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Can the Toll-like receptors 4 expression in peripheral blood mononuclear cells help assess the effectiveness of immunosuppression and the chance of a future good renal transplant function?

Slawomir C. Zmonarski[a], Katarzyna Madziarska[a,*], Tomasz Golebiowski[a], Miroslaw Banasik[a], Oktawia Mazanowska[b], Marcin Madziarski[a], Magdalena Krajewska[a]

[a] Dept. of Nephrology and Transplantation Medicine, Medical University Wroclaw, Poland
[b] Faculty of Medicine and Dentistry, Dept. of Nephrology and Transplantation Medicine, Medical University Wroclaw, Poland

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ABSTRACT

Background: A small percentage of peripheral blood mononuclear cells (PBMCs) circulating during the kidney transplantation (KT) period remain in the blood long after transplantation. A part of the PBMCs penetrates the graft.

Aim: To examine if the choice of immunosuppression may change TLR4ex and how TLR4ex affects the transplant function in the future.

Material: The study population-143 transplanted patients (pts) (55 females, 88 males), mean age on recruitment day 50.33 ± 12.8 years old, mean BMI 25.04 ± 4.18. 41 pts. experienced delayed graft function (DGF+). 55 pts. were treated with cyclosporine A (CsA) and 88 with tacrolimus (Tac). All were treated with mofetil mycophenolate (MMF). The PBMCs acquisition and starting point of the follow-up (TLR-day) was at least one month after KT.

Method: We investigated averaged mRNA expression of Toll-like receptors 4 (TLR4ex) in non-stimulated peripheral blood mononuclear cells with the use of real-time polymerase chain reaction. The KT pts. (All, Tac, CsA, DGF+) were divided by the respective median of their TLR4ex (lower: L-TLR4ex, higher: H-TLR4ex). Main clinical parameters and transplant biopsy files (if available) were assessed on TLR-day and post follow-up.

Results: We found that TLR4ex was reduced for a long time in patients who experienced delayed graft function. L-TLR4ex had a higher proportion of DGF+ patients, and patients treated with CsA but lower of those treated with Tac than in H-TLR4ex. The amplitude of changes in renal function parameters (ΔEGFR%/ΔsCr/ΔsCr%) was clearly less favorable for L-TLR4ex. Tacrolimus expressed a stabilizing effect. Both the positive vasculitis score and chronic graft nephropathy were more frequent in the L-TLR4ex group. On TLR-day an association of renal function and Tac concentration with TLR4ex was clear only in the tacrolimus population. The TLR4ex was lower in patients with a future deterioration of the graft function.

Conclusion: In kidney transplant recipients the occurrence of DGF results in a long-term reduction of the averaged TLR4ex in PBMC. Tacrolimus exerts a clear, stabilizing, positive and dose-dependent effect on TLR4ex. An improvement in renal transplant function may be expected in KT patients with high TLR4ex. Evaluation of the averaged TLR4ex can be used to assess the efficacy of immunosuppression in the treatment with tacrolimus and to estimate the likelihood of deterioration in renal function.

1. Introduction

The mononuclear cells population in peripheral blood is largely variable, subject to a continuous, varying intensity of renewal and exchange. Only a small percentage of peripheral blood mononuclear cells (PBMC) circulating in the transplantation period remain present from a few to several dozen months after transplantation. From embryogenesis, in a certain balance with the cells circulating in the blood, part of the population of monocytes penetrates into tissues, where they are transformed into macrophages in a specific manner for particular organs, taking the form of, e.g., Kupffer cells in the liver or mesangial cells in the kidney [1]. The migration of monocytes later in life is usually

* Corresponding author at: Dept. of Nephrology and Transplantation Medicine, Medical University Wroclaw, Borowska St. 213, 50-556 Wroclaw, Poland.
E-mail address: kmadziarska@wp.pl (K. Madziarska).

