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Early Macrophage Infiltration and Sustained Inflammation in Kidneys From Deceased Donors Are Associated With Long-Term Renal Function

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Kidney transplants from living donors (LDs) have a better outcome than those from deceased donors (DDs). Different factors have been suggested to justify the different outcome. In this study, we analyzed the infiltration and phenotype of monocytes/macrophages and the expression of inflammatory and fibrotic markers in renal biopsy specimens from 94 kidney recipients (60 DDs and 34 LDs) at baseline and 4 months after transplantation. We evaluated their association with medium- and long-term renal function. At baseline, inflammatory gene expression was higher in DDs than in LDs. These results were confirmed by the high number of CD68-positive cells in DD kidneys, which correlated negatively with long-term renal function. Expression of the fibrotic markers vimentin, fibronectin, and α–smooth muscle actin was more elevated in biopsy specimens from DDs at 4 months than in those from LDs. Gene expression of inflammatory and fibrotic markers at 4 months and difference between 4 months and baseline correlated negatively with medium- and long-term renal function in DDs. Multivariate analysis point to transforming growth factor-β1 as the best predictor of long-term renal function in DDs. We conclude that early macrophage infiltration, sustained inflammation, and transforming growth factor-β1 expression, at least for the first 4 months, contribute significantly to the difference in DD and LD transplant outcome.

Abbreviations: DD, deceased donor; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; ICAM, intercellular adhesion molecule; IL, interleukin; IRI, ischemia–reperfusion injury; LD, living donor; MCP-1, monocyte chemoattractant protein-1; MDRD, Modification of Diet in Renal Disease; NF-κB, nuclear factor κB; TGF, transforming growth factor; TNF, tumor necrosis factor

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Introduction

Kidney transplantation is the treatment of choice for the majority of patients with end-stage renal disease (ESRD). Dialysis extends life expectancy but has drawbacks. Waiting time on dialysis is associated with worse outcomes after living donor (LD) and deceased donor (DD) kidney transplantations, resulting in decreased patient and graft survival (1,2). In clinical renal transplantation, allografts from related LDs have superior graft function and survival compared with those from DDs (3). Kidney transplantation entails a period of ischemia–reperfusion injury (IRI) during organ retrieval, preservation, and implantation, and it is possible that early damage to DD organs may render them more susceptible to harmful physiological and immunological events after transplantation (4,5). Immediately after transplantation, deceased renal allografts may display a nonspecific inflammatory response associated with prolonged cold IRI (6). IRI induces recruitment of inflammatory cells within the allograft, which may cause amplify organ damage. Renal cell death and ischemia create a proinflammatory environment and generate reactive oxygen species, driving cytokine release and further inflammation (7). Macrophages infiltrate the graft during IRI and acute and chronic rejection (8,9) and are associated with poor short-term outcome during rejection (10,11). However, infiltrating macrophages are functionally heterogeneous. Evidence suggests that there is a temporal association between

M1 and M2 phenotypes during IRI (12). Chemokines, such as monocyte chemoattractant protein-1 (MCP-1), actively recruit monocytes into the injured organ (13). MCP-1 is hardly detectable in normal human kidneys, but its expression increases in kidneys from patients with acute renal transplant rejection (14), indicating its involvement in macrophage infiltration and renal graft damage. Additional cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β activate the transcription of nuclear factor κB (NF-κB), which upregulates MCP-1 expression (15). Macrophages can mediate injury in the context of reactive oxygen species, creating a proapoptotic environment. Tubular cells also contribute to inflammation in response to ischemia, generating proinflammatory and chemotactic cytokines, which activate inflammatory cells (16,17). This inflammation initiates the repair process, but sometimes repair can result in the development of tubulointerstitial fibrosis, when transforming growth factor (TGF)-β1 and other fibrotic factors are overactivated (18–21). However, there are scarce human data exploring the relationship between baseline and early persistent kidney inflammation from DDs and LDs and their relationship to kidney outcomes.

We studied the dynamics of the inflammatory status and expression of fibrotic factors in kidneys from DDs and LDs at the time of donation and 4 months after transplantation. We assessed its impact of early kidney inflammation on medium- and long-term renal outcome.

Methods

Patients and samples

This is a prospective observational study. The study was approved by the ethics committee of the Fundació Puigvert. We collected protocol biopsy specimens from kidney donors and recipients who underwent transplantation in our institution between 2008 and 2011, all of whom signed an informed consent. A total of 94 patients were enrolled in the study. We aimed to take, from each transplanted kidney, two biopsy cores at the time of transplantation: just before implantation (baseline) and 4 months after surgery. One core was processed for mRNA analysis, and the second was paraffin embedded. Due to technical problems or medical prescriptions, however, we could not collect all the samples at each time point. Therefore, for the present mRNA expression study, we included 68 baseline samples, 47 from DDs and 21 from LDs. At 4 months, we analyzed 26 samples from DDs and 23 from LDs. Histology and CD68 staining were performed in 47 baseline samples (30 DDs and 17 LDs) and 22 samples at 4 months (10 DDs and 12 LDs).

Blood and urine were collected at different time points for clinical analysis, and we retrospectively reviewed the clinical charts of 94 kidney recipients. Proteinuria was assessed by measurement of a 24-h urine sample or by assessing the urine protein:creatinine ratio. Estimation of the glomerular filtration rate (eGFR) was performed using the simplified Modification of Diet in Renal Disease (MDRD) formula from traceable creatinine. Renal failure was defined by an eGFR of < 60 mL/min/1.73 m^2, and the stage of chronic kidney disease was defined according to the Kidney Disease Outcomes Quality Initiative classification.

