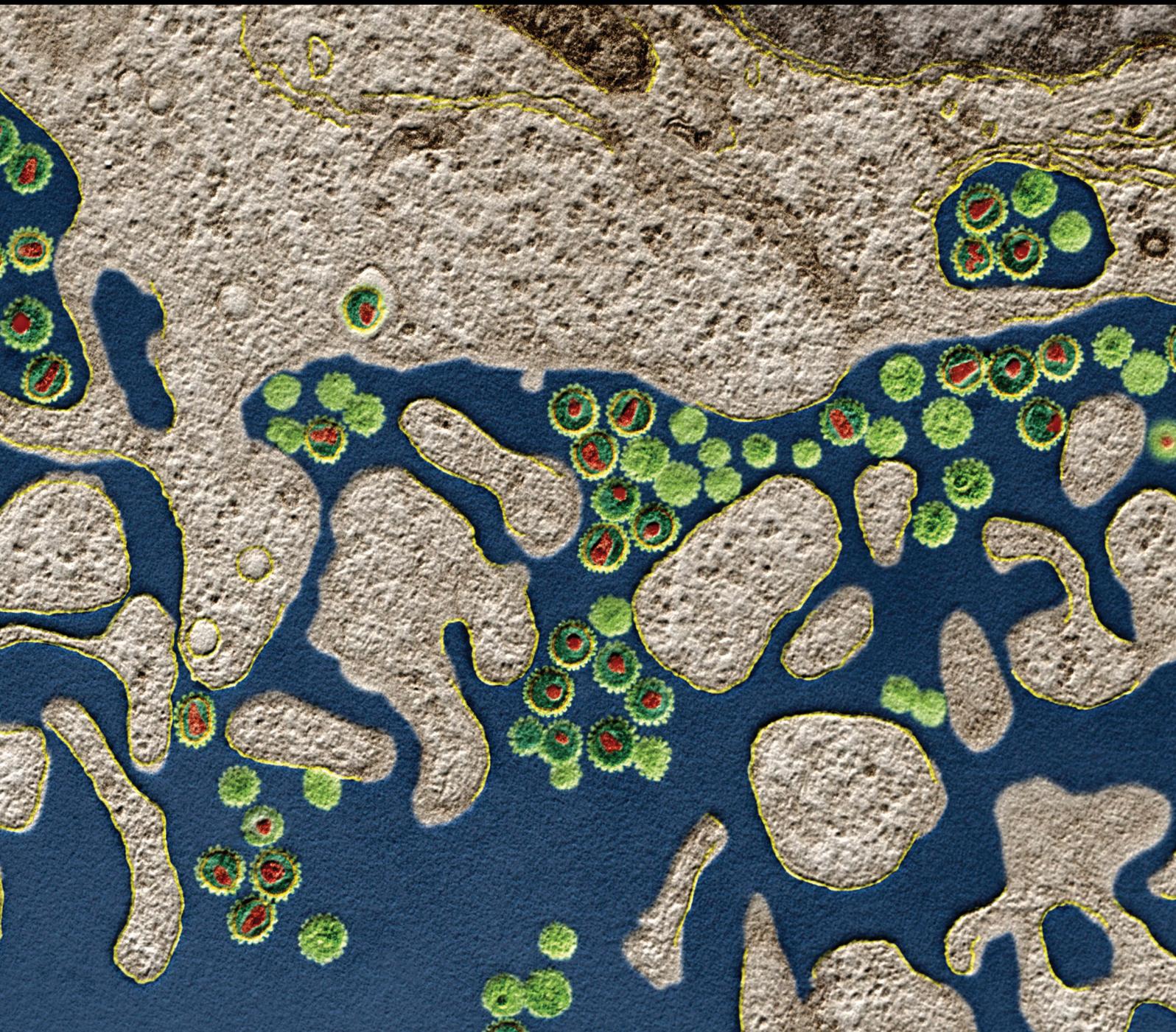


Immunological Aspect on Late Allograft Dysfunction

Guest Editors: Qiquan Sun, Akinlolu O. Ojo, and Xian C. Li





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Journal of Immunology Research

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Editorial

Immunological Aspect on Late Allograft Dysfunction

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Organ transplantation is a well-accepted treatment for patients with end-stage organ failure. Despite continued improvement in short-term graft survival, late allograft dysfunction remains a significant problem in the clinic, especially in kidney transplant patients. Many factors contribute to late graft dysfunction, and among them immunological factors are the leading cause of late grafts loss. In this special issue, we have solicited a set of interesting papers addressing some aspects of this important topic in the field.

Here we focused on antibody-mediated rejection (AMR), as AMR is emerging as a major barrier to long-term graft survival, and the donor-specific antibodies are directly involved in AMR. The paper by Q. Sun and Y. Yang provides a comprehensive overview of the characteristics of AMR, both in acute rejection and late chronic rejection, and the current strategies in managing AMR. S. Lionaki et al. provide us with an excellent review on the incidence and clinical significance of de novo donor specific antibodies (DSA) after renal transplantation. It is well recognized that transplant glomerulopathy is a very special entity of late AMR, and Dr. W. Hanf from Australia discussed the effect of donor HLA antibodies on endothelium in transplant settings. These three review papers provide an extensive overview on antibody reactivity and its correlation with late graft injury.

On the mechanistic side, while C4d remains a marker of a humoral response, recent evidence indicates that, in renal allografts, microvascular injury in the presence of donor antibodies is indicative of antibody mediated graft injury. X. Li et al. confirmed that AMR is characterized with early

capillary dilation, which is strongly correlated with intracapillary inflammation. They also discussed the diagnostic value of transcription factors T-bet/GATA3 ratio in predicting AMR, which may be of value in diagnosis of C4d-negative AMR.

The treatment of chronic ongoing AMR remains controversial. A group headed by C. W. Yang investigated the effect of combination therapy with rituximab and intravenous immunoglobulin on the progression of chronic AMR. They found that this therapy can delay the progression of chronic AMR. However, higher baseline proteinuria levels are associated with poor response to the treatment.

In another paper, H. Petra et al. evaluated differences in the intrarenal expression patterns of immune related genes in acute and chronic rejections. They found that Banff 2007 chronic rejection categories did not differ in intrarenal expression of 376 selected genes associated with immune response.

There are evidences that characters in early immune responses may also affect late graft function. L. Ma et al. reported that arterial lesions of donor kidneys had significant effects on the renal allograft function 2 years after transplantation, and correlations between donor age and arterial lesions were significant in this regard. Y. Wu and colleagues found that a novel tissue protective peptide, helix B surface peptide (HBSP) derived from erythropoietin, can effectively improve renal function by reducing tissue damage caused by ischemia reperfusion or cyclosporine A. The mechanism might be related to reduced caspase-3 activation, a key trigger in cell apoptosis and inflammation. Whether HBSP

can display similar effect in clinical practice remains to be determined.

It should be noted that liver transplantation invokes relatively weak immunological responses. E. Muñoz-Sáez et al. reported the experimental design of an in utero hepatocellular transplantation model in rats, which is an interesting tool for investigating immune responses in other settings as well. Concerning clinical liver transplantation, data from S. Mizuno et al. suggested that peripheral blood CD4+ adenosine triphosphate activity (ATP) assay (ImmuKnow assay) can be useful in monitoring immunological aspects of transplant responses, while Y. Hu et al. documented that APACHE IV is superior to MELD scoring system in prognosis of transplant outcomes in patients with liver transplants, which may be used to improve the outcome of liver transplantation.

We anticipate that these outstanding papers will provide the readers with a comprehensive view on some of the challenging problems facing the transplant field. It is our hope that, by understanding the fundamental immune mechanisms in late allograft dysfunction, new and much improved strategies could be developed to further enhance transplant outcomes.

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Review Article

Transplant Glomerulopathy: The Interaction of HLA Antibodies and Endothelium

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Transplant glomerulopathy (TG) is a major cause of chronic graft dysfunction without effective therapy. Although the histological definition of TG is well characterized, the pathophysiological pathways leading to TG development are still poorly understood. Electron microscopy suggests an earlier appearance of TG and suggests that endothelial cell injury is the first sign of the disease. The pathogenic role of human leukocyte antigen (HLA) antibodies in endothelial cells has been described in acute vascular and humoral rejection. However the mechanisms and pathways of endothelial cell injury by HLA antibodies remain unclear. Despite the description of different causes of the morphological lesion of TG (hepatitis, thrombotic microangiopathy), the strong link between TG and chronic antibody mediated rejection suggests a major role for HLA antibodies in TG formation. In this review, we describe the effect of classes I or II HLA-antibodies in TG and especially the implication of donor specific antibodies (DSA). We update recent studies about endothelial cells and try to explain the different signals and intracellular pathways involved in the progression of TG.

1. Introduction

Since the 1970s, kidney transplantation has served as the strong hold to cure chronic kidney disease. However more than 50% of transplant recipients experience late allograft rejection after 5 to 10 years which presents as a significant clinical problem and remains a major barrier to maximizing the utility of transplanted kidneys. Recurrent primary disease, toxicity of immunosuppressive therapy, and late renal rejection all contribute to late transplant loss and significantly reduce the transplant half-life. Whilst acute antibody mediated rejection (AMR) is well recognised as an early cause of graft dysfunction, the chronic late lesion of AMR is less well studied and therapeutic strategies to treat this entity are lacking. With the improvement in management of acute rejection and acute rejection rates now being less than 15% in many centres, management of chronic antibody mediated rejection and its final pathological entity transplant

glomerulopathy (TG) has become a major unmet need of transplant nephrology, for which new treatment strategies are urgently required. Prior to 2005 the term “chronic allograft nephropathy” was used to cover a variety of pathological lesions without specific cause. Transplant glomerulopathy itself is a form of chronic allograft nephropathy with poor graft outcomes and a distinctive pathological appearance [1–5]. However, a recent study showed different outcomes between these 2 entities [6].

The pathological features of TG include a multilamination and double contour formation of glomerular basement membrane (GBM) in the absence of immune-complex deposit and are identifiable by Periodic Acid Schiff or silver staining using light microscopy.

Patients with a TG histological diagnosis present frequently with a nephrotic range proteinuria and/or hypertension and/or kidney graft function deterioration as illustrated in Table 1. Peritubular capillary C4d staining has

TABLE 1: Studies including TG patients a/reporting different class I or II antibodies anti HLA a/DSA are expressed on percentage.

Year	Number of TG patients	Time to biopsy diagnosis	Proteinuria	Anti-HLA Class I	Anti-HLA Class II	Both	Class I DSA	Class II DSA	Both DSA	Antibody detection method
Sun et al. [6]	43	4.53 years	2.01 g/24 h	30	56.7	/	/	/	/	FlowPRA
Wavamunno et al. [8]	7	2.3 years (LM)	70%	/	/	/	/	/	71.4	Luminex SA
Sjpkens et al. [9]	18	8.3 years	3.1 g/24 h	/	/	/	7.6	23	7.6	ELISA
Haas and Mirocha [15]	8	10.1 months	/	/	/	/	13	38	50	Luminex SA
Gloor et al. [19]	55	21 months	1.48 g/24 h	/	/	76	7	22	46	Luminex SA
Eng et al. [22]	61	4 years	/	54	32	/	53	47	58	Luminex SA
Lopez Jimenez et al. [25]	30	7.1 years	1.9 g/24 h	/	/	40	/	/	23.3	/
Sis et al. [26]	41	5.5 years	Dipstick 26+	6.3	27.6	36.2	12.1	42.4	30.3	FlowPRA
Rostaing et al. [48]	14	9.3 years	2.35 g/24 h	/	/	/	7	36	43	ELISA Luminex ND

SA: single antigen; ND: not defined.

also been considered recently for the diagnosis of antibody mediated kidney rejection but is not correlated well with TG. Interestingly, study using electron microscopy showed early modification of endothelial cells (EC) suggesting earlier appearance of TG [6–10].

The new concept EC injury predefining TG raises the question about the possible crosstalk between these cells and HLA antibodies [11]. Despite different medical strategies (based on acute humoral rejection treatment strategies) to treat TG, none of them appear effective [12]. Some trials with new drugs like eculizumab or bortezomib are ongoing and suggest a noncomplement pathway in TG [13].

This review will cover the morphology and clinical outcomes of TG, the role of HLA antibodies, and a focus on EC injury as a key concept in the TG process.

2. Definition of Transplant Glomerulopathy (TG)

TG was first recognised in the early 1980s with characteristic features defined as mesangial and EC changes in transplantation kidney graft biopsies [14]. The cardinal features observed in biopsy series included (i) light microscopy identifying a duplication of glomerular basement membrane (GBM), mesangial matrix expansion, and glomerulitis, (ii) electron microscopy (EM) identifying a loss of endothelial fenestration, endothelial cell swelling, and mesangial matrix expansion, and (iii) immunofluorescence identifying mesangial IgM and C3 staining \pm C4d in glomerular cells and peritubular capillaries [15, 16]. The manifestations of all these changes are only observed in the lesions of advanced TG but are frequently absent in the early TG lesion. Basement membrane lamellation, which is the earliest sign of TG can be detected by EM within the first 3 months posttransplantation [8] which supports use of early protocol biopsies to survey the allograft to predict its decline in function [17]. Figure 1 summarizes histological lesions observed in TG. Being associate with these light and electronic microscopic lesions, our group frequently observed the presence of PCT inflammation which raises the question about the earliest target in TG. It is tantalising to predict that EC are the first target of HLA class I Ab as they are present in the surface of glomerular and juxtaposed to the PCT cells. Is it possible that lymphocytes use the same process to aggress the glomerular and/or PCT ECs via a possible link with HLA Ab and thereby increases the risk of TG development?

Despite TG being recognised for more than 30 years ago, the mechanisms involved in the development of TG remain largely unknown. Other histological appearances in graft recipients (e.g., thrombotic microangiopathy, membranoproliferative glomerulonephritis, or lupus nephritis) make the diagnosis difficult [18].

3. TG Risk Factors and Clinical Outcome

The development of TG is associated with poor kidney graft outcomes [1–5]. A number of associated factors for the development of TG have been identified of the recipients

including age, presence of antibodies directed towards HLA molecules especially class II donor specific antibodies (DSA), C hepatitis positive serology (HCV), and last acute rejection beyond 3 months post-Tx [9, 19]. Unfortunately de novo DSA appearance has been identified in younger recipients and attributed to their noncompliance to immunosuppressive therapy [20]. De novo DSA after transplant result in the increase of chronic humoral injury with development of TG [21].

Gloor et al. showed also that subclinical TG detected in protocol biopsies affects long-term graft outcomes [19]. Our previous study showed that allografts presenting with TG 10 years after transplantation experienced a 33% reduction in graft survival versus 63% in the matched control group [22]. This has been confirmed in other reports [4, 10]. It has been observed that around 4% and 20% of transplant patients present with TG at 1 and 5 years, respectively, after transplantation [19] but some studies showed a lower cumulative incidence of TG [9, 10, 16, 23]. In a previous 16-year retrospective registry analysis we observed a TG diagnosis in 4% of patients [22]. The true frequency of TG is probably underestimated because many centers do not perform protocol systematic biopsy and likely miss the subclinical TG [24]. The mean duration from transplant to diagnosis of TG is 2 to 9 years in clinically indicated biopsies [9, 19, 22, 25, 26]. The main presentation of the earlier stage of clinical TG is the late appearance of subnephrotic range proteinuria [9, 10]. In some cases it is associated with hypertension and ultimately with decline renal function as a late and inevitable complication. A recent study confirms that the appearance of proteinuria one year after transplantation without evidence of TG may predict the development of TG 5 years after transplantation in sensitized patients [4].

4. HLA Antibody and Association with TG

It is well accepted that HLA DSA are associated with poor kidney allograft outcomes and that their presence is strongly associated with the development of TG. Preformed DSA, which are present at the time of transplant, persisting after transplantation despite desensitization therapy or de novo DSA are considered a risk factor contributing to TG [27, 28].

We previously showed that 50% of patients presenting with TG had DSA and poor graft survival [22]. Moreover it is well documented that TG is associated with humoral rejection according to the Banff criteria [29, 30]. The presence of HLA antibodies and/or the presence of peritubular capillary inflammation were/was often associated with GBM thickening [30]. The term of chronic allograft nephropathy disappeared after the 2005 Banff meeting [31]; TG term still remains applicable with an overlapping between TG and chronic humoral rejection [26] in part mediated by DSA. More recently a French study confirmed the association of TG anti-HLA antibodies and a new defined antibody-mediated vascular rejection in kidney allograft [32]. The second part of chronic humoral rejection diagnosis is based on peritubular capillary (PTC) C4d deposits [29] similar to the acute humoral rejection criteria [31].

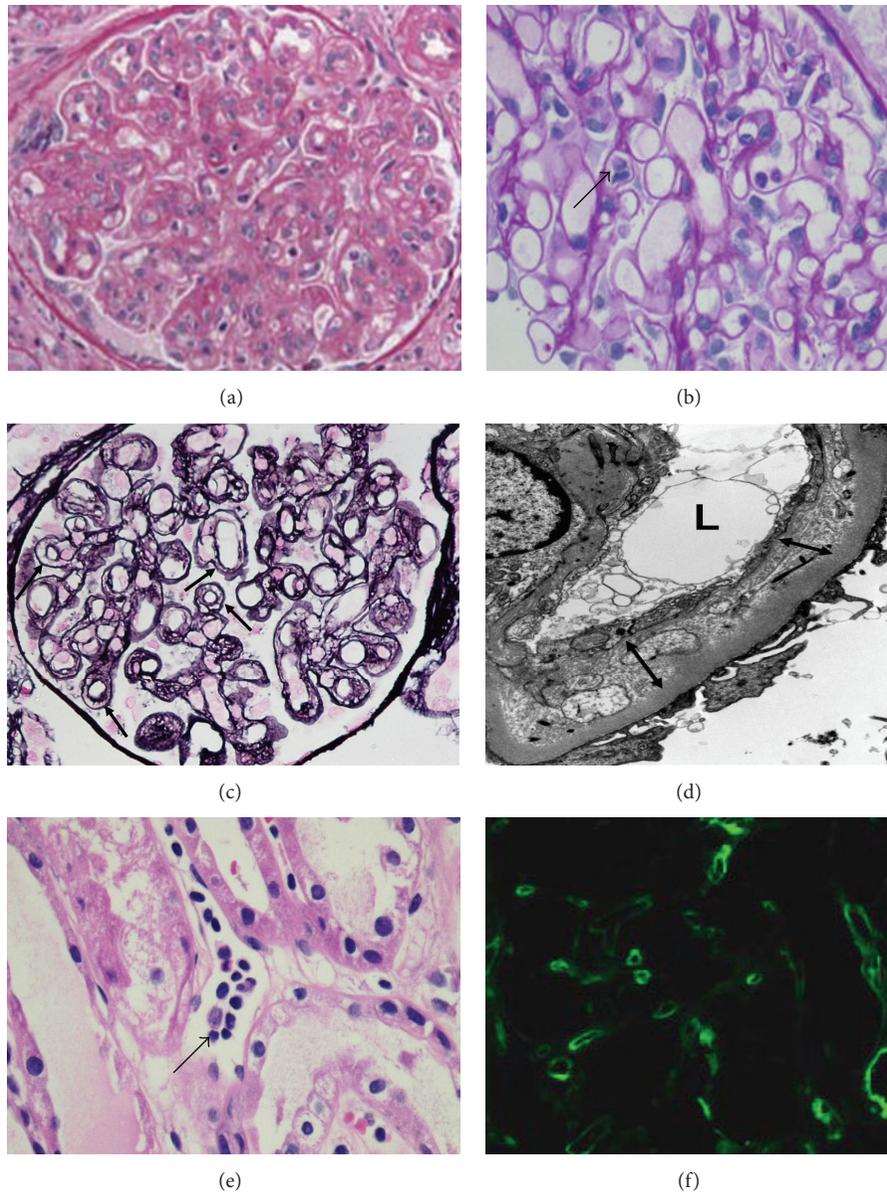


FIGURE 1: Histopathology of transplant glomerulopathy. (a) Light microscopy showing TG with a glomeruli showing sclerosis (periodic-acid-Schiff stain $\times 400$). (b) Light microscopy showing glomerulitis (presence of mononuclear cells in glomerular capillaries) in a biopsy specimen (periodic-acid-Schiff stain $\times 600$) (c) Silver staining highlighting double contouring of the glomerular capillary wall ($\times 400$). (d) Electron microscopy showing duplication of the glomerular basement membrane—arrows ($\times 8000$) (e) Peritubular capillitis (arrow) in a TG biopsy specimen (periodic-acid-Schiff $\times 600$) (f) Peritubular C4d staining in a TG biopsy (immunofluorescence $\times 400$).

5. C4d Staining in Transplant Glomerulopathy

The demonstration of antibody deposition in the graft is an important component of making a diagnosis of antibody mediated allograft injury. The last Banff working group meeting revised in 2011 the definition of antibody mediated rejection (AMR) and acknowledged that the chronic humoral rejection and/or TG development could be C4d negative [7, 33]. PCT C4d staining biopsies is strongly associated with DSA in TG biopsies [19, 26]. Glomerular C4d may occur without C4d staining in PTC in recipients who frequently presented with DSA [9, 19, 34, 35].

Glomerular C4d deposition is a useful additional marker for making the diagnosis of TG and has been proposed as an index of severity in TG. Indeed Batal et al. observed significant higher chronic glomerular lesion score (cg) in paraffin-embedded biopsies that were associated with positive glomerular basement membrane staining as well as a trend toward a significant difference in frozen specimens with glomerular staining [36]. The interpretation of glomerular C4d staining is further complicated by the choice of tissue in which it is detected. Normal glomeruli examined by immunofluorescence in frozen section may show peripheral C4d staining and therefore use of paraffin section is

preferred when seeking evidence of glomerular deposition [37]. Loupy et al. described different stages of TG mediated by preformed DSA according to early kidney graft biopsies [27]. The remaining question about the DSA threshold and subsequent development of TG is still unclear and needs to be addressed with further studies including patients with DSA after transplantation [38]. Despite its usefulness when present, C4d staining can be problematic in that its detection varies depending on biopsy series and technique employed for detection [37]. Chronic antibody mediated injury likely occurs in wave like patterns, with variable antibody production, and therefore biopsy may potentially miss periods of peak antibody production and therefore C4d positivity. These pathogenic variations in disease activity and variation in technical detection techniques therefore largely account for the range of C4d positivity reported in biopsy series, which ranges between 25–61% of TG cases described in the literature. Avoiding overdependence on C4d as a diagnostic cornerstone of antibody-mediated rejection and taking other factors into consideration, for example, presence of DSA and other histological evidence of TG (glomerulitis/PTCitis), are likely to contribute to better diagnosis in the future, but this will require further studies to confirm the utility of these factors. Recently the concept of C4d negative antibody mediated rejection has been proposed, which recognises that C4d staining is problematic and proposes a new classification which includes evidence of allograft injury based on light microscopic features of glomerulitis (Banff “g” lesions) and peritubular capillitis (“ptc” lesions). This new classification from the Banff 2013 meeting has not yet been published and validated but it may alter the diagnostic criteria for antibody mediated rejection. Finally, newer techniques including microarray analysis of TG biopsies may provide molecular signals and intracellular pathway activation to allow the diagnosis of TG without histological controversy and may potentially identify new pathways that can be targeted in intervention strategies.

6. HLA Antibody Specificity and TG

Table 1 summarized the percentage of class I and class II HLA Abs in the different studies including TG patients. The link between anti-HLA DSA and TG is well recognised. Moreover, studies report the main risk factor of TG being DSA HLA class II antibodies. However HLA class I antibodies have also been identified [21]. A recent study demonstrated a correlation between HLA class II Ab in de novo DSA after transplantation and poor graft survival [20]. However complete dominance of HLA class II Ab in TG is unlikely even if some explanation has been proposed [20, 26, 39–41]. The important question regarding the risk of TG according to the time of DSA appearance needs to be addressed. For example, are pretransplant DSA as deleterious as posttransplantation DSA for TG lesion development? The majority of studies on TG focused on DSA after transplantation [6, 9, 21] and in fact excluded patients with DSA pretransplantation [19, 20]. We observed that pretransplant DSA were a risk factor of TG but statistical analyses were not performed according to the

small number of TG patients. We also described a higher risk of TG appearance in de novo DSA patient group [22]. Due to the low number of recipients susceptible to develop TG, the possibility to lead a prospective randomized controlled trial is still unexpected. The high sensitized living kidney transplantation should be a good model to analyse the risk of DSA and TG.

The presence or absence of C4d staining deposition in TG biopsies specimen correlates poorly with the presence of HLA antibodies [26]. In a recent study, 31/48 AMR associated with TG was C4d negative and the AMR was due to anti-HLA class I and/or II in the same proportion [21]. Even if TG occurred via either HLA class I antibodies or HLA class II antibodies, 5-year graft survival in a large series of living donor kidneys with positive cross match decreased widely in class II sensitized patients as compared to that of class I sensitized patients (85.3% versus 62.6%) [4]. The presence of TG one year after transplantation results in graft loss in 30% of anti-HLA class II patients and approximately 20% of those with anti-HLA class I antibodies [4]. These authors also demonstrated a higher rate of chronic glomerulopathy (defined by a cg score > 0), which is frequently associated with chronic antibody mediated injury in the anti-HLA class II patient group [4]. The reason explaining the greater effect of anti-class II antibody as compared to class I antibody in AMR and also in TG remains unknown.

Anti-DP antibodies have been reported in TG as an only immunological cause in one patient presenting with TG [42]. Anti-DQ DSA are increasingly recognized as the predominant HLA class II DSA produced [43]. A recent study showed the clinical relevance of anti-DQ antibodies in kidney graft outcomes and confirms the pathogenic effect in cardiac and liver transplantation [44, 45]. Patients with DQ-DSA are at a higher TG risk. Interestingly, an association of Cw-DSA in TG patients was not observed suggesting a major effect of DQ DSA [46]. Issa et al. demonstrated a strong correlation between anti-class II antibody titre and the risk of TG but did not observe differences between anti-DR or anti-DQ subset effects [47].

Notably, as half of the biopsies performed 5 years after transplantation did not present evidence of TG, this raises the question of the crucial crosslink of HLA antibodies and thus suggests the involvement of other aetiologies. To this end, some studies did not confirm an association between HLA antibodies and TG suggesting another possible mechanism in TG formation [15]. Examples include patients positive for HCV and those presenting with thrombotic microangiopathy [18, 48].

7. Non-HLA Antibody Involvement in TG

Despite strong evidence for a correlation between HLA antibodies and TG, the implication of non-HLA antibodies has been suggested as a mechanism of glomerular damage occurring after transplantation. The percentage of TG cases in which HLA antibodies have not been identified is variable, with one large series from Canada suggesting that up to 27% of cases had no demonstrable antibody [26]. In our own

series over 50% of cases of TG were associated with donor specific HLA antibodies [22]. Some of TG cases presented without anti-HLA antibodies or C4d staining [49] and recent reports demonstrated the role of non-HLA antibody. This autoreactivity has been well described in heart transplantation with antibody directed against myosin or vimentin [50]. Angiotensin II type 1 receptor (AT1R) antibody strongly correlated with AMR in kidney transplantation independently of DSA and confirming the rule of non-HLA Ab in acute graft injury [51]. In our well-characterized cohort of TG patients [22] we have recently identified AT1R antibodies in 52% of cases with biopsy proven TG and importantly a group of HLA antibody negative TG cases in whom AT1R antibodies were identified indicating for the first time that AT1R antibodies by themselves are associated with TG (Hanf et al. submitted). In pediatric cohort, recipients with antibodies to protein kinase C ζ developed rejection and increased the risk of allograft loss but TG was not described [52]. Using a protein screening microarray Dinavahi et al. showed that transplantation induced changes in antibody repertoires reactive to non-HLA Ag and isolated three possible pretransplant serum antibodies to peptidyl-propyl-isomerase-A, peroxisomal-trans-2-enoyl-coA-reductase (PECR), and serine threonine kinase 6 correlating with TG development. Notably, only PECR was confirmed by ELISA with a strong association between it and TG [53]. Moreover a recent study using the same process describes four new biomarkers predicting the future development of chronic allograft injury in pretransplant sampling: chemokine ligand 9 (MIG), interferon gamma (IFN γ), chemokine ligand 11 (ITAC), and Glial-derived neurotrophic factor [54]. Importantly, these recent pieces of data were derived from a retrospective cohort and thus require confirmation in large prospective studies.

8. Endothelial Cells and HLA Antibody: Crosstalk to Develop TG?

Binding HLA antibodies to HLA molecules may cause endothelial cell injury via the complement cascade and/or may induce endothelial cell proliferation and survival via intracellular signalling. Donor-specific anti-HLA alloantibodies initiate renal allograft rejection through complement-mediated and antibody-dependent cell-mediated cytotoxicity as previously described [55]. In a sensitized kidney allograft cohort, 68% of tested sera were found to contain complement-fixing alloreactivity. IgG1 type panel reactivity was predominant (detectable for HLA class I and II reactivity), followed by IgG3, and both were independently correlated. Complement fixation was also favored by the simultaneous presence of alloreactive IgG1, IgG3, and IgM [56]. IgG2 and IgG4 were more weakly involved in complement-fixing activity [57].

Electron microscopy studies show that the endothelium exhibits the first signs of injury, well before development of TG [8]. The concept of endothelial injury is supported by EC gene analysis showing a strong correlation of high expression of endothelial cell-associated transcript (ENDAT) and the presence of DSA correlating with late antibody

mediated rejection. In 2009, Sis et al. described that more than 10 ENDAT genes increased ABMR predicting late graft loss. Importantly, patients presenting with DSA without high expression of ENDAT had better graft outcomes [57]. However, high ENDAT expression was also seen in borderline TG changes, T cell rejection, or polyomavirus infection [58]. Thus this marker should be interpreted in association with the presence of DSA to improve sensitivity [59]. These data suggest the correlation between HLA antibodies and EC injury mediated by complement dependent or independent pathways in chronic ABMR.

Previous studies showed that binding antibodies to class I molecules on the surface of endothelial cells results in tyrosine phosphorylation of various intracellular proteins (AKT, ERK...). The two major consequences of class I-mediated phosphorylation are cell proliferation via upregulation of fibroblast growth factor receptors (FGFR) on the surface of endothelial cells and cell survival stimulation by increased endothelial cells expression of antiapoptotic proteins Bcl-2 and Bcl-xL via the PI3K/Akt pathway [60, 61]. These observations raise an important question—what are the factors that determine the outcomes of HLA class I antibody-mediated phosphorylation?

As illustrated in Figure 2, studies by the Reed's group showed that stimulation of endothelial cell proliferation was observed at concentrations of HLA antibodies ranging from 0.1–10 $\mu\text{g}/\text{mL}$ with maximal cell proliferation at concentrations of 10 $\mu\text{g}/\text{mL}$. On the other hand, treatment of endothelial cells with HLA class I antibodies for 24 hours induced a prominent increase in the prosurvival proteins Bcl-2 and Bcl-xL protein levels; with maximum increases observed with lower antibody concentrations antibodies were used (0.01–1 $\mu\text{g}/\text{mL}$) [61]. These findings suggest that concentration of HLA antibodies is one of the factors contributing to the outcomes of HLA class I antibodies-mediated phosphorylation and gene expression. Furthermore, Iwasaki et al. showed that low dose HLA class I antibody activates the AKT pathway leading to the induction of antiapoptotic genes (Bcl-2) as well as the cytoprotective genes HO-1 and ferritin H [62] (Figure 2).

Recent *in vitro* work using human aortic EC addressed the question of EC cytoskeleton and antibody mediated rejection or transplant vasculopathy. In 2012 Zhang and Reed demonstrated a mutual dependency between HLA I and integrin subunit β 4 to stimulate signal transduction and EC proliferation [63]. Ziegler et al. improved the understanding on HLA class I ligation EC pathway using both mass spectrometry analysis on cytoskeleton structure [64] as well as analysis on stress fiber formation [65]. As depicted in Figure 3, results suggest a major contribution of ERK and MLC phosphorylation in a calcium independent manner and that the remodelling in EC structure may be involved in chronic allograft rejection. Monocyte infiltration occurs during graft injury and may also contribute to the risk of TG. An elegant study by Valenzuela et al. showed, *in vitro* and *in vivo*, a role for p-selectin in monocyte recruitment induced by HLA class I Ab [66] (Figure 3). Taken together, these results confirm the corner stone role of HLA antibodies in

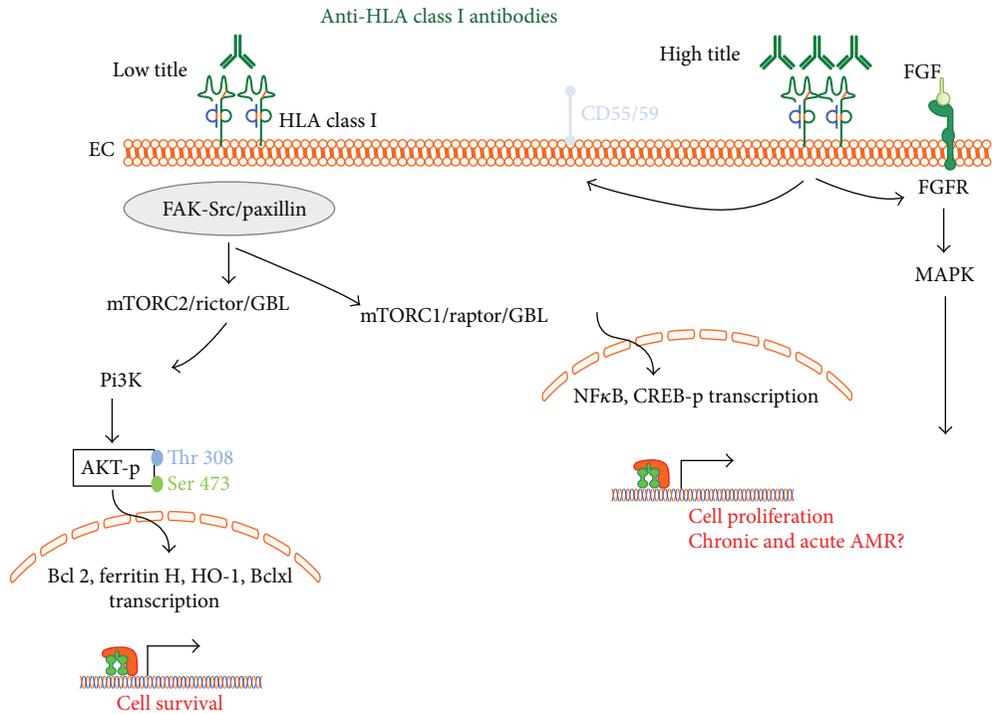


FIGURE 2: Intracellular signalling pathways mediated by high/low titre HLA class I antibodies after endothelial binding. Putative cell survival and proliferation pathways are illustrated.

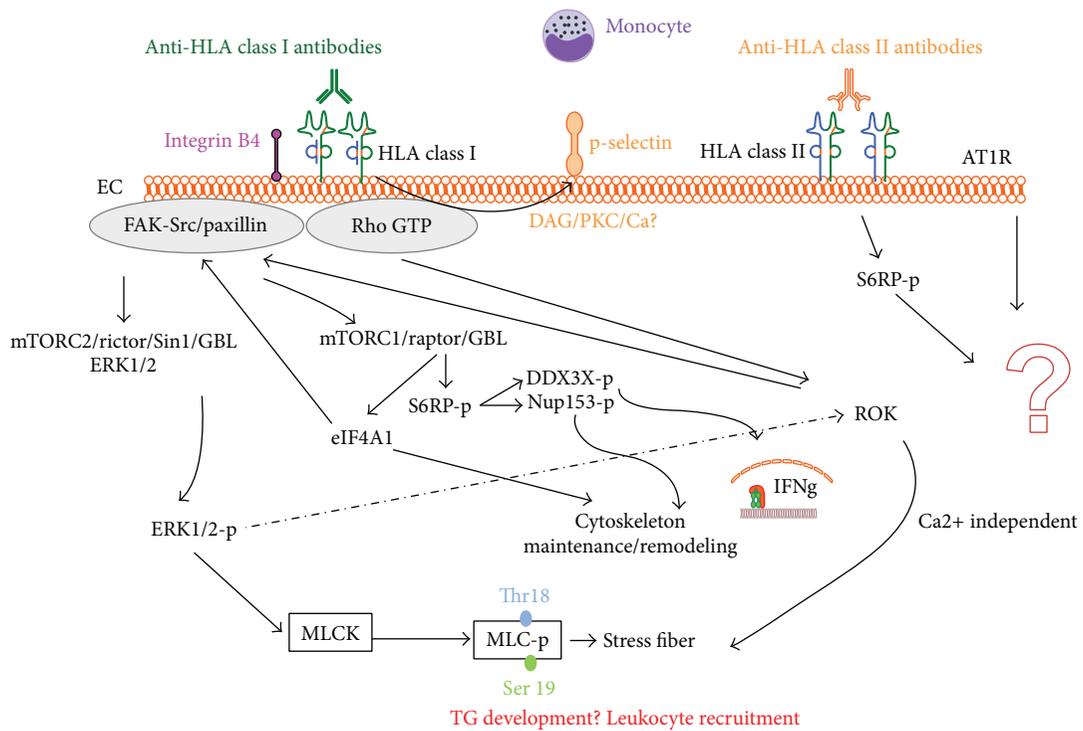


FIGURE 3: Intracellular signalling pathways mediated by HLA class I or II antibody binding to endothelial cells. The observation that the main HLA class I antibody binding stimulates intracellular EC pathways via the integrin β4 subunit is summarized in this figure. After stimulation cytoskeleton remodelling and stress fiber formation via ERK pathway occur with calcium dependent and independent signalling leading to possible TG development and inflammatory cell recruitment.

the chronic rejection and thus warrant further investigation in TG formation.

Interestingly, the majority of studies investigating antibody activity in EC injury were mediated by HLA class I monoclonal antibodies leaving clear need to investigate class II antibodies. One study investigated the effects of class I and II antibodies on EC on cardiac allografts and showed that phosphorylation of S6 ribosomal protein (S6RP), a downstream target of the PI3K/Akt/mTOR pathway, was a biomarker of antibody mediated rejection [67]. The authors demonstrated that ligation of HLA class I and class II molecules on EC resulted in increased phosphorylation of S6RP. Then they showed in a cardiac biopsy that antibody production to class II antigens was positively associated with p-S6RP-positive staining and confirmed a strong association between generation of DSA to class II antigens and EC staining of p-S6RP staining. These observations were not significant with class I Ab or DSA. These data suggest crosstalk between class II Ab and EC but at present there is a lack of *in vitro* or animal studies with class II DSA or class II antibodies. Le Bas-Bernardet et al. showed that DR expression was sufficient to trigger intracellular signaling in EC isolated from human deceased donor, in response to HLA-DR ligation. Crosslinking of HLA-DR on ECs promotes Akt activation and phosphorylation, suggesting that the PI3-K pathway, involved in EC survival, was activated. These two studies on class II antibodies raise the question of survival signalling pathways contributing to EC changes. However the EC used (from human large vessels) are quite different from those of glomerular EC likely specifically involved in the TG process [68, 69]. HLA antibodies tested in glomerular EC subset demonstrated that these were able to produce complement component (C3 and C4) [70].

Non-human primate studies in cynomolgus monkeys have further added to our knowledge of the pathogenesis of TG suggesting that there are 4 stages of the process. The initiating stage is increased donor-specific antibodies, followed by C4d deposition, then development of tissue injury, and finally decrease in allograft function [71].

9. Treatment Options

Interestingly, the current recommendations in TG are not based on randomized controlled trials or level I evidence but rather on expert advice. Moreover, there is no efficient treatment to limit TG progression with treatment based on preventive recommendations (e.g., monitoring DSA, avoiding and controlling antibody mediated rejection, and reinforcing medication compliance) [72, 73]. The use of antiproteinuric agents (e.g., ACE and ARB) is currently ongoing [74]. Different desensitization protocols have been used in sensitized patients at risk for antibody mediated rejection [73, 74] and transplantation teams replayed these different strategies in chronic ABMR and/or TG without major significance benefits [12].

The diagnosis of TG in early stage should probably be a goal but it requires systematic biopsies with electron microscopy to dissect endothelial change. Thus it should

be recommended for electron microscopy to be performed on all transplant biopsies in patients at risk of developing TG to enable early detection of these changes. In line with the recent consensus guideline on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation protocol biopsies should be performed once de novo DSA have been detected [38]. A recent study showed a benefit in the rate of development of TG in patients presenting glomerular ultrastructural changes and DSA and receiving IG IV + plasmapheresis and/or rituximab [15]. However a pilot study using rituximab did not show efficacy in TG with stabilisation of TG in 50% of the cases [48] but these results and other strategies should be performed in prospective trials with TG and DSA monitoring as key end points in the trial design [38]. The use of rituximab or splenectomy in incompatible ABO transplantation was efficient to treat chronic ABMR in ABO incompatible kidney transplantation with reduction of DSA titre [74]. Lefaucheur et al. suggested that using plasma exchange in association with high dose of intravenous immunoglobulin's and rituximab reduced DSA level and AMR three years after transplantation and thus reducing the risk of chronic AMR [75].

Based on B cells depletion efficacy in experimental models [76, 77], bortezomib showed an efficacy in chronic AMR case studies [78] and in AMR treatment [79]. A controlled trial is ongoing to investigate if bortezomib will prevent TG in patients who are at high risk of developing the condition due to high donor-specific alloantibody in posttransplant kidney recipients (NCT01349595 on ClinicalTrials.gov).

The other new drug in the field is the C5 inhibitor eculizumab (anti-C5 humanized monoclonal antibody; Alexion, Cheshire, Connecticut). This agent was initially developed for paroxysmal nocturnal hemoglobinuria, the implication of complement pathway in AMR lead transplantation team, to test eculizumab in sensitized patients with excellent results [80]. It is also suggested that one of the pathways resulting in TG is complement dependent due to the presence of C4d deposition in biopsies. Stegall et al. showed that sensitized patients receiving eculizumab decreased the rate of AMR [81]. Even if the protocol biopsy at one year after transplantation tends to show efficacy in TG development, the same group present, during the American Transplantation Congress 2012, results until 2 years after transplantation from positive crossmatch patients receiving eculizumab. They demonstrated similar rate of TG in both groups at two years posttransplantation (50% versus 55% in eculizumab versus control group, resp.) [82]. These results suggest that some case of early TG may involve with independent complement pathway.

10. Conclusion

TG remains a major cause of graft loss. We described a strong correlation between EC injuries and HLA antibody likely involved in TG process. The recent knowledge in intracellular pathways involved in transplant vasculopathy after HLA antibody ligation to their receptor in EC will

likely improve in the future years and we hope that could be extended to the concept of TG to develop new TG formation blockade strategies. Treatment recommendations are mainly preventive because treatment targeting HLA Ab or their consequences did not show encouraging results, especially for eculizumab. The main and only prospective study devoted to TG is ongoing and should be an alternative to the current therapy based on HLA Ab removal.

The unraised question about allo- or auto-non-HLA antibody and TG remains open and warrants address to determine the contribution of these antibodies in TG as well as the HCV status and thrombotic microangiopathy disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Capillary Dilation and Rarefaction Are Correlated with Intracapillary Inflammation in Antibody-Mediated Rejection

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Antibody-mediated rejection (ABMR) remains one of the major causes of graft loss after renal transplantation. It is dominated by endothelial damage in microcirculation. Clarifying the mechanism of microcirculating damage is obviously a key step to understand the pathogenesis of ABMR. Here we characterized capillary variation in ABMR and its possible mechanisms. Compared with T cell-mediated rejection and stable grafts, there was a significant dilation and rarefaction in peritubular capillaries (PTCs) of the ABMR group; Image-Pro Plus revealed a significantly larger intra-PTC area. Interestingly, the dilation of PTCs was strongly correlated with the intra-PTC cell counting. Moreover, peritubular capillary inflammation is correlated with *in situ* T-bet expression, and there was a good correlation between the intra-PTC expression of T-bet and the PTC diameter. HIF-1 α up-regulation could be observed in ABMR but it was not necessary for capillary dilation. In general, ABMR is characterized with early capillary dilation and rarefaction; our data confirmed that the dilation is strongly correlated with intracapillary inflammation, which in turn is correlated with *in situ* T-bet expression. T-bet plays an important role in the development of microcirculating injury, and thus it is a potential target for the treatment of ABMR.

1. Introduction

Antibody-mediated rejection (ABMR) is a recalcitrant entity with great impact on patient and graft survival [1, 2]. In the past decade, improvements in HLA technology along with the recognition of the role of C4d in ABMR have revolutionized the understanding of this important entity, and significant advances have occurred in the treatment of ABMR [3–5]. However, the mechanism of ABMR is far from being fully elucidated, and the long-term survival of these allografts is greatly reduced when compared to that of grafts without rejection or history of T cell-mediated rejection (TCMR) [2, 6].

ABMR is dominated by endothelial damage in microcirculation [7, 8]. Microcirculation inflammation, including glomerulitis and peritubular capillaritis (PTCitis), has been recognized as a cardinal feature in the diagnosis of ABMR [9, 10]. Peritubular capillary dilation is another important side of microcirculation changes, and although it has been noticed

in the ABMR for years [2], it is far from being clearly demonstrated and its pathogenesis remains unclear. The assessment of capillary dilation will be helpful to clarify the mechanism of ABMR.

T-bet is a member of the T-box family of transcription factors regulating Th1 lineage commitment [11]. In a recent study, we found that transplant glomerulopathy, a principal form of late ABMR, had a significant increase in T-bet expression in peritubular capillaries (PTC), and this expression was strongly correlated with the count of intra-PTC inflammation cells. Furthermore, PTC dilation was also strongly correlated with the intra-PTC inflammation [12]. In a previous study, we found intraglomerular inflammation correlated with *in situ* expression of T-bet in patients with ABMR [13]. We hypothesize that T-bet expression might be also correlated with PTC injury in early ABMR.

HIF-1 is a transcription factor which acts as a master regulator coordinating oxygen homeostasis [14], and the HIF system is ubiquitous which is instantaneously up-regulated

upon hypoxia [15]. In ABMR, whether PTC injury can cause tissue damage via hypoxia is unknown. In this study, we explored the dilation of PTC in relation to inflammation, T-bet expression, and hypoxia. Our data provide novel insight into the development of antibody-mediated graft injury.

2. Materials and Methods

2.1. Patient Selection. The patients were retrospectively selected from among 226 renal allograft recipients who had performed renal biopsy between June 2008 and May 2012 at Jinling Hospital, Nanjing University School of Medicine, Nanjing, China. Among them, 18 recipients were diagnosed as having C4d-positive acute rejection episodes according to clinical manifestations and histological features. The diagnosis was based on the following: (1) clinical evidence of acute rejection, manifested as rapid renal dysfunction and/or decrease of urine volume; (2) C4d deposition in the PTC area; and (3) pathologic features that met Banff's 1997 criteria for acute rejection grade I, II, or III. Additionally, rejection episodes occurring beyond the first month after transplantation are more likely to offer a mixed histologic picture, often demonstrating acute and chronic, vascular and tubulointerstitial, pathology. Thus, we examined only biopsies from recipients in the first posttransplant month. This group was compared with a group of TCMR patients who were diagnosed within the same time period. Since PTcitis (ptc) and glomerulitis (g) are often associated with ABMR and $g + ptc = 0$ was confirmed to be a useful diagnostic algorithm for TCMR exclusion [16], we excluded all the recipients with PTcitis and glomerulitis in TCMR group. These patients were randomly matched with a group of recipients with stable graft function who received protocol biopsies as controls. In addition, we also included a TG group to compare PTC variation with ABMR. We applied the following inclusion criteria: (1) biopsy confirmation of the presence of a duplication of the glomerular basement membrane on periodic acid-Schiff or silver stain, and (2) 1 year of follow-up after the diagnosis. An electron microscopic evaluation was performed to exclude membrane duplication that was caused by recurrent or de novo glomerular disease. Informed consent was obtained from all patients, and the Human Subjects Committee of Jinling Hospital, Nanjing University School of Medicine approved all of the study protocols.