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associated with the process of activation of the primary immune response by pathogen associated molecular patterns (PAMP) or danger associated molecular patterns (DAMP). The transfer of a kidney from the donor to the recipient includes a number of unfavorable stages, leading to ischemia-reperfusion injury with sterile inflammation [2,3] and increased graft immunogenicity [4,5]. The condition of transplanted kidney affects the PBMC behavior, but also the PBMC status influences the transplant function [6,7]. The penetration of PBMC from the blood to the tissues depends on the degree of their attraction to the blood vessel wall, which is dependent on the intensity of the local inflammatory response [3,8]. Toll like receptor 4 (TLR4) activation is a double-edged sword and can accelerate or alleviate the inflammation [6,7]. The chance of being attracted to the vascular wall increases in proportion to the susceptibility to stimulation detected by appropriate receptors, e.g., TLR4, and this depends on their expression on the PBMC [9]. Thus, proper functioning of a transplanted kidney does not so much depend on the part of the PBMC that remained in the peripheral blood but on the one that effectively disappeared from it and entered the graft [2,4,10–12]. Neutrophils often cluster around graft-infiltrating monocytes [9]. Monocytes infiltrating the graft are considered a major cell-damaging component in chronic allograft injury (CAI) [12] linking an endothelial dysfunction with TLR 4 expression [13]. Successful treatment of acute rejection in the past seems to have a rapidly decreasing effect on the averaged TLR4 expression in PBMC [4,10].[0]

2. We looked for the answers to the following questions

1. Can the choice of immunosuppression (IS) cause a change in the percentage of mononuclear cells with low or high TLR4 expression in the peripheral circulation resulting in a shift in the distribution of the overall averaged TLR4 expression?

2. To what extent does the quality change in the overall TLR4 PBMC expression (estimated by a single measurement) affect the transplant function in the future?

2.1. Material

Patients who received a supplementary induction of immunosuppression (incl.: photopheresis, anti-thymocyte globulin, basiliximab) were excluded from the study. The study population was recruited from patients who underwent kidney transplantation (KT) between the years 1996 and 2013 and were registered to the transplant outpatient clinic at the University Hospital in Wroclaw, Poland. The time from transplantation to the recruitment day and first blood sample acquisition (KT-TLR) was at least one month (> 1 m). The criterion of delayed graft function (DGF) was the need for at least 1 hemodialysis from the 3-rd day after KT. All patients were treated with mofetil mycophenolate (MMF). In all patients, except for emergencies, the aim was to maintain stable IS during the follow-up period (F-up). A sex – and age matched control group without any known systemic disorder was also recruited. The demographic and clinical data for the study groups are presented in Table 1. All the participants expressed an informed consent according to the guidelines of the local ethic committee.

3. Method

We investigated the averaged mRNA expression of Toll-like receptors 4 (TLR4 expression, TLR4ex) in non-stimulated PBMC with the use of real-time polymerase chain reaction (RT-PCR). PBMCs were separated from heparinized blood samples, mRNA was isolated with RNeasy Mini Kit (QIAGEN) and reversely transcribed with High Capacity RNA to cDNA kit (Applied Biosystems). The cDNA amount for TLR genes and GAPDH was assessed using TaqMan PCR Master Mix on Taqman 9700HT real-time PCR system. All the procedures were performed according to the manufacturer protocols. The control group data (Contr., 46 volunteers) were used only for PCR data normalization. The TLR4-ex data are presented as $\Delta\Delta Ct$ = mean ΔCt_{Contr} - ΔCt_{sample}, where $\Delta Ct = Ct_{gene} - Ct_{GAPDH}$, Ct is the cycle threshold value and defines the calculated cycle number in which the fluorescence measured during PCR reaction increases over the preset threshold value. The starting point of follow-up (recruitment day, TLR-day) was at a different time after kidney transplantation (KT-TLR; at least one month) when blood samples for the PBMC separation for TLR-mRNA analysis were taken. The biochemical, hematologic, viral/bacteriologic status and diagnostic kidney transplant biopsy (if available) parameters were assessed at the same time-point. Serum creatinine concentration (sCr) and estimated glomerular filtration rate values (EGFR) were used to assess renal function. In order to assess the bi-directional association of TLR4 expression with renal function more efficiently, both the EGFR/sCr variability was evaluated in a qualitative way and an arbitrary, three-step qualitative scale of the sCr range was used: normal (Normal) with sCr ≤ 1.3 mg/dL, intermediate renal failure (IRF) with sCr from 1.3 to 2.0 mg/dL and renal failure (RF) with sCr > 2,0 mg/dL. For qualitative comparisons we assumed that the transplanted kidney function deteriorated when the EGFR at the end of the follow-up period decreased by > 10% or 15% (i.e., ΔEGFR > 15%) from the baseline value or when the creatinine concentration increased by > 10% or 15% (i.e., ΔsCr > 15%), depending on the nature of the comparison and the size of respective population. The KT pts. (All, Tac, CsA, DGF +) were divided by the respective median of TLR4 expression into two groups of low-TLR4 expression (L-TLR4ex) and of high-TLR4 expression (H-TLR4ex). For detailed comparison of L-TLR4ex with H-TLR4ex see Tables 2-5.

