Pretransplant Transcriptome Profiles Identify among Kidneys with Delayed Graft Function Those with Poorer Quality and Outcome

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Robust biomarkers are needed to identify donor kidneys with poor quality associated with inferior early and longer-term outcome. The occurrence of delayed graft function (DGF) is most often used as a clinical outcome marker to capture poor kidney quality. Gene expression profiles of 92 preimplantation biopsies were evaluated in relation to DGF and estimated glomerular filtration rate (eGFR) to identify preoperative gene transcript changes associated with short-term function. Patients were stratified into those who required dialysis during the first week (DGF group) versus those without (noDGF group) and subclassified according to 1-month eGFR of >45 mL/min (eGFR$_{hi}$) versus eGFR of ≤45 mL/min (eGFR$_{lo}$). The groups and subgroups were compared in relation to clinical donor and recipient variables and transcriptome-associated biological pathways. A validation set was used to confirm target genes. Donor and recipient characteristics were similar between the DGF versus noDGF groups. A total of 206 probe sets were significant between groups ($P < 0.01$), but the gene functional analyses failed to identify any significantly affected pathways. However, the subclassification of the DGF and noDGF groups identified 283 probe sets to be significant among groups and associated with biological pathways. Kidneys that developed postoperative DGF and sustained an impaired 1-month function (DGF$_{lo}$ group) showed a transcriptome profile of significant immune activation already preimplant. In addition, these kidneys maintained a poorer transplant function throughout the first-year posttransplant. In conclusion, DGF is a poor marker for organ quality and transplant outcome. In contrast, preimplant gene expression profiles identify "poor quality" grafts and may eventually improve organ allocation.

INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal disease. However, despite the significant survival benefit, poor long-term outcomes and insufficient number of donor organs remain the major problems in organ transplantation (1,2). This result has lead to the increasing pressure to use kidneys of potentially inferior quality with likely compromised short- and long-term function. Hence, reliable kidney quality and outcome markers are needed.

A wide number of publications have studied and suggested single as well as combinations of clinical- and histopathology-based variables and derived algorithms to assess organ tissue quality and to predict short- and long-term outcomes. However, none of these biomarkers have so far been proven to be useful in routine clinical decision-making for the individual donor organ and recipient (3,4). This result is partly due to the multitude of factors affecting donor organ quality, such as nephron supply, pre- and peritransplant insults and posttransplant immunologic and nonimmunologic variables (4). In addition, the lack of robust outcome measures further prevents the identification of predictive markers (5). Traditionally, the occurrence of delayed graft function (DGF) is used as a reference marker for poor organ quality and associated impaired short- and long-term outcome.

However, a major problem with DGF is the multitude of definitions itself, usually incorporating the need for hemodialysis during the first week after transplantation (6). The indication for dialysis,

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however, is subjective, and a number of factors such as postoperative hemodynamics independent of organ quality may affect the need for dialysis treatment. The lack of standardized definitions of DGF as well as the rather weak association between DGF and organ quality are reflected in the controversial results linking DGF with long-term allograft performance and contributes to the current paucity of biomarkers and therapies to affect early and long-term allograft outcome (3,7).

Recently, the value of molecular profiles in biopsies taken at time of transplantation to provide the missing information for a more complex understanding and evaluation of organ quality and long-term function have been reviewed (4). Earlier studies have investigated differences in gene expression between living and deceased donors (8–10), the effects of donor age on gene expression patterns (11) and genes associated with DGF (8–12). The identified transcriptome profiles have significantly enriched our understanding of the pathomechanisms associated with ischemia-reperfusion injury. However, none of these studies have so far produced a robust set of gene-associated transcripts applicable in the clinical routine to capture organ quality and outcome.

In the present study, a large patient cohort including a validation set are analyzed in regard to standard DGF diagnosis and glomerular filtration rate (GFR)-based outcome measures to discover new biomarkers in deceased donor biopsies associated with donor quality and short- and longer-term outcomes.

MATERIALS AND METHODS

Kidney Samples and Patient Enrollment

The study included 92 consecutive adult kidney transplant recipients of deceased donor kidneys (ages 17–70 years). The Institutional Review Board at Virginia Commonwealth University

(VCU-IRB Protocol #HM11454) approved the study protocol. Written informed consent was obtained from all patients. No living donors, human immunodeficiency virus (HIV)-positive patients or retransplantation patients were included in the study. Allograft biopsies from kidneys preserved using both cold preservation and pump perfusion preservation were included. Kidney allograft tissue was obtained through an 18-gauge biopsy needle, and all samples were placed in RNA*later* (Ambion) immediately after collection. Biopsies were collected at preimplantation time (post–cold ischemia time; n = 92). Estimated GFR (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease formula (13). DGF was defined as the need of dialysis during the first 7 d after kidney transplantation. The patients were stratified into patients undergoing posttransplant DGF versus patients without DGF (DGF versus noDGF) and then further subdivided into patients with an eGFR >45 mL/min versus patients with an eGFR ≤45 mL/min (eGFR$_{hi}$ versus eGFR$_{lo}$) following the criteria described by Kainz *et al.* (14).

RNA Isolation, cDNA Synthesis and *In Vitro* Transcription for Labeled cRNA Probe

The sample preparation protocol follows the Affymetrix GeneChip® Expression Analysis Manual (Affymetrix, Santa Clara, CA, USA). Briefly, total RNA was reverse-transcribed using T7-polydT primer and converted into double-stranded cDNA (One-Cycle Target Labeling and Control Reagents; Affymetrix), with templates being used for an *in vitro* transcription reaction to yield biotin-labeled antisense cRNA. The labeled cRNA was chemically fragmented and made into the hybridization cocktail according to the Affymetrix GeneChip protocol, which was then hybridized to HG-U133A 2.0 GeneChips. The array image was generated by the high-resolution GeneChip® Scanner 3000 by Affymetrix.

Microarray Data Processing

Gene expression microarray data for 92 preimplantation (PI) samples were available. Probe level data were read into the R programming environment using the *affy* Bioconductor package, and the robust multiarray average method was used to obtain probe set expression summaries. Quality assessment of each GeneChip was performed by assessing the average background; scaling factor; percent present calls; the 3′:5′ ratio for GAPDH, β-actin and signal transducer and activator of transcription 1 (STAT1); and the overall 3′:5′ ratio with associated 95% confidence interval using the publicly available application (15,16). One microarray did not pass quality control, leaving a total of 91 arrays for the analysis. Probe level data were read into the R programming environment using the *affy* Bioconductor package, and the robust multiarray average method was used to obtain probe set expression summaries (17,18). All control and probe sets declared absent for all samples were removed, leaving 17,529 probe sets for statistical analysis of the PI biopsies.

Analysis of Differentially Expressed Genes among the Patient Groups

To identify genes differentially expressed between transplant patients with and without dialysis during the first week after kidney transplantation, for each probe set, a two-sample *t* test comparing the DGF versus noDGF groups was performed. Probe sets having a raw *P* value <0.01 were considered significant.

