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Immunosuppressive Drug-Free Operational Immune Tolerance in Human Kidney Transplant Recipients: Part I. Blood Gene Expression Statistical Analysis

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Abstract Survival of solid organ grafts depends on life-long immunosuppression, which results in increased rates of infection and malignancy. Induction of tolerance to allografts would represent the optimal solution for controlling both chronic rejection (CR) and side effects of immunosuppression. Although spontaneous ''operational tolerance'' can occur in human kidney transplantation, the lack of noninvasive peripheral blood biological markers of this rare phenomenon precludes the identification of potentially tolerant patients in whom immunosuppression could be tapered as well as the development of new tolerance inducing strategies. Here, the potential of high throughput microarray technology to decipher complex pathologies allowed us to study the peripheral blood specific gene expression profile and corresponding EASE molecular pathways associated to operational tolerance in a cohort of human kidney graft recipients. In comparison with patients with CR, tolerant patients displayed a set of 343 differentially expressed genes, mainly immune and defense genes, in their peripheral blood mononuclear cells (PBMC), of which 223 were also different from healthy volunteers. Using the expression pattern of these 343 genes, we were able to classify correctly >80% of the patients in a cross-validation experiment and classified correctly all of the samples over time. Collectively, this study identifies a unique PBMC gene signature associated with human operational tolerance in kidney transplantation by a classical statistical microarray analysis and, in the second part, by a nonstatistical analysis. J. Cell. Biochem. 103: 1681–1692, 2008.

Key words: gene expression; DNA microarray; kidney transplant; transplant tolerance; chronic rejection

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Induction of operational tolerance [Ashton-Chess et al., 2006], clinically defined as "*the absence of acute and chronic rejection and indefinite graft survival with normal function in an immunosuppressive free and fully immunocompetent host*" [Ansari and Sayegh, 2004], is regarded as the optimal solution for both chronic rejection (CR) and side effects of standard immunosuppression [Dantal et al., 1998; Soulillou, 2001] in patients with solid organ transplantation. Several therapeutic strategies have been shown to induce operational tolerance in experimental models [Cobbold et al., 2006; Ballet et al., 2007], but are turning out to be difficult to apply in the clinic. However, accumulating evidence indicates that a state of operational tolerance can indeed occur in human graft recipients: immunosuppression can be completely withdrawn in 25% of liver recipients [Takatsuki et al., 2001; Martinez-Llordella et al., 2007] and some kidney recipients display spontaneous operational tolerance years after immunosuppressive treatment interruption [Roussey-Kesler et al., 2006]. A better understanding of this rare but biologically and medically important phenomenon may help to guide progressive immunosuppressive drug minimization in selected patients as well as to develop monitoring of new tolerance induction protocols in humans [Fudaba et al., 2006].

Several mechanisms may contribute to the spontaneous acceptance of a kidney graft. We have previously described the clinical features of drug free operational tolerance in kidney transplantation [Ballet et al., 2006; Roussey-Kesler et al., 2006]. We have shown that most of these patients display an altered T cell receptor (TCR) repertoire usage with a unique pattern of inflammatory cytokine transcripts in T cells with skewed TCR usage [Brouard et al., 2005] as well as CD4 and CD8 lymphocyte phenotypes distinct from patients with CR but close to those of normal individuals [Baeten et al., 2006; Louis et al., 2006]. However, none of these observations have yet been convincingly validated as being functionally involved in operational tolerance in humans. Therefore, the identification of the peripheral blood molecules associated with drug-free operational tolerance in kidney graft recipients remains an important and challenging issue.

High throughput methods such as microarrays have expanded our ability to study complex pathological situations such as cancers [Alon et al., 1999; Alizadeh et al., 2000; van de Vijver et al., 2002; Glas et al., 2005; Becker et al., 2006], cardiovascular disease [Hiltunen et al., 2002; Tuomisto et al., 2003], autoimmune disorders [Chabas et al., 2001; Lock et al., 2002; Baechler et al., 2003; Bennett et al., 2003; Peterson et al., 2004; Kasperkovitz et al.,], glomerular kidney diseases [Luckow et al., 2004], and organ transplantation [Stegall et al., 2002; Man et al., 2003; Sarwal et al., 2003; Flechner et al., 2004; Horwitz et al., 2004; Hotchkiss et al., 2006]. Recently, Martinez-Llordella et al. [2007] reported a specific gene signature in blood from liver transplant recipients who had been successfully weaned from immunosuppressive drugs. In the current study, we report a differential transcriptional pattern, consisting mainly of immune and defense genes, in peripheral blood of patients who are operationally tolerant to kidney grafts compared to patients with CR and healthy volunteers (HV). Data of nonstatistical bioinformatics gene analysis based on classification of "key genes" are presented in the twin article [Sivo et al., 2007].

MATERIALS AND METHODS

Patients

Thirty-four individuals were included in the study. The protocol was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks of the University Hospital. All patients signed a written informed consent before inclusion.

The study included:

(a) Eight drug-free operationally tolerant (TOL) patients, defined as kidney graft recipients with stable graft function (blood creatinemia < 150 μmol/L, proteinuria < 1 g/24 h) for at least 1 year (mean 6.4 years, range 1.6–17.2 years) after complete interruption of immunosuppressive therapy. Thus, this disruption is based on clinical and stable graft function years after withdrawal of all immunosuppressive drugs. Stable graft function was defined by the absence of 30% or more variation of the baseline creatinemia. Biopsies were not recommended by the ethical committee and refused by the patients. Immunosuppressive treatment, including corticosteroids, was

stopped due to non-compliance (TOL01, 04, 05, 06, 07 and TOL08), post-transplant lymphoproliferative disorder (PTLD) (TOL03), or calcineurin inhibitor toxicity (TOL02). The clinical and biological characteristics of the operationally tolerant patients have been described in detail previously [Roussey-Kesler et al., 2006]; the most relevant demographic and clinical data are summarized in Table I. Four out of the 8 TOL patients (TOL01 to TOL04) were sampled and analyzed at two different time points with an interval of at least 16 months (mean 25.8 months, range 16–31 months). These patients fulfilled all clinical and biological criteria of drug-free operational tolerance at the different studied time points. Serially harvested samples are denoted by an asterisk.