3.1. Statistical analysis

Due to lack of data normality the results are expressed as median and interquartile range (x, y to z) unless it is stated otherwise. The comparison of quantitative variables between different study groups was performed using Mann-Whitney test or Sign/Wilcoxon test. Chi-

Table 1
Study population.

Population	Patients	Clinical parameter	Mean	SD
Control	46	Donors: KT Age (y)	44,9	13,2
All pts	143	Recipients: KT Age (y)	47,8	12,2
Females	55	KT -TLR (m)	28,8	34,6
Males	88	Follow-up time (m)	51,13	13,3
DGF +	41	Recipients: TLR Age (y)	50,3	12,8
TLR-Day: Tac	88	Recipients: TLR Age Tac (y)	47,2	12,4
TLR-Day: CsA	55	Recipients: TLR Age CsA (y)	55,4	12,0
Transplant biopsy	44	TLR4 expression (TLR4ex)	MEDIAN	Q25 - Q75
TLR-Day: sCr < 1,3 mg/dL	50	All pts	0,400	−0,813 to 1339
TLR-Day: sCr 1,3–2,0 mg/dL	59	Low- TLR4-ex	-0,813	−1,53 to −0,027
TLR-Day: sCr > 2,0 mg/dL	34	High-TLR4-ex	1,33	0,76 to 2,15
		DGF +	−0,103	−1,3 to 0,677

Table 2
Comparison of qualitative parameters (All pts).

Compared All pts.:	Low-TLR4ex	High-TLR4ex	Chi2	P =
Hemodialysis before KT	90,6%	81,2%	3,69	0,0546
History of ARJ	21,0%	21,3%		NS
History of DGF	32,8%	19,0%	8,04	0,0047
Treatment: cyclosporine A (CsA)	43,7%	28,1%	7,72	0,0054
CsA concentration > median 139 ng/mL	51,8%	61,1%	5,96	0,015
Treatment: tacrolimus (Tac)	56,3%	71,9%	7,72	0,0054
Tac concentration > median 7,30 ng/mL	26,4%	60,4%	27,11	0,000001
KT Biopsy. BANFF score: v > 0	44,44 (8/18)	14,28 (3/21)	11,88	0,00057
KT Biopsy. BANFF criteria of chronic nephropathy	27,78 (5/18)	5,26 (1/19)	17,89	0,00003
Follow-up: New proteinuria	40,4%	27,9%	4,78	0,029
TLR-Day: sCr RF (> 2 mg/dL)	29,7%	20,3%	3,49	0,062
Follow-up: ΔsCr exceeds +15%	25,0%	12,5%	9,14	0,0025
Follow-up: ΔsCr exceeds −10%	20,3%	28,1%		
Follow-up: sCr stabile	46,9%	54,7%		
Follow-up: ΔsCr exceeds +10%	32,8%	17,2%	11,19	0,037
TLR-Day: EGFR ≥ 3 Class	18,8%	9,4%	6,33	0,012
Follow-up: ΔEGFR exceeds −15%	26,6%	15,6%	5,81	0,0159
Follow-up: ΔEGFR exceeds −10%	32,8%	18,2%		
Follow-up: EGFR stabile	43,8%	48,5%		
Follow-up: ΔEGFR exceeds +10%	23,4%	33,3%	9,47	0,0087

Table 3
TLR-Day comparison of continuous variables.