Subjects were subsequently classified into two groups on the basis of their GFR at 1 month, individuals having eGFR >45 (GFR$_{hi}$) and individuals having eGFR ≤45 (GFR$_{lo}$). A moderated *t* test was used to compare the two groups using the *limma* package, and a *P* value from the moderated *t* test <0.01 was used to identify significant probe sets. To identify probe sets exhibiting significant differential expression between the four groups, probe set level analysis of variance models were fit, considering probe set expression as

the response and group (noDGF with 1 month eGFR >45; noDGF with 1 month eGFR ≤45; DGF with 1 month eGFR >45; and DGF with 1 month eGFR ≤45) as the fixed effect of interest. A group means parameterization of group was included as the fixed-effect term to facilitate extraction of linear contrasts of interest. Again, the *limma* package was used to fit the mixed-effects models using restricted maximum likelihood procedure, and an empirical Bayes method was applied to moderate probe set standard errors by borrowing information across the entire set of probe sets. To adjust for multiple comparisons, probe sets with a *P* value from the overall *F* test <0.01 were considered significant. Thereafter, among probe sets identified as differentially expressed via the *F* test, pair-wise comparisons of interest were performed. Specifically, we examined the following four pair-wise comparisons: (i) no DGF with 1 month eGFR ≤45 versus noDGF with 1 month eGFR >45, (ii) DGF with 1 month GFR ≤45 versus DGF with 1 month eGFR >45, (iii) DGF with 1 month eGFR ≤45 versus noDGF with 1 month GFR ≤45, and (iv) DGF with 1 month eGFR >45 versus noDGF with 1 month eGFR >45.

Biological and Functional Analysis

Gene ontology and gene interaction analyses were executed using ToppGene (http://toppgene.cchmc.org) (19). Gene lists containing Entrez GeneID numbers were used as input. Identification of significant biological processes and pathways was determined using the false discovery rate method (*P* < 0.01). The results obtained with ToppGene were confirmed using Ingenuity Pathway (IPA, www.ingenuity.com).

Validation of Five Genes in an Independent Set of Patients Using RT-qPCR

Initially, to validate our microarray findings, we carried out a reverse transcriptase–quantitative real-time polymerase chain reaction (RT-qPCR) for *CCL5* (*chemokine [C-C motif] ligand 5*),

ITGB2 (*integrin β2 [complement component 3, receptor 3 and 4 subunit]*) and *EGF* (*epidermal growth factor*) mRNAs from the same RNA samples that were subjected to microarray study. Moreover, an independent set of samples (validation set) was tested for *CCL5*, *ITGB2*, *EGF*, *VCAN* (*versican*) and *CXCR4* (*chemokine [C-X-C motif] receptor 4*) mRNAs. The genes for validation were selected based on the following: (a) statistical differential expression between DGF_{hi} versus DGF_{lo} groups and statistical differential expression between $eGFR_{hi}$ versus $eGFR_{lo}$ at 1 month posttransplantation, and (b) the genes were among the top scoring networks and pathways when using interaction networks and functional analysis for the different comparison analysis. Total RNA was subjected to reverse transcription using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Real-time PCRs were then carried out in an ABI Prism 7700 Sequence Detection System, using TaqMan® Gene Expression Assays (Applied Biosystems). Data were analyzed according to the comparative cycle threshold (Ct) method and were normalized with a housekeeping gene (*GAPDH* [*glyceraldehyde-3-phosphate dehydrogenase*]). Pearson correlation coefficient (*r*) was calculated to examine the relation between microarray and real-time PCR results. *P* < 0.05 was considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Study Cohort Characteristics

A total of 92 patients were enrolled in the study. Patients were transplanted between January 2008 and March 2010. All patients received a deceased donor kidney transplant and had a minimum follow-up of at least 12 months. Of the kidney transplant recipients included in the analysis, only one graft was lost during the first year after kidney trans-

plant because of rapid progressive chronic allograft dysfunction with interstitial fibrosis and tubular atrophy. We first compared the two groups of DGF versus noDGF patients. The clinical definition of DGF used was the need for dialysis within the first week after kidney transplant (12). A total of 25 patients developed post–kidney transplant DGF (27%) and 67 patients did not (73%). Within the DGF group, the period of dialysis before recovery for the individual patient showed a wide range (23 ± 36 d). The clinical indications for dialysis in these 25 patients are described in Supplementary Table 1. For patients with DGF extended >3 wks, allograft biopsy was performed. In the vast majority of the performed biopsies, the histological diagnosis was acute tubular necrosis. Clinically, the two patient groups were similar regarding recipient and donor age, recipient and donor sex, last donor creatinine, cold ischemia time, warm ischemia time, incidence of acute rejection (AR) during the first year after kidney transplant or the use of pump perfusion preservation. A total of 57% of the donor kidneys underwent pump perfusion preservation, and 26.4% of the donors were extended criteria donors (including 13.2% non–heart-beating donors). The two groups only differed significantly in serum creatinine levels at 1 month post–kidney transplant and eGFR at 1-month and 1-year post–kidney transplant (Table 1 and Figure 1). The histology of the biopsies at preimplantation time is shown in Supplementary Table 2.

Gene Expression Profiling of DGF Versus noDGF Grafts in Preimplantation Biopsies

One microarray failed our quality-control assessment so that preimplantation gene expression microarray data were available for 91 patients from our cohort. Gene expression microarray analysis comparing DGF (n = 25) to noDGF (n = 66) patients identified 206 probe sets (*P* < 0.01) differentially expressed. How-

Table 1. Demographic and relevant clinical information for the enrolled study patients separated according to DGF versus noDGF after transplantation.

	DGF	noDGF	DGF versus noDGF (*P*)
n	25	66	
Recipient age	52.6 ± 11.2	49.6 ± 13.8	0.29
Recipient sex (n) (M (F))	17 (8)	41 (25)	0.395
Recipient race (n)			
Caucasian	2	17	<0.001
African American	22	45	
Other	1	4	
Recipient hepatitis C virus status (positive, n)	3	9	0.269
CMV disease (positive, n)	0	0	N/A
Recipient CMV status (positive, n)	18	46	0.144
Donor age (years)	42.8 ± 13.0	38.2 ± 15.0	0.150
Donor sex (n) (M (F))	18 (7)	35 (30)	0.091
Donor race (n)			
Caucasian	17	36	0.177
African American	5	23	
Other	2	1	
Donor hepatitis C virus antibody (positive, n)	3	8	0.278
Donor CMV status (positive, n)	15	33	0.139
Cold ischemia time (min)	1271 ± 530	1207 ± 532	0.607
Warm ischemia time/revascularization time (min)	29.6 ± 7.8	30.5 ± 7.7	0.647
Pump perfusion preservation time (min)	450 ± 508	488 ± 491	0.746
Last donor serum creatinine (mg/dL)	1.3 ± 0.6	1.0 ± 0.3	0.175
Acute rejection episodes (n)	4	90.275	
HLA MM-A	1 ± 1	2 ± 1	0.067
HLA MM-B	2 ± 1	2 ± 1	0.889
HLA MM-DR	1 ± 1	1 ± 1	0.462
HLA MM total	4 ± 2	4 ± 2	0.200
Panel reactive antibodies at transplant–T cells (%)	43.3 ± 42.9	40.7 ± 34.9	0.782
Panel reactive antibodies at transplant–B cells (%)	17.8 ± 27.6	22.3 ± 32.1	0.510
Serum creatinine (mg/dL)			
1 month	2.3 ± 1.7	1.4 ± 0.5	0.019
3 months	1.7 ± 0.8	1.4 ± 0.5	0.052
6 months	1.7 ± 0.7	1.4 ± 0.6	0.117
9 months	1.6 ± 0.6	1.6 ± 0.7	0.900
12 months	1.9 ± 1.0	1.4 ± 0.5	0.065
Average eGFR (mL/min)			
1 month	48.4 ± 24.4	62.2 ± 21.5	0.017
3 months	55.1 ± 24.2	61.9 ± 18.9	0.229
6 months	55.7 ± 25.4	60.2 ± 18.4	0.448
9 months	57.3 ± 23.5	62.4 ± 20.1	0.408
12 months	50.7 ± 22.1	61.7 ± 18.5	0.064