Remuzzi and Banff scores

Light microscopic morphology was assessed on 3-μm histologic sections stained with hematoxylin and eosin, methenamine silver–periodic acid, Masson trichrome, and periodic acid–Schiff. Immunohistochemical staining was made on paraffin -mbedded tissue sections, and staining was performed in a Dako Autostainer Link, Glostrup, Denmark.

Remuzzi score (22) was applied to analyze graft fibrosis at the moment of transplantation, and Banff score (23) was performed to assess fibrosis and inflammation after 4 months of transplantation.

In this study, Remuzzi score was evaluated only for research purpose but not for implantation decision. This score was performed to obtain an approximation about the sample chronicity state at the time of implantation. As described, four different parameters were considered in the scoring system: glomerular global sclerosis (0–3), tubular atrophy (0–3), interstitial fibrosis (0–3), and arterial and arteriolar narrowing (0–3).

Banff score was performed in 4-months renal biopsy specimens. Banff classification was applied, evaluating and scoring "t" (tubulitis), "i" (interstitial inflammation), "v" (arteritis), "g" (glomerulitis), "ptc" (peritubular capillarity), and C4d peritubular capillary deposition and chronic damage ("cg," "ci," and "cv"). The scoring ranges from 1 (mild) to 3 (severe).

CD68 immunohistochemistry

Tissue was fixed in 4% neutrally buffered formalin. Formalin-fixed, paraffin-embedded sections at 2 μm thick were deparaffinized and rehydrated followed by pretreatment with heat-induced epitope retrieval (Dako PT Link). CD68 staining was performed in a Dako Autostainer Link. Staining steps include tissue blocking and addition of primary antibody (Dako). Samples were incubated with horseradish peroxidase and labeled, and reaction was visualized using the diaminobenzidine substrate system (FLEX-DAB+-Sub-Chromo, Dako). Staining was counterstained with hematoxylin. CD68 interstitial infiltrating cells in the cortex were counted blindly in at least 10 high-power fields, excluding subcapsular, glomerular, and vascular areas. Nodular CD68 infiltrates were also excluded from counting.

RNA extraction

Kidney tissue biopsy specimens immediately before and 4 months after transplantation were stabilized in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and frozen at -80°C until processed. Biopsy specimens were immersed in 800 μL of chilly TRI Reagent (Sigma-Aldrich, St. Louis, MO) and homogenized with Tissuelyser LT (Qiagen) in accordance with manufacturers' instructions. Briefly, 160 μL of chloroform was added and samples were centrifuged. The aqueous phase containing RNA was recovered and gently mixed with 1:1 vol:vol of 100% ethanol. Samples were transferred into an RNeasy column (RNeasy Plus Micro kit, Qiagen). After several washing steps, RNA was eluted with 14 μL of RNase-free water. The integrity of total RNA was assessed on a denaturing agarose gel allowing visual assessment of the 28S and 18S rRNA bands. The concentration of RNA was adjusted to 200 ng/μL, followed by storage at -80°C.

cDNA retrotranscription

Retrotranscription of total RNA to cDNA was done according to the user guide of Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System, OpenArray® Experiments (Life Technologies, Waltham, MA). Briefly, 10 μL of 2× reverse transcription mix of the High Capacity cDNA Reverse Transcription kit (Life Technologies) was added to a well with 10 μL of total RNA of a 96-well reaction plate. Plates were incubated at room temperature for 10 min, then incubated at 37°C for 2 h and placed on ice for 5 min, incubated at 75°C for 10 min, placed on ice for 5 min, and spun down.

Real-time PCR

The experiment was done in accordance with the TaqMan® gene expression assays protocol (Life Technologies). The setup for PCR was 2× TaqMan OpenArray Real Time PCR Master Mix (Cat No. 4.462.159 Life Technologies), 3.6 μL of cDNA, and 3.9 μL of water. This mix was prepared in a 384-well OpenArray sample loading plate. The mix was then loaded on each OpenArray plate using the OpenArray AccuFill System. Each OpenArray plate can analyze 168 assays for 16 samples. These plates were run on the computer QuantStudio 12K Flex Real-Time PCR system. In each run the computer can process four OpenArray plates (64 samples simultaneously).

Statistics

GraphPad Prism software was used to perform statistical analysis. Results are expressed as the mean ± SD (lower and upper extremities) and percentages, as appropriate. Student's parametric t-test was applied to compare means. When our data did not follow a Gaussian distribution, nonparametric tests such as Mann–Whitney U test and Spearman correlation were used for data analysis. Univariate, bivariate (ANOVA), and multivariate analyses with SPSS were used to evaluate the effects of all variables on the eGFR response. All p values were two-sided, and $p < 0.05$ was considered significant.