2.2. Renal Biopsies. Protocol biopsies were performed between days 12 and 17 posttransplantation (so-called 2-week protocol biopsy), and diagnostic biopsies were performed upon clinical indication and according to local standard of practice. All rejection episodes were proved by biopsy. Two needle biopsy cores were obtained from each renal allograft for morphologic study: one for formalin fixation and the other for quick-freezing. Hematoxylin and eosin, periodic acid Schiff, methenamine-silver, and Masson stains were routinely used on the formalin-fixed tissue. The residual biopsy tissues were stored for future use. Fresh-frozen tissues were analyzed by immunofluorescence microscopy using a conventional panel of antibodies against IgG, IgM, IgA, C3, C4, C1q, and C4d. C4d staining was routinely performed

on frozen slides using an indirect immunofluorescence technique with a primary affinity-purified monoclonal antibody (mouse anti-human; Quidel, San Diego, CA, USA) and an FITC-labeled affinity-purified secondary rabbit anti-mouse IgG antibody (Dako, Denmark). The staining was performed using standard procedures. Positive C4d staining was defined as a bright linear stain along the capillary basement membranes that involved over half of the sampled capillaries in accordance with the 2001 Banff Meeting [17].

2.3. Immunohistological Analysis. CD4, CD8, CD68, and CD20 were regularly detected when a biopsy was performed. The intragraft expression of HIF-1 α and CD31 was retrospectively studied via immunohistochemistry using stored residual biopsy tissues. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. The antibody regimens were conducted as follows: mouse monoclonal antibody HIF-1 α (Novus Biologicals, Littleton, CO, USA); mouse monoclonal antibody against T-bet (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse monoclonal antibodies against CD4 (Novocastra, Newcastle upon Tyne, UK), CD8 (Novocastra), CD68 (Dako, Carpinteria, CA, USA), CD20 (Dako), and CD31 (Dako). The sections were reviewed by two pathologists, and the results are expressed as the total number of positive cells per glomerulus or per square millimeter in the cortex.

2.4. Microvascular and Intracapillary Cell Counting. To assess the number and size of PTCs, a CD31 costaining was used as previously described [18, 19]. The PTC density was calculated by counting the total number of PTCs within the confines of each of 10 random 0.25 mm² fields (each of these fields was delineated by a 1 cm² ocular grid that was viewed at $\times 400$ magnification), and the result is expressed as the mean per field. The diameter of these PTCs was also measured, and their mean was calculated in micrometers. To measure the PTC spaces, slides from each case were examined using an Olympus IX70 inverted system microscope (Olympus America, Melville, NY, USA) connected to a Hewlett Packard computer with Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA) [20]. The intra-PTC area is expressed as the proportion of total PTC spaces over the entire cortex field. Intraglomerular capillaries were measured for their quantity (capillary counts per glomerulus) and diameter on cross-section of the glomeruli. To calculate the intracapillary inflammation cells, costaining of CD31 and inflammation markers (e.g., CD4, CD8, and CD68) were performed, and the results are expressed as the mean per PTC or glomerulus. Glomerular cross-sectional area was also determined using the Image-Pro Plus software.

2.5. Statistical Methods. Statistical analyses were conducted using SPSS (v16.0) and GraphPad Prism (v5) software. Pairwise comparisons of variables based on proportions were done by Fisher's exact test with Bonferroni correction for *P* value. Continuous variables were presented as mean \pm s.d. and compared using one-way analysis of variance (ANOVA) followed by post hoc pairwise comparisons using LSD tests, or analyzed using nonparametric method if the data were

TABLE 1: Clinical characteristics of patients who participated in this study.

Characteristics	ABMR ($n = 18$)	TCMR ($n = 13$)	SG ($n = 14$)	P value
Gender, male (%)	9 (50.00)	10 (76.92)	10 (71.42)	0.244
Age (years)	40.72 \pm 7.09	39.15 \pm 9.64	44.21 \pm 14.10	0.393
Donor age (years)	40.17 \pm 7.81	44.46 \pm 9.37	44.36 \pm 7.87	0.349
Positive pretransplant PRA (n)	0	0	0	—
Previous transplant	0	0	0	—
Previous rejection, n (%)	0	0	0	—
Cold ischemic time (h)	8.22 \pm 1.06	7.77 \pm 1.17	8.00 \pm 0.88	0.471
Warm ischemic time (min)	6.61 \pm 1.65	6.31 \pm 1.32	6.71 \pm 1.27	0.728
Induction with IL-2R antibody, n (%)	18 (100)	13 (100)	14 (100)	—
Baseline immunosuppressants				0.224
MMF + Tac + Pred	13	10	8	
MMF + CsA + Pred	5	3	3	
Others	0	0	3	
Time of biopsy (day)	8 (5–20)	15 (11–25)	14 (12–15)	0.14

Abbreviations: ABMR: antibody-mediated rejection; TCMR: T cell-mediated rejection; SG: stable graft; PRA: panel-reactive antibody; IL: interleukin; MMF: mycophenolate mofetil; Pred: prednisolone; Tac: tacrolimus; CsA: cyclosporine A.

not normally distributed. Ordered categorical data were presented as median (25th–75th percentiles) and compared using the nonparametric Kruskal-Wallis ANOVA on ranks for global comparison, followed by Duncan's analysis for multiple comparisons. Spearman's correlation was used in analysis correlation. The level of statistical significance was set at $P \leq 0.05$ (two-sided).

3. Results

3.1. Baseline Patient Characteristics. Forty-five renal allograft recipients were included in this study, including 18 cases of ABMR, 13 cases of TCMR, and 14 cases of stable grafts as controls. The diagnosis of ABMR and TCMR was based on Banff 05 [21]. The baseline patient characteristics are listed in Table 1. None of the recipients had previously received an organ transplant. There were no significant differences among the three groups with respect to patient age, gender, time of prior transplantation, time of biopsy, or incidence of positive panel-reactive antibody. Each patient received anti-IL-2 receptor monoclonal antibody for the induction of immunosuppressive therapy and was subsequently maintained on a similar immunosuppressive protocol after transplantation (Table 1).

3.2. Pathology Findings. A comparison of histological lesions in the three groups is given in Tables 2 and 3. Recipients with lymphocytes infiltration in PTCs or glomeruli were excluded from TCMR group while PTCitis was observed almost in all patients in ABMR group. Tubulitis was seen in all patients of acute rejection enrolled in the study, compared with 42.9% of stable graft. Significantly more patients (88.9%) have intimal arteritis in the ABMR group compared with either of the other two groups. We used immunohistochemistry to detect CD4, CD8, CD68, and HLA-DR expression. In the ABMR group, the average values for CD4, CD8, CD4/CD8 and CD68, were all similar to the TCMR group. However, when

compared with stable grafts group, every kind of lymphocyte is significantly higher in both ABMR and TCMR groups.

3.3. ABMR Is Associated with Peritubular Capillary Dilation and Rarefaction. Labeling the endothelial cells for CD31 made it possible to calculate the number and diameters of the capillaries. Figures 1(a) and 1(b) show that CD31 staining labeled the capillaries notably well. Overall, ABMR was correlated with an increased PTC diameter and a decreased PTC density.

We compared the PTC density and capillary diameter among the three groups (Figures 1(c) and 1(d), Table 4). In the ABMR group, the density of PTCs was significantly lower compared with the stable graft group (22.22 ± 2.51 versus $25.64 \pm 1.82/\text{field}$, resp., $P < 0.001$), whereas the diameter of the existing PTCs was significantly larger (2.34 ± 0.81 versus $1.09 \pm 0.29 \mu\text{m}$, resp., $P < 0.001$), suggesting a rarefaction and dilation of the capillaries (Figure 1(a)). However, in the TCMR group, we found no such rarefaction and dilation of PTCs as observed in the ABMR group (Figure 1(b)). The capillary density was similar between the TCMR group and protocol biopsies (27.23 ± 2.49 versus $25.64 \pm 1.82/\text{field}$, resp., $P = 0.105$). In addition, Image-Pro Plus revealed a significantly larger intra-PTC area in the ABMR group (0.035 ± 0.018) compared with the TCMR (0.023 ± 0.012 , $P = 0.026$) and stable graft (0.020 ± 0.006 , $P = 0.006$) groups (Figure 1(e)). In addition to PTCs, capillary dilation was also observed within the glomeruli. However, there were no significant differences between the three groups in the diameter of the intraglomerular capillaries and intraglomerular capillaries' area.

3.4. PTC Dilation Is Correlated with Microcirculation Inflammation. In the ABMR group, we detected a significant correlation between PTC dilation and microcirculation inflammation. The dilation of PTCs was strongly correlated with the quantity of intra-PTC infiltrating cells ($r = 0.664$, $P = 0.004$),

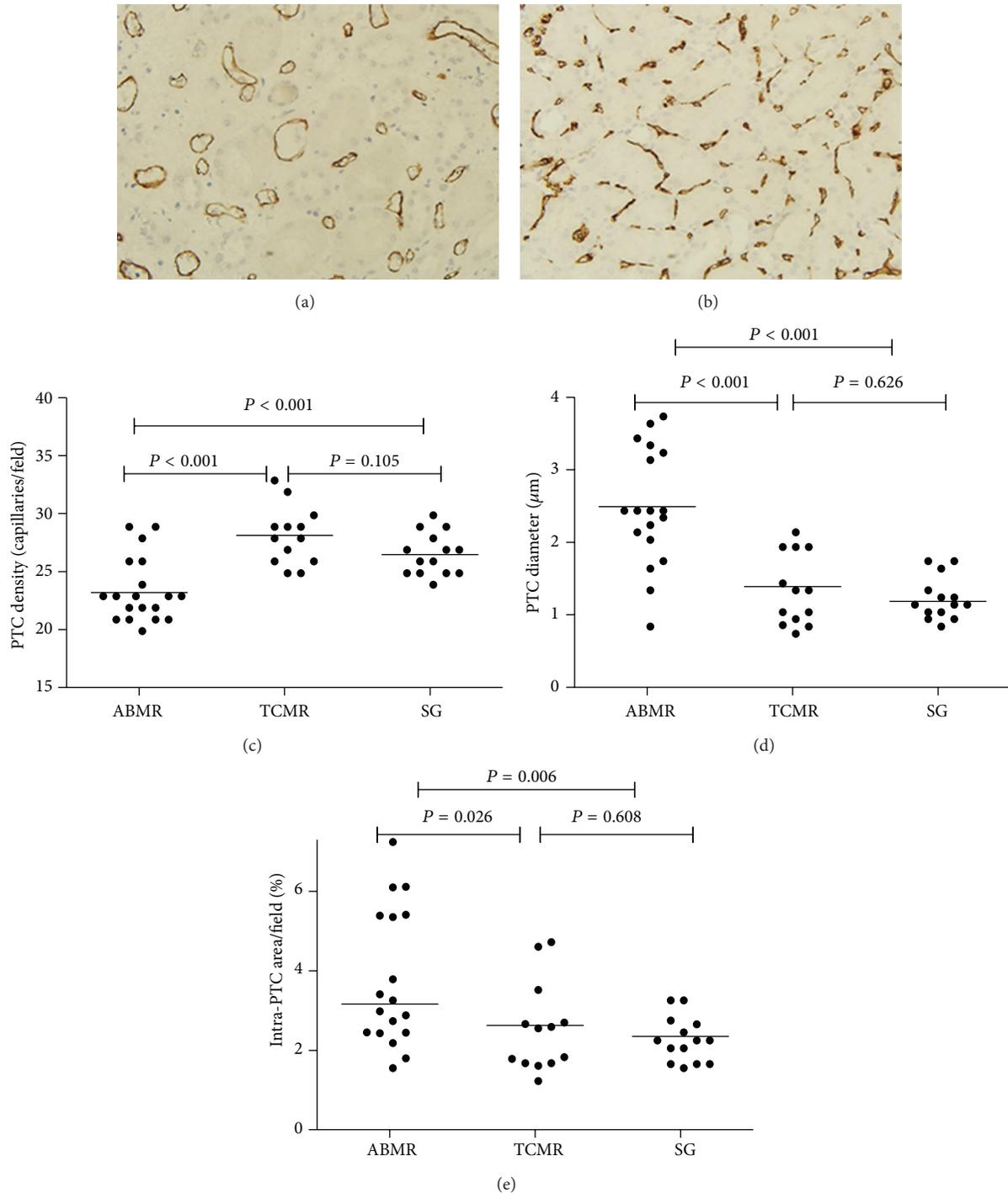


FIGURE 1: Peritubular capillary (PTC) dilation and rarefaction in antibody-mediated rejection (ABMR). Labeling the endothelial cells for CD31 showed PTC dilation and rarefaction in ABMR group (a), compared with PTC staining in T cell-mediated rejection (TCMR) group (b). (c) PTC density among the three groups revealed a rarefaction of PTCs in ABMR group. (d) PTC diameters among the three groups revealed significant PTC dilation in ABMR group. (e) Intra-PTC areas among the three groups revealed significant PTC enlargement in ABMR group. (a, b) Original magnification, $\times 400$.

as shown in Figure 2(a). In contrast, as shown in TCMR group (Figure 1(b)) and SG group (not shown), capillary dilation was rare, and it was also found to mostly coexist with luminal inflammatory cells. We also calculated the ratio of the total

intra-PTC area within the cortex to the area of the entire cortex in ABMR group, and found that this ratio was also strongly correlated with intra-PTC cell counting ($r = 0.578$, $P = 0.006$) (Figure 2(b)).

TABLE 2: Histological characteristics in different groups.

	ABMR (<i>n</i> = 18)	TCMR (<i>n</i> = 13)	SG (<i>n</i> = 14)	<i>Post hoc</i>		
				<i>P</i> ₁₂	<i>P</i> ₁₃	<i>P</i> ₂₃
Histological lesions						
PTC inflammation, <i>n</i> (%)	18 (100)	0	0	—	—	—
Glomerulitis, <i>n</i> (%)	18 (100)	0	0	—	—	—
Tubulitis, <i>n</i> (%)	18 (100)	13 (100)	6 (42.9)	—	<0.001	<0.001
Intimal arteritis, <i>n</i> (%)	16 (88.9)	5 (38.5)	0	<0.001	<0.001	0.005
Intraglomerular infiltrating cells count (cells/mm ²)	5.90 (1.76–9.30)	0.30 (0.00–2.03)	0.00 (0.00–0.60)	0.001	<0.001	0.518
Interstitial						
CD4 (cells/mm ²)	322 (165–426)	276 (181–372)	98 (69–147)	0.477	<0.001	<0.001
CD8 (cells/mm ²)	266 (171–352)	204 (160–424)	96 (45–129)	0.863	<0.001	<0.001
CD4/CD8	1.16 (0.78–1.54)	1.15 (0.97–1.23)	1.18 (0.95–1.33)	0.766	0.849	0.655
CD68 (cells/mm ²)	624 (398–924)	476 (202–614)	94 (50–194)	0.110	<0.001	<0.001
HLA-DR	0.36 (0.12–0.67)	0.20 (0.00–0.67)	0.00 (0.00–0.06)	0.365	0.004	0.049

Abbreviations: ABMR: antibody-mediated rejection; TCMR: T cell-mediated rejection; SG: stable graft; HLA: human lymphocyte antigen; PTC: peritubular capillary.

*P*₁₂ means *P* value for ABMR group and TCMR group; *P*₁₃ means *P* value for ABMR group and SG group; *P*₂₃ means *P* value for TCMR group and SG group.

TABLE 3: BANFF scoring in different groups.

	ABMR (<i>n</i> = 18)	TCMR (<i>n</i> = 13)	SG (<i>n</i> = 14)	<i>Post hoc</i>		
				<i>P</i> ₁₂	<i>P</i> ₁₃	<i>P</i> ₂₃
PTC inflammation						
0	0	13 (100%)	14 (100%)	<0.001	<0.001	—
1	2 (11.1%)	0	0			
2	11 (61.1%)	0	0			
3	5 (27.8%)	0	0			
Glomerulitis						
0	0	13 (100%)	14 (100%)	<0.001	<0.001	—
1	1 (5.6%)	0	0			
2	8 (44.4%)	0	0			
3	9 (50.0%)	0	0			
Tubulitis						
0	0	0	8 (57.1%)	0.126	<0.001	<0.001
1	3 (16.7%)	5 (38.5%)	6 (42.9%)			
2	12 (66.7%)	7 (53.8%)	0			
3	3 (16.7%)	1 (7.7%)	0			
Intimal arteritis						
0	2 (11.1%)	8 (61.5%)	14 (100%)	<0.001	<0.001	0.020
1	6 (33.3%)	5 (38.5%)	0			
2	10 (55.6%)	0	0			
3	0	0	0			

Abbreviations: ABMR: antibody-mediated rejection; TCMR: T cell-mediated rejection; SG: stable graft; PTC: peritubular capillary.

*P*₁₂ means *P* value for ABMR group and TCMR group; *P*₁₃ means *P* value for ABMR group and SG group; *P*₂₃ means *P* value for TCMR group and SG group.

3.5. Microcirculation Inflammation Is Correlated with In Situ T-bet Expression. A significant increase in T-bet expression was detected in the PTCs, and the majority of T-bet⁺ cells were typically located within the capillary lumen. Intra-PTC T-bet expression was also detected in the TCMR group; however, its expression was much lower than that in the ABMR group (1.34 ± 0.56 versus 0.15 ± 0.22 /capillary, $P < 0.001$)

(Figure 3(a)). In the ABMR group, the T-bet expression correlated well with the quantity of infiltrating cells ($r = 0.768$, $P < 0.001$) (Figure 3(b)). Moreover, since PTC dilation is correlated with microcirculation inflammation as above mentioned, we also find a good correlation between the intra-PTC expression of T-bet and the PTC diameter ($r = 0.491$, $P = 0.038$) (Figure 3(c)).

TABLE 4: Capillary variation in different groups.

	ABMR (<i>n</i> = 18)	TCMR (<i>n</i> = 13)	SG (<i>n</i> = 14)	<i>Post hoc</i>		
				<i>P</i> ₁₂	<i>P</i> ₁₃	<i>P</i> ₂₃
Peritubular capillary						
Density (capillaries/field)	22.22 ± 2.51	27.23 ± 2.49	25.64 ± 1.82	<0.001	<0.001	0.105
Diameters (μm)	2.34 ± 0.81	1.21 ± 0.49	1.09 ± 0.29	<0.001	<0.001	0.626
Intra-PTC area/field (%)	3.52 ± 1.76	2.30 ± 1.23	2.01 ± 0.56	0.026	0.006	0.608
Glomerulus						
Area (×10 ⁴ μm ²)	2.16 ± 0.36	2.29 ± 0.29	2.06 ± 0.44	0.274	0.512	0.097
Diameters of loops	165.50 ± 13.59	171.58 ± 11.18	162.14 ± 16.28	0.248	0.503	0.093

Abbreviations: ABMR: antibody-mediated rejection; TCMR: T cell-mediated rejection; SG: stable graft.

*P*₁₂ means *P* value for ABMR group and TCMR group; *P*₁₃ means *P* value for ABMR group and SG group; *P*₂₃ means *P* value for TCMR group and SG group.

TABLE 5: Capillary variation between ABMR and TG.

	ABMR (<i>n</i> = 18)	TG (<i>n</i> = 32)	<i>P</i>
Peritubular capillary			
Density (capillaries/field)	22.22 ± 2.51	23.87 ± 3.92	0.115
Diameters (μm)	2.34 ± 0.81	6.35 ± 2.16	<0.001
Intra-PTC area/field (%)	3.52 ± 1.76	9.53 ± 3.16	<0.001
Glomerulus			
Area (×10 ⁴ μm ²)	2.16 ± 0.36	3.48 ± 0.64	<0.001
Diameters of loops	165.50 ± 13.59	212.43 ± 25.20	<0.001

Abbreviations: ABMR: antibody-mediated rejection; TG: transplant glomerulopathy; PTC: peritubular capillary.

3.6. HIF-1α Expression Is Correlated with PTC Dilation in Both ABMR and TCMR. Similar to the findings in experimental acute renal failure [22], the strongest and most abundant signals were detected in the medulla, and signal intensity and abundance increased from the outer medulla to the deep papilla. Moreover, cortical tubular immunostaining for HIF-1α was positive and even signals can be detected in some glomeruli. According to the results of double staining of CD31 and HIF-1α, capillary dilation and HIF-1α expression occurred somewhat in parallel both in ABMR and TCMR. It is noteworthy that, as shown in Figure 4, PTC dilation could be observed where HIF-1α was up-regulated while HIF-1α expression was not necessary for capillary dilation.

3.7. Capillary Variation between ABMR and TG. In order to compare the capillary variation between ABMR and TG, 32 cases of TG were selected as control group (the baseline characteristics previously described in [12]). We found that the PTC loss is similar in two groups, while the PTC diameters were significantly larger in TG group (2.34±0.81 versus 6.35±2.16, *P* < 0.001), leading to much more severe PTC dilation. Moreover, the diameters of capillary loops within glomerulus were also increased, and the intraglomerulus area was also significantly increased and showed an enlarged glomerulus. Anyway, the sizes of glomerulus as well as capillary loops within glomerulus were similar between ABMR and TCMR cases. (Figure 5 and Table 5).

4. Discussion

This study revealed that ABMR is associated with peritubular capillary dilation and rarefaction. We confirmed that PTC dilation is correlated with microcirculation inflammation, and PTC inflammation is in turn strongly correlated with *in situ* T-bet expression. These data suggest that T-bet plays an important role in the pathogenesis of microcirculation injury in ABMR. These findings shed a new light on the pathogenesis of ABMR.

Though it is the general belief that ABMR is associated with peritubular capillary dilation [23–25], it is not until this study that dilation has been demonstrated clearly in this setting. Our study clearly demonstrates that the PTC diameter and intra-PTC area were significantly increased in the ABMR. Shortly after the development of ABMR, the diameters of PTCs are increased to twice of the control group, which leads to significantly increased intra-PTC area. Nevertheless, the dilation is diffused and can be seen in most of the PTCs, no matter in cortex or medulla. This variation can be regarded as a feature of ABMR as it cannot be observed in TCMR. As the diameter of PTC is easy to measure and evaluate throughout the whole specimen, it may be applicable to biopsy interpretation and may be of good diagnostic utility for ABMR.

Moreover, our data also revealed a rarefaction of PTC during ABMR. To our knowledge, this is the first report on

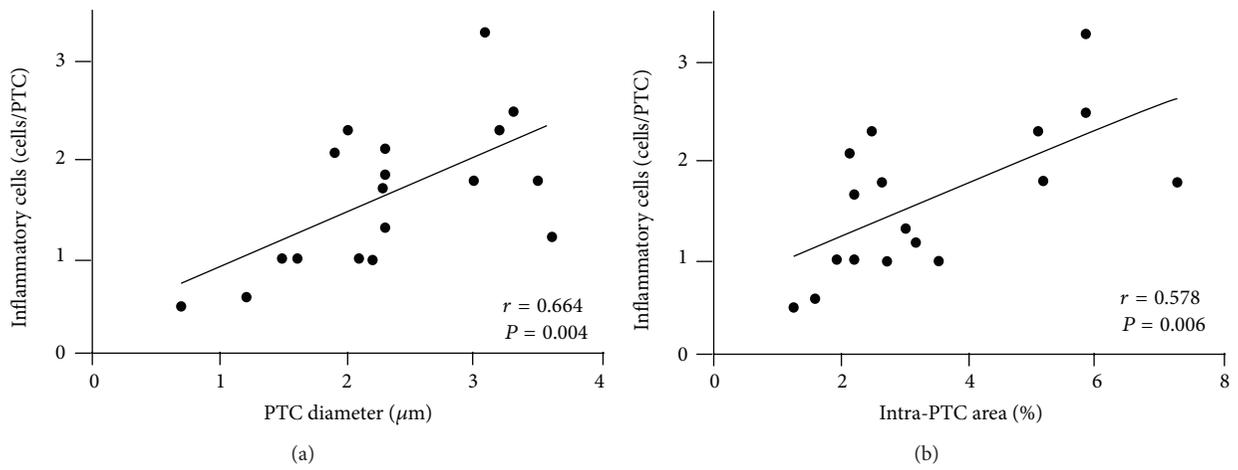


FIGURE 2: The relationship between microcirculation inflammation and peritubular capillary (PTC) dilation. In the antibody-mediated rejection group, PTC diameter (a) and intra-PTC area (b) were strongly correlated with the number of intra-PTC cells.

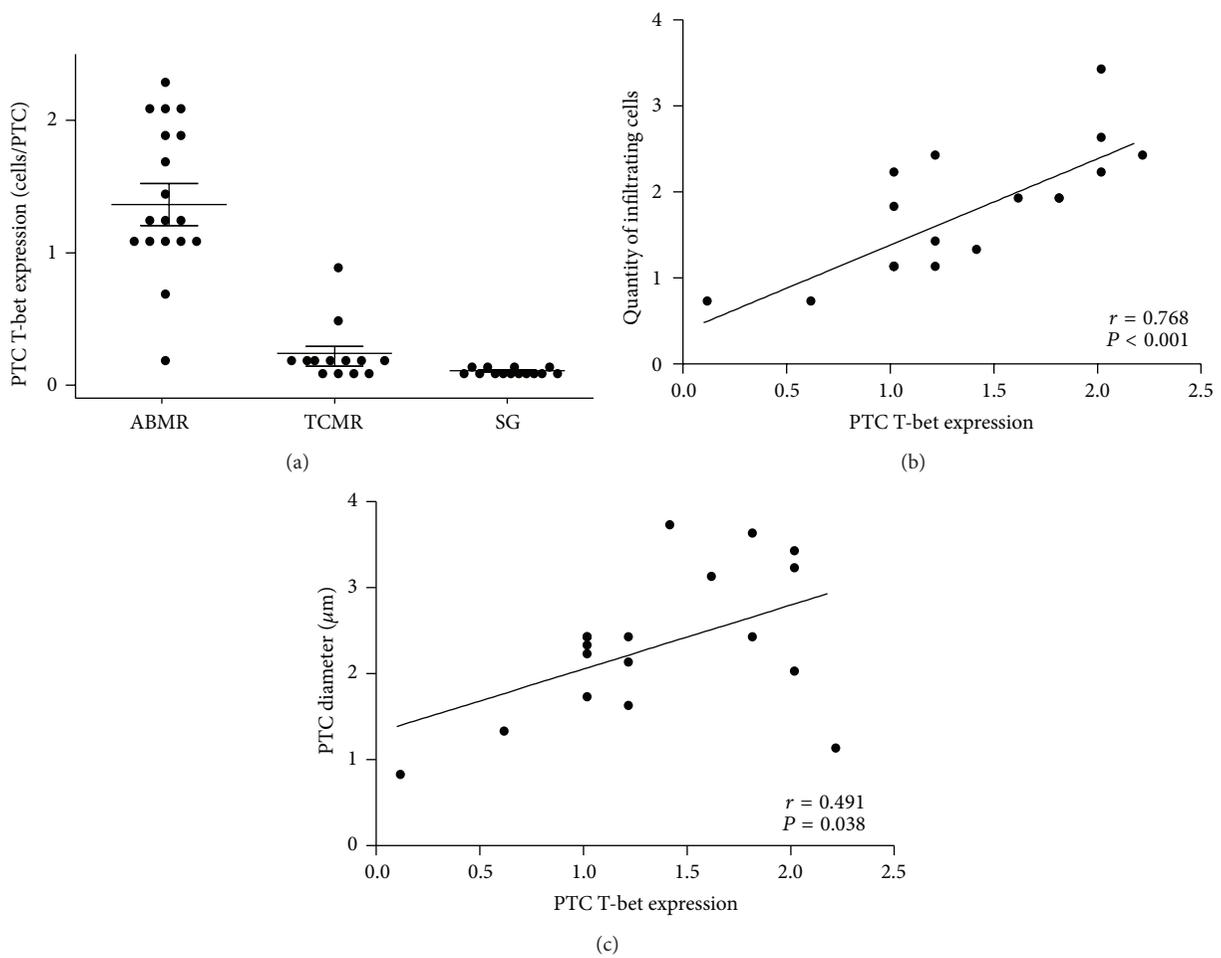


FIGURE 3: T-bet expression in antibody-mediated rejection (ABMR). (a) Quantitative measurement of the number of intra-PTC T-bet-expressing cells in the ABMR, T cell-mediated rejection (TCMR), and stable graft (SG) groups. In the ABMR group, T-bet expression was strongly correlated with the quantity of infiltrating cells (b) and peritubular capillary (PTC) diameter (c).

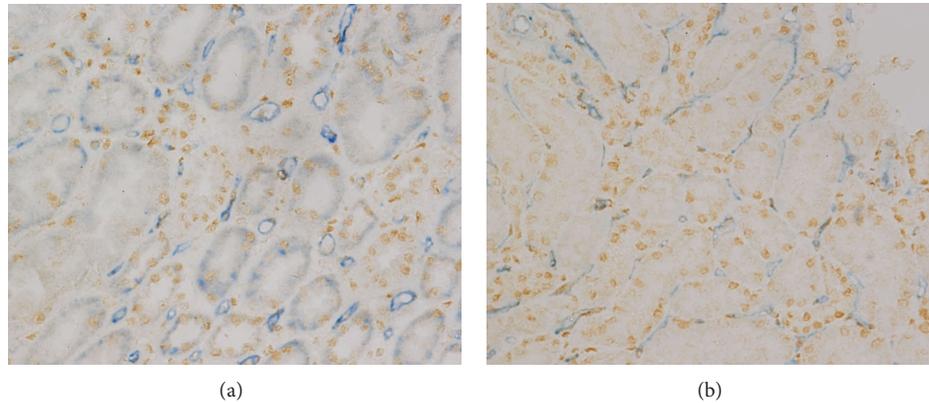


FIGURE 4: HIF-1 α expression in antibody-mediated rejection (ABMR) and T cell-mediated rejection (TCMR). Double staining of CD31 and HIF-1 α showed capillary dilation, and HIF-1 α expression occurred somewhat in parallel both in ABMR (a) and TCMR (b) while HIF-1 α expression was not necessary for capillary dilation. Original magnification, $\times 400$.

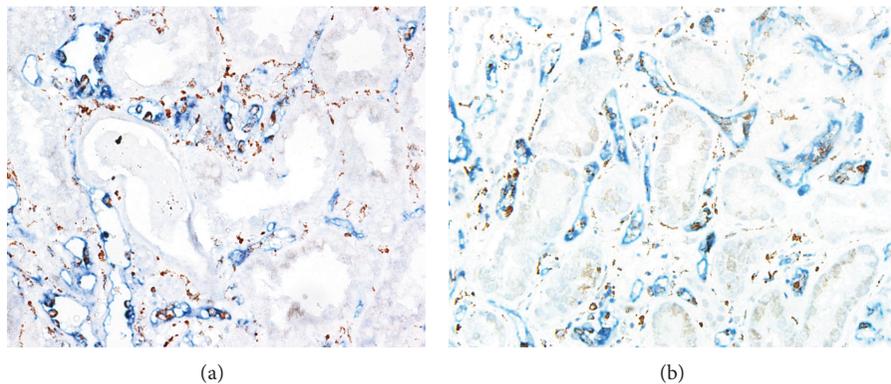


FIGURE 5: Capillary variation between antibody-mediated rejection (ABMR) and transplant glomerulopathy (TG). CD31 and CD68 (labeled in brown) costaining showed peritubular capillary (PTC) dilation was much more severe in the TG group (a) than in the ABMR group (b).

PTC loss when ABMR occurs. Although the diameter of each PTC is increased, the count of PTC is significantly decreased (Figure 1, Table 4) which can be observed in early biopsies immediately after the ABMR occurs. The quick rarefaction of PTC seems to be unique in early ABMR as it is not observed in TCMR or TG. PTC loss has been demonstrated in models involving ageing, cyclosporine, angiotensin II infusion, chronic catecholamine infusion, glomerulonephritis, radiation-induced injury, and potassium depletion [26], which mostly are involved in a local alteration in the balance between angiogenic and angiostatic factors [27, 28]. However, the quick PTC rarefaction during the rejection suggests a dramatic process, which is most probably caused by direct damage to the capillary wall. PTC loss after renal transplantation usually associates with increased interstitial fibrosis/tubular atrophy, and predicts reduced renal function [19]; thus the dramatic PTC loss during ABMR is an important process to graft dysfunction.

The pathogenesis of PTC variation during ABMR is not clear; however, as the dilation can be observed quickly when

ABMR occurs, it should be a response to the antibody activity. Our data revealed that the PTC dilation is strongly correlated with intra-PTC inflammation. Although interstitial inflammation might cause quick vessel dilations in the early phase [29], the PTC dilation is more likely to be related to intra-PTC inflammation, as the dilation cannot be observed in TCMR cases, which have more severe interstitial inflammation. Moreover, the dilation of the PTC lumen was strongly correlated with the degree of microcirculation inflammation, whereas PTC dilation was mostly observed in areas with PTC inflammation. This finding demonstrates a strong correlation between capillary dilation and PTC cellular infiltration. This phenomenon can be seen in TG as well and it is quite likely that the capillary dilation is caused by *in situ* inflammation. Those data suggest a central role of inflammation in the development of microcirculating variation during ABMR.

Furthermore, we find that intra-PTC inflammation is strongly correlated with *in situ* expression of T-bet. This correlation is unique to the ABMR group, as the TCMR and stable graft groups, which rarely have microcirculation

inflammation, only rarely exhibited T-bet expression in the PTC areas. T-bet is a member of the T-box family of transcription factors and is a key determinant of T-helper cell differentiation into Th1. Our previous study showed that T-bet expression is correlated with glomerulitis in ABMR, further study revealed that both glomerulitis and PTCitis are correlated with T-bet expression in TG, which is a chronic form of ABMR. This study proved that both kinds of microcirculating inflammation, PTCitis and glomerulitis, are correlated with T-bet expression, no matter in acute ABMR or chronic type. Higher T-bet expression is correlated with more PTC inflammation, and in turn is correlated with larger PTC diameters. This study suggests that T-bet pathway might be a potential target in the management of ABMR.

As capillary damage may lead to local hypoxia, it is possible that hypoxia is involved in the pathogenesis of ABMR. At experimental level, there has also been described an intense dilation of the peritubular capillaries in chronic allograft dysfunction [26, 30], and the morphological changes were thought to be related to chronic ischemia [26]. We hypothesized that hypoxia takes part in the development of PTC dilation. Since HIF allows for hypoxia detection at a single cell resolution [31–33], and as previously described *in vivo* [34], renal HIF-1 α immunostaining was almost exclusively found in tubular segments, HIF-1 α immunostaining had been performed in this cohort. Somewhat surprising, PTC dilation could be observed where HIF-1 α was up-regulated while some dilation had nothing to do with HIF-1 α expression, which suggests that hypoxia is not necessary for PTC dilation in ABMR. Obviously the PTC dilation in ABMR has a pathway that is different to chronic allograft dysfunction.

Current data also showed that the microcirculating variation in ABMR is similar to its chronic pattern, TG. Both ABMR and TG have significant PTC dilation, which is correlated with PTC inflammation, and in turn with *in situ* T-bet expression. It suggests that both acute and chronic patterns of humeral rejection share a common mechanism of PTC variation; T-bet expression might account for the development of microcirculation inflammation. However, in TG, the PTC dilation is much more severe than in ABMR, and there are enlarged glomerulus loops and glomerulus size, which are not shown in the ABMR group. Those changes might be caused by continuous antibody activity.

The diagnosis of ABMR has largely depended on the C4d staining in the past decade. However, in spite of high specificity, C4d is lacking sensitivity, and many cases of rejection with anti-HLA are C4d negative [7, 35–37]. The variation of PTC during ABMR, including PTC dilation and rarefaction, differs to TCMR and may be used in the diagnosis of ABMR. Moreover, our previous study had reported that the predominance of T-bet over GATA-3 may distinct ABMR from TCMR [13]; the current data even showed that the predominance of T-bet expression in PTC is a feature of ABMR and can also be regarded as a diagnosis marker.

In summary, this study shows that ABMR is associated with PTC dilation and the latter is correlated with microcirculation inflammation, and microcirculation inflammation is strongly correlated with *in situ* T-bet expression. These results

suggest that inflammation may take part in the pathogenesis of PTC dilation, and they warrant further investigation.

Abbreviations

ABMR: Antibody-mediated rejection
TCMR: T cell-mediated rejection
TG: Transplant glomerulopathy
PTC: Peritubular capillary
SG: Stable graft
HLA: Human lymphocyte antigen.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

The Effect of Combination Therapy with Rituximab and Intravenous Immunoglobulin on the Progression of Chronic Antibody Mediated Rejection in Renal Transplant Recipients

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The treatment for chronic active antibody-mediated rejection (CAMR) remains controversial. We investigated the efficacy of rituximab (RTX) and intravenous immunoglobulin (IVIg) for CAMR. Eighteen patients with CAMR were treated with RTX (375 mg/m²) and IVIg (0.4 g/kg) for 4 days. The efficacy of RTX/IVIg combination therapy (RIT) was assessed by decline in estimated glomerular filtration rate per month (Δ eGFR) before and after RIT. Patients were divided into responder and nonresponder groups based on decrease and no decrease in Δ eGFR, respectively, and their clinical and histological characteristics were compared. Response rate to RIT was 66.7% (12/18), and overall Δ eGFR decreased significantly to 0.4 ± 1.7 mL·min⁻¹·1.73 m⁻² per month 6 months after RIT compared to that observed 6 months before RIT (1.8 ± 1.0 , $P < 0.05$). Clinical and histological features between the 12 responders and the 6 nonresponders were not significantly different, but nonresponders had a significantly higher proteinuria levels at the time of RIT (2.5 ± 2.5 versus 7.0 ± 3.5 protein/creatinine (g/g), $P < 0.001$). The effect of the RIT on Δ eGFR had dissipated in all patients by 1 year post-RIT. Thus, RIT delayed CAMR progression, and baseline proteinuria level was a prognostic factor for response to RIT.

1. Introduction

Circulating alloantibodies are found in a substantial number of renal allograft recipients, and the presence of these alloantibodies is significantly correlated with the development of allograft injury and later graft loss [1–3]. In renal allograft tissue, chronic injury is represented microscopically as transplant glomerulopathy and diffuse C4d deposition in peritubular capillaries (PTCs); recently, it was included as new disease entity named chronic antibody-mediated rejection (CAMR) in the update of the Banff 05 classification

[4]. Usually the prognosis of CAMR is poor, and conventional immunosuppressants mainly targeting T cell-mediated immunity cannot prevent or reverse it [5–7]. Therefore, some researchers have suggested that therapies directed at the humoral response may be required for the treatment of CAMR [3].

Recently, some reports have suggested that the combined use of rituximab (RTX) and intravenous immunoglobulin (IVIg) therapy may be useful for the treatment of CAMR. Billing et al. published their experience with the RTX and IVIg combination protocol for treatment of CAMR in 6

pediatric patients, and they subsequently reported the long-term effects of this protocol [8, 9]. In adult renal transplant recipients, only a few studies have been published. Fehr et al. demonstrated that allograft function of CAMR was improved or stabilized with the RTX and IVIg combination therapy in 4 cases [10]. Our preliminary study also showed that the combination therapy was effective in delaying the progression of CAMR, especially in its early stages [11]. However, the above studies were conducted with small numbers of adult patients during periods of relatively short duration.

For these reasons, we decided to perform a study investigating the efficacy of the RTX and IVIg protocol for the treatment of CAMR, using a larger group of adult patients and with a longer period of followup.

2. Patients and Method

2.1. Diagnosis of CAMR. The diagnosis of CAMR was based on the update on Banff classification: (1) transplant glomerulopathy and severe peritubular capillary basement membrane multilayering (PTCBMM), interstitial fibrosis (IF) and tubular atrophy (TA) with or without peritubular capillary loss, and fibrous intimal thickening in arteries without internal elastic duplication; (2) diffuse C4d deposition in PTCs; and (3) presence of donor-specific anti-HLA antibody (DSA) [4]. Among allograft biopsies done between September 2009 and December 2012, in Seoul St. Mary's Hospital, 16 cases met the above Banff criteria. We also included 2 patients who did not fully satisfy with the criteria (negative HLA-DSA and C4d score 0 and score 1) but showed typical transplant glomerulopathy with slowly deteriorating graft function. Finally 18 patients were included in this study.

2.2. Patient Characteristics. Patient characteristics are shown in Table 1. The mean age of the patients was 44.0 ± 7.1 years at the time of CAMR diagnosis; 13 patients (72%) were male. Of the 18 patients, 11 (61%) received kidneys from living donors and 2 patients had histories of retransplantation. Eight of the 18 patients (44%) experienced acute rejection, including both antibody-mediated and T cell-mediated rejections, before CAMR. The median time posttransplant until the diagnosis of CAMR by renal graft biopsy was 93.2 months (range: 8.2–214.9). The follow-up duration after treatment was 14.1 months (range: 1.4–31.9). This study was approved by the Institutional Review Board of our institution (KC12RISI0070).

2.3. Protocol of Rituximab/IVIg Combination Therapy for CAMR. The protocol in our institution for the treatment of CAMR has been described previously (RIT protocol) [11]. Briefly, all patients were treated with IV RTX (375 mg/m^2) once on day 1 followed by IVIg, 0.4 g/kg , once daily for 4 days. Pulse methylprednisolone at a dose of 500 mg IV was administered daily for the first 3 days, followed by oral prednisolone, tapered to 30 mg/day . We measured anti-HLA antibody using Luminex solid-phase assays (LSA; Tepelex Lifecodes Corp., Stamford, CT) at the time of biopsy. If the type of anti-HLA antibody detected in the patient corresponded to the HLA

TABLE 1: Baseline characteristics of patients populations at treatment of CAMR.

Clinical parameters	All patients ($n = 18$)
Age (years)	44.0 ± 7.1
Male gender, n (%)	13 (72)
BMI (Kg/m^2)	24.3 ± 2.7
Primary renal disease	
cGN, n (%)	7 (39)
HBP, n (%)	6 (33)
DM, n (%)	1 (6)
Unknown, n (%)	4 (22)
Dialysis type before KT	
Hemodialysis, n (%)	13 (72)
Peritoneal dialysis, n (%)	5 (28)
Dialysis duration, month	24.6 ± 24.5
Donor type, Living, n (%)	11 (61)
Multitransplant History, n (%)	2 (11)
Main immunosuppressant	
Cyclosporine, n (%)	6 (33)
Tacrolimus, n (%)	12 (67)
Previous acute rejection, n (%)	8 (44)
Serum Cr (mg/dL)	2.3 ± 0.9
MDRD eGFR ($\text{mL/min}/1.73 \text{ m}^2$)	35.8 ± 16.1
Proteinuria (g/day)	4.3 ± 3.6
Time posttransplant until diagnosis, month	93.2 ± 61.5
Time posttreatment, month	14.1 ± 9.3
HLA mismatch number	3.2 ± 1.4
HLA-DSA	
Not done, n (%)	2 (11)
Positive, Class I, n (%)	2 (11)
Positive, Class II, n (%)	5 (28)
Negative, n (%)	9 (50)

CAMR: chronic antibody mediate rejection; BMI: body mass index; cGN: chronic glomerulonephritis; Cr: creatinine; MDRD eGFR: estimated GFR using the Modification of Diet in Renal Disease Study equation; HLA-DSA: donor specific anti-HLA antibody.

type of the donor, it was regarded as a donor-specific anti-HLA antibody (HLA-DSA). The results were presented as 4 levels, according to the median fluorescent intensity (MFI) value: strong, $>10,000$; moderate, $5000\text{--}10,000$; weak, $1000\text{--}5000$; and negative, <1000 .

2.4. Efficacy of Treatment Protocol. The primary outcome of this study was improvement in allograft function after treatment. Allograft function was assessed on the basis of serum creatinine levels and estimated glomerular filtration rate (eGFR), using the modification of the diet in renal disease (MDRD) formula ($\text{eGFR} = 186.3 \times \text{serum creatinine}^{-1.154} \times \text{age}^{-0.263} [\times 0.742 \text{ if female}] \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^2$) [12]. We calculated the decline in the rate of eGFR per month (ΔeGFR) during the 6 months before and after RIT and at 6-month intervals until the last followup. We also evaluated the amount of proteinuria ($\text{g protein/g creatinine (g/g)}$) in random urine chemistry, collected 6 months before RIT, at the time of RIT,

TABLE 2: Histopathology of allograft biopsy and grading according to Banff 05'.

Characteristics (total $n = 18$)*	Score	N (%)
Transplant glomerulopathy (cg)	0	7 (41)
	1	0 (0)
	2	0 (0)
	3	10 (59)
PTC BMM	(-)	7 (41)
	(+)	10 (59)
C4d in PTC	0	3 (18)
	1	3 (18)
	2	9 (53)
	3	2 (11)
Peritubular capillaritis (ptc)	0	4 (24)
	1	1 (6)
	2	5 (29)
	3	7 (41)
Interstitial fibrosis (ci)/Tubular atrophy (ct)	0	1 (6)
	1	9 (50)
	2	7 (38)
	3	1 (6)

PTC: peritubular capillary; BMM: basement membrane multilayering.

*17 subjects had available data about transplant glomerulopathy, PTC BMM and C4d in PTC.

6 months after 6 RIT, and at 6-month intervals thereafter. Finally, we divided the patient populations into 2 groups, according to their response to the therapy. The responder group was comprised patients who showed a decrease of Δ eGFR during the 6 months after RIT; the patients who showed an increase or no decrease of Δ eGFR after treatment were regarded as nonresponder group. To investigate the factors associated with the response to the therapy, we compared (1) the clinical characteristics, (2) the change in allograft function and amount of proteinuria, (3) histopathologic findings, and (4) alloantibodies between the two groups.

2.5. Statistical Analysis. Data were expressed as mean and standard deviation (SD). Means of normally distributed data were compared using Student's t -test; a χ^2 -test was used to compare proportions. The changes in eGFR before and after treatment were evaluated by paired comparison. Graft survival rates after RIT were calculated using Kaplan-Meier analysis, and we used the log-rank analysis to compare survival rates between groups. The association of the degree of histological lesions with the response to RIT was explored with Fisher's exact test. In all analyses, $P < 0.05$ (two-tailed) was taken to indicate statistical significance.

3. Results

3.1. Histologic and Immunologic Characteristics. Table 2 shows the histological characteristics at diagnosis of CAMR. Transplant glomerulopathy and PTCBMM were found in 10 patients (59%). Advanced chronic changes such as interstitial

fibrosis and tubular atrophy were detected in most patients (17/18, 94%), and the staining for C4d was diffusely positive in 14 out of 18 patients (82%). In 16 patients who were examined for HLA-DSA using LSA at the time of biopsy, anti-HLA antibody was detected in 10 patients, and of these, 6 patients were identified to have HLA-DSA. HLA-DSA showed strong MFI in only 1 patient, moderate intensity in 3, and weak intensity in 2.

3.2. The Response to RTX/IVIG Treatment in Terms of Allograft Function. All patients tolerated RIT well and completed treatment without immediate adverse effects. Before RIT, progressive deterioration of allograft function was found in all patients. At 6 months before RIT, eGFR was $48.1 \pm 17.5 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ and progressively declined to $37.1 \pm 15.6 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ at the time of RIT ($P < 0.001$). The calculated Δ eGFR was $1.8 \pm 1.0 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month during that period. Six months after RIT, eGFR was $34.7 \pm 19.2 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$, which is similar to that at the time of RIT ($P = 0.40$), and Δ eGFR 6 months after RIT was $0.4 \pm 1.7 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month, which is significantly lower than that at 6 months before RIT ($P < 0.05$). Proteinuria increased significantly from $3.0 \pm 3.7 \text{ g/g}$ at 6 months before RIT to $4.3 \pm 3.6 \text{ g/g}$ at the time of RIT ($P < 0.05$). The amount of proteinuria showed a decreasing trend at 6 months since RIT ($3.0 \pm 2.2 \text{ g/g}$, versus that at the time of RIT, $P = 0.129$) compared to the value at the time of RIT; this trend was observed even at the last followup ($2.9 \pm 2.7 \text{ g/g}$, versus at the time of RIT, $P = 0.136$).