CMV, cytomegalovirus; N/A, not applicable; MM, mismatch.
Data are averages ± standard deviation (SD) unless otherwise stated. eGFR was calculated using the abbreviated Modification of Diet in Renal Disease formula. *P* values were calculated using Fisher exact test.

ever, gene ontology analyses failed to identify any significantly affected biological processes or pathways overrepresented by these genes ($P < 0.01$). Furthermore, unsupervised hierarchical clustering did not show any clear separation between the DGF and noDGF preimplantation biopsies (not shown).

Gene expression microarray analysis comparing the two groups defined as 1 month eGFR >45 (GFR$_{hi}$, N = 62) and 1 month eGFR ≤45 (GFR$_{lo}$, N = 29) iden-

tified 208 differentially expressed probe sets ($P < 0.01$) (Supplementary Table 3). Expression for 80 probe sets was higher in the GFR$_{hi}$ group, whereas expression for 128 probe sets was higher in the GFR$_{lo}$ group. Core analysis was performed to interpret the data set in the context of biological processes, pathways and molecular networks. From the analysis of the differentially expressed genes, the top network associated biological functions were cellular compromise; molecular transport; inflammatory disease (network 1, score 38); and cell-to-cell signaling and interaction, cell-mediated immune response and cellular development (network 2, score 29). Moreover, the top biological functions associated with the statistical differentially expressed genes were inflammatory disease (P = 9.4E-10–9.5E-03) with overexpression of the following genes: *ADA* (*adenosine deaminase*) *CD28* (*T-cell-specific surface glycoprotein CD28*), *CD48* (*CD48 antigen [B cell membrane protein]*), *CCL5*, *CD2* (*T-cell surface antigen CD2*), *CD27* (*T cell activation antigen CD27*) and *CXCR4*, among others. Also, immune cell trafficking (P = 2.6E-08–9.5E-03) and cell-mediated immune response (P = 3.7E-08–9.3E-03) were among the top biological functions. The top canonical pathway associated with these genes was B-cell development.

Combination of DGF and eGFR Values Identifies Biologically Relevant Subgroups of Patients

The comparison between DGF and noDGF did not yield any robust difference in gene expression profiles. In addition, clinically, the DGF group showed a high degree of heterogeneity regarding severity of kidney injury, reflected by the wide range in recovery time needed (Figure 2). Thus, we further segregated the DGF and the noDGF patient groups each into two subgroups according to the degree of kidney function at 1 month after kidney transplant. These four patient subgroups were as follows: DGF$_{hi}$, DGF patients with 1-month

eGFR >45 mL/min (rapid recovery); DGF_{lo}, DGF patients with 1-month eGFR ≤45 mL/min (slow recovery); $noDGF_{hi}$, noDGF patients with 1-month eGFR >45 mL/min (stable eGFR at 1 year); and $noDGF_{lo}$, noDGF patients with 1-month eGFR ≤45 mL/min (declining eGFR at 1 year).

Figure 2 shows that each subgroup displays a persistent and stable transplant function for at least the first year; the subgroups within the DGF as well as within the noDGF patients remain statistically different at 3, 6, 9 and 12 months (Table 2). This supports the biological validity of selecting the 1-month eGFR as the stratification variable.

The 1-month function is not associated with any of the clinical donor and recipient variables (Table 2). In addition, 1-month transplant function is independent of the occurrence of DGF. Kidneys can have a high or low eGFR in the noDGF as well as in the DGF group, and longer-term function is not associated with early hemodialysis treatment or not. Function throughout the first year is not statistically different between DGF_{hi} and $noDGF_{hi}$ allografts. DGF_{lo} versus $noDGF_{lo}$ kidneys again show overall no statistical difference during the first 12 posttransplant months; however, the 13 DGF_{lo} allograft showed poorer early function recovery and worse 1-year function compared to the 16 $noDGF_{lo}$ transplants (Figure 2 and Table 2).

