ORIGINAL ARTICLE

T helper 1, 2 and 17 cell subsets in renal transplant patients with delayed graft function

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Summary

Ischemia-reperfusion injury (IRI) in kidney transplantation is the major cause of delayed graft function (DGF), an event associated with an increased risk of acute rejection. The aim of this study was to evaluate T helper (Th) cell phenotype in renal transplants with DGF. T-bet (Th1), GATA-3 (Th2) and IL-17 (Th17) protein expression was investigated in pretransplant biopsies, DGF and acute tubular damage (ATD) caused by calcineurin-inhibitor toxicity. Intracytofluorimetric analysis of IFN-γ, IL-4 and IL-17 was performed to analyze Th1, Th2 and Th17 responses in peripheral blood mononuclear cells of recipients with early graft function (EGF) and DGF, before (T0) and 24 h after transplantation (T24). In pretransplant biopsies, T-bet$^+$, GATA-3$^+$ and IL-17$^+$ cells were barely detectable. In DGF, T-bet$^+$ and IL-17$^+$ cells were significantly increased compared with pretransplant and ATD. More than 90% of T-bet$^+$ and less then 5% of IL-17$^+$ cells were CD4$^+$. GATA-3$^+$ cells were increased to a lower extent. T-bet$^+$/GATA-3$^+$ cell ratio was significantly higher in DGF. Peripheral CD4$^+$ IFN-γ/IL-4 ratio was significantly decreased in DGF, while CD4$^+$/IL-17$^+$ cells did not differ between T0 and T24 in DGF. Our data suggest that DGF is characterized by a prevalent Th1 phenotype within the graft. This event might represent a link between DGF and acute rejection.

Introduction

Delayed graft function (DGF) is a form of acute renal failure in the immediate post-transplant period. It is associated with an increased incidence of acute rejection and with poor long-term graft outcome [1]. The reported frequency of DGF in deceased donor kidney transplants varies greatly, from 2% to 50%. It is strictly dependent upon the features of the graft and the length of cold ischemia time [1–3]. Prolonged hypothermic ischemia and subsequent reperfusion may activate a complex sequence of events that promote renal injury and cause DGF [4,5]. Ischemia-reperfusion injury (IRI) is characterized by a significant activation of innate immunity [3–9].

In the last decade also T and B cells have been identified as important mediators of renal IRI [10–13]. Several studies in experimental models of IRI in the kidney, as well as in other organs, support a pathogenic role of T cells in this setting [14–16]. Mice lacking T cells are functionally and structurally protected from kidney IRI. CD4$^+$ T cells play the major role in this scenario, as CD4-deficient mice, but not CD8-lacking mice, had significantly improved recovery of renal function after IRI compared with wild-type control mice [17]. Naïve CD4$^+$ helper T (Th) cells, upon activation by antigen-presenting cells, develop into different Th cell subsets, Th1, Th2 and Th17, with different cytokine profiles and distinct effector functions [18].

The differentiation of Th1 cells is promoted by the IFN-γ-induced activation of STAT-1 and T-bet, the Th1 specific transcription factor. T-bet promotes Th1 lineage commitment [19] and forms an auto-regulatory positive feedback loop with IFN-γ. By contrast, the differentiation of Th2 cells is induced by IL-4 and governed by the Th2 specific transcription factor GATA-3. Engagement of the IL-4 receptor leads to the phosphorylation of STAT-6 which binds to the IL-4 promoter and further induces IL-4 production, thus establishing a positive feedback loop to increase Th2 differentiation [20]. Several studies indicate that a prevalence of Th1 over Th2 characterizes acute renal allograft rejection [21,22].

Recently, a new CD4^{+} effector T-cell subset that produces IL-17 (Th17) has emerged. These cells express a transcription factor different from those of Th1 and Th2 cells, the retinoic acid-related orphan receptor γt (ROR γt) [23]. The combination of the acute phase protein IL-6 together with TGF-β induces the differentiation of Th17 cells from naïve T cells. Once induced, Th17 cells produce large quantities of IL-17. IL-17 is produced by a variety of cell types including subsets of CD4^{+} T cells, CD8^{+} T cells, γδ-T cells, NK cells and neutrophils [24]. Nevertheless, the predominant source of IL-17 remains the CD4^{+} T-cell population [19]. In the absence of IFN-γ and IL-4, also IL-23 may induce naive precursors to differentiate into Th17 cells independently of the transcription factors STAT-1, T-bet, STAT-4 and STAT-6 [25]. A growing body of evidence suggests a close association between Th17 cells and acute allograft rejection [25–29]. Indeed, in the absence of Th1-mediated alloimmune responses, CD4 Th17 cells mediate an aggressive pro-inflammatory response culminating in severe accelerated allograft rejection and vasculopathy [29].

The influence of ischemia-reperfusion on the Th subsets within the graft and at the peripheral blood level in kidney transplantation is still poorly defined. Thus, the aim of this study was to characterize the phenotype of graft-infiltrating immune cells and to evaluate the role of Th cell phenotype during IRI at tissue and peripheral level in graft recipients with DGF.

