

# Increased Expression of Toll-Like Receptors 2 and 4 in Renal Transplant Recipients that Develop Allograft Dysfunction: A Cohort Study

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## ABSTRACT

**Background:** The incidence of ischemic reperfusion injury (IRI) in early phase post-transplantation and activation of toll-like receptor (TLR-2) and TLR-4 remarkably impact the outcome of a renal allograft. **Objective:** To investigate whether the expression of TLRs in peripheral blood mononuclear cells (PBMCs) can predict the clinical outcome of kidney allografts. **Methods:** We obtained blood samples from 52 renal transplant patients before transplant, and 2, 90, and 180 days post-transplantation in order to analyze the surface expressions of TLR-2 and TLR-4 on peripheral blood monocytes. The expression patterns of TLR-2 and TLR-4 were compared between patients with graft dysfunction (GD) and those with well-functioning graft (WFG). **Results:** Significantly different mean dynamic changes in surface expression of TLR-2, according to percentage of TLR-2<sup>+</sup> cells, between (the GD and WFG) groups existed at most time-points before and after renal transplantation ( $p=0.007$ ) with the exception of day 2 post-transplantation. We observed significantly higher mean fluorescence intensities of TLR-2 and TLR-4 on CD14<sup>+</sup> cells in the GD group compared to the WFG group. This finding was particularly observed 180 days post-transplantation ( $p=0.001$ ). Based on TLR-2 and TLR-4 protein expression for each step, multiple logistic regression and ROC curve analysis revealed that an increase in CD14<sup>+</sup> TLR-2<sup>+</sup> monocytes within the 90 days post-transplantation was associated with increased risk of GD at 180 and 365 days post-transplantation [odds ratio (OR)=1.27,  $p=0.005$ ]. **Conclusion:** Sequential monitoring of TLR-2 and TLR-4 expression patterns in peripheral blood monocytes appear to be prognostic and predictive biomarkers for early and late kidney allograft outcomes.

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## INTRODUCTION

Kidney transplantation is the latest standard treatment for end-stage renal disease (ESRD), however, adaptive and innate immune responses to graft alloantigens are the highest threats to graft survival (1). Evaluation of renal function after transplantation is a very important process for graft survival (2). For this reason, different diagnostic protocols include serial monitoring of non-invasive samples, in addition to the study of gene expressions, cytokines, chemokines and mediators associated with graft rejection (2). A protocol biopsy (PB) is one method for early detection of renal damage despite its normal function. PB is beneficial for the study of kidney graft conditions such as interstitial fibrosis and tubular atrophy (IF/TA), acute rejection (AR), subclinical rejection (SCR), graft vasculopathy, and glomerulopathy. PB can be used to confirm molecular studies, although the risk of bleeding is 0.5% (3-7). Numerous evidences exist regarding the important role of the innate immune system, such as pathogen recognition receptors in the establishment and development of different mechanisms of rejection (8). Recent findings suggest that adaptive immunity and T cells without innate immune activity lack the capability to induce an allograft rejection (9). Immunological events inside the kidney graft begin at the time of donor nephrectomy. In response to anoxia / ischemia and ischemic reperfusion injury (IRI) lesions, some of the kidney cells lyse and release their intracellular contents into the interstitial space (10). The lysed kidney cell contents, defined as "damage-associated molecular pattern" (DAMP) bind to innate immunity receptors such as toll-like receptors (TLRs) trigger intracellular signaling pathways, (especially MyD88), and activate dendritic cells in the tissues (10-13). These cells process alloantigens and present them to naïve T-cells in the draining lymph nodes, which in turn activates the adaptive immune system against the graft. Therefore, the kidney allograft already expresses inflammatory conditions at the time of transplantation (11-16).

Preclinical studies on knockout mice for *TLR-4*, *TLR-2*, *MYD-88* and *TRIF* genes have clearly shown a severe reduction in immune reaction against the allograft, in addition to maturation and function of dendritic cells, generation of Th1 effector cells, expressions of  $\alpha$ -SMA and collagen types I and II, and finally inhibition of development (17-19). The role of TLRs in alloimmunity and allograft rejection is evident in MyD88 and TRIF signaling pathways which lead to the production of nuclear factors (NF $\kappa$ B, IRF3), the release of pro-inflammatory cytokines (IL-6, TNF- $\alpha$ , IFN- $\alpha$ - $\beta$ ) and chemokines (CCL2 or MCP-1), as well as adaptive immune stimulation (20,21).