3.3. Comparison between Responder and Nonresponder Groups. According to the change in Δ eGFR during 6 months after RIT compared to that observed 6 months before RIT, 12 patients (67%) met the criteria for the responder group, and the other 6 patients, for the nonresponder group. The eGFR at 6 months before RIT (45.4 ± 16.4 versus $54.5 \pm 20.2 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$, $P = 0.347$) and that at the time of RIT (34.2 ± 14.3 versus $39.0 \pm 20.3 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$, $P = 0.568$) were not significantly different between 2 groups. The Δ eGFR (1.9 ± 1.1 versus $1.8 \pm 0.9 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month, $P = 0.83$) 6 months before RIT did not differ between the 2 groups, as well. Δ eGFR decreased to $-0.3 \pm 1.2 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month 6 months after RIT in the responder group compared to that observed 6 months before RIT ($1.9 \pm 1.1 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month, $P < 0.01$). In contrast, nonresponders showed relatively higher Δ eGFRs 6 months after RIT ($2.5 \pm 0.8 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month) compared to that before 6 months, which suggests that the allograft function was still rapidly deteriorating ($P = 0.105$; Figure 1). In comparison, the amount of proteinuria at the time of RIT was significantly higher in the nonresponder group ($7.0 \pm 3.5 \text{ g/g}$) than in the responder group ($2.8 \pm 2.8 \text{ g/g}$, $P < 0.05$). However, the histological features and other clinical parameters did not show any significant differences. The positivity of HLA-DSA at biopsy did not differ either (Table 3).

TABLE 3: Comparison of parameters between responder and nonresponder groups at treatment of CAMR.

Clinical parameters	Responder (<i>n</i> = 12)	Nonresponder (<i>n</i> = 6)	<i>P</i> value
Age (years)	44.0 ± 7.0	44.3 ± 7.9	0.928
Male gender, <i>n</i> (%)	8 (67)	5 (83)	0.615
BMI (Kg/m ²)	24.0 ± 3.2	24.9 ± 1.8	0.518
Multitransplant History, <i>n</i> (%)	1.0 ± 0.0	1.3 ± 0.5	0.175
Previous acute rejection, <i>n</i> (%)	0.5 ± 0.7	0.7 ± 0.8	0.650
Serum Cr (mg/dL)	2.3 ± 0.7	2.4 ± 1.3	0.809
MDRD eGFR (mL/min/1.73 m ²)	34.2 ± 14.3	39.0 ± 20.3	0.568
Proteinuria (g/day)	2.8 ± 2.8	7.0 ± 3.5	0.015
Time posttransplant before CAMR diagnosis, month	106.1 ± 65.6	67.3 ± 46.8	0.217
Time posttreatment, month	13.9 ± 7.8	14.6 ± 12.6	0.889
HLA-DSA, MFI*			0.629
Strong, <i>n</i> (%)	1 (10)	0 (0)	
Moderate, <i>n</i> (%)	1 (10)	2 (50)	
Weak, <i>n</i> (%)	2 (20)	0 (0)	
Negative, <i>n</i> (%)	6 (60)	2 (50)	
Histologic parameters			
Transplant glomerulopathy	1.75 ± 1.5	1.8 ± 1.6	0.953
PTCBMM (+/-)	7/5	3/2	0.951
Peritubular capillaritis	1.9 ± 1.3	1.8 ± 1.1	0.864
IF/TA	1.4 ± 0.8	1.5 ± 0.5	0.821
C4d in PTC	2.25 ± 1.4	2.8 ± 0.8	0.436

CAMR: chronic antibody mediate rejection; BMI: body mass index; Cr: creatinine; MDRD eGFR: estimated GFR using the Modification of Diet in Renal Disease Study equation; HLA-DSA: donor specific anti-HLA antibody; PTC: peritubular capillary; BMM: basement membrane multilayering.

*16 out of 18 subjects take HLA-DSA and 14 had available data.

3.4. The Clinical Outcome during Long-Term Followup after Treatment. During long-term followup, only 1 case developed herpes zoster infection; no other serious complications were detected. Four patients (39%) exclusively in the nonresponder group experienced allograft loss at 1.4, 5.1, 8.6, and 11.9 months since treatment with RIT, and no allograft loss was noted in the responder group (Figure 2). In 7 patients with a follow-up duration of >12 months, the Δ eGFR observed 6–12 months after RIT ($0.5 \pm 0.7 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month) was still lower than that observed 6 months before RIT ($1.6 \pm 1.1 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month, $P < 0.05$). However, Δ eGFR showed an increasing trend over the final 12 months until the last followup ($1.2 \pm 0.8 \text{ mL}\cdot\text{min}^{-1}\cdot 1.73 \text{ m}^{-2}$ per month), at which it showed a value similar to that 6 months before RIT (Figure 3).

4. Discussion

In this study, 18 adult patients who were diagnosed as CAMR or suspicious of CAMR were treated with RTX and IVIg combination protocol. After this combination treatment, the rate of decline in allograft function decreased significantly in most patients, which suggests that this combination therapy is effective in delaying the progression of CAMR.

The effect of the combination therapy with RTX and IVIg on CAMR in pediatric patients has been reported in previous studies [8, 9]. However, the effect of the combination therapy

in adult renal transplant recipients has not been established. We previously reported the beneficial effect of that therapy in 6 adult patients [11]. In this study, we investigated the effect of our protocol in larger patient group with longer follow-up period. The detailed mechanism for the development of CAMR has not been fully elucidated; however, in nature, antibody-mediated injury may be the main pathogenetic mechanism of CAMR [2, 3]. IVIg can suppress immunoglobulin synthesis, has anti-idiotypic activity against DSA (with resultant neutralization of DSA), blocks the Fc receptor, inhibits complement activation, and has anticytokine activity [13]. RTX, a chimeric anti-CD20 monoclonal antibody, can induce antibody-dependent cytotoxicity, complement-dependent cell killing, and apoptotic cell death, especially in B cells. Consequently RTX depletes B cells and interferes with antigen-presenting cell activity of B cells [14]. For this reason, RTX and IVIg, which target humoral immunity by different action mechanism, have been proposed as a therapeutic option for CAMR [8].

At first, we investigated the effect of this combination therapy on the progression of CAMR by comparing the rate of decline in eGFR before and after RIT. After RIT, the overall Δ eGFR slopped down, and in particular, in 12 out of 18 patients (67%), the Δ eGFR showed a significant decrease, which is similar to the result from a previous report [9]. The amount of proteinuria, which is poor prognostic factor for allograft outcome, showed a decrease after RIT, as well [15–17]. In addition, this protocol is well tolerated, and fatal

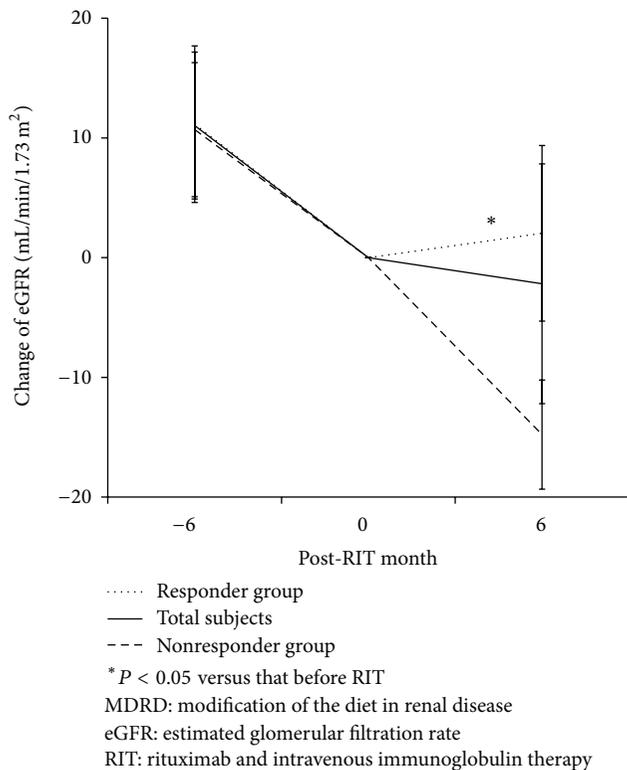


FIGURE 1: Comparison of changes in allograft function between the responder and nonresponder groups. eGFR showed sustained decline during 6 months before RIT in both responder and nonresponder groups. After RIT, eGFR in the responder group showed significant increase; however, decline of eGFR was persisted in the nonresponder group.

infectious complications were not detected during the long-term follow-up period. All the above findings suggest that this protocol is not only effective but also safe for treating patients with CAMR.

However, 6 patients did not show a significant response to therapy and 4 out of the 6 patients in the nonresponder group experienced allograft failure within 1 year since treatment with RIT. To investigate the risk factors associated with this lack of response to the RIT protocol, we compared the clinical parameters between the responder and nonresponder groups. We did not find any significant differences in clinical characteristics. Of note, however, the amount of proteinuria at the time of RIT was significantly higher in nonresponder group than in the responder group. This finding is consistent with a previous study that showed that proteinuria is associated with more severe acute and chronic allograft rejection [18]. In contrast, allograft function at the time of RIT and the rate of decline in eGFR observed 6 months before RIT did not differ between the two groups. This suggests that the severity of allograft dysfunction does not predict the response to treatment.

In contrast to some previous reports, histological features were not associated with clinical outcomes in our study. For example, the proportion of transplant glomerulopathy and

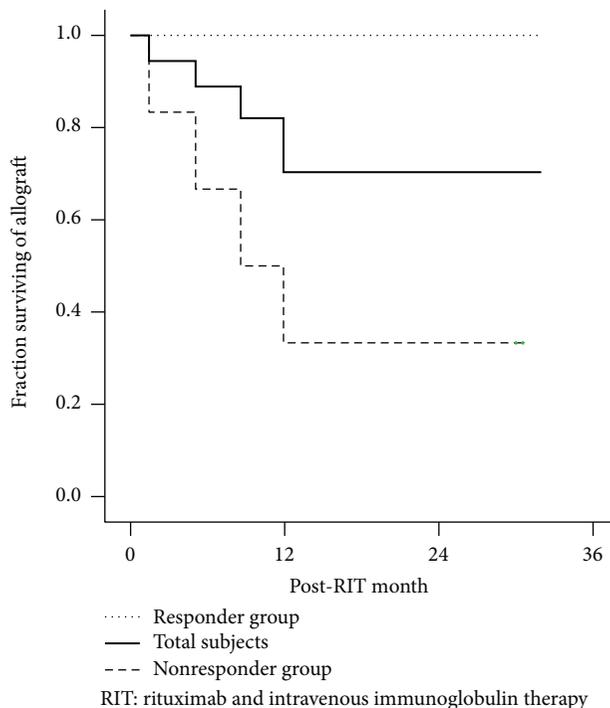
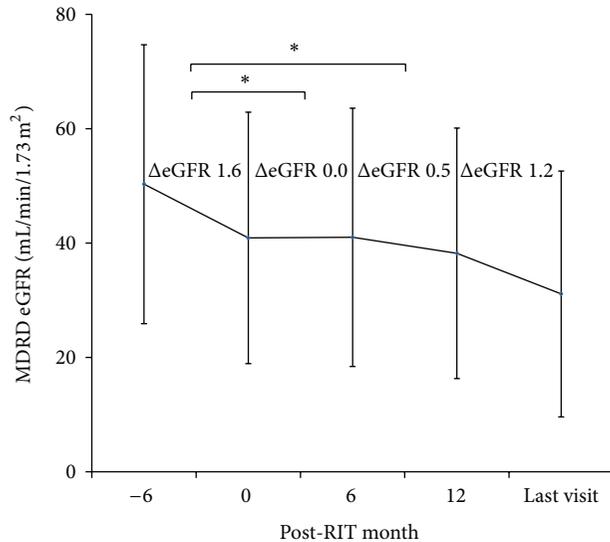


FIGURE 2: Allograft survival rate of the total number of patients and of those in the responder and nonresponder groups. During followup, the survival rate was 89%, 82%, and 70%, at 6, 12, and 24 months, respectively, in all patients. The responder group showed a significantly higher survival rate compared to the nonresponder group ($P = 0.005$).

the severity of IF/TA, which is an important morphologic pattern of chronic kidney allograft injury, did not differ between the two groups [5, 19–21]. This result suggests that the histologic pattern is a prognostic factor in CAMR that progresses without intervention; however, it may not be an accurate prognostic indicator with the use of antihumoral therapy. Indeed, a previous study reported that pathological correlations that predicted the response to therapy were not identified [22]. Further investigation may be required to clarify this issue.

In the long-term followup, the therapeutic effect of RIT showed a decreasing trend with time, especially after 1 year since RIT initiation. In this study, 4 patients with a follow-up duration >2 years were included, and the time-dependent decrease in eGFR was detected. Interestingly, this pattern was found 6 months after RIT treatment not only in the nonresponder group but also in the responder group. The decrease may be associated with the duration of the B cell-depleting effect of RTX. A previous study showing RTX-induced B cell depletion in the peripheral blood indicated that patients recover approximately 6 months since RTX infusion [23], which suggests that the therapeutic effect of RIT on the progression of CAMR may be limited to this time period; accordingly, repeated RIT therapy or other additional strategies for humoral immunity such as bortezomib may be necessary to prolong the therapeutic effect [24–27].



* $P < 0.05$ versus that observed 6 months before RIT
 MDRD: modification of the diet in renal disease
 eGFR: estimated glomerular filtration rate
 Δ eGFR: decline rate of eGFR per month ($\text{mL}\cdot\text{min}^{-1}\cdot 1.73\text{ m}^{-2}$ per month)
 RIT: rituximab and intravenous immunoglobulin therapy

FIGURE 3: Changes in Δ eGFR values in 7 patients with long-term followup over 12 months. With time, Δ eGFR gradually decreased at every 6-month interval and the Δ eGFR at 12 months from RIT and that at the last followup showed similar values compared to that observed 6 months before RIT.

The combination of RTX and IVIg showed a relatively long-term effect in pediatric renal transplant recipients with CAMR over 2 years, in contrast to this study [8, 9]. The possible reason is that the response to RIT may differ between adult and pediatric CAMR patients. The hematopoietic bone marrow contains mostly naive B cells of diverse specificities and has only a small number of memory B cell clones in childhood. Usually, memory B cells and plasma cell, which are responsible for the development of CAMR, accumulate with age [28]. Hence, these different immunologic characteristics, the higher memory B cell, and plasma cell pool in adult patients, may be associated with the limited long-term effect to RIT [29].

In our study, we included two patients who were not satisfied with the diagnostic criteria of CAMR; they did not show C4d deposition on biopsy tissue and DSA was not detected. We enrolled those patients for two reasons. First, it is strongly suggested that typical transplant glomerulopathy on allograft biopsy is responsible for slowly deteriorating allograft function. Second, there is a possibility that C4d or alloantibody is not detected even in the presence of morphologic evidence of antibody-mediated rejection [30]. It suggests that CAMR is a dynamic process and is difficult to make a clear-cut diagnosis.

This study has some limitations. First, we did not perform follow-up biopsies. Despite a significant decrease in Δ eGFR, we could not prove this benefit in the allograft tissue, for example, in the reduction of positive C4d or transplant

glomerulopathy. Second, we did not include an untreated control group with CAMR. A larger randomized study, including treated subjects and untreated controls, may be required to prove the efficacy of RIT.

In conclusion, this study showed that the combination of RTX and IVIg is an effective treatment in delaying the progression of CAMR. In addition, the amount of proteinuria at the time of treatment is the most important prognostic factor for predicting the patient's response to RTX/IVIg combination therapy. However, the therapeutic effect showed a decreasing pattern over 1 year after RIT, which indicates that additional therapeutic strategy may be required in such patients.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Incidence and Clinical Significance of *De Novo* Donor Specific Antibodies after Kidney Transplantation

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Kidney transplantation has evolved over more than half a century and remarkable progress has been made in patient and graft outcomes. Despite these advances, chronic allograft dysfunction remains a major problem. Among other reasons, *de novo* formation of antibodies against donor human leukocyte antigens has been recognized as one of the major risk factors for reduced allograft survival. The type of treatment in the presence of donor specific antibodies (DSA) posttransplantation is largely related to the clinical syndrome the patient presents with at the time of detection. There is no consensus regarding the treatment of stable renal transplant recipients with circulating *de novo* DSA. On the contrast, in acute or chronic allograft dysfunction transplant centers use various protocols in order to reduce the amount of circulating DSA and achieve long-term graft survival. These protocols include removal of the antibodies by plasmapheresis, intravenous administration of immunoglobulin, or depletion of B cells with anti-CD20 monoclonal antibodies along with tacrolimus and mycophenolate mofetil. This review aims at the comprehension of the clinical correlations of *de novo* DSA in kidney transplant recipients, assessment of their prognostic value, and providing insights into the management of these patients.

1. Introduction

Despite all advances in the development of effective immunosuppressive regimens in kidney transplantation, chronic allograft dysfunction remains a major problem [1]. Humoral immune response contributes to the development of this entity. Pretransplantation unsensitized kidney transplant recipients may develop *de novo* antibodies against donor human leukocyte antigens (HLA) or non-HLA as are the polymorphic MHC system class I chain-related gene A (MICA) molecules [2]. *De novo* formation of donor specific antibodies (DSA), directed against HLA, has been recognized as one of the major risk factors for reduced allograft survival. First observation was in 2002, when anti-HLA antibodies were shown to appear 6 months to 8 years before graft failure in a serial longitudinal study [3]. Now, we have substantial evidence, showing that formation of *de novo* DSA after kidney transplantation is associated with antibody-mediated graft injury that may lead to graft failure [4, 5]. Anti-HLA class II DSA are considered the predominant *de novo* produced

antibodies posttransplantation in unsensitized pretransplantation renal transplant recipients [6–8]. The introduction of more sensitive and specific assays [9], as well as serial evaluation of multiple samples [10] from the same patient, allowed the detection of DSA after transplantation and comprehend of their interference to graft dysfunction. The aim of this paper is to review the incidence of *de novo* detected anti-HLA and anti-MICA DSA after kidney transplantation, underlining their clinical impact and pathologic correlations and assess their prognostic ability to transplantation outcome. Insights into the management of patients with posttransplant formation of DSA are also within the scope of this review.

2. *De Novo* Developed Posttransplantation Donor Specific Antibodies

Patient's exposure to "nonself-" HLA molecules as after blood transfusion, pregnancy, or organ transplantation can lead to the development of anti-HLA antibodies [11, 12]. Thus, a

transplant candidate may present with preformed anti-HLA antibodies, while being in the waiting list. The antibodies that do not preexist but develop after transplantation and are directed against foreign graft HLA are considered as *de novo* anti-HLA DSA. The distinction as donor specific is crucial, when considering clinical relevance of anti-HLA antibodies, as the DSA are for the graft. However, the Major Histocompatibility (MHC) system is highly polymorphic, with “private” epitopes that characterize each specific allele and “public” epitopes occurring between alleles, not only in the same locus, but also in different loci [13]. Humoral sensitization on the other hand is a matter of anti-HLA antibodies that recognize epitopes expressed on specific HLA molecules. An epitope is defined as the physical area of an antigenic molecule that an antibody binds to. In the case of proteins, epitopes are defined by the tertiary conformation of amino acid sequences. As a result, the primary sequence of amino acids of a protein does not necessarily define an epitope. HLA epitopes are structurally defined with the usage of an algorithm [14] which is important when classifying a given anti-HLA antibody as DSA. Another crucial characteristic is the difference between the antigenicity of epitopes (i.e., the reactivity with the anti-HLA antibody) and the immunogenicity of epitopes (i.e., the capacity of inducing anti-HLA antibody) [15]. Yet, partial denaturation of antigens may lead to false positive results. Thus, it is essential to elucidate the difference between HLA epitopes and antigens in the light of understanding humoral immune response in transplant recipients.

The MICA genes were first described in 1994 [16]. They are located in the 46 kb centromeric to HLA-B region and encode molecules similar in conformation to HLA class I proteins. MICA genes are polymorphic and several studies have found that immune response against MICA may correlate with a decrease in graft survival after transplantation. MICA antigens are expressed in epithelial cells [17], in fibroblasts, in endothelial cells, and in monocytes and dendritic cells [18, 19]. Lymphocytes are devoid of MICA and thus cross-matching with lymphocytes obtained from the blood does not work for the detection of antibodies against MICA. Only activated lymphocytes have been reported to express MICA [20], indicating them as danger signals in helping activating innate immunity through binding of NKG2D on natural killer cells and certain T cells [21]. A collaborative study of more than 200 patients and their donors, with typing for MICA alleles by sequenced-based typing to determine antibodies against MICA, showed that antibodies against MICA are donor specific [22]. Anti-MICA antibodies also recognize nonself-“private” and “public” epitopes on MICA molecules [22].

3. Incidence of *De Novo* Donor Specific Antibodies

Several investigators have searched the incidence rate of *de novo* formation of anti-HLA DSA among kidney transplant recipients. Nevertheless, there is considerable variation in the reported rates, basically related to the diversity of methods

used to detect anti-HLA antibodies [2, 23]. During the past decade, HLA antibody tests have moved from CDC to solid-phase assays, which show increased sensitivity and specificity to detect HLA antibodies [24]. Using Luminex technology one can detect and define low levels of these antibodies, which has been a substantial help in clinical practice. Early in 2002, antibodies directed against HLA were shown to appear 6 months to 8 years before graft failure of kidney transplants in a serial longitudinal study [3]. Following this observation, another study showed that antibodies appear to HLA class I and HLA class II, within approximately 2.7 to 3.9 years, respectively, before failure [6]. We know now that the incidence of anti-HLA antibodies developing 6 months after transplantation is roughly the same as after 10 years [25]. In a prospective design study, Terasaki and Cai evaluated 2231 patients and found that 21.4% of them were positive for anti-HLA antibodies 1 year after transplantation [12]. Lachmann et al. [9] studying a large cohort of 1014 kidney transplant recipients from deceased donors, monitored in a cross-sectional manner for the development of anti-HLA antibodies, found that 29% of them became positive and 31% of these antibodies were DSA. Another study of 72 patients, who were also negative for anti-HLA antibodies before transplantation, showed that 22.2% of them developed antibodies after transplantation, while 75% of them had DSA [26]. Wiebe et al. [27] evaluating a cohort of low risk patients found that 15% of them developed *de novo* DSA, in a mean follow up time of 6.2 ± 2.9 years. The mean time to development of these antibodies was 4.6 ± 3.0 years. In a retrospective analysis of 505 patients Willicombe et al. reported a rate of *de novo* production of DSA in 18.2% of patients [5]. The mean time to detection of DSA after transplantation was 9.98 ± 12.48 months [5]. In our center [8], periodical screening of 597 kidney transplant recipients revealed that 15.4% of them produced DSA after transplantation, with similar rates recorded between individuals sensitized not against the donor before transplantation and those without anti-HLA antibodies. Another recent study of 82 previously negative pediatric patients showed that 23% of them developed DSA in a mean follow-up time of 4.3 years [28]. The mean time to appearance of *de novo* DSA in this population was 24 months after transplant. Wang et al. measured the incidence of *de novo* DSA in the serum of 620 kidney recipients one year after transplantation and found that 7.3% of them had developed anti-HLA antibodies, with 84.4% of them being DSA [29]. Likewise, 32% of previously nonsensitized patients developed *de novo* DSA in the study by Gingu et al. [30]. Everly et al. [31] reported that 11% of the patients without detectable DSA at the time of transplantation will have detectable DSA 1 year later, and over the next 4 years, the incidence of *de novo* DSA will increase to 20%. After *de novo* DSA development 24% of the patients will fail within 3 years [31].

Antibodies against MICA antigens have been found in transplant patients [32, 33] and in about 10% of the patients in the waiting list for a first kidney [22]. When these antibodies are donor specific they correspond to mismatched MICA epitopes [22]. According to Zou and Stastny, about 20% of kidney transplant recipients may present with anti-MICA antibodies [22]. The authors also report a higher frequency,

accounting for 30% of the patients who have rejected a previous transplant [22]. Another study, which employed integrative genomics analysis of ProtoArray data, showed that antibody responses against MICA antigens are modulated after transplantation, irrespective of the graft rejection, and may be very high at the time of humoral rejection, or simply elevated in cellular rejection [34]. They report that 73% of the patients showed an increase in MICA specific antibody response after transplant, regardless of the presence or absence of biopsy proven graft rejection [34]. Furthermore they found that MICA is preferentially localized to the glomerulus [34].

4. Clinical Correlations of *De Novo* Donor Specific Antibodies

Recent studies have provided substantial evidence that the development of *de novo* DSA is associated with antibody mediated injury and allograft failure [4, 5]. Studies from the past also support a significant role for anti-HLA antibodies in chronic allograft rejection [35]. Posttransplant production of anti-HLA antibodies, especially in the presence of circulating antibodies to donor HLA antigens, is highly associated with the incidence of acute rejection and graft loss [3, 6, 36, 37]. Patients with *de novo* DSA may be classified according to the clinical syndrome they present with at the time of detection, as follows: (i) acute allograft dysfunction, that is, patients with a rise in serum creatinine $\geq 25\%$ from baseline in ≤ 2 months. In this group of patients the onset of *de novo* DSA was shown concurrent with the onset of clinical dysfunction. (ii) Indolentallograft dysfunction, that is, patients with graft dysfunction (proteinuria ≥ 0.5 g/day or increase in serum creatinine $\geq 25\%$ in >2 months). This group includes all patients in whom the onset of *de novo* DSA preceded the start of clinical dysfunction by an average of 9 months [27], (iii) stable renal function in the allograft, including patients with no graft dysfunction, in whom DSA were detected by routine surveillance [27]. Results from a study which categorized a cohort of 315 low risk patients using the above scheme showed that independent predictors of *de novo* DSA production were HLA-DRB1 (OR: 5.66, $P < 0.006$) and nonadherence (OR: 8.75, $P < 0.001$). Specifically, nonadherence was documented in 100% of the acute dysfunction group, in 53% of the indolent dysfunction *de novo* DSA group, and in only 6% of the stable function group. In the group with acute dysfunction, the onset of *de novo* DSA was essentially concurrent with the onset of clinical dysfunction and the mean serum creatinine at the time of kidney biopsy was 5.57 mg/dL [27]. Graft loss occurred in 22/315 patients during the study period and 14 of them (63.7%) had *de novo* DSA [27]. In the study by Willicombe et al. *de novo* production of DSA, of any specificity, was found to be associated with acute mediated rejection (AMR) and transplant glomerulopathy. Only HLA-Cw DSA were found not to be significantly associated with allograft failure [5]. The major risk factor for the development of these DSA posttransplant, revealed from this study, was the higher mean HLA mismatch [5]. More detailed analysis showed that the antigen mismatches were associated with the

anti-HLA DQ DSA, while patients mismatched at the HLA-DR locus were at significant risk for the developing DQ DSA ($P = 0.0021$) [5]. Thus, there is an enhanced immunogenicity with mismatching at both the HLA-DR and HLA-DQ loci, which is associated with increased production of *de novo* anti-HLA DQ DSA [5]. The high incidence of anti HLA DQ DSA is probably related to the high number of polymorphic epitopes that are expressed on both α and β chains of the HLA-DQ molecule [38].

In our experience [8], *de novo* appearance of DSA was significant in that anti-HLA class II DSA, mostly directed against HLA-DQ molecules, were predominant in HLA class II incompatible grafts (Table 1). Moreover, recipients of HLA-class II incompatible grafts developed DSA more frequently than those receiving HLA-class II compatible (17.9% versus 7.9%, $P = 0.003$) [8]. Over the follow-up time, 48/597 (8%) patients lost their graft and 28/48 (58.3%) of these had *de novo* formation of DSA. The presence of detectable anti-HLA antibodies, either DSA or non-DSA, was the only independent predictor for graft loss in the study coming from our center. Hazards ratios for DSA positive and DSA negative patients were found in 22.54, 95% CI: 6.69–75.89, $P < 0.001$ and 5.94, 95% CI: 1.67–21.06; $P = 0.006$, respectively [8]. A retrospective study [28], evaluating the *de novo* formation of DSA in a pediatric population of 82 patients, showed that renal function, measured as serum creatinine, was significantly different between patients with and without *de novo* formation of DSA. Specifically, patients who did not develop anti-HLA antibodies, and those with non DSA had comparable serum creatinine levels at discharge, and throughout the follow-up time (mean time 4.3 years). Conversely, a significant increase in creatinine levels was observed in the DSA group at the end of follow-up period, when compared with values at discharge and at the time of first DSA appearance [28]. Interestingly, Lee et al. studying serial sera collected in a period of 17 years from two groups of patients, one whose allograft failed due to chronic rejection and a control group consisting of patients with functioning grafts matched by transplant date, found that DSA appeared in 96% of the patients with graft failure versus 48% of the controls [42]. Importantly, this study provided clear evidence that time to development of posttransplant antibody is a critical factor in determining allograft survival. According to these findings, antibodies which were developed within a year after transplantation resulted in graft failure in a mean time of 5.1 years [42]. In contrast, antibodies, which were formatted after the first year, were associated with a slow rate of failure and 80% of patients had functioning grafts one decade after transplantation [42]. This difference was mostly attributed to the condition of the graft and probably the response of the host to the graft. It is possible that antibodies forming within the first year react rapidly on the endothelium initiating a cascade of events leading to rejection. In the same study it was shown that HLA class I DSA are produced sooner (median time to detection 6.6 months) and are associated with rapid graft loss, while class II DSA occur later (median time to detection 12.5 months) and may be associated with chronic transplant glomerulopathy [42]. Huang et al. [43] monitoring performed and *de novo*

TABLE 1: Studies searching the incidence of *de novo* anti-HLA DSA and their impact on graft survival.

Author Publication year	Cohort size, <i>N</i>	Incidence of <i>de novo</i> anti-HLA DSA	<i>De novo</i> anti-HLA abs, class, DSA frequency	Followup (years)	Incidence of AMR in pts. with <i>de novo</i> anti-HLA Abs	Incidence of GF in total	Incidence of GF in pts. with <i>de novo</i> anti-HLA and anti-HLA DSA
Worthington et al. 2003 [6]	76	10.5%	Class I, 7.9% Class II, 6.6% Class I and II, 1.3%	10		23.7%	91.7%
Hourmant et al. 2005 [23]	1229	5.5%	Class I, 0.1% Class II, 5.4%	5	8%	8.2%	16.8% 4.8%
Terasaki and Cai 2005 [37]	1564			2		8%	16.7%
Zhang et al. 2005 [39]	49	22.4%	Class I, 10.2% Class II, 6.1% Class I + II, 6.1%	2	26.7%		
Mihaylova et al. 2006 [26]	72	16.7%	Class I, 9.7% Class II, 5.6% Class I + II, 1.4%	1-5	8.3%	18%	56.25%
Mao et al. 2007 [40]	54	27.8%		5		46.3%	65.6% 40.6%
Lachmann et al. 2009 [9]	1014	9.2%	Class II, 6%	5.5	3.6% 2.4%	20.9%	14.7%
Ntokou et al. 2011 [8]	597	15.4%	Class I, 3.2% Class II, 11.4% Class I + II, 0.8%	1.2-10	6% 3.7%	8%	15.6% 9.7%
Wang et al. 2012 [29]	620	6.2%	Class I, 1.5% Class II, 3.9% Class I + II 0.8%, DSA	5		18.4%	60% 63.2%, DSA
Ginevri et al. 2012 [28]	82	23.1%	Class I 2.4%, DSA Class II 13.4%, DSA Class I + II 7.3%, DSA	4.3	40% 36.7%, DSA		13.5%, DSA
Alberu et al. 2012 [41]	53	32%	Class I 20.7%, DSA Class II 7.5%, DSA Class I + II 3.8%, DSA	2	5.7%, DSA	9.4%	23.5%, DSA
Wiebe et al. 2012 [27]	315	14.9%	Class I 0.9%, DSA Class II 10.2%, DSA Class I + II 3.8%, DSA	2.9 ± 6.2	5.3%, DSA	7%	13.6%, DSA
Willicombe et al. 2012 [5]	505	18.2%	Class I 5.5%	5	30.6%, DSA		14.4%, DSA

Abs: antibodies; DSA: donor specific antibody; HLA: human leucocyte antigen; AMR: antibody-mediated rejection; GF: graft failure.

HLA DSA found the incidence of acute rejection in 34%, 48%, and 70% for patients with no DSA, with performed DSA and with *de novo* DSA, respectively. Notably, in all recipients with *de novo* DSA and rejection the first rejection episode preceded or was concurrent with the emergence of *de novo* DSA. Likewise DeVos et al. [44] reported that patients who developed DSA after transplant had increased rate of acute rejection episodes, higher serum creatinine, and worst graft survival. Moreover patients with persistent DSA had increased rates of rejection and worst renal function [44]. However, a prospective DSA screening protocol failed to identify patients at risk for acute rejection or poor short term graft outcomes [45]. Specifically, although DSA was detected in 27% of all patients by protocol or indication searching,

and patients with DSA were significantly more likely to have experienced acute rejection compared with those without DSA, only 3 out of 19 with DSA had DSA detected before the rejection episode [45].

MICA antibodies are also becoming increasingly recognized as critical in the pathogenesis of organ allograft outcomes. Prospective studies of patients with MICA antibodies have shown that they experience lower allograft survival accounting for 83% versus 94% for patients with HLA antibodies or 96% for those without HLA antibodies ($P = 0.0005$, $P = 0.0004$, resp.) [46]. Notably, the multivariate analysis from that study revealed hazard ratios for patients with MICA antibodies as high as 6.1 opposed to 3.6 for patients with HLA antibodies ($P < 0.00001$) [46]. An earlier

retrospective study had identified MICA in kidneys which had been rejected [47]. They were also found in the sera of patients who eventually experience graft failure at a higher frequency than in those who had functioning grafts [48]. Moreover, MICA antibodies were found before transplantation in about 25% of the 85 patients in the waiting list [44] and associated with hyperacute rejection and in the absence of HLA antibodies [48]. Several studies have shown that kidney allograft recipients undergoing both acute and chronic rejection may have measured antibodies against MICA antigens [32, 49–52].

5. Pathologic Correlations

A key to understanding the effects of antibody-mediated graft damage is to define the relationship between donor specific antibody in the recipients' sera and the histopathological lesions in their grafts. The current Banff criteria define the diagnosis of AMR as the presence of DSA along with certain histological changes [48] including the C4d deposition [50]. In this regard, Hidalgo et al. [53] studied the frequency of *de novo* DSA in the sera of patients and their associations with specific histologic lesions and prognosis. They found that *de novo* DSA were more frequent in patients having late biopsies (34%) versus early biopsies (4%) [53]. Microcirculation inflammatory changes, such as glomerulitis, or capillitis, and damage such as glomerulopathy, or capillary basement membrane multilayering and C4d staining were associated with *de novo* DSA [53]. Thus, in late biopsies, *de novo* DSA detection is frequent and associated with microvascular lesions but not with scarring lesions. This study, performed in late biopsy population, indicates that *de novo* DSA stresses microcirculation in the allograft, causing these kidneys to present with clinical indications for biopsy at a median of about 6 years after transplant. Weibe et al. on the other hand, studying 315 consecutive renal transplants without pretransplant DSA, with protocol and for cause biopsies, correlated the patients' clinical phenotypes by graft function at the time of *de novo* DSA detection. They found that 0–6-month clinical rejection episodes (borderline or Banff 1A/1B cellular rejections) occurred more commonly in the *de novo* DSA group compared with the patients without *de novo* DSA (28% versus 13%, $P = 0.015$). In addition, despite a median acute glomerulitis score of zero in both groups, the *de novo* DSA group had significantly higher peritubular capillaritis scores in 0–6-month clinical rejection biopsies compared to the group without *de novo* DSA (2 versus 1, $P = 0.049$). Moreover, both the clinical rejection frequency and the grade of capillaritis were higher independent of adherence in the *de novo* DSA group [27]. Despite the widespread use of C4d staining in the clinical management of kidney transplant recipients [54–59], over time it has become clear that C4d is neither completely specific nor sufficiently sensitive for the diagnosis of AMR [54–56, 60]. The current Banff diagnostic criteria [57] however require positive C4d peritubular capillary staining for a definite diagnosis of AMR, which might exclude some patients from such a specific diagnosis. Interestingly, C4d-negative AMR, a clinical entity

which emerged in 2009 [56], was shown to be associated with microcirculation changes and the presence of anti-HLA class II DSA. As predictors of progression to chronic mediated rejection, these findings confirmed the notion that independently of C4d positivity, C4d-negative kidneys could share features of antibody-mediated injury, and C4d staining alone may not be sensitive enough to establish a diagnosis of acute mediated rejection. Another study demonstrated that high endothelial-specific gene expression in biopsies from kidney transplant recipients with DSA but without C4d, indicating ongoing antibody-mediated damage [56]. Most cases of C4d-negative AMR tend to occur more than 1 year after transplantation and represent chronic or acute-on-chronic AMR episodes [56].

6. The Methods of Measuring DSA in Serum and Diagnosis of DSA in Serum and Diagnosis of DSA-Associated Graft Rejection

Although the complement-dependent cytotoxicity (CDC) [61] crossmatch has been the gold standard assay for many years in kidney transplantation, the need for alternative HLA antibody screening was also clear. The target in the CDC assay is the lymphocyte, and thus not only HLA molecules but also other unrelated cell membrane components may be targets for antibody reactivity. Moreover, autoantibodies, immune complexes, and immunoglobulin allotypes may also interfere in this assay [62]. Because the assay is based on complement activation, HLA specific IgG antibodies, not able to fix complement, such as IgG2 and IgG4, cannot be detected. This problem was solved by the development of solid phase assays using isolated HLA molecules as targets for antibody detection. They are based on ELISA or fluorescence and can detect both complement fixing and noncomplement fixing IgG antibodies [63]. A positive reaction in this occasion is by definition caused by the reactivity with an HLA class I or a class II molecule, and not with another unrelated cell membrane molecule.

The ELISA assay was developed first, in which HLA class I and HLA class II molecules were used as target molecules. Later studies showed that kidney transplant recipients who were transplanted with a negative CDC crossmatch with both HLA class I and HLA class II antibodies present before transplantation with this assay had a significantly poorer graft survival [63] compared with patients without HLA antibodies. Yet, patients with HLA class I or HLA class II antibodies did not experience this worst graft survival. This effect was attributed to donor specific antibodies as the impact on graft survival was shown greater with the number of HLA mismatches between donor and recipient [61]. Subsequently, Lefaucheur et al. showed a significantly lower graft survival in patients with DSA compared with patients without DSA [62, 63]. In this regard, when the presence of DSA was associated with the occurrence of AMR graft survival was worse in the DSA group, while in the absence of AMR graft survival was similar to that of patients without DSA. Therefore, although the DSA detected

by ELISA are a risk factor for some patients, it is not feasible to assign the risk on a specific patient [56]. More recently, assays based on antibody reactivity against HLA molecules attached to Luminex beads are used. The availability of single HLA antigen beads facilitates the determination of the antibody specificity enormously compared to previous panel analyses [64]. There are several retrospective studies showing that the presence of DSA is associated with a significantly decreased graft survival even if no AMR takes place [64–66]. Yet, donor HLA class II specific antibodies detected by Luminex were shown to be clinically relevant with a positive B-cell crossmatch [67]. Other studies reported that DSA detected in Luminex are irrelevant in patients transplanted with a negative CDC crossmatch [68, 69] or with a negative flow cytometric crossmatch [69]. Incidence of rejection, renal function and graft survival were found similar between patients with and without DSA BY Luminex.

Late AMR is a major cause of late kidney transplant failure and major assistance in its understanding was provided by the 1997 Banff classification. It was created basically in order to classify rejection prior to the meeting in 2001, which further defined pathological classification of AMR [49]. Incidence of AMR has been reported as 0–8% in renal transplant recipients in larger centers largely due to increased recognition, detection of DSA, retransplanted patients, and increase in positive crossmatch and ABO incompatible transplantation for highly sensitized patients. A few studies have indicated C4d staining is around 93–96% specific but 31–95% sensitive [49]. Lately quantification of DSA has been introduced as a comarker of AMR to be taken into consideration in diagnosis and treatment. After the Banff meeting in 2001 it was determined that AMR has 3 cardinal features upon biopsy findings: (i) acute tubular injury; neutrophils and/or mononuclear cells in peritubular capillaries and/or glomeruli, and/or capillary thrombosis; or arteritis/fibrinoid necrosis in the intima along with intramural/transmural arterial inflammation. (ii) C4d evidence for antibody action and/or immunoglobulin in peritubular capillaries, immunoglobulin and complement in arterial fibrinoid necrosis. (iii) anti-HLA antibody (DSA) circulation in serum or other anti-donor endothelial antigens.

After the Banff meeting in 2009, positive C4d deposition without evidence of rejection was added to criteria suggesting antibody-mediated changes. Several studies had reported C4d deposition in biopsies without evidence of rejection [56]. In protocol biopsies from ABO incompatible grafts, 21/37 had C4d deposition without evidence of AMR lesions or T-cell-mediated rejection, which can suggest accommodation. Another addition to Banff criteria indicating antibody-mediated changes was determined to be positive C4d with presence of circulating anti-donor antibodies (no signs of acute or chronic T-cell-mediated rejection or AMR/no ATN-like minimal inflammation) [70].

7. Management of the Patient with *De Novo* DSA after Kidney Transplantation

Patients with *de novo* DSA may present with a spectrum of clinical syndromes and various pathological features.

The clinical phenotypes of the patients who develop DSA after kidney transplantation vary significantly, from acute allograft dysfunction to stable renal function [27]. In this regard, the best therapeutic approach for the kidney transplant recipient with *de novo* DSA will depend on the clinical picture at the time of detection. Between episodes of acute AMR and late chronic AMR there is a dynamic and progressive process of injury and repair standing, which ultimately contributes to failure of the allograft. Therefore, it is believed that the most important clinical criterion of which protocol to use is the rapidity of onset of graft dysfunction. Therapeutic protocols include removal of the antibodies by plasmapheresis (or immunoadsorption), suppression of antibody production by intravenously administered immunoglobulin (IVIG), depletion of the antibody-secreting plasma cells with anti-CD20 treatment with rituximab, along with tacrolimus and mycophenolate mofetil. Most recent protocols employ Campath-1H and Bortezomib [70]. Patients with *de novo* formation of DSA after transplantation are treated by the clinical syndrome they present with at the time of detection: (i) Acute allograft dysfunction with histological evidence of antibody mediated injury (C4d+) with minimal pathologic features, acute tubular necrosis-like changes, are treated with a combination of pulse methylprednisolone, a course of plasma exchange therapy, 6–8 sessions, IVIG, and one pulse of rituximab, 375 mg/m². However, patients with more severe clinical picture, that is rapid deterioration of renal function and diffuse C4d+ staining, evidence of capillary and/or glomerular inflammation with thrombosis require therapy with longer courses of plasma exchange, 8–10 sessions, followed by IVIG and rituximab. The new agent, which has emerged in the treatment of AMR episodes, is bortezomib [71]. It has been shown effective in reducing anti-HLA antibody levels and reversing both AMR episodes in substantial numbers of treated patients.

In both occasions, formation of *de novo* DSA is typically documented in the light of worsening of renal function and typically at the same time with the biopsy showing acute AMR. It is essential to note that for this group of patients institution of therapy should be rapid to avoid irreversible graft loss. Despite the fact that a biopsy result is not a requirement for institution of treatment, a graft biopsy is required to avoid treating patients with advanced degrees of interstitial fibrosis and tubular atrophy who are unlikely to benefit. (ii) Indolent allograft dysfunction represents a slower, gradual decline of renal function, which occurs without acute compromise of renal function or significant proteinuria and cannot be explained by any other cause. Kidney transplant recipients who develop *de novo* DSA often show pathologic features of indolent and slowly progressive microvascular abnormalities. Donor specific antibodies, formed *de novo*, bind to allogenic targets expressed by graft endothelium activate the system of complement system and modulate the biology of rejection. The appearance of these antibodies results from inadequate immunosuppression and thus prevention is synonymous with sufficient immunoregulation and/or enhancing of the level of as needed. (iii) Detection of *de novo* DSA in a routine test in patients with stable allograft function represents a

step behind in the continuum of the natural history of acute AMR. It is largely unknown how to treat these patients. A closer monitoring of these patients, in addition to the advancement of immunosuppressive therapy, which typically includes tacrolimus and mycophenolate mofetil is generally suggested. We tend to maintain higher trough level of tacrolimus in such patients (>6 ng/dL) and usually administer 1.5–2 g of mycophenolate mofetil per day, depending on the body weight. However, recent investigation has shown other potential pathways that might be beneficial in such patients. For instance, the effect of the proteasome inhibitor bortezomib was evaluated as a desensitization strategy in recent study [72]. They administered one cycle of bortezomib (1.3 mg/m² × 4 doses), used as the sole desensitization therapy. Bortezomib treatment did not significantly decrease DSA within the 150-day posttreatment period in any of the 4 patients [72].

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Clinical Study

APACHE IV Is Superior to MELD Scoring System in Predicting Prognosis in Patients after Orthotopic Liver Transplantation

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This study aims to compare the efficiency of APACHE IV with that of MELD scoring system for prediction of the risk of mortality risk after orthotopic liver transplantation (OLT). A retrospective cohort study was performed based on a total of 195 patients admitted to the ICU after orthotopic liver transplantation (OLT) between February 2006 and July 2009 in Guangzhou, China. APACHE IV and MELD scoring systems were used to predict the postoperative mortality after OLT. The area under the receiver operating characteristic curve (AUC) and the Hosmer-Lemeshow C statistic were used to assess the discrimination and calibration of APACHE IV and MELD, respectively. Twenty-seven patients died during hospitalization with a mortality rate of 13.8%. The mean scores of APACHE IV and MELD were 42.32 ± 21.95 and 18.09 ± 10.55 , respectively, and APACHE IV showed better discrimination than MELD; the areas under the receiver operating characteristic curve for APACHE IV and MELD were 0.937 and 0.694 ($P < 0.05$ for both models), which indicated that the prognostic value of APACHE IV was relatively high. Both models were well-calibrated (The Hosmer-Lemeshow C statistics were 1.568 and 6.818 for APACHE IV and MELD, resp.; $P > 0.05$ for both). The respective Youden indexes of APACHE IV, MELD, and combination of APACHE IV with MELD were 0.763, 0.430, and 0.545. The prognostic value of APACHE IV is high but still underestimates the overall hospital mortality, while the prognostic value of MELD is poor. The function of the APACHE IV is, thus, better than that of the MELD.

1. Background

Liver transplantation has become exclusive and feasible treatment for various end-stage liver diseases, including liver cirrhosis, acute liver failure and tumor [1]. Although liver transplantation is widely conducted, the mortality remains significantly high as much as 5%~8% [2].

As there was no an objective and accurate evaluation tool available for prediction of the outcome for liver transplantation till now [3]. Acute physiology and chronic health evaluation (APACHE) is one of the most widely used and authoritative scoring system for evaluation of the severity and prognosis of critically ill diseases, including liver transplantation, and APACHE IV showed better predicting value against APACHE II and APACHE III [4].

The Model for End-Stage Liver Disease (MELD) as another important scoring system for prediction of the mortality of critical ill patients is a survival model. This

model has been adopted for donor liver allocation systems in the United States in 2002 [5]. Additionally, MELD has been reported to be used for prediction of the outcome for liver transplantation [6].

Therefore, this aims to explore and compare the effects of APACHE IV, MELD and combination APACHE IV with MELD for predicting the mortality risk after orthotopic liver transplantation.

2. Methods and Patients

2.1. Patients. A retrospective cohort study was performed. At a liver transplant center in Guangzhou, a total of 195 patients admitted to the ICU after orthotopic liver transplantation (OLT) between February 2006 and July 2009 were included when meeting the inclusion criteria. The inclusion criteria included age > 18 years, patients with OLT for the first time,

cadaveric donor (cardiac death) and brain-dead donor liver transplant patients, and patients who had been more than four hours in the ICU after OLT operation. Living donor liver transplantation, multiple organ transplantation, and previous organ transplantation were excluded.

2.2. Data Collection. The data were collected by the researchers independently, and double check was conducted. We recorded the data of the previous day if the data used to calculate the APACHE IV scores was missing, and we recorded the data of the first two days when the data of the previous day was missing.