Patients and methods

Patients

Seventy-two primary kidney transplant recipients from deceased donors were included in this observational study. Twenty of them were randomly selected from 557 patients with early graft function (EGF) and 40 from 183 patients with DGF. The presence of DGF was defined as the need for dialysis in the first week after transplantation. All DGF patients underwent a graft biopsy between day 7–10 post-transplant (mean 8.7 ± 1.8 days) according to our standard clinical practice. For ethical reasons, we do not routinely perform a time-matched graft biopsy in transplant recipients with EGF. Therefore, twelve graft biopsies with acute tubular damage (ATD) caused by calcineurin inhibitor (CNI) toxicity performed within 1 month from transplantation (mean 17.1 ± 3.2 days) were used as post-transplant control group. Twenty cadaveric donor kidneys were used as pretransplant control group, of these, 10 presented DGF after transplantation and 10 ATD. All patients with pretransplant panel reactive antibodies (PRA) >0% (Luminex; OneLambda, Canoga Park, CA, USA) [30] or biopsy-proven acute rejection, according to Banff 2007 criteria [31], were excluded from the study.

All patients enrolled received 500 mg of methylprednisolone intra-operatively, 250 mg of prednisone daily, with the dose tapered to 25 mg by day 8; 20 mg of a chimeric monoclonal anti-CD25 antibody (Simulect; Novartis, Basel, Switzerland) intravenously on day 0 and day 4; mycophenolate mofetil (Cell-Cept; Roche, Basel, Switzerland) 1000 mg b.i.d and either cyclosporine A (Neoral; Novartis, C2 levels 800–1200 ng/ml; $n = 25$ in DGF, $n = 13$ in EGF and $n = 4$ in ATD groups) or tacrolimus (Prograf; Astellas, Staines, UK, through levels 8–12 ng/ml; $n = 15$ in DGF, $n = 7$ in EGF and $n = 8$ in ATD groups) starting 24 h after reperfusion. Thus, at T24 both EGF and DGF patients were on the same immunosuppressive regimen.

All pretransplant biopsies were evaluated by two independent pathologists. None of these biopsies presented tubular necrosis. A blood sample, for peripheral blood mononuclear cells (PBMC) isolation, was collected both before and 24 h after transplantation.

In DGF patients, the graft biopsy revealed a diffuse acute tubular necrosis with no evidence of acute rejection according to Banff 2007 criteria [31]. ATD biopsies were characterized by mild-moderate tubular damage and focal tubular necrosis, with histological signs of acute CNI nephrotoxicity and without evidence of acute rejection. At the same time no patient in both groups presented an increase of PRA (Luminex). The mean recovery time from DGF after the graft biopsy was 18.1 ± 8.2 days.

The study was carried out according to the Declaration of Helsinki and was approved by our Ethics Committee. Every patient signed an informed consent form agreeing to participate in the study.

Immunohistochemistry

CD4, CD8, CD20 and CD68

Paraffin-embedded sections of graft biopsies after specific epitope unmasking were incubated with protein block solution (Dako, Glostrup, Denmark) and with the primary antibodies: anti-CD4 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-CD8, anti-CD20 (Dako

Cytomation, Carpintera, CA, USA) and anti-CD68 (1:100, Santa Cruz Biotechnology). Binding of the secondary biotinylated antibody was detected by the Dako EnVision G/2 System/AP kit (Dako).

T-bet, GATA-3, IL-17

Paraffin-embedded human and swine renal sections after antigen retrieval were incubated with H_2O_2 (3%), Triton (0.05%), anti-T-bet (1:400), anti-GATA-3 (1:300) and anti-IL-17 (1:50) antibodies (Santa Cruz Biotechnologies). The sections were then incubated with the biotinylated secondary antibody, ABC complex streptavidin/horse-radish peroxidase and diaminobenzidine Chromogen Solution.

Immunohistochemistry quantification

The infiltrating cells number was measured in at least 15 high power (×200) fields (hpf) of cortical areas/section (mean 18.3 ± 3.1 hpf/section) by two independent observers. The final counts were the mean of the two measures. In no case the inter-observer variability was higher than 15%.

Tissue immunofluorescence and confocal laser scanning microscopy

Paraffin-embedded human kidney sections from DGF patients were double-stained for CD4 and IL-17. The slides were incubated with the blocking solution (BSA 2%), primary antibodies (anti-CD4 1:50, and anti-IL-17, 1:50) and secondary antibodies (AlexaFlour 555 anti-mouse and 488 goat anti-rabbit; Molecular Probes, Eugene, OR, USA). Specific fluorescence was acquired using the confocal microscope Leica TCS SP2 (Leica, Wetzlar, Germany). The number of IL-17$^+$ and CD4$^+$ cells was measured in at least 10 high power (×630) fields/section by two independent observers blinded to the origin of the slides.

PBMC isolation and RNA extraction

Blood was collected before and 24 h after transplantation from DGF ($n = 6$) and EGF ($n = 6$) patients. PBMCs were isolated by density separation over a Ficoll-Hypaque (Flow Laboratories, Irvine, UK) gradient. Total RNA was isolated using RNeasy mini kit (Qiagen, Milan, Italy), quantified by NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and its quality was assessed by electrophoresis on agarose gel (1%).

