

Fibrosis with Inflammation at One Year Predicts Transplant Functional Decline

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ABSTRACT

Lack of knowledge regarding specific causes for late loss of kidney transplants hampers improvements in long-term allograft survival. Kidney transplants with both interstitial fibrosis and subclinical inflammation but not fibrosis alone after 1 year have reduced survival. This study tested whether fibrosis with inflammation at 1 year associates with decline of renal function in a low-risk cohort and characterized the nature of the inflammation. We studied 151 living-donor, tacrolimus/mycophenolate-treated recipients without overt risk factors for reduced graft survival. Transplants with normal histology ($n = 86$) or fibrosis alone ($n = 45$) on 1-year protocol biopsy had stable renal function between 1 and 5 years, whereas those with both fibrosis and inflammation ($n = 20$) exhibited a decline in GFR and reduced graft survival. Immunohistochemistry confirmed increased interstitial T cells and macrophages/dendritic cells in the group with both fibrosis and inflammation, and there was increased expression of transcripts related to innate and cognate immunity. Pathway- and pathologic process-specific analyses of microarray profiles revealed that potentially damaging immunologic activities were enriched among the overexpressed transcripts (e.g., Toll-like receptor signaling, antigen presentation/dendritic cell maturation, IFN- γ -inducible response, cytotoxic T lymphocyte-associated and acute rejection-associated genes). Therefore, the combination of fibrosis and inflammation in 1-year protocol biopsies associates with reduced graft function and survival as well as a rejection-like gene expression signature, even among recipients with no clinical risk factors for poor outcomes. Early interventions aimed at altering rejection-like inflammation may improve long-term survival of kidney allografts.

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It is surprising that advances in early treatment of kidney transplant (KTx) recipients have not more dramatically improved long-term graft survival.¹⁻⁴ Barriers to understanding the causes for this include difficulties in prospectively following KTx recipients over long periods, clinical heterogeneity within retrospective studies, and unclear mechanistic links between early and late graft pathology.¹⁻⁷ We and others have sought to understand better the causes of late KTx loss through intensive clinical testing and sequential surveillance ("protocol") allograft biopsies up to ≥ 5 years after transplantation.^{5,8-14}

One important observation from these studies has been that a large proportion of graft losses within the first 5 years after transplantation is due to specific biopsy-proven processes such as recurrent

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primary disease, polyomavirus-associated nephropathy, and the sequelae of overt acute rejection.⁵ Significantly, some of these causes may be erroneously ascribed to nonspecific chronic allograft nephropathy if not diagnosed by early-stage surveillance biopsy.^{4,15} In other studies, we showed that two histologic abnormalities of 1-year (T12) surveillance biopsies—transplant glomerulopathy (an indicator of antibody-mediated damage¹⁶) and interstitial fibrosis (IF) with cellular infiltration (IF+i)—are linked to increased risk for functional decline and graft loss by 5 years.^{8,9} IF alone, although common, was associated with comparable 5-year outcome to KTx with normal T12 histology.⁸ Although similar findings have been reported from other centers,^{11,14} important questions remain unanswered: (1) Do early histologic abnormalities predict reduced long-term graft survival in the absence of specific clinical risk factors? (2) Can therapeutically relevant mechanistic insights be derived from early histologic patterns that are associated with poor long-term prognosis?

In this study, we addressed these questions by focusing on whether IF alone and IF+i in T12 surveillance biopsies are associated with subsequent decline in renal function and with characteristic molecular profiles. To exclude grafts for which other, readily identifiable factors contributed to functional decline, the study was limited to living-donor (LD) KTx recipients who lacked defined complications during the first year and were treated with a single immunosuppressive regimen. For this cohort, we showed, first, that IF+i at T12 was associated with declining renal function whereas patients with normal histology and IF alone retained stable GFR. Second, we

found that IF+i was associated with upregulation of immune/inflammatory pathways linked with acute cellular rejection.^{17–23} The results lend credence to the view that early surveillance histology with or without targeted molecular analysis provides important prognostic information.^{10,24} They also suggest that analysis of intra-graft innate and adaptive immune pathways during early posttransplantation years²⁵ may provide the basis for interventions to subvert chronic deterioration in a subset of recipients with clinical expectations of excellent long-term outcome.

RESULTS

Histologic Analysis

A total of 151 KTx recipients who had T12 surveillance biopsies and lacked clinical risk factors for poor graft survival (see the Concise Methods section) were classified on the basis of T12 Banff '97 ci and i scores into normal histology (ci = 0, i = 0; n = 86, 57%), IF alone (ci ≥ 1, i = 0; n = 45, 30%), and IF+i (ci ≥ 1, i ≥ 1; n = 20, 13%). Relevant histologic and clinical variables are summarized in Table 1. There were no between-group differences for recipient age and gender, donor age and gender, proportion with three or more HLA mismatches, and calculated predonation GFR (50% of donor uncorrected iothalamate clearance [C_{ioth}]). The large majority (91%) of the cohort had not undergone additional, clinically indicated biopsies during the first posttransplantation year, and those who did were evenly divided among the three groups (data not

Table 1. T12 histologic and baseline demographic characteristics of 151 noncomplicated, HLA-mismatched, LD KTx