(b) Eighteen kidney graft recipients with CR: All patients had a progressive degradation of their renal function (blood creatinemia $\geq$150 μmol/L, proteinuria $\geq$1 g/24 h). CR was diagnosed on a graft biopsy according to the updated Banff criteria [Solez et al., 2007]. CR was defined by histological signs of chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell infiltration) and/or transplant glomerulopathy with glomerular double contours: 15/18 patients had a transplant glomerulopathy, 10/18 had an "active humoral component" [Solez et al., 2007] as demonstrated by the presence of C4d together with circulating anti-HLA antibodies. One of the patients had C4d deposits but no circulating anti-HLA antibodies and another patient did not show C4d deposits but did have circulating anti-HLA antibodies. Three CR patients (CR001 to CR003) were also sampled and analyzed at two time points with an interval of at least 12 months (mean 25.8 months, range 12–36 months). To assess the role of maintenance immunosuppressive treatment on the gene expression pattern, this population of 18 patients consisted of 15 patients under immunosuppressive treatment (CR001 to CR006, CR010 to CR013) and three patients with CR (CR007 to CR009) in whom immunosuppression was completely stopped and dialysis was recently restarted. Detailed demographic and clinical data are shown in Table I.

(c) Eight age-matched HV were also included after informed consent. They all had a normal blood formula and no infectious or other concomitant pathology for at least 6 months prior to the study (Table I).

RNA Preparation

Ten milliliters of peripheral blood were collected in EDTA tubes. Peripheral Blood Mononuclear Cells (PBMC) were separated on a Ficoll layer (Eurobio, Les Ulis, France) and frozen in Trizol® reagent (Invitrogen, Life Technologies, CA). One microgram of total RNA (tRNA) of each sample was subjected to amplification using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Austin, TX) according to the manufacturer's protocols. This method of RNA amplification has been extensively tested and validated for our microarray platform (see: http://cardioserve.nantes.inserm.fr/ptf-puce/publis_en.php#_4). In accordance with previous studies reporting that RNA amplification may induce significantly different expression ratios in approximately 10% of the genes [Nygaard et al., 2003], preliminary validation assays with 14 samples subjected to two independent amplifications showed differential expression of 13.7% of the analyzed genes (data not shown). In order to reduce this technical variability in the present study, each tRNA sample was amplified in duplicate and both duplicates were subsequently used for microarray hybridization and analysis. Quality and quantity of tRNA and amplified RNA (aRNA) was tested by an Agilent 2100 Bioanalyser® (data not shown). All aRNA samples were subsequently fragmented using RNA fragmentation Reagents (Ambion) according to the manufacturer's protocols. Fragmented aRNA of each sample was labeled with Cyanine 3 (Cy3; Amersham Biosciences, Buckinghamshire, UK). As the reference for the hybridization, aRNA were prepared as described from PBMC of 169 kidney graft recipients with stable graft function and under standard immunosuppressive treatment. This referential aRNA was pooled and labeled with Cyanine 5 (Cy5). Cyanine incorporation and aRNA quantities were confirmed using a Nanodrop ND1000 spectrometer (data not shown).

Microarray Hybridizations

Six thousand eight hundred sixty-four genes of interest in immunology, apoptosis and cell signaling were selected to be used on a dedicated "immunology microarray" spotted on glass-slides.

TABLE I. Detailed Demographic and Clinical Definition of the Patients

ID	Class	Age (years)	Gender	Previous episode of acute rejection	Lymphoma status, cancer	Episode of acute infection (CMV)	Time between graft and analysis (months)	Creatinemia (μmol/L)	Proteinuria (g/24 h)	Donor (living vs. deceased)	Number of HLA mismatches	Medical treatment	Time between immunosuppression withdrawal and analysis (months)	Reason of immunosuppression withdrawal	Banff/histology	C4d staining	Anti-HLA antibody	Allograft glomerulopathy
TOL01[a]	TOL	38	M	0	0	0	55.9	115	0	Liv	0	0	37	Uncompliance	—	—	—	—
TOL02[a]	TOL	74	F	2	0	0	127.0	96	0.50	Dec	3	0	110	CsA toxicity	—	—	—	—
TOL03[a]	TOL	25	M	0	1	0	135.6	103	0.35	Dec	3	0	39	PTLD	—	—	—	—
TOL04[a]	TOL	57	M	0	0	0	185.8	82	0	Dec	4	0	25	Uncompliance	—	—	—	—
TOL05[a]	TOL	37	M	1	0	0	371.6	139	0.93	Dec	3	0	147	Uncompliance	—	—	—	—
TOL06	TOL	39	F	0	0	0	138.0	131	0.10	Dec	4	0	20	Uncompliance	—	—	—	—
TOL07	TOL	43	F	0	0	0	60.0	10	0.12	Dec	1	0	26	Uncompliance	—	—	—	—
TOL08	TOL	75	M	1	0	NA	400.0	134	0.22	Dec	1	0	207	Uncompliance	—	—	—	—
CR001[a]	CR	58	M	0	0	0	41.7	244	0.56	Dec	5	CNI + CC	—	—	2–3	C4d−	−	+
CR002[a]	CR	57	M	0	0	0	45.5	127	2.96	Dec	1	CNI + CC + CS	—	—	2	C4d+	+	+
CR003[a]	CR	73	F	0	0	1	38.7	202	0.86	Dec	2	CNI	—	—	3	C4d+	+	−
CR004	CR	55	F	1	0	0	39.4	71	2.75	Dec	5	CNI + CC	—	—	1–2	C4d+	+	+
CR005[a]	CR	52	M	0	0	0	132.0	492	11.75	Dec	2	CNI + CS	—	—	NA	C4d+	+	NA
CR006[a]	CR	27	M	1	0	0	183.2	301	7.34	Dec	3	CNI + CC	—	—	1	C4d+	+	+
CR007[a]	CR	72	M	0	0	0	67.7	HD	NA	Dec	4	0	—	—	3	C4d+	−	+
CR008[a]	CR	30	M	0	0	0	58.7	HD	NA	Dec	5	0	—	—	2	C4d+	+	−
CR009[a]	CR	50	F	0	0	0	42.8	HD	NA	Dec	4	0	—	—	3	C4d−	−	+
CR010	CR	51	F	0	0	0	47.7	160	0.58	Dec	5	CNI + CC	—	—	1	C4d−	−	+
CR011	CR	58	M	0	0	0	157.9	344	9.56	Dec	2	CNI + CC	—	—	3	C4d+	+	+
CR012	CR	59	F	1	0	1	97.1	254	2.46	Dec	2	CNI + CC	—	—	2	C4d−	−	+
CR013	CR	42	F	1	0	1	126.3	195	2.56	Dec	1	CNI + CC + CS	—	—	NA	C4d+	+	+
CR014	CR	63	F	0	0	0	37.0	224	3.22	Dec	6	CNI	—	—	2	C4d−	−	+
CR015	CR	70	F	0	0	0	49.0	254	0.28	Dec	2	CNI	—	—	2	C4d−	−	+
CR016	CR	50	F	0	0	NA	220.0	312	4.03	Dec	2	CNI + CC	—	—	2	C4d−	+	+
CR017	CR	58	M	0	0	0	25.0	267	4	Dec	5	CNI + CC	—	—	2	C4d+	+	+
CR018	CR	27	M	0	0	0	50.0	185	1	Dec	0	CNI + CC + CS	—	—	2	C4d+	+	+
HV001	HV	42	F															
HV002	HV	44	M															
HV003	HV	41	F															
HV004	HV	39	F															
HV005	HV	54	F															
HV006	HV	51	F															
HV007	HV	56	F															
HV008	HV	50	M															