Cytometric analysis of intracellular cytokines

Intracytofluorimetric analysis of IFN-γ, IL-4 and IL-17 was performed to analyze Th1, Th2 and Th17 responses. In brief, 10^6 PBMCs were stimulated with 10 ng/ml phorbol-12-myristate-13 acetate (PMA) (Sigma-Aldrich,

St. Louis, MO) plus 1 M ionomycin (Sigma-Aldrich, St. Louis, MO) for 6 h, the last four of which in the presence of 5 g/ml brefeldin A (Sigma-Aldrich). After stimulation, cells were washed twice with PBS pH 7.2, fixed 15 min with formaldehyde (2% in PBS pH 7.2), washed twice with 0.5% BSA in PBS pH 7.2, permeabilized with PBS pH 7.2 containing 0.5% BSA and 0.5% saponin, and then incubated for 15 min at room temperature with FITC-, PE- or PE-Cy-5-conjugated mAbs: anti-CD3, anti-CD4, anti-IFN-γ, anti-IL4 and anti-IL-17. Cells were then washed and analyzed on a "EPICS XL" Flow Cytometer (Beckman Coulter, Brea, CA) and analyzed using WinMDI Version 2.8 software. The area of positivity was determined using an isotype-matched mAb, a total of 10^4 events for each sample were acquired.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical differences between groups were assessed by analysis of variance (ANOVA) or *t*-test. A *P*-value <0.05 was considered statistically significant. Statistical analysis was performed using the STATVIEW Software package (5.0 version; SAS, Cary, NC, USA).

Results

Characterization of infiltrating inflammatory cells in pre/post renal transplant with DGF and acute tubular damage

Table 1 summarizes the main clinical features of the patients included in the study. The first step was to characterize the graft infiltrating cells; we observed very few

Table 1. Main demographic and clinical features of the patients included in the study.

	EGF	DGF	ATD
Patients (*n*)	20	40	12
Recipient age (years)	44.2 ± 9.7	49.1 ± 9.0*	45.5 ± 11.0
Donor age (years)	33.4 ± 13.4	52.0 ± 14.9**	35.4 ± 10.2
Donor cause of death (trauma)	6/20	15/40	4/12
Estimated GFR (ml/min)	86.4 ± 16.3	78.6 ± 19.2	87.1 ± 18.6
Donor hypertension (Y/N)	0/20	21/40***	1/12
HLA mismatches (*n*)	3.4 ± 1.1	3.5 ± 0.9	3.1 ± 1.1
Cold ischemia time (h)	11.2 ± 4.1	17.1 ± 5.2****	10.6 ± 6.1
Length of DGF (days)	0	18.1 ± 13.1	0

EGF, early graft function; DGF, delayed graft function; ATD, acute tubular damage; HLA, human leukocyte antigen; GFR, glomerular filtration rate.
*$P = 0.03$, **$P = 0.001$, ***$P = 0.001$, ****$P = 0.01$ by ANOVA.

infiltrating cells in pretransplant biopsies (Fig. 1a–f). Both DGF and ATD were characterized by an increase of CD4^{+}, CD8^{+} and CD68^{+} cells within the graft tubulointerstitial area (Fig. 1g–l). Quantification of these cells demonstrated a significant increase in the DGF group compared with ATD and pretransplant biopsies (Fig. 1 m–o). Moreover, the number of infiltrating CD20^{+} cells was higher in the DGF group compared with ATD, although the difference did not reach a statistical significance (data not shown).

IL-17, T-bet and GATA-3 protein expression before/after transplantation in renal biopsies patients with DGF and ATD

IL-17^{+} cells were barely detectable in pretransplant biopsies (Fig. 2a and b). We observed a modest increase of IL-17^{+} cell number only in graft biopsies with DGF (Fig. 2c and d). The increase was statistically significant when compared with ATD ($P = 0.02$) and pretransplant biopsies ($P = 0.03$) (Fig. 2e). We, then, characterized the phenotype of IL-17^{+} graft-infiltrating cells within the

tubulointerstitial area of grafts with DGF and only $4.5 \pm 1.2\%$ of IL-17^{+} cells were also CD4^{+} (Fig. 2f and i). In addition, very few IL-17^{+} cells were also CD8^{+} ($2.1 \pm 1.1\%$) (data not shown).

T-bet^{+} cells, absent in pretransplant biopsies (Fig. 3a and b), were significantly increased in DGF graft tissue compared with pretransplant ($P = 0.005$) and ATD biopsies ($P = 0.0001$) (Fig. 3c–e). The majority of the cells expressing T-bet ($92.1 \pm 6.1\%$) were CD4^{+} (Fig. 3f and g).

Finally, we observed an increase of GATA-3^{+} cells in both DGF and ATD compared with pretransplant biopsies (Fig. 4a–d). DGF was characterized by a lower number of GATA-3^{+} cells when compared with ATD, although this difference failed to reach statistical significance (Fig. 4e). As shown in Fig. 4f, T-bet[+]/GATA-3^{+} cell ratio was significantly increased in DGF biopsies compared with ATD ($P = 0.001$) and pretransplant biopsies ($P = 0.0001$).