The APACHE IV scoring system takes age, chronic health conditions, and the acute physiology score (APS) into account. The APS is based upon the worst measurement during the first 24 hrs in the ICU. The Glasgow Coma Scale (GCS) score, whether sedation or paralysis resulted in an inability to assess GCS, and $\text{PaO}_2/\text{Fio}_2$ were recorded as part of the data collection for APS. All the following were also recorded: ICU admission diagnoses; admission source; length of stay before ICU admission; whether a patient received mechanical ventilation on day 1, had emergency surgery, or was an ICU readmission; and whether a patient with acute myocardial infarction received thrombolytic therapy in the 24 hrs before or after ICU admission. These data were collected over the first 24 hrs of admission to the ICU and were entered into a computer-based APACHE IV calculator. The calculator returns values that include the APACHE score, predicted mortality rate, and predicted ICU length of stay.

The MELD score was determined prior to OLT using the following equation:

$$\begin{aligned} \text{MELD} = & 9.57 \times \log_e (\text{Creatinine}) + 3.78 \times \log_e (\text{Bilirubin}) \\ & + 11.2 \times \log_e (\text{INR}) + 6.43, \end{aligned} \quad (1)$$

where INR is international normalized ratio and creatinine and bilirubin are expressed in mg/dL. The values lie between 6 and 40, depending on the severity of the clinical conditions.

2.3. Statistical Analysis. The outcomes of all patients were presented by frequencies, percentages, mean values, and standard deviations. The correlation between the predicted and the actual ICU LOS was tested using the Spearman test, and the differences were tested using the Wilcoxon test. The discrimination and accuracy of APACHE IV and MELD to predict the early mortality of OLT patients were described by receiver operating characteristic curves (ROC) and the Hosmer-Lemeshow test. The discrimination of a prognostic model is defined as the ability to distinguish between survivors and nonsurvivors. The discrimination of APACHE IV and MELD to predict hospital mortality was analyzed by calculating the area under the receiver operating characteristic curves (AUC). An AUC of >0.9 was considered to be outstanding; 0.7 to 0.9, acceptable; 0.5 to 0.7, poor. The calibration of the model is the degree of agreement between the predicted mortality and actual mortality. The Hosmer-Lemeshow C statistic was used to determine the

calibration of the model. A model with good calibration should have a Hosmer-Lemeshow statistic with degrees of freedom, approximately, equal to the number of categories minus 2 as well as a P value > 0.05 . Standardized mortality ratios (SMRs) were calculated by dividing the actual rates by the rates predicted by APACHE IV. The significance level was set at $P < 0.05$. All statistical analyses were performed using SPSS 13.0.

3. Results

This retrospective study included 195 adult patients, comprising 171 males and 24 females with an overall mean age of 48.18 ± 11.13 years, who were admitted to the ICU during the immediate OLT postoperative period. Twenty-seven died during hospitalization with a mortality rate of 13.8%; the overall mean APACHE IV and MELD scores were 42.32 ± 21.95 , and 18.09 ± 10.55 . The mean APACHE IV and MELD scores of nonsurvivors and survivors were 75.26 ± 25.47 versus 35.86 ± 15.58 and 25.70 ± 12.92 versus 16.87 ± 9.61 . The mean predicted hospital mortality rates of nonsurvivors and survivors were $(12.84 \pm 16.18)\%$ and $(2.30 \pm 3.77)\%$, respectively ($P < 0.05$). The data was shown in Tables 1, 2, and 3.

The AUC of the APACHE IV and MELD predictions of mortality during hospitalization was 0.937 (95%CI, 0.892 to 0.981) and 0.694 (95%CI, 0.51 to 0.817), respectively; $P < 0.05$ for both models. The two models were well-calibrated (with Hosmer-Lemeshow C statistics of 1.568 and 6.818 for APACHE IV and MELD, resp.; $P > 0.05$ for both models). The data was shown in Figure 1.

The highest Youden index was 0.763 when the APACHE IV score was 55.5 at the cutoff value, demonstrating a specificity of 0.911, a sensitivity of 0.852, a positive predictive value (PPV) of 0.605, and a negative predictive value (NPV) of 0.975. The highest Youden index was 0.430 when the MELD score was 20.7 at the cutoff value, demonstrating a specificity of 0.726, a sensitivity of 0.704, a positive predictive value (PPV) of 0.292, and a negative predictive value (NPV) of 0.938. In the combination test, the predicted mortality rate was classified as a positive result when APACHE IV score ≥ 55.5 , and MELD score ≥ 20.07 . The data was shown in Table 4.

The median predicted ICU LOS of survivors was 3.21 (2.39, 4.82) days, and the median actual ICU LOS was 3.71 (2.38, 5.47). There was a relationship between the predicted ICU LOS and the actual ICU LOS ($r = 0.467$, $P < 0.05$). Comparing the predicted ICU LOS to the actual ICU LOS of survivors, we noticed that the former was shorter than the latter ($Z = -3.760$, $P < 0.05$).

4. Discussion

The study put APACHE IV and MELD to evaluate their validity on posttransplantation of liver. We found that the AUC of APACHE IV was higher than that of MELD. We also demonstrated that nonsurvivors were higher in the mean APACHE IV score than survivors. Thus, our results may

TABLE 1: Comparison of survivors until hospital discharge and nonsurvivors ($n = 195$).

	All (195)	Survivors ($n = 168$)	Nonsurvivors ($n = 27$)	<i>P</i> values
Age, yrs, mean (S.D.)	48.18 (11.13)	47.83 (10.92)	50.41 (12.33)	0.265
Male	171	149	22	
Female	24	19	5	
MELD, mean (S.D.)	18.09 (10.55)	16.87 (9.61)	25.70 (12.92)	0.002
APACHE IV, mean (S.D.)	41.32 (21.95)	35.86 (15.58)	75.26 (25.47)	<0.001
Surgical time, hrs, mean (S.D.)	7.41 (1.61)	7.27 (1.58)	8.26 (1.60)	0.002
Anhepatic time, hrs, mean (S.D.)	42.30 (10.14)	41.76 (8.84)	45.59 (16.09)	0.370
Cold ischemia time, hrs, mean (S.D.)	7.13 (2.71)	7.11 (2.81)	7.23 (3.20)	0.688
Pred. hosp. death%, mean (S.D.)	3.76 (7.79)	2.30 (3.77)	12.84 (16.18)	<0.001
Pred.ICU LOS days, median (IQR)	3.49 (2.39, 4.82)	3.21 (2.21, 4.21)	5.5 (5.05, 6.03)	<0.001
Actual ICU LOS days, median (IQR)	3.71 (2.38, 5.47)	3.5 (2.34, 32.33)	8 (2.67, 32.75)	0.001
SMR; 95% CI		3.68; 2.38~4.96		

Hosp.: Hospital.

ICU: Intensive care unit.

Pred.: Predicted.

LOS: Length of stay.

Yrs: Years.

Hrs: Hours.

TABLE 2: Comparison of the mortality in APACHE IV score groups ($n = 195$).

	Group total	Nonsurvivors	APACHE IV	Actual mortality rates (%)	Actual mortality rates 95% CI	Pred. mortality rates (%)
All	195	27	41.32 ± 21.95	13.85	8.96~18.64	3.76
<30	65	0	21.77 ± 5.65			0.69
30~60	104	8	42.82 ± 8.18	7.69	2.57~12.81	2.62
>60	26	19	84.19 ± 21.62	73.08	52.00~88.00	16.00

provide some guidance in the outcome judgment of patients after liver transplant.

Here, the hospital mortality was underestimated by using APACHE IV scoring system (SMR was 3.68, 95%CI: 2.38 to 4.96). In contrast to the original study by Zimmerman et al. [4], the SMR of 0.997 showed little difference between the predicted hospital mortality and the actual hospital mortality. Several reasons might account for this difference. First, our data might not be nationally representative because the collection was limited to patients who were admitted to the ICU after OLT. In addition, the recovery process of OLT patients was affected by the characteristics of the donors and the experience of the surgeons. At last, the different levels of ICUs may account for this discrepancy.

The ROC curves scoring system is used for predicting the sensitivity and specificity of death. The area under the receiver operating characteristic curve of APACHE IV was 0.937, however, higher than the value of 0.88 reported in the original study by Zimmerman et al. [4], suggesting that the APACHE IV score system has a good ability to distinguish possible nonsurvivors from survivors. This difference may be because the original data of Zimmerman for APACHE IV were derived mostly from integrated ICUs selected for complex diseases, while the subjects of this study were specific for ICU patients with OLT. The APACHE IV score was well-calibrated (Hosmer-Lemeshow was 1.568; $P = 0.980$). In contrast, other

studies reported poorly calibrated APACHE IV scores that overestimated hospital mortality in integrated ICUs [7, 8]. According to our study, the APACHE IV score had a better calibration when it was applied to specialized ICUs, such as OLT patients; this scoring system is sensitive to distinguish possible nonsurvivors from survivors. Comparing with data from integrated ICUs, the APACHE IV score showed better predictive validity in a specialized ICU, which has been demonstrated by Knaus [9]. These results suggested that the APACHE IV scoring system is more appropriate for prediction of the prognosis of patients in specialized ICUs than in the integrated ICUs.

The MELD score is based on 3 biochemical variables that are objective and easy to obtain, which are the international normalized ratio of prothrombin, serum creatinine, and serum bilirubin. Renal function is often recognized as a major determinant of patient survival and is given a heavy weight in MELD scoring system. In our study, the MELD score values in nonsurvivors (25.70 ± 12.92) were higher than in survivors (16.87 ± 9.61), $P < 0.05$. This result showed that the MELD score can predict early outcomes of transplantation and, as previously reported, hospital mortality. The pretransplantation MELD scores were 15~25, and the mortality rate was 6.12% at the lowest level; at <15, the mortality rate was 8.08%, and at >25, the mortality rate was 34.04%, which was the highest among the groups. The selection of proper patients

TABLE 3: Comparison of the mortality in MELD score groups ($n = 195$).

Group	Total	Nonsurvivors	MELD score values	Actual mortality rate (%)
all	195	27	18.09 ± 10.55	13.85
<15	99	8	9.96 ± 2.72	8.08
15~25	49	3	19.10 ± 2.64	6.12
>25	47	16	34.02 ± 6.18	34.04

TABLE 4: APACHE IV, MELD, and combined APACHE IV and MELD score prognostic utility.

	Sensitivity	Specificity	PPV	NPV	Youden index	Cutoff value
APACHE IV	0.852	0.911	0.605	0.975	0.763	55.5
MELD	0.704	0.726	0.292	0.938	0.430	20.07
Combination	0.593	0.952	0.667	0.936	0.545	

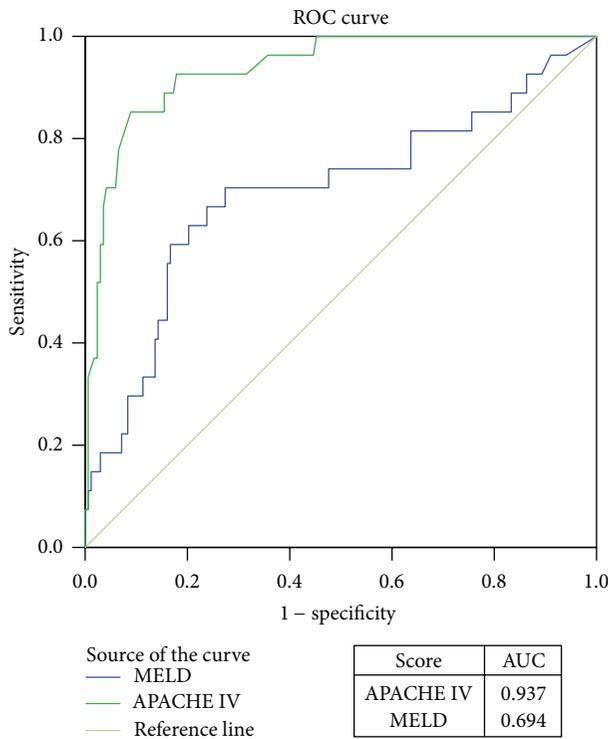


FIGURE 1: Receiver operating characteristic curves for the death risks of the acute physiology and chronic health evaluation IV (APACHE IV) and the Model for End-stage Liver Disease (MELD) at preorthotopic liver transplantation (OLT). AUC, area under the receiver operating characteristic curve.

and timing for OLT is complex and depends on multiple factors, such as survival, morbidity, resource utilization, and quality of life. Our results suggested that patients with lower death risks were not suitable for liver transplantation; in such cases, the survival times may be short. Therefore, the medium MELD scores (15~25) were the best fit for the operation; this confirms the results of Merion et al. that the low or high MELD score is not the most promising indicator [10].

The ROC curves scoring system was used to predict the sensitivity and specificity of death. The area under the receiver operating characteristic curve for MELD was 0.694,

a relatively low prognostic value. The MELD scoring system was well-calibrated (Hosmer-Lemeshow was 6.818; $P = 0.556$). Basile-Filho et al. reported that the area under the receiver operating characteristic curve of MELD is only 0.5 [11]. The prognostic value to predict hospital mortality postoperation was low, which was also demonstrated in other studies [12, 13].

As there was no an objective and accurate evaluation tool available for prediction of the outcome for liver transplantation till now The highest Youden index was 0.430 when the MELD score was at a 20.07 cutoff value, demonstrating a specificity of 0.726, a sensitivity of 0.704, a positive predictive value (PPV) of 0.292, and a negative predictive value (NPV) of 0.938. This result indicated that APACHE IV was higher than MELD regarding sensitivity and specificity, which was mainly due to that APACHE IV takes the diagnosis at admission and objective data into account. Barie et al. reported that it would be better to combine APACHE IV with another critical scoring system to provide more accurate prediction [14]. In this study, although the combination of APACHE IV and MELD had the highest specificity and PPV, its Youden index was only 0.545. Vincent considered that different critical scoring systems may assist each other in their assessment, rather than compete with each other. The combined APACHE IV and MELD improved the predictive accuracy for postoperative mortality against MELD, but declined the accuracy when compared with APACHE IV. Thus, the Youden index was highest when using APACHE IV scores alone. Therefore, the function of the APACHE IV is better than that of others.

Prediction of ICU LOS by APACHE IV is used to evaluate and compare the overall efficient use of the ICU in medical center. Care in the ICU accounted for approximately 13% of hospital costs and 4.2% of national health expenditures [16]. These costs were largely explained by the LOS in the ICU [17, 18]. We found that the difference between the predicted ICU LOS and the actual ICU LOS was significant ($P < 0.05$), but the correlation between them was poor ($r = 0.473$, $P < 0.05$). The APACHE IV model provides clinically useful ICU LOS predictions for critically ill patient groups, but its accuracy and utility are still limited, as demonstrated in the study of Vasilevskis et al. [19].

In summary, the prognostic value of APACHE IV is higher than that of MELD scoring system; thus, it needs to be validated in multiple ICU centers.

Authors' Contribution

Yueyun Hu and Xianling Zhang made equal contributions to this work.

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Research Article

Molecular Profiling of Acute and Chronic Rejections of Renal Allografts

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Both antibody mediated (AMR) and T-cell mediated (TCMR) rejections either acute or chronic represent the main reason for late graft dysfunction. In this study we aimed to evaluate differences in the intrarenal expression patterns of immune system related genes in acute and chronic rejections. Graft biopsies were performed and evaluated according to Banff classification. Using the TaqMan Low Density Array, the intrarenal expressions of 376 genes relating to immune response (B-cell activation, T-cell activation, chemokines, growth factors, immune regulators, and apoptosis) were analyzed in the four rejection categories: chronic AMR, chronic TCMR, acute AMR, and acute TCMR. The set of genes significantly upregulated in acute TCMR as compared to acute AMR was identified, while no difference in gene expressions between chronic rejections groups was found. In comparison with functioning grafts, grafts that failed within the next 24 months after the chronic rejection morphological confirmation presented at biopsy already established severe graft injury (low eGFR, higher proteinuria), longer followup, higher expression of CDC20, CXCL6, DIABLO, GABRP, KIAA0101, ME2, MMP7, NFATC4, and TGFB3 mRNA, and lower expression of CCL19 and TRADD mRNA. In conclusion, both Banff 2007 chronic rejection categories did not differ in intrarenal expression of 376 selected genes associated with immune response.

1. Introduction

Both acute and chronic rejections have been shown to affect the long-term outcome of kidney transplantation. Chronic rejection is thought to be associated with both cellular and humoral alloimmune responses [1]. Chronic active antibody mediated rejection (CAMR) is characterized by C4d deposition in peritubular capillaries, the presence of circulating anti-donor antibodies, and morphologic evidence of chronic tissue injury such as glomerular double contours and peritubular capillary basement membrane multilayering and interstitial fibrosis/tubular atrophy (IF/TA) and fibrous arterial intimal thickening. The diagnosis of this entity is problematic since C4d deposits are not permanent and antibody mediated rejection was described to be associated also with different pathways where C4d is not involved [2]. Similarly, the chronic T-cell mediated rejection, albeit well described at Banff scheme, is of unclear pathogenesis.

Moreover, the therapy of both processes remains to be insufficient.

Beside conventional morphological evaluation, molecular histology offers better insight into rejection pathogenesis and prognosis. Moreover, molecular phenotype may better predict the graft outcome [3, 4].

In this study we aimed for evaluation of molecular signatures of acute and chronic rejections categories and for evaluation of association of gene transcripts with kidney graft loss due to chronic rejection.

2. Materials and Methods

2.1. Patients. For the purpose of this study, 41 case biopsies revealing early acute AMR ($n = 9$), early acute T-cell mediated rejection (TCMR) ($n = 10$), chronic AMR ($n = 13$), and chronic TCMR ($n = 9$) performed in 2007–2009 were evaluated. Basic demographic parameters of patients are

TABLE 1: Basic patient characteristics.

	AMR ^a	TCMR ^b	CAMR	CTCMR
N	9	10	13	9
Age	39.56 ± 9.91	49.63 ± 13.96	50.13 ± 11.63	52.60 ± 14.80
Female gender	1 [11.1%]	2 [20.0%]	5 [38.5%]	3 [33.3%]
HLA mismatches: total	4.0 ± 1.3	3.6 ± 1.1	3.4 ± 1.2	3.1 ± 1.1
HLA-A	1.4 ± 0.5	1.2 ± 0.7	1.2 ± 0.7	1.0 ± 0.5
HLA-B	1.5 ± 0.8	1.6 ± 0.5	1.3 ± 0.6	1.2 ± 0.4
HLA-DR	1.1 ± 0.6	0.8 ± 0.4	1.0 ± 0.6	0.9 ± 0.6
PRA at Tx [%]	17.4 ± 22.0	17.3 ± 36.0	17.5 ± 21.9	13.7 ± 12.1
IS: triple therapy based on FK	9 [100%]	10 [100%]	7 ^c [53.8%]	8 [88.9%]
triple therapy based on CsA	0 [0.0%]	0 [0.0%]	4 [30.8%]	0 [0.0%]
mTORi	0 [0.0%]	0 [0.0%]	1 [7.7%]	1 [11.1%]
other	0 [0.0%]	0 [0.0%]	1 [7.7%]	0 [0.0%]
First/second/third/fifth transplants	1/6/1/1 ^d	8/2/0/0	8/4/1/0	7/1/1/0
Induction therapy	9 [100%]	3 [30.0%]	5 [38.5%]	3 [33.3%]
Time to biopsy (months)			91.81 ± 66.99 ^e	33.84 ± 46.65 ^e
(days)	12 ± 4 ^f	8 ± 3 ^f		
sCr at Bx (μmol/L)	358.07 ± 148.55	398.66 ± 210.44	213.47 ± 105.15	272.99 ± 98.74
eGFR at Bx (mL/s/1.73 m ²)	0.34 ± 0.16	0.36 ± 0.26	0.49 ± 0.22	0.34 ± 0.11
Proteinuria at Bx (g/day)	1.77 ± 1.43	2.26 ± 1.94	2.8 ± 3.9	1.3 ± 1.1
C4d +	9 [100%]	0 [0.0%]	13 [100%]	0 [0.0%]
Graft loss during the followup (n)	1 [11.1%]	2 [20.0%]	4 [30.8%]	3 [33.3%]

Continuous variables are means ± SD.

^aIncluding combined AMR and TCMR (n = 3).

^bType IA (n = 2), IIA (n = 4), IB (n = 2), and IIB (n = 2).

^cSignificantly fewer FK treatment than in other groups (P < 0.05).

^dSignificantly more retransplantation in AMR (P < 0.05).

^eNo significant difference between CAMR and CTCMR.

^fSignificantly longer time to rejection in AMR compared to TCMR (P < 0.05).

shown in Table 1. All patients were treated with maintenance immunosuppression based on either tacrolimus (TAC, 82%) or cyclosporine A (CsA, 10%), along with mycophenolate mofetil and corticosteroids, or using mTOR inhibitors (5%) or CNI with azathioprine (3%). Patients received induction therapy with rATG (Thymoglobulin, Genzyme) or daclizumab (Zenapax, Roche) in a case of PRA > 50% and 20%, respectively. All patients were followed up for at least 24 months after the biopsy. Graft failure was defined as a return to dialysis treatment. All patients gave their written informed consent to participate in the study, and the Ethics Committee of the Institute for Clinical and Experimental Medicine in Prague approved the study protocol.

2.2. Renal Biopsy. All biopsies were performed using a 14-gauge Tru-Cut needle (Uni-Cut Nadeln, Angiomed, Germany) guided by ultrasound (Toshiba, Power Vision 6000, Japan). Small portions of renal tissue from the cortical or juxtamedullary zone were immediately stored in preserve solution (RNA later, Qiagen) for expression analysis, while the majority of renal tissue taken by core biopsy was used for routine histology performed by the standard method. Samples were routinely stained according to the protocol of our laboratory (H&E, PAS, Sirius red with elastin, AFOG, and PASM). Immunofluorescence detection of C4d was

performed in all cases. Biopsy tissue was scored on the basis of the Banff '07 working classification [1].

2.3. RNA Isolation and Gene Expression Analysis. The renal tissue was homogenized. Total RNA was extracted by RNA Blue (Top-Bio) and reversely transcribed into cDNA, using the SuperScript II Reverse Transcriptase (Invitrogen). Complementary DNA samples from each biopsy were analyzed on TaqMan Low Density Array Cards containing primers and probe sets for targets by 7900HT Fast Real-Time PCR System (Applied Biosystems). The set of targets was chosen on the basis of potential relevance to the study of renal allograft rejection according to the existing literature data (see Supporting Table S1 available online at <http://dx.doi.org/10.1155/2013/509259>). Specific gene expression was calculated relative to that of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the calibrator sample (FirstChoice Human Kidney Total RNA, Ambion) by comparative threshold cycle method ($2^{-\Delta\Delta C_T}$). RQ Manager 1.2 software for automated data analysis (Applied Biosystems) was used and results were expressed as relative quantity (RQ).

2.4. Statistical Analysis. After gene expression data were collected and the number of missing values was assessed.

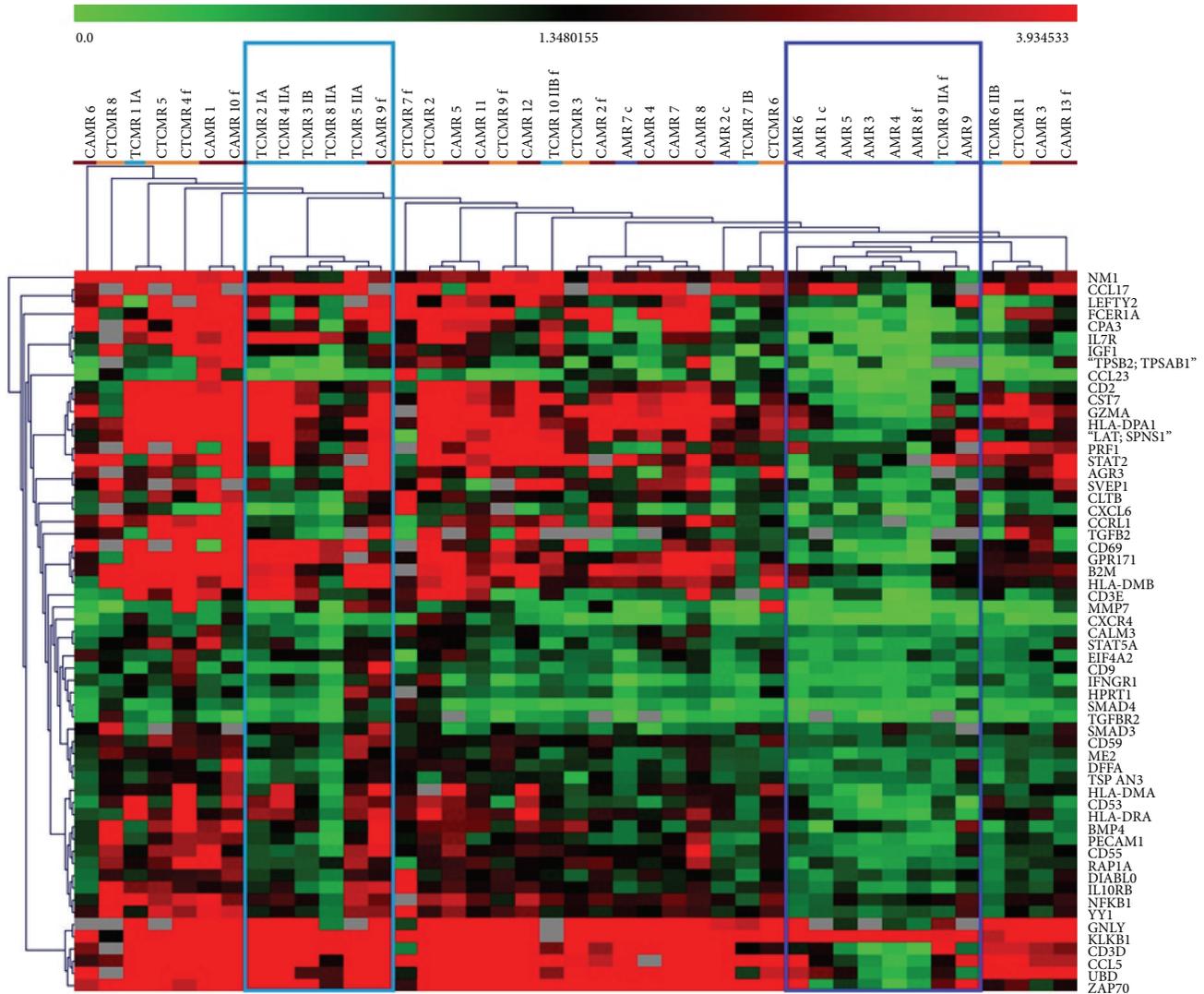


FIGURE 1: Unsupervised hierarchical clustering of samples with acute or chronic allograft rejection. Marked clusters contain 50% of TCMR samples or 78% of AMR samples, respectively. AMR samples out of the marked cluster had combined AMR and TCMR histological findings. Samples with CAMR and TCMR were mixed in different clusters. “f” in the sample name means failed; “c” in the sample name means combined AMR with TCMR.

Two low quality samples with less than 50% of successfully measured genes were excluded from all other analyses. Similarly, genes successfully measured in less than 45% of biopsy samples were excluded from all other analyses. Gene expression was statistically analyzed for the remaining 305 genes.

Basic statistical parameter data are given as absolute or relative frequency, average and SD, or median and range. Nonparametric test was used for analyzing data because of non normal distribution. Differences in mRNA expression or clinical parameters between groups were analyzed using the Kruskal-Wallis test and pairwise comparisons with Holm-Sidak correction and chi-square test for discrete variables. The differences in gene expression between groups expressed as RQ were considered to be biologically significant, if their means ratio reaches at least 1.5. PASW Statistics 18 software was used for statistical analyses. Unsupervised hierarchical

clustering was performed using MeV software. Variables which can assess the graft outcome were defined by discriminant function analysis. The variables which were significantly different between failed and survived grafts with chronic AMR and chronic TCMR and had no missing values were included in that analysis after log transformation. A P value < 0.05 was considered to be statistically significant in all tests.

3. Results

3.1. Patient Data. The clinical characteristics of patients are listed in Table 1. Patient age, gender, HLA mismatches, maximal or actual panel reactive antibodies (PRA), ischemia time, and number of failed grafts during 24 months followup did not differ significantly among patient groups. There were significantly more patients with retransplantation in the AMR group compared to others ($P < 0.05$). Significantly

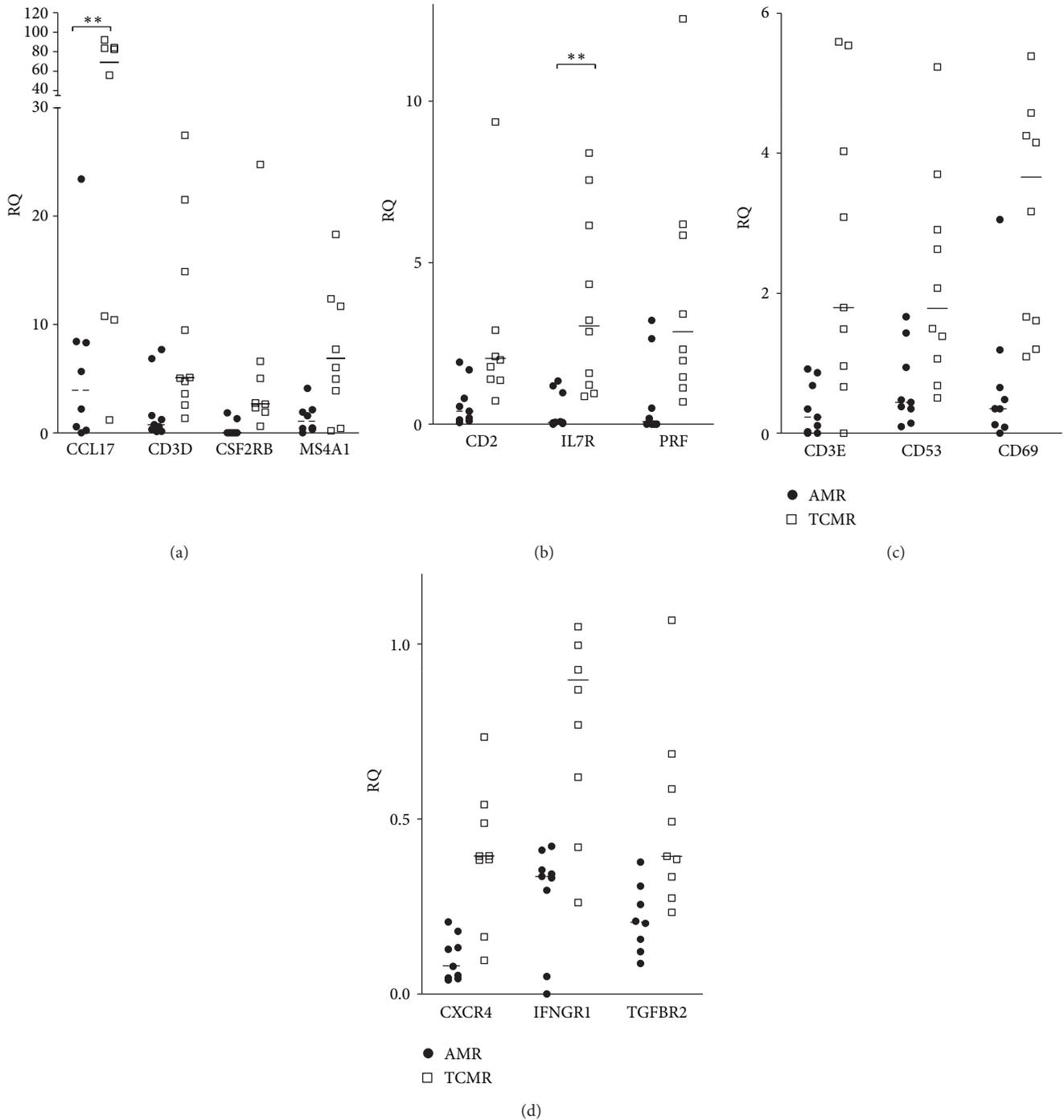


FIGURE 2: Genes differentially expressed between AMR and TCMR. Lines show medians of RQ. ** $P < 0.01$; all unmarked plots: $P < 0.05$.

more patients received ATG induction therapy in AMR group as compared to other groups. Followup to biopsy was longer in acute AMR as compared to acute TCMR ($P < 0.05$); however, no differences were observed between chronic AMR and chronic TCMR.

3.2. Gene Transcripts in Acute and Chronic Rejections. We compared the molecular profile among all rejection groups. In hierarchical clustering, samples with AMR were clustered

in one cluster, except for two samples in which the AMR was combined with TCMR (Figure 1). The cluster included only one sample with different histological diagnoses (TCMR, type IIA). Fifty percent of samples with TCMR were grouped together in another cluster. The rest of samples with TCMR (majority with type IB or IIB) were mixed together with chronic rejection samples. Cluster analysis did not distinguish chronic AMR from chronic TCMR. Similarly, the pairwise comparison did not reveal any difference in gene

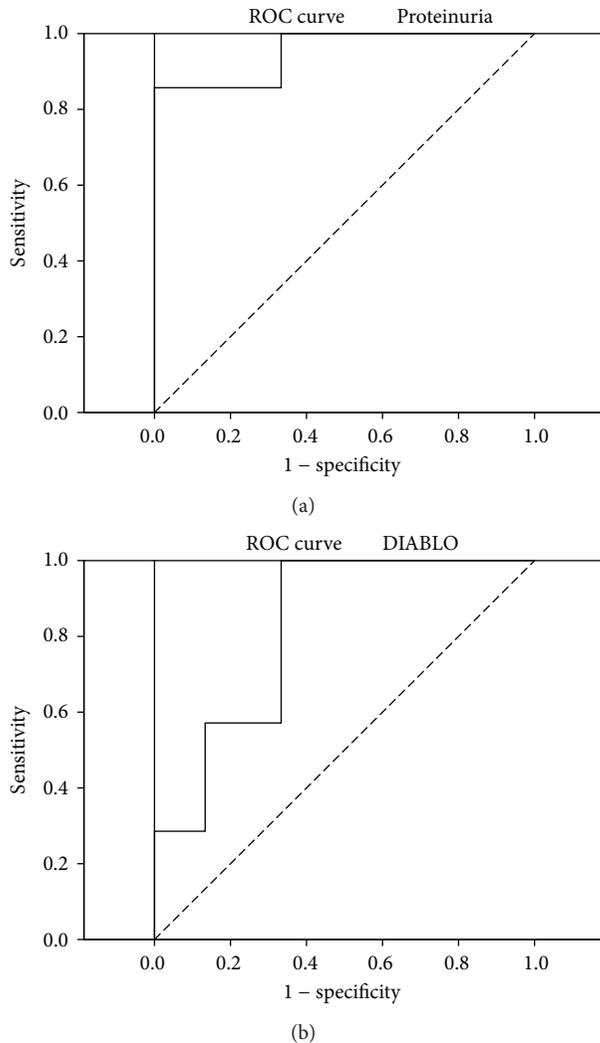


FIGURE 3: ROC curve analyzing graft failure after CAMR and CTCMR dependent on proteinuria at the time of biopsy (a) or intrarenal expression of DIABLO (b).

expression between chronic AMR and chronic TCMR. On the contrary, in acute rejection the set of genes that were differently regulated in acute TCMR and acute AMR was identified (Figure 2).

3.3. Chronic Rejection Outcome Prediction. The effect of gene expression patterns on the chronic rejection outcome was analyzed. There were no differences in expressions of evaluated genes between chronic AMR and chronic TCMR. In 7 cases, graft function deteriorated during the 24-month followup after biopsy to CKD5T and dialysis therapy was initiated. These patients exhibited significantly worse renal function and proteinuria at the biopsy. Similarly, they had longer followup after transplantation, higher expression of CDC20, CXCL6, DIABLO, GABRP, KIAA0101, ME2, MMP7, NFATC4, and TGFB3 mRNA, and lower expression of CCL19 and TRADD mRNA (Table 2). All patients whose grafts failed as a consequence of chronic rejection underwent first

renal transplantation. All different variables between failed and survived grafts after chronic rejection diagnosis were included in discriminant function analysis. This analysis revealed the proteinuria at the biopsy and DIABLO mRNA expression to discriminate failed and survived grafts. Next, classification functions containing these variables classified 100% of samples correctly. ROC analysis confirmed that proteinuria and DIABLO can predict the graft failure after chronic AMR or chronic TCMR (Figure 3, Table 3).

4. Discussion

In this study, both acute and chronic rejections were shown to be associated with several patterns of immune system related gene transcripts. Recently, despite different pathologies, both T-cell mediated and antibody mediated rejections were shown to be associated with similar molecular features [2, 5]. In both rejection types, interferon gamma (IFNG) related gene transcripts were shown to play a role. IFNG-inducible or cytotoxic T-cell associated transcripts distinguished rejection from nonrejection and were elevated in both acute T-cell mediated and antibody mediated rejections [6]. In our study, the upregulation of a large set of immune system related genes in acute T-cell mediated rejection was observed. Of note, IFNG-inducible and cytotoxic T-cell associated transcripts were upregulated in the acute TCMR that is in line with the observation of others [5, 6]. However, gene transcripts in both chronic rejection types were similar in our study. Neither paired test nor hierarchical clustering distinguished these types of chronic rejection on the molecular basis. There are few data in the current literature dealing with gene expression in chronic rejection of renal allograft. Changes in histological classification that passed off in the last several years could have contributed to the lack of such studies. While in Banff '97 classification [7] the chronic changes were represented by non specific chronic allograft nephropathy category, Banff '05 Meeting substituted it by interstitial fibrosis and tubular atrophy (IF/TA) and the terms chronic active antibody mediated rejection and chronic active T-cell-mediated rejection were included [8]. Another complication seems to be the relative rarity of biopsies diagnosed as chronic active T-cell-mediated rejection. Studies dealing with molecular phenotyping of chronic antibody mediated rejection focused on comparison with samples from patients with stable renal function and normal histopathology [9]. However, our study is one of the first studies focusing on comparison of gene expression in precisely defined histological findings, described by Banff 2007 classification as antibody-mediated and T-cell-mediated chronic rejections. It was not possible to find out differences between those two diagnoses without prior whole-genome microarray screening only on the basis of the literature-based selection of 305 analyzed genes. Several genes indicating inflammation were significantly upregulated in grafts that failed after chronic rejection in our study. Other upregulated genes in failed grafts belonged to cytotoxic T cells associated transcripts, ENDATs, chemotactic transcripts, or apoptosis markers. Some of them were referred to predict graft failure also in the study of Einecke et al. [10]. In the discriminant

TABLE 2: Significant differences between failed and survived grafts after chronic rejection (both CAMR and CTCMR). Only variables that reach statistical significance are listed.

	Failed grafts ($n = 7$)	Survived grafts ($n = 15$)	P
sCr at Bx [$\mu\text{mol/L}$]	298.1 [197.3–456.8]	193.5 [91.2–485.6]	0.005
eGFR at Bx [mL/s/1.73 m^2]	0.28 [0.18–0.37]	0.47 [0.13–0.98]	0.008
Proteinuria at Bx [g/day]	3.47 [0.61–12.58]	0.47 [0–2.35]	0.001
CCL19	1.5197 [0.1042–11.1551]	8.6061 [0.4848–82.2367] ^b	0.044
CDC20	2.4168 [1.8262–3.1978]	0.9337 [0.4203–3.0410]	0.007
CXCL6	2.6814 [0.3292–14.7608] ^a	0.4933 [0.0000–1.9457] ^b	0.039
DIABLO	1.9110 [1.6832–6.2070]	1.3873 [0.7627–2.0413]	0.018
GABRP	3.5970 [0.5333–13.8507] ^a	0.2527 [0.0000–7.2719] ^b	0.013
KIAA0101	5.9674 [2.8230–17.7875]	3.7627 [0.2982–6.2863]	0.032
ME2	1.7962 [1.0032–2.5815]	1.1111 [0.6937–2.5290]	0.038
MMP7	1.4519 [0.8888–4.4876]	0.4838 [0.0026–4.3613]	0.004
NFATC4	2.3609 [0.8072–7.0016] ^c	0.7314 [0.0000–2.3032] ^b	0.012
TGFB3	1.1726 [0.1405–2.0126] ^c	0.1292 [0.0000–15.6462] ^d	0.034
TRADD	2.3305 [0.9925–16.0450] ^e	9.6845 [3.1361–45.8793] ^e	0.037

Variables are presented as median (min–max).

^a $n = 6$.

^b $n = 14$.

^c $n = 5$.

^d $n = 12$.

^e $n = 10$.

TABLE 3: Cutoff values from ROC curve analysis that discriminate failed and survived grafts with the best combination of sensitivity and specificity.

	Optimal cutoff	Sensitivity	Specificity	AUC	95% CI
Proteinuria	2.35	85.7	100	0.952	0.768–0.993
DIABLO	1.43	100	66.7	0.819	0.598–0.947

function analysis, however, the outcome of chronic rejection in our cohort of patients depended only on the state of disease and graft injury (proteinuria) in combination with intrarenal expression of DIABLO, the caspase activator playing the key role in apoptosis. It was clearly shown that proapoptotic mechanisms have been implicated in ischemia induced acute kidney injury [11]. Similarly, chronic rejection of kidney allograft is associated with small vessels narrowing causing local ischemia.

In conclusion, in this study, beside transcripts differences observed between acute T-cell mediated and antibody mediated rejections, both chronic rejections did not differ in 305 analyzed genes.

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Research Article

Protective Effects of HBSP on Ischemia Reperfusion and Cyclosporine A Induced Renal Injury

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Ischemia reperfusion (IR) and cyclosporine A (CsA) injuries are unavoidable in kidney transplantation and are associated with allograft dysfunction. Herein, the effect and mechanism of a novel tissue protective peptide, helix B surface peptide (HBSP) derived from erythropoietin, were investigated in a rat model. The right kidney was subjected to 45 min ischemia, followed by left nephrectomy and 2-week reperfusion, with or without daily treatment of CsA 25 mg/kg and/or HBSP 8 nmol/kg. Blood urea nitrogen was increased by CsA but decreased by HBSP at 1 week and 2 weeks, while the same changes were revealed in urinary protein/creatinine only at 2 weeks. HBSP also significantly ameliorated tubulointerstitial damage and interstitial fibrosis, which were gradually increased by IR and CsA. In addition, apoptotic cells, infiltrated inflammatory cells, and active caspase-3+ cells were greatly reduced by HBSP in the both IR and IR + CsA groups. The 17 kD active caspase-3 protein was decreased by HBSP in the IR and IR + CsA kidneys, with decreased mRNA only in the IR + CsA kidneys. Taken together, it has been demonstrated, for the first time, that HBSP effectively improved renal function and tissue damage caused by IR and/or CsA, which might be through reducing caspase-3 activation and synthesis, apoptosis, and inflammation.

1. Introduction

Kidney transplantation is the best treatment for patients with end-stage renal disease. Ischemia reperfusion (IR) injury is associated with delayed graft function, acute rejection, and chronic allograft dysfunction [1, 2]. The principle approaches to improve allograft survival have focused largely on the inhibition of immune cell activation in recipients [3], as the release of immune adjuvants initiating by IR injury promotes adaptive alloimmune responses and rejection. Cyclosporine A (CsA) is a mainstay immunosuppressant following kidney transplantation, but its nephrotoxicity limits clinical application [4].

The mechanism IR and/or CsA induced injury has been intensively studied but still not been fully understood. IR

and CsA directly damage tubular epithelial cells and cause interstitial fibrosis through upregulating TGF- β 1 dependent pathways, which consequently results in apoptosis and inflammation [5]. The activation of caspase-3 was revealed in the injury induced by IR and/or immunosuppressants including CsA [6], which plays crucial roles in apoptosis and inflammation, either improving renal function and structure through resolving inflammation and remodeling or leading to renal cell deletion and fibrosis [7, 8].

Since the early 1990s, erythropoietin (EPO) has been found to protect different organs including the brain, heart, and kidney against IR injury [9]. EPO protects tissues through a heterodimer composed of EPO receptor and β -common receptor (β cR), which is pharmacologically distinct

from the homodimer receptor that is known to mediate erythropoiesis [10]. Our own studies demonstrated the renoprotection of EPO against IR injury by decreasing tubular cell apoptosis but promoting inflammatory cell apoptosis [11, 12]. The tissue protection, however, requires large dosage of EPO, which often causes hypertension and thrombosis *in vivo* [13, 14]. Therefore, a novel helix B surface peptide (HBSP) that interacts only with the heterodimer receptor has been developed, which is composed of 11 amino acids (QEQLERALNSS) derived from the aqueous face of helix B in EPO 3D structure. The tissue protective activities of HBSP comparable with EPO were demonstrated in a variety of biological settings [15]. HBSP has been shown to reduce apoptotic cardiomyocytes [16] and to activate critical survival signaling pathways [17].

In this study, the effects of HBSP were further evaluated on the kidneys subjected to an initial IR followed by CsA induced injuries mimicking a clinical posttransplant setting in a 2-week rat model. It has been hypothesized that HBSP improves renal function and structure through modifying caspase-3, apoptosis, and inflammation.

2. Materials and Methods

2.1. Renal IR Injury Model. Male Sprague-Dawley rats weighing 180–200 g were obtained from the Experimental Animal Center of Nantong University, China, and housed at constant temperature (25°C) and humidity (55%) on a 12-hour light/dark cycle, fed *ad libitum* on standard laboratory rat chow with free access to tap water. All animal procedures were performed according to the guidelines of the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee.

For the renal IR injury, the rat was anesthetized by 50 mg/kg chloral hydrate, with no sign of pain during surgical procedures without using analgesia. The abdominal cavity was exposed through a midline incision, and the renal pedicles were carefully isolated. The right renal pedicle occlusion was performed using nontraumatic vascular clamps for 45 min and the efficacy of occlusion was confirmed by color changing in the entire kidney. The left nephrectomy was performed before reperfusion for 2 weeks. To minimize the number of experimental animals, the tissues immediately collected from 6 left nephrectomized kidneys were used as the normal control, while the tissues collected after 45 min renal pedicle occlusion from another 6 left nephrectomized kidneys were used as the ischemia only (I only) control.

Rats were randomly divided into 4 groups ($n = 6$): (1) IR group: IR injury, (2) IR + CsA group: IR injury with 25 mg/kg microemulsion CsA (Novartis Pharma GmbH, Eberbach, Germany) dissolved in pure olive oil and administered by gavage daily, (3) IR + HBSP group: IR injury with 8 nmol/kg HBSP (Shanghai Science Peptide Biological Technology Co., Ltd, Shanghai, China) dissolved in 0.9% saline intraperitoneally injected after reperfusion once a day, and (4) IR + CsA + HBSP group: IR injury treated with CsA and HBSP.

2.2. Sample Collection. Blood samples were collected from orbital venous plexus before surgery, 1 and 2 weeks after

reperfusion to obtain serum. At the same time points, 24 h urine samples were also collected using metabolic cages. Animals were ethically sacrificed at 2 weeks after IR injury. The kidneys were harvested, either fixed with 10% (wt/vol) neutral buffered formalin for histology and immunohistochemistry or snap frozen in liquid nitrogen and stored at -80°C for western blot and qPCR analysis. Urea nitrogen, creatinine, and albumin in both serum and urine were measured using an automatic biochemistry analyzer (Siemens, Berlin, Germany).

2.3. Histological Assessment. The paraffin embedded kidneys were sectioned and stained by H&E and Masson's trichrome to evaluate tubulointerstitial damage (TID) and interstitial fibrosis. H&E stained sections were semiquantitatively scored for tubular damage (dilation and vacuolation), interstitial expansion (edema or inflammatory cell infiltration), and cells or cell debris in tubular lumens, respectively, based on a scale of 0–3. The damage affecting no more than 5% of the field was scored 0; mild damage affecting 5%–25% of the field was scored 1; moderate damage affecting 25%–75% of the field was scored 2; and severe damage exceeding 75% of the field was scored 3. The examiners were blinded to the experimental groups, and 12 randomly selected cortical fields were scored at 200x magnification. The scores from three compartments assessed were then summed up to obtain an average score per field for each group.

Masson's trichrome staining was performed according to a standard protocol to evaluate collagen deposition. Four μm paraffin sections were deparaffinized, stained with Weigert's hematoxylin for 10 min, and then stained with solution containing chromotropic acid, light green, phosphotungstic acid, and glacial acetic acid for another 10 min, followed by 0.5% light green for 5 min. Collagen deposition in the cortex was quantified in 20 fields at 400x magnification using the Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) incorporating a Leica microscope (Leica, Solms, Germany).