Characteristic	Normal	IF Alone	IF+i	P ^a
n (%)	86 (57)	45 (30)	20 (13)	
T12 surveillance biopsy scores, original report (mean ± SD) ^b				
g	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.6	NS, 0.002, 0.002
i	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.6	NS, 0.0001, 0.0001
t	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.0	NS, 0.0001, 0.0001
ci	0.0 ± 0.0	1.2 ± 0.5	1.2 ± 0.5	0.0001, 0.0001, 0.9
ct	0.0 ± 0.0	1.2 ± 0.5	1.2 ± 0.7	0.0001, 0.0001, 0.7
cv	0.3 ± 0.5	0.8 ± 0.7	0.5 ± 0.8	0.0001, 0.4, 0.1
ah	0.1 ± 0.3	0.3 ± 0.5	0.5 ± 0.8	0.001, 0.1, 0.9
T12 surveillance biopsy scores, blinded reread (mean ± SD [% with score ≥ 1])				
total i	ND	0.07 ± 0.26 (6%)	1.81 ± 0.93 (100%)	–, –, 0.0001
Recipient				
age at transplantation (years, mean ± SD)	52 ± 12	51 ± 15	47 ± 17	0.5
female gender (%)	45	36	55	0.3
Donor				
age at transplantation (years, mean ± SD)	40 ± 11	44 ± 12	46 ± 14	0.1
female gender (%)	55	47	55	0.7
GFRu/2 (C _{ioth} ml/min; mean ± SD) ^c	59 ± 11	60 ± 12	55 ± 11	0.2

ND, not done.

^aP values for histology scores based on Wilcoxon rank sum test of normal versus IF alone; normal versus IF+i, and IF alone versus IF+i, respectively. P values for clinical indices given for ANOVA.

^bBy definition, v and cg scores were 0 for all biopsies.

^cCalculated predonation allograft function.

Table 2. Graft function and graft survival of 151 noncomplicated, HLA-mismatched, LD KTxs between 1 and 5 years after transplantation

Parameter	Normal	IF Alone	IF+i	P ^a
Length of follow-up after T12 surveillance biopsy (days; mean ± SD)	1515 ± 535	1677 ± 586	1416 ± 806	0.2, 0.4, 0.1
Graft survival at most recent follow-up (%)	97	91	85	0.2, 0.04, 0.5
Death-censored graft survival at most recent follow-up (%)	99	98	85	0.6, 0.003, 0.05
GFRu by C _{ioth} (ml/min; mean ± SD [n])				
1 month	66 ± 16 (82)	61 ± 17 (41)	61 ± 20 (17)	0.08, 0.04, 0.49
12 months	71 ± 18 (83)	67 ± 23 (43)	55 ± 17 (19)	0.20, 0.01, 0.09
24 months	68 ± 18 (76)	62 ± 16 (30)	53 ± 13 (12)	0.11, 0.01, 0.14
36 months	70 ± 23 (66)	67 ± 25 (33)	52 ± 18 (12)	0.28, 0.01, 0.10
48 months	68 ± 24 (50)	64 ± 23 (27)	44 ± 20 (10)	0.57, 0.01, 0.05

^aP values based on Wilcoxon rank sum tests of normal versus IF alone; normal versus IF+i, and IF alone versus IF+i, respectively.

shown). Patients with previous biopsy-proven acute rejection or BK nephropathy were not included. Biopsies assigned to IF-alone and IF+i groups were reanalyzed in a blinded manner by a renal pathologist (L.D.C.) to determine reproducibility of ci and i scoring and to examine the degree of overlap between the groups for cellular inflammation in areas of IF (assessed by “total inflammation” [total-i] score). This reanalysis did not result in any incident of reassignment among the groups (data not shown). Furthermore, only three (6.8%) of 44 IF-alone biopsies had a total-i score of >0 (all total-i = 1) compared with 100% of IF+i biopsies (mean total-i = 1.8 ± 0.9; Table 1), indicating that use of this score to categorize for inflammation would not have substantially altered the grouping. In total, 127 (84%) and 140 (93%) had matching T0 and T4 surveillance biopsies, Banff ’97 scoring of which are summarized in Supplemental Table S1. Mean ci scores were higher at T0 for IF-alone and IF+i groups compared with the normal group, but the differences were not evident at T4.

Graft Survival and Function after T12 Surveillance Biopsy

We next examined associations between T12 histology and subsequent KTx function and survival. Mean duration of follow-up after T12 biopsy was approximately 4 years for all groups (Table 2). Overall graft survival during follow-up was lower for IF+i than for normal (85 versus 97%; $P = 0.04$). More notable, as shown in Figure 1A, death-censored graft survival was lower for IF+i than for normal (85 versus 99%; $P = 0.003$) and IF alone (85 versus 98%; $P = 0.05$). In total, one graft each failed for reasons other than death in the normal and IF-alone groups and three in the IF+i group. These failures occurred 2.6, 4.6, 6.7, 7.4, and 7.9 years after T12 biopsy. Of interest, four of five failed grafts had subsequent histologic evidence suggestive of chronic antibody-mediated injury that was not present at T12.