Circulating Th1, Th2 and Th17-specific T-cell subsets

We, then, evaluated the circulating Th1, Th2 and Th17 subsets before and after transplantation in the two
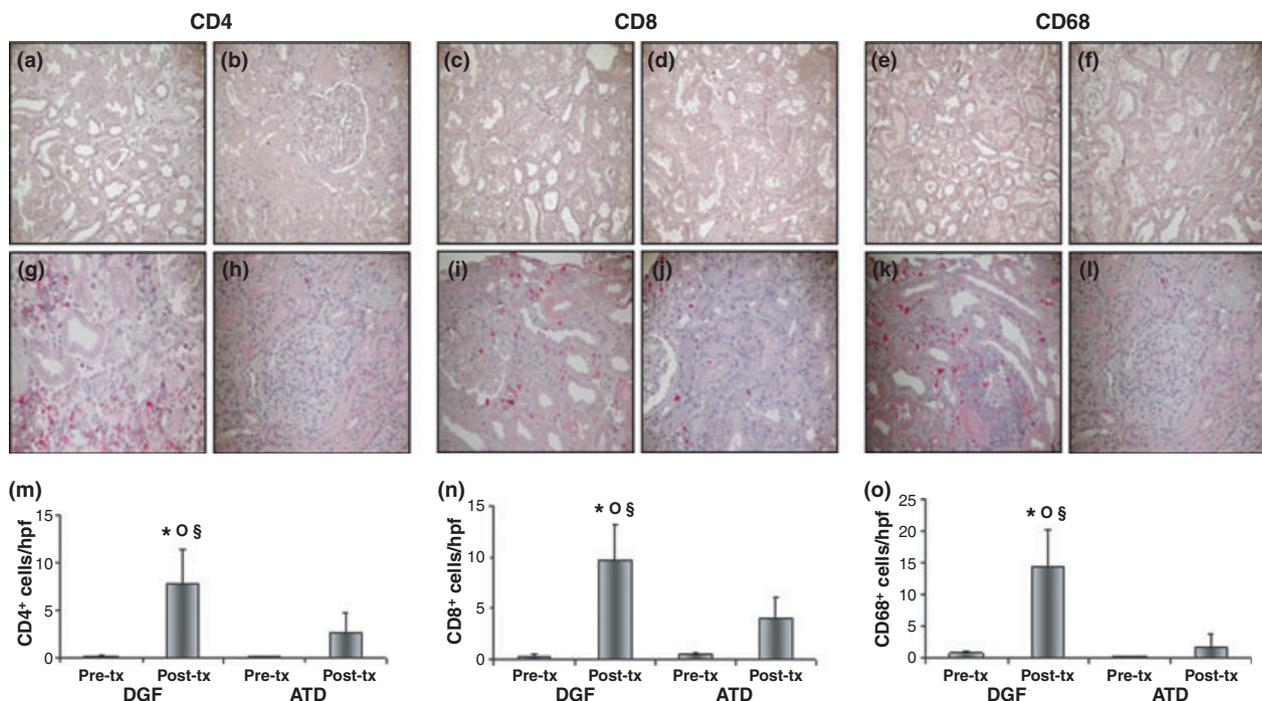
Figure 1 Characterization of infiltrating inflammatory cells in pretransplant renal tissue samples (a–f) and in graft biopsies of patients with delayed graft function (DGF) and post-transplant acute tubular damage (ATD) (g–l). The presence of CD4^{+} (a, b, g, h), CD8^{+} (c, d, i, j) and CD68^{+} (e, f, k, l) cells was investigated in pretransplant biopsies (a–f) ($n = 20$) and in graft biopsies of patients with DGF (g, i and k) ($n = 40$) and ATD (h, j and l) ($n = 12$) by immunohistochemistry. The number of infiltrating CD4^{+} (m), CD8^{+} (n) and CD68^{+} (o) cells was measured as described in Patients and Methods. DGF was characterized by a statistically significant increase of CD4^{+}, CD8^{+} and CD68^{+} cell number. For each group, all images (×200) are from a single patient and are representative of the whole group of patients. Results are expressed as mean ± SD of number of cells/high power field (hpf). (CD4^{+} cells *$P = 0.012$ vs. ATD, °$P = 0.02$ and §$P = 0.02$ vs. pre-Tx); (CD8^{+} cells *$P = 0.015$ vs. ATD, °$P = 0.01$ and §$P = 0.01$ vs. pre-Tx); (CD68^{+} cells *$P = 0.0001$ vs. ATD, °$P = 0.003$ and §$P = 0.003$ vs. pre-Tx).
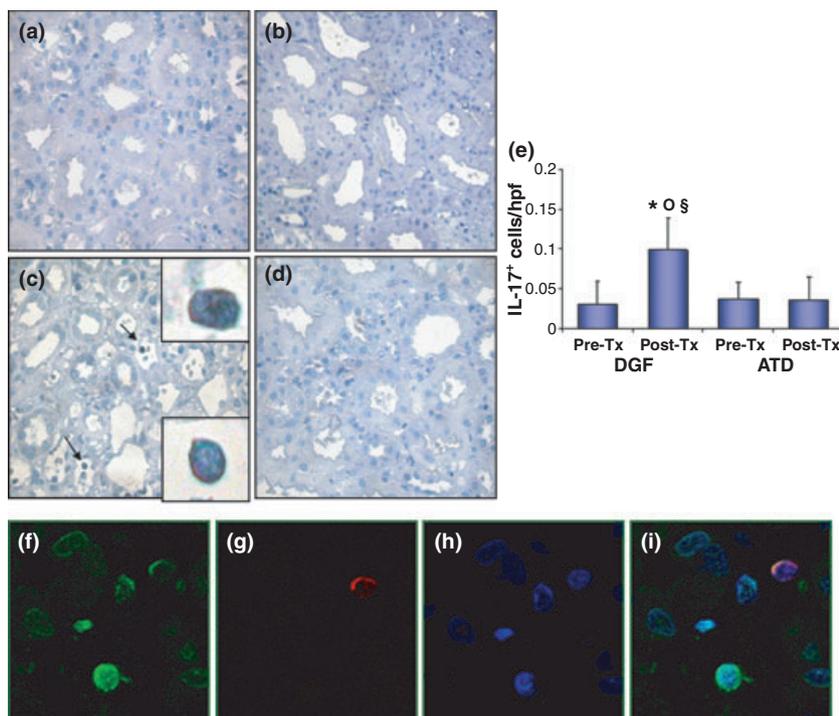
Figure 2 Interleukin-17 expression before (a, b) and after transplantation in patients with delayed graft function (DGF) (c) and acute tubular damage (ATD) (d). Paraffined human kidney section were examined for expression of interleukin-17 by immunohistochemistry in pretransplant cadaveric donor kidney (a, b) ($n = 20$), graft kidney with DGF (c) ($n = 40$) and graft kidney with ATD (d) ($n = 12$). Immunoperoxidase staining was carried out as described in the Patients and Methods section. (e) In the graft recipients with DGF, we detected a significantly increase of Th17+ cells compared with patients with ATD (*$P = 0.02$) and to pretransplant biopsies (°$P = 0.03$ and §$P = 0.03$). Nuclei were counterstained by hematoxylin. Magnifications: (×400). Arrows indicate IL-17+ cells. In the graft kidney with DGF the co-localization of IL-17/CD4 protein was investigated by immunofluorescence/confocal microscopy. Only few (4.5 ± 1.2%) IL-17+ cells (green) (f) were also CD4+ (red) (g); nuclei were stained with TO-PRO-3 (blue) (h); merge (i).