TLR-Day comparison (Mann-Whitney test)				
Parameter	Group	Median	Q25 - Q75	p =
All pts	CsA	1,29	1,19 to 1,76	
sCr (mg/dL)	Tac	1,53	1,34 to 2,23	0,038
All pts	CsA	0,19,860	−1,17 to 0,81	
TLR4-ex	Tac	0,538	0,65 to 1,55	NS
All pts	DGF: No	0,53	−0,65 to 1,71	
TLR4-ex	DGF: Yes	−0,103	−1,3 to 0,67	0,025
All pts	Low-TLR4-ex	8,0	4 to 13	
DGF: YES (days)	High-TLR4ex	7,5	3,5 to 12	NS
All pts	Low-TLR4-ex	15,9	12,73 to 22,75	
IS MMF (mg/kg)	High-TLR4ex	16,6	12,98 to 22,85	NS
All pts	Low-TLR4-ex	0,08	0,06 to-0,12	
IS Pred. (mg/kg)	High-TLR4ex	0,12	0,07 to 0,16	0,033
All pts	Low-TLR4-ex	144	94 to 217	
IS CsA (ng/mL)	High-TLR4ex	161,5	116 to 202	NS
All pts	Low-TLR4-ex	6,45	4,7 to 8,0	
IS Tac. (ng/mL)	High-TLR4ex	8,1	7,0 to 10,4	0,0023
All pts	Low-TLR4-ex	1,42	1,23 to 2075	
sCR (mg/dL)	High-TLR4ex	1,49	1,2 to 1,92	NS
CsA	Low-TLR4-ex	1,29	1,17 to 1,92	
sCR (mg/dL)	High-TLR4ex	1,69	1,3 to 2,09	NS
Tac.	Low-TLR4-ex	1,54	1,33 to 2,08	
sCR (mg/dL)	High-TLR4ex	1,35	1,17 to 1,88	0,047
All pts	Low-TLR4-ex	51	34 to 61	
EGFR (mL/min)	High-TLR4ex	47	38 to 58	NS
CsA	Low-TLR4-ex	52	42 to 63	
EGFR (mL/min)	High-TLR4ex	42,5	30 to 50	0,043
Tac.	Low-TLR4-ex	46	34 to 59	
EGFR (mL/min)	High-TLR4ex	52	42 to 61	0,1
All pts	Low-TLR4-ex	6,85	6,2 to 7,6	
Uric acid (mg/dL)	High-TLR4ex	6,75	5,7 to 7,7	NS
All pts	Low-TLR4-ex	68	64 to 74	
Total protein (g/L)	High-TLR4ex	65	59 to 70	0,014

square or Fishers exact tests (depending on the quantities of subgroups) were applied to categorical variables. Univariate or multivariate analyses were done using a uni- or multivariate regression respectively. *P* value < .05 was considered statistically significant, and < 0.1 marginally significant.

4. Results

The choice between tacrolimus (Tac) and cyclosporin A (CsA) was marginally related to the age of the recipient. Only patients treated with CsA had a negative correlation of calcineurin inhibitor concentration with a total cumulative dose of intravenous methylprednisolone. Strong correlations between KT-TLR and the concentrations of both main immunosuppressive drugs: cyclosporin A ($R = 0.77$, $p < .001$) and tacrolimus ($R = 0.67$; $p < .001$) were noticed. Therefore, for the analysis KT-TLR was considered to be a dependent factor. There was no difference in TLR4ex between patients treated with cyclosporin A or tacrolimus. However, the CsA population was marginally younger ($p = .066$), had lower current sCr (Table 3). A correlation between concentration of calcineurin inhibitor and TLR4ex was found only in the Tac-treated patients ($R = 0.314$, $p = .004$). TLR4ex correlations with various kidney function parameters (KFp) on the TLR day or with KFp at the end of the follow-up were roughly similar in patients with CsA and Tac: positive correlation coefficients for EGFR and negative for sCr. (data not shown). We found no correlation between TLR4ex and the donor's age. No correlation was found between the actual age of the recipient on the TLR-day and the dose per kilogram body weight or the concentration of both Tac and CsA. On the TLR-day L-TLR4ex had a higher proportion of patients treated with CsA and a lower one of those treated with Tac than in H-TLR4ex. Also among people treated with tacrolimus and classified as L-TLR4ex there was a greater proportion of renal recipients with prednisone doses per kilogram body weight below the median. For both drugs: CsA and Tac, in L-TLR4ex a higher proportion of patients had concentrations and doses of drugs per kilogram of body weight below the median for the general population (Table 2).