Recent studies have shown a key role of TLR-2 and TLR-4 in the development of adaptive and innate immune responses, In particular, their decisive role in the progress of IRI after transplantation and determining the paths of immune responses toward graft tolerance or rejection has been proposed. Expressions of these markers are used as predictive and therapeutic targets to follow up allograft recipient patients (25). The main purpose of this cohort study was to determine the sequential dynamic changes in the surface expressions of TLR-4 and TLR-2 on peripheral blood monocytes of kidney transplant patients before transplantation and at 2, 90 and 180 days after transplantation. Next, we compared the expression patterns of both TLRs between patients with well-functioning graft (WFG) and those with graft dysfunction (GD). This, in turn, could lead to the identification of innate immunity genes and markers associated with graft inflammation and pathological conditions as well as an early prediction of graft rejection in order to carry out necessary therapeutic interventions.

## MATERIALS AND METHODS

This cohort enrolled 52 patients, 31 (59.6%) men and 21 (40.4%) women from November, 2014 January 2016. All patients received their first renal transplants from living related (2%) or unrelated donors (98%). All participants provided informed consent and the University Hospital Ethical Committee approved the study. All patients received a standard triple immunosuppressive protocol that consisted of mycophenolate mofetil, cyclosporine or tacrolimus, and Prednisolone without antibody induction therapy. We collected the available demographic, clinical and laboratory data from patients' hospital records (Table 1).

**Table 1. Demographic and clinical data from patients who underwent renal transplantation (N=52).**

Age, y (mean $\pm$ SD)	41.79 $\pm$ 12.09
Gender (male/female) (%)	59.6 / 40.4
Type of allograft (%) Living	100
Type of donors (%)	
Related	2
Non-related	98
Serum creatinine baseline (mg/dl)	6.78 $\pm$ 2.95
Cause of end-stage renal disease (%) (n)	
Hypertension	46.1 (24)
Diabetes	15.3 (8)
Polycystic kidney disease	9.6 (5)
Glomerulonephritis	5.7 (3)
Reflux	3.8 (2)
Proteinuria	1.9 (1)
Alport syndrome	1.9 (1)
Other	15.3 (8)
HLA incompatibility (%) (n)	
1	5.8 (3)
2	9.6 (5)
3	26.9 (14)
4	40.4 (21)
5	11.5 (6)
6	5.8 (3)

**Blood Samples and Biopsies.** Venous blood samples were collected in EDTA vacutainers for flowcytometry analysis at the following time points: before

transplantation and 2, 90 and 180 days after transplantation. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation (Inno-Train, Germany) and frozen in freezing media reagent that included FBS (Gibco) with DMSO (Sigma-Aldrich USA): (1/9) Samples were placed in a liquid nitrogen tank until use. 180 days after transplantation, a biopsy sample of the kidney (PB) was taken from those patients (n=24) who consented to the procedure. Biopsy samples were also taken from six patients who experienced increased a creatinine levels before the 90 days of transplantation (cause biopsy).

We subdivided the study patients into two groups according to GFR, at 90 and 180 days post-transplant those who had WFG with GFR rates higher than 60 ml/min/1.7m<sup>2</sup>, and patients with GD whose GFR rates were lower than 60 ml/min/1.7m<sup>2</sup>. Patients with an increase of 20% in serum creatinine or an increase of 10% in sequential daily sampling were candidates for cause biopsy.