Results

Donor and recipient characteristics

Ninety-four kidney biopsy specimens from donors (60 DDs/34 LDs; 46 male/48 female) were collected and analyzed. Baseline characteristics of patients and donors and their clinical background are summarized in Table 1(A and B). We observed significant differences in donor age ($p = 0.001$) and ischemia time (14.8 ± 4.7 h DDs versus 2.4 ± 1.3 h LDs, $p < 0.0001$), which was significantly longer in DD kidneys. Furthermore, DD recipients were significantly older ($p = 0.0009$) and had spent more time on dialysis than LD recipients before transplantation ($p < 0.0001$). Significant differences between the groups were also observed with respect to donor eGFR ($p = 0.03$) and delayed graft function (<0.0001). Only one presensitized LD patient was found. Immunosuppressive therapies were prescribed following the standard protocols of our institution. All patients received triple immunosuppressive therapy (mycophenolate, prednisone, and tacrolimus), and 40 DD (66.6%) and 27 LD (79.4%) patients were also treated with antilymphocyte serum (basiliximab or thymoglobulin).

As shown in Figure 1, kidney graft survival was higher in LD than in DD graft recipients (89.3% versus 63.3%, respectively), at 88.2 months of follow-up.

Histology

Figure 2(A) shows the percentage of LD and DD biopsy specimens according to Remuzzi total score. A total

Table 1: Baseline characteristics of donors (A) and recipients (B) (N = 94)

	DD (n = 60)	LD (n = 34)	p value
(A) Donors			
Gender (male/female)	35/25	11/23	0.01
Age (mean ± SD)	58.5 ± 14.4	49.9 ± 10.1	0.001
Hours of cold ischemia time (mean ± SD)	14.8 ± 4.7	2.4 ± 1.3	<0.0001
eGFR (mL/min/1.73 m^2)	92.2 ± 31.3	105 ± 26.6	0.03
Causes of death (n)			
Intracranial hemorrhage/thrombosis	40		
Hypoxic brain damage	1	–	–
Head trauma	11		
Cardiovascular	4		
Other	4		
(B) Recipients			
Gender (male/female)	39/21	23/11	n.s.
Age at the moment of transplantation (mean ± SD)	59 ± 17	48 ± 17	0.0009
Time on dialysis before transplantation (months)	44.3 ± 28	6.9 ± 15.2	<0.0001
Causes of chronic kidney disease			
Cardiovascular	13	5	
Glomerulopathy	20	8	
Hereditary	7	3	
Interstitial nephropathy	5	3	
Obstruction	0	1	
Unknown	15	12	
Other	0	2	
Immunosuppressive therapy			
Induction[1]/triple therapy[2]	40/20	27/7	n.s.
Delayed graft function (n)	24	1	<0.0001

eGFR, estimated glomerular filtration rate.
[1]Induction therapy: thymoglobulin or basiliximab followed by triple therapy.
[2]Triple therapy: tacrolimus, mycophenolate mofetil, and prednisone.

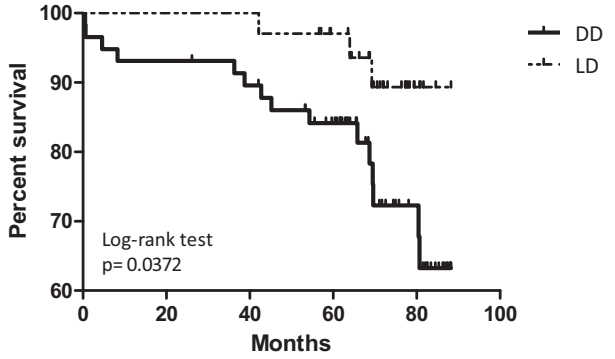


Figure 1: Kaplan–Meier curves plotting percent survival since renal transplantation in months censored at death, doubled creatinine, or graft loss (χ^2 = 4.339, p = 0.0372). Follow-up lasted 88.2 months, and survival at this point was 63.3% in deceased donors (red line) and 89.3% in living donors (black line).

score ≤ 3 was observed in 88.8% of LD and in 81.9% of DD samples. This score was related to medium-term renal function in DDs; thus, those samples with a score ≤ 3 showed better eGFR at 12 months (p = 0.040) and 24 months (p = 0.026) than those with a score ≥ 4.

According to 4-month Banff score (Figure 2B) in DD and LD samples, 41% and 20% inflammation and 50% and 32% fibrosis were found, respectively. Banff 4-month inflammation was related with a worse eGFR at 12 months (p = 0.026), 24 months (p = 0.017), and last control (p = 0.011), with high expression of TGF-β1 (p = 0.032) and IL-1β (p = 0.045) at 4 months and with baseline CD68-positive cells (p = 0.004). Banff score evidenced acute rejection in samples from 2 LDs and 9 DDs.