Tolerant (TOL) patients presented a highly stable kidney function for years after complete withdrawal of immunosuppression. Only one tolerant patient was a recipient of a deceased mismatched graft. Chronic rejection was confirmed by histology in all patients. The histological definition was based on the new Banff 2005 recommendations (Solez et al., 2007). HD, hemodialysis; Liv, living donor; Dec, deceased donor; CNI, calcineurin inhibitor; CS, corticosteroids; CC, CellCept; CsA, cyclosporin A; PTLD, post-transplant lymphoproliferative disorders.

[a]Patients used in the previous pangenomics experiments.

The genes were selected based on literature data, on data obtained in other immunological and cancer fields (IFR26, West Genopole, Nantes, France), and on our previous experiments with pangenomic arrays on 6 TOL and 8 CR, which had allowed us to select 520 unique genes (data not shown). These patients are denoted by superscript "a" in Table I. Detailed information on the microarray is available online (http://cardioserve.nantes.inserm.fr/ptf-puce/cancerochips_en.php). A set of 50-mer oligonucleotides corresponding to these 6,864 genes was spotted on the arrays, with each oligonucleotide being spotted in triplicate in order to reduce the technical variability of the assay. Subsequently, 1 μg of Cy5-labeled reference aRNA and 1 μg of Cy3-labeled sample aRNA were hybridized on the array for 16 h at 42°C in a sealed chamber (Telechem Int., Sunnyval, CA). Validation assays on 28 samples indicated a high correlation between the two hybridizations arising from the same sample, with an R^2 of 0.764 (±0.09) (data not shown). Since each RNA sample was amplified in duplicate, one hybridization per aRNA was performed (one for each aRNA) leading to a total of six data points per sample. All replicates were used in order to reduce variability [Yang and Speed, 2002].

Microarray Analysis

Hybridized slides were scanned by fluorescence confocal microscopy (ScanArray 3000, GSI-Lumonics) using independent laser excitation of the two fluorescent dyes at 532 and 633 nm at a 10 μm/pixel resolution. Fluorescence values and ratios were extracted with the GenePix Pro 5.1 software. For each fluorescent spot representing the hybridization with a nucleotide of a single gene, the average pixel intensity of the spot and the local background were computed and subtracted to obtain the net fluorescence intensity. The data were normalized using a lowest fit approach independently applied to each log ratio distributions [Golfier et al., 2004]. Significance levels (scaled fold) were computed by comparing the fluorescence intensity of the tested sample with that of the reference RNA for each spot of the microarray, using the VARAN error models (http://www.bionet.espci.fr/varan/). VARAN performs a signal intensity based analysis of the log 2 expression ratio variability deduced from the DNA microarray data. Considering the duplicates for the amplification and the triplicates for each gene spotted on the microarray, the median of the six-scaled fold values for each gene was used for statistical analysis. The technical details of the analysis of the microarrays are available on the website: http://www.bionet.espci.fr/varan/. Functional annotation of the genes selected as differentially expressed was performed using the Expression Analysis Systematic Explorer (EASE; http://niaid.abcc.ncifcrf.gov/content.jsp?file=/ease/ease1.htm&type=1). EASE provides a statistical significance of gene families using standardized Kyoto Encyclopedia of Genes and Genomes (KEGG) or Gene Ontology database terms, and a normalized gene enrichment score is reported for each functional category.

Statistics

Unsupervised clustering of gene expression data was performed by hierarchical clustering (Pearson uncentered distance and average linkage) with Cluster/TreeView software [Eisen et al., 1998] (http://rana.lbl.gov/EisenSoftware.htm). Cross-validation was performed using the Predictive Analysis of Microarray (PAM) software [Tibshirani et al., 2002] (http://www-stat.stanford.edu/~tibs/PAM/).

RESULTS

Identification of a Specific Gene Expression Pattern in Drug-Free Operational Tolerant Kidney Graft Recipients

We first analyzed the transcriptional profile in PBMC of the 8 TOL versus the 18 CR by two-class Significance Analysis of Microarray (SAM software) using a False Discovery Rate (FDR) <0.05 and running 1,000 permutations of the 6,864 oligonucleotides. As shown in Table II, 343 oligonucleotides corresponding to 331 distinct genes were found to be significantly differentially expressed between TOL and CR sets. Of these, 275 were downregulated in TOL with a fold change of 0.475–0.896. The other 68 genes were upregulated in TOL compared to CR, with a fold change of 1.143–2.529 (Supplemental Table I).

In order to ascertain that the 343 genes set was associated with operationally tolerance and not due to the absence of immunosuppressive drugs in these patients, we reanalyzed the expression data of the 343 genes in comparison with healthy individuals (HV) by SAM analysis. Among the 68 upregulated genes in TOL versus

TABLE II. Functional Classification of the 343 Differentially Expressed Genes Using EASE

Gene Category	Number of genes	EASE score (*P*-values)
Upregulated genes in TOL		
Response to wounding	10	4.10E – 05
Immune response	14	1.84E – 04
Defense response	14	3.34E – 04
Response to biotic stimulus	14	6.44E – 04
Response to pest/pathogen/parasite	10	1.14E – 03
Response to external stimulus	15	2.38E – 03
Response to stress	11	7.56E – 03
Downregulated genes in TOL		
Immune response	49	1.67E – 05
Defense response	51	1.75E – 05
Response to biotic stimulus	52	5.12E – 05
Response to pest/pathogen/parasite	34	6.68E – 05
Response to external stimulus	60	3.08E – 04
Response to stress	44	3.87E – 04
Cell motility	18	2.75E – 03
Response to wounding	21	4.18E – 03
Inflammatory response	16	6.77E – 03
Receptor activity	45	7.19E – 03
Innate immune response	16	9.30E – 03
Defense/immunity protein activity	13	1.84E – 02
Transmembrane receptor activity	31	2.20E – 02
Obsolete molecular function	15	3.86E – 02
Carbohydrate metabolism	14	4.91E – 02

Functional categories with a *P*-value <0.05 and a number of genes >10 were selected (http://niaid.abcc.ncifcrf.gov/content.jsp?file=/ease/ease1.htm&type=1).