Trends in renal function (uncorrected GFR [GFRu], measured by C_{ioth}) were compared among the groups before, at the time of, and for 3 years after T12 surveillance biopsy (Table 2). GFRu at 1 month after transplantation was moderately lower for the IF+i group than for the normal group (61 ± 20 versus 66 ± 16 ml/min; $P = 0.04$) but not different for the

IF-alone group (61 ± 20 versus 61 ± 17 ml/min; $P = 0.5$). At T12, the difference in GFRu between the IF+i and normal groups was more marked (55 ± 17 versus 71 ± 18 ml/min; $P = 0.01$). Subsequent GFRu measurements were progressively lower for the IF+i group, having fallen to 44 ± 20

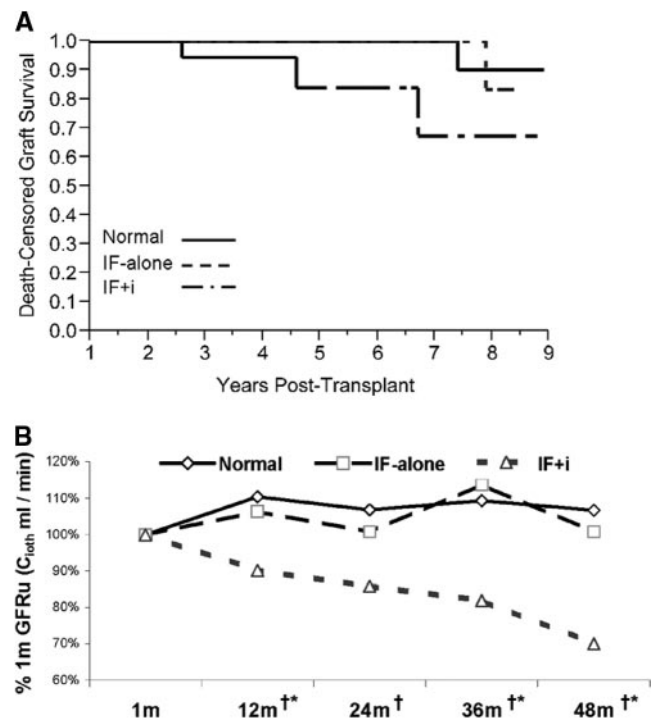


Figure 1. Death-censored graft survival and longitudinal trends in GFR indicate progressive renal functional loss in KTx recipients with IF+i on T12 surveillance biopsies. (A) A Kaplan-Meier plot is shown for death-censored graft survival after T12 surveillance biopsy for KTx recipients with normal histology, IF alone, and IF+i at T12. Time of graft loss = time of return to dialysis or decline in estimated GFR to <50% of the 12-month estimated GFR. (B) Group mean values for GFRu, measured by C_{ioth} and expressed as a percentage of the baseline value at 1 month (1m) are shown for 1, 12, 24, 36, and 48 months after transplantation. Groups were categorized by Banff ’97 scoring of T12 surveillance biopsies as having normal histology (Normal), IF alone, and IF+i. † $P \leq 0.05$ for normal versus IF+i; * $P \leq 0.05$ for IF alone versus IF+i.

ml/min by 4 years after transplantation compared with 68 ± 24 ($P = 0.01$) and 64 ± 23 ($P = 0.05$) for normal and IF-alone groups, respectively. Representation of GFRu values as a percentage of the baseline (1 month) value (Figure 1B) demonstrated that only those with IF+i had undergone a decline in function between 1 and 12 months and that this group continued to experience progressive functional loss during the subsequent 3 years. Thus, in contrast to grafts with normal histology or IF alone at T12, those with IF+i had lower graft survival and were at increased risk for progressive functional decline. We next sought to compare the groups using immunohistochemistry (IHC) and gene expression studies.

IHC, Targeted Molecular Profiling, and Microarray Analyses

Sections of T12 biopsies from subsets of each group were stained and analyzed for T cells (CD3) and macrophages/dendritic cells (DCs; CD68) with results expressed as cells/mm² of the total cortical interstitial space (Figure 2). The normal and IF-alone groups did not differ for CD3 or CD68 immunostaining, whereas biopsies from the IF+i group had significantly higher interstitial positivity for both. The results indicated that IF+i was distinguished from normal histology and IF alone by increased numbers of inflammatory cells, including T cells and macrophages/DCs, in the cortical interstitium.

Low-density quantitative reverse transcriptase-PCR arrays (TaqMan Low Density Array [TLDA]) were next carried out using frozen T12 biopsy cores. Gene targets were chosen on the basis of the results of a previous study²⁶ and on other published work.^{17,18,27-29} Between-group comparisons of relative expression levels revealed five patterns (Table 3): (1) Increase in IF alone and IF+i compared with normal; (2) increase in IF+i compared with both normal and IF alone; (3) increase in IF+i

compared with the normal; (4) no difference for any intergroup comparison; and (5) reduced expression in IF+i compared with the normal and IF-alone groups. These patterns suggested that IF+i (and, to a limited extent, IF alone) was associated with increased activity of innate (tissue injury) immune pathways including IFN- γ and TLR responses as well as increased T cell immunity^{18,30-33} and downregulation of potentially protective gene products (toll interacting protein, vascular endothelial growth factor, Bcl2).³³⁻³⁵

To explore further, we performed whole-genome microarrays with pathway analyses on T12 biopsies from each group. Of 13,611 detectable genes, only 374 demonstrated altered expression between the normal and IF-alone groups. In contrast, expression of 4367 genes was altered between normal and IF+i and 4295 between IF-alone and IF+i groups. A canonical pathway analysis of genes overexpressed in IF-alone and IF+i groups was carried out (Table 4). Although several pathways were overrepresented in the IF-alone compared with the normal group, only limited numbers of gene products were overexpressed within these pathways. In contrast, IF+i, compared with both the normal and IF-alone groups, was characterized by overrepresentation of multiple transcripts from numerous pathways, the majority of which were linked to innate and adaptive immune responses (Table 4).