**Flowcytometry.** PBMCs from renal transplant patients were isolated by Ficoll-Hypaque density gradient centrifugation. Briefly, the cells were washed twice and resuspended in RPMI 1640 cell culture medium. A total of 10 µL of each antibody which included FITC-labeled anti-human CD14, (FAB3832F), Phycoerythrin (PE)-labeled anti-human TLR4 (FAB6248P), and PE-labeled anti-human TLR2 (FAB2616P) was added to tubes that contained  $1 \times 10^6$  cells, and the mixture was suspended in 100 µL of cell culture medium, after which they were allowed to incubate at room temperature for 30 minutes. PE and FITC isotype-matched antibodies were used as the negative controls. FACS Calibur flowcytometer was used to separately determine the frequencies of CD14+TLR2+ and CD14+TLR4+ cells by gating CD14+ monocytes. FACS analysis was performed using a FACS Calibur flowcytometer (Becton Dickinson, Cowley, UK). Data analysis was performed with flow-jo software.

**Statistical Analysis.** Data were represented as means  $\pm$  standard deviations, or median and inter-quartile range (IQR) for numeric variables and frequencies or percentages for categorical variables. Repeated-measure analysis of variance was conducted to compare the mean dynamic change of cell surface markers in the normal allograft versus dysfunctional grafts. ROC curve analysis was conducted to determine the cutoff points that had the highest sensitivity and specificity for the diagnosis of GD. Multiple logistic regression analyses were performed to predict the risk of GD represented by odds ratio (OR) and a confidence interval (CI) of 95%. All statistical tests were two-sided, and a p-value 0.05 was considered statistically significant. The Benjamini-Hochberg method was used to adjust the p-values for multiple comparisons. Data were analyzed using SPSS software version 20 (IBM Corporation, Armonk, NY, USA).

## RESULTS

**The Main Causes of End-Stage Renal Disease (ESRD).** As depicted in Table 1 the most common causes for ESRD in the study groups included hypertension (46.1%) diabetes (15.3%), polycystic kidney disease (9.6%), glomerulonephritis (5.7%), reflux (3.8%), Alport syndrome (1.9%), and proteinuria (1.9%). Diseases with unknown etiology or congenital disorders were attributed to other factors that caused ESRD (15.3%). We classified 27 of the 52 patients as the (WGF) group and the remaining 25 patients as the GD group.

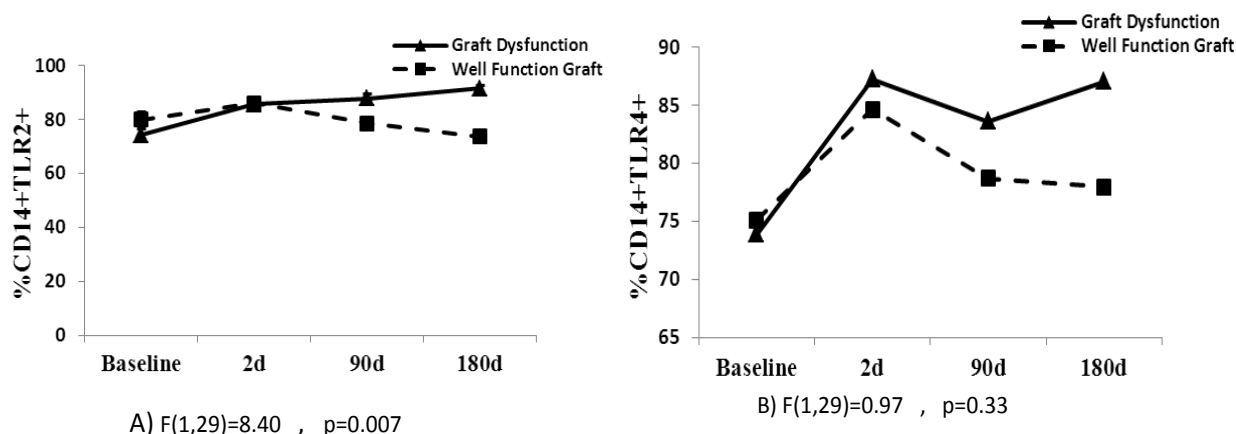
We obtained 15 biopsy samples from the 25 GD patients. From these, there were 9 and 6 obtained from patients who needed a cause biopsy. Histological evaluation of the 9 PB patients showed 1 with acute cellular rejection (classified as Banff grade 4 type IB), 5 mononuclear interstitial inflammations, 1 BK virus infection, 1 acute pyelonephritis and 1 chronic pyelonephritis. However, the remarkable histological findings noted in 6 causes biopsy samples included 2 patients with antibody-mediated rejection (Banff grade 2 type I), 2 with cell-mediated rejection (Banff grade 4 type IB) and 2 patients with pyelonephritis. Other cases obtained from the WFG group did not show any symptoms or signs of rejection according to histological analysis.