groups of patients. In DGF, but not in EGF, patients after transplantation we observed a significant decrease of circulating IFN-γ-producing CD4+ T cells ($P = 0.002$) (Fig. 5a and b). On the other hand, IL-4+/CD4+ T cells were significantly reduced in EGF, but not in DGF patients ($P = 0.009$) (Fig. 5a and c). Thus, after transplantation, in EGF patients the IFN-γ/IL-4 ratio was significantly higher ($P = 0.004$) than that in DGF patients where it decreased ($P = 0.004$) (Fig. 5d). Finally, circulating IL-17+/CD4+ T cells were significantly reduced in EGF patients after transplantation, while their number did not change in DGF patients ($P = 0.001$) (Fig. 5a and e).

Discussion

We report the first description of the T-cell phenotypes in transplant recipients with DGF and we demonstrate that DGF is associated with an increased T-bet/GATA-3 ratio in graft infiltrating immune cells and a reduction of IFN-γ/IL-4 ratio in circulating CD4+ T cells, suggesting a

significant recruitment of T-bet+ cells into the graft of DGF patients. The modest presence of IL-17-producing T cells confirms the hypothesis that DGF is characterized by a Th1-driven immune response.

Delayed graft function is a common event in the early post-transplant period and has been shown to be an independent risk factor for chronic transplant dysfunction and graft loss. Several studies observed a close association between DGF and acute rejection, suggesting a direct influence of this complication on the modulation of the alloimmune response [1,32,33].