Gene Expression and Functional Analysis of DGF/GFR Subgroups

We next compared the gene expression profiles of the PI biopsies from the four subgroups to identify any potential differences associated with the observed segregation of samples. Four comparisons were carried out: (i) DGF_{lo} (n = 13) versus DGF_{hi} (n = 12), (ii) $noDGF_{lo}$ (n = 16) versus $noDGF_{hi}$ (n = 50), (iii) DGF_{hi} versus $noDGF_{hi}$, and (iv) DGF_{lo} versus $noDGF_{lo}$. Statistical analyses identified 155 probe sets differentially expressed between DGF_{hi} and DGF_{lo}; 79 probe sets between $noDGF_{hi}$ and $noDGF_{lo}$; 32 probe sets between $noDGF_{hi}$ and DGF_{hi}; and
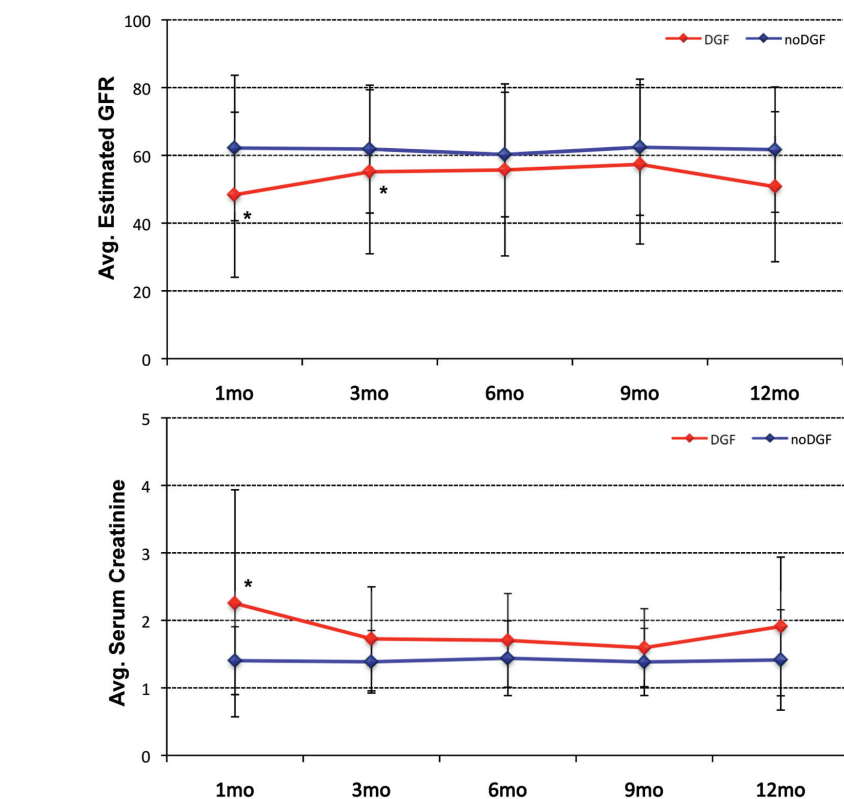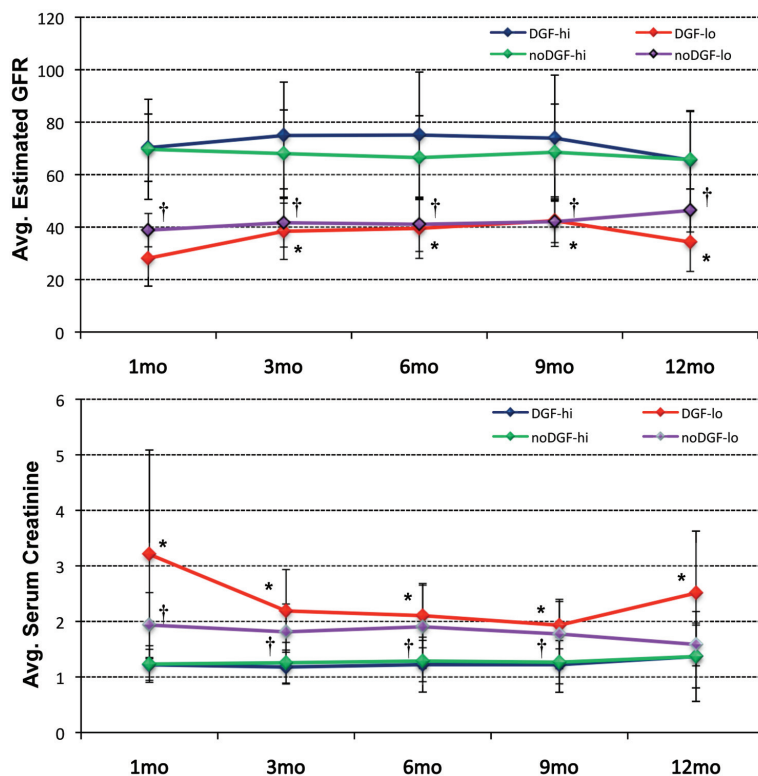

Figure 1. Course of eGFR (top) and serum creatinine levels (bottom) for the DGF and noDGF patient groups throughout the first posttransplant year. Asterisks indicate a statistically significant difference ($P \leq 0.05$) between the DGF and noDGF groups.

130 probe sets between $noDGF_{lo}$ and DGF_{lo} ($P < 0.01$). A total of 27 genes identified as differentially expressed among groups were also present in the GFR_{hi} versus GFR_{lo} gene list. A complete list of the differentially expressed probe sets can be found in Supplementary Table 4.

Functional analyses of genes differentially expressed between DGF_{hi} and DGF_{lo} indicate that these genes are involved in antigen processing and presentation via major histocompatibility complex (MHC) class I/II, regulation of T-cell–mediated cytotoxicity and positive thymic T-cell selection (Supplementary Table 5). Moreover, biological pathways overrepresented by the affected genes included the allograft rejection/graft versus host disease, antigen processing and presentation and cell adhesion molecules. Example genes identified in these categories included *PSMB8*, *FCGR2B*, *HLA-G*, *HLA-F*, *HLA-E*, *HLA-DRB1*,

HLA-DRA, *HLA-DPB1*, *HLA-DPA1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-B*, *HLA-DMA*, *IL7R*, *PTPRC*, *CD3D*, *CXCL6*, *CCL19*, *CCL5* and *LAT2* (Table 3). Between $noDGF_{hi}$ and $noDGF_{lo}$, intracellular transport and protein localization were identified as significant.

Finally, functional analyses between $noDGF_{lo}$ and DGF_{lo} showed that differentially expressed genes between these groups, similar to the comparison between DGF_{lo} and DGF_{hi}, are involved in antigen processing and presentation via MHC class I/II (Figure 3) and regulation of T-cell–mediated cytotoxicity, whereas biological pathways overrepresented by the affected genes included allograft rejection/graft versus host disease, antigen processing and presentation and cell adhesion molecules. Genes identified included *HLA-G*, *HLA-E*, *HLA-DRB1*, *HLA-DRA*, *HLA-DPB1*, *HLA-DPA1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-B*, *HLA-C*,

Figure 2. Course of eGFR (top) and serum creatinine levels (bottom) for the four subgroups of DGF$_{hi}$, DGF$_{lo}$, noDGF$_{hi}$ and noDGF$_{lo}$ during the first year after transplantation. Statistically significant difference ($P \leq 0.05$) between the DGF and noDGF $\leq$45 mL/min group with its corresponding >45 mL/min counterpart are indicated by * (for DGF) or † (for noDGF). Statistical differences were identified by analysis of variance followed by Tukey-Kramer honestly significant difference (HSD) testing ($\alpha = 0.05$).

HLA-DMA, *PSMB8*, *PSME1*, *HSP90AB1*, and *PRDX1* (see Table 3). The top-scoring network of interactions among the probe sets identified as significantly differentially expressed when comparing noDGF$_{lo}$ versus DGF$_{lo}$ is shown in Supplementary Figure 1. Also, from the analysis of the canonical pathways, cytokine signaling involved in this comparison between groups is shown the Figure 4.

Microarray results were validated for a number of genes using the remaining stock RNA from samples used in the microarray study. All genes tested showed a similar trend to that observed in the microarray data with a Pearson correlation of 0.928 (Supplementary Figure 2).

Markers Validation Using RT-qPCR

Five genes were also validated in an independent group of patients including

DGF$_{hi}$ (n = 15) and DGF$_{lo}$ (n = 20) (validation set). Supplementary Figure 3 shows that the significance of the identified genes was validated in the independent set of patients.

DISCUSSION

To identify robust biomarkers capable of predicting graft outcome, a better understanding of the mechanisms leading to DGF and its impact on long-term function is necessary, in particular as more and more marginal organs are accepted to increase the donor pool (2123). The heterogeneity in the causes of DGF; the multitude of donor, recipient and procedural factors influencing early graft function; and the variety of definitions used for DGF reflect its limitations as a robust clinical end point and the failure to find reliable biomarkers of kidney

quality (4,6,7). In the present report, we evaluated in 91 consecutive deceased donor kidney transplants clinical variables and gene expression profiles in relation to the development of DGF as well as transplant function throughout the first year.