**Table 2. Frequency and Standard errors of CD14<sup>+</sup> Peripheral blood monocytes expressing TLR-2 and TLR-4 (Mean ± SE).**

Markers	Days	WFG <sup>a</sup>	GD <sup>b</sup>	P-Value
CD14 <sup>+</sup> / TLR2 <sup>+</sup>	D0 <sup>c</sup>	79.85±2.92	74.23±2.32	0.936
	D2	85.85±2.36	85.37±1.88	0.782
	D90	77.85±1.95	87.58±1.55	<b>0.001</b>
	D180	74.25±1.68	91.33±1.33	<b>0.001</b>
CD14 <sup>+</sup> / TLR4 <sup>+</sup>	D0	77.53±2.72	70.87±2.16	0.653
	D2	85.39±2.68	85.69±2.13	0.250
	D90	78.78±2.05	83.13±1.63	0.096
	D180	78.38±1.73	87.35±1.37	<b>0.001</b>

a: WFG: Well-functioning graft

b: GD: graft dysfunction



**Figure 1.** Frequency of TLR-2 and TLR-4 immunopositive peripheral blood monocytes in both groups of renal transplant patients (GD & WFG) as determined by flowcytometry analysis before (Baseline) and 2, 90 and 180 days after transplantation.(A): Repeated measure analysis revealed That TLR-2 Protein expression during the study period was significantly increased in GD patients compared to WFG patients,  $F(1, 29) = 8.40$ ,  $p=0.007$ ). (B): TLR-4 protein expression in the Peripheral blood monocytes was not significantly different in patients with GD compared to those with WFG at before and 2 and 90 days after transplantation, but this significant increase for TLR-4 in GD patients only worse seen at 180 days after transplantation ( $P=0.001$ ).

**Frequency of Peripheral Blood Monocytes that Expressed Toll-Like Receptor (TLR)-2 and TLR-4 in Renal Transplant Recipients.** Flowcytometry analysis results indicated the extent of dynamic changes in the cell surface expressions of TLR-2 and TLR-4 at four time points before and after (2, 90, 180 days) renal transplantation (Table 2 and Figure 1). As seen in Figure 2A, repeated measure analysis revealed that TLR-2 protein expression during the study period significantly increased in GD patients compared to WFG patients,  $F(1, 29) = 8.40, p = 0.007$  (Figure 2A). TLR-4 protein expression in the Peripheral blood monocytes had shown no significant difference in patients with GD compared to those with WFG before as well as 2 and 90 days post-transplantation samples, however a significant increase occurred for TLR-4 in GD patients at 180 days after transplantation ( $p = 0.001$ , Figure 2-B).