Ischemia-reperfusion-induced renal injury is the main pathogenic mechanism underlying DGF, since the incidence of this event is strictly dependent upon the length of cold ischemia time [1]. IRI is characterized by a dramatic activation of the innate immune system, including the local recruitment of monocytes/macrophages, granulocytes and dendritic cells [1,4,34]. In our DGF patients, the innate immune system involvement was confirmed by the extensive infiltration of CD68+ cells within the tubulointerstitial area of kidney grafts.
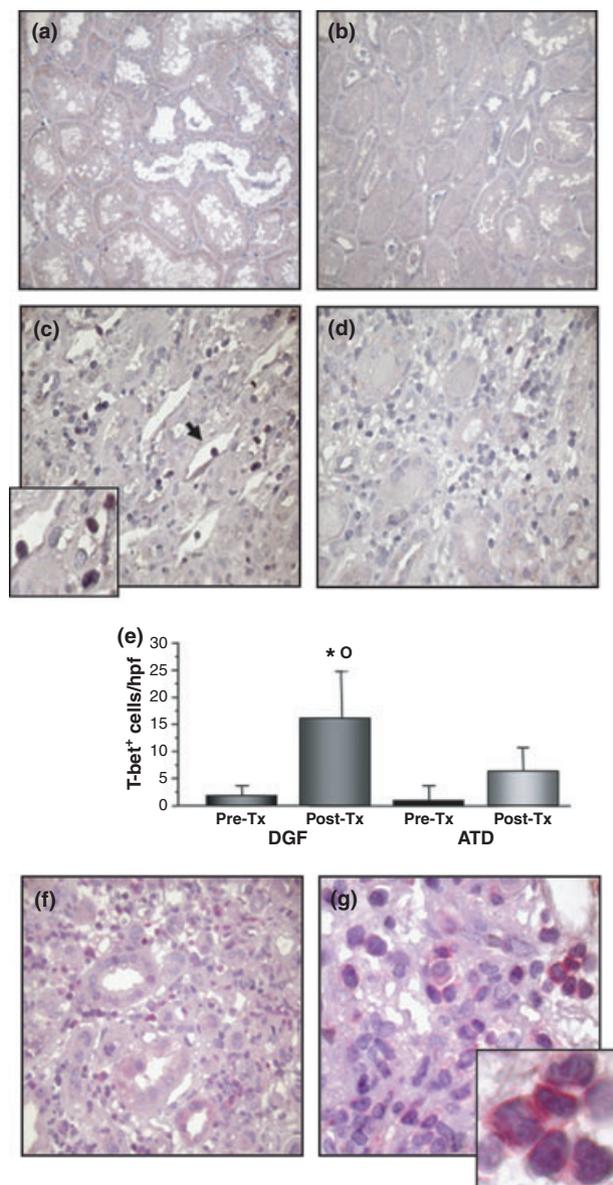
Figure 3 T-bet protein expression before and after transplantation in graft kidney with delayed graft function (DGF) and acute tubular damage (ATD) T-bet (a–e) protein expression was evaluated by immunohistochemistry in pretransplant cadaveric donor kidney (a, b) ($n = 20$), graft kidney with DGF (c) ($n = 40$) and graft kidney with ATD (d) ($n = 12$). The number of T-bet[+] (e) infiltrating cells was measured as described in Methods section and was significantly increased in DGF compared with ATD and to pretransplant kidney (*$P = 0.005$ vs. ATD; °$P = 0.0001$ vs. pre-Tx). The majority of the T-bet^{+} cells were also CD4^{+} (red) (f, g). For each group, all images (×400) are from a single patient and are representative of the whole group of patients. Results are expressed as mean ± SD of number of cells/high power field (hpf). Arrows indicate T-bet^{+} cells.

In the last years, however, an increasing body of evidence has suggested the prominent role of the adaptative branch of the immune system in the pathogenesis of renal

IRI. Indeed, a key role for T-cell has been shown in several experimental models [16,17]. Ascon *et al.*, reported an increased T- and B-cell trafficking in mouse kidney early after ischemia-reperfusion [35]. Although in our study, the renal biopsies were performed 7–10 days after transplantation, we confirmed this observation in our patients, clearly demonstrating an increased influx of T and B lymphocytes.

The functional role of T cells was clarified by Burne *et al.* [11]. They demonstrated that nu/nu mice, a murine strain lacking T cells, were protected from kidney IRI and this effect was completely abolished by reconstitution with wild-type T cells. They reported that CD4^{+}, but not CD8^{+}, T cells play a key role in the pathogenesis of IRI. Indeed, CD4, but not CD8, deficient animals were partially protected from renal damage. In addition, the infusion of wild-type CD4^{+} T cells in this model restored the IRI [11]. Nevertheless, other groups reported a role for CD8^{+} T cells in the pathogenesis of IR-dependent tissue injury [36]. In our DGF population, we observed an equal recruitment of CD4^{+} and CD8^{+} T cells, although we cannot discriminate in the human setting the functional role of these two different T-cell subpopulations. However, our further characterization was mainly focused on the CD4^{+} cell population.

The next question is whether ischemia-reperfusion-induced T-cell activation is characterized by a specific Th1/Th2 bias. This is a key point in the transplantation setting, as the Th1/Th2 bias may represent the link between the IRI featuring DGF and acute allograft rejection. D'Elios *et al.* [37], after functional characterization of infiltrating T cells in graft biopsies with acute rejection, concluded that this condition is defined by a Th1 alloresponse. Yokota *et al.* [38] reported that STAT6 null mice, characterized by a reduction in the Th2 response, presented a significant worsening of IRI, whereas STAT4 null mice, characterized by a reduction in the Th1 response, were partially protected by the consequences of IRI on renal function. Our observation of a higher T bet/GATA-3 ratio in DGF graft biopsies supports the hypothesis of a specific role for Th1 cells in IRI.