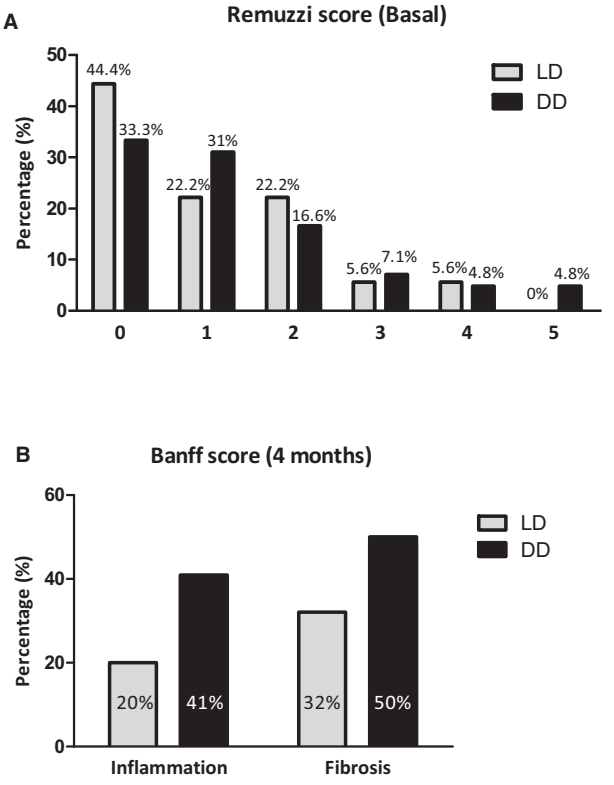


Figure 2: Histological results of baseline and 4-month biopsy specimens. Remuzzi score was applied to baseline samples to evaluate grafts condition. Results were divided according different Remuzzi scores and differentiated between LDs and DDs (A). Banff score of 4-month biopsy specimens showed a greater inflammation and fibrosis in DD than LD (B). DD, deceased donor; LD, living donor.

Inflammation and monocyte/macrophage infiltration in baseline and 4-month kidney biopsy specimens from DDs and LDs

Gene expression studies disclosed differences between DD and LD kidneys. In baseline DD kidneys, there was higher expression of proinflammatory markers, including IL-1β (p = 0.0106), MCP-1 (p = 0.003), intercellular adhesion molecule (ICAM)-1 (p < 0.0001), and TNF-α (p = 0.0308), than in LD samples. The profibrotic cytokine TGF-β1 was also more expressed in baseline DD than in LD samples (p = 0.0005). Some inflammatory markers decreased at 4 months after transplantation in DD samples (IL-1β; p = 0.0326, ICAM-1; p < 0.0001). On the other hand, TGF-β1 and TNF-α expression remained elevated in DD and started to increase in LD samples (Figure 3).

Moreover, baseline DD kidneys had higher expression of macrophage genes such as CD163 (p < 0.0001), CD209 (p < 0.0001), CD14 (p = 0.0011), CD16 (p < 0.0001), and IL4-R (p = 0.0046) than kidneys from LDs (Figure 3). Expression of the M1 macrophage marker CD86 was similarly higher in kidneys from DDs (p = 0.0454). Furthermore, macrophage infiltration in baseline biopsy specimens measured by CD68 staining was higher in kidneys from DDs than in those from LDs (p = 0.0002). At 4 months post-transplantation, CD68-positive cells remained elevated in DD recipients and had increased in LD recipients (Figure 4A), but despite the latter increase the differences between the groups remained significant.

Expression of fibrotic and M2 macrophage markers in kidney biopsy specimens in baseline and 4 months after transplantation

Figure 3 shows gene expression levels at baseline and 4 months in LD and DD samples. Expression of the M2 macrophage marker CD206 had increased at 4 months in kidneys from both DDs (p < 0.0001) and LDs (p = 0.0046). Expression in samples from DD kidneys was higher than that in LD samples (p = 0.0337). CD163 and CD209 gene expression remained high, but while we observed an increase in these markers in LD samples (p = 0.0017 and p = 0.0100, respectively), DD levels were not reached. Fibrotic markers such as vimentin (p < 0.0001), fibronectin (p < 0.0001), and α-SMA

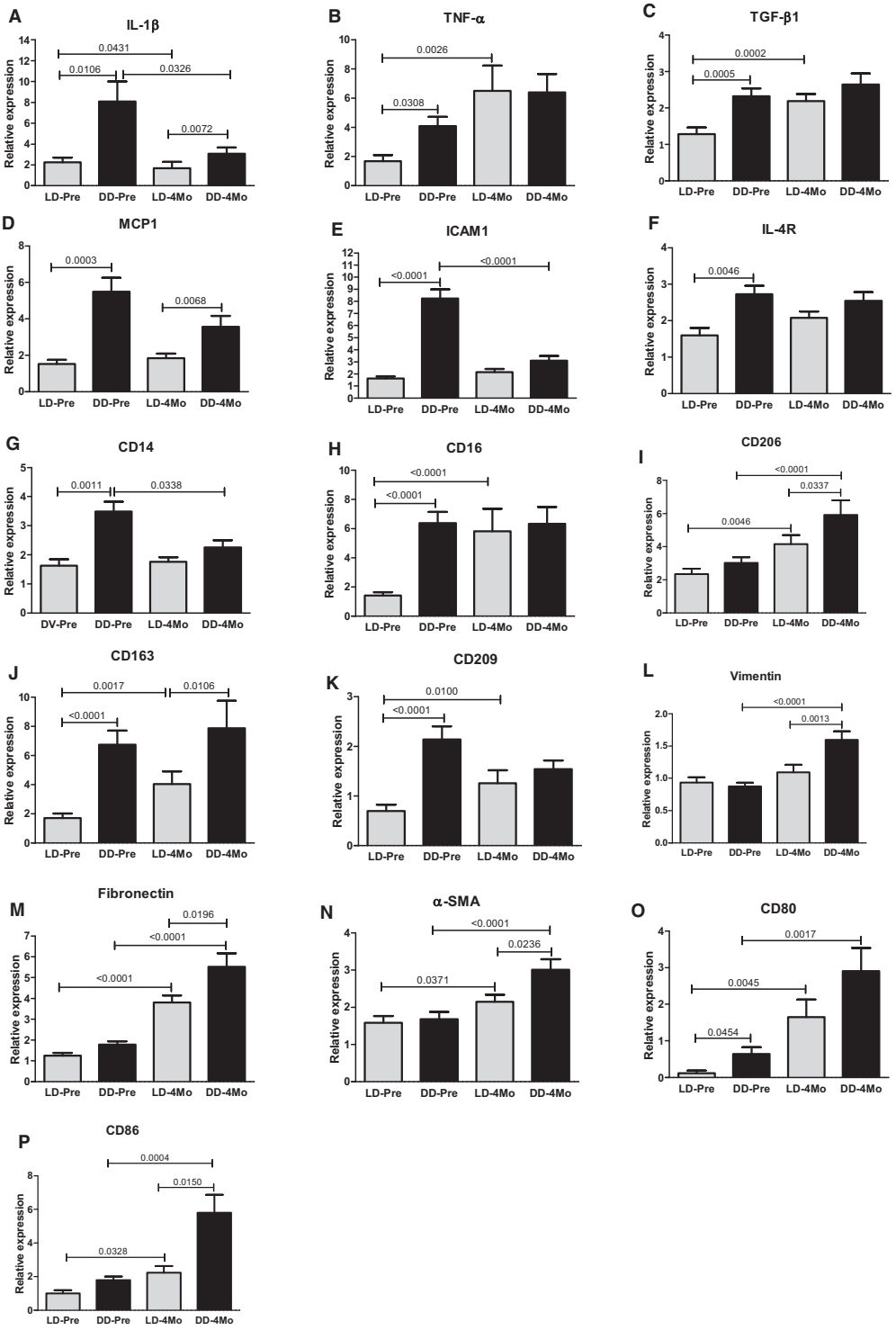
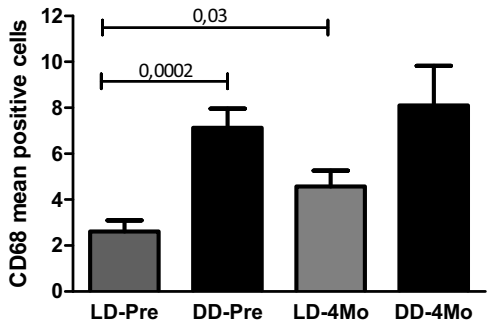