The occurrence of DGF was neither associated with any of the tested clinical variables, including ischemia times, pump perfusion, donor and recipient sex, age and race or incidence of rejections, nor with kidney function beyond 3 months after kidney transplant. In addition, despite 206 probe sets differentially expressed in the preimplantation biopsies of kidneys that developed DGF versus those that did not, the functional analyses failed to identify any overrepresented biological pathways from this list of probe sets separating DGF from noDGF. These findings corroborate the clinical experience that the DGF classification is subjective, lacks biological relevance for longer-term outcome and is as such not reflecting organ quality and a poor reference point to identify reliable biomarkers (24).

However, the classificataion of patients in groups according with the eGFR at 1 month posttransplantation (GFR$_{hi}$ versus GFR$_{lo}$) showed an important set of genes associated with inflammation and immune response differentially expressed between groups.

To better capture the "quality" of the kidneys and dissect the intrinsic heterogeneity when using the DGF definition, we further subclassified the patient cohort according to 1-month eGFR values. This however identified four subgroups of patients with distinct and persistent clinical 1-year transplant function. Patients in the DGF$_{lo}$ and noDGF$_{lo}$ group remained statistically different from patients in their counterpart group throughout the first year after transplantation. Furthermore, the DGF$_{hi}$ and noDGF$_{hi}$ groups were not statistically different post–kidney transplant, whereas the DGF$_{lo}$ and noDGF$_{lo}$ groups were only statistically different from each other in serum creatinine at 1 month

Table 2. Demographic and relevant clinical information for the 91 study patients when separated on the basis of DGF versus noDGF classification in combination with eGFR values at 1 month posttransplantation.

	DGF_{lo}	DGF_{hi}	DGF_{lo} versus DGF_{hi} (P)	$noDGF_{lo}$	$noDGF_{hi}$	$noDGF_{lo}$ versus $noDGF_{hi}$ (P)	DGF_{lo} versus $noDGF_{lo}$ (P)	DGF_{hi} versus $noDGF_{hi}$ (P)
N	13	12		16	50			
Recipient age (years)	56.2 ± 8.1	47.7 ± 13.1	0.474	53.3 ± 9.1	48.5 ± 14.7	0.573	0.928	0.999
Recipient sex (n) (M (F))	10 (3)	7 (5)	0.286	11 (5)	30 (20)	0.375	0.474	0.582
Recipient race (n)						0.051	0.018	0.037
Caucasian	1	1		7	10			
African American	12	10		8	37			
Other	0	1		1	3			
Recipient hepatitis C virus status (positive)	0	3		0	7			
CMV disease (positive)	0	0		0	0			
Recipient CMV status (positive, n)	10	8		9	39			
Donor age (years)	47.1 ± 11.7	38.3 ± 13.3	0.418	42.3 ± 17.7	37.3 ± 13.9	0.549	0.809	1.000
Donor sex (n) (M (F))	9 (4)	9 (3)		6 (10)	30 (20)	0.228	0.092	0.263
Donor race (n)						0.232	0.232	0.109
Caucasian	7	10		7	29			
African American	3	2		6	19			
Other	3	0		3	2			
Donor HBV antibody (positive)	0	1		0	1			
Donor hepatitis C virus antibody (positive)	0	3		1	7			
Donor CMV status (positive)	9	6		9	24			
Cold ischemia time (min)	1,328 ± 458	1,208 ± 616	0.980	1,201 ± 480	1,209 ± 550.3	1.000	0.924	0.996
Warm ischemia time/revascularization time (min)	30.8 ± 8.3	28.3 ± 7.5	0.893	30.1 ± 6.7	30.6 ± 8.1	0.995	0.994	0.848
Pump perfusion preservation time (min)	611.5 ± 522.6	428.70 ± 511.7	0.904	500.1 ± 490.0	494.8 ± 496.6	1.000	0.949	0.999
Last donor serum creatinine (mg/mL)	1.5 ± 0.8	1.1 ± 0.4	0.223	0.9 ± 0.2	1.0 ± 0.3	0.941	0.029	0.999
Acute rejection episodes (n)	4	0		2	7			
HLA MM-A (n)	1 ± 1	1 ± 1	0.924	1 ± 1	2 ± 1	0.295	0.933	0.334
HLA MM-B (n)	2 ± 1	2 ± 1	0.871	1 ± 1	2 ± 1	0.541	0.701	0.811
HLA MM-DR (n)	1 ± 1	1 ± 1	1.000	1 ± 1	1 ± 1	0.721	1.000	0.874
HLA MM total (n)	4 ± 2	4 ± 2	1.000	4 ± 2	5 ± 1	0.296	0.997	0.544
Panel reactive antibodies at transplant–T (%)	40.0 ± 42.4	43.0 ± 44.9	0.997	33.1 ± 34.1	43.5 ± 35.1	0.791	0.960	0.989
Panel reactive antibodies at transplant–B (%)	14.7 ± 24.4	21.1 ± 31.1	0.956	26.0 ± 38.7	21.1 ± 30.1	0.946	0.767	1.000
Serum creatinine (mg/dL)								
1 month	3.2 ± 1.9	1.2 ± 0.3	<0.0001	1.9 ± 0.6	1.2 ± 0.3	0.013	<0.0001	0.999
3 months	2.2 ± 0.7	1.2 ± 0.3	<0.0001	1.8 ± 0.5	1.3 ± 0.4	0.001	0.135	0.958
6 months	2.1 ± 0.6	1.2 ± 0.5	0.001	1.9 ± 0.7	1.3 ± 0.4	0.001	0.731	0.983
9 months	1.9 ± 0.4	1.2 ± 0.5	0.668	2.5 ± 3.3	1.3 ± 0.4	0.032	0.729	0.999
12 months	2.5 ± 1.1	1.4 ± 0.6	0.011	1.6 ± 0.4	1.4 ± 0.8	0.844	0.048	1.000
eGFR (mL/min)								
1 month	28.1 ± 10.6	70.3 ± 12.8	<0.0001	38.8 ± 6.3	69.6 ± 19.1	<0.0001	0.329	0.893
3 months	38.4 ± 10.7	74.9 ± 20.4	<0.0001	41.7 ± 9.3	68.0 ± 16.6	<0.0001	0.942	0.818
6 months	39.5 ± 11.5	75.1 ± 24.0	<0.0001	41.1 ± 10.4	66.5 ± 16.0	<0.0001	0.994	0.323
9 months	42.4 ± 8.3	73.9 ± 24.0	0.001	40.5 ± 13.3	68.5 ± 18.4	<0.0001	1.000	0.849
12 months	34.3 ± 11.2	65.5 ± 18.8	0.001	46.3 ± 8.2	65.7 ± 18.4	0.006	0.386	0.969

CMV, cytomegalovirus; HBV, hepatitis B virus; MM, mismatch.
Data are averages ± SD unless otherwise stated. eGFR was calculated using the abbreviated Modification of Diet in Renal Disease formula. Statistical differences were determined by Tukey-Kramer honestly significant difference (HSD) ($\alpha = 0.05$) for multiple comparison testing or by Fisher exact test.