This role is closely linked to our previous observation that DGF is characterized by an extensive infiltration of myeloid dendritic cells [8]. Indeed, these cells have been suggested to induce a Th1 immune response [3]. The finding of a Th1 predominance within the renal tubulointerstitium of human grafts with DGF is directly due to ischemia-reperfusion. Our observation that T-bet[+], GATA-3^{+} and IL-17^{+} cells were only barely detectable in pretransplant biopsies plausibly exclude any influence of pretransplant events, including donor's brain death in the modulation of Th cell subsets.
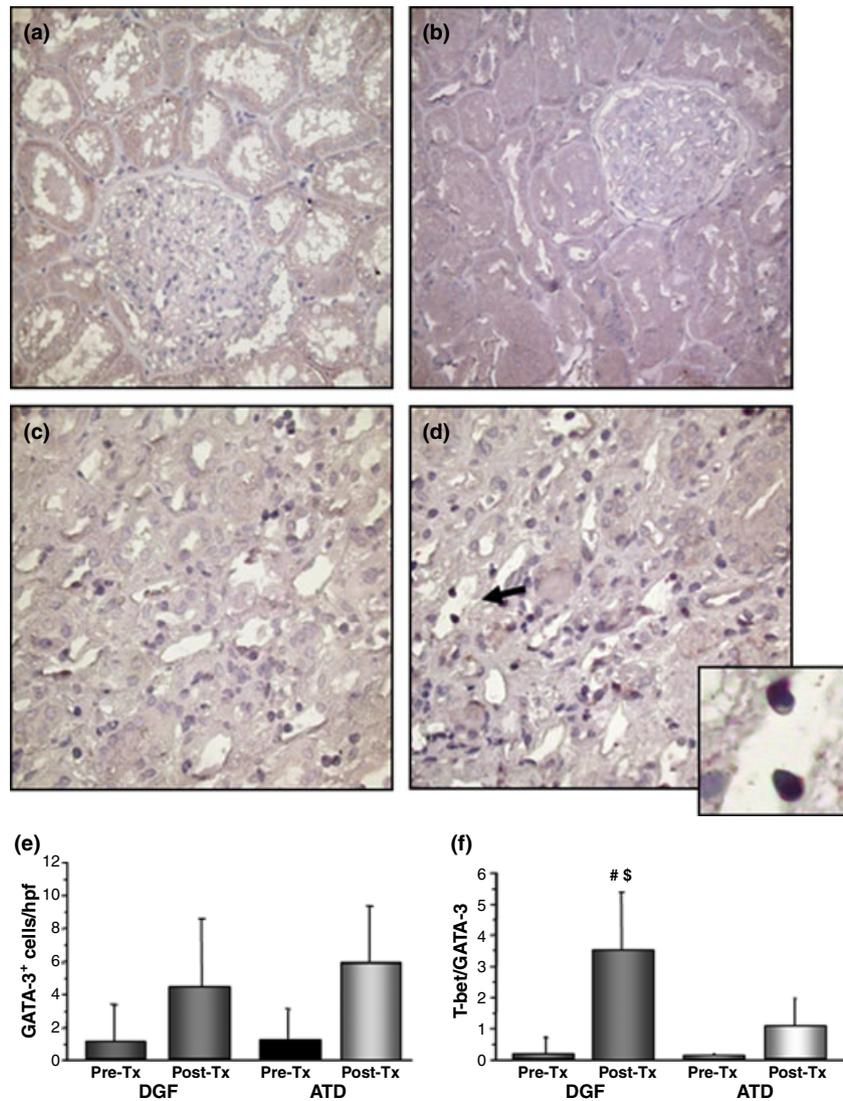
Figure 4 GATA-3 protein expression before and after transplantation in graft kidney with delayed graft function (DGF) and acute tubular damage (ATD). GATA-3 (a–e) protein expression was evaluated by immunohistochemistry in pretransplant cadaveric donor kidney (a, b) (n = 20), graft kidney with DGF (c) (n = 40) and graft kidney with ATD (d) (n = 12). DGF was characterized by a lower number of GATA-3$^+$ cells when compared with ATD, although this difference failed to reach statistical significance (e). (f) Graft T-bet/GATA-3 ratio was significantly increased in DGF compared with ATD and pre-Tx ($^#P$ = 0.001 vs. ATD; $^$P$ = 0.0001 vs. pre-Tx).

The Th1/Th2 paradigm in renal transplantation has been recently challenged by the finding that IL-17 is strongly associated with allograft rejection [39;26–28]. Yuan et al. [40] reported that Th17 cells mediate accelerated vascular rejection in T-bet$^{-/-}$ mice, which exhibit profound Th1 deficiency. Concurrently, these mice exhibit a Th2 switch characterized by increased production of IL-4, IL-5, IL-10 and IL-13, along with an increased production of pro-inflammatory cytokines, including IL-6, IL-12p40 and IL-17 [38]. We decided, then, to investigate the role of Th17 in DGF. We did not observe a significant increase in circulating Th17 cells. In addition, very few IL-17$^+$ graft-infiltrating cells were also CD4$^+$, suggesting that most of IL-17 expression within the graft was due to infiltrating polymorphonuclear cells. It is conceivable that graft infiltrating CD8$^+$ T cells may represent the source of IL-17 [41,42].

Indeed, these cells have been shown to produce IL-17, although in our model very few IL17$^+$ cells were also CD8$^+$.