CR, 43 were also differentially expressed between TOL and HV. Among the 275 downregulated genes in TOL versus CR, 180 were also differentially expressed between TOL and HV. Taken together, these 223 genes (Supplemental Table II) are differentially expressed in TOL compared to CR but also different compared to HV. These 223 genes constitute a unique pattern for operational tolerance. We employed EASE in order to identify functional categories for the 343 genes list and the 223 gene list (Tables II and III, respectively). For each analysis, we selected only those functional categories with a $P < 0.05$ and a number of genes >10. For the 343 genes list, EASE identified 7 and 16 functional groups among the upregulated and the downregulated genes, respectively (Table II). For the 223 genes list, EASE identified no functional family among the upregulated and 12 functional groups among the downregulated genes (Table III). In both analysis, the functional categories corresponded to genes involved in immune responses and host defense.

Hierarchical Classification Using the Gene Expression Profiles

We next analyzed the capacity of the gene expression profiles to classify the diagnostic category (TOL vs. CR) of a sample using PAM

TABLE III. Functional Classification of the 223 Differentially Expressed Genes Using EASE

Gene Category	Number of genes	EASE score (*P*-values)
Downregulated genes in TOL		
Response to wounding	15	2.12E – 05
Immune response	23	1.13E – 04
Defense response	23	2.70E – 04
Response to biotic stimulus	23	6.90E – 04
Inflammatory response	10	1.33E – 03
Innate immune response	10	1.70E – 03
Response to pest/pathogen/parasite	15	2.09E – 03
Cell motility	10	2.47E – 03
Response to stress	19	4.30E – 03
Response to external stimulus	24	8.81E – 03
Calcium ion binding	12	9.05E – 03
Biological process unknown	10	3.73E – 02

Functional categories with a *P*-value <0.05 and a number of genes >10 were selected (http://niaid.abcc.ncifcrf.gov/content.jsp?file=/ease/ease1.htm&type=1).

software [Tibshirani et al., 2002]. PAM analysis of the 343 differentially expressed genes was performed in the 8 TOL and the 18 CR patients. As shown in Figure 1A, 5 out of 8 TOL and 16 out 18 CR were classified correctly. Collectively, these data indicate we are able to classify TOL and CR patients correctly in this cross-validation experiment with a positive predictive value of 80.8%.

The same analysis was performed using the 223 genes differentially expressed in TOL compared to both CR and HV that constitute the unique gene expression pattern of operational tolerance. As shown in Figure 2A, again 5 out of 8 TOL and 16 out of 18 CR were classified correctly by PAM, yielding the same positive predictive value.

Using the list of 343 differentially expressed genes between TOL versus CR, the corresponding expression matrix was classified by an unsupervised hierarchical clustering approach where the degree of similarity between samples and genes are visualized. As shown in Figure 3, two clearly distinct clusters of patients were identified solely on the basis of the gene expression data: one cluster contained 6 out of 8 TOL, whereas the other contained 16 out of the 18 CR as well as two TOL (TOL06 and TOL08). This result is in accordance with what is observed in Figure 2.

Stability of the Gene Expression Profile Over Time

In order to confirm that the TOL and CR gene signature was reproducible and stable over time, 4 TOL (TOL01 to TOL04) and 3 CR (CR001 to CR003) patients were sampled and analyzed at a second time point with an interval of at least 12 months (mean 25.7 months, range 16–31 months for TOL and mean 25.8 months,