Differentially expressed transcripts from this study were next compared with published gene expression signatures for experimental and human KTx complications.^{22,23} Most striking, gene expression signatures associated with cytotoxic T lymphocytes, IFN- γ response, B cells, and acute rejection were heavily enriched in IF+i compared with normal and IF-alone groups (Supplemental Tables S2 and S3). Microarrays were also carried out on T4 biopsies from the three groups and were compared in a paired manner with the results of T12 microarrays to determine expression changes over time of acute rejection-associated genes.²³ Between T4 and T12, only the IF+i group demonstrated altered expression of a substantial proportion (38%) of the acute rejection-associated genes (Supplemental Table S3).

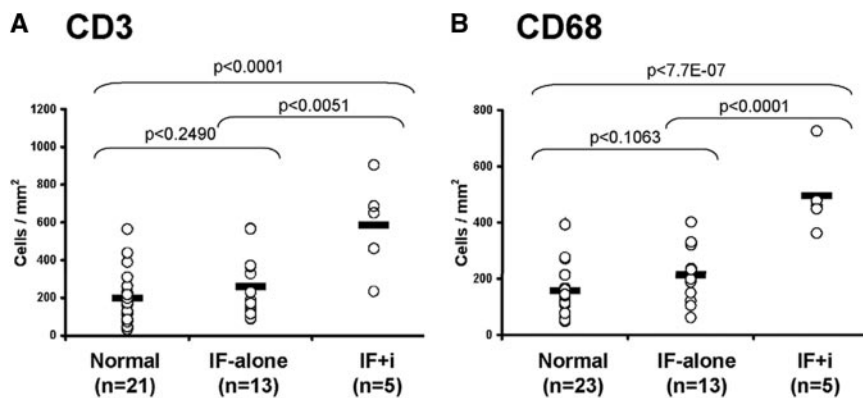


Figure 2. Increased immunostaining for T cells and macrophages/DCs in T12 surveillance biopsies with IF+i by Banff '97 criteria. (A and B) Results are shown graphically for digital image analysis of tissue sections from T12 surveillance biopsies stained by immunoperoxidase for T cells (CD3; A) and macrophages/DCs (CD68; B). Sections were stained from groups categorized by Banff '97 scoring as having normal histology (Normal), IF alone, and IF+i. Results are shown as cells/mm² of the total cortical interstitial space. ○, results for individual biopsies; thick bars, group mean results. P values indicate results of between-group comparisons by unpaired t test.

Finally, we sought to determine whether the biological responses of renal parenchymal cells could be distinguished from those of infiltrating leukocytes. By overlaying gene expression profiles of normal transplanted kidneys on those of individual leukocyte populations, a set of 1395 gene products predicted to be specific for non-inflamed kidney (termed "low proinflammatory profile kidney associated transcripts" [lowPIPKAT] genes) were derived. As shown in Table 5, there was no consistent pattern of altered expression of low-PIPKAT genes in any between-group comparison. In contrast, when IF+i was compared with normal and IF-alone

Table 3. Comparative results of quantitative reverse transcriptase–PCR for selected injury response, inflammation, and fibrosis-related genes in T12 biopsies

Gene Product	Normal (n = 39) versus IF Alone (n = 28)	P ^a	Normal (n = 39) versus IF+i (n = 16)	P	IF Alone (n = 28) versus IF+i (n = 16)	P
Pattern 1						
IL-6	1.65	0.01	1.69	0.02	−1.01	0.96
CD106 (VCAM-1)	1.33	0.02	1.36	0.01	1.00	0.98
CD62L (L-selectin)	1.75	0.01	3.69	0.00001	1.83	0.01
CD28	1.43	0.02	2.30	0.0001	1.47	0.04
Pattern 2						
CD14	1.15	0.22	1.43	0.01	1.29	0.05
IFN- γ	1.10	0.77	2.60	0.001	2.18	0.04
CXCL9 (MIG)	−1.26	0.53	2.59	0.01	3.47	0.0006
CXCL10 (IP-10)	−1.02	0.93	1.90	0.03	1.91	0.02
ubiquitin D (FAT10, diubiquitin)	1.23	0.29	2.32	0.0007	1.69	0.02
FOXP3	1.06	0.68	2.10	0.01	1.60	0.005
Pattern 3						
TNF	1.26	0.16	1.87	0.001	1.37	0.16
TGF- β 1	1.23	0.11	1.42	0.02	1.15	0.33
TLR-4	1.21	0.06	1.44	0.02	1.27	0.20
TLR-2	1.18	0.17	1.45	0.01	1.11	0.48
Pattern 4						
CD74	1.10	0.31	1.23	0.13	1.04	0.75
fibronectin 1	1.27	0.20	1.74	0.14	1.38	0.42
collagen type IV α 1	1.18	0.12	1.10	0.59	−1.10	0.56
Pattern 5						
VEGF	−1.07	0.52	−1.36	0.02	−1.29	0.05
TOLLIP	−1.03	0.75	−1.61	0.001	−1.67	0.004
BCL2	1.06	0.60	−1.42	0.04	−1.62	0.01