2.4. In Situ End-Labeling Apoptotic Cells. Four μm paraffin sections were used for ISEL with digoxigenin-deoxyuridine (dUTP) by terminal deoxynucleotidyl transferase (TdT) using an ApopTag peroxidase kit (Appligene Oncor, Illkirch, France). Briefly, sections were digested by 40 $\mu\text{g}/\text{mL}$ proteinase K for 15 min at 37°C , incubated with TdT and digoxigenin-dUTP at 37°C for 60 min, and transferred to "stop" buffer for 30 min. After adding antidigoxigenin-peroxidase complex for 30 min, these sections were developed by 3'-amino-9-ethylcarbazole (AEC, dark red color) substrate. Apoptotic cells were separately examined in the tubular, interstitial, and tubular lumen areas in 20 fields at 400x magnification.

2.5. Immunostaining of Myeloperoxidase (MPO) and Active Caspase-3. Immunohistochemical staining of MPO and active caspase-3 (detecting the 17 kD subunit of caspase-3) was undertaken on 4 μm paraffin sections using a DAKO ChemMate EnVision Detection Kit (DAKO, Glostrup, Denmark). Antigen retrieval was performed by 40 $\mu\text{g}/\text{mL}$ proteinase K digestion at 37°C , 10 min for MPO; immersion of the sections in 10 mM sodium citrate buffer, pH 6.0, in

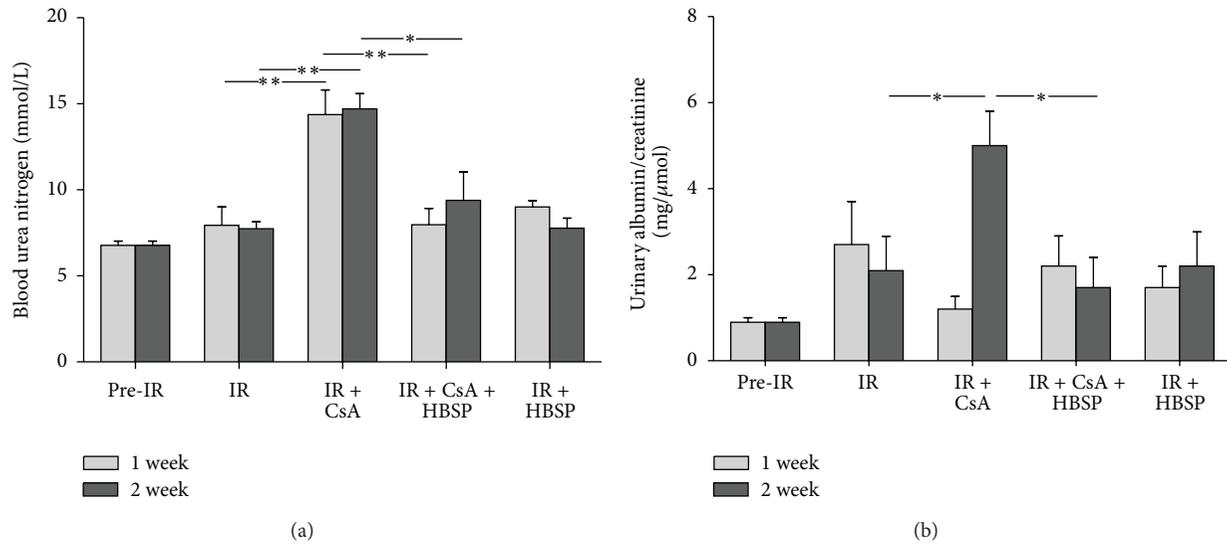


FIGURE 1: The level of BUN was decreased by HBSP treatment in the IR kidneys exposed to CsA at both 1 and 2 weeks (a). The ratio of urinary albumin/creatinine was significantly increased by CsA in the kidneys subjected to IR injury but decreased by HBSP treatment only at 2 weeks (b). Data are expressed as mean value of each group (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

a steam bath maintained by high power microwave, 5 min, twice, cooled at room temperature, 20 min for active caspase-3, and then blocked by peroxidase-blocking reagent. The sections were labeled by anti-MPO antibody (1:600 dilution, DAKO) or antiactive caspase-3 antibody (1:100 dilution, R&D System, Abingdon, UK) at 4°C overnight. The sections were incubated with anti-rabbit/mouse secondary antibody (DAKO) for 30 min at room temperature. The antibody binding was revealed by AEC with hematoxylin counterstaining. MPO+ cells and active caspase-3+ in the tubular, interstitial, and tubular lumen areas were semiquantitatively scored in 20 fields at 400x magnification, respectively.

2.6. Western Blot Analysis. Thirty micrograms of protein from kidney cortex was separated on a 15% (weight/volume) polyacrylamide denaturing gel and electroblotted onto a PVDF membrane on 12 volts for 16 h at 4°C. This was blocked with 5% (weight/volume) milk and probed with a polyclonal full length caspase-3 antibody (Santa Cruz Biochemicals, Santa Cruz, USA) or a monoclonal β -actin antibody (Abcam, Cambridge, UK) at 1:500 or 1:10000 dilution. The peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, USA) was incubated for 2 h at room temperature. Antibody binding was revealed using ECL substrate (Thermo Fisher Scientific, Rockford, USA) and a Molecular Imager Chemi Doc XRS+ system (Bio-Rad, Berkeley, USA). Developed images were semiquantitatively analyzed by scanning volume density using Alpha View Software 3.3 (Cell Biosciences, Inc. Santa Clara, USA). Optical volume density values for caspase-3 were corrected for loading with use of β -actin and expressed as the percentage of average control volume density.

2.7. Real-Time qPCR. The detection of caspase-3 mRNA in renal tissues was performed by qPCR using an ABI StepOne

PCR system. The probes of caspase-3 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were both 6-carboxy-fluorescein (FAM) labeled (Life Technologies, Paisley, UK). Total RNA was extracted using TRIZOL reagent (Life Technologies). For complementary DNA synthesis, 5 μ g of total RNA was used for reverse transcription (Fermentas, Glen Burnie, USA). Two μ L of reverse transcription product was amplified with Taq polymerase (Life Technologies) in qPCR reaction buffers containing 900 nM forward or reverse primer and 250 nM probe at 95°C for 10 min followed by 45 cycles of 95°C for 30 seconds and 60°C for 1 min. The sequences of forward primer, reverse primer, and probe were previously used [7, 8]. The expression of caspase-3 mRNA in kidneys normalized with GAPDH was calculated against relative noninjured kidneys (the normal control) using a $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical Analysis. Data were presented as mean \pm standard error of the mean (SEM). Statistical analysis (SPSS 18.0 software, SPSS Inc., Armonk, NY, USA) of the data was performed with a one-way ANOVA after the demonstration of homogeneity of variance. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Renal Function. The level of blood urea nitrogen (BUN, mmol/L) was increased by CsA compared with the IR group at 1 week and 2 weeks (14.4 ± 1.4 versus 8.0 ± 1.1 ; 14.7 ± 0.9 versus 7.7 ± 0.5 , $P < 0.01$, Figure 1(a)). HBSP decreased the level of BUN in the IR + CsA group at both time points (8.0 ± 0.9 , $P < 0.01$; 9.4 ± 1.7 , $P < 0.05$). The IR injury, however, did not significantly change BUN in contrast to the Pre-IR group.

The ratio of urinary protein/creatinine (mg/ μ mol) was also increased by CsA compared with the IR group,

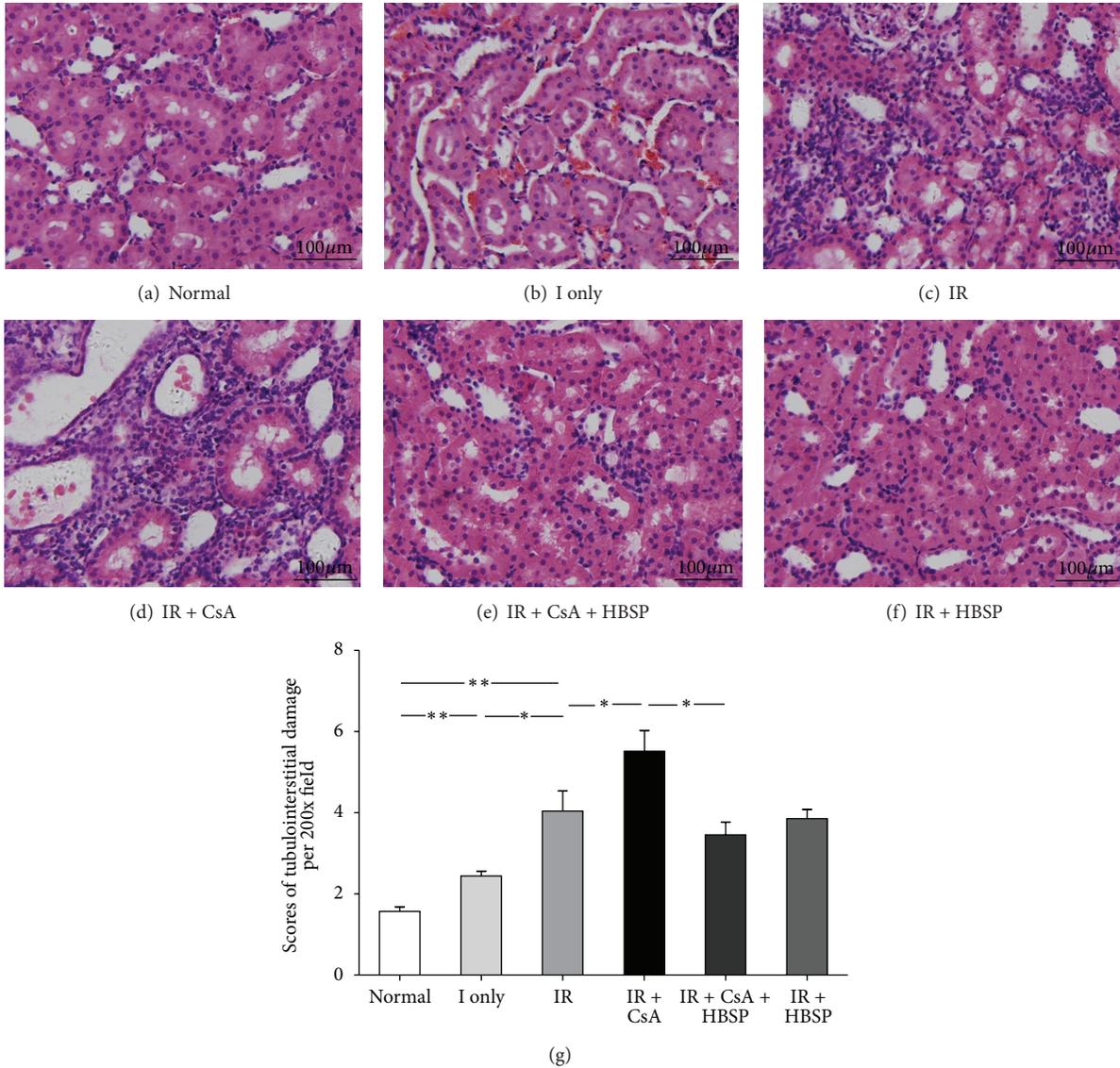


FIGURE 2: TID was assessed in H&E stained sections. Tubular dilation, epithelial cell vacuolation, interstitial expansion with edema or inflammation, and cells or cell debris in tubular lumens were mainly seen in IR kidneys and kidneys exposed to CsA (a–f). Semiquantitative scores revealed that the score of TID was significantly higher in the CsA exposed IR kidneys but reduced by HBSP (g). Data are expressed as mean number in the high power field of each group (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

but reduced by HBSP only at 2 weeks (0.50 ± 0.08 versus 0.21 ± 0.08 or 0.17 ± 0.07 , $P < 0.05$, Figure 1(b)), without significant changes between any other groups.

There was, however, no statistically significant difference between groups at 1 week and 2 weeks in either serum creatinine (Pre-IR: $71.2 \pm 1.4 \mu\text{mol/L}$; IR: 51.4 ± 3.9 ; 55.7 ± 5.3 ; IR + CsA: 66.8 ± 5.2 ; 62.6 ± 4.2 ; IR + CsA + HBSP: 81.6 ± 4.8 ; 68.8 ± 4.6 ; and IR + HBSP: 63.7 ± 2.7 ; 58.0 ± 6.8) or serum albumin (Pre-IR: $39.0 \pm 0.9 \text{ g/L}$; IR: 35.5 ± 0.4 ; 34.1 ± 1.2 ; IR + CsA: 36.2 ± 0.6 ; 29.7 ± 2.6 ; IR + CsA + HBSP: 38.4 ± 1.1 ; 31.7 ± 1.5 ; and IR + HBSP: 38.7 ± 0.6 ; 33.8 ± 1.2).

3.2. Histological Findings. To assess the degree of TID in H&E stained sections (Figures 2(a)–2(f)), the score of tubular damage, and interstitial expansion and cells or cell debris in

tubular lumens were semiquantitatively analyzed separately (data not shown). The total score of TID (Figure 2(g)), summed up from the 3 individual scores, was increased by the IR injury compared to the ischemia only or the normal group (4.0 ± 0.5 versus 2.5 ± 0.1 or 1.6 ± 0.1 , $P < 0.01$) and further increased by CsA (5.5 ± 0.5 , $P < 0.05$) but decreased by HBSP (3.5 ± 0.3 , $P < 0.05$). However, HBSP (3.9 ± 0.2) did not significantly change the score of TID induced by the IR injury. In addition, Masson's trichrome staining (Figures 3(a)–3(f)) demonstrated that IR injury caused more collagen deposition in tubulointerstitial areas compared to the normal group or ischemia only group (0.024 ± 0.001 versus 0.007 ± 0.000 or 0.005 ± 0.000 , $P < 0.01$). Collagen deposition was further increased by CsA (0.047 ± 0.003 , $P < 0.01$), but decreased by HBSP (0.029 ± 0.005 , $P < 0.05$, Figure 3(g)).

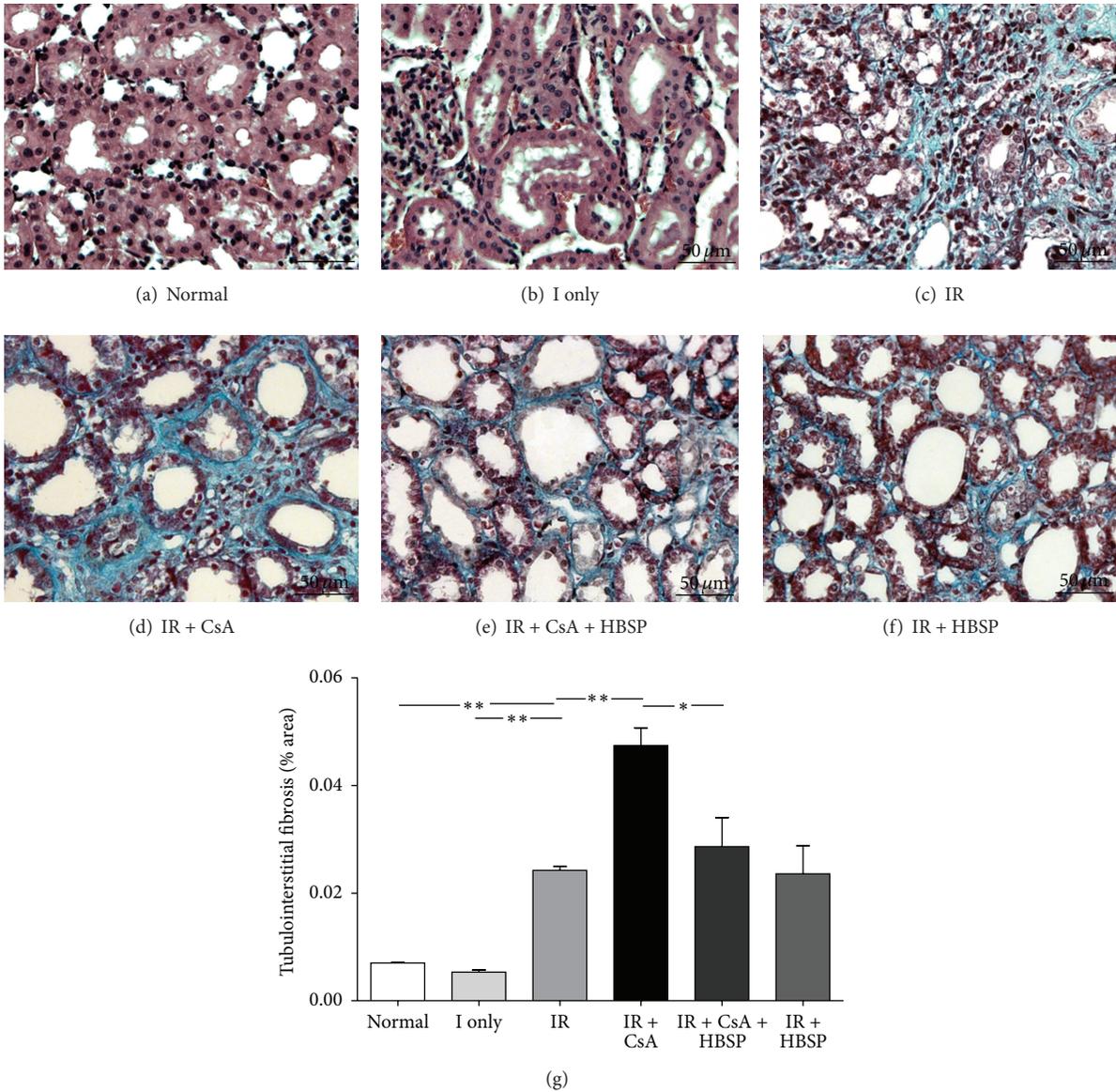


FIGURE 3: Tubulointerstitial fibrosis was detected by Masson's Trichrome staining (a–f). IR did cause more collagen deposition compared with the normal or ischemia only group. The typical striped interstitial fibrosis and significantly increased extracellular collagen deposition in the cortex (400x) were shown in the IR + CsA group in comparison to the IR group, which was significantly lowered by HBSP (g). Data are expressed as the percentage of stained area per high power field of each group (mean ± SEM; n = 6). *P < 0.05; **P < 0.01.

3.3. Cellular Apoptosis. Apoptotic cells, detected by *in situ* end labelling fragmented DNAs (ISEL), were mainly located in the tubular and interstitial areas (Figures 4(a)–4(f)); some of them had polymorphic nuclei (Figure 4(f)). There were very few apoptotic cells seen in glomerular areas. The total number of apoptotic cells (Figure 4(g)) summed up from that in tubular, interstitial, and tubular lumen areas separately (data not shown) was greatly increased in the IR group compared the ischemia only and normal group (48.5 ± 1.2 versus 11.3 ± 1.6 or 4.7 ± 1.4 , $P < 0.01$, Figure 4(g)). CsA (55.0 ± 2.6 , $P < 0.01$) further increased apoptotic cells in the IR kidneys, which were decreased by HBSP (35.3 ± 2.5 , $P < 0.05$). HBSP also reduced apoptotic cells in the IR kidneys

(34.0 ± 1.5 , $P < 0.01$), with the most predominant effect in the tubular areas.

3.4. Inflammation Assessment. The inflammation was assessed by immunostaining of MPO+ cells (Figures 5(a)–5(f)), a marker mainly for neutrophil granulocytes. The total number of MPO+ cells in each field (Figure 5(g)) was the sum of that in 3 individual compartment (data not shown). There were limited MPO+ cells in the normal (21.3 ± 1.3) and the ischemia only kidneys (25.2 ± 2.7). However, MPO+ cells were dramatically increased by IR and CsA (35.3 ± 2.4 ; 50.3 ± 2.6 , $P < 0.01$), especially in peritubular and expanded interstitial areas (Figures 5(c),

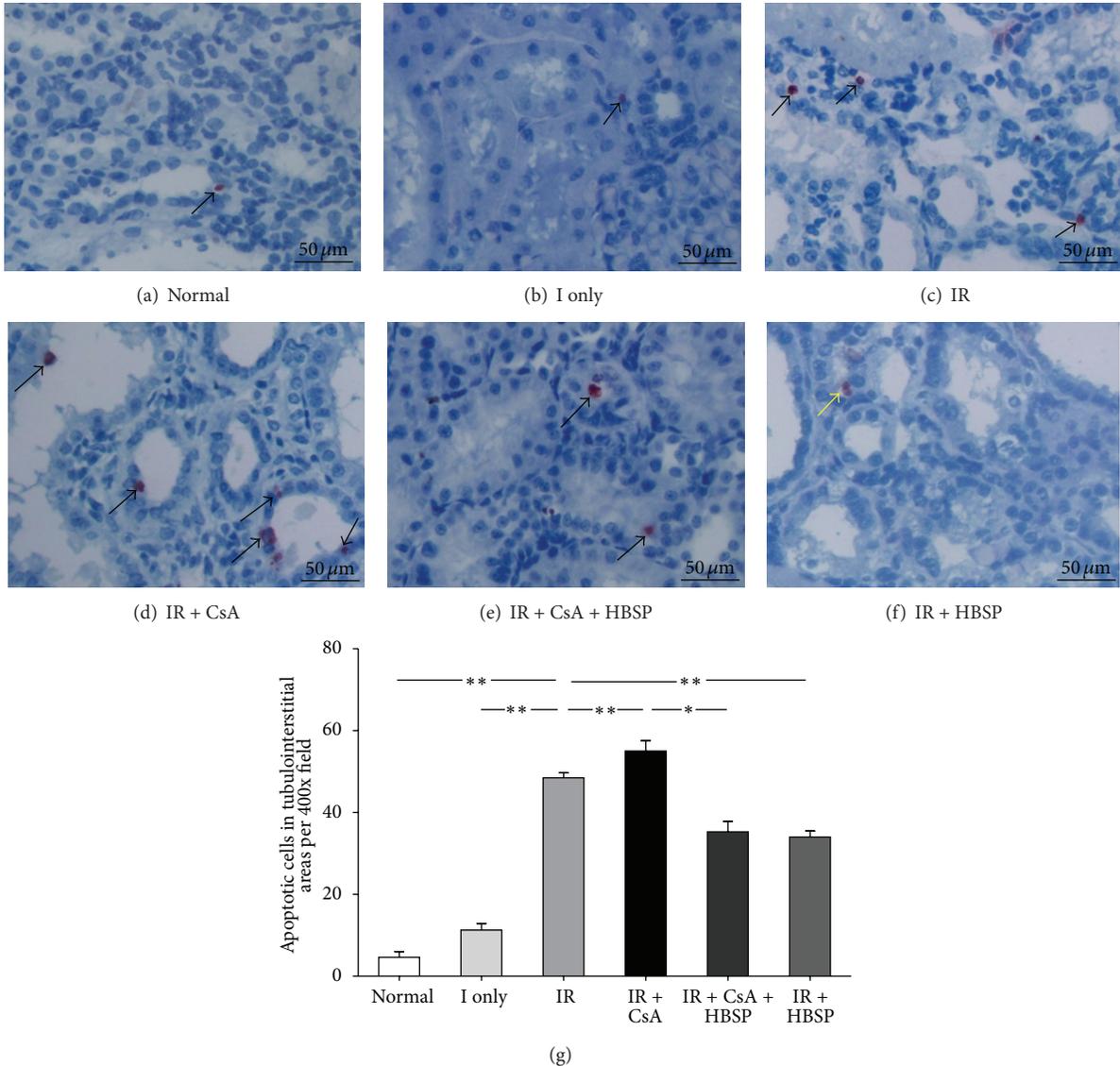


FIGURE 4: Apoptotic cells, labeled by fragmented DNAs, were mainly shown in tubular areas (a, d) and interstitial areas (b, c); some in tubular lumens (e); others with polymorphous nuclei ((f), yellow arrow). The number of apoptotic cells was significantly increased in the IR and IR + CsA kidneys but decreased by HBSP (g). Data are expressed as mean number per high power field of each group (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

5(d), and 5(e)). HBSP decreased MPO+ cells induced by not only CsA (29.2 ± 4.8 versus 50.3 ± 2.6 , $P < 0.05$) but also IR (27.5 ± 2.0 versus 35.3 ± 2.4 , $P < 0.01$), mainly in the interstitial areas. Some MPO+ cells demonstrated condensed nuclei, morphologic features of apoptosis (Figure 5(d)), or shed into tubular lumens (Figures 5(e) and 5(f)).

3.5. Active Caspase-3+ Cells. The distribution of active caspase-3+ cells detected by immunostaining, recognizing 17 kD subunit, was mainly in the tubular and interstitial areas (Figures 6(b)–6(f)), fewer in glomerular areas. Almost all positive cells showed condensed nuclei, the morphologic feature of apoptosis. The total number of active caspase-3+ cells in each field (Figure 6(g)) was the sum of that in 3

individual compartments (data not shown). Active caspase-3+ cells were increased by ischemia only (34.5 ± 1.6) and IR (33.0 ± 2.6) compared with the normal group (23.5 ± 0.9 , $P < 0.05$) further increased by CsA (39.5 ± 1.4 , $P < 0.05$) but decreased by HBSP (25.0 ± 0.6 , $P < 0.05$). The similar change of active caspase-3+ cells was seen in tubular areas, less significant changes in interstitial areas, but no difference in tubular lumens.

3.6. Caspase-3 Protein Expression. The expression of caspase-3 protein subunits in rat kidneys was detected by western blot (Figure 7(a)). The numerically lowest expression of 32 kD caspase-3 precursor was shown in the IR + CsA group (8.5 ± 0.9) but significantly reversed by HBSP (28.9 ± 2.1 , $P < 0.01$,

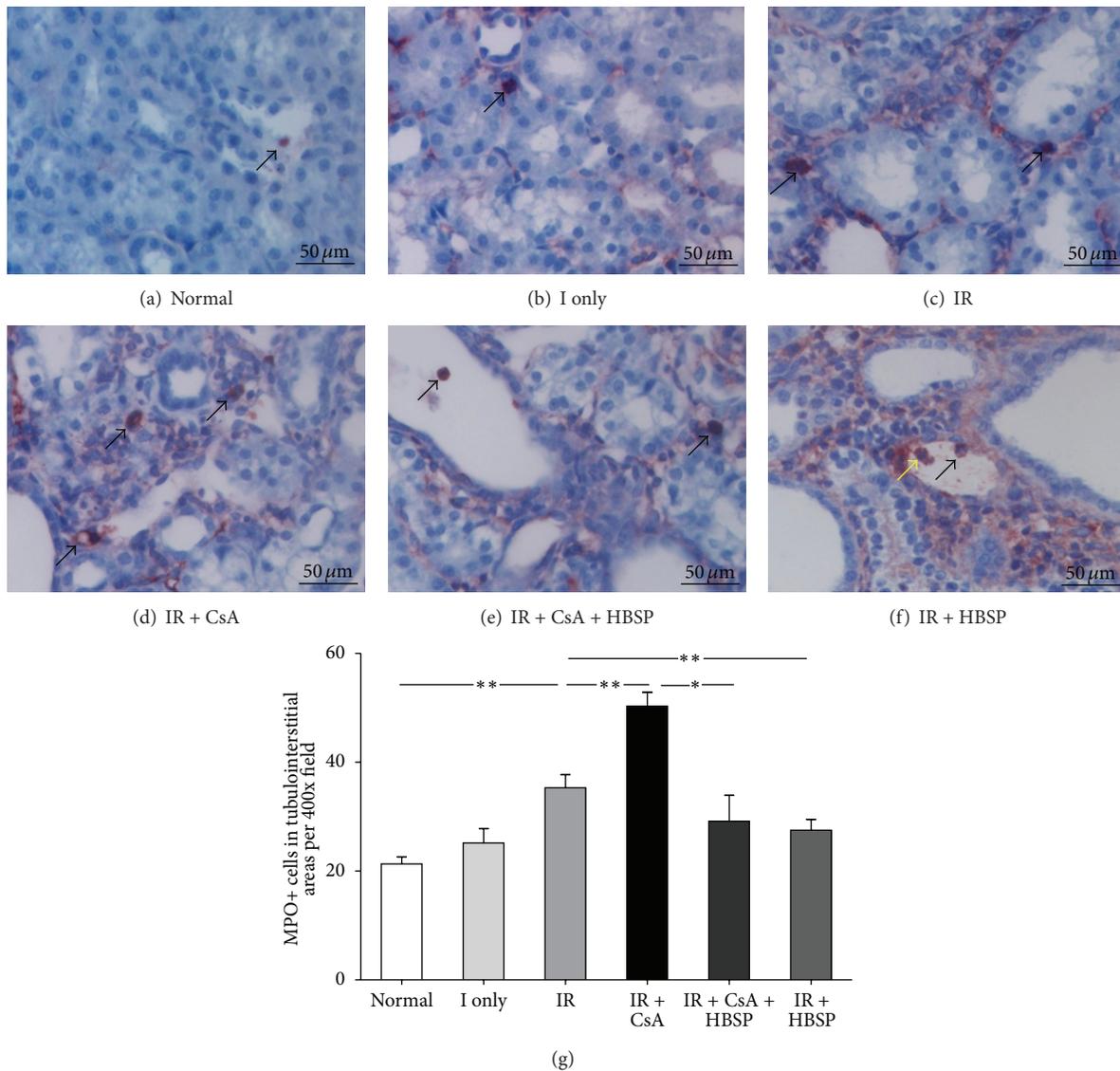


FIGURE 5: Myeloperoxidase (MPO)+ cells (dark red color), detected by Immunostaining, were mainly located in tubular lumens (e, f) and interstitial areas (b–e); and a few were also seen in tubular areas (a, c) and glomerular areas. MPO+ cells were significantly increased by IR and CsA but decreased by HBSP (g). The yellow arrow indicated a MPO+ cell with apoptotic feature (f). Data are expressed as mean number in the high power field of each group (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

Figure 7(b)). The 17 kD active subunit of caspase-3 was significantly upregulated by ischemia only (18.9 ± 2.6 , $P < 0.01$) and IR (16.2 ± 2.7 , $P < 0.05$) compared to the normal group (7.4 ± 0.5 , Figure 7(c)), and further increased by CsA (28.2 ± 4.3 , $P < 0.05$) but decreased by HBSP in both IR and IR + CsA groups (4.9 ± 1.0 , $P < 0.01$; 12.4 ± 2.3 , $P < 0.05$, Figure 7(c)). The 12 kD active caspase-3 was mainly expressed in the ischemia only kidneys, which was decreased after reperfusion in the IR kidneys (17.3 ± 1.9 versus 7.1 ± 0.7 , $P < 0.01$, Figure 7(d)).

3.7. Caspase-3 mRNA Expression. To further understand the transcription of caspase-3, the expression of caspase-3 mRNA was measured by real-time quantitative reverse-transcriptase

polymerase chain reaction (qPCR). The level of caspase-3 mRNA was increased by CsA compared in the normal group (3.5 ± 0.9 versus 1.2 ± 0.2 , $P < 0.05$) but decreased by HBSP (1.0 ± 0.4 , $P < 0.05$, Figure 8) in the IR + CsA group.

4. Discussion

A disassociation between IR and CsA induced injuries was revealed at the end of 2 weeks in this study, with the recovery of acute IR injury and the accumulation of CsA toxicity. Most interestingly, the protection of HBSP at 2 weeks was shown mainly against CsA toxicity for the first time, with improved renal function and tissue structure, but also against IR injury with less inflammation and apoptosis associated with caspase-3 activation and synthesis inhibition.

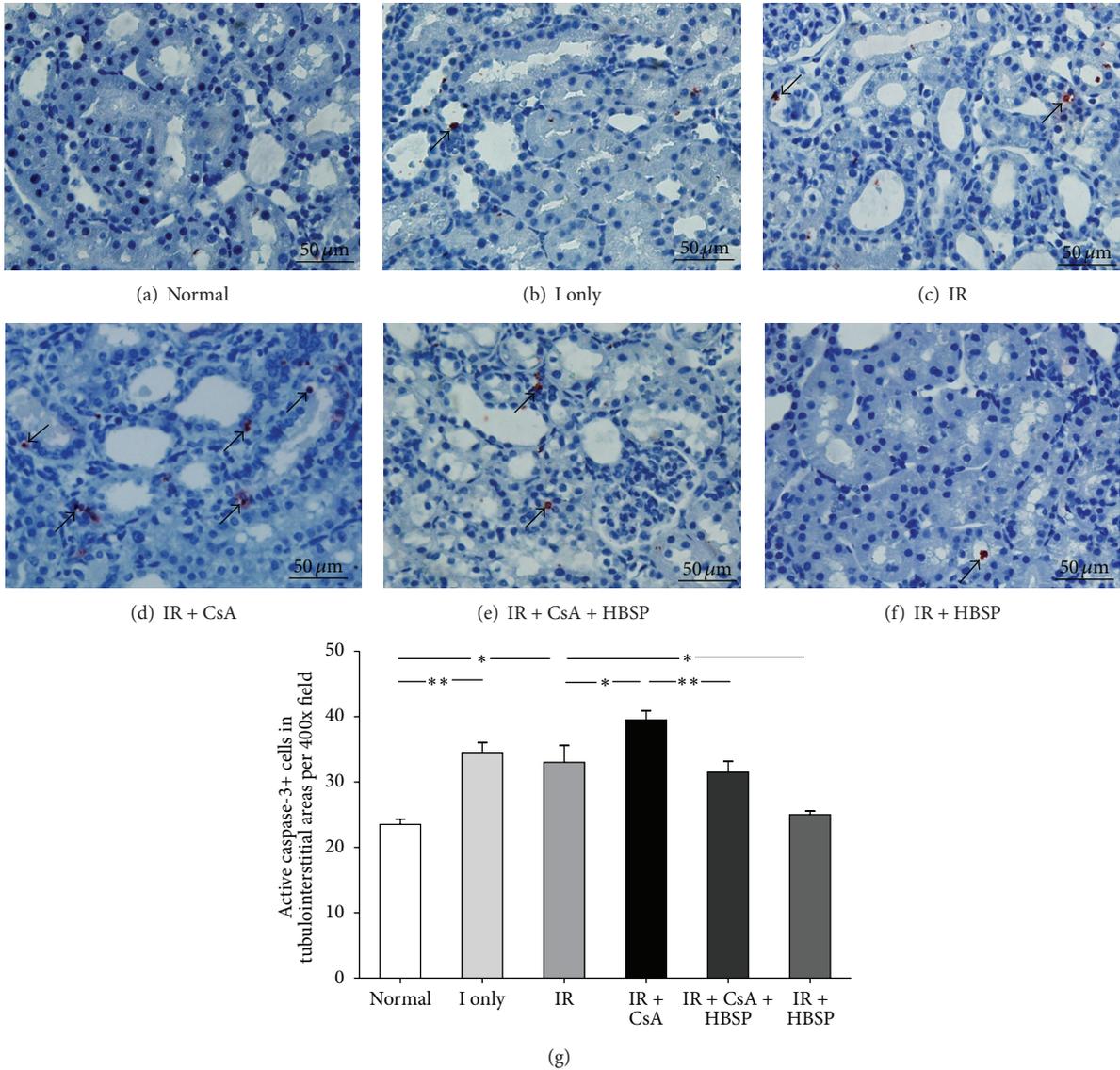


FIGURE 6: The majority of active caspase-3+ cells, detected by immunostaining (a–f), were demonstrated morphologic features of apoptosis such as condensed nuclei. HBSP significantly decreased active caspase-3+ cells in both the IR kidneys and IR + CsA kidneys (g). Data are expressed as mean number per high power field of each group (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

To disclose the clinical impact of HBSP, renal function was firstly assessed. The level of BUN remarkably increased by CsA at both 1 and 2 weeks, while urinary protein/creatinine was also further increased by CsA at 2 weeks. However, there were no significant changes in serum creatinine or albumin. For monitoring CsA toxicity, therefore, both BUN and urinary protein/creatinine appeared to be more sensitive, and the former changed a week earlier than the latter. These phenomena might reflect the characteristics of CsA toxicity predominated by tubulointerstitial damage other than glomerular injury and the natural recovery of acute IR injury. HBSP significantly decreased BUN at 1 and 2 weeks and urinary protein/creatinine at 2 weeks in the IR + CsA group. These imply that the renoprotection of HBSP on renal function might be based on the certain threshold of injury

such as accumulated CsA toxicity over 1-2 weeks upon the IR injury.

CsA-induced long-term nephrotoxicity is pathologically characterized by tubular atrophy and interstitial fibrosis [18]. TID in ischemic kidneys was not obvious, which may be due to lack of time for the change to occur, and also suggests reperfusion injury might be more meaningful. TID score was significantly aggravated by CsA but restrained by HBSP treatment with no improvement in the IR kidneys, suggesting that HBSP attenuated CsA induced TID more effectively than IR-induced TID at 2 weeks. Consistent with TID, in Masson's trichrome stained sections typical striped interstitial fibrosis and more extracellular collagen deposition were observed in the IR + CsA kidneys compared with the IR kidneys, which was decreased by HBSP. Therefore, the establishment of IR

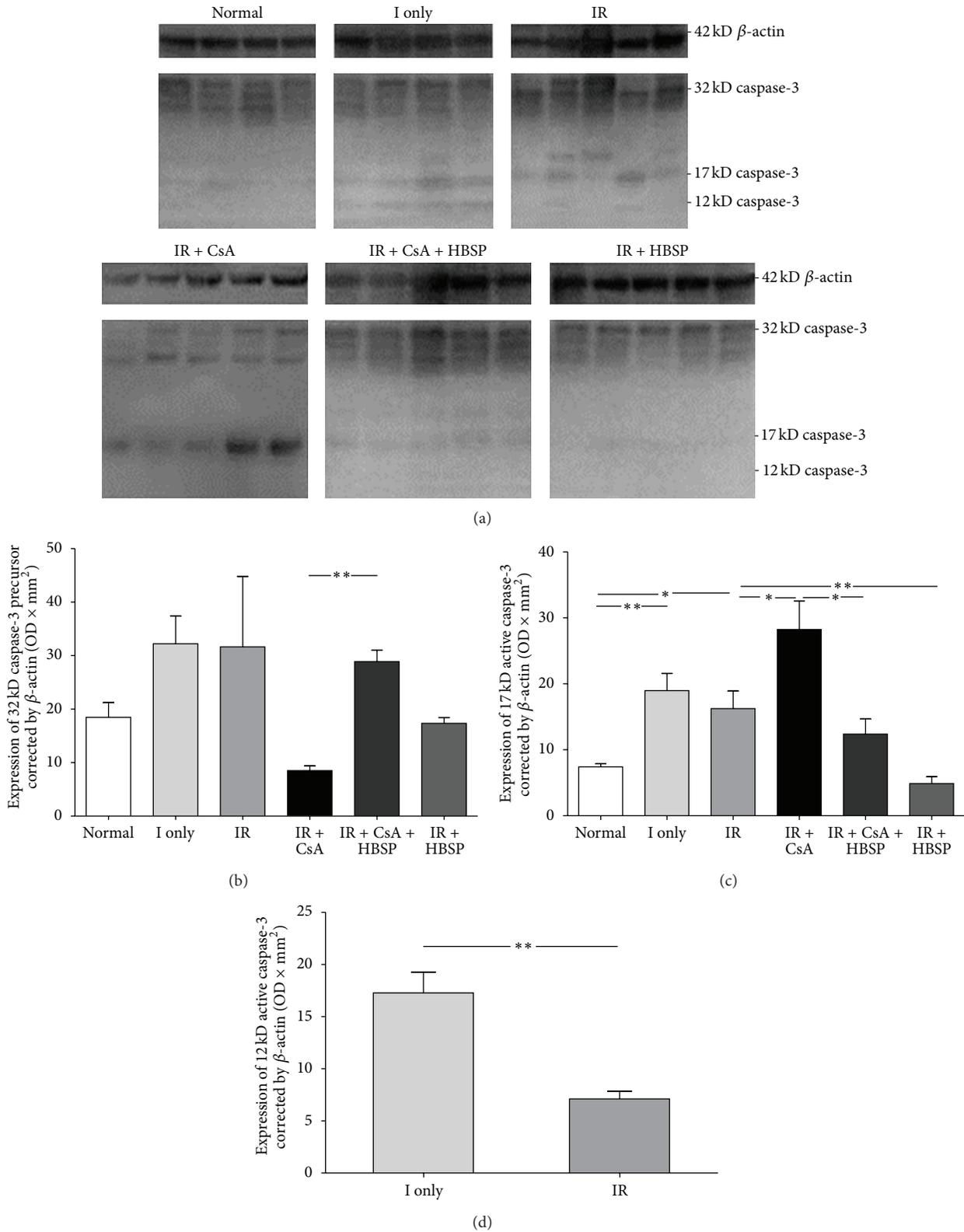


FIGURE 7: The expression of caspase-3 protein was measured by western blot (a). There was much more 32 kD precursor transformed to active subunits in the IR + CsA kidneys than that in the normal, ischemia only, or IR kidneys (b) but this was reversed by HBSP treatment. The expression of 17 kD active caspase-3 was upregulated by IR and furthered by CsA and decreased by HBSP (c). Another active subunit of 12 kD caspase-3 was predominantly expressed in the ischemia only kidneys, which was decreased after reperfusion in the IR kidneys (d). Data are expressed as the volume density corrected against the loading control of 42 kD β -actin (mean \pm SEM; $n = 6$). * $P < 0.05$; ** $P < 0.01$.

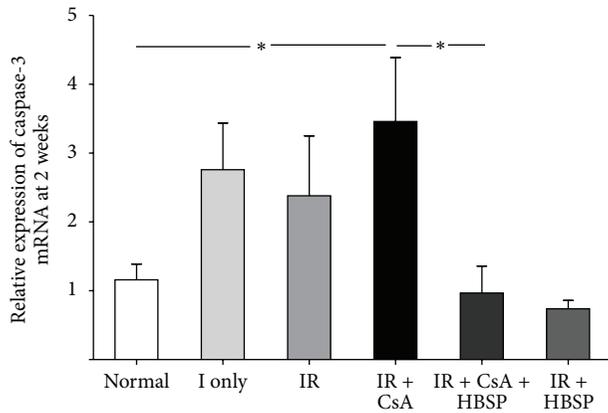


FIGURE 8: The level of caspase-3 mRNA in the kidneys, measured by qPCR, was predominantly increased by IR + CsA compared with the normal group but significantly decreased by the treatment of HBSP. Data are expressed as mean \pm SEM, $n = 6$. * $P < 0.05$.

and CsA injury model and the efficacy of HBSP treatment were further confirmed.

The cellular mechanism of renoprotection induced by HBSP was further investigated. The number of apoptotic cells was raised in the IR kidneys and furthered by CsA in this study, while increased tubular cell apoptosis was also observed in the patient with CsA nephrotoxicity [19]. HBSP reduced apoptotic cells in the tubulointerstitial area of the IR kidneys with or without exposed to CsA. Apoptosis of different type of cells could lead to different outcomes; excessive apoptosis in tubular epithelial cells results in tubular atrophy and loss of functional mass, whereas inflammatory cells cleared away by apoptosis facilitates renal structure remodeling and functional recovery [20]. Our previous study demonstrated that EPO promoted inflammatory cell apoptosis, drove inflammatory and apoptotic cells into tubular lumens, eventually led to inflammation clearance and renoprotection in isolated haemoperfused porcine kidneys [11]. It has been proved here that HBSP, derived from EPO, remains the renoprotective property of EPO via reduced apoptosis in tubulointerstitial areas that was mainly ascribed to less apoptosis in tubular areas.

In addition, HBSP minimized MPO+ cells in the interstitial areas, which were increased by IR and/or CsA. HBSP might ameliorate renal damage by decreasing neutrophil infiltration, and subsequently attenuated the IR [21] and CsA [22] induced tissue damage and renal dysfunction. Massive infiltration of neutrophils in the interstitial area is a characteristic pathological phenomena in acute IR injury, while CsA exposure further increased MPO+ cells in the tubulointerstitial areas. The local robust synthesis of proinflammatory cytokines could launch defensive physiologic activities to aggravate tissue injury and dysfunction by producing oxygen free radicals and recruiting more inflammatory cells [23]. It has been undoubtedly revealed that the nephrotoxicity caused by CsA was associated with neutrophil infiltration, which was inhibited by HBSP in this study.

Caspase-3 associated with apoptosis and inflammation was involved in the progression of renal injury caused by

CsA, as well as the treatment of EPO [24, 25]. Additional investigation in this study using active caspase-3 immunostaining demonstrated that active caspase-3+ cells mainly located in the tubulointerstitial areas and tubular lumens of injured kidneys. Most positively stained cells showed the morphologic features of apoptosis, condensed nuclei, and/or neutrophils and polymorphic nuclei, which reflected the downstream biological involvement of caspase-3 in apoptosis and inflammation. The gradually increased active caspase-3+ cells by ischemia, IR, and CsA were significantly suppressed by HBSP in the IR kidneys with or without exposure to CsA. Furthermore, the level of 17 kD active caspase-3 protein was upregulated by ischemia only and IR, and furthered by CsA with lowest 32 kD caspase-3 precursor but reduced by HBSP in the both IR and IR + CsA kidneys. Another active subunit of 12 kD caspase-3 was predominantly expressed in the ischemia only kidneys and reduced after reperfusion, which confirms that the cleavage of caspase-3 can occur in the period of 45 min ischemia with 12 and 17 kD subunits both increased. This result was consistent with what was seen in our previous study using a renal IR rat model [26]. The change trend in 17 kD active caspase-3 between the groups is similar to that in active caspase-3 immunostaining, using an antibody against 17 kD subunits. The mRNA level of caspase-3 detected by qPCR was increased in the IR + CsA kidneys, but reduced by HBSP as well. These results indicate that not only the activation but also the synthesis of caspase-3 was affected by HBSP treatment at 2 weeks after the IR and CsA injury. The reversed expression of caspase-3 precursor after HBSP treatment in comparison to the IR + CsA kidneys was more likely due to its cleavage reduced but not its synthesis increased, as the 17 kD subunit and its mRNA level were both decreased. However, in the IR + CsA kidneys, the synthesis of caspase-3 appeared to be slower than its cleavage as the lowest expression of caspase-3 precursor was revealed, although the caspase-3 mRNA and its 17 kD active subunit were both raised. The detection of caspase-3 at different level demonstrates dynamic changes in the IR and CsA injury model and responses to HBSP treatment.

5. Conclusion

Upon IR injury CsA further damaged renal function and tissue structure with increased interstitial fibrosis, apoptosis, inflammation, and caspase-3 activation, which are better biomarkers for detecting IR and/or CsA induced injuries. BUN, urinary albumin/creatinine, and T1D are more sensitive than serum creatinine and albumin in monitoring CsA nephrotoxicity. HBSP well preserved the renoprotective action of EPO, effectively improved CsA induced renal dysfunction and tissue injury, and limited both IR and CsA caused apoptosis and inflammation via downregulated caspase-3 synthesis and activation. These data provide valuable evidence for the potential clinical application of HBSP on later allograft dysfunction associated with immunosuppressants.

Disclosure

There is no financial conflict in the information contained in this paper.

Authors' Contribution

Yuanyuan Wu, Junlin Zhang, and Feng Liu contribute equally to this study.

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Research Article

The Diagnostic Value of Transcription Factors T-bet/GATA3 Ratio in Predicting Antibody-Mediated Rejection

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Background. Previous data showed that the predominance of intraglomerular T-bet or GATA3 is correlated with different mechanisms of rejection, suggesting that the ratio of T-bet/GATA3 might be used to distinguish antibody-mediated rejection (ABMR) and T-cell-mediated rejection (TCMR). **Methods.** We compared the intraglomerular T-bet/GATA3 ratio in ABMR and TCMR. The intragraft expression of T-bet and GATA3 was studied via immunohistochemistry. The correlation of the diagnosis of AMR with the ratio of T-bet/GATA3 was examined. **Results.** Both intraglomerular T-bet- and GATA3-expressing cells were increased during acute rejection. T-bet/GATA3>1 was strongly correlated with ABMR (93.3% versus 18.2%). The incidence of positive HLA-I/II antibodies and glomerulitis is significantly higher in T-bet/GATA3>1 group ($P < 0.001$, 0.013 , resp.). The scores of peritubular capillary inflammation and glomerulitis were also higher in T-bet/GATA3>1 group ($P = 0.052$, $P < 0.001$, resp.). Nevertheless, T-bet/GATA3>1 is also correlated with C4d-negative ABMR and resistance to steroid treatment. Compared with C4d deposition, T-bet/GATA3>1 had a slight lower (90% versus 100%) specificity but a much higher (87.5% versus 68.8%) sensitivity. **Conclusion.** Our data suggested that intraglomerular predominance of T-bet over GATA3 might be used as diagnosis maker of ABMR in addition to C4d, especially in C4d-negative cases.