**Table 3. The MFI of CD14<sup>+</sup> Peripheral blood monocytes expressing TLR-2 and TLR-4 (Mean  $\pm$  SE).**

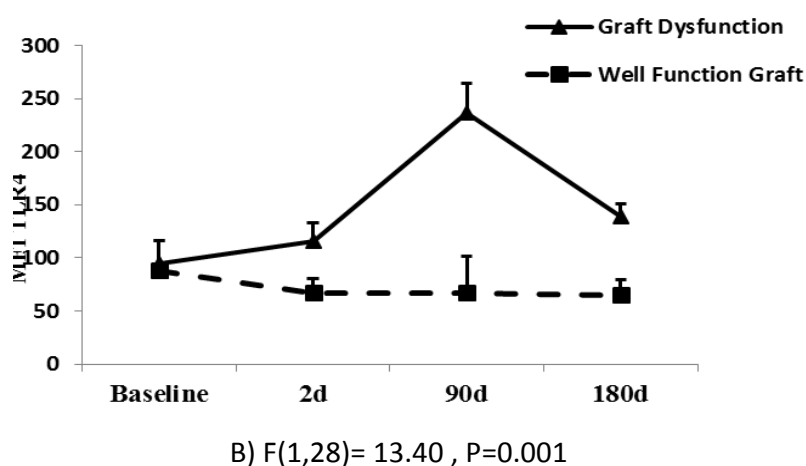
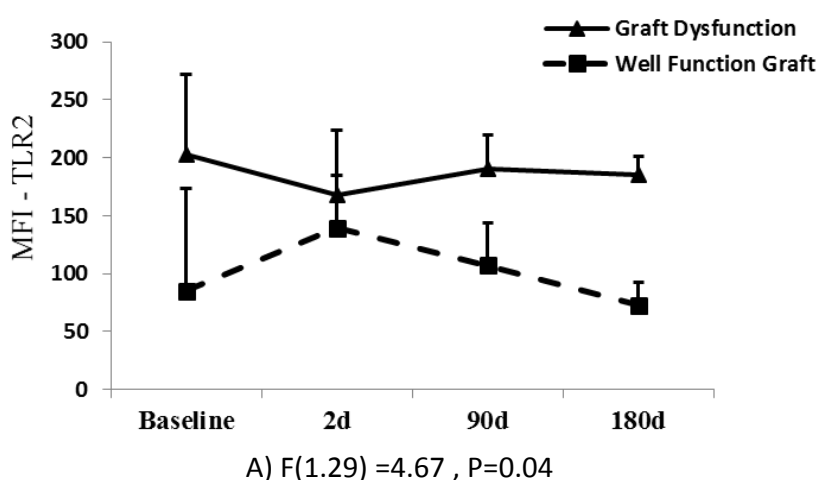
Markers	Days	WFG <sup>a</sup>	GD <sup>b</sup>	P-Value
CD14 <sup>+</sup> / TLR2 <sup>+</sup>	D0 <sup>c</sup>	85.07 $\pm$ 27.92	201.59 $\pm$ 69.83	0.347
	D2	139.37 $\pm$ 44.88	167.58 $\pm$ 56.47	0.988
	D90	77.85 $\pm$ 1.95	190.34 $\pm$ 29.71	<b>0.267</b>
	D180	72.63 $\pm$ 19.90	185.25 $\pm$ 15.81	<b>0.001</b>
CD14 <sup>+</sup> / TLR4 <sup>+</sup>	D0	88.20 $\pm$ 27.46	94.17 $\pm$ 22.42	0.618
	D2	66.34 $\pm$ 13.62	115.74 $\pm$ 16.76	0.096
	D90	67.05 $\pm$ 34.36	236.48 $\pm$ 24.06	0.01
	D180	64.66 $\pm$ 14.91	139.10 $\pm$ 12.17	<b>0.001</b>

a: WFG: Well-functioning graft

b: GD: graft dysfunction

We assessed mean fluorescent intensity (MFI) at sequential times for the TLR-2 and TLR-4 and CD14<sup>+</sup> cell populations in both groups. A significantly higher MFI of TLR-2 and TLR-4 on gated CD14<sup>+</sup> cells existed in patients with GD compared to WFG at 180 days post-transplantation ( $p = 0.001$ , Figure 2, Table 3).

We performed ROC curve analysis of the surface expressions of TLR-2 and TLR-4 on Peripheral blood monocytes. Table 4 and Figure 3 show the calculated area under the curve (AUC), CI, sensitivity (85%) specificity (65%) cut off point (80.6), and p-value ( $p = 0.001$ ) for TLR-2 expression on day 90, that predicted the likelihood, for a GD diagnosis at day 180 as well as the day 180, values of sensitivity (73%), specificity (68%), cut-off point (86.5), and p-value ( $p = 0.002$ ) that predicted a GD diagnosis at the first year (Table 4 and Figure 3).