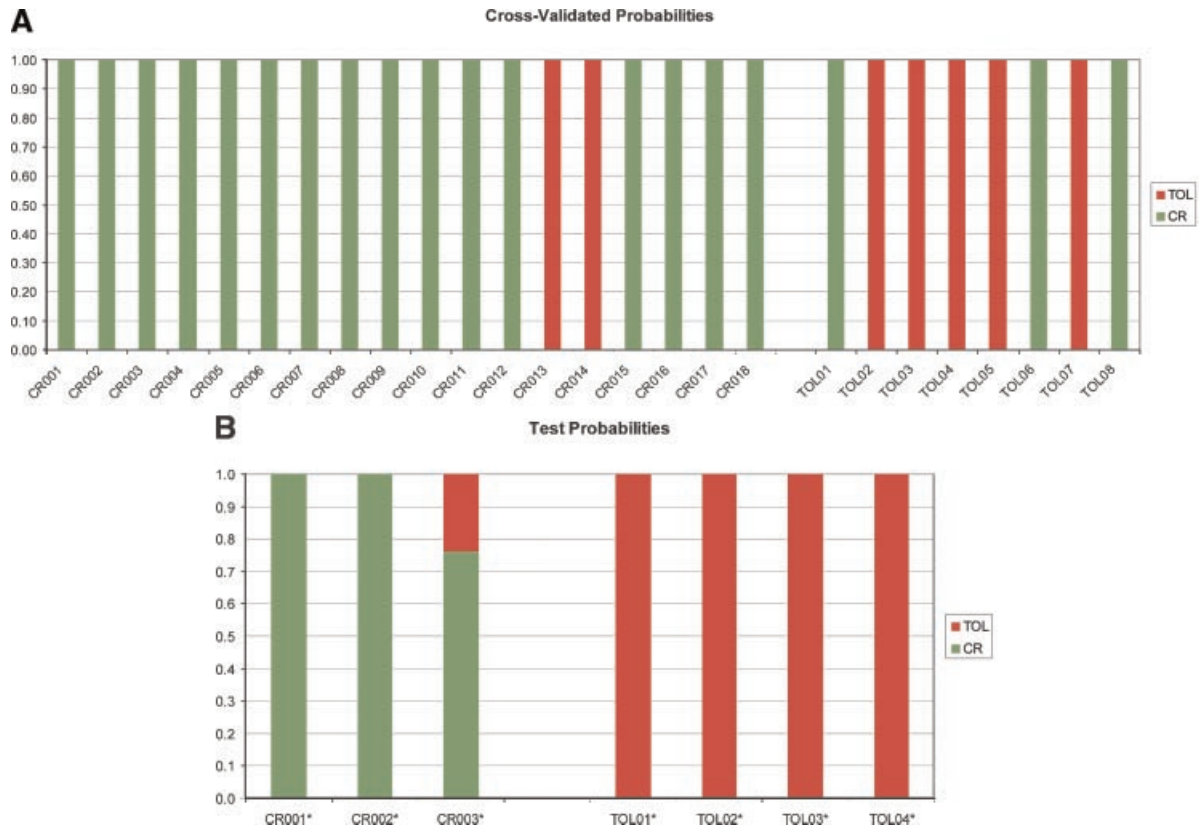

Fig. 1. Classification probabilities of individual patients by Predictive Analysis of Microarray (PAM) based on the 343 differentially expressed genes between operationally tolerant kidney graft recipients (TOL) and patients with chronic rejection (CR). Each patient sample is shown by a bar, as labeled in the X-axis. The color codes indicate the probability (0–1, as indicated in Y-axis) that the sample belongs to TOL (red) or CR (green). **A**: Cross-validated probabilities on the 8 TOL and 18 CR that were used to set up the 2-class (TOL/CR) classification algorithm. Among the 26 patients, 5 samples (CR013, CR014, TOL01, TOL06 and TOL08) were misclassified. **B**: Using the PAM algorithm defined with 8 TOL and 18 CR patients, 7 serially harvested samples (4 TOL and 3 CR) at a time interval of more than 1 year after the first sample were classified. The algorithm correctly classified all samples, with a probability of 100% for TOL and 92.0% for CR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

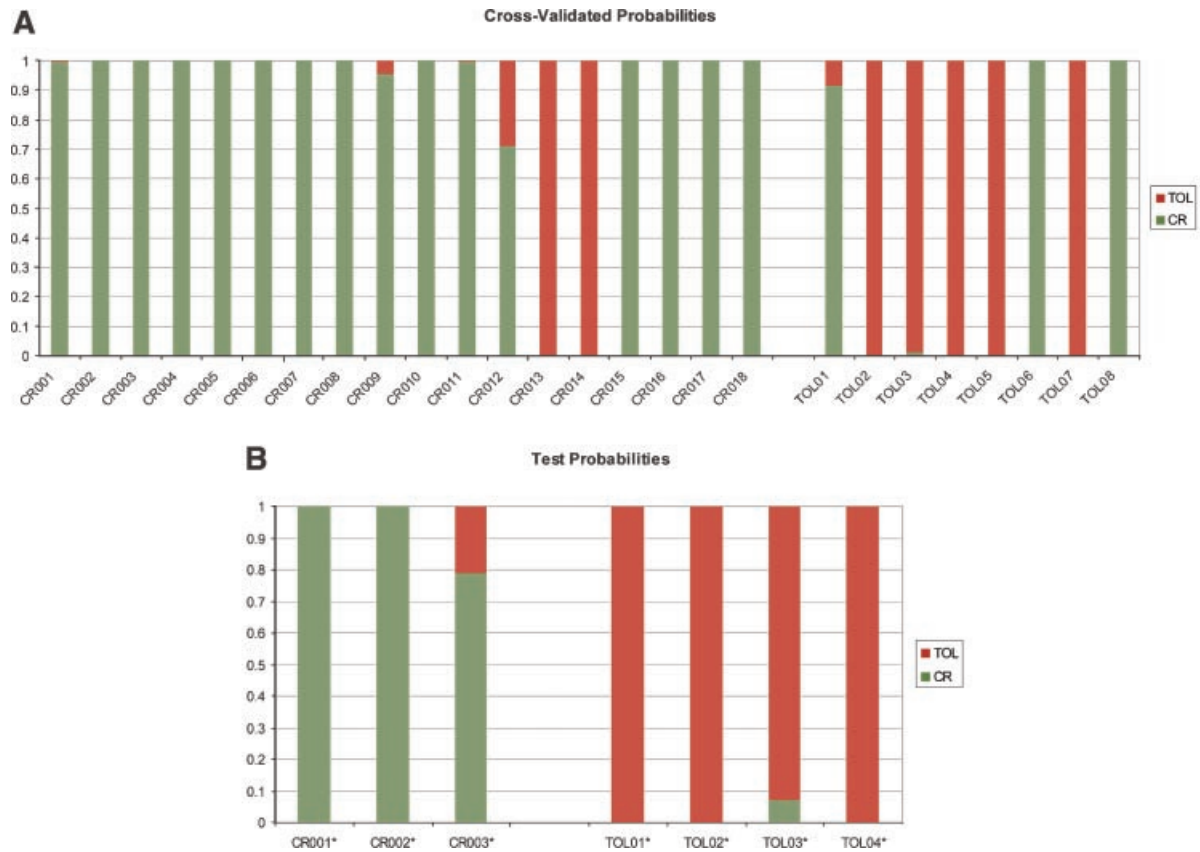

Fig. 2. Classification probabilities of individual patients by Predictive Analysis of Microarray (PAM) based on the 223 specific genes differentially expressed between operationally tolerant kidney graft recipients (TOL), patients with chronic rejection (CR), and HV. Each patient sample is shown by a bar, as labeled on the X-axis. The color codes indicate the probability (0–1, as indicated in Y-axis) that the sample belongs to TOL (red) or CR (green). **A**: Cross-validated probabilities on the 8 TOL and 18 CR that were used to set up the 2-class (TOL/CR) algorithm. Among the 26 patients, 5 samples (CR013, CR014, TOL01, TOL06, and TOL08) were misclassified. **B**: Using the PAM algorithm defined with 8 TOL and 18 CR patients, 7 serially harvested samples (4 TOL and 3 CR) at a time interval of more than 1 year after the first sample were classified. The algorithm correctly classified all samples, with a probability of 98.2% for TOL and 92.9% for CR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

range 12–35 months for CR). The gene expression data were analyzed using the PAM algorithm defined in the previous paragraph by the 8 TOL and 18 CR samples. All the serial TOL and CR samples were classified correctly with a probability of 100% and 92.0% for TOL and CR, respectively (Fig. 1B). Also the PAM algorithm based on the unique 223 gene set classified all TOL and CR correctly with a probability of 98.2% and 92.9%, respectively (Fig. 2B). Accordingly, the gene expression levels of the serial samples showed a good correlation over time in 6 out of 7 patients (Table IV).