Table 3. List of differentially expressed genes with corresponding average fold changes detected between DGF_{lo} and DGF_{hi} as well as DGF_{lo} and $noDGF_{lo}$ by microarray gene expression analysis.

AffyID	Entrez ID	Gene symbol	DGF_{lo} versus DGF_{hi}		DGF_{lo} versus $noDGF_{lo}$	
			P	Average fold change	P	Average fold change
200001_at	826	CAPNS1			0.00047	1.56
200814_at	5720	PSME1			0.00056	1.23
201030_x_at	3945	LDHB	0.00090	−1.21		
201044_x_at	1843	DUSP1			0.00038	1.52
201060_x_at	2040	STOM			0.00036	1.49
201137_s_at	3115	HLA-DPB1	0.00003	2.42	0.00170	1.84
201519_at	9868	TOMM70A	0.00065	−1.29		
201621_at	4681	NBL1			0.00064	1.31
201666_at	7076	TIMP1	0.00081	2.12		
201721_s_at	7805	LAPTM5	0.00042	1.97		
201743_at	929	CD14	0.00077	2.38		
201781_s_at	9049	AIP			0.00078	1.39
201841_s_at	3315	HSPB1			0.00052	1.73
201988_s_at	1389	CREBL2			0.00083	1.36
202207_at	10123	ARL4C	0.00031	2.25		
202237_at	4837	NNMT	0.00031	3.39	0.00513	2.39
202238_s_at	4837	NNMT	0.00012	3.46	0.00013	3.16
202540_s_at	3156	HMGCR			0.00068	−1.63
202791_s_at	9701	SAPS2			0.00022	1.26
202803_s_at	3689	ITGB2	0.00056	1.66		
202959_at	4594	MUT	0.00067	−1.54	0.00406	−1.40
202960_s_at	4594	MUT	0.00025	−1.46		
203392_s_at	1487	CTBP1			0.00027	2.35
204118_at	962	CD48	0.00093	1.53		
204198_s_at	864	RUNX3	0.00019	1.25		
204259_at	4316	MMP7	0.00024	2.51		
204655_at	6352	CCL5	0.00056	1.74		
204661_at	1043	CD52	0.00006	1.75	0.00422	1.44
205683_x_at	64499	TPSAB1	0.00056	1.52	0.00222	1.41
205798_at	3575	IL7R	0.00009	1.93		
206045_s_at	8715	NOL4			0.00013	−1.20
206254_at	1950	EGF	0.00048	−2.90		
206336_at	6372	CXCL6	0.00006	2.23	0.00114	1.82
206343_s_at	3084	NRG1	0.00064	−1.76		
206392_s_at	5918	RARRES1			0.00085	2.68
206783_at	2249	FGF4			0.00027	−1.26
207134_x_at	64499	TPSB2	0.00016	1.56	0.00195	1.40
207174_at	2262	GPC5	0.00139	−2.72	0.00037	−2.84
207238_s_at	5788	PTPRC	0.00057	1.75		
208306_x_at	3123	HLA-DRB1	0.00026	1.62	0.00092	1.50
208675_s_at	1650	DDOST			0.00054	1.49
208679_s_at	10109	ARPC2	0.00062	1.24	0.00430	1.18
208729_x_at	3106	HLA-B	0.00052	1.71	0.00153	1.57
208845_at	7419	VDAC3	0.00701	−1.27	0.00017	−1.37
208894_at	3122	HLA-DRA	0.00469	1.60	0.00038	1.74
208971_at	7389	UROD			0.00098	1.30
209040_s_at	5696	PSMB8	0.00100	1.66	0.00354	1.52
209073_s_at	8650	NUMB			0.00043	1.39
209138_x_at	3535	IGL@	0.00019	5.34		
209217_s_at	11152	WDR45			0.00085	1.31

Continued

posttransplantation. This analysis shows that grafts with DGF and fast recovery (DGF_{hi}) behave clinically similar, at least for the first year, to grafts from the noDGF group.

These findings suggest that the occurrence of DGF in kidneys with good quality has little or no clinical impact on long-term survival of the graft. This stratification further identified subsets of kidneys that have a poorer posttransplant function (DGF_{lo} and $noDGF_{lo}$), independent of whether they had experienced DGF or not. The clinical variables available before transplant did not identify these groups of kidneys with better or poorer function during the first posttransplant year. Gene expression profiling obtained from preimplantation biopsies identified the subset of grafts (DGF_{lo}, n = 13) that developed DGF and continued to have a low GFR throughout the first year. Patients in this DGF_{lo} group showed increased expression of a number of HLA and immune-related genes when compared with the other groups. Immune response genes have been shown to be overexpressed with renal ischemia in implantation biopsies (9,11). However, these studies included a mix of living and deceased donor biopsies in their profile analysis. Donor factors have been estimated to account for 35–45% of the variability in early allograft function (25). There is a high likelihood that the molecular mechanisms associated with ischemia-reperfusion injury differ depending on the donor type, as suggested by the independent segregation of the samples in the unsupervised cluster analysis presented in the previously described articles. Moreover, one study identified 132 transcripts including coagulation and complement genes that separated deceased and living donor kidneys (8). In concordance, Mueller *et al.* (10) identified 3,718 probe sets differentially expressed at an adjusted P value of <0.01 from the comparison between deceased versus living donors kidneys, showing high differences between groups also after reperfusion.

Table 3. *Continued*.

