or tolerogenic DCs as therapy to promote peripheral tolerance on organ transplantation has been an area of active research [121]. Recent studies have used immature donor DCs with the addition of costimulation blockade, revealing a synergistic effect in promoting long-term allograft survival [122]. It has been demonstrated that precursors of plasmacytoid DC (pDC2) are increased in functionally tolerant liver transplant recipients and in those being weaned from immunosuppressive drugs relative to those receiving chronic immunosuppression [35]. The ratio of pDC2:pDC1 also seems to be increased in tolerant patients relative to those maintained on immunosuppression. Immunosuppressive drugs themselves were not shown to affect the number of pDC2 or the pDC2:pDC1 ratio, thereby supporting the validity of this assay for the identification of tolerant liver transplant recipients [35]. A novel macrophage population [123], IFN γ -induced monocyte-derived cells (IFN γ -MdC), may also be an interesting pool of cells to study in

tolerant patients as they are capable of deleting activated T cells and enriching Treg.

A different approach for identifying tolerance-driven biomarkers has been used by moving from animal models of tolerance to human clinical trial studies [124]. Gene expression data were analyzed in conventional animal models of tolerance induction, and then tested in models in which tolerance was challenged by memory cells and heterologous immunity [125]. The evaluated biomarkers were then examined for their ability to predict tolerance abrogation in human clinical trials. With this strategy, the investigators were able to identify at least two biomarkers, Toag-1 and the ratio of Foxp3 to α -1,2-mannosidase, suitable for monitoring patients after transplantation [124]. Encouragingly, by whole-blood expression analysis of markers identified with this strategy, the researchers were able to correctly categorize quasi-tolerant patients in the European Union-funded Indices of Tolerance project [108,109].

There is still no clear understanding if the mechanisms leading to the operational tolerance are the same between different organs. Some preliminary findings were reported at American Transplant Congress-2008 by Dr. Martinez-Llordella and colleagues [95], who have compared gene expression patterns of 22 operationally tolerant and 31 nontolerant liver recipients with Affymetrix data obtained from nontransplanted healthy individuals and with our previously published cDNA array dataset [41] comprising 11 tolerant kidney recipients. This analysis concluded that liver- and kidney-tolerant states seem to be fundamentally different biologic processes, although some common pathways (cell cycle and carboxylic acid metabolism pathways) could be relevant to both settings (see Table 2). Nevertheless, additional work on more patient numbers is required to substantiate these data as the array datasets were from different laboratories and array platforms, and on relatively small patient numbers. It is important to support, in the conduct of microarray-based studies, that all published microarray studies should be made publicly available through the Internet, and should follow the minimum information about a microarray experiment (MIAME) compliance format [126] or microarray gene-expression markup language (MAGE-ML) [127]. Many freely software tools are now available for microarray data analysis and many of the currently used analytic methods have been recently described [75].

In conclusion, longitudinal human studies in a large cohort of stable immunosuppressed patients and studies to examine the rare occurrences of operational tolerance and induced transplant tolerance are important directions for unraveling the mysteries of the development and maintenance of stable human tolerance. Such studies can provide an *in vivo* means to analyze various molecular mechanisms and cellular compartments, and the impact of the frequency and suppressive capacities of regulatory T cells toward maintaining immune homeostasis. Harnessing the ability to promote transplantation tolerance will be a major advance for promoting positive

recipient health without the toxicity of maintenance immunosuppression and extended graft survival with the anticipation of limiting chronic rejection.

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