1. Introduction

With the introduction of new strong immunosuppressants, the incidence of early acute rejection is decreasing; however, antibody-mediated rejection (ABMR) remains an important barrier to successful renal transplantation [1–5]. Peritubular capillaries (PTC) C4d deposition was found to have high sensitivity and specificity for circulating antidonor antibodies and is regarded as a marker for ABMR in renal allograft recipients [4, 6]. It has been proposed that an early posttransplant biopsy displaying diffuse C4d-positive staining is suggestive of ABMR, even in the absence of detectable serum antihuman leukocyte antigen (HLA) antibodies [7].

However, accumulating evidence suggests that C4d deposition occurs only in some but not all ABMRs [8, 9]. A number of C4d-negative ABMR had been recognized. Conversely,

C4d can also be detected in grafts with stable function [10]. Therefore, C4d is no longer a reliable maker of ABMR. Seeking a new marker for the diagnosis of ABMR is becoming more and more important. T-box expressed in T cells (T-bet) and GATA3 are two transcription factors that determine the T-helper cell differentiation into Th1 or Th2, respectively [11, 12]. The expression of T-bet had been reported to be increased in renal allograft developing acute rejection, and predominance of intraglomerular T-bet has also been observed in patients with antibody-mediated chronic rejection and transplant glomerulopathy [13, 14]. Our previous study [15] examined the expression of T-bet/GATA3 within the renal allografts. We found a predominant intraglomerular expression of T-bet in ABMR patients that was distinct from that in TCMR patients. In ABMR, there is a predominant expression of intraglomerular T-bet over GATA3,

while GATA3 expression is predominant in TCMR. The predominance of intraglomerular T-bet expression relative to GATA3 expression was associated with poor response to bolus steroid treatment. These data suggest that the ratio of T-bet/GATA3 might be used to distinguish between ABMR and TCMR. This study was performed to evaluate the significance of intraglomerular T-bet/GATA3 >1 as marker of ABMR, especially in the diagnosis of C4d-negative ABMR.

2. Concise Methods

2.1. Patients Selection. This study included twenty-six renal allograft recipients who were diagnosed as having acute rejection during 2006–2009. The diagnoses of ABMR and TCMR were based on Banff, 2001 [6]. Acute rejection which meets the diagnosis criteria of ABMR except for C4d deposition was diagnosed as C4d-negative ABMR; in addition, all C4d-negative ABMR episodes were required to occur within the first week after transplant and have severe graft dysfunction. All the rejection episodes were proven by renal biopsy. Informed consent was obtained from all patients, and the Human Subjects Committee of Jinling Hospital, Nanjing University School of Medicine, approved all study protocols.

2.2. Renal Biopsies. Renal biopsies were performed after onset of presumed rejection. Two needle biopsy cores were obtained from each renal allograft for morphologic study: one for formalin fixation and the other for quick freezing. Hematoxylin and eosin, periodic acid Schiff, methenamine-silver, and Masson stain were routinely used on the formalin-fixed tissue. The residual biopsy tissues were stored for future use. Fresh frozen tissue was analyzed by immunofluorescence microscopy using a conventional panel of antibodies against IgG, IgM, IgA, C3, C4, C1q, and C4d. C4d staining was routinely performed on frozen slides, using an indirect immunofluorescence technique with a primary affinity-purified monoclonal antibody (mouse antihuman; dilution, 1:50; 1.5-hour incubation at room temperature; Quidel, San Diego, CA) and an FITC-labeled affinity-purified secondary rabbit anti-mouse IgG antibody (1:20; 40 min incubation at room temperature; DAKO, Denmark). Staining was performed according to standard procedures. A positive C4d staining was defined as bright linear stain along capillary basement membranes, involving over half of sampled capillaries according to the 2001 Banff Meeting [6].

2.3. Immunohistological Analysis. The intragraft expression of T-bet and GATA3 was retrospectively studied via immunohistochemistry using stored residual biopsy tissues (Figure 1). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue. Regimens included mouse monoclonal antibody to T-bet (H-210, sc-21003; Santa Cruz Biotechnology, Santa Cruz, CA) rabbit polyclonal to GATA3 (ab61168; Abcam, Cambridge, UK) and mouse monoclonal antibodies to CD68 (KPI; Dako, Carpinteria, CA). Sections were reviewed by two separate pathologists, and the results were expressed as total number of positive cells per glomerulus or per square millimeter in the cortex.

2.4. Treatment of Acute Rejection. Once a rejection episode had occurred, bolus corticosteroid therapy (methylprednisolone 500 mg/day for 3 days) was selected as first-line treatment. Concomitantly, all the patients were given MMF (1.5 g/day) and Tac (trough levels maintained at 8–15 ng/mL). For patients being treated with Tac, MMF, and steroids as primary immunosuppression, the dose of Tac was increased so that trough levels were maintained at 8–15 ng/mL. If patients needed dialysis, continuous venovenous hemofiltration (CVVH) was performed. Immunoabsorption was used for patients with high level of antibodies or very strong and diffuse C4d staining.

2.5. Statistical Methods. Descriptive statistical values are expressed as mean \pm SD. Between-group differences in frequencies of clinical characteristics were determined using the Fisher exact test. The analyses were done using SPSS 15.0 (SPSS, Chicago, IL, USA). A *P* value of 0.05 or less was considered significant.

3. Results

3.1. General Information. Twenty-six renal allograft recipients who developed acute rejection were included in this study, including 11 cases of C4d-positive ABMR, 10 cases of T-cell-mediated acute rejection (TCMR), and 5 cases of C4d-negative ABMR. All the rejection episodes were proven by renal biopsy. The diagnoses of C4d-positive ABMR and TCMR were based on Banff, 2001 [6]. The diagnosis of C4d-negative ABMR was also based on Banff, 2001, except for C4d deposition. There were no retransplant cases in this cohort, and no patients were positive for panel-reactive antibodies (PRA) pretransplant. All the cases received IL-2R antibody as induction therapy and mycophenolate mofetil (MMF) + tacrolimus (Tac) + prednisolone as baseline immunosuppressants. There were no significant differences among groups in recipients' age, cold and warm ischemia time (Table 1).

3.2. T-bet and GATA3 Expressions Were Increased during Acute Rejection. We used immunohistochemistry to detect T-bet and GATA3 expressions; cells (excluding tubular epithelial cells) expressing T-bet and GATA3 were counted. In protocol biopsies from recipients with normal graft function ($n = 6$), neither T-bet nor GATA3 positive cells could be detected. However, in patients with acute rejection, both T-bet- and GATA3-expressing cells were significantly increased. All the patients had increased T-bet expression in interstitial area and 80.8% in glomerulus. GATA3 could be detected in 46.2% patients in interstitial area and 88.5% in glomerulus. Our observations were focused on the intraglomerular expression of T-bet and GATA3.

3.3. T-bet/GATA3 >1 Distinguishes ABMR from TCMR. According to the ratio of intraglomerular T-bet/GATA3 positive cells, we divided the patients into two groups: T-bet/GATA3 >1 and T-bet/GATA3 ≤ 1 . There were no significant differences between patients' age, onset time of rejection, and

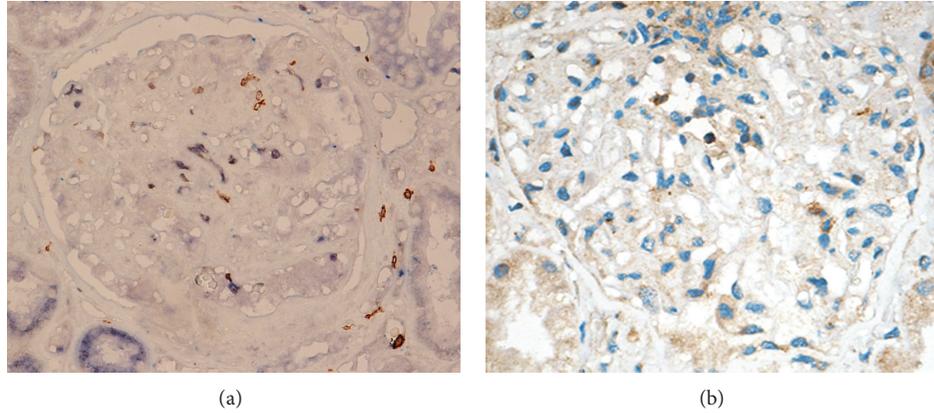


FIGURE 1: Immunohistological analysis of intraglomerular T-bet and GATA3. Positive staining is labeled in brown color. (a) Intraglomerular T-bet expression. (b) Intraglomerular GATA3 expression (magnification 400x).

TABLE 1: Clinical characteristics of patients participating in this study.

Characteristics	C4d(+) ABMR ($n = 11$)	C4d(-) ABMR ($n = 5$)	TCMR ($n = 10$)	P value
Gender, male (%)	2 (18.2)	4 (80.0)	9 (90.0)	0.002
Age (years)	40.18 \pm 8.38	39.60 \pm 12.95	38.80 \pm 11.74	0.957
Donor age (years)	44.00 \pm 9.89	44.90 \pm 11.92	41.20 \pm 10.84	0.771
Positive pretransplant PRA (n)	0	0	0	—
Previous transplant	0	0	0	—
Cold ischemic time (h)	8.18 \pm 1.40	8.40 \pm 1.14	8.93 \pm 1.57	0.866
Warm ischemic time (min)	6.36 \pm 1.20	6.20 \pm 1.30	6.80 \pm 1.31	0.623
Induction with IL-2R antibody, n (%)	11 (100)	5 (100)	10 (100)	—
Baseline immunosuppressants				0.118
MMF + Tac + Pred	11 (100)	5 (100)	10 (100)	—
Time of biopsy after Tx (day)	6.36 \pm 5.16	3.40 \pm 0.89	11.50 \pm 4.90	0.008

PRA: panel-reactive antibody; IL: interleukin; MMF: mycophenolate mofetil; Pred: prednisolone; Tac: tacrolimus; Tx: transplantation; ABMR: antibody-mediated rejection; TCMR, T-cell-mediated rejection.

induction and maintenance of immunosuppressants. However, we found that T-bet/GATA3>1 was strongly correlated with ABMR (93.3% versus 18.2%) and related lesions. The incidence of positive HLA-I/II antibodies ($P < 0.001$) and glomerulitis ($P = 0.013$) is significantly higher in T-bet/GATA3>1 group. The scores of PTC ($P = 0.052$) and glomerulitis ($P < 0.001$) were also higher in T-bet/GATA3>1 group (Table 2).

3.4. T-bet/GATA3>1 Is Strongly Correlated with C4d-Negative ABMR. We compared the characteristics of C4d-positive ABMR and C4d-negative ABMR (Table 3) and found that there were no differences between two groups in clinical and histological characteristics, such as incidences of HLA-I/II antibodies, incidence and severity of PTC inflammation as well as glomerulitis, and most importantly, the resistance of steroid treatment. When compared with TCMR group, C4d-negative ABMR group had significantly higher incidences of PTC inflammation and higher PTC score and glomerulitis score, which were very similar to C4d-positive ABMR. There was no difference in intraglomerular ratio of T-bet/GATA3

between the C4d-positive and -negative ABMR groups, while it was significantly higher in both groups compared with TCMR group. On the other hand, T-bet/GATA3 ratio >1 was 80% in the C4d-negative ABMR group, suggesting a diagnosis value of T-bet/GATA3 ratio in this special type of rejection.

3.5. T-bet/GATA3>1 Is Strongly Correlated with Steroid-Resistant Acute Rejection. In T-bet/GATA3>1 group, only 1 rejection episode had positive response to steroid treatment, even for the patient in TCMR group, while, in the T-bet/GATA3 \leq 1 group, 100% of the rejection episodes can be reversed by steroid treatment ($P < 0.0001$), including two cases of ABMR. Obviously, T-bet/GATA3>1 was strongly correlated with steroid-resistant acute rejection.

3.6. Sensitivity and Specificity. We compared the sensitivity and specificity of C4d and T-bet/GATA3 ratio as markers of ABMR; T-bet/GATA3>1 had a slight lower (90% versus 100%) specificity but a much higher (87.5% versus 68.8%) sensitivity compared with C4d.

TABLE 2: Patients' demography and histologic characters with different T-bet/GATA3 ratios and status of C4d deposition.

	T-bet/GATA3>1 (n = 15)	T-bet/GATA3≤1 (n = 11)	P	C4d+ (n = 11)	C4d- (n = 15)	P
Gender (female %)	8 (53.3%)	3 (27.3%)	0.193	9 (81.8%)	2 (13.3)	<0.001
Age	39.40 ± 8.72	39.73 ± 12.48	0.938	40.18 ± 8.38	39.07 ± 11.69	0.790
Rejection type						
ABMR	14 (93.3%)	2 (18.2%)	<0.001	11 (100%)	5 (33.3%)	0.001
TCMR	1 (6.7%)	9 (81.8%)	<0.001	0	10 (66.7%)	0.001
Positive HLA-I or II Ab	14 (93.3%)	2 (18.2%)	<0.001	11 (100%)	5 (33.3%)	<0.001
Histological features						
Peritubular capillaritis	14 (93.3%)	7 (63.6%)	0.163	11 (100%)	10 (66.7%)	0.037
PTC score	1.93 ± 1.03	1.09 ± 1.04	0.052	2.00 ± 0.89	1.27 ± 1.16	0.094
Glomerulitis	15 (100%)	7 (63.6%)	0.013	11 (100%)	11 (73.3%)	0.068
Glomerulitis score	2.20 ± 0.94	0.73 ± 0.65	<0.001	2.18 ± 0.98	1.13 ± 0.99	0.013
Tubulitis	12 (80.0%)	11 (100%)	0.339	8 (72.7%)	14 (93.3%)	0.158
Tubulitis score	1.07 ± 0.80	1.64 ± 0.92	0.106	0.91 ± 0.70	1.60 ± 0.91	0.047
Intraglomerular CD68	9.03 ± 7.86	1.89 ± 4.09	0.006	9.49 ± 8.79	3.45 ± 5.01	0.059
Intraglomerular T-bet	2.66 ± 2.74	0.42 ± 0.48	0.007	2.67 ± 3.20	1.02 ± 1.18	0.077
Intraglomerular GATA3	0.69 ± 0.61	2.20 ± 1.41	0.001	0.73 ± 0.71	1.77 ± 1.41	0.034

ABMR: antibody-mediated rejection; TCMR, T-cell-mediated rejection.

TABLE 3: Histologic lesion of renal allograft.

	C4d(+) ABMR (n = 11)	C4d(-) ABMR (n = 5)	TCMR (n = 10)	P12	P13	P23
PTC inflammation, n (%)	11 (100%)	5 (100%)	4 (40%)	—	0.003	0.031
PTC score	2 ± 0.89	2.20 ± 1.10	0.80 ± 0.92	0.704	0.007	0.021
Glomerulitis, n (%)	11 (100%)	5 (100%)	6 (60%)	—	0.023	0.111
Glomerulitis score	2.18 ± 0.98	2.00 ± 1.00	0.70 ± 0.67	0.738	0.001	0.010
Tubulitis, n (%)	8 (72.7%)	4 (80%)	10 (100%)	0.763	0.082	0.157
Tubulitis score	0.91 ± 0.70	0.80 ± 0.45	2.00 ± 0.82	0.756	0.004	0.010
Intimal arteritis, n (%)	7 (63.6%)	3 (60%)	3 (30%)	0.893	0.133	0.280
Interstitial inflammation score	1.73 ± 0.65	2.4 ± 0.55	1.7 ± 0.67	0.064	0.926	0.067
Intraglomerular immunohistological analysis						
T-bet (cells/glomeruli)	2.67 ± 3.20	2.30 ± 1.19	0.37 ± 0.42	0.808	0.039	0.020
GATA3 (cells/glomeruli)	0.73 ± 0.71	1.32 ± 0.78	2.00 ± 1.63	0.156	0.042	0.401
T-bet/GATA3>1	9 (81.8%)	4 (80%)	1 (10%)	0.933	0.001	0.009
Interstitial immunohistological analysis						
T-bet (cells/mm ²)	63.64 ± 65.88	77.6 ± 47.72	124.40 ± 112.66	0.679	0.158	0.280
GATA3 (cells/mm ²)	20 ± 37.65	11.2 ± 20.86	27.2 ± 41.17	0.636	0.680	0.434
T-bet/GATA3>1	9 (81.8%)	5 (100%)	9 (90%)	0.324	0.602	0.480

ABMR: antibody-mediated rejection; TCMR, T-cell-mediated rejection.

P12 means P value for C4d(+) ABMR group and C4d(-) ABMR group, P13 means P value for C4d(+) ABMR group and TCMR group, P23 means P value for C4d(-) ABMR group and TCMR group.

4. Discussion

C4d deposition in PTC area has been regarded as a marker of ABMR for years [16, 17]. C4d-positive has been widely

accepted as one of the diagnosis criteria [6, 18] and has contributed to the diagnosis and treatment of ABMR. However, as the recognition of a group of C4d-negative ABMR, C4d is no longer a reliable marker for ABMR diagnosis; thus, seeking

a diagnosis marker that can distinguish C4d-negative ABMR is very important.

In our previous observation [15], we found that predominance of intraglomerular T-bet or GATA3 is correlated with different mechanisms of acute renal allograft rejection, suggesting that ratio of T-bet/GATA3 might be useful to distinguish ABMR from TCMR. T-bet expression is strongly correlated with peritubular capillaritis and glomerulitis, which are typical lesions of ABMR. The predominance of T-bet can also be found in transplant glomerulopathy, which is a chronic form of ABMR [19]. This study investigated the value of intraglomerular T-bet/GATA3 ratio in the diagnosis of ABMR. We found that the ratio of intraglomerular expression of T-bet/GATA3 can be used as a marker of ABMR.

Intraglomerular expression of T-bet/GATA3>1 is strongly correlated with ABMR. In acute rejection episodes with intraglomerular T-bet/GATA3>1, 93.3% of patients were ABMR, and only 1 patient (6.7%) was diagnosed as developing TCMR. Nonetheless, intraglomerular T-bet/GATA3>1 is strongly correlated with positive HLA-I/II antibodies and antibody-related lesions, such as glomerulitis, PTC score, and glomerulitis score. We need to point out that the correlation between T-bet/GATA3>1 and incidences of glomerulitis, PTC score, and glomerulitis score is even stronger than C4d deposition.

In order to evaluate the significance of T-bet/GATA3 in the diagnosis of C4d-negative ABMR, we compared the characteristics of C4d-positive ABMR, C4d-negative ABMR, and TCMR and found that there were no significant differences between C4d-positive and -negative ABMR in either incidence or severity of antibody-related lesions. The expression of T-bet and GATA3 and their ratio were very similar between the two groups. When compared with TCMR, there were significant differences between C4d-negative ABMR and TCMR, which is comparable to C4d-positive ABMR. These data proved that C4d-positive ABMR and C4d-negative ABMR shared the same characteristics.

As C4d-positive and -negative ABMR share similar T-bet and GATA3 expression, the ratio of T-bet/GATA3>1 is correlated with C4d-negative ABMR as well. The correlation of T-bet/GATA3>1 with C4d-negative ABMR resulted in a higher sensitivity in diagnosis of ABMR. In this group, compared with C4d deposition, T-bet/GATA3>1 had a similar specificity but a higher sensitivity in the diagnosis of overall ABMR. This suggests that T-bet/GATA3>1 is potentially a valuable maker for the diagnosis of ABMR, especially for the diagnosis of C4d-negative ABMR.

ABMR is featured to be resistant to steroid treatment [20]. Our data showed that intraglomerular ratio of T-bet/GATA3 is also correlated with response to the treatment of rejection. T-bet/GATA3>1 is associated with steroid resistance. It is interesting that, even in ABMR group, T-bet/GATA3<1 is correlated with a good response to steroid treatment. The resistance of steroid treatment in T-bet/GATA3>1 group is consistent with nature of ABMR.

The exact role of T-bet and GATA3 in the pathogenesis of ABMR remains unclear. It is possible that the high expression of T-bet expression in glomerulus will induce Th1 activity and resulted in intraglomerular macrophages infiltration, which

is the feature of ABMR. As the sample size is rather small in this study, a prospective multiple centers study will be helpful to prove our findings in this preliminary study.

Overall, intraglomerular Th1/Th2 transcription factors T-bet/GATA3>1 are correlated with both C4d-positive and -negative ABMR. T-bet/GATA3>1 might be used as a diagnosis maker of ABMR in addition to C4d deposition.

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Review Article

Late and Chronic Antibody-Mediated Rejection: Main Barrier to Long Term Graft Survival

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Antibody-mediated rejection (AMR) is an important cause of graft loss after organ transplantation. It is caused by anti-donor-specific antibodies especially anti-HLA antibodies. C4d had been regarded as a diagnosis marker for AMR. Although most early AMR episodes can be successfully controlled or reversed, late and chronic AMR remains the leading cause of late graft loss. The strategies which work in early AMR have limited effect on late/chronic episodes. Here, we reviewed the lines of evidence that late/chronic AMR is the leading cause of late graft loss, characteristics of late AMR, and current strategies in managing late/chronic AMR. More effort should be put on the management of late/chronic AMR to make a better long term graft survival.

1. Introduction

Organ transplantation now represents the treatment of choice for patients developing end stage organ failure [1]. However, despite the now routine nature of clinical transplantation, even well-matched transplants are recognized and eventually destroyed by the host immune system [2]. The emerging of a new immunosuppressant has decreased the incidence of early graft loss, and even T-cell-mediated rejection occurs; it is usually easily controlled. However, the long term graft survival remains to be improved [3]. Although it was formerly held that alloreactive T cells are solely responsible for graft injury, it is now well recognized that antidonor alloantibodies are also an important barrier to long term graft survival [4, 5]. More and more lines of evidence suggest that antibody-mediated rejection (AMR) is predominance cause of late term graft loss [6, 7], especially late occurring AMR and chronic AMR (CAMR). Thus, strategies targeting alloantibody reactivity will be helpful in prolonging long term graft survival.

2. Antibody-Mediated Rejection

AMR is caused by anti-donor-specific antibodies, mostly anti-HLA antibodies [8, 9]. Some non-HLA antibodies also have been reported to induce AMR in rare cases. The phenotype of AMR ranges from hyperacute rejection, acute AMR, and chronic AMR. The diagnosis of AMR depends on typical histological lesions, C4d staining, and serum DSA detection. C4d, a protein from the classical complement activation cascade that remains attached to the site of complement activation, is regarded as a diagnosis marker for AMR. The introduction of C4d as marker of AMR aroused an ever-increasing interest in recognizing mechanisms of allograft rejection. However, C4d has several limitations in the diagnosis of AMR. For instance, it can be found in the majority of grafts with stable function in ABO-incompatible transplantations. On the other hand, a group of C4d-negative AMR has been recognized based on endothelial gene expression [10, 11]. About 40% of patients with endothelial-associated transcripts expression and chronic AMR features demonstrated no C4d

TABLE 1: Early versus late AMR in renal transplant recipients.

	Early AMR	Late AMR
Main risk factor	Positive panel reactivity antibody before transplantation, including factors causing sensitization	Withdrawal or reduction of immunosuppressants Noncompliance with immunosuppressive therapy, young age
Antibody	Mostly pre-existing donor-specific antibodies	Mostly de novo donor-specific antibodies, especially HLA class-II antibodies
Clinical features	Very rapid graft dysfunction, significantly decreased urine output, and rapid graft dysfunction	Proteinuria, hypertension, progressive functional deterioration, and overt graft failure
Histology	ATN-like minimal inflammation; capillary and/or glomerular inflammation and/or thrombosis; arterial—v3	May have chronic tissue injury, such as glomerular double contours, peritubular capillary basement membrane multilayering, interstitial fibrosis/tubular atrophy, and/or fibrous intimal thickening in arteries
Outcome	Good, mostly reversible	Usually poor

staining. Similarly, C4d staining is only positive in about half of patients with transplant glomerulopathy [12, 13], which is a special form of chronic AMR. C4d-positive and -negative AMR share similar degrees of glomerulitis and peritubular capillaritis, similar frequencies of concurrent cell-mediated rejection, and both may occur early or late after transplantation, thus needing to be treated equally [14].

Obviously, a new marker for AMR is extremely necessary. It is reported that microcirculating inflammation is strongly correlated with alloantibody reactivity; however, whether it is can be used as maker of AMR is still of contradictory [15]. T-box expressed in T cells (T-bet), transcription factor for Th1, has been reported to be correlated with microcirculating inflammation both in acute and chronic AMR [16, 17], and the predominance of T-bet over GATA3 (transcription factor for Th2) is strongly correlated with AMR [16]. However, whether the ratio of T-bet/GATA3 can be used as a diagnosis maker for AMR needs further investigation.

2.1. Late/Chronic AMR. The importance of CAMR is increasingly recognized. It has been known as a major cause of late graft dysfunction in renal transplantation. Banff 07 consensus conference [18] described that the characteristics of chronic AMR were C4d deposition in the capillary basement membrane, the presence of circulating anti-donor antibodies, and morphologic evidence of chronic tissue injury such as glomerular double contours compatible with transplant glomerulopathy, peritubular capillary basement membrane multilayering, interstitial fibrosis/tubular atrophy, and fibrous arterial intimal thickening. Late occurring AMR may manifest as CAMR; however, according to Banff 07 meeting, the term “chronic” is not related to a certain time after transplantation but indicates morphological changes of remodeling seen in the allograft due to antibody-mediated injury [18], for example, double contours of glomerular basement membranes. Thus, it is not strange that late AMR can be acute AMR. However, both CAMR and late AMR have poor response to regular anti-AMR treatment, and they are sometimes discussed together.

2.2. Late AMR, a Special Clinical Entity? AMR episodes occurring at different time periods seem to be different

clinical subtentities [19–21]. They have different risk factors, different clinical manifestations, and different outcomes (Table 1). Early AMR are usually correlated with sensitization, pre-existing alloantibodies, and rapid graft dysfunction and are usually easy to be controlled; while late AMR mostly correlated with withdrawal or reduction of immunosuppressants, noncompliance with immunosuppressive therapy. There is a relatively slow but progressive graft dysfunction; some patients have anemia and hypoalbuminemia. Late AMR have little response to antirejection strategies and thus correlate with poor graft outcomes [19, 20]. The significantly poorer outcome of late AMR is also observed in simultaneous pancreas-kidney transplantation [22], even under combined treatment of steroids, intravenous immunoglobulin (IVIG), and rituximab.

2.3. Late/Chronic AMR, Main Cause to Late Graft Loss. A eliminated term, chronic allograft nephropathy, had been regarded as a main cause of late graft loss [23]. However, chronic allograft nephropathy is actually like a can, which includes both immunological and nonimmunological parameters caused graft damage; thus, this term has been eliminated in Banff 2005 meeting [24]. Recent studies revealed that AMR is the leading cause of late grafts loss. In 2009, researchers from Dr. Holloran’s group in Edmonton studied the phenotype of late kidney graft failure [6]; they found that patients with late index biopsies (>1 year) frequently displayed donor-specific HLA antibody (particularly class II) and microcirculation changes, including glomerulitis, glomerulopathy, capillaritis, capillary multilayering, and C4d staining. T-cell-mediated rejection rarely leads to graft failure. However, they found that 63% of late kidney failures after biopsy were attributable to AMR.

Further prospective study from the same group [7] investigated kidney transplants that progressed to failure after a biopsy for clinical indications. Similarly, they found that graft failure was rare after T-cell-mediated rejection and acute kidney injury while was common after AMR or glomerulonephritis. The majority of graft loss had evidence of AMR by the time of failure. Interestingly, pure T-cell-mediated rejection, acute kidney injury, and drug toxicity were not causes of loss. These findings are interesting and, however,

are not strange as they are consistent with an early study which reported that all chronic rejection failures of kidney transplants preceded by development of HLA antibodies [25]. Recent study from Terasaki Foundation Laboratory revealed that 11% of the patients without detectable DSA at transplantation will have detectable DSA at 1 year; and over the next 4 years, the incidence of de novo DSA will increase to 20%. After de novo DSA development, 24% of the patients will fail within 3 years [26]. Given these findings, de novo DSA, especially anti-HLA class II antibodies [27], have to be considered as a primary risk factor for late allograft loss.

2.4. Natural History of Chronic AMR. The development of chronic AMR, especially TG usually takes years, and there still lacks of a perfect animal model to mimic the lesions during chronic AMR; thus, the natural history of chronic AMR is still not clear. Recently Wiebe et al. [28] have monitored a group of renal allograft recipients with de novo DSA, they proposed that posttransplant de novo DSA is probably preceded by an antibody-free period. Then, inflammatory events such as cellular rejection or graft infection might upregulate HLA expression on endothelial cells and stimulate B-cell allorecognition and subsequent long-lived plasma cells producing de novo DSA. De novo DSA binding vascular endothelium could induce injury through the activation of complement or recruitment of neutrophils, macrophage, or natural killer cells. Sustained microvascular inflammation eventually leads to progressive tissue damage and graft dysfunction. Cellular inflammation is often concomitant of AMR [29, 30] in either its acute or chronic form. However, the pathogenesis remains to be determined.

2.5. Antibody-Mediated Vascular Rejection. A recent population based study [31] revealed a new type of kidney rejection not presently included in classifications, which is an antibody-mediated vascular rejection. This study included 302 cases of acute biopsy-proven rejection. Four distinct patterns of kidney allograft rejection were identified, including T-cell-mediated vascular rejection (26 patients (9%)), antibody-mediated vascular rejection (64 (21%)), T-cell-mediated rejection without vasculitis (139 (46%)), and AMR without vasculitis (73 (24%)). The graft survival is very poor in antibody-mediated vascular rejection, which has a 9 times higher graft loss incidence compared with T-cell-mediated rejection without vasculitis. Unfortunately, the authors did not provide how many of the AMR episodes were late AMR.

3. Management of Late/Chronic AMR

3.1. An Ounce of Prevention Is Worth a Pound of Cure. Chronic AMR is a B-cell-mediated production of immunoglobulin (Ig) G antibody against a transplanted organ. Based on this pathophysiologic condition, rituximab, IVIG, and bortezomib have been used as treatment for chronic AMR recently. However, till now, there is no standardized treatment for late/chronic AMR. The strategies that can effectively reverse early AMR do not work as well in late episodes; thus, an ounce of prevention is really worth a

TABLE 2: Strategies to treat AMR.

Strategies	Mechanisms
Plasmapheresis [37], immunoadsorption [38]	Removal of donor-specific antibodies
IVIG [39, 40]	Multiple mechanisms, basically pleiotropic immunomodulation
Rituximab [41, 42]	Chimeric anti-CD 20 monoclonal antibody, depleting B cells
Bortezomib [43, 44]	Proteasome inhibitor, may cause apoptosis of normal plasma cells which in turn decreases alloantibody production
Eculizumab [45, 46]	Humanized monoclonal antibody anti-C5
Mycophenolic acid, tacrolimus [34, 35]	Inhibit production of DSA
Splenectomy [47, 48]	Immediate reduction of the B-cell and plasma cell pool

pound of cure [32]. As late AMR usually is caused by de novo DSA, posttransplant HLA alloantibody monitoring is of great importance for the prevention of antibody-mediated allograft injury [33]. Prevention of nonadherence and insufficient immunosuppression are additional important issues in the prevention of antibody-mediated allograft injury, as these factors are risk factor for late AMR. A recent study based on ABO-incompatible renal transplantation revealed that B-cell depletion protocols, such as splenectomy or rituximab administration, could reduce chronic AMR after kidney transplantation. Finally, the triple immunosuppressants protocol including mycophenolic acid, tacrolimus, and steroid can control antidonor antibody production in renal allograft recipients with chronic rejection [34] and seem to be superior to others in treating AMR [35, 36]; however, whether it can prevent the development of late AMR is not clear, see Table 2.

3.2. Rituximab/IVIG. Several single center studies showed that the combination treatment with rituximab/IVIG may be a useful strategy for the treatment of chronic AMR. In 2008, a German group [49] published their pilot study in six pediatric renal transplant recipients with chronic AMR. Their treatment regimen was four weekly doses of IVIG (1g/kg body weight per dose), followed by a single dose of rituximab (375 mg/m² body surface area) 1 week after the last IVIG infusion. Four of the six patients had good response to this treatment; they had improved or stabilized eGFR. Further prospective studies from the same group showed that under this treatment, in the means of eGFR, 70% patients responded to treatment as measured 6 months after intervention, and this response persisted over a 24-month observation period. The rationale for the rituximab/IVIG treatment was to use IVIG for its immunomodulatory action and then rituximab for prevention of further antibody production.

At the year 2009, Fehr et al. [50] published his work of using rituximab/IVIG treatment on four adult patients with chronic AMR. The result showed that rituximab/IVIG treatment improved kidney allograft function in all four

patients, and donor-specific antibodies were reduced in 2 of 4 patients. The treatment regimen of this study was that on diagnosis of chronic AMR, all patients received intravenous steroid pulses (500–1000 mg once daily for 3 to 5 days) and rituximab (375 mg/m² once on day 1), whereas IVIG (0.4 g/kg once daily on day 2 to 5) was given only to 3 patients. About the treatment safety, 3 out of 4 patients underwent therapy with rituximab/IVIG without side effects. One patient had severe, possibly rituximab-associated lung toxicity. Their study showed that rituximab/IVIG may be a useful strategy for the treatment of chronic AMR. Another pilot study showed that rituximab/IVIG treatment took effect in 3 out of 4 patients. Early stage of chronic AMR has better response than advanced stage [51]. Anyway, although rituximab/IVIG treatment takes effects in some CAMR cases, it is far from comparable to early AMR cases [22]. A retrospective study from Massachusetts General Hospital [52] studied the effect of rituximab followed by standard maintenance immunosuppression, they found that this protocol shows a therapeutic effect in 8 out of 14 CAMR. Response to rituximab was defined as decline or stabilization of serum creatinine for at least one year in this study.

3.3. Bortezomib. Bortezomib is a proteasome inhibitor that leads in vitro to apoptosis of alloantibody-producing plasma cells [53]. It has shown promising effect in early AMR cases [43]. Early reports of bortezomib-based AMR treatment demonstrated the ability of bortezomib to deplete plasma cells producing DSA, reduce DSA levels, provide histological improvement or resolution, and improve renal allograft function [54]. Initial results from a multicenter study showed [55] that bortezomib-based regimen reversed AMR in adult kidney, kidney/pancreas, and pediatric heart transplant recipients; a common bortezomib-based regimen demonstrated substantial DSA reductions, with more than half of the patients achieving a 45.0% reduction in DSA level. However, plasmapheresis has been performed every third day immediately before bortezomib therapy. In a chronic AMR rat cardiac transplant model, administration of bortezomib 60 or 80 days after transplantation may reduce antidonor MHC classes I and II Abs. Histological improvements were also observed with bortezomib administration, including reduction in C4d expression, interstitial fibrosis, and vasculopathy [56]. Unfortunately, it is not as effective in late AMR cases. Walsh et al. treated 30 episodes of AMR, and they found that early AMR patients demonstrated greater reduction in DSA and histologic resolution/improvement. They concluded that early and late AMR exhibit distinct immunologic characteristics and respond differently to proteasome inhibitor therapy.

3.4. Eculizumab. As complement plays an important role in the pathogenesis of AMR, complement-blocking agents could be used for the treatment of AMR. Eculizumab is a humanized monoclonal antibody against complement C5. It can bind to the C5 protein and inhibit conversion of C5 to C5b, thus preventing formation of the membrane attack complex (C5b-9). Eculizumab has been used to rescue atypical

hemolytic uremic syndrome after renal transplantation [57]. A prospective study showed that eculizumab can reduce the incidence of AMR and transplant glomerulopathy in highly sensitized individuals when administered immediately after transplant [58]. Cases had been reported that eculizumab reverse AMR is associated with thrombotic microangiopathy [59]; it can even reverse severe AMR episode refractory to salvage splenectomy and daily plasmapheresis in ABO incompatible (ABOI) living donor kidney transplantation [45]. However, there is no evidence that eculizumab can be used for late AMR, and clinical trials are necessary to determine the optimal use of C5 inhibition.

3.5. Splenectomy. The spleen acts as a repository for memory B cells and plasma cells; thus, splenectomy is supposed to be effective in treating AMR. There is data suggesting that splenectomy alone can lead to rapid diuresis and immediate restoration of renal function [47]. Rescue splenectomy is currently regarded as last salvage option for AMR. There is a case that reported [60] that splenectomy is effective for treatment of CAMR after renal transplantation. However, clinical trials are needed to prove this finding.

4. AMR in Liver Transplantation

The liver allograft is generally regarded as relatively resistant to AMR. The resistance is attributed to a variety of characteristic features of liver which contribute to the clearing and dilution of antibodies or antigen-antibody complexes, such as Kupffer cell phagocytosis, large sinusoidal surface area, dual afferent hepatic blood supply, and secretion of soluble MHC class I antigen [61]. For many years, hyperacute rejection was thought not to occur, even when the ABO incompatible graft was used. However, subsequent increasing studies have shown that liver transplantation across the ABO blood type barrier (ABOi) is prone to AMR, which often leads to a poor clinical outcome. Unlike a reliable tissue marker of AMR in renal and cardiac allografts, the diagnostic utility and functional significance of C4d immunostaining in the liver allograft are controversial and less clearly formed. There are reports that showed that extensive C4d deposition is associated with AMR and correlated with graft survival. However, C4d deposition in liver was also detected in several other conditions, such as acute cellular rejection, chronic rejection, and recurrent diseases including hepatitis B, hepatitis C, and autoimmune hepatitis, and even preservation injury [62–64]. Therefore, the diagnosis of AMR in liver cannot be made on the basis of histological finding alone and requires other supportive features as well as the exclusion of other causes of graft dysfunction that can mimic the pathological changes occurring in AMR. However, the presence of diffuse C4d immunostaining (involving endothelium or stroma in >50% of portal tracts or sinusoids) provides supportive evidence for a diagnosis of AMR. Similar to renal transplantation, conventional T-cell-based immunosuppression usually seems less effective for cases with strictly defined AMR. Treatment with aggressive B-cell directed immunosuppression, including IVIG, plasmapheresis, and rituximab, is recommended to be

used [61]. In animal models, antibody-mediated responses might play important roles in the development of chronic liver allograft rejection. However, the role of AMR in late liver graft loss is still underdetermined.

5. AMR in Other Organ Transplantation

AMR is also involved in other organ transplantation, especially for heart transplantation. DSA binding to the heart allograft causes myocardial injury predominantly through immune complex activation of the classical pathway of the complement cascade [65], and thus is a significant risk for allograft failure, cardiac allograft vasculopathy, and poor survival. C4d is accepted as a marker for AMR in cardiac allografts. The diagnosis of AMR has evolved from a clinical diagnosis to a primarily pathologic diagnosis based on histopathology and immunopathology. The ISHLT 2005 Working Formulation [66] recommended that AMR be diagnosed on the basis of (1) evaluation of the routinely processed and stained paraffin sections for endothelial-cell swelling and accumulations of intravascular macrophages; (2) immunophenotypic evidence of immunoglobulin (IgG, IgM, and/or IgA) and complement (C3d, C4d and/or C1q) deposition in capillaries by immunofluorescence (IF) on frozen sections and/or CD68 staining of intravascular macrophages in capillaries and C4d staining of capillaries by paraffin immunohistochemistry (IC). The final clinical diagnosis of AMR required evidence of allograft dysfunction and circulating donor-specific antibodies together with the histopathologic and immunophenotypic findings. Therapies include plasmapheresis, immunoadsorption columns, intravenous immune globulin, rituximab, and bortezomib. The combinations of steroid, IVIG, and plasmapheresis are suggested as initial therapies [67]. Late cardiac AMR caused by de novo DSA is also a serious problem; despite treatment consistent with current best practice, 46% of patients developed persistent cardiac dysfunction and their medium-term survival was poor [68].

Besides renal and heart transplantation, AMR is also a major complication causing graft injury after lung [69], pancreas [70], and intestinal [71] transplantation. Similarly, all the AMR are caused by DSA, and C4d is accepted as diagnosis marker. Antibody removal strategies are also used for these episodes. More studies are needed to understand these terms and improve their outcomes.

Abbreviations

AMR: Antibody-mediated rejection
 CAMR: Chronic antibody-mediated rejection
 DSA: Donor-specific antibody
 HLA: Human lymphocyte antigen
 IVIG: Intravenous immunoglobulin.

Conflict of Interests

The authors declared that they have no conflict of interests.

Authors' Contribution

Qiquan Sun and Yang Yang contributed equally to this work.

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Clinical Study

Immunological Aspects in Late Phase of Living Donor Liver Transplant Patients: Usefulness of Monitoring Peripheral Blood CD4+ Adenosine Triphosphate Activity

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Aim. To evaluate whether the combination of the peripheral blood CD4+ adenosine triphosphate activity (ATP) assay (ImmuKnow assay: IMK assay) and cytochrome P450 3A5 (CYP3A5) genotype assay is useful for monitoring of immunological aspects in the patient followup of more than one year after living donor liver transplantation (LDLT). **Methods.** Forty-nine patients, who underwent LDLT more than one year ago, were randomly screened by using IMK assay from January 2010 to December 2011, and the complete medical records of each patient were obtained. The CYP3A5 genotypes were examined in thirty-nine patients of them. **Results.** The mean ATP level of the IMK assay was significantly lower in the patients with infection including recurrence of hepatitis C (HCV) ($n = 10$) than in those without infection ($n = 39$): 185 versus 350 ng/mL ($P < 0.001$), while it was significantly higher in the patients with rejection ($n = 4$) than in those without rejection ($n = 45$): 663 versus 306 ng/mL ($P < 0.001$). The IMK assay showed favorable sensitivity/specificity for infection (0.909/0.842) as well as acute rejection (1.0/0.911). CYP3A5 genotypes in both recipient and donor did not affect incidence of infectious complications. **Conclusions.** In the late phase of LDLT patients, the IMK assay is very useful for monitoring immunological aspects including bacterial infection, recurrence of HCV, and rejection.

1. Introduction

In liver transplantation (LT), the introduction of a variety of immunosuppressive agents, the advances in surgical technique, and the short-term graft survival have been greatly improved in the last two decades. However, long-term graft survival remains unsatisfactory and one of the leading causes is the difficulty in the maintenance of an adequate level

of immunosuppressive agents. Too little immunosuppressive status can lead to increasing the risks of acute and chronic rejection [1], whereas too much immunosuppression may cause malignant tumors, opportunistic infections, and drug toxicities [2, 3]. The most transplant centers assess the immunological status of the graft liver by measuring trough levels of calcineurin inhibitors (CIs) combined with laboratory data [4, 5], although neither of them is sensitive

or specific for determining the current immunosuppressive status.

In 2002, the Food and Drug Administration approved an *in vitro* assay, the ImmuKnow (IMK) assay, which measures the ability of CD4+ T cells to respond to mitogenic stimulation by phytohemagglutinin-L *in vitro*. This assay is developed as an additional tool to adjust the immunosuppressive treatment [6–9], and this has a possibility to predict the mortality as well as the status of immunosuppressant agent condition in LT recipients [10]. However, little is known about the usefulness of this assay as a monitoring tool of immunological aspects in late phase of LT patients.

Tacrolimus, which is extensively used as an immunosuppressive drug in LT and has a narrow therapeutic window, is mainly metabolized by cytochrome P450 (CYP) 3A4 and CYP3A5 in the small intestine and the liver [6, 7], and CYP3A5 plays a key role in the pharmacokinetics of tacrolimus especially in LT patients [8, 9]. Although a previous study [10] reported that the CYP3A5 genotype in both recipients and donors affected infectious complications within 6 months following living donor LT (LDLT), the influence of CYP3A5 genotype on late allograft dysfunction remains unclear.

We hypothesized that the IMK assay can be useful for monitoring of immunological aspects in the late phase after LDLT, and that the CYP3A5 genotypes in both recipients and donors affect late allograft dysfunction. In this study, we focused on the patients more than one year after LDLT. We decided to use the IMK assay for studying the frequency of infectious complications in patients with the results of this assay below a target immunological response zone and the frequency of rejection in patients with IMK assay above a target zone. We also decided to study the influence of the combination of donor's and recipient's CYP3A5 genotype on infectious complications and/or rejection.

2. Patients and Methods

2.1. Patients. In LDLT patients who were treated at the Mie University Hospital between March 2002 and December 2011, 89 patients, who had been followed as outpatients for more than one year ago after LDLT, were candidates for this study. The inclusion criteria were LDLT patients, who happened to be treated either as inpatients or who had return visits to the clinic on a Thursday during the period from January 2010 to December 2011. This study was retrospective cohort study. A total of 49 LDLT patients were screened using the IMK assay and were observed clinically. The only exclusion criterion was if the patient was followed at other center than the Mie University Hospital. The complete medical records of each patient were obtained. Acute cellular rejection was defined by the 9-point Banff rejection activity index [11] as mild, moderate, or severe based on a liver biopsy at the time of undergoing the IMK assay when rejection was suspected clinically. Table 1 shows patient characteristics in this study. The CYP3A5 genotypes were examined in 39 patients who underwent LDLT after September 2005 [12]. This study (IMK assay and CYP3A5 genotypes) was approved by institutional

TABLE 1: Characteristics of the 49 patients.

Age	51 (20–69)
Male/female	33/16
Etiology of LDLT	
HCV (HCC)	11 (6)
HBV (HCC)	11 (3)
NBNC (HCC)	10 (1)
PBC	6
Others	11
Median months after LDLT	54.6 (12.2–111.5)
Laboratory data	
AST (U/L)	41.4 (14–257)
TB (mg/dL)	1.5 (0.3–3.7)
WBC (mm ⁻³)	6161 (2480–10480)
Dose of tacrolimus (mg/day)	2.3 (0.5–8)
Blood concentration of tacrolimus (ng/mL)	5.4 (0.4–10.3)
C/D ratio (ng/mL per mg/kg/day)	170.3 (40.3–599.5)

LDLT: living donor liver transplantation, HCV: hepatitis C virus, HCC: hepatocellular carcinoma, HBV: hepatitis B virus, PBC: primary biliary cirrhosis, LC: liver cirrhosis, FH: fulminant hepatitis, ALT: alanine aminotransferase, TB: total bilirubin, WBC: white blood cell count, C/D ratio: tacrolimus concentration/dose ratio.

review board of Mie University Hospital and patients' consent was obtained.

2.2. ImmuKnow (IMK) Assay. Blood samples were collected in sodium heparin tubes, and the intracellular adenosine triphosphate activity (ATP) level was measured. Blood samples were processed on the day of sample collection. Briefly, 250 μ L of anticoagulated whole blood was diluted with the provided sample diluent to make a final volume of 1000 μ L. Samples were added to wells of a 96-well plate and incubated from 15 to 18 h with phytohemagglutinin at 37°C and 5% CO₂ atmosphere. After enrichment of CD4+ T cells by addition of magnetic particles coated with an anti-human CD4 monoclonal antibody (Dynabeads, Dynal, Oslo, Norway), cells were washed and lysed to release intracellular ATP. Released ATP was measured with a luciferin/luciferase assay in a luminometer. The patient's level of immune response was expressed as the amount of ATP (ng/mL). According to a previous report [8], we used the cutoff ATP level of 225 ng/mL for identifying risk of infection and 525 ng/mL for rejection, and we defined a target immunological response zone ranging from 226 to 525 ng/mL.