209312_x_at	100133484	HLA-DRB1	0.00025	1.81	0.00088	1.64
209312_x_at	100133484	HLA-DRB1			0.00088	1.27
209417_s_at	3430	IFI35			0.00076	1.61
209625_at	5283	PIGH	0.00049	−1.40		
210072_at	6363	CCL19	0.00043	3.74		
210084_x_at	7177	TPSAB1	0.00069	1.48	0.00213	1.39
210397_at	1672	DEFB1	0.00446	−1.44	0.00079	−1.50
210764_s_at	3491	CYR61	0.00055	2.00		
210889_s_at	2213	FCGR2B	0.00026	1.87		
210915_x_at	28639	TRBC1	0.00004	1.81		
210982_s_at	3122	HLA-DRA	0.00260	1.64	0.00037	1.73
211395_x_at	9103	FCGR2C	0.00020	1.52	0.00104	1.41
211430_s_at	28396	IGH@	0.00061	6.08		
211645_x_at	0	Affy_211645_x_at	0.00044	3.53		
211654_x_at	3119	HLA-DQB1	0.00025	2.28	0.00011	2.26
211796_s_at	28638	TRBC1	0.00044	1.74		
211911_x_at	3106	HLA-B	0.00090	1.66	0.00051	1.65
212510_at	23171	GPD1L	0.00019	−1.51		
212587_s_at	5788	PTPRC	0.00023	2.44		
212588_at	5788	PTPRC	0.00063	2.68		
212592_at	3512	IGJ	0.00031	5.70		
213193_x_at	28639	TRBC1	0.00084	1.39		
213321_at	594	BCKDHB	0.00006	−1.59		
213539_at	915	CD3D	0.00047	1.46		
213603_s_at	5880	RAC2	0.00012	1.76		
214164_x_at	771	CA12	0.00005	−1.38		
214382_at	54346	UNC93A			0.00090	−1.33
214669_x_at	3514	IGKC	0.00023	3.92		
214677_x_at	3535	IGL@	0.00027	6.02		
214836_x_at	3514	IGKC	0.00053	2.76		
215121_x_at	28815	IGL@	0.00033	4.51		
215176_x_at	100130100	LOC100130100	0.00038	4.37		
215193_x_at	100133484	HLA-DRB1	0.00018	1.86	0.00038	1.73
215379_x_at	28815	IGL@	0.00017	4.69		
215646_s_at	1462	VCAN	0.00099	2.94		
215867_x_at	771	CA12	0.00028	−1.34		
215946_x_at	91353	IGLL3	0.00030	2.71		
215952_s_at	4946	OAZ1			0.00097	1.34
216474_x_at	64499	TPSAB1	0.00019	1.60		
216576_x_at	28299	IGKC	0.00091	2.96		
217022_s_at	100126583	IGH@	0.00008	6.43		
217148_x_at	3535	IGL@	0.00039	2.30		
217235_x_at	28813	IGL@	0.00084	1.72		
217456_x_at	3133	HLA-E	0.00049	1.47	0.00843	1.31
217478_s_at	3108	HLA-DMA	0.00037	1.76	0.00270	1.55
217747_s_at	6203	RPS9	0.00032	1.38		
217774_s_at	51504	HSPC152			0.00085	1.25
218024_at	51660	BRP44L	0.00010	−1.41	0.00381	−1.27
218170_at	51015	ISOC1	0.00087	−1.44		
218192_at	51447	IP6K2			0.00092	1.24
218546_at	79762	C1orf115	0.00031	−1.38		
219076_s_at	5827	PXMP2	0.00015	−1.90		
219118_at	51303	FKBP11	0.00074	1.66		
221523_s_at	58528	RRAGD	0.00281	−1.72	0.00025	−1.88
221524_s_at	58528	RRAGD			0.00092	−1.56
221581_s_at	7462	LAT2	0.00083	1.31		

Continued

It is intriguing to find immune activation already before implantation in kidneys that will undergo DGF and will maintain a poor 1-year graft function (DGF$_{lo}$ group). The biopsy time point excludes any impact of recipient factors on these transcriptome findings. The major difference between the two low GFR groups is the donor serum creatinine (1.5 versus 0.9 in the DGF$_{lo}$ versus noDGF$_{lo}$ groups, respectively; $P = 0.034$). Hence, the signature of immune activation might be reflecting increased injury to an older tissue with impaired repair capacity resulting in significantly poorer 1-month (serum creatinine 3.2 versus 1.9 or 1.2 mg/dL in DGF$_{lo}$ versus noDGF$_{lo}$ versus DGF$_{hi}$, respectively) and 12-month graft function (serum creatinine 2.5 versus 1.6 versus 1.4 mg/dL in DGF$_{lo}$ versus noDGF$_{lo}$ versus DGF$_{hi}$, respectively).

These findings were further corroborated by an analysis on the basis of the 1-month GFR alone. Kidneys with lower GFR again expressed genes associated with inflammation and immune response, overlapping with the identified genes and pathways associated with poorer outcomes in the analyses of the four subgroups.

Overall, these findings suggest that a subset of allografts may have increased expression of immune-related genes before transplantation. It is possible that early immune activation within the grafts and possible prolonged upregulation of these and other immune-related genes may lead to long-term detrimental effects, an issue that needs to be further investigated.

It remains unclear why there is segregation between patients within the noDGF group. From the analysis of the differentially expressed genes in this group, most of these genes were downregulated in the noDGF$_{lo}$ group and were found to be involved in intracellular localization and cellular transport. These gene changes may provide a biological explanation to what has been described in the literature as "slow graft function" (26–28). Finally, few differentially ex-

Table 3. *Continued*.

221651_x_at	3514	*IGK@*	0.00036	3.84		
221671_x_at	3514	*IGK@*	0.00030	3.97		
221875_x_at	3134	*HLA-F*	0.00075	1.55		
34210_at	1043	*CD52*	0.00011	2.13	0.00918	1.59
217028_at	7852	*CXCR4*	0.00097	1.53		

Only genes with a *P* value <0.001 in at least one of the comparisons are shown.

pressed probe sets were identified between the DGF$_{hi}$ and noDGF$_{hi}$ subgroups, indicating that transcriptionally these two groups of patients are very similar.