Although a growing body of evidence is available on the role of T cells in IRI murine models, so far there are no reports either in humans or in larger animals [16,17]. One of the reasons for the lack of human data is represented by the difficulty, for ethical reasons, to obtain graft biopsies within the first few days after transplantation in patients with EGF. We attempted to overcome this issue with two approaches. We included a control group represented by transplant recipients with ATD observed early after transplantation. This control group is extremely interesting, as it gives us the opportunity to define the difference in the pathogenesis of ATD caused by IRI compared with a similar histological picture characterized by a different etiology.
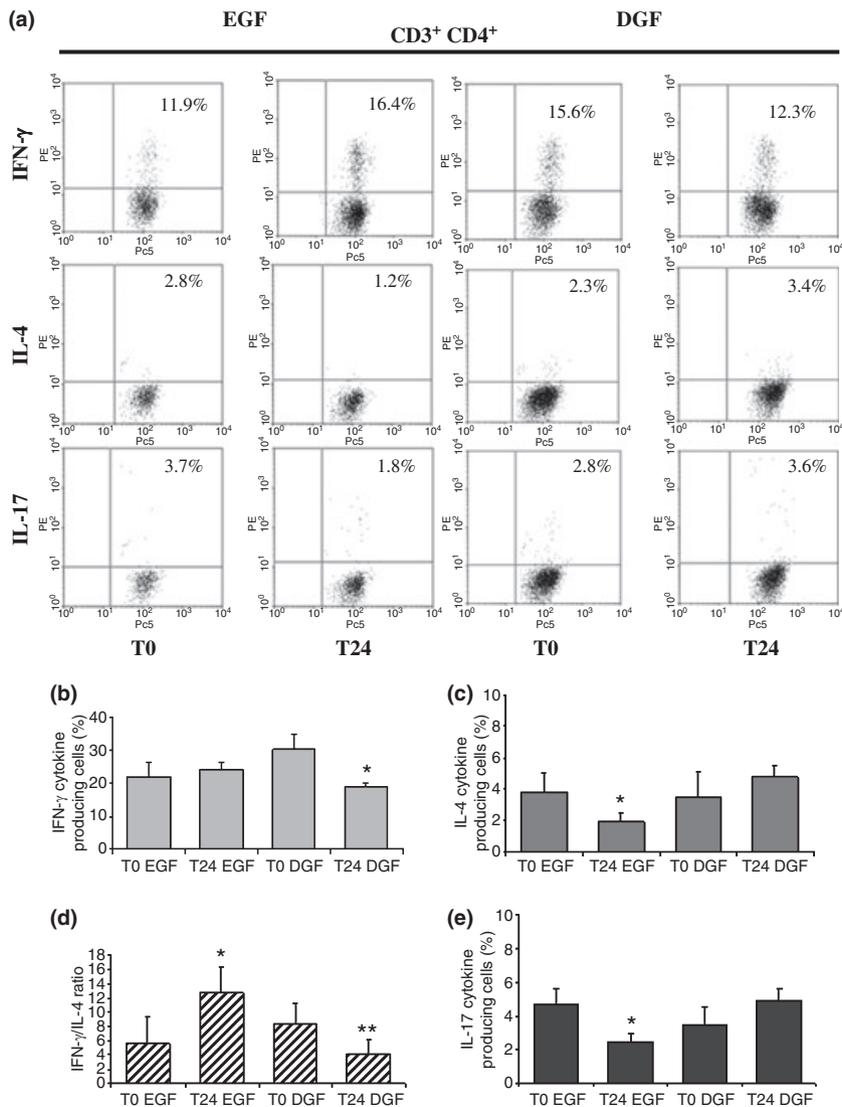
Figure 5 Circulating IFN-γ-, IL-4- and IL-17-producing CD4+ T cells in early graft function (EGF) and delayed graft function (DGF) patients. The frequency of IFN-γ-, IL-4- and IL-17-producing T cells was evaluated using flow cytometry on peripheral blood mononuclear cells isolated from EGF ($n = 6$) and DGF ($n = 6$) patients, before (T0) and 24 h after transplantation (T24), following stimulation for 6 h with PMA plus ionomycin in the presence of brefeldin (a). A representative flow-cytometric analysis of the one experiment is reported and the percentages of gated cytokine-producing cell are shown (b–e). Columns represent mean ± SD of cytokine cell production (Panel b *$P = 0.002$ vs. T24 DGF); (Panel c *$P = 0.009$ vs. T24 EGF); (Panel d *$P = 0.004$ vs. T24 EGF and **$P = 0.004$ vs. T24 DGF); (Panel e *$P = 0.001$ vs. T24 EGF).

The reduction in circulating CD4+/IFN-γ+ cell number in DGF patients, 24 h after kidney transplantation, seems in disagreement with the increased in T-bet+ cells within the graft. However, it is conceivable that the lower number of circulating CD4+/IFN-γ+ cells may reflect the recruitment of T-bet+ cells into the graft of DGF patients. On the other hand, the data on circulating CD4+/IL-17+ cells confirm the results on graft biopsies, suggesting that the Th17 subset may not play a critical role in the DGF setting. Finally, recent findings show that plasticity is one of the major features of CD4+ T cells and there is growing evidence that different CD4+ cell subsets share some functional characteristics [43]. Thus, our findings may represent an oversimplification of the immune events characterizing the transplant recipients with DGF. However, the observation that DGF is associated with a bias toward a Th1-driven immune response might represent a possible pathogenic mechanism of the well-established link between DGF and acute graft rejection.

Authorship

AL: performed research, analyzed data and wrote the manuscript; CD: participated in the performance of the research and contributed to statistical analysis; GC, MR and GZ: participated in research design; TT: participated in the performance of the research; PD: provided study material and supervised sample collection; MB: provided study material and supervised sample collection; SP: supervised sample collection; MG: performed part of the experiments; ER and FPS: contributed to the conception of the study; GG: contributed to the conception of the study, designed experiments and wrote the manuscript.

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