Figure 3: Real-time quantitative PCR analysis of mRNA in kidney biopsy specimens from living and deceased donors (LD and DD) before transplantation and after 4 months. Relative expression data in respect of IL-1β (A), TNF-α (B), TGF-β1 (C), MCP-1 (D), ICAM-1 (E), IL-4R (F), CD14 (G), CD16 (H), CD206 (I), CD163 (J), CD209 (K), vimentin (L), fibronectin (M), α-SMA (N), CD80 (O), and CD86 (P) were normalized using the mean expression value of three endogenous controls (GUSB, GAPDH, and β-tubulin). Mann–Whitney U test was used to perform comparative analysis between different groups and times. Pre, baseline, before transplantation; 4 mo, 4 months after transplantation. α-SMA, α–smooth muscle actin; ICAM, intercellular adhesion molecule; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TGF, transforming growth factor; TNF, tumor necrosis factor.

A



B

CD 68	MDRD (ml/min/1,73m^2)			
	24M		Last follow-up (±5 years)	
	p value	rho Spearman	p value	rho Spearman
BASELINE DD	**0.01**	**-0,663**	**0,005**	**-0,551**
BASELINE LD	0.136	-0.714	0.139	-0.643
4 MONTHS DD	0.149	0.424	0.122	0.433
4 MONTHS LD	0.318	-0.377	0.488	-0.267
Δ4M-Baseline DD	0.906	-0.071	0.582	-0.238
Δ4M-Baseline LD	**0.047**	**-0.638**	0.112	-0.483

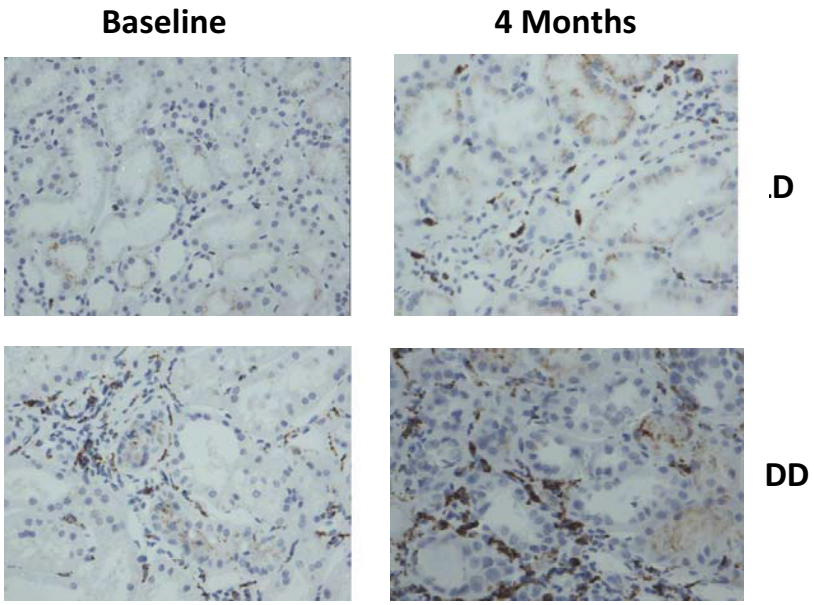


Figure 4: CD68-positive cells in kidney grafts from living donors (LD) and deceased donors (DD) before transplantation (Pre) and after 4 months (4 mo). (A) Inflammatory infiltration is higher in DD than in LD kidneys at both time points, although there is a significant increase in CD68-positive cells in LD kidneys at 4 months. (B) Spearman's ρ correlation analysis of the relationships between the number of CD68-positive cells at baseline and 4 months, and changes in the number during this period, and medium and long term. (C–F) Representative images of CD68 staining: (C) LD kidney at baseline; (D) LD kidney after 4 months; (E) DD kidney at baseline; (F) DD kidney after 4 months.