It is clear from the data presented (see Figure 2) that patients with 1-month
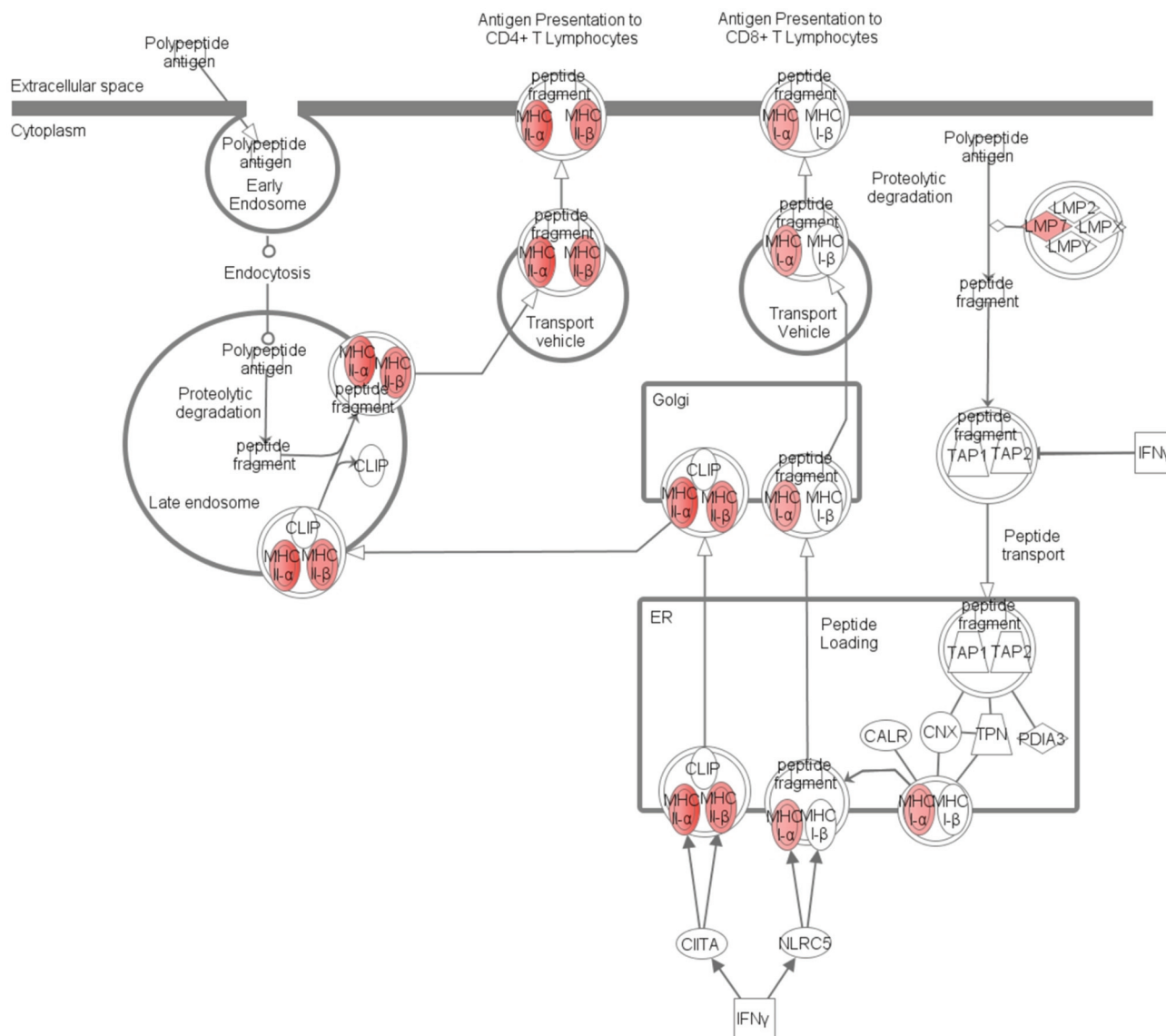
Figure 3. Graphic representation from canonical pathways using ingenuity pathways analysis of the antigen presentation signaling showing (in red) all the differentially expressed genes between noDGF$_{lo}$ versus DGF$_{lo}$ that were overexpressed in noDGF$_{lo}$. IFNγ, interfon-γ. LMP, proteasome (prosome, macropain) subunit, beta type, 9 (PSMB9); TAP, transporter associated with antigen processing; CLIP, class II–associated invariant chain peptides; CIITA, class II, major histocompatibility complex, transactivator; NLRC, NLR family; CALR, calreticulin; CNX, calnexin; TPN, TAP binding protein; PDIA, protein disulfide isomerase family A.
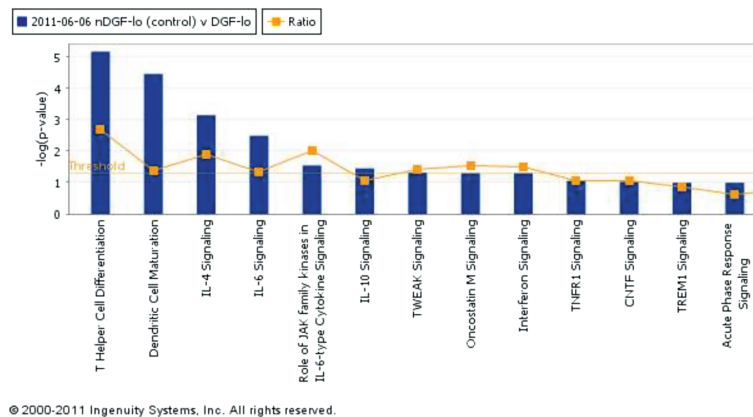
Figure 4. Canonical pathways identified from the analysis of the differentially expressed genes between noDGF$_{lo}$ versus DGF$_{lo}$. Canonical pathways are displayed along the *x* axis; the *y* axis displays the –log of *P* value, which is calculated by the Fisher exact test right-tailed. The orange points represent the ratio of the number of genes in a given pathway that meet cutoff criteria divided by the total number of genes in that pathway. TWEAK, TNF-like weak inducer of apoptosis; TNFR1, tumor necrosis factor receptor superfamily, member 1A; CNTF, ciliary neurotrophic factor; TREM1, triggering receptor expressed on myeloid cells 1.

eGFR values ≤45 mL/min have a tendency to remain as such by 1 year post-transplantation and that eGFR may be in fact a more accurate clinical marker of short- and longer-term function than DGF (29,30). Further follow-up of these patients will reveal whether this tendency continues beyond the 1-year posttransplantation mark and whether the graft's long-term function has been significantly affected by development of DGF or the persistent lower graft function.

In the present analyses, gene expression changes effectively separated the DGF$_{lo}$ group from the other three groups of patients. Upregulated genes differentially expressed in this group included various genes involved in T-cell activation (*DDOST, IL7R, CXCR4, ITGB2, PTPRC, HLA-G, HLA-DQA1, CD48* and *HLA-DMA*) and/or chemotaxis (*CXCL6, CCL19, CCL5, CXCR4, ITGB2, RAC2* and *VCAN*).

The gene expression signals identified in this subset of patients (DGF$_{lo}$) suggest the presence of active donor antigen-presenting cells with the potential of attracting and activating recipi-

ent T cells. Additionally, some of the gene signals identified may also suggest the presence of activated T cells already present on the graft at preimplantation. These kidney grafts may represent a set of real "poor-quality" grafts, reflected in the poorer medium-term graft function observed at 12 months after transplant. If such is the case, then these donor organs would require early identification, ideally before implantation, to identify kidney transplant recipients at risk of lower long-term allograft function. Further investigation of these donor organs may eventually help improve organ allocation and posttransplant management.

Kainz *et al.* (31) recently showed in a randomized control trial that steroid pretreatment of the deceased organ donor suppressed inflammation in the transplant organ but did not reduce the rate or duration of DGF. This study sought to elucidate those factors that caused DGF in the steroid-treated subjects. Genome-wide gene expression profiles were used from 20 steroid-pretreated donor organs and were analyzed on the level of regulatory

protein–protein interaction networks (32). A total of 63 significantly down-regulated sequences associated with DGF that could be functionally categorized according to Protein Analysis Through Evolutionary Relationships ontologies into two main biological processes (transport and metabolism) were identified. The recognized genes suggested hypoxia as the cause of DGF, which cannot be compensated by steroid treatment.

In summary, organ quality and clinical performance does not appear to be accurately captured by the DGF/noDGF classification. Stratification of patients on the basis of a combination of 1-month eGFR and DGF classification appears to have more predictive value and be more relevant to medium-term (12-month) graft performance than DGF classification alone. Comparative GE profiling of preimplantation biopsies using this classification has identified a subgroup of DGF kidney transplant recipients with elevated expression of immune-related genes. A better understanding of molecular pathways affected early on in the graft (that is, at preimplantation) may signal high risk for graft dysfunction, giving the chance for potential therapeutic approaches to be taken.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine,* or other interests that

might be perceived to influence the results and discussion reported in this paper.

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