Data are expressed as fold change in RNA amount for second compared with first named group. TOLLIP, toll interacting protein; VEGF, vascular endothelial growth factor.

^aP values based on t test for between-group comparisons.

groups, we observed overexpression of 28 to 67% of genes predicted to be confined to leukocytes (broad inflammatory cell genes) or shared by parenchymal kidney cells and leukocytes (combined kidney and inflammatory cell genes).

DISCUSSION

Because most failing KTx have severe IF,^{36–40} one might assume that the presence of IF early after transplantation would identify allografts destined for decline in function leading to graft loss.^{5,9,40} This study, however, demonstrates that IF alone on T12 surveillance biopsies is typically associated with stable graft function up to 5 years after transplantation. In contrast, we found that when IF was accompanied by an interstitial cellular infiltrate (termed “inflammation” by the Banff schema^{41–43}), a progressive decline in GFR commonly occurred. Importantly, the histologic severity of inflammation on T12 biopsies in the IF+i group studied was generally mild (mean i score was 1.4) and below the threshold for diagnosis of acute cellular rejection.⁴²

Although the Banff i score omits cellular infiltrates in areas of IF,⁴² it has recently been reported, for clinically indicated

biopsies, that a “total inflammation” score that incorporates all cellular infiltrates better predicts the risk for future graft deterioration.⁴¹ For our cohort, we observed that the severity of inflammation among biopsies designated as IF+i was greater when expressed as total-i (mean score 1.8), but use of this scoring criterion did not greatly alter the distinction between IF+i and IF alone. Nonetheless, because the combined presence of fibrosis and inflammation is clearly associated with adverse outcomes, it will be of interest in subsequent studies to identify surveillance biopsies for which i and total-i scores are divergent. Less clear for the present is the value of surveillance biopsy–guided treatment for subclinical inflammation among KTx recipients who are treated with tacrolimus/mycophenolate.^{12,44} Of the 20 patients with IF+i, nine received bolus corticosteroid therapy after T12 biopsy on the basis of clinical judgment and 11 did not. Formal analysis of the effect on graft outcome, however, was precluded by case number and non-random assignment of treatment. Nonetheless, immunohistochemical analysis of the graft infiltrates confirmed higher numbers of T cells and macrophages/DCs in the IF+i group, and gene expression profiles were consistent with the presence of activated T cells and monocyte/macrophages, which would likely be susceptible to conventional antirejection therapy. In

Table 4. Biological pathways differentially represented in T12 surveillance biopsy samples from the three groups on the basis of whole-genome microarrays

Parameter	Gene Number ^a	Normal (n = 25) versus IF Alone (n = 24)			Normal (n = 25) versus IF+i (n = 16)			IF Alone (n = 24) versus IF+i (n = 16)		
		Gene Number P < 0.05 ^b	% Increased ^c	P ^d	Gene Number P < 0.05	% Increased ^c	P	Gene Number P < 0.05	% Increased ^c	P
All detectable pathway-assigned genes	13,601	528			5469			5380		
Death receptor signaling	51	7	86	0.003	24	83	0.2	24	75	0.2
CCR5 signaling in macrophages	43	6	100	0.005	21	76	0.2	22	64	0.08
Starch and sucrose metabolism	47	6	100	0.009	15	67	0.9	17	41	0.8
α-Adrenergic signaling	65	7	100	0.015	29	59	0.3	32	50	0.1
Macropinocytosis	57	6	100	0.02	30	67	0.04	23	61	0.5
CCR3 signaling in eosinophils	80	7	100	0.04	34	53	0.4	39	38	0.07
Chemokine signaling	53	5	80	0.05	24	67	0.3	28	50	0.03
Antigen presentation pathway	28	2	100	0.3	26	92	0.00001	20	90	0.0006
CTLA4 signaling in cytotoxic T lymphocytes	66	1	100	0.9	41	68	0.0003	38	66	0.002
T helper cell differentiation	22	2	100	0.2	17	100	0.0005	14	100	0.02
IL-4 signaling	56	2	100	0.6	35	77	0.0006	31	74	0.01
CD28 signaling in T helper cells	81	2	100	0.8	47	79	0.0009	44	75	0.004
CTL-mediated apoptosis of target cells	16	1	100	0.5	13	85	0.001	11	82	0.02
Dendritic cell maturation	98	6	100	0.1	55	87	0.001	50	74	0.01
Toll-like receptor signaling	40	2	100	0.5	25	64	0.004	19	58	0.2
Role of PKR in IFN induction and antiviral response	33	0	–	1.0	21	67	0.006	21	57	0.004
NK cell signaling	72	3	100	0.5	40	73	0.006	44	64	0.0002
IFN signaling	24	3	100	0.06	16	94	0.008	12	100	0.2
T cell receptor signaling	72	2	100	0.8	39	74	0.01	38	68	0.01
Calcium-induced T lymphocyte apoptosis	33	2	100	0.3	20	75	0.01	18	72	0.06
Role of pattern recognition receptors in recognition of bacteria and viruses	60	3	100	0.4	33	85	0.01	31	81	0.04
Hepatic fibrosis/hepatic stellate cell activation	99	6	83	0.2	51	78	0.02	40	68	0.5
NF-κB signaling	102	3	100	0.8	52	69	0.02	40	63	0.6
Fcγ receptor-mediated phagocytosis in macrophages and monocytes	78	1	100	1.0	41	71	0.02	42	64	0.007
Glycosphingolipid biosynthesis, neolactoseries	16	1	0	0.5	11	55	0.02	10	60	0.05
Role of NFAT in regulation of the immune response	123	5	100	0.5	61	72	0.02	61	66	0.01
B cell receptor signaling	116	6	83	0.3	57	70	0.03	57	60	0.02
EGF signaling	42	1	100	0.8	23	48	0.04	19	42	0.3
FcεRI signaling	69	3	100	0.5	34	56	0.08	37	46	0.02
fMLP signaling in neutrophils	82	5	100	0.2	39	64	0.1	41	56	0.03
GM-CSF signaling	56	2	50	0.6	29	62	0.05	29	59	0.04
CXCR4 signaling	114	6	100	0.3	48	54	0.4	54	43	0.05