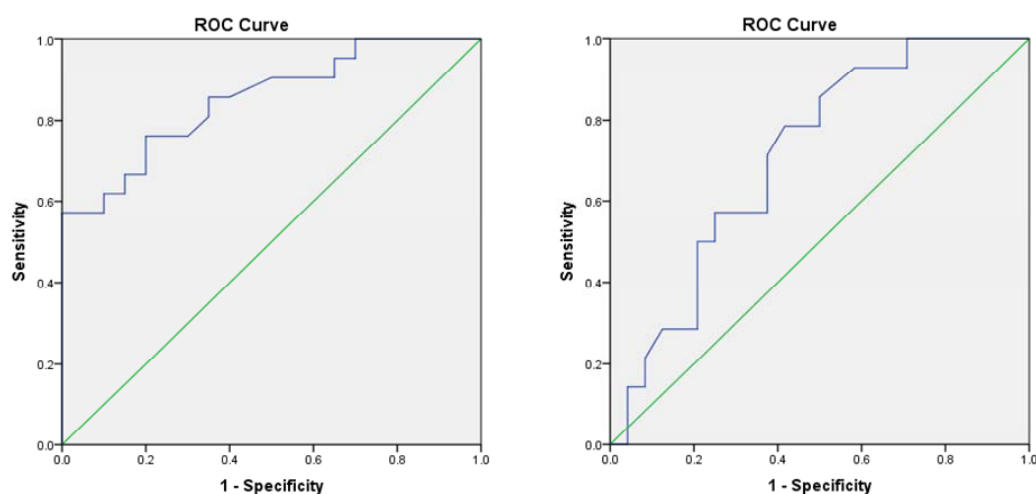


**Figure 2.** MFI of TLR-2 and TLR-4 immunopositive peripheral blood monocytes in both groups of renal transplant patients (GD & WFG) as determined by flowcytometry analysis before (Baseline) and 2, 90 and 180 days after transplantation.(A): Repeated measure analysis revealed that TLR-2 MFI during the study period was significantly Increased in GD patients compared to WFG patients,  $F(1, 29) = 4.67, p = 0.04$ . (B): TLR-4 MFI in the peripheral blood monocytes was significantly higher in patients with GD compared to those with WFG at 90 and 180 days after transplantation ( $F(1,28) = 13.4, P = 0.001$ ).

**Table 4. Cut off point of TLR-2 expression on the peripheral blood monocyte of renal transplant patients, for diagnosis of graft dysfunction on next phase based on GFR Index, with ROC curve.**

TLR	Days	Cut off	Sensitivity	Specificity	AUC <sup>a</sup> (95% CI <sup>b</sup> )	P Value
TLR-2	90-180	80.6	85%	65%	0.85(0.73-0.96)	0.001
TLR-2	180-360	86.5	73%	68%	0.78(0.64-0.93)	0.002

a: Area under the curve.  
b: Confidence interval.



A) ROC curve analysis at the 3<sup>rd</sup> for the 6<sup>th</sup> months    B) ROC curve analysis at the 6<sup>th</sup> months for the 1<sup>st</sup> year

**Figure 3.** ROC curve analysis for TLR-2 immunopositive peripheral blood monocytes in diagnosis and prediction of graft dysfunction. (A): Roc curve analysis revealed that TLR-2 expression at the 3<sup>rd</sup> months for GD at the month 6 (AUC =0,85 , 95%CI= 0,73-0,96, sensitivity=0.85 , specificity=0.65) B: Roc curve analysis revealed that TLR-2 expression at the 6<sup>th</sup> months for GD at the month 12 (AUC =0.78, 95%CI= 0.64-0.93, sensitivity=0.73, specificity=0.68)

Based on TLR-2 and TLR-4 protein expressions at each step, a multiple logistic regression analysis was performed to predict GD in the next step. An increase in CD14+ TLR-2+ monocytes, at day 90, resulted in an increased risk of GD (OR=1.27, p=0.005) at day 180 as well as for the first year post-transplantation (OR=1.11 p=0.028, Table 4).

## DISCUSSION

Activity of the immune system following solid organ transplantation and formation of sterile inflammation is one of the reasons of breakdown of tolerance toward grafts and increased risk of acute and chronic graft rejection (11). TLR molecules are among the most important innate immune receptors that identify the pathogen-associated molecular patterns (PAMPs) and DAMPs (26). On the other hand, TLR molecules play an essential role in IRI mechanisms and pathogenesis, such as changes in energy metabolism, mitochondrial and cell membranes, and development of various forms of cell death, such as apoptosis, necrosis that lead to the destruction of renal tubules, degradation of the extracellular matrix and infiltration of monocytes and macrophages into the interstitial areas. They are considered the key reasons in AR and delayed graft function (22-24).