($p < 0.0001$) increased more in DD than in LD samples (Figure 3).

Correlation of macrophage infiltration and gene expression with medium- and long-term renal function

Correlation of macrophage infiltration with renal function: Baseline macrophage infiltration in DD kidneys correlated inversely with medium- and long-term eGFR (24 months: $\rho = -0.663$, $p = 0.001$; last control [mean 5.8 ± 1.0 years]: $\rho = -0.551$, $p = 0.005$), while the number of CD68-positive cells in LD samples at baseline and at 4 months failed to correlate with MDRD (Figure 4B). A correlation was seen between medium-term eGFR at 24 months and the increase in CD68-positive cells between baseline and 4 months in LD kidney samples (Figure 4B).

Correlations between gene expression and medium- and long-term renal function differed between DDs and LDs: Baseline expression of none of the assessed genes did correlate with eGFR at any time point in the DDs (data not shown). In contrast, expression of several genes at 4 months after transplantation in DD recipients exhibited a statistically significant negative correlation with both medium- and long-term renal function (Table 2A). In general, in DD samples, the correlation was stronger with long-term eGFR, and this was especially noticeable because fibrosis-related genes (TGF-β1, fibronectin, vimentin, and α–smooth muscle actin) correlated better with long-term than 4-month eGFR. TGF-β1 gene expression and the combination of fibronectin, CD206, and IL-1β ($r = 0.822$, $p = 0.001$) appeared to be potential variables to predict last control eGFR. By contrast, only TGF-β1, MCP-1, and CD163 correlated with 24-month eGFR among LDs, and no gene correlated with long-term eGFR (Table 2B). In addition, an increase in the expression from baseline to 4 months also correlated with medium- and long-term eGFR for most of these genes in DDs (Table 3A). In LDs, only the difference between 4 months and baseline in vimentin, CD209, and CD86 correlated with long-term eGFR (Table 3B).

Clinical variables correlation with long-term renal function in DDs

Initially, a univariate regression linear model with DD results at 4 months after transplantation indicates that donor age ($r = -0.536$, $p < 0.0001$), recipient age ($r = -0.348$, $p = 0.013$), and ischemia time ($r = -0.305$, $p = 0.035$) are also variables that predict last control eGFR. However, the multivariate analysis, including clinical, histological, and gene expression variables, finally showed that the best model that explains eGFR in DD recipients is the one that only uses TGF-β ($r = 0.795$, $p < 0.0001$).

Table 2: Correlations between 4-month gene expression and medium- and long-term renal function

	MDRD (mL/min/1.73 m^2)			
	24 months		Last follow-up (±5 years)	
	p value	ρ Spearman	p value	ρ Spearman
(A) 4-month DD				
α-SMA	0.0426	−0.4171	0.0070	−0.5358
TGF-β	0.0026	−0.5860	0.0000	−0.8287
Fibronectin	0.1182	−0.3275	0.0007	−0.6437
CD16	0.0181	−0.4780	<0.0001	−0.7404
CD206	0.0035	−0.5725	0.0012	−0.6204
MCP-1	0.0212	−0.4677	0.0011	−0.6239
CD163	0.0231	−0.4619	0.0010	−0.6281
Vimentin	0.1086	−0.3359	0.0233	−0.4613
TNF-α	0.0209	−0.4686	0.0202	−0.4709
IL-1β	0.0187	−0.4760	0.0003	−0.6739
CD14	0.0513	−0.4023	0.0003	−0.6790
ICAM-1	0.0473	−0.4089	0.0007	−0.6429
IL-4R	0.0214	−0.4672	<0.0001	−0.7112
CD209	0.0323	−0.4380	0.0471	−0.4091
CD80	0.2065	−0.2674	0.0421	−0.4179
CD86	0.0357	−0.4306	0.0007	−0.6442
(B) 4-month LD				
α-SMA	0.6289	−0.1064	0.1132	0.3394
TGF-β	0.0079	−0.5395	0.9643	−0.0099
Fibronectin	0.0537	−0.4073	0.9002	0.0277
CD16	0.0506	−0.4123	0.7095	0.0821
CD206	0.2823	−0.2341	0.5958	−0.1167
MCP1	0.0111	−0.5192	0.5556	−0.1296
CD163	0.0264	−0.4621	0.2747	−0.2378
Vimentin	0.4146	−0.1787	0.5902	−0.1185
TNF-α	0.6778	−0.0915	0.3286	0.2132
IL-1β	0.1427	−0.3154	0.6102	−0.1122
CD14	0.1062	−0.3456	0.8488	−0.0421
ICAM1	0.0708	−0.3836	0.2853	0.2327
IL-4R	0.3159	−0.2188	0.8683	0.0366
CD209	0.8912	−0.0302	0.5676	−0.1257
CD80	0.2655	0.2422	0.4783	0.1556
CD86	0.1699	−0.2963	0.7825	−0.0609

α-SMA, α–smooth muscle actin; DD, deceased donor; ICAM, intercellular adhesion molecule; IL, interleukin; LD, living donor; MCP-1, monocyte chemoattractant protein-1; TGF, transforming growth factor; TNF, tumor necrosis factor.