DISCUSSION

Kidney transplantation remains the major treatment for end-stage renal diseases, but is often complicated either by acute or CR or by side effects of long-term immunosuppression. The molecular basis of these processes have been analyzed by gene expression profiling in various studies focusing on acute [Sarwal et al., 2001; Gimino et al., 2003; Hippen et al., 2005; Hoffmann et al., 2005] or CR [Donauer et al., 2003; Scherer et al., 2003; Flechner et al., 2004; Cheng et al., 2006; Hotchkiss et al., 2006], response to treatment [Flechner et al., 2004; Melk et al., 2005], and lung cancer [Chen et al., 2007], demonstrating the unique potential of this approach to decipher complex pathological processes in human disease. Recently, the feasibility and value of microarray analysis of operational tolerance has been demonstrated by Martinez-Llordella et al. [2007] in liver transplantation and by our-selves in kidney transplantation [Brouard et al., 2007].

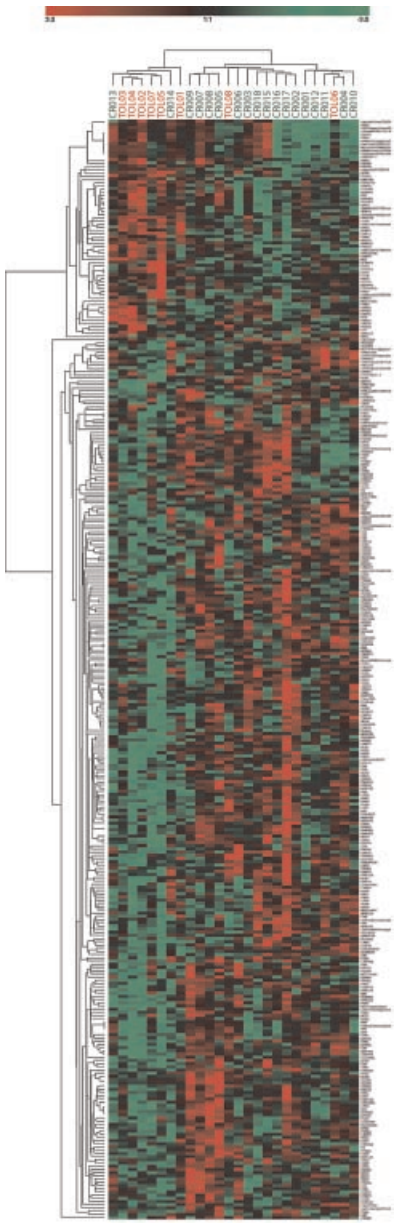

Fig. 3. Schematic representation of the clustering analysis of the 343 genes which were differentially expressed between operationally tolerant kidney graft recipients (TOL) and patients with chronic rejection (CR). The color scale ranges from green, representing downregulation of genes in TOL, to red, representing upregulation of genes in TOL. Each column of the matrix corresponds to a single patient sample and each row to a gene. Patients' samples were clustered according to the similarity in their expression profile across the genes. The dendrogram tree summarizes the results of this clustering. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In the present study, we identified a list of 343 genes, mainly immune and defense genes, differentiating operational tolerance from CR in human kidney recipients. We demonstrated that the obtained gene expression profile correctly classified more than 80% of the samples in a cross-validation experiment and separated well on the unsupervised hierarchical clustering. More importantly, the expression algorithm still yielded a positive predictive value for all samples harvested over time of both operationally tolerant and CR patients, demonstrating the stability of the gene expression pattern. Interestingly, one patient (TOL06) was classified as CR in the cross-validation test. This patient, who fulfilled the criteria of operational tolerance at the time of the study two years after total withdrawal of immunosuppressive drugs, had a declining graft function (creatinemia: 165 μm/l, proteinuria: 1 g/day) and displayed anti-donor class II (anti-DR4) antibodies 6 months later. Although single cases should be interpreted with caution, this suggests that the blood gene profiling may contribute to identifying patients at risk of losing the status of tolerance. Two other patients (TOL01 and TOL08), however, were classified as CR by their gene expression profile but still fulfilled the criteria of operational tolerance upon follow-up. Therefore, no conclusions can be made about the potential prognostic value of the identified expression signature without large, prospective and longitudinal trials.

Of major importance in a clinical perspective is the fact that the described gene expression profile has been obtained from PBMC and can thus easily be used and validated in other settings. Several studies have reported that gene profiling of peripheral blood cells may be useful in the prediction of clinical events and the management of immunosuppressive treatment in heart [Deng et al., 2006] and kidney transplantation [Flechner et al., 2004] as well as in other pathological conditions [Steinman and Zamvil, 2003; Achiron and Gurevich, 2006]. In an experimental rodent model of infectious tolerance, regulatory lymphocytes from peripheral blood were able to transfer long-term survival [Fujino et al., 2004], indicating that peripheral regulatory mechanisms really exist. Thus, even in the presence of compartment-specific differences [Lair et al., 2007], gene expression profiling of peripheral blood may be useful to monitor the state of the immune response to kidney transplants.

Besides the clinical implications of the presented data, a major topic of interest is the identification of the molecular pathways underlying the

TABLE IV. Pattern Stability Over Time

Patients	Spearman r	*P*-values
CR001	0.5872	<0.0001
CR002	0.8785	<0.0001
CR003	0.5699	<0.0001
TOL01	0.06869	NS
TOL02	0.7168	<0.0001
TOL03	0.7273	<0.0001
TOL04	0.4481	<0.0001

The gene expression of 4 serial TOL and 3 serial CR samples were analysed. Correlations between the two samples of each patient using the non-parametric Spearman test indicated that the gene expression pattern is stable for 6 out of the 7 patients over a period of at least 12 months ($P < 0.001$).

identified expression profile and their functional involvement in tolerance. Although stringently significant at the statistical level, all these genes showed a relatively moderate fold-change compared to CR, thereby indicating that a complex interaction between a multitude of genes and pathways rather than a massive upregulation of a few single genes is relevant for operational tolerance. This expression pattern is consistent with previous studies in autoimmune diseases [Bennett et al., 2003] and even highly specific oncology gene signatures are composed of sets of 70–100 genes with relatively small fold-changes [van 't Veer et al., 2002; Glas et al., 2006]. A detailed functional analysis of the obtained gene expression profiles using two different statistical and nonstatistical methods is therefore presented in the second part of this article.

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REFERENCES

Achiron A, Gurevich M. 2006. Peripheral blood gene expression signature mirrors central nervous system disease: The model of multiple sclerosis. Autoimmun Rev 5:517–522.

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Jr., Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403:503–511.

Alon U, Barkai N, Notterman DA, Gish K, Ybarra S, Mack D, Levine AJ. 1999. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. Proc Natl Acad Sci U S A 96:6745–6750.

Ansari MJ, Sayegh MH. 2004. Clinical transplantation tolerance: The promise and challenges. Kidney Int 65:1560–1563.