^aRefers to total number of detectable genes within each biological pathway. Biological pathway nomenclature derived from Ingenuity Pathways Analysis software (Ingenuity Systems).

^bRefers to the number of pathway genes with significantly altered expression (by t test) between groups.

^cRefers to the proportion of the significantly altered genes that demonstrated increased expression in IF alone compared with normal, IF+i compared with normal, or IF+i compared with IF alone, respectively.

^dRefers to the result of Fisher exact test for overrepresentation of pathway-specific genes in one group compared with the other.

addition, that a number of the grafts that failed within the follow-up period eventually developed evidence of chronic antibody-mediated injury suggests that the link between persistent subclinical T cell activation and subsequent antidonor antibody production requires further investigation.

Immunohistochemical and molecular analyses of T12 surveillance biopsies were performed with a view to correlating specific histologic patterns with more mechanistically informative assays. Although assignment of $i \geq 1$ may vary for individual pathologists,^{11,41,42,45} the results of IHC, TLDA, and microarrays confirmed that biopsies assigned to IF+i had distinct characteristics compared with those interpreted as having normal histology or IF alone. Targeted reverse transcriptase-PCR analysis demonstrated elevated expression of multiple innate

and adaptive immune mediators consistent with tissue injury response, Th1-type T cell response, and suppression of counterregulatory pathways.^{18–20,22,23,26,30,32–34,46} Microarray analyses confirmed and extended this profile, revealing the overexpressed pathways and gene clusters in the IF+i group to be heavily enriched for immune activation and identifying the process as being closely linked with IFN-γ-induced, cytotoxic T lymphocyte-associated, and acute rejection signatures.^{17,18,23} Pathway analysis of microarray data for the IF+i group also provided evidence of active participation of a range of immunologic cell types, including T cells, B cells, monocyte/macrophages, DCs and NK cells. The strong overlap between gene expression patterns observed for KTx with IF+i on protocol biopsies and reported profiles for experimental and clin-

Table 5. Differential gene expression in T12 surveillance biopsy samples from the three groups on the basis of gene specificity for lowPIPKAT, broad inflammatory cells, and combined kidney and inflammatory cells

Parameter	Gene Number ^a	Normal (n = 25) versus IF Alone (n = 24)			Normal (n = 25) versus IF+i (n = 16)			IF Alone (n = 24) versus IF+i (n = 16)		
		Gene Number	%	P ^d	Gene Number	%	P	Gene Number	%	P
		P < 0.05 ^b	Altered ^c		P < 0.05	Altered		P < 0.05	Altered	
All included genes	8473	188	2		2522	30		2487	29	
lowPIPKAT genes	1395	30	2	0.8	292	21	1.0	268	19	1.0
Broad inflammatory cell genes	499	4	1	0.01	150	30	2 × 10 ⁻³²	141	28	2 × 10 ⁻²⁴
Common kidney and inflammatory genes	6475	154	2	0.5	2063	67	0.002	2064	32	5 × 10 ⁻⁷

^aRefers to total number of detectable genes within each categorization (see Supplemental Methods for details).

^bRefers to the number of genes in each category with significantly altered expression (by t test) between groups.

^cRefers to the proportion of the genes in each category with significantly altered expression between groups.