2.3. Immunosuppression. The immunosuppression protocol consisted of tacrolimus and low-dose steroids. The target whole-blood trough level for tacrolimus was from 10 to 12 ng/mL during the first 2 weeks, approximately 10 ng/mL thereafter, and from 5 to 10 ng/mL from the second month after LDLT. Methylprednisolone (1 mg/kg per day, intravenously) was given on postoperative days 1 to 3, followed by 0.5 mg/kg per day on postoperative days 4 to 6. Steroid administration was then switched to oral prednisolone (0.3 mg/kg per day) on postoperative day 7, and the dose was

reduced to 0.1 mg/kg per day at 1 month after LDLT. If their liver function was stable, recipients were weaned off steroids at 3 to 6 months after LDLT.

2.4. Evaluation of Tacrolimus Blood Concentration and Concentration/Dose (C/D) Ratio. We collected 1 mL of blood treated with EDTA for anticoagulation at 12 h after the previous dose, and the tacrolimus blood concentration was then measured by using a semiautomated microparticle enzyme immunoassay (IMx, Abbott Co., Ltd., Tokyo, Japan). The daily dose of tacrolimus was recorded and its weight-adjusted dosage (mg/kg per day) was calculated. Then, the measured blood tacrolimus concentration was normalized by the corresponding dose per body weight 24 h before blood sampling to obtain the concentration/dose (C/D) ratio, which was then used for estimating the tacrolimus dose needed to achieve the target trough concentration.

2.5. Genotyping of Cytochrome P450 3A5. Genomic DNA was isolated by using the QIAamp Blood Kit (Qiagen, Hilden, Germany). A fragment containing the A6986G polymorphism was amplified as follows. The PCR samples contained 10× PCR buffer, 2 mM deoxyribonucleoside triphosphate, 0.1 mM primers, and Taq polymerase (Applies Biosystems, Foster City, CA, USA). The primer sequences were as follows: forward 5'-taccacgtatgtaccacc-3' and reverse 5'-gcactgtctgatacgtcg-3'. PCR conditions were denaturation at 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 7 min. After purification using calf intestine alkaline phosphatase (CIP, Promega, Madison, WI, USA), 1.0 μL of purified PCR product was mixed with 2.5 μL of SNaPshot ready Reaction Mix (ABI) and 20 pmol/μL of SNaPshot primer (5'-aagagctctttgtcttca-3'). The cycling program was 25 cycles consisting of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. Postextension products were purified with CIP and incubated at 37°C for 45 min and at 75°C for 10 min. Then, 1 μL of the final reaction samples containing the extension products was added to 9 μL Hi-Di formamide (Applied Biosystems). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then analyzed by electrophoresis on an ABI Prism 3730 DNA analyzer. Results were analyzed using GeneScan Analysis 3.1 (Applied Biosystems) software. The CYP3A5 A6986G (rs776746) polymorphism was analyzed for the detection of the *3 allele, since previous reports suggested that CYP3A5*3 is the major defective allele and that other functional exonic SNPs are rare in the Japanese population [13]. With regard to the CYP3A5 genotype, patients were allocated into 2 groups: CYP3A5*1/*1 or CYP3A5*1/*3 (expressors) and CYP3A5*3/*3 (nonexpressors).

2.6. Statistical Analyses. All values were expressed as the mean ± standard deviation (SD) and median as appropriate. Fisher's exact tests were used for categorical factors. Student's *t*-test was used to compare ATP levels and tacrolimus C/D ratio between infection versus no infection, Hep C recurrence versus no recurrence, and rejection versus no rejection. The Pearson correlation coefficient was used to determine the

relationship between the blood concentration of tacrolimus and the dosage of tacrolimus, and between the blood concentration of tacrolimus and the IMK ATP levels. Data were analyzed using statistics computer software Pharmaco Analyst II (Hakuhousha Co., Tokyo, Japan). A *P* value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Pharmacokinetics of Tacrolimus and IMK ATP Level. The blood concentration of tacrolimus ranged from 0.4 to 13.9 ng/mL, and the dosage of tacrolimus ranged from 0.4 to 8.0 mg/day in each recipient. There was no statistically significant relationship between the blood concentrations of tacrolimus and the dosage of tacrolimus in LDLT recipients ($R = 0.0806$, $P = 0.1162$) (Figure 1(a)). There was also no statistically significant relationship between the blood concentrations of tacrolimus and the ATP levels in LDLT recipients ($R = 0.1473$, $P = 0.2745$) (Figure 1(b)). Clinically, there were no samples that behave like outliers in both figures.

3.2. IMK ATP Level and Tacrolimus C/D Ratio in Patients with or without Infection. Posttransplant bacterial and viral infection occurred in 10 of 49 patients (20.4%). The mean tacrolimus C/D ratios were 218.4 ± 129.2 ng/mL per mg/kg/day in patients with infection and 149.3 ± 99.1 in patients without infection. There was no significant difference between two groups ($P = 0.132$) (Figure 2(a)). The mean ATP levels in patients with infection ($n = 10$) was significantly lower than that in patients without infection ($n = 39$) (185.5 ± 64.5 ng/mL versus 350 ± 159.7 ng/mL, $P < 0.001$) (Figure 2(b)).

3.3. IMK ATP Level and Tacrolimus C/D Ratio in Patients with or without Rejection. Histologically proven rejection occurred in 4 cases (8.2%). The mean tacrolimus C/D ratios were 134.1 ± 71.9 ng/mL per mg/kg/day in patients with rejection ($n = 4$) and 179.2 ± 133.6 in patients without rejection ($n = 45$), showing no significant difference between two groups ($P = 0.641$) (Figure 3(a)). The mean ATP levels in patients with rejection was significantly higher than that in patients without rejection (663.2 ± 63.6 ng/mL versus 306.6 ± 138.7 ng/mL, $P < 0.001$) (Figure 3(b)).

3.4. IMK ATP Level and Tacrolimus C/D Ratio in Patients with Hepatitis C. Histologically proven recurrence of hepatitis C occurred in 5 cases (45.5%) out of 11 patients with hepatitis C. The mean tacrolimus C/D ratios were 166.4 ± 94.2 ng/mL per mg/kg/day in patients with recurrence ($n = 5$) and 170.4 ± 56.8 in patients without recurrence ($n = 6$). There was no significant difference between two groups ($P = 0.944$) (Figure 4(a)). The mean ATP levels in patients with recurrence of hepatitis C was significantly lower than that in patients without recurrence (205.6 ± 73.4 ng/mL versus 387.7 ± 137.5 ng/mL, $P = 0.0262$) (Figure 4(b)).

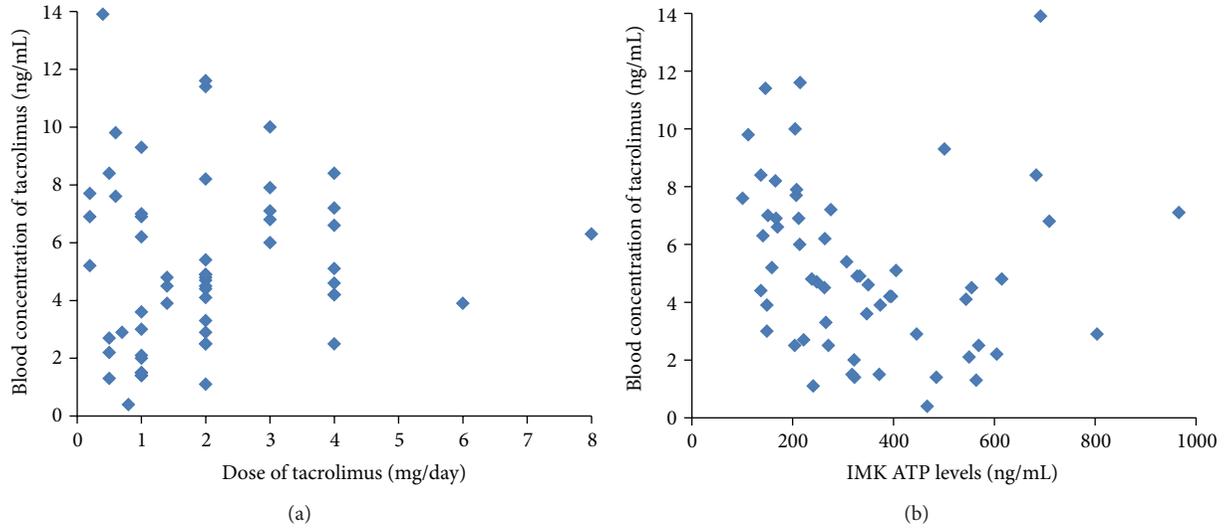


FIGURE 1: (a) Relationship between the blood concentration of tacrolimus and dosage of tacrolimus after LDLT ($R = 0.0806, P = 0.1162$). (b) Relationship between blood concentration of tacrolimus and ImmuKnow ATP level after LDLT ($R = 0.1473, P = 0.2745$).

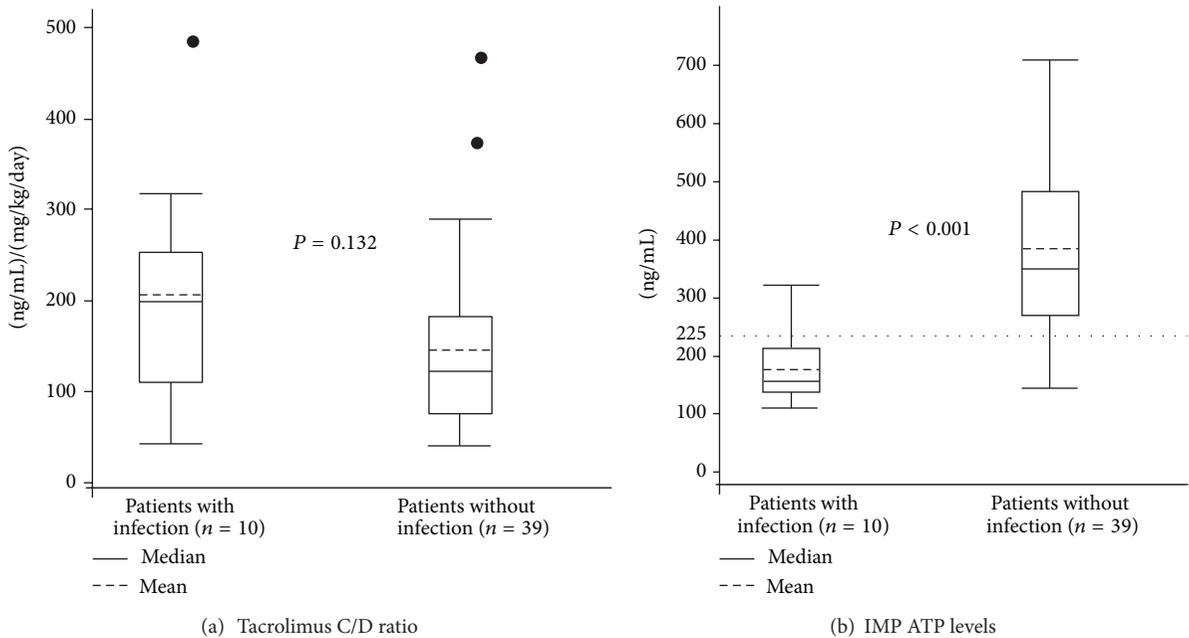


FIGURE 2: IMK ATP levels and tacrolimus C/D ratio in patients with or without infection. (a) The ATP was 218.4 ± 129.2 (range 112–312) ng/mL and 149.3 ± 99.1 (range 146–706) ng/mL, respectively. (b) The ATP was 185.5 (range 112–312) ng/mL and 350 (range 146–706) ng/mL, respectively.

3.5. IMK ATP Level in the Patients Who Developed Special Clinical Events. During this survey, 14 of the 49 patients experienced special clinical events, such as bacterial infectious complications, recurrence of hepatitis C (RHC), and acute cellular rejection (ACR), as shown in Table 2. All of the patients suffered from bacterial infectious complications and

4 out of 5 patients who developed RHC showed ATP levels lower than 225 ng/mL. On the other hand, the ATP levels in all patients with ACR were higher than 525 ng/mL.

When we used cut-off ATP level of 225 ng/mL for identifying risk of infection and 525 ng/mL for rejection according to a previous report [8], diagnostic accuracy of IMK for

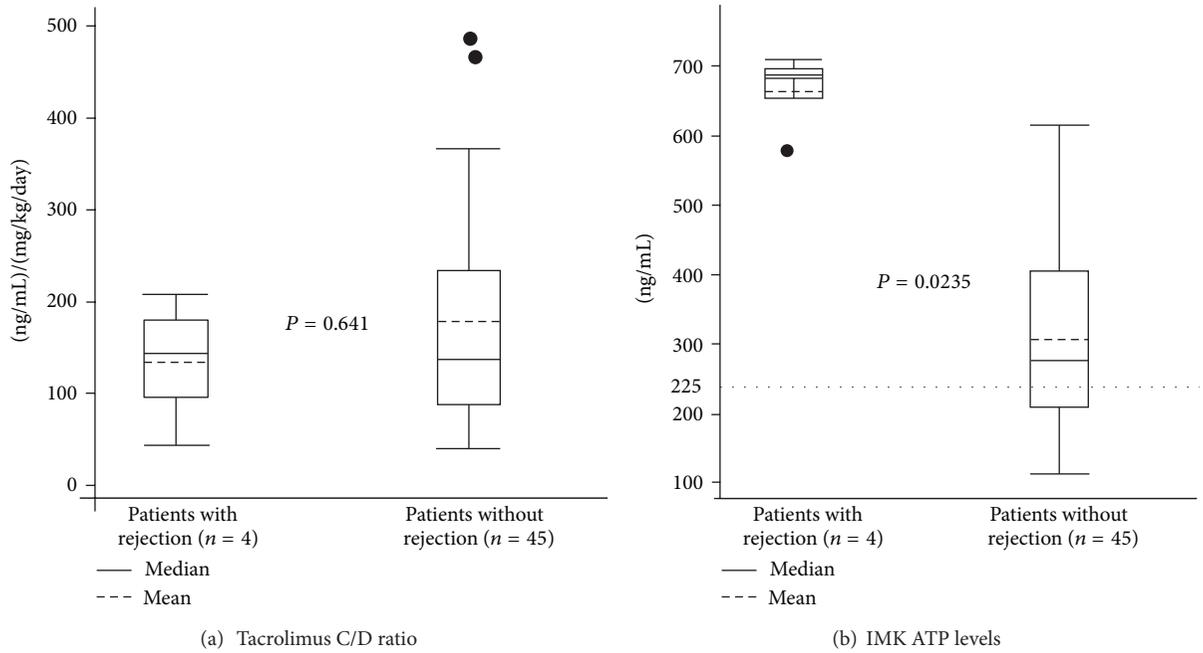


FIGURE 3: IMK ATP levels and tacrolimus C/D ratio in patients with or without rejection. (a) 134.1 ± 71.9 (range 112–312) ng/mL and 179.2 ± 133.6 . (b) The ATP was 663.2 (range 569–709) ng/mL and 306.6 (range 146–615) ng/mL, respectively.

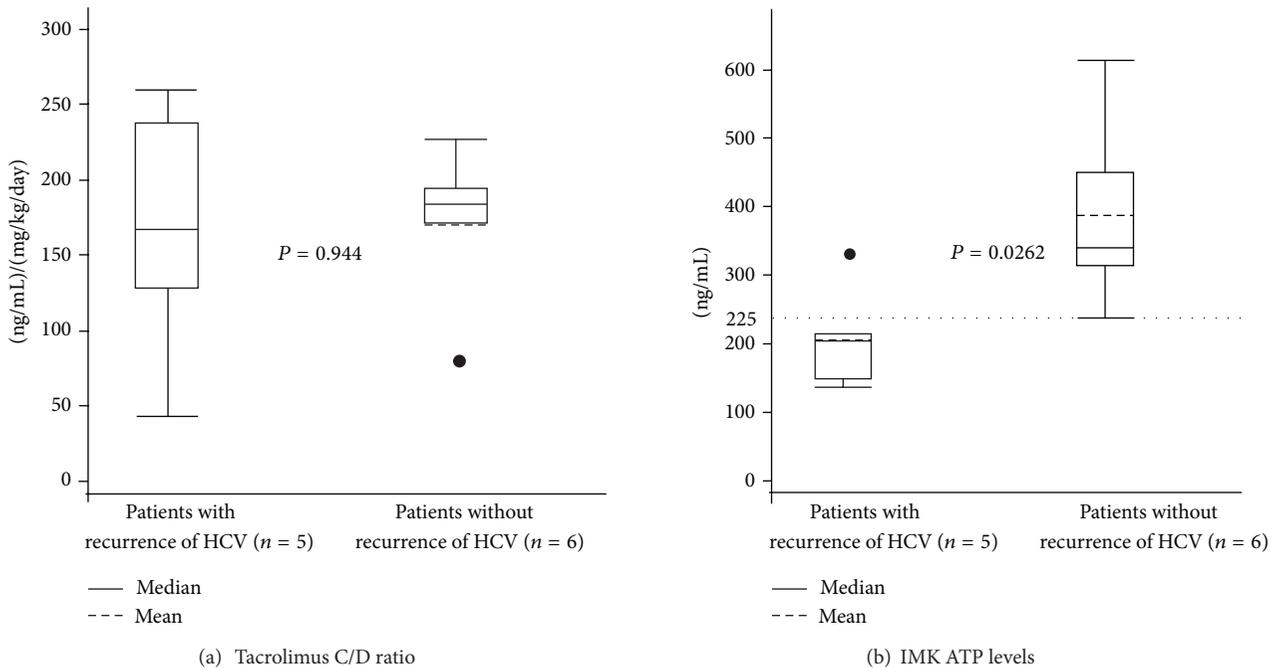


FIGURE 4: IMK ATP levels and tacrolimus C/D ratio in patients with hepatitis C. (a) 166.4 ± 94.2 (range 112–312) ng/mL and 170.4 ± 56.8 . (b) The ATP was 215.0 (range 141–322) ng/mL and 398.4 (range 238–615) ng/mL, respectively.

TABLE 2: IMK ATP levels in the patients who experienced late clinical events.

Age	Gender	Etiology of LDLT	Clinical events	Months after LDLT	Tacrolimus concentration (ng/mL)	C/D ratio (ng/mL/mg/kg/day)	IMK ATP levels (ng/mL)
46	F	LC	Phlegmon	89.5	9.8	99.76	112
60	M	LC	Cholangitis	103.3	8.4	478.80	113
58	M	HCV, HCC	RHC	57.9	6.3	43.31	137
20	F	PSC	Cholangitis	92.1	3.9	105.86	141
60	M	HCV	RHC	27.1	8.2	259.12	149
61	F	LC, HCC	UTI	34.9	10	229.00	166
68	M	HCV, HCC	RHC	52.4	7.9	166.95	205
62	F	HCV, HCC	RHC	86.3	8.6	237.79	215
52	M	PBC	Pneumonia	55.5	2.7	316.44	222
50	M	HCV	RHC	17.3	2.0	128.80	322
51	M	PBC	Rejection	38.8	2.5	43.25	569
43	M	FH	Rejection	45.4	8.4	171.57	683
58	F	HBV, HCC	Rejection	19.9	9.9	207.90	692
25	M	BA	Rejection	104.7	6.8	113.33	709

IMK: ImmuKnow, LDLT: living donor liver transplantation, C/D ratio: concentration/dosage ratio, LC: liver cirrhosis, HCV: hepatitis C virus, HCC: hepatocellular carcinoma, PSC: primary sclerosing cholangitis, PBC: primary biliary cirrhosis, HBV: hepatitis B virus, FH: fulminant hepatitis, BA: biliary atresia, RHC: recurrence of hepatitis C, UTI: urinary tract infection.

identifying risk of infection was favorable with sensitivity of 0.909 and specificity of 0.842, and that of rejection was also satisfactory with sensitivity of 1.0 and specificity of 0.911.

3.6. Influence of CYP3A5 Genotype on the Incidences of Post-operative Infectious Complication and Acute Cellular Rejection. Table 3 shows the relationship between the patients with infectious complication or acute rejection and those divided by CYP3A5 genotype. The incidence rate of infectious complications did not differ among 4 groups. Additionally, incidence of acute cellular rejection was also similar among 4 groups.

4. Discussion

One of the most important things to improve long-term graft survival is the maintenance of an adequate level of immunosuppressive agents. Neuberger [13] analyzed the common causes of death in 617 patients more than five years after transplantation and concluded that cardiovascular disease (22%), *de novo* malignancy (19%), infection (19%), and chronic rejection (5%) were responsible for the majority of deaths among them, which means that the major causes of late death were immunosuppression-related diseases.

The IMK assay is considered as a useful tool of monitoring of immune activity in transplant recipients; however, the predictive capability of IMK for identification of infection and rejection during the late period after LT remains unclear. Till now, there are two studies of meta-analysis regarding IMK. Rodrigo et al. [14] reported that the IMK is a valid tool for determining the risk of further infection in LT recipients, but the ability of IMK for detecting of rejection was controversial because of significant heterogeneity across studies. Ling et al. [15] suggest that IMK is not able to identify

individuals at risk of infection or rejection and additional studies are still needed to clarify the usefulness of this test. However, regarding the individual studies included in these meta-analyses, IMK was measured at different time points after transplantation. In this present study, we focused on late allograft dysfunction, especially in patients more than one year after LT.

In our study, we had five bacterial infectious disease cases, five recurrent HCV cases, and four patients with rejection during the period of our surveillance, and almost all of these patients showed the IMK ATP levels out of the target immunological response zone, suggesting the usefulness of this test for identifying risks of infection and rejection. Fortunately, none of them were lethal, and bacterial infections were cured by administration of the antibiotics and rejections were successfully treated by steroid pulse and increase of dosage of immunosuppressive agents. In LT patients with HCV, we have to decide very carefully the adequate level of immunosuppressive agents because the use of strong immunosuppression during the treatment of acute rejection causes early and severe recurrence of HCV in the post-LT setting [16–18].

Recurrence of HCV in LT leads to cirrhosis in 30% of the patients within 5 years following LT [19], and 41% have severe fibrosis or cirrhosis 6 years after LT [20], so our transplant physicians have to reduce immunosuppressive agents as much as possible. Although liver biopsy is considered to be the gold standard for assessing fibrosis progression of HCV, it is an invasive procedure with some limitations, such as coagulopathy and thrombocytopenia. Several authors reported that the IMK assay can be a helpful tool to monitor the immune status in LT patients with HCV: HCV-positive recipients with recurrent disease had significantly lower ATP levels than those without recurrence [21], the sensitivity and specificity for distinguishing recurrent HCV

TABLE 3: Relationship between the patients with infectious complication or acute rejection and those divided by CYP3A5 genotype.

	EX-R/EX-D (n = 5)	EX-R/NEX-D (n = 6)	NEX-R/EX-D (n = 10)	NEX-R/NEX-D (n = 18)	Value
Infection	0	1 (16.7%)	3 (30%)	5 (27.7%)	n.s.
Rejection	1 (25%)	1 (16.7%)	1 (10%)	1 (5.5%)	n.s.

EX: expressor (*1/*1 or *1/*3), NEX: nonexpressor (*3/*3).

EX-R: recipient with EX, NEX-R: recipient with NEX.

EX-D: donor with EX, NEX-D: donor with NEX.

n.s.: nonsignificant.

from acute cellular rejection were 88.5% and 90.9% [22], and the progression of fibrosis in HCV-positive recipients has been correlated with lower IMK ATP levels [23]. We also suggested the IMK assay reflected the immunological aspects of the LT patients with HCV; however, the small sample size and lack of a validation group of patients should be taken into consideration.

CYP3A5 is known to play a key role in the pharmacokinetics of tacrolimus in LT patients and to be responsible for the interindividual variations in the pharmacokinetics of tacrolimus in the recipients until 12 months after LDLT [24]. We revealed that the tacrolimus C/D ratio was significantly lower in subjects with CYP3A5*1 alleles (expressor) than in those with the CYP3A5*3 allele (nonexpressor), suggesting that high dose of tacrolimus is required in expressors to achieve the target trough levels [7]. We also previously suggested that there was higher risk for the development of infectious complications in patients with the CYP3A5*1 allele (expressor) than in those with the CYP3A5*3 allele (nonexpressor) until 6 months after LDLT, because the patients with expressors tend to be overimmunosuppressed [10]. However, our study suggested that there was no association between CYP3A5 and infectious complication or acute rejection in patients more than one year after LDLT. These results suggest that CYP3A5 in both recipient and donor mainly affects tacrolimus pharmacokinetics during early postoperative period but not late phase after operation.

Our study has some limitations. The IMK assays were undergone at some haphazard times and no clear reasons for this assay could be identified, which means either inadequate ancillary studies or questionable indications for the IMK assays. The small number of allograft rejection episodes rendered the analysis of this subgroup very difficult. In addition, the CYP3A5 genotypes were examined in only 39 patients (79.5%). Although our results are interesting, their broad applicability in other transplantation centers, in which immunosuppression and management protocols are different, should be justified as a part of future studies, because of the small sample size, its retrospective nature, and the use of single time point measurements of IMK ATP levels in some patients.

5. Conclusion

In conclusion, our study identifies the IMK assay as a useful tool for monitoring immunological aspects on late allograft dysfunction, including bacterial infection, recurrence of

HCV, and ACR. CYP3A5 in both recipient and donor did not affect tacrolimus pharmacokinetics during late phase after operation.

Abbreviations

IMK: ImmuKnow immune cell function assay
 CYP3A5: Cytochrome P450 3A5
 LDLT: Living donor liver transplantation
 LT: Liver transplantation
 C/D ratio: Concentration/dose ratio.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Clinical Study

Latent Abnormal Pathology Affects Long-Term Graft Function in Elder Living Renal Allograft Recipients

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Objective. This study evaluated the long-term effects and clinical significance of latent abnormal pathology on elder living donor kidney graft function after renal transplantation in China. **Methods.** One-hundred and thirty-eight living donor renal transplantations have been carried out at our hospital in recent years. Of these, 72 Time-Zero biopsies were performed and used in this analysis. Clinical data were retrospectively measured at 3, 6, 12, and 24 months after renal transplants. Relationships and effects from biopsy results taken from implanted donor kidney grafts were analyzed. **Results.** Time-Zero biopsy pathology results from donor kidneys showed that 48.61% of donor kidneys had latent abnormal changes; arterial lesions of donor kidneys had significant effects on the renal function of grafts after 2 years' transplantation; correlations between donor age and arterial lesions were significant; and Time-Zero biopsy pathology results could help predict the long-term function of a renal graft. **Conclusions.** Existing latent pathological changes of an elder living donor kidney before transplantation could affect long-term renal function. Whether a senior donor is used should be very carefully considered.

1. Introduction

The shortage of organ donors presents a major obstacle for adequate treatment of patients with end-stage renal disease. Living-related kidney donor is turning to be an important organ resource for transplantation. In China, this problem is even more serious. As “the one family one child” family plan has been carried out for 30 years, most of the young generations do not have brothers or sisters. Parents are one of the most possibilities to be a donor. Thus more and more elder donor and marginal living-related kidneys have been received in China in recent years. Whether the quality of kidneys from these sources has an effect on the long-term survival of renal grafts and to what extent has become a focus of concern.

“Time-Zero biopsy” [1] or “baseline biopsy” [2] pathological examinations are conventionally carried out before renal transplants. They have become an important way to detect donor kidney status and to investigate renal graft function in long term. Many centers have included donor kidney biopsy pathological examinations into their routine diagnosis and treatment regimens. Research has suggested that Time-Zero

biopsies can identify pathological changes to donor kidneys that cannot be observed by routine noninvasive examinations [3]. Moreover, some pathological changes identified by a kidney Time-Zero biopsy may be related to a recipient's long-term renal function after transplantation. There were a few centers summarizing the experience on the relationship with the latent pathologic changes of Time-Zero biopsy and long-term function of the living donor kidney in China although this examination has been carried out by some transplantation centers in China. This paper observed the long-term results of renal function from 72 living donor renal transplantation cases that have received Time-Zero biopsy at our center and analyzed the impact of existing latent pathological changes to donor kidney grafts on long-term renal function.

2. Material and Methods

2.1. Study Population. Patients who had received a renal transplant operation from a living donor and had undergone a donor kidney Time-Zero biopsy at our department between

November 2007 and November 2008 were examined. All donors were strictly screened using a comprehensive physical examination before donation, including routine biochemistry, blood, urine, stool examinations, infectious disease screening, radioactive isotope renography glomerular filtration rate, renal artery computerized tomography angiography, abdomen type-B ultrasound, X-ray chest examination, and electrocardiography. Donor kidney criteria were in accordance with living donor kidney donation recommended principles of the Transplantation Society, Live Donor Kidney Transplantation Amsterdam Forum, 2004 [4], and the *Clinical Practice Guidelines of Living-Related Donor Kidney Transplantation* [5]. Both donors and recipients were required to sign kidney donation volunteer forms and informed consent forms. Donation and transplantation operations were approved by the ethics committee at our hospital.

2.2. Time-Zero Biopsy Method of Renal Graft. Donor nephrectomy approaches were all retroperitoneal open-loop nephrectomy. After dissection of the kidney and before the interrupted renal pedicle, a biopsy of the inferior pole of the kidney was performed with a 16 G bard biopsy needle. Biopsy specimens were put into ice cold saline (0°C) for 30–45 min before tests. Pathological examination preparation involved routine paraffin embedding and sectioning prior to hematoxylin and eosin and immunochemical staining. Sections were observed with microscopy. Diagnosis of pathological abnormalities complied with relevant criteria of urinary disease in *Diagnostic Pathology* [6]. Pathological examinations were performed at the Renal Pathology Laboratory of the Nephrology Department at the Affiliated Beijing Friendship Hospital of Capital Medical University.

2.3. Clinical Research Methods. According to donor kidney biopsy pathological results, donor kidneys were grouped into an abnormal group and a normal group. Cases with pathological abnormalities were subdivided further according to the pathological type. Relationships were observed between recipient renal function and different pathological abnormalities of the donor kidneys and the result of long-term recipient renal function. Renal function evaluation parameter in clinical examination data of participants were collected at 3, 6, 12, and 24 months after transplants.

Exclusion criteria were (1) to avoid any influence on pathological observations, occurrence of severe acute rejection reaction without reversion during the perioperative period; (2) cases without recovery of renal function or primary renal graft dysfunction; (3) severe infection or other complications that led to irreversible renal dysfunction; and (4) death within the observation period.

2.4. Renal Function Evaluation Criteria. Serum creatinine (serum Cr), blood urine nitrogen (BUN), and blood uric acid were tested using a UniCel DxC 800 system (Beckman Coulter, Los Angeles, CA, USA). Normal ranges for biochemistry testing are taken from *Laboratory Diagnosis* [7]. After quality control analysis, ranges were adjusted to serum Cr, 60–115 $\mu\text{mol/L}$; BUN, 3.5–6.7 mmol/L; and blood uric acid, 350–440 mmol/L.

2.5. Statistical Analysis. Data were evaluated using SPSS 13.5 statistical software. Data are presented as arithmetic mean values \pm SD. Comparative analysis of histological and pathological changes and clinical data between groups was carried out using *t*-tests. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical Demographic Data. A total of 138 patients with uremia received living-related donor kidney transplants between November 2007 and November 2008. One-hundred and twenty donor kidneys underwent a Time-Zero biopsy. Three patients died within 1 month after transplantation; one patient succumbed to alimentary tract hemorrhaging, and two to cerebral hemorrhages. Two years after transplantation, 21 cases (17.5%) were lost to followups. Seventy-two patients were periodically monitored with followups, with complete data recording and qualified pathological specimens taken in accordance with observation criteria, and were enrolled in retrospective analysis.

Seventy-two living-related kidney donors were qualified according to physical examinations without contraindications or other illness histories that might affect renal function. Of the 72 recipients, primary diseases included primary purpura nephritis (one case), diabetic nephropathy (two cases), and chronic glomerulonephritis and uremia in all other cases. Because of limited medical and economic conditions, only five cases received biopsy examinations of their kidneys and conformed to the diagnostic criteria for chronic glomerulonephritis. Two patients received a second renal transplantation. Donor-recipient gender mismatches occurred in 45 cases. Parents donated a kidney to their child in 28 cases; siblings donated in 18 cases; couples donated in four cases; a nonlinear senior donated in 10 cases; and cousins donated in 12 cases. There were no significant differences observed in the body mass index of donors and recipients, and there were no significant differences observed in the choice immunosuppressive agent between with and without latent pathological exchanges in donated graft. Detailed demographic data are presented in Table 1.

3.2. Immunosuppressive Therapy. All patients received a postoperative routine triple immunosuppressive maintenance regimen after transplantation. Thirty-seven patients received a combination regimen of tacrolimus (FK506; initial dose: 0.1 mg/kg/day), mycophenolate mofetil (initial dose: 1.5 g/day), and prednisone (initial dose: 10 mg/day). Thirty-five patients received a regimen of cyclosporin A (initial dose: 6 mg/kg/day), mycophenolate mofetil (initial dose: 1.5 g/day), and prednisone (initial dose: 10 mg/day). Tacrolimus and cyclosporin A doses were adjusted according to plasma drug concentrations. Antithymocyte globulin induction therapy was carried out in 58 patients at a dosage of 0.75–1 mg/kg/day for 3–5 days. One patient received induction therapy using basiliximab at day 0 and day 4 after transplantation. No other patient received induced therapy. Among all 72 cases, there was no occurrence of irreversible renal dysfunction caused by severe complications during the perioperative period.

TABLE 1: Demographic data of donors and recipients.

Pathology	n	Parent donor (%)	FK/CSA*	Donor			Recipient		
				Age	M/F	BMI	Age	M/F	BMI
With path	37	10 (27.0%)	23/13	37 ± 8	22/15	22.53	35 ± 13	28/9	21.4
Without path	35	18 (51.43%)	15/19	44 ± 12	20/14	23.17	30 ± 10	27/7	22.5

*Triple immunosuppressive agent, basic FK, or CSA; BMI: body mass index; FK/CSA: tacrolimus/cyclosporin A; M/F: male/female.

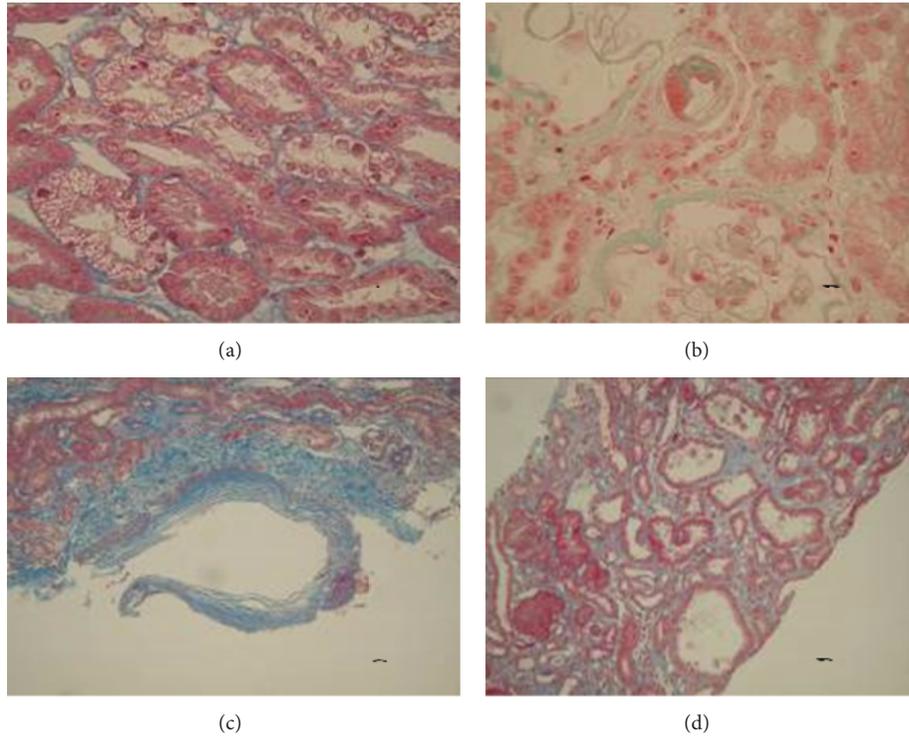


FIGURE 1: Discovering the latent pathological changes in elder donor kidney by Time-Zero Renal Biopsy. (a) Tubular cavity degeneration. (b) Capillary arteriolar pathological changes. (c) Intimal thickening and arteriosclerosis. (d) Focal interstitial fibrosis.

3.3. *Pathological Examination.* Of the original 120 biopsy specimens, instances of death, missed followups, and disqualified specimens (in cases where less than seven glomeruli were defined, as per prior criteria) reduced the final specimen count to 72. These specimens were qualified by meeting all criteria and were enrolled in the study.

Of the 72 donor kidney Time-Zero biopsies, 37 cases were without abnormal pathological change and 35 cases had pathological changes to a varying extent and type; the abnormal rate was 48.61%. Multiple abnormal pathological changes existed on one patient’s biopsy sample in partial patients. Abnormal pathological changes were observed in glomeruli, tubules, capillaries, arterioles, and the renal interstitium of nephrons (Figures 1(a)–1(d)). The main issues were (1) glomerulosclerosis: 1/3–1/14 of total glomeruli of biopsy specimens had glomerulosclerosis; (2) renal tubular injury: there were losses in the tubule epithelial brush border, slight vacuolar degeneration of the tubular epithelium, tubular ectasia, granular degeneration, and focal tubular atrophy; (3) capillary and arteriole change: slight capillary arteriolar hyalinosis, intimal thickening, and arteriosclerosis; and (4) focal

renal interstitial fibrosis. Data of the various pathological changes are shown in Table 2.

3.4. *Relationships between Donor Kidney Pathological Abnormalities and Postoperative Renal Functions of Recipients.* Of the 72 cases, there were statistically significant differences in age between those with or without pathological changes ($P = 0.02$). There were no significant differences in total renal function, regardless of whether viewed over the short or long term (Table 3). Of the various pathological changes in donor kidneys, the most noticeable were arterial lesions contributing to renal function deterioration in the long term; a statistically significant observation (Table 4).

3.5. *Relationships between Donor Kidney Pathological Changes and Recipient Renal Functions.* (i) Time-Zero biopsy results showed 22 cases with renal tubule lesions, which included epithelial vacuolation, loss of brush border, slight renal tubular ectasia, and focal renal tubular atrophy. When compared with 50 cases that did not show any tubule lesions, serum Cr

TABLE 2: Pathological changes of donor kidney.

Pathological change	<i>n</i>	F/M	Age	D and R*
Capillary and arteriola	15	7/8	49 ± 9	Parents 9/sibling 3/cousins 3
Glomerulosclerosis	4	4/0	54 ± 3	Parents 3/sibling 1
Interstitial fibrosis	14	10/4	46 ± 12	Parents 7/sibling 4/cousins 3
Tubular injury**	16	11/5	43 ± 12	Parents 7/sibling 1/cousins 8

*D: donor, R: recipients, D and R relationship: a parent donated a kidney to a son or daughter, sibling: a sibling donated a kidney to their brother or sister. Other relationships were cousin-german relationships; **renal tubular injury: loss of the kidney tubule epithelial brush border, vacuolar, and granular degeneration of the tubular epithelium, tubular ectasia, and focal tubular atrophy. F/M: female/male.

concentrations in the tubule lesion cases showed an increase after 2 years (tubule lesions: 156 $\mu\text{mol/L}$; no tubule lesions: 128 $\mu\text{mol/L}$). After excluding the effects of all arteriopathy and microangiopathy, there were 17 cases with simple tubule injuries, with a mean serum Cr concentration at 2 years after transplantation of 115 $\mu\text{mol/L}$. The mean serum Cr concentration at 2 years after transplantation was 111 $\mu\text{mol/L}$ in 41 cases in which there were no tubule injuries and no arteriopathy and microangiopathy. Differences in serum Cr concentrations between the two groups at 2 years after transplantation were not significant.

Loss of brush border in renal tubule injury represented typical ischemic damage. Of these cases, puncture biopsy was performed before there was any circulation interruption; therefore, changes were considered to be related to biopsy sampling, preservation, and shipment processes rather than as a result of any renal pathological change. It is of note that the mean age of donors with renal tubule pathological changes was more than that of donor without the same condition, although the difference was statistically significant (44 years versus 37 years, resp.; $P = 0.045$). It is possible that at the same condition, an increase in the age of a donor may be related to a worsened tolerance to ischemic damage.

(ii) Fifteen cases received a donor kidney that developed focal interstitial fibrosis. The mean serum Cr concentration at 2 years after transplantation was 154 $\mu\text{mol/L}$. Compared with the 50 cases in which there was no such pathological change (serum Cr: 125 $\mu\text{mol/L}$), there was no difference in renal function 2 years after transplantation. When patients with and without fibrosis were compared, a statistically significant difference in ages between the two groups was observed (46 years versus 37 years, resp.; $P < 0.05$). It is therefore suggested that focal renal fibrosis changes could be a sign of renal physiological degeneration.

(iii) It is possible that latent vascular lesions in donor kidneys could be the main reason affecting long-term renal function of recipients. There were significant differences in renal function data of serum Cr ($P < 0.05$) and BUN ($P < 0.05$) concentrations between recipients who received renal

grafts with or without latent arteriopathy and microangiopathy after transplanted operation. There were also significant differences between the ages of donors in these two groups. Those donors with latent pathological changes were older than those donors without such changes ($P = 0.001$). Deteriorating renal function was more serious in cases in which the patient had higher grades of pathological change in arteriopathy and microangiopathy.

(iv) Sclerosis changes of glomeruli involving capillaries could affect renal function directly. In the current study, there were four cases with renal glomerulosclerosis. The mean age of the four donors was 54 years, which is 14 years older than those donors without glomerulosclerosis. Serum Cr concentrations were also significantly higher in those patients with glomerulosclerosis compared with those patients without glomerulosclerosis after transplanted operation. It is therefore suggested that donor kidneys with arteriopathy and microangiopathy can lead to abnormal long-term renal function in recipients. These two pathological changes were significantly related to donor age (Table 5). The levels of uric acid were normal when each group was compared with and without pathological changes. This result suggests that deterioration in long-term graft function may be closely related to arteriopathy and microangiopathy.

4. Discussion

To our knowledge, this is the first study investigating the correlation of the latent pathological changes of living donor with two-year graft function in China. The living kidney donors in this study underwent strict preoperative examinations and were a healthy population as qualified by clinical tests. However, Time-Zero biopsy results showed that a considerable number of donor kidneys had latent pathological changes that could not be detected by clinical noninvasive tests, especially the elder donated kidney in our study. Cosio et al. reported that 5% of kidneys in 7% of donors show interstitial fibrosis at the time of kidney donation [8]. Progression of fibrosis in such a kidney could continue for 4 months after a transplant and could progressively affect over 7% of kidneys 2 years after transplantation, with the severity of fibrosis being significantly related to renal graft loss. All kidney interstitial fibrosis in the current study's cohort was slight and sporadic focal fibrosis. It was therefore not a threat to renal function at 2 years after transplantation.

Many researchers studying renal allograft Time-Zero biopsies have reported that latent pathological changes in donor kidneys affect the postoperative renal function of the recipient, and this could help predict long-term survival [9, 10]. A meta-analysis analyzed 16 clinical research reports on renal allograft biopsy in Europe and concluded that glomerulosclerosis, arteriopathy, and renal interstitial lesions affect long-term survival of a renal graft [11]. The study cited results of 2300 donor kidneys from the United Network for Organ Sharing between 1999 and 2002 by Cicciarelli et al. [12], and a Time-Zero biopsy was conducted in 25% of cases. The conclusion was that the severity of sclerosis of renal glomeruli was significantly related to the survival of a renal graft, delayed graft function, and primary renal dysfunction

TABLE 3: Comparisons between postoperative renal function in cases with or without pathological changes.

Group	Average age		SCr			BUN [§]			UA ^Δ					
	D	R	3**	6	12	24	3	6	12	24	3	6	12	24
Total	40 ± 7	33 ± 5	115 ± 26	120 ± 19	124 ± 13	138.8 ± 67	6.2 ± 1.6	6.6 ± 1.1	6.6 ± 1.8	7 ± 3.3	324 ± 91	350 ± 130	334 ± 13	349 ± 121
Normal	37 ± 8	35 ± 13	112 ± 18	106 ± 17	124.7 ± 19.8	113 ± 9.3	6.6 ± 1.4	6.4 ± 2.6	6.4 ± 3	6.4 ± 2.1	320 ± 46	329 ± 33	345 ± 37	344 ± 30
Change	44 ± 12	30 ± 10	111 ± 23	110 ± 19	109.6 ± 18	154 ± 140	7.1 ± 3.4	6.9 ± 1.9	6.7 ± 2	7.9 ± 3.6	336 ± 99	343 ± 87	42 ± 91	365 ± 98

Compared with and without pathological changes: * age of donor, P = 0.02; [†] serum Cr (umol/L), P = 0.550; [§] BUN (mmol/L), P = 0.13; ^Δ uric acid, P = 0.11; ** postoperative months; D: donor; R: recipient; UA: uric acid (mmol/L).

TABLE 4: Postoperative renal functions of patients who received a donor kidney with different pathological changes.

Path. change	n (%)	Average age		Serum Cr (umol/L)**			BUN (mmol/L)			Uric acid (mmol/L)					
		D	R	3 (mon)	6	12	24	3	6	12	24	3	6	12	24 ^{&}
Normal	37 (51.39)	37 ± 8	35 ± 13	112 ± 18	106 ± 17	124.7 ± 19.8	113 ± 9.3	6.6 ± 1.4	6.4 ± 2.6	6.4 ± 3	6.4 ± 2.1	320 ± 46	329 ± 33	345 ± 37	344 ± 30
Tubule injury	22 (30.56)	44 ± 12	32 ± 11	113 ± 23	114 ± 18	112 ± 16.7	156 ± 144	7.9 ± 3.9	7.5 ± 2	7.1 ± 1.9	7.6 ± 3.8	334 ± 108	361 ± 83	376 ± 103	363 ± 90
Inte. fib.	15 (20.13)	46 ± 12	33 ± 10	109 ± 24	110 ± 20	111 ± 17	154 ± 171	7.2 ± 5	6.6 ± 2	6.7 ± 2	7.4 ± 4	366 ± 122	351 ± 88	46 ± 12	374 ± 98
GS	4 (5.56)	54 ± 3	31 ± 10	103 ± 17	98 ± 19	103 ± 13	270 ± 333	5.5 ± 1.1	6.9 ± 1.8	8.0 ± 1	10.9 ± 7.8	272 ± 43	321 ± 81	317 ± 72	336 ± 67
A pathy [#]	15 (20.83)	49 ± 9	29 ± 8	116 ± 20	112 ± 17	113.5 ± 13.2	208 ± 200	6.7 ± 2.1	7.0 ± 1.2	7.0 ± 1.5	9.6 ± 4.6	340 ± 79	340 ± 93	310 ± 54	366 ± 94

** Compared with normal, age, and serum Cr in 24 months were significantly different ($P < 0.05$) in GA and A. pathy; [#] A: arteriolar pathology included degrees 1 and 2; compared with donor age, serum Cr were significantly different ($P < 0.05$). [&] All groups of uric acid not different ($P > 0.05$).

TABLE 5: Long-term graft function in various arteriopathy and microangiopathy compared with and without pathological changes.

Path. change	n	Average age*		Serum Cr (umol/L) [#]			BUN (mmol/L) [§]			Uric acid (mmol/L) ^Δ					
		D	R	3 (m)	6	12	3 (m)	6	12	3 (m)	6	12	24		
Normal	37	37 ± 8	35 ± 13	112 ± 18	106 ± 17	124.7 ± 19.8	113 ± 9.3	6.6 ± 1.4	6.4 ± 2.6	6.4 ± 3	6.4 ± 2.1	320 ± 46	329 ± 33	345 ± 37	344 ± 30
Arteriopathy I	9	46 ± 6	33 ± 9	119 ± 16	111 ± 9.8	113.5 ± 14.9	196 ± 268	7.1 ± 1.4	7.3 ± 1	7.2 ± 1.3	9.6 ± 6.2	360 ± 88	310 ± 77	329 ± 70	358 ± 91
Arteriopathy II	6	53 ± 8	33 ± 9	113 ± 29	108 ± 21	112.3 ± 13.5	227 ± 192	6.1 ± 1.8	6.1 ± 0.7	6.5 ± 1.9	10 ± 4.4	310 ± 77	335 ± 106	282 ± 31	379 ± 126
Glomerulosclerosis 4 [§]	4 [§]	54 ± 3	31 ± 10	103 ± 17	98 ± 19	103 ± 13	270 ± 333	5.0 ± 1.1	6.9 ± 1.8	8.0 ± 1	10.9 ± 7.8	272 ± 43	321 ± 81	317 ± 72	336 ± 67

Compared with normal: * age, P < 0.05; # serum Cr at 24 months, P < 0.05; §BUN at 24 months, P < 0.05; Δ uric acid at 24 months, P > 0.05; § three cases of complicated arteriopathy were observed.