Discussion

Our group showed that circulating monocytes could be useful as a prognostic tool with respect to graft dysfunction in living kidney recipients (24). In this work, we focus on gene expression of inflammatory and fibrotic markers and macrophage infiltration in kidneys from DDs and LDs at the time of transplantation and after 4 months. It is known that inflammation has a pathogenic role in acute kidney damage after ischemic injury (25), and there is evidence suggesting that macrophage infiltration in kidney tissue is involved in graft outcome and interstitial fibrosis and tubular atrophy (26,27).

Table 3: Correlations of difference between 4-month and baseline gene expression with medium- and long-term renal function

	MDRD (mL/min/1.73 m^2)			
	24 months		Last follow-up (±5 years)	
	p value	ρ Spearman	p value	ρ Spearman
(A) Δ4 months–baseline DD				
α-SMA	0.0958	−0.4814	0.1618	−0.4121
TGF-β	0.0210	−0.6300	0.0490	−0.5549
Fibronectin	0.0258	−0.6135	<0.0001	−0.9231
CD16	0.0398	−0.5750	0.0037	−0.7418
CD206	0.0500	−0.5530	0.1497	−0.4231
MCP-1	0.0003	−0.8473	0.0024	−0.7637
CD163	0.0233	−0.6217	0.1744	−0.4011
Vimentin	0.0196	−0.6355	0.0367	−0.5824
TNF-α	0.0241	−0.6190	0.0346	−0.5879
IL-1β	0.0102	−0.6823	0.0007	−0.8132
CD14	0.1323	−0.4402	0.2309	−0.3571
ICAM-1	0.0026	−0.7593	0.0673	−0.5220
IL-4R	0.0034	−0.7455	0.0112	−0.6758
CD209	0.0315	−0.6200	0.3541	−0.2937
CD80	0.1972	−0.3824	0.0252	−0.6154
CD86	0.8373	−0.0633	0.3344	−0.2912
(B) Δ4 months–baseline LD				
α-SMA	0.9661	0.0167	0.9661	−0.0167
TGF-β	0.7650	−0.1167	0.8647	0.0667
Fibronectin	0.4600	−0.2833	1.0000	0.0000
CD16	1.0000	0.0000	0.8984	−0.0500
CD206	0.9661	−0.0167	0.7650	−0.1167
MCP1	0.6368	−0.1833	0.7650	0.1276
CD163	1.0000	0.0000	0.6059	−0.2000
Vimentin	0.4064	−0.3167	0.0424	−0.6833
TNF-α	0.7980	−0.1000	0.4064	−0.3167
IL-1β	0.4175	−0.3096	0.2950	−0.3933
CD14	0.8984	−0.0500	0.4328	0.3000
ICAM1	0.3558	−0.3500	0.3807	−0.3333
IL-4R	0.9661	−0.0167	0.7980	0.1000
CD209	0.2861	0.4000	0.0298	0.7167
CD80	0.5996	0.2034	0.7946	0.1017
CD86	0.0671	0.6333	0.0418	0.6830

α-SMA, α–smooth muscle actin; DD, deceased donor; ICAM, intercellular adhesion molecule; IL, interleukin; LD, living donor; MCP-1, monocyte chemoattractant protein-1; TGF, transforming growth factor; TNF, tumor necrosis factor.

Moreover, it was suggested that presence of M2 macrophages in 1-year renal allograft biopsy specimens could be associated with inflammation, tubular injury, and progression of fibrosis (27). On the other hand, tubular injury in kidneys from DDs is followed by greater systemic inflammatory activity in the postoperative period (28).

Our results with human kidney biopsy specimens show that inflammation inducers and monocyte recruitment molecules such as TGF-β, TNF-α, IL-1β, ICAM-1, and MCP-1 exhibit higher expression in kidneys from DDs than in kidneys from LDs at the time of transplantation. This indicates that at donation, kidney grafts from DDs show more inflammation than those from LDs, a finding in agreement with a previous observation that the expression of inflammatory factors in the kidney at the time of donation is increased in brain-dead donors (29,30).

Gene expression of CD14 and the number of CD68-positive cells were also more elevated in DD than in LD samples at this time, which is indicative of greater macrophage infiltration in DD kidneys. These results are consistent with published findings from a rodent model of ischemia in which proinflammatory and chemokine gene expression induced by IRI were elevated and sustained over time and accompanied by an increase in kidney macrophage infiltration (31). A nice microarray analysis with kidney transplant biopsy specimens showed that impaired renal function correlated with the expression of tissue injury genes but not with inflammation (32), whereas another study suggested that subclinical inflammation together to progressive interstitial fibrosis, analyzed in kidney allografts, can lead to graft failure (33). Our results in renal recipients suggest that persistent inflammation plays an important role in the graft function of DD renal transplanted patients.