Ashton-Chess J, Brouard S, Soulillou JP. 2006. Is clinical tolerance realistic in the next decade? Transpl Int 19:539–548.

Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V, Gregersen PK, Behrens TW. 2003. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 100:2610–2615.

Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, Pallier A, Giral M, Brouard S, Soulillou JP. 2006. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol 17:294–304.

Ballet C, Roussey-Kesler G, Aubin JT, Brouard S, Giral M, Miqueu P, Louis S, van der Werf S, Soulillou JP. 2006. Humoral and cellular responses to influenza vaccination in human recipients naturally tolerant to a kidney allograft. Am J Transplant 6:2796–2801.

Ballet C, Brouard S, Soulillou JP. 2007. Revisiting tolerance induction by donor cells priming. Curr Opin Organ Transplant 24:335–339.

Becker D, Mihm MC, Hewitt SM, Sondak VK, Fountain JW, Thurin M. 2006. Markers and tissue resources for melanoma: Meeting report. Cancer Res 66:10652–10657.

Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V. 2003. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 197:711–723.

Brouard S, Dupont A, Giral M, Louis S, Lair D, Braudeau C, Degauque N, Moizant F, Pallier A, Ruiz C, Guillet M, Laplaud D, Soulillou JP. 2005. Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. Am J Transplant 5:330–340.

Brouard S, Mansfield ES, Braud C, Li L, Giral M, Hsieh SC, Baeten D, Zhang M, Ashton-Chess J, Braudeau C, Hsieh F, Sherlock G, Dupont A, Pallier A, Moreau A, Louis S, Ruiz C, Salvatierra O, Soulillou JP, Sarwal M. 2007. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. Proc Natl Acad Sci U S A (in press).

Chabas D, Baranzini SE, Mitchell D, Bernard CC, Rittling SR, Denhardt DT, Sobel RA, Lock C, Karpuj M, Pedotti R, Heller R, Oksenberg JR, Steinman L. 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 294:1731–1735.

Chen HY, Yu SL, Chen CH, Chang GC, Chen CY, Yuan A, Cheng CL, Wang CH, Terng HJ, Kao SF, Chan WK, Li HN, Liu CC, Singh S, Chen WJ, Chen JJ, Yang PC. 2007. A five-gene signature and clinical outcome in non-small-cell lung cancer. N Engl J Med 356:11–20.

Cheng O, Thuillier R, Sampson E, Schultz G, Ruiz P, Zhang X, Yuen PS, Mannon RB. 2006. Connective tissue growth factor is a biomarker and mediator of kidney allograft fibrosis. Am J Transplant 6:2292–2306.

Cobbold SP, Adams E, Graca L, Daley S, Yates S, Paterson A, Robertson NJ, Nolan KF, Fairchild PJ, Waldmann H. 2006. Immune privilege induced by regulatory T cells in transplantation tolerance. Immunol Rev 213:239–255.

Dantal J, Hourmant M, Cantarovich D, Giral M, Blancho G, Dreno B, Soulillou JP. 1998. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: Randomised comparison of two cyclosporin regimens. Lancet 351:623–628.

Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, Kobashigawa J, Johnson FL, Starling RC, Murali S, Pauly DF, Baron H, Wohlgemuth JG, Woodward RN, Klingler TM, Walther D, Lal PG, Rosenberg S, Hunt S. 2006. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. Am J Transplant 6:150–160.

Donauer J, Rumberger B, Klein M, Faller D, Wilpert J, Sparna T, Schieren G, Rohrbach R, Dern P, Timmer J, Pisarski P, Kirste G, Walz G. 2003. Expression profiling on chronically rejected transplant kidneys. Transplantation 76:539–547.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95:14863–14868.

Flechner SM, Kurian SM, Head SR, Sharp SM, Whisenant TC, Zhang J, Chismar JD, Horvath S, Mondala T, Gilmartin T, Cook DJ, Kay SA, Walker JR, Salomon DR. 2004. Kidney transplant rejection and tissue injury by gene profiling of biopsies and peripheral blood lymphocytes. Am J Transplant 4:1475–1489.

Fudaba Y, Spitzer TR, Shaffer J, Kawai T, Fehr T, Delmonico F, Preffer F, Tolkoff-Rubin N, Dey BR, Saidman SL, Kraus A, Bonnefoix T, McAfee S, Power K, Kattleman K, Colvin RB, Sachs DH, Cosimi AB, Sykes M. 2006. Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: In vivo and in vitro analyses. Am J Transplant 6:2121–2133.

Fujino M, Kitazawa Y, Kawasaki M, Funeshima N, Kimura H, Nakajima T, Saito H, Li XK. 2004. Differences in lymphocyte gene expression between tolerant and syngeneic liver grafted rats. Liver Transpl 10:379–391.

Gimino VJ, Lande JD, Berryman TR, King RA, Hertz MI. 2003. Gene expression profiling of bronchoalveolar lavage cells in acute lung rejection. Am J Respir Crit Care Med 168:1237–1242.

Glas AM, Kersten MJ, Delahaye LJ, Witteveen AT, Kibbelaar RE, Velds A, Wessels LF, Joosten P, Kerkhoven RM, Bernards R, van Krieken JH, Kluin PM, van't Veer LJ, de Jong D. 2005. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 105:301–307.

Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, Lahti-Domenici JS, Bruinsma TJ, Warmoes MO, Bernards R, Wessels LF, Van't Veer LJ. 2006. Converting a breast cancer microarray signature into a high-throughput diagnostic test. BMC Genomics 7:278.

Golfier G, Dang MT, Dauphinot L, Graison E, Rossier J, Potier MC. 2004. VARAN: A web server for variability analysis of DNA microarray experiments. Bioinformatics 20:1641–1643.

Hiltunen MO, Tuomisto TT, Niemi M, Brasen JH, Rissanen TT, Toronen P, Vajanto I, Yla-Herttuala S. 2002. Changes in gene expression in atherosclerotic plaques analyzed using DNA array. Atherosclerosis 165:23–32.

Hippen BE, DeMattos A, Cook WJ, Kew CE, Gaston RS. 2005. Association of CD20+ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. Am J Transplant 5:2248–2252.

Hoffmann SC, Hale DA, Kleiner DE, Mannon RB, Kampen RL, Jacobson LM, Cendales LC, Swanson SJ, Becker BN, Kirk AD. 2005. Functionally significant renal allograft rejection is defined by transcriptional criteria. Am J Transplant 5:573–581.