Preclinical studies by Goldstein *et al.* (17), McKay *et al.* (28) and Wang *et al.* (29) demonstrated that endogenous ligands for TLR-2 and TLR-4 significantly increased in mouse model of a kidney graft. Transplantation from wild-type mice to mutant recipients for *TLR-4*, *TLR-2 MYD88*, and *TRIF* genes resulted in a sharp reduction in



the infiltration of macrophages, T cells, and DCs at the site of the transplant along with better graft function (29). Kown *et al.* reported a significant increase in mRNA expression of TLR-2 and TLR-4 in the allograft groups compared to normal healthy controls (12). Thus far, numerous studies researched the relationship between activity and signaling of the innate immune components with allograft outcome.

The results of the present study showed that the surface expression of TLR-2 increased significantly at 90 and 180 days post-transplantation. ROC curve analysis revealed that TLR-2 with cut-off points at 180 days post-transplantation of (80.60, sensitivity of 85% and specificity of 65% along with the 12 month post-transplantation cut-off value of 83.4), 83.4), sensitivity of (71%) and specificity (63%) diagnosed GD at 6 and 12 months post-transplantation. In addition, as depicted in Table 4 multiple logistic regression analysis showed that TLR-2 surface expression 90 days post-transplantation predicted GD at days 180. In addition, TLR-2 surface expression at 180 days also predicted GD at one year post-transplantation. Surface expression of TLR-4 did not increase in our study patients except at 180 days where it was (78.3% in WFG and 87.3% in GD patients,  $p=0.001$ ). ROC curve and logistic regression analysis were not informative for TLR-4.

The decline in expression of TLR-4 from day 2 to day 90 post-transplantation in both groups could be related to administration of immunosuppressive drugs and the use of hydrocortisone which has been shown to reduce the expression level of TLR-4 in peripheral blood monocyte cells (30). Thus analysis of the surface expression of TLRs in peripheral blood monocytes in the early stages after transplantation clearly showed that TLR-2 has a higher predictive value compared to TLR-4. Preclinical studies indicated that subsequent to IRI, expression levels of the TLRs; in particular TLR-2 and TLR-4, increased significantly in either epithelial cells, endothelial cells, or infiltrated cells of the allograft (24). These were associated with symptoms such as increased infiltration of immune cells (DCs, macrophages, T cells) to the areas of inflammation, increased secretion of inflammatory cytokines and chemokines within tissues and urine, acute rejection of allografts, and renal dysfunction (31). Interestingly, the use of knockout animal models for MYD88, TLR-2, and TLR-4 genes or the use of antibodies against each of the above markers prevented sterile inflammation, cellular infiltration, inflammatory cytokine and chemokine secretions, renal tubular cell apoptosis, and destruction of the allograft (18,24,32,33).

Park *et al.* showed that allograft inflammation and the incidence of allograft fibrosis in the first year after kidney transplantation was associated with increased expression of TLR-2 and TLR-4 and cellular infiltration (monocytes, macrophages, DCs, and T cells) (7). These studies supported our results on the clinical relevance of the dynamic changes in TLRs expression in peripheral blood monocytes of the kidney allograft patients.

Taken together, patients that faced renal dysfunction for any reason and had lower GFR levels at 90 and 180 days after transplantation, showed increased activities of genes and molecules involved in the innate immune system. This finding confirms the increased activities of TLR-2 and TLR-4 in the endogenous ligands binding process and stimulation of the MyD88 signaling pathway. Thus, we propose that profiling the surface expression of TLRs may be used as biomarkers to predict early and even late allograft outcomes.

The results of this study suggest that renal allograft dysfunction is associated with increased expression of TLR-2 and TLR-4 on peripheral blood monocytes. More importantly, these dynamic changes in the innate immunity players can be simply

monitored in addition to monitoring adaptive immunity for the early detection of allograft lesions even before histopathological and clinical manifestations. This, in turn, may lead to more appropriate and timely therapeutic interventions which will potentially eliminate the need for invasive methods (biopsy) to monitor allograft function. However, additional studies are needed to confirm the predictive role of these molecules.

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