In renal transplantation, early inflammatory events are considered essential in the recruitment and differentiation of myofibroblasts. Macrophages are a central component of the early inflammatory response, and macrophage infiltration in glomerular capillaries in both acute and chronic rejection has been associated with antibody-mediated rejection and subsequent graft failure (34,35). Similarly, early macrophage infiltration in renal allograft biopsy specimens is associated with subsequent interstitial fibrosis (27). We found that markers of M2 macrophages, CD163 and CD209, and the M1 marker CD80 are also expressed more in DD than in LD kidneys, which corroborates the coexistence of M1 and M2 macrophages in the kidney immediately before transplantation. The type of donation, therefore, entails infiltration of macrophages with distinct phenotypes, which impact differently on inflammation and kidney repair. Lee et al revealed the coexistence of inflammatory and reparative responses in renal macrophage infiltrate (12). We have shown that inflammation, monocyte recruitment and coexistence of M1 and M2 macrophages occur in the kidneys of DDs immediately before transplantation and that these events are not detected in kidneys from LDs. Several groups have shown that macrophage depletion decreases kidney damage (36,37), suggesting that macrophage infiltration augments the inflammatory response and promotes tubular injury. Macrophages may accumulate and could be actively recruited into the rejection organ by the chemokine MCP-1, a potent chemoattractant. Indeed, studies have found that blockade of MCP-1 leads to prolonged islet allograft survival (38) and limited interstitial inflammation and renal dysfunction in polycystic kidney disease in rats (39). In the present study, expression of high levels of MCP-1 at 4 months and the difference in expression between 4 months and baseline

correlated inversely with medium- and long-term MDRD, indicating that an increase in MCP-1 worsens renal function. In fact, MCP-1 seems to be involved not only in monocyte recruitment but also in the initiation and progression of tubulointerstitial damage (40). Moreover, we found that the elevated numbers of CD68-positive cells at baseline in DD kidneys correlated inversely with medium- and long-term renal function. This is indicative of the impact of kidney inflammatory status before transplantation on graft outcome. On the other hand, our results in LD kidneys reveal that the increase in CD68-positive cells that occurs after transplantation is highly probably associated with post-transplant events and only affects medium-term renal function. Because protein and mRNA represent different steps of the cellular genetic information flow process, mRNA expression does not always agree with the protein, although in our study these discrepancies may also be explained because qPCR is quantitative and immunohistochemistry is not. Once in the allograft, macrophage proliferation may occur and contribute to the accumulation of these cells. We observed that expression of CD14 decreases in DD biopsy specimens at 4 months after transplantation, whereas CD206 expression increases and CD163 remains elevated. These results are consistent with those obtained by other groups, suggesting that it is the effector phenotype of the recruited macrophages, rather than their presence alone, that determines the extent of renal injury (41,42). Taken together, these findings indicate that macrophage infiltration in kidneys immediately before transplantation plays an essential role in graft outcome, whereas after 4 months it is the macrophage phenotype that impacts renal function. In fact, at 4 months, we observed increases in epithelial mesenchymal transition and fibrotic markers such as vimentin, fibronectin, and α–smooth muscle actin, which are indicative of the onset of fibrosis. It is known that kidney fibrosis is almost always preceded by chronic interstitial inflammation (43,44). Repair is a normal process that is activated in response to injury. When this process is deregulated, because of prolonged injury, it may result in an excess deposition of extracellular matrix with progressive fibrosis and loss of function (45). Moreover, the increase in fibrotic gene expression coincides with a decline in most of the proinflammatory genes studied (an exception is TNF-α) and an increase in M1 and M2 macrophage markers. High levels of TNF-α were found in DD and LD kidney biopsy specimens at 4 months, but expression at baseline was elevated more in DDs than in LDs. Despite this similarity at 4 months, only the TNF-α expression level at 4 months in DD kidneys and the difference between baseline and 4 months in DDs correlated with medium- and long-term renal function. M1 macrophages, via TNF-α, can perpetuate inflammation, leading to impaired wound healing (46). This suggests that is the maintenance of inflammation that is involved in graft outcome. Expression of TGF-β1, a cytokine produced by macrophages that has anti-inflammatory as well as profibrotic functions (47), shows similar results to those for TNF-α, which supports the idea of the coexistence of M1 and M2 macrophage populations. In fact, our results confirmed previous observations that TGF-β1 is important in graft outcome, and this is explained by the macrophage phenotype involved in TGF-β1 production. Moreover, our results suggested that in DD early macrophage infiltration is associated with 4-month Banff inflammation, which in turn is related to those 4-month samples with high expression of TGF-β1 and Il-1β.

In this study, baseline DD kidney samples showed a high level of inflammation, macrophage recruitment and infiltration, and expression of adhesion molecules that can lead to chronic injury. Injury can activate the innate immune system, inducing inflammatory cell recruitment and an increase in chemoattractants that enhance inflammatory responses. On the other hand, anti-inflammatory macrophages are involved in the resolution of inflammation. At 4 months after transplantation, DD samples showed an increase in M2 macrophage and fibrotic markers, implying a repair mechanism that can lead to fibrosis, whereas inflammation appeared to decrease or remain at the same level in the renal graft.

All these findings should be validated in an independent validation cohort.

In summary, the results of this study suggest that macrophages play an important role in mediating persistent inflammation and fibrosis through TGF-β1 production in DD kidney recipients, leading to development of chronic kidney disease. The initial inflammatory status of the graft from DDs is essential and indicative of renal function outcome. Control of donor inflammation therefore offers great therapeutic potential and would assist in inhibiting progressive kidney fibrosis. We conclude that strategies targeting macrophage infiltration or activation can be useful in the prevention of development of chronic kidney disease in recipients of transplants from deceased donors.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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