($P < 0.001$). Other research has also indicated that if the extent of glomerulosclerosis of a donor kidney is greater than 10%, long-term survival of the renal graft decreases significantly. Escofet et al. reported that every 1% increase in donor kidney glomerulosclerosis results in a 0.8 mL/min decrease in glomerular filtration rate 4 years after transplantation [13]. Each single acute rejection episode would lead to a 7.5 mL/min decrease in the glomerular filtration rate. Ten percent glomerulosclerosis would be equal to the pathological damage of a single acute rejection episode.

Results from the present study's cohort accord with the historical literature. There were four donor kidneys with glomerulosclerosis and a degree range in 7.69–33.33% (mean: 17.02%). Long-term renal function in these cases was significantly lower than that in those cases without glomerulosclerosis. Of all the pathological changes, glomerulosclerosis has the most profound effect on long-term renal function, and long-term renal function of recipients with glomerulosclerosis was significantly more harmful than that of those recipients without glomerulosclerosis.

Glomerulosclerosis appeared mainly in cases in which a parent or a senior aunt or uncle donated a kidney. In such cases, the donors' ages were much older than the ages of those donors in whom there were no pathological vessel changes. It is therefore suggested that the effect of glomerulosclerosis on long-term renal function is related to a donor's age. In these cases, preoperative physical examination and various test results were normal, but biopsy pathologically verified that the donor kidneys had thickened renal arteriolar walls, capillary hyalinization, and glomerulosclerosis of varying severity.

Lubuska et al. [9] observed various graded pathological changes of living donor kidneys and classified arterial pathological changes into four grades according to the extent of thickening and hyalinization of the arteriolar wall. Grade 0 is defined as no pathological change; Grade 1 as pathological change not exceeding 25%; Grade 2 as pathological change of 25–50%; and Grade 3 as pathological change exceeding 50%. Although cases with a grade higher than Grade 1 were not observed in the current study, a review of the literature suggests that (1) pathological change of arterioles could certainly affect immediate postoperative and long-term renal function and (2) the age of a donor is positively correlated with the occurrence rate and severity of arterial pathological changes.

Similar to the results of Lubuska et al., in the current study, there were 15 cases with arterial pathological changes. Nine cases were evaluated as Grade 1. The mean age of donors was 46 years. The mean 2-year serum Cr concentration was 196 $\mu\text{mol/L}$ for these nine cases. A comparison with those cases that did not show arterial pathological changes showed a significant difference ($P < 0.05$). Six cases were evaluated as Grade 2. The mean age of donors was 53 years. The 2-year mean serum Cr concentration was 227 $\mu\text{mol/L}$ for these six cases. A comparison with those cases that did not show arterial pathological changes showed a significant difference ($P = 0.038$). The mean age of donors without arterial pathological changes was 40 years. The mean age of the four cases with glomerulosclerosis in this group was 54 years.

The 2-year mean serum Cr concentration was 270 $\mu\text{mol/L}$. Comparison of these four cases with those cases without arterial pathological changes showed a significant difference ($P = 0.004$). It is possible that pathological changes in small vessels in donor kidneys were directly related to donor age; arteriopathy and microangiopathy possibly directly affect long-term function of renal grafts.

In the past 10 years, owing to serious deficiencies in sourcing kidneys, the age of marginal kidney donors has increased in all of the world. In current implemented expanded criteria for donor kidneys [14], the upper age limit for marginal healthy kidney donors without complications is determined as 60 years. Some investigations have reported that, in some cases, there has been no upper limit for a donor's age [15]. Studies have reported that long-term renal function of older donor renal grafts is much lower than that of younger donors. Despite such reports, the previous upper age limit of 50 years (which had been maintained for 30 years) has risen to around 65 years. It has been suggested that the reason for this increase is due to the status of elder donors in recent years. That is, they are generally healthier and pay closer attention to their lifestyle choices, and medical insurance coverage is now better after donation [16]. Researchers from the Tokyo Women's Medical University in Japan reported 242 donors with persistent hematuria and albuminuria after donation, with a mean age of 57 years [17]. The study reported that 8.3% of donors had albuminuria and 5.2% had microscopic hematuria after donation. The earliest abnormal urine test case occurred 3 months after donation. Di Cocco et al. summarized clinical data, such as blood pressure, body weight index, ischemic time, and accompanied complications, of 32 cases who received kidneys from donors aged over 60 years [18]. The conclusion reached by the researchers in that study is similar to that of the current study. That is, long-term renal function of kidneys from elder donors is significantly lower than long-term renal function of kidneys from younger donors. Time-Zero pathological results from the current study's cohort were 15 cases with obvious microangiopathy (mean age of 49 years), which accounted for 20.83% of all cases. It is therefore suggested that a cautious approach should be taken when considering expansion of donor age limits, with full consideration being given to subclinical pathological changes caused by age-related factors. Long-term renal function changes and survival status should be strictly monitored at the same time.

Previous research has reported that the serum Cr clearance rate decreases 7 mL/min·1.73 m every 10 years after a person reaches the age of 30 and renal blood flow decreases 10% every 10 years after a person reaches the age of 40 years [19]. Corresponding histological changes were glomerulosclerosis, tubular atrophy, and interstitial fibrosis. All of these micropathological changes could not be detected by non-invasive tests. Therefore, it is a physiological regularity that renal function would progressively deteriorate with increased age. Donor kidneys with such slight and hidden pathological changes could accelerate in their progression to clinical pathological status by ischemic injury, adverse effects of a drug, or immune reaction, for example, which could affect function and long-term survival of the renal graft. Surveys

on Chinese chronic kidney disease by Zhang et al. found that, in several provinces of the China, for example, the incidence of chronic kidney disease in those aged older than 40 years reached 10.8% [20]. Although many people underwent a routine annual physical examination, the disease awareness rate was only 12.5% that could be explained partly by our data. Some patients are healthy kidney donor candidates but have hidden kidney diseases, which creates two potential hazards for the long-term renal function of recipients and the future healthy status of the donor.

This study had some limitations. The study was a retrospective case analysis, in which random subgroups and controls were lacking. In future studies, the methodology can be improved to be included in. As physical examination items are qualified for donors, this study was based on pathological data as the major evaluation criteria. Specific tests, such as immunofluorescence, immunohistochemistry, and electronic microscopy, should be used in future research but may not be able to be conducted on all participants owing to limitations in laboratory conditions and the economic status of patients.

5. Conclusions

The results of this study suggest that basic latent pathological changes, especially pathological changes in arteries of the donor kidney, could affect long-term renal function of the recipient, the extent of which is positively correlated with the pathological grade and scale. The age of donors may also be positively correlated with pathological changes. It is therefore suggested that doctors should give considerable thought to the age of the donor when deciding on marginal kidney donors. In particular, they should consider possible latent pathological changes due to the age of a donor. A Time-Zero biopsy can help doctors understand the status of the basic structure of the donor kidney and if any latent lesions may be present, especially for marginal kidney donation from an older donor. It may also assist with recovery prognosis of a renal graft after transplantation and long-term graft function. A Time-Zero biopsy may provide important evidence so doctors can accurately and confidently prescribe the correct immunosuppressive regimen for a patient and may assist doctors to provide reasonable advice to donors with regard to a healthy lifestyle over the long term following a donation.

The results indicate that latent pathological changes that cannot be detected by routine noninvasive tests may exist in healthy populations, so that a cautious approach should be taken when considering expansion of donor age limit. This could possibly help explain the high incidence of kidney disease and the low disease awareness status in the general Chinese population.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Linlin Ma and Lei Zhang contributed equally to this work.

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Research Article

In Utero Hepatocellular Transplantation in Rats

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This work represents a step forward in the experimental design of an *in utero* hepatocellular transplantation model in rats. We focused on the enrichment optimization of isolated fetal hepatocytes suspension, arranging the surgery methodology of *in utero* transplantation, monitoring the biodistribution of the transplanted hepatocytes, and assessing the success of the transplants. Rat fetuses have been transplanted at the 17th embryonic day (ED17) with fetal hepatocytes isolated from rats at the end of pregnancy (ED21). We assessed possible differences between lymphocyte population, CD4 positive, CD8 positive, double-positive T-cells, and anti-inflammatory cytokines interleukins 4 and 10 (IL4 and IL10) as well. Cellular viability reached the rates of 90–95%. Transplanted groups had a limited success. Transplanted hepatocytes were not able to pass through the hematoplacental barrier. The hepatocytes injected were primarily located in the liver. There was an upward trend in the whole amount of T CD4 and T CD8 cells. There was an increased IL4 in the transplanted groups observed in the pregnant rats. The possibility to induce tolerance in fetuses with a hepatocyte transplant *in utero* could be a key point to avoid the immunosuppression treatments which must be undergone by transplanted patients.

1. Introduction

Terminal hepatic diseases are the final consequence of many chronic liver diseases, damaging irreversibly its functions [1, 2]. The prevalence of these pathologies in Europe is estimated to represent about 6% of the whole number of diseases.

Classically, these hepatic diseases have been treated by means of orthotopic liver transplantation [3]. Nevertheless, the balance between advantages and risks of this therapy must be evaluated, and unfortunately the number of patients who can benefit is very limited [3], and this procedure is associated with several problems [4].

The idea of using hepatocytes or fragments of liver as a treatment was proposed for the first time by Eiseman in 1967 [5]. Thus, the cellular transplantation using hepatocytes is an emergent field in the clinical therapy to treat hepatic disorders [6]. Since the first hepatocellular transplant in 1992 [3], more

than 80 patients have been treated using this approach [7]. Moreover, the transplantation of hepatocyte mass equivalent to 10% of the patients' livers should be sufficient to normalize the metabolic situation [8]. The cellular transplant has some advantages: it is cheaper, it is a less invasive technique, it is associated with smaller mortality and morbidity, and it is less immunogenic [8].

It has been demonstrated that adult hepatocytes have a limited capacity to proliferate [9]. In contrast, the early fetus is exceptionally tolerant to foreign antigens accepting cells even when the major histocompatibility complex (MHC) does not match [6, 10]. In addition, the transplantation of cells *in utero* to cure fetuses with birth defects has several advantages [11] and does not involve the immune system of the mother [12]. In fact, *in utero* transplantation has been performed in different animal models besides the rat, like for instance sheep [13], chimpanzees [14], and pigs [15].

Since it is difficult to culture mature hepatocytes *in vitro*, cells derived from other tissues are also being investigated as potential candidates in some hepatic diseases [16]. There are different animal models that express as a marker an enzymatic deficiency to differentiate the isolated and transplanted cells from the host's hepatocytes [6] like the Gunn rat model [17] and the most used model F344 rats [18].

The possibility of generating the induction of tolerance with an *in utero* transplantation of hepatocytes is a key point to avoid the immunosuppression treatments undergone by the transplanted people [19]. The majority of studies have focused on T cells as the most important effectors of this response, including T CD4 and CD8 [20]. The condition to achieve a successful transplantation consists of inducing immunological tolerance to the grafts [21, 22]. Therefore, the hepatocellular transplantation can be an option for patients with inherited metabolic diseases [8, 20].

For these reasons, we develop a model of hepatocellular transplantation *in utero* to avoid immunosuppression in future treatments. Our aims are to optimize the enrichment of the suspension of isolated fetal hepatocytes, arranging the surgery methodology of *in utero* transplantation, monitoring the biodistribution of the transplanted hepatocytes, assessing the success of the transplants, to analyze the lymphocyte populations (T CD4 and T CD8 cells), and to analyze the anti-inflammatory cytokines (interleukins 4 and 10).

2. Materials and Methods

2.1. Experimental Animals. Pregnant females of the *Rattus norvegicus* species, albinus variety, Wistar, and Fischer (F344) strains (Harlan Laboratories) were used. Animal donors were fetuses on day 21 of gestation (ED21), and the hepatocellular transplantation is performed in fetuses on day 17 (ED17). In order to determine the day of gestation, the rats were mated in a room with controlled photoperiod cycles (12 hours light and 12 hours darkness from 8:00 a.m. to 15:00 p.m.). Next, a vaginal smear was taken and visualized under optical microscope looking for the presence of sperm. If smears were positive, that was considered the day 0 of gestation (ED0) from which the days of gestation for the animal donors and the receivers were counts. The rats were maintained in the Animal Facility of the Faculty of Biological Sciences of the Complutense University of Madrid under controlled photoperiod conditions, with 12 hours light and 12 hours darkness per day. In any case, food and water were supplied *ad libitum*. Animals were maintained in accordance with the principles set forth in the National Institute of Health (NIH) guide for the care and use of laboratory animals.

2.2. Experimental Groups. Four experimental groups were made as described in Figure 1. No surgery was carried out to those pregnant rats belonging to group 1. The second experimental group was transplanted with 10 μ L EMEM + SFB without cells. For group 3, the hepatocytes of Wistar fetuses were isolated at embryonic day 21 and subsequently resuspended in 10 μ L medium; for transplantation to Wistar fetuses on ED17: 10⁶ cells were transplanted to them. In contrast, in the fetuses included in group 4, the hepatocytes were

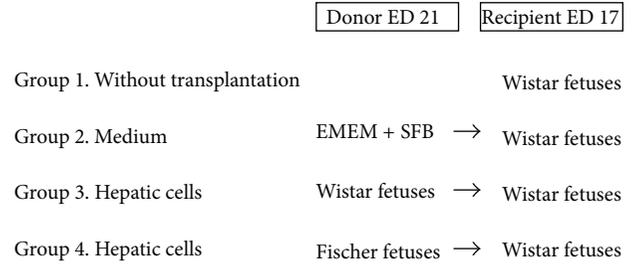


FIGURE 1: Scheme of the four experimental groups (group 1: $n = 60$, group 2: $n = 113$, group 3: $n = 153$, and group 4: $n = 163$).

isolated from Fischer fetuses on day 21 of gestation and afterwards transplanted *in utero* to fetuses of Wistar rat on embryonic day 17.

2.3. Fetal Hepatocytes Isolation. After anesthetizing the pregnant rat at ED21 with 4% isoflurane in O₂, a medial laparotomy by planes was done, both uterine horns were externalized, and the fetuses were extracted removing the vitelline membrane and the placenta. Next, the fetuses were sacrificed, and the livers were obtained with curved tweezers by mechanical traction in the abdominal region of the fetus. The isolation of fetal hepatocytes was carried out according to the protocol described by Berry and Friend [23] and modified by Arahetes et al. [24]. The suspension of cells obtained was filtrated through a mesh of Nylon of 50 μ m pore size, then it was centrifuged at 1000 g for 5 minutes at 4°C, and the supernatant was discarded. An ammonium chloride buffer, or lysis buffer (NH₄Cl 0,155 M, KHCO₃ 0,01 M, EDTA 10–6 M, add distilled water to 500 mL), was added to the pellet obtained. With this method, the hepatocytes suspension is enriched since the erythrocytes are lysates. Lysis was carried out in ice, adding 0.5 mL lysis buffer per 1 mL cellular suspension, followed by an incubation during 5 minutes. The reaction was stopped adding the same volume used for the lysis buffer of EMEM + bovine fetal serum (BFS). Afterwards, the suspension was centrifuged at 1000 g for 1 minute at 4°C, the supernatant was discarded, and the pellet was washed twice with EMEM + BFS and eventually resuspended in a known volume.

The percentage of hepatocytes in this suspension was around 70% in all cases. As the purification with biomagnetic technics damaged the hepatocyte membrane, we assumed that the major part of our suspension were fetal hepatocytes [25].

Then, the cellular viability was tested by exclusion of the vital dye trypan blue (0.2%), and the cells were counted in a Neubauer chamber. It is important that the suspension of fetal hepatocytes remains in ice until the moment of transplantation.

2.4. Transplanted Fetal Hepatocyte 111In-Oxine Labeled and Biodistribution. In order to track the transplanted hepatocytes, they were labeled incubating them 30 minutes at 37°C with 14.8 MBq 111Indium-oxine. The 111Indium linked the intracellular proteins as a result of oxina which promotes the entrance of 111Indium-oxine at intracellular section. This was



FIGURE 2: Pictures taken during transplantation. (a) A uterine horn can be observed; in this step, the disposition of the fetuses in the uterus was visualized. (b) The place where the intraperitoneal injection to a fetus *in utero* was made is shown.

not a specific marker, all of our cells were going to express the ^{111}In -oxine. Since we were interested in cell homing over several days, we selected ^{111}In as the radionuclide with a half-life of 67 h [26].

The cells were transplanted as described in the following item. Gammagraphic images of the pregnant rats and the fetuses were obtained at 6, 24, and 48 hours posttransplantation. Later, they were sacrificed, and placentas as well as liver, spleen, heart, lungs, and digestive apparatus of the fetuses were collected.

2.5. Methodology of the Hepatocellular Transplantation. The pregnant rat was anaesthetized at ED17 with 4% isoflurane in O_2 and placed on a heating blanket at a sustained temperature of 37–38°C. Next, a medial laparotomy by planes was performed trying to prevent a long incision, in order to externalize uterine horns. However, the operation was carried out horn by horn, maintaining it always moisturized and at 37°C. The disposition of the fetuses was determined, and 10^6 fetal hepatocytes (contained in 10 μL EMEM SFB) were injected to them through uterus with a Hamilton hypodermic syringe of 10 μL of 30 G (6 pk 30/10 mm7pst2) and intraperitoneally in the fetuses, that is, between the hindlimb and the dark area which represents the fetal liver (Figure 2). Once all fetuses were transplanted, the uterine horn is reintroduced into the abdominal cavity, and we proceed the same way with the other one. Finally, transplanted fetuses were counted (to contrast the viability of the surgery with respect to the newborn), and the pregnant rat was sutured with continuous stitches in the muscular layer and discontinuous stitches in the skin layer. Both sutures were completed with absorbable braided thread.

2.6. Sample Collection. At postnatal day 15 (P15), blood and liver samples were taken and the pups were sacrificed. The liver samples were frozen to -80°C , and the blood was separated into two aliquots. One of the aliquots was kept at room temperature, as complete blood sample, to assess the lymphocyte populations by flow cytometry. The other one was centrifuged to obtain serum, which was frozen to -20°C before quantifying interleukins 4 and 10.

2.7. Assessment of Lymphocyte Populations. The complete blood aliquot was divided into three subaliquots of 100 μL each, to carry out flow cytometry analysis. First, each subaliquot underwent lysis, at room temperature and in the dark, using BD FACS Lysis solution (BD Bioscience). Then we labeled the different populations of lymphocytes with antibodies, following manufacturer indications. The first aliquot is used as negative control, without any additional antibody. The second one contains mouse antiRat-CD4 FITC/CD8 RPE (AbD Serotec) and mouse antiRat-CD3APC (BD Bioscience, Pharmingen). The third one contains mouse antiRat-CD4 FITC/CD25 RPE (AbD Serotec) and mouse antiRat-CD3 APC (BD Bioscience, Pharmingen). Then the labeled populations were determined by flow cytometry.

2.8. Interleukin Assessment. The interleukins 4 and 10 were quantified in the serum samples collected from the pups at P15. A commercial ELISA in 96-well plate was done, Rat IL4 or IL10 Platinum ELISA (eBioscience), following the manufacturer's protocol. A standard curve was constructed for each cytokine using the suitable standards. The absorbance at 450 nm is displayed in the y -axis and concentrations (in pg/mL) in the x -axis.

The following standard curves were obtained. For IL4; $y = 3,792e^{-0,56x}$; $R^2 = 0,993$. For IL10; $y = 2,417e^{-0,79x}$; $R^2 = 0,969$.

When the results of absorbance (y) yielded by each sample is extrapolated in these equations, the concentration of IL4 or 10 in pg/mL (x) of the sample can be obtained.

2.9. Statistical Analysis. The results were processed with the GraphPad Prism 4 software, selecting variables within a single group, and performing one-way ANOVA with normalized data. For repeated and nonparametric measurements, the Friedman's test was used. The data were considered significant if the yielded P value was smaller than 0.05.

3. Results

3.1. Hepatocyte Enrichment and Viability. The number of cells obtained in every isolation was around 15×10^6 cells/liver

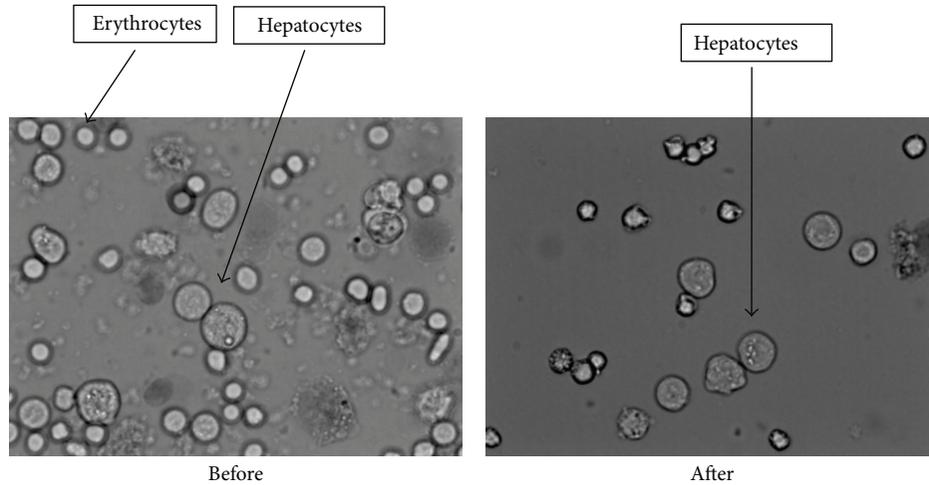


FIGURE 3: Pictures of the cellular suspension dyed with trypan blue and visualized with an optical microscope. We can see how the suspension looks before and after the lysis. Hepatocytes are bigger and have vesicles in their cytoplasm, while erythrocytes are smaller, round, and refringent.

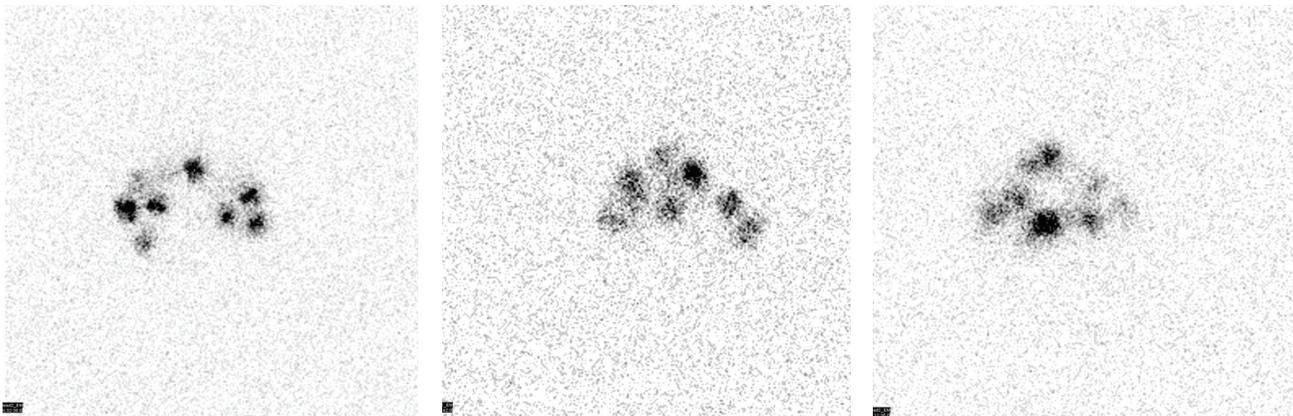


FIGURE 4: Gammagraphic images of the pregnant rats. There are deposits of ^{111}In -oxine in the abdominal cavity in similar number to transplanted fetuses.

weight (g), and the average viability was about 90–95% (Figure 3).

3.2. Hepatocellular Transplantation Viability. In the case of individuals without transplantation, the total newborn pups coincide with the born-alive pups. This is logical, because they have not suffered surgery of any kind. The transplantations performed with medium show the negative effect the surgery can have, since there is a slight decrease in the number of newborn pups and born-alive pups with respect to the number of transplanted fetuses. So that the possible damage the vehicle in which cells are re-suspended could cause is discarded. In the transplantation groups 3 and 4, the total offspring and the born-alive pups decreased considerably to yield significant differences ($P = 0.02$ for both groups).

3.3. Tracking of Labeled Hepatocytes. The gammagraphic images of the pregnant rats exhibit important deposits of

^{111}In -oxine in the abdominal cavity, similar in number to the number of transplanted fetuses (Figure 4).

Gammagraphic images of the uterus *ex vivo* revealed more clearly the different fetal deposits (Figure 5). Gammagraphic images of the rats after uterus extraction did not show any deposit (image not shown). There was no significant activity in other organs from the pregnant rat. Nevertheless, the evaluation of the placentas showed significant activity. Also, the intensity of the deposits decreased while the implanted cells proliferate.

The gammagraphic images taken from different fetal organs had little or no activity, except for some isolated livers and spleens. The activity was determined for each organ (percentage of ^{111}In -oxine incorporated by the organ with respect to the total amount in the fetus). Livers incorporate more amount than spleen, heart, lung, and digestive apparatus (Figure 6). The rest of the body and placentas exhibited a high activity.

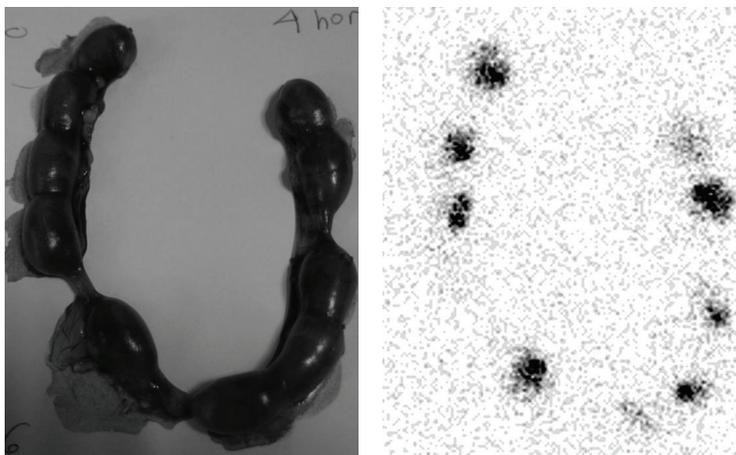


FIGURE 5: Gammagraphic image of the uterus *ex vivo*. Different fetal deposits of ¹¹¹In-oxine are clearly visualized after transplantation with labeled hepatocytes.

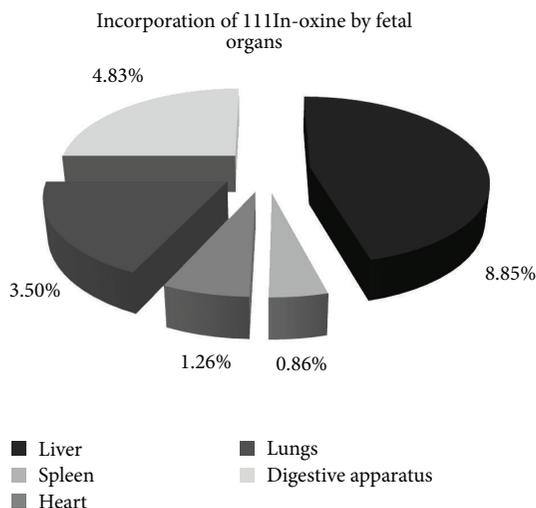


FIGURE 6: Incorporation of ¹¹¹In-oxine by different fetal organs. Hepatocytes were mostly located in the liver. There were other deposits found at the digestive apparatus, lungs, heart, or spleen, although the proportion of these was smaller than the amount in the liver.

3.4. Assessment of Lymphocyte Populations. Here, we show the individual results of one of the flow cytometry analyses (Figure 7).

The averages of all the analyzed samples were calculated and graphically represented, detailing the lymphocyte population studied and the percentage of positive cells for that population in each experimental group (including in the group both mothers and pups). There were no significant differences between groups and the lymphocyte populations analyzed in the case of mothers (data not shown). In the pups there were no significant differences either, although a trend can be seen.

The population of positive lymphocytes T CD4 does not show significant differences in any of groups of treatment in

the newborn ones. The same happens in the case of activated lymphocytes T CD4 (Figure 8).

No statistically significant differences were observed between groups of pups for total and activated T CD8 lymphocytes. In this case, there is no relevant significance between experimental groups either (Figure 9).

The percentage of double positive lymphocyte cells (i.e., positive for both CD4 and CD8) is very low, although there are no significant differences between the different experimental groups (Figure 10).

3.5. Interleukin Assessment in Serum. IL4 and the IL10 were quantified both in mothers and transplanted pups. Although there is a clear tendency in the concentration of both interleukins, there were no significant differences between experimental groups (Figures 11 and 12).

The concentration of IL4 in mothers that underwent surgery tends to increase with respect to mothers that were not operated. However, this tendency is not observed in pups (Figure 11).

No significant differences were observed for the concentration of IL10, neither in mothers nor pups between groups (Figure 12).

4. Discussion

The hepatocellular viability obtained after the isolation process (approx. 90–95%) is high enough to determine that the cellular suspension is suitable for the transplantation (Figure 3) [27]. However, the success decreases when the injections contain hepatocytes. This could be due to an immune reaction triggered in the recipient fetuses [28]. It has been seen that allogeneic transplantation of hepatocytes in mouse is limited by immunological responses to the cells transplanted, even more when the transplantation is carried out *in utero* [29]. Nevertheless, many studies have reported that group 3 similar transplantations have been better tolerated if the procedure was carried out *in utero* [28].

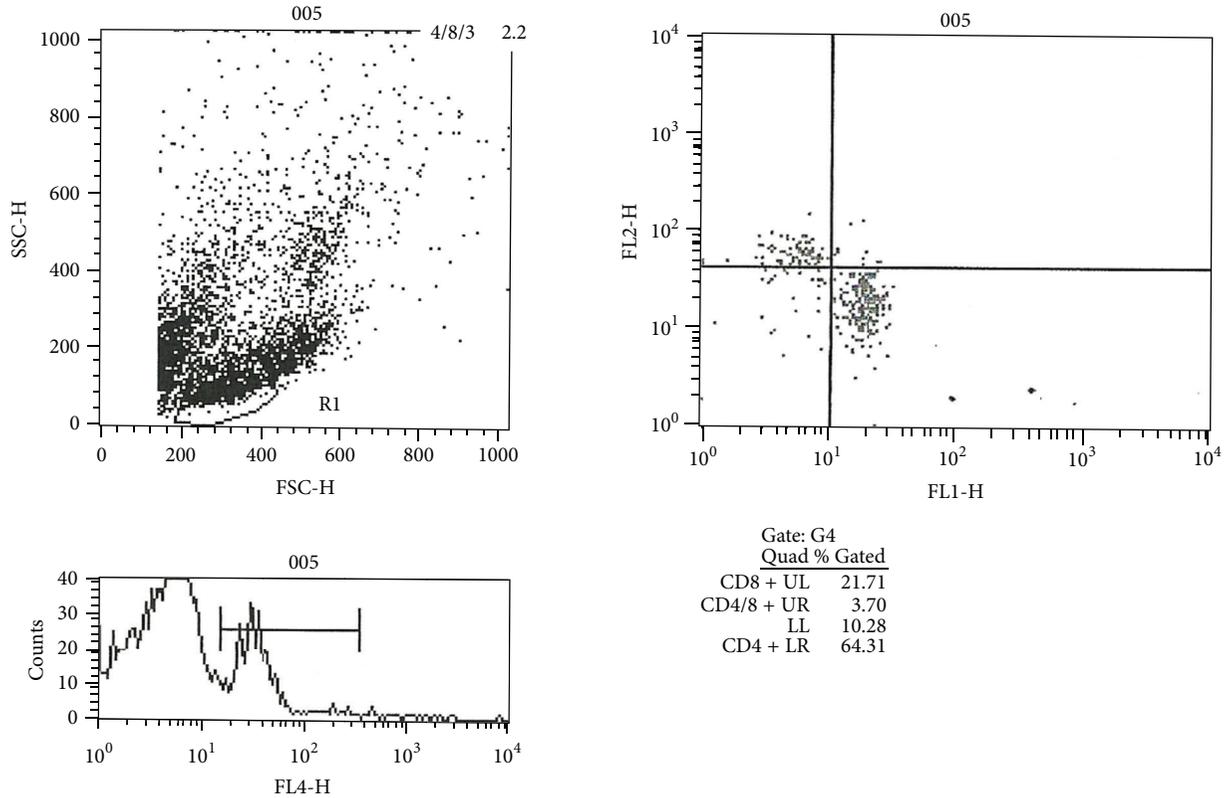


FIGURE 7: Example of one of the flow cytometry analyses where the percentages of each analyzed lymphocyte population (of a pup at postnatal day 15 belonging to the experimental transplantation group 3) are shown.

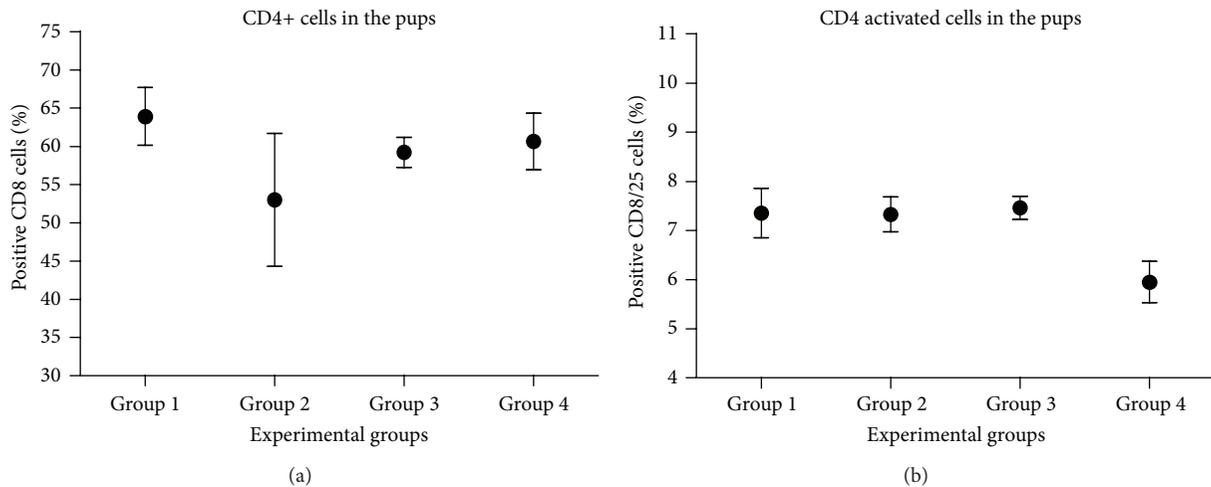


FIGURE 8: Evaluation of the total CD4 positive lymphocyte population (a) and the activated population (b). In the x -axis, it showed the experimental groups and in the ordinates the average percentage of positive CD4 cells without activating or activated with his bar of error.

In this work, we chose fetal hepatocytes instead of adult ones because, in previous studies of our laboratory, we found that these hepatoblasts keep all their potentialities and characteristics of low immunogenicity and high proliferativity [6, 17]. Moreover, the transplantation of cells *in utero* to cure fetuses with birth defects has several advantages; namely, (i) the fast growth of fetuses provides an unique opportunity for the settlement and expansion of the implanted cells; (ii) the

fetal immunological immaturity and the potential to induce specific tolerance to the donor; (iii) the protective and sterile fetal environment contributes to isolate environmental pathogens; and (iv) the fact that precocious treatments are beneficial and critical to assure effectiveness [11].

The purification of hepatocellular suspensions from fetal rat is justified mainly by two factor; first, the fetal liver has a very important role in hemopoiesis; and second,

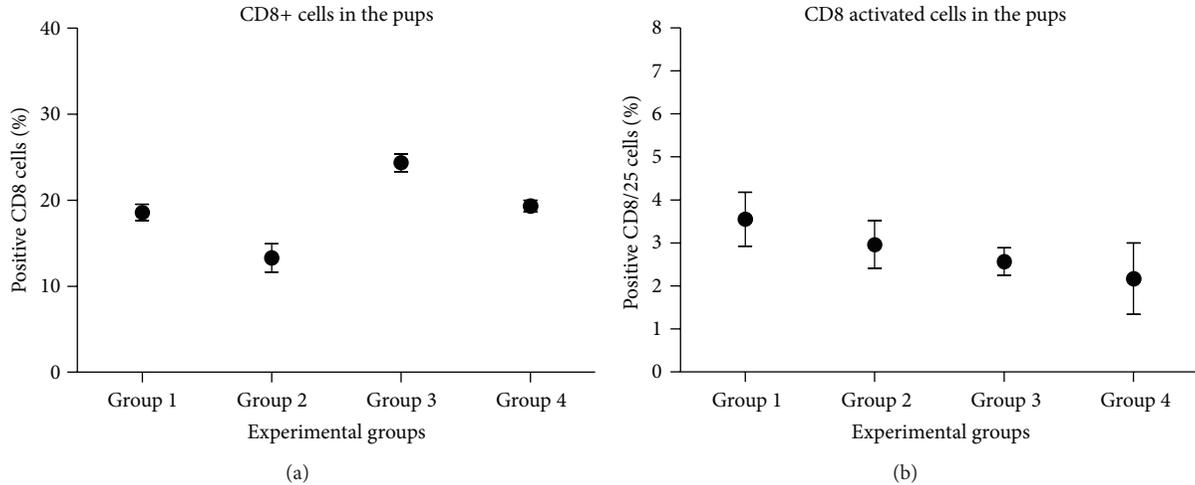


FIGURE 9: Assessment of total CD8 positive lymphocyte population (a) and the activated population (b). The experimental groups are displayed in the x-axis, and in the y-axis, the average percentage of positive CD8 cells is displayed.

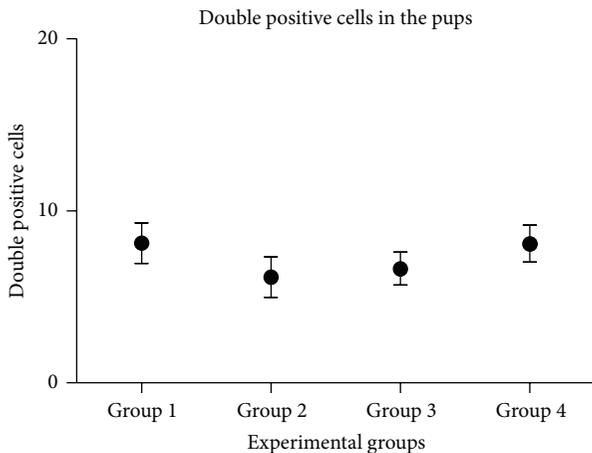


FIGURE 10: Double positive lymphocyte population. The experimental groups are displayed in the x-axis it showed, and in the y-axis, the average percentage of double positive cells is displayed.

the contamination of the suspension with blood cells is usual [20]. Previous to the present work, we performed a compared study on the purification of fetal hepatocytes using a biomagnetic isolation and measured the results by flow cytometry [6, 25]. As the cells suffer a lot with the purification, we assume that about 70% of our cell suspensions were hepatocytes.

Determining the location of transplanted hepatocytes is important to assess if the failures in the transplants are due to the erratic situation of the hepatocytes and also to check if transplantation had been correctly performed; that is, if the cells had been injected in the peritoneal cavity of the fetuses (Figure 5). We demonstrated that transplanted hepatocytes to the fetuses are not able to cross the placental barrier, and therefore they do not appear in the pregnant rat (Figures 4 and 5). This fact provides more safety about this type of therapeutic procedures and constitutes a step forward for their clinical application [6].

Since most of the transplanted hepatocytes were in the liver, the surgical process was demonstrated to be correct (Figure 6). Suckow et al. [28] showed that, after a hepatocellular transplant *in utero*, the hepatocytes were essentially located in the liver and some of them in the lungs [28], coinciding with our results. The localization of the transplanted hepatic cells is relevant, since the ectopic transplantation of hepatocytes has been reported to have therapeutic effectiveness [6]. We think it would be interesting to evaluate the signals involved in the spatial settlement of the transplanted hepatocytes somehow. Indeed, there were more cellular types apart from fetal hepatocytes in the transplantation mixture. This mixture also can include stem-like cells; this could be another explanation for the biodistribution of the labeled cells.

We also assume that the transplant was successful and our transplanted cells were dividing because the radiolabel signal from deposits of ¹¹¹In-oxine decreases with increasing time after transplant.

When we assessed the lymphocyte populations in the pups at P15 day, we did not find significant differences in the number of T CD4 and T CD8 positive cells, both total and activated.

There was an important increase of CD4 positive cells in group 2 (Figure 8). This fact was possible to be explained because the culture medium includes SFB containing a lot of proteins which were able to activate the immune cell response.

A trend to increase can be seen in T CD4 positive cells and total T CD8, in the transplanted groups 3 and 4 (Figures 8(a) and 9(a)). We could not see any significant difference between groups in the double positive cells percentage either (Figure 10).

Significant differences were not obtained in the quantification of interleukins 4 and 10 between groups. For the IL4, we have seen that there are increased levels of this cytokine in the blood of the mothers of every group (Figure 11). IL4 is an anti-inflammatory cytokine; therefore, its increase after

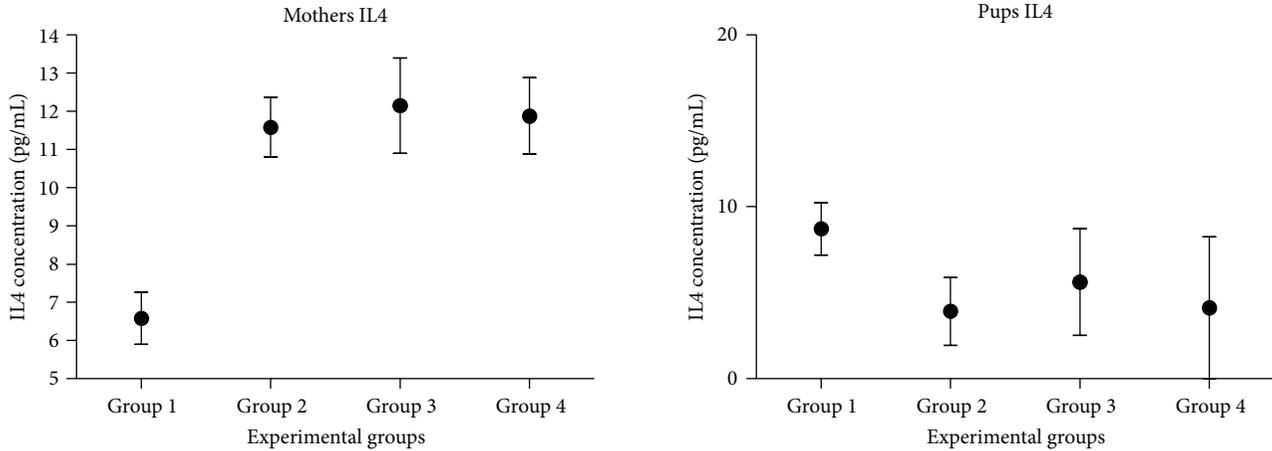


FIGURE 11: Quantification of interleukin 4 in the serums of mothers and pups. In the *x*-axis, the different experimental groups are displayed; in the *y*-axis, the average concentration of IL4 in pg/mL is represented.

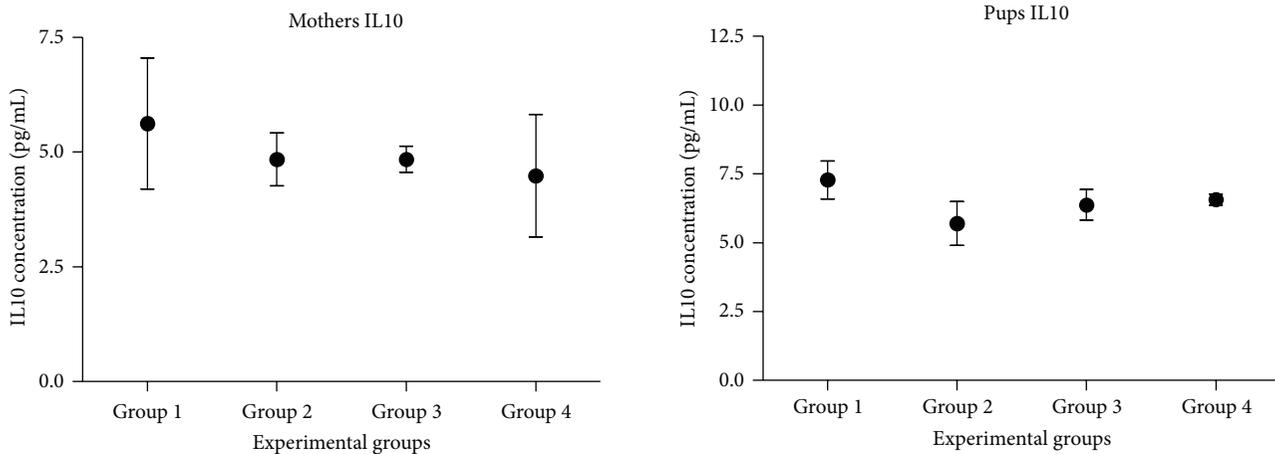


FIGURE 12: Quantification of interleukin 10 in the serums of mothers and pups. In the *x*-axis, the different experimental groups are displayed; in the *y*-axis, the average concentration of IL10 in pg/mL is represented.

transplantation is beneficial for the animal, because they can avoid the subsequent inflammation. This case only happens in the mothers, because their immune system is completely developed and their Th2 cells, IL4 producers (namely, activated basophils, mast cells, and eosinophils), are functionally active [30]. In the pups, there are smaller levels of IL4. This could lead to more inflammation and therefore could maybe cause (or be one of the causes of) the high morbidity of the transplanted fetuses, because they will not be able to inhibit inflammatory substances such as IL1, TNF α , IL6, and the inflammatory protein of the macrophage [31]. It would be interesting to quantify these inflammatory cytokines to contrast this hypothesis.

About the data obtained for the IL10, also known as cytokine synthesis inhibitory factor (CSIF) (Figure 12), no significant differences were found between experimental groups, neither in mothers nor in pups. Since there are no high levels of IL10, it is probable that the balance between

Th1 cells and Th2 cells is not altered [30]. Depending on the conditions of the transplant, tolerance or allosensitization can be developed. The factors that may influence the response are: time taken for transplantation, transplanting cell dose and composition, the trauma caused by the prenatal surgery, the condition of the immune system during the cell injection, and the level of postnatally induced chimerism [31]. Moreover, it has been seen that hepatocyte transfusion increases lifespan of rats that undergo a hepatic transplantation and improves the production of hepatic proteins [32].

Finally, it is important to mention that this work contributes with interesting tools for hepatocellular transplantation *in utero*. Indeed, we consider that it is necessary to further research in this field in order to achieve a deeper knowledge, because of the relevance to unveil the mechanisms involved in tolerance induction and allosensitization, to allow the development of safe prenatal transplantation protocols making a success in individuals with congenital disorders.

5. Conclusion

We obtained rates of 90–95% cellular viability making the hepatocyte suspension isolated from rat fetuses at ED 21 suitable for transplantation. Also, both types of transplantation have a limited success. Indeed, transplanted hepatocytes are not able to pass through the blood-placenta barrier. Moreover, the hepatocytes injected to fetuses of pregnant rats at ED17 were primarily located in the liver, although they show some other ectopic locations. In transplanted experimental groups, there is an upward trend in the whole amount of T CD4 and T CD8 cells in transplanted experimental groups. In addition, there is an increase of IL4 in the transplanted groups in the pregnant rats. As it is an anti-inflammatory cytokine, this increment is positive because it protects the mother from inflammatory processes caused by the surgery.

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