^dRefers to the result of Fisher exact test for increased expression of category-specific genes in one group compared with the other.

ical acute rejection^{10,17,23,46,47} indicate that a subgroup of KTx recipients with seemingly excellent clinical indicators are at risk for breakthrough alloimmunity that may progress to early graft loss. The basis for this risk may derive from polymorphisms for immune-related genes of donors and recipients or from pharmacogenomic or behavioral variability among recipients.^{48,49}

Because there were trends toward lower initial graft function (1-month C_{10th}) for both IF-alone and IF+i groups compared with normal, it is possible that variations in the severity of immediate tissue injury responses to transplantation played a role in the development and persistence of intra-graft fibrosis and inflammation at T12.^{26,50} In keeping with this, we previously reported increased expression of inflammation-related gene transcripts between T0 and T12 among grafts with IF alone compared with those with normal histology.²⁶ Although the histology of T4 biopsies did not demonstrate striking differences for fibrosis and/or inflammation among the three groups at this earlier time point, this model of heightened or prolonged tissue injury response leading to IF+i and graft deterioration during the first year may be better addressed through further molecular analyses of sequential biopsy specimens.

Unlike IF+i, the IF-alone group exhibited relatively subtle gene expression changes compared with allografts with normal histology. This included overexpression of a limited number of innate response genes by TLDA (IL-6, vascular cellular adhesion molecule 1, L-selectin) and microarray analysis results compatible with a macrophage-mediated repair response (e.g., increased alternative macrophage activation and IFN- γ -suppressed/repair-induced transcripts).^{50,51} Coupled with the excellent 5-year graft survival for this group, the results suggest that early IF of LD KTx, although common and linked with increased inflammatory activity,^{9,26,52} may be self-limiting or associated with alternative immunologic pathways in the majority of cases.⁵³ In contrast to some gene expression studies of biopsies for cause,^{29,35,36,40,54} we found in early surveillance biopsies with IF alone or IF+i that neither TLDA nor microarray

analyses demonstrated broadly increased expression of transcripts related to extracellular matrix or profibrotic pathways. Furthermore, although in some settings alterations in “kidney response to injury” genes (termed lowPIPKAT genes in this study) have been linked with early graft damage,^{10,50,52} we found that virtually all of the altered gene expression in the IF+i grafts may be attributed to the infiltrating cells. This may indicate that cellular infiltration/activation and fibrosis/atrophy occur episodically³⁵ at separate time points or, alternatively, that secondary responses of the renal parenchyma to inflammatory activity are a relatively late component of a progressive process. In this regard, it is important to note the possibility that early renal parenchymal response to injury is more marked in categories of KTx recipients not studied here, including those with deceased donor source, cyclosporine-based immunosuppression, a history of delayed graft function, or other early complications.^{35,52,55}

During the past 10 years, surveillance biopsy and other monitoring protocols have greatly enhanced our understanding of the spectrum of pathologic abnormalities among functioning KTx and of the cellular biology underlying specific allograft complications.^{5,8,10,11,13,14,17,24–26,36,37,52} For molecular analysis studies in particular, an important concern is the degree to which results from carefully selected patient cohorts may apply to the broader population of KTx recipients. The similarity of the gene expression profiles we observed for grafts with IF+i to those reported by others for less highly selected grafts with acute rejection suggests that our results, to some degree, can be generalized beyond the tightly defined recipient population that we studied here.^{17,18,20,23} We also propose, however, that selection of specific histologic and clinical scenarios for the application of molecular techniques represents a viable model for the eventual clinical translation of these emerging analysis tools. Although it has been suggested that gene expression assays could eventually supplant conventional graft histology for the purpose of making therapeutic decisions,^{10,21,22,43} we believe it more likely that pathologic classification of graft abnormalities will remain the initial step toward

diagnosing injurious processes. Furthermore, for overt complications (e.g., BK nephropathy, recurrent primary disease, severe acute rejection), the diagnostic and prognostic information provided by histology may prove not to be greatly enhanced by gene expression analysis. In contrast, for KTx with subclinical or mechanistically ill-defined histologic abnormalities, it is feasible that the ability to detect signature transcriptional profiles within the tissue can be used to identify subgroups at highest predicted risk for subsequent loss of function. Thus, in specific circumstances, the information provided by gene expression analyses may be used to enhance rather than replace that provided by graft pathology. Finally, in common with the work published by others in the field,^{18,20,23,25,29,40,54,56} our TLDA and microarray analyses contribute to a growing publicly available databank of expression profiles from KTx recipients with diverse characteristics and provide a basis for ongoing mechanistic investigation of renal allograft injury and repair.

CONCISE METHODS

Biopsy Acquisition and Case Selection

All study procedures were approved by the Mayo Clinic institutional review board (07-002743). Cases were selected from KTx recipients at Mayo Clinic (Rochester, MN) between May 4, 2000, and September 28, 2007. During this time, consenting recipients underwent surveillance KTx biopsy intraoperatively (T0) and at 4 months (T4) and 12 months (T12) after transplantation, as described previously.⁹ One tissue core (“histology core”) was paraffin-embedded with sections subjected to conventional histologic stains and scored by a consultant renal pathologist using Banff ’97 criteria.⁴² A second core (“research core”) was placed in RNA stabilizer (RNALater; Ambion, Austin, TX), then snap-frozen in liquid nitrogen and stored in a -80°C freezer. Selection criteria for this study were as follows:

1. Nonsensitized, HLA-mismatched LD KTx
2. Adequate T12 surveillance biopsy histology and research cores acquired⁴²
3. Oral immunosuppression with tacrolimus, mycophenolate mofetil, and prednisone for the entire first year after transplantation
4. No occurrence of delayed graft function, acute rejection (clinical or subclinical), polyomavirus (BK)-associated nephropathy, or recurrent primary renal disease before T12 surveillance biopsy
5. Absence of transplant glomerulopathy (cg >0) or recurrent glomerular disease on T12 biopsy

Cases meeting entry criteria were divided into three groups using the original clinical report of the responsible renal pathologist for the T12 biopsy. Grouping was based on reported Banff ’97 scores for IF (ci) and interstitial inflammation (i).⁴² During the period covered by the study, all i scores were assigned according to the “classic” Banff ’97 criterion of scoring cellular infiltrate only within nonfibrotic areas of cortical interstitium with thresholds of 10 to 25%, 26 to 50%, and $>50\%$ interstitial involvement for scores of i1, i2, and i3, respectively.

For ci scores, the Banff ’97 thresholds of 6 to 25%, 26 to 50%, and $>50\%$ fibrosis of scorable cortex were applied.⁴² Subsequently, all T12 biopsies assigned to IF-alone and IF+i groups were reanalyzed in a blinded manner by a single renal pathologist (L.D.C.) for Banff ’97 scores and for “total inflammation” (total i) to include information regarding cellular infiltrates contained within areas of cortical fibrosis.^{41,43} As we described previously,⁸ T12 biopsies with $i >0$ and $ci = 0$ (“interstitial cellular infiltrates alone”) were very infrequent (2% of cases screened) and, as a result, were not included in the study. Clinical data were abstracted from patient medical records using a dedicated transplant database as described previously.^{8,9} GFR was accurately measured by short C_{ioth} ⁵⁷ in LDs during evaluation and in KTx recipients at 3 to 4 weeks and 12 months after transplantation and annually thereafter. C_{ioth} was expressed in the uncorrected (ml/min) format. Graft failure was defined as return to dialysis or decline in estimated GFR to $<50\%$ of the 12-month estimated GFR.

IHC and Digital Image Analysis

Freshly prepared tissue sections were stained by immunoperoxidase technique with anti-human CD3 (F7.2.38; Dako, Carpinteria, CA) or anti-human CD68 antibodies (PG-M1; Dako) then counterstained with hematoxylin. Stained sections were scanned using a NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu City, Japan). IHCscore software (Bacus Laboratories, Lombard, IL) was used to analyze positively stained cells as a proportion of the total cortical interstitial cells, and the data were converted to cells/mm² using measured nuclear size.

RNA Extraction from Tissue Cores and Low-Density Arrays

Total RNA was extracted from frozen research biopsy cores using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), then cleaned using the RNeasy Mini Kit (Qiagen, Valencia, CA) and analyzed for quality using an Agilent Bioanalyzer (Agilent, Palo Alto, CA). First-strand cDNA synthesis was carried out on 1 μg of total RNA for each sample using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed for a panel of 30 transcripts using a customized 384-well TLDA (Applied Biosystems) with primer/probe sets selected from the Applied Biosystems expression assay catalog (details provided in Supplemental Table S4). Amplifications were carried out in the Mayo Advanced Genomics Technology Center using an ABI 7900HT (Applied Biosystems). Reactions were performed in triplicate; normalized to six replicates of the housekeeping gene 18S and to a four-point, 2.5-fold total RNA standard curve; and expressed as arbitrary units.

Human Whole-Genome Microarrays

Whole-genome microarrays were carried out in the Mayo Advanced Genomics Technology Center using GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA). Labeling, hybridization, and scanning of microarrays were carried out by manufacturer-recommended instructions as described previously.^{26,58}

Statistical Analysis of Clinical Data, IHC, and TLDA

Results

Data were expressed as means \pm SD and compared between groups using JMP software (SAS Institute, Cary, NC). Means of normally distributed continuous data were compared by *t* test and ANOVA. Non-normally distributed variables were compared by nonparametric tests. The χ^2 test was used to compare proportions between binary variables. Significance was assigned to $P \leq 0.05$.

Statistical Analysis of Whole-Genome Microarrays

For global gene expression analyses, microarray data were analyzed using a Perfect Match–only model, then subjected to FASTLO normalization. Intergroup comparisons were tested using a generalized linear model in which $P < 0.05$ was considered significant.^{26,58,59} For biologic pathway-specific analyses, significant microarray data were overlaid onto 235 previously annotated biologic pathways (Ingenuity Systems, Mountain View, CA) and analyzed for overrepresentation by Fisher exact test. For analyses related to pathologic processes and cell compartment–specific alterations, significant between-group expression changes were compared with relevant publicly available gene lists⁴¹ and with novel gene lists generated for this study. In the latter case, gene lists were derived to separately examine between-group gene expression changes specific for kidney-resident cell populations (lowPIPKATs), for total or individual infiltrating leukocytes. Details of how these lists were generated are provided in the Supplemental Methods section. Microarray data, including .cel files, from all T12 samples in this study are available on the GEO web site (<http://www.ncbi.nlm.nih.gov/gds>; GEO: GSE22459).

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DISCLOSURES

None.

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