In case of All pts. on the TLR-Day L-TLR4ex and H-TLR4ex did not differ by distributions of continuous parameters (sCr, EGFR) of renal transplant function assessment (Table 3). Differences were seen in the qualitative analysis, which indicated that a higher proportion of patients from the L-TLR4ex group placed in the third and higher class of renal failure according to EGFR scale (Table 2). Prospective analysis including post follow-up sCr (F-up sCr) and EGFR (F-up EGFR) showed a marginal difference between L-TLR4ex and H-TLR4ex (Table 5). The numerical and proportional amplitude of changes in renal function parameters (ΔEGFR%/ΔsCr/ΔsCr%) was clearly less favorable for L-TLR4ex. In the L-TLR4ex group a greater proportion of patients experienced a change that exceeded a 10% or even 15% limit of decrease in EGFR or a respective increase in serum creatinine accompanied by the nearly equal inverse proportion of favorable changes in H-TLR4ex. The comparison between L-TLR4ex and H-TLR4ex did not show differences in biopsy results for most of the parameters included in the BANFF classification, except for vasculitis ("v") index. Both the positive "v" (v ≥ 1) and the diagnosis of chronic graft nephropathy were more frequent in the L-TLR4ex group than in the H-TLR4ex one (Table 3). With respect to the L-TLR4ex group, in All pts. and in the Tac the comparison showed no changes in graft function evaluated by sCr/eGFR between the KT-TLR and F-up points. In the CsA, an analogical comparison showed deterioration of the graft function. Similarly, in the H-TLR4ex group no changes in graft function were observed in the Tac group and there was an improvement in All pts. and CsA. The univariate regression analysis (Table 6) pointed that in all transplanted patients TLR4ex was affected by TLR-day recipient's age and a history of delayed graft function. Also current (on TLR-Day) TLR4ex influenced expected post follow-up sCr, EGFR and EGFR percentage change (Table 7). The regression analysis indicated that qualitative dividing parameter L-TLR4ex/H-TLR4ex contributed to the way of the follow-up EGFR change and marginally affected the qualitative assessment of F-up sCr or F-up EGFR change (a change that exceeds the 10% limit).

After narrowing the study group to tacrolimus-only population, both the uni- and multiple-regression analyses showed a statistically stronger association of the TLR-day renal function with TLR4ex. The one-way regression analysis revealed the importance of a set of clinical

Table 4
TLR-Day vs follow-up comparison of continuous variables.

TLR-Day vs Follow-up comparison											
Descriptive data								Sign test		Wilcoxon test	
Group	Parameter	L/H-TLRex:	Mean	Median	Q25	–	Q75	Z	p =	Z	p =
L-TLR4ex	sCr	TLR-Day	1,79	1,42	1,23	–	2,08	0,744	NS	1,51	NS
		Follow-up	2,35	1,53	1,21	–	2,27				
All Pts	eGFR	TLR-Day	47,4	51,0	34	–	61	0,75	NS	1,34	NS
		Follow-up	43,6	43	30	–	61				
H-TLR4ex	sCr	TLR-Day	1,60	1,49	1,2	–	1,91	2,92	0,004	2,22	0,026
		Follow-up	1,59	1,39	1,13	–	1,79				
All Pts	eGFR	TLR-Day	48,5	47,0	38,0	–	58,5	2,08	0,037	2,09	0,036
		Follow-up	50,4	49,5	41,5	–	61,5				
L-TLR4ex	sCr	TLR-Day	1,75	1,54	1,33	–	2,08	0,79	NS	0,53	NS
		Follow-up	2,22	1,4	1,32	–	2,11				
Tac	eGFR	TLR-Day	47,1	46,0	34,0	–	59,0	0,48	NS	0,26	NS
		Follow-up	45,1	46,5	34,5	–	58,5				
L-TLR4ex	sCr	TLR-Day	1,65	1,29	1,17	–	1,92	2,08	0,037	2,05	0,04
		Follow-up	2,3	1,58	1,15	–	2,15				
CsA	eGFR	TLR-Day	50,1	52,0	42,0	–	63	1,67	0,095	1,87	0,06
		Follow-up	43,7	45,0	29,0	–	61				
H-TLR4ex	sCr	TLR-Day	1,52	1,35	1,17	–	1,88	1,73	0,08	1,22	NS
		Follow-up	1,54	1,38	1,08	–	1,7				
Tac	eGFR	TLR-Day	52,3	52,0	42,0	–	63,0	0,81	NS	0,85	NS
		Follow-up	53,0	53,0	43,0	–	64,0				
H-TLR4ex	sCr	TLR-Day	1,76	1,69	1,3	–	2,09	2,18	0,029	1,75	0,08
		Follow-up	1,75	1,63	1,17	–	2,05				
CsA	eGFR	TLR-Day	41,8	42,5	30,0	–	50,0	2,01	0,044	2,07	0,038
		Follow-up	45,6	43,5	32,0	–	62,0				