Horwitz PA, Tsai EJ, Putt ME, Gilmore JM, Lepore JJ, Parmacek MS, Kao AC, Desai SS, Goldberg LR, Brozena SC, Jessup ML, Epstein JA, Cappola TP. 2004. Detection of cardiac allograft rejection and response to immunosuppressive therapy with peripheral blood gene expression. Circulation 110:3815–3821.

Hotchkiss H, Chu TT, Hancock WW, Schroppel B, Kretzler M, Schmid H, Liu Y, Dikman S, Akalin E. 2006. Differential expression of profibrotic and growth factors in chronic allograft nephropathy. Transplantation 81: 342–349.

Kasperkovitz PV, Timmer TC, Smeets TJ, Verbeet NL, Tak PP, van Baarsen LG, Baltus B, Huizinga TW, Pieterman E, Fero M, Firestein GS, van der Pouw Kraan TC, Verweij CL. 2005. Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: Evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis. Arthritis Rheum 52:430–441.

Lair D, Degauque N, Miqueu P, Jovanovic V, Guillet M, Merieau E, Moreau A, Soulillou JP, Brouard S. 2007. Functional compartmentalization following induction of long-term graft survival with pregraft donor-specific transfusion. Am J Transplant 7:538–549.

Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, Langer-Gould A, Strober S, Cannella B, Allard J, Klonowski P, Austin A, Lad N, Kaminski N, Galli SJ, Oksenberg JR, Raine CS, Heller R, Steinman L. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat Med 8:500–508.

Louis S, Braudeau C, Giral M, Dupont A, Moizant F, Robillard N, Moreau A, Soulillou JP, Brouard S. 2006. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation 81:398–407.

Luckow B, Joergensen J, Chilla S, Li JP, Henger A, Kiss E, Wieczorek G, Roth L, Hartmann N, Hoffmann R, Kretzler M, Nelson PJ, Perez de Lema G, Maier H, Wurst W, Balling R, Pfeffer K, Grone HJ, Schlondorff D, Zerwes HG. 2004. Reduced intragraft mRNA expression of matrix metalloproteinases Mmp3, Mmp12, Mmp13 and Adam8, and diminished transplant arteriosclerosis in Ccr5-deficient mice. Eur J Immunol 34:2568–2578.

Man K, Lo CM, Lee TK, Li XL, Ng IO, Fan ST. 2003. Intragraft gene expression profiles by cDNA microarray in small-for-size liver grafts. Liver Transpl 9:425–432.

Martinez-Llordella M, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, Lerut J, Latinne D, Margarit C, Bilbao I, Brouard S, Hernandez-Fuentes M, Soulillou JP, Sanchez-Fueyo A. 2007. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant 7:309–319.

Melk A, Mansfield ES, Hsieh SC, Hernandez-Boussard T, Grimm P, Rayner DC, Halloran PF, Sarwal MM. 2005. Transcriptional analysis of the molecular basis of human kidney aging using cDNA microarray profiling. Kidney Int 68:2667–2679.

Nygaard V, Loland A, Holden M, Langaas M, Rue H, Liu F, Myklebost O, Fodstad O, Hovig E, Smith-Sorensen B. 2003. Effects of mRNA amplification on gene expression ratios in cDNA experiments estimated by analysis of variance. BMC Genomics 4:11.

Peterson KS, Huang JF, Zhu J, D'Agati V, Liu X, Miller N, Erlander MG, Jackson MR, Winchester RJ. 2004. Characterization of heterogeneity in the molecular pathogenesis of lupus nephritis from transcriptional profiles of laser-captured glomeruli. J Clin Invest 113:1722–1733.

Roussey-Kesler G, Giral M, Moreau A, Subra JF, Legendre C, Noel C, Pillebout E, Brouard S, Soulillou JP. 2006. Clinical operational tolerance after kidney transplantation. Am J Transplant 6:736–746.

Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, Clayberger C, Sibley R, Krensky AM, Pavlakis M. 2001. Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. Hum Immunol 62:21–31.

Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, Salvatierra O. 2003. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med 349:125–138.

Scherer A, Krause A, Walker JR, Korn A, Niese D, Raulf F. 2003. Early prognosis of the development of renal chronic allograft rejection by gene expression profiling of human protocol biopsies. Transplantation 75:1323–1330.

Sivozhelezov V, Braud C, Giacomelli L, Pechkova E, Giral M, Soulillou J-P, Brouard S, Nicolini C. 2007. Immunosuppressive Drug-Free Operational Immune Tolerance in Human Kidney Transplant Recipients. Part II. Non-statistical gene microarray analysis. J Cell Biochem (in press).

Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, Drachenberg CB, Gibson IW, Grimm PC, Haas M, Lerut E, Liapis H, Mannon RB, Marcus PB, Mengel M, Mihatsch MJ, Nankivell BJ, Nickeleit V, Papadimitriou JC, Platt JL, Randhawa P, Roberts I, Salinas-Madriga L, Salomon DR, Seron D, Sheaff M, Weening JJ. 2007. Banff '05 Meeting Report: Differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 7:518–526.

Soulillou JP. 2001. Immune monitoring for rejection of kidney transplants. N Engl J Med 344:1006–1007.

Stegall M, Park W, Kim D, Kremers W. 2002. Gene expression during acute allograft rejection: Novel statistical analysis of microarray data. Am J Transplant 2:913–925.

Steinman L, Zamvil S. 2003. Transcriptional analysis of targets in multiple sclerosis. Nat Rev Immunol 3:483–492.

Takatsuki M, Uemoto S, Inomata Y, Egawa H, Kiuchi T, Fujita S, Hayashi M, Kanematsu T, Tanaka K. 2001. Weaning of immunosuppression in living donor liver transplant recipients. Transplantation 72: 449–454.

Tibshirani R, Hastie T, Narasimhan B, Chu G. 2002. Diagnosis of multiple cancer types by shrunken centroids of gene expression. Proc Natl Acad Sci U S A 99:6567–6572.

Tuomisto TT, Korkeela A, Rutanen J, Viita H, Brasen JH, Riekkinen MS, Rissanen TT, Karkola K, Kiraly Z, Kolble K, Yla-Herttuala S. 2003. Gene expression in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions as studied by laser microdissection and DNA array: Overexpression of HMG-CoA reductase, colony stimulating factor receptors, CD11A/CD18 integrins, and interleukin receptors. Arterioscler Thromb Vasc Biol 23:2235–2240.

van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. 2002. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347:1999–2009.

van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. 2002. Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530–536.

Yang YH, Speed T. 2002. Design issues for cDNA microarray experiments. Nat Rev Genet 3:579–588.