parameters such as an occurrence of delayed renal graft function, total cumulative dose of intravenous steroids and co-expression of other TLRs (not shown) as factors influencing TLR4ex. In addition, the multiple regression analysis pointed a collective influence of serum creatinine, total protein, tacrolimus concentration and borderline significance of general body mass index (BMI). The regression analysis indicated a link between TLR4ex and post-follow-up continuous parameters (serum creatinine/EGFR and % EGFR change). The one-way regression analysis points that after the qualitative breakdown of L-TLR4ex/H-TLR4ex separation, the inclusion into L-TLR4ex influenced the transplanted kidney function at the end of the follow-up period (Table 6 and Table 7).

5. Discussion

We assumed that the interaction of the kidney transplant with its recipient is an important element shaping the PBMC function and expression of TLR4. The interaction between the transplanted kidney and PBMC is probably asymmetrical. In contrast to many other studies examining the association between different clinical parameters

Table 5
Follow-up comparison of continuous variables.

FOLLOW - UP comparison (Mann-Whitney test)				
Parameter	Group	Median	Q25–Q75	p =
All pts	Low-TLR4ex	1,48	1,19 to 2,22	
F.- up: sCR (mg/dL)	High-TLR4ex	1,4	1,13 to 1,8	0,057
Tac pts.:	Low-TLR4ex	1,4	1,32 to 2,11	
F.- up: sCR (mg/dL)	High-TLR4ex	1,38	1,08 to 1,7	0,049
CsA pts.:	Low-TLR4ex	1,58	1,15 to 2,15	
F.- up: sCR (mg/dL)	High-TLR4ex	1,63	1,17 to 2,05	NS
All pts	Low-TLR4ex	44,0	30,5 to 61,0	
F.- up: GFR (mL/min)	High-TLR4ex	49,5	41,5 to 61,5	NS
Tac pts.:	Low-TLR4ex	46,5	34,5 to 58,5	
F.- up: GFR (mL/min)	High-TLR4ex	53	43 to 64	0,059
CsA pts.:	Low-TLR4ex	45	32,0 to 62,0	
F.- up: GFR (mL/min)	High-TLR4ex	43,5	32,0 to 62,0	NS
All pts	High-TLR4ex	0,79	−8,56 to 15,58	
F.- up: ΔsCr %	High-TLR4ex	−4,56	−13,06 to 3,69	0,0096
All pts	Low-TLR4ex	−2,67	−14,34 to 9,37	
F.- up: ΔEGFR %	High-TLR4ex	4,91	−3,93 to 16,77	0,0085
All pts	F-up sCr: Δ > +15%	−0,45	−1,36 to 0,63	
TLR4-ex	F-up sCr: Δ < +15%	0,538	−0,634 to 1514	0,021
All pts	F-up sCr: Δ exceeds +10%	−0,08	−1360 to 0,712	
TLR4-ex	F -up sCr: Δ exceeds −10%	0,53	−0,57 to 1,10	0,056
All pts	F-up EGFR: Δ exceeds −15%	−0,299	−1,41 to 0,772	
TLR4-ex	F-up EGFR: Δ does not exceed −15%	0,530	−0,615 to 1398	0,043
All pts	F-up EGFR: Δ exceeds −10%	−0,10	−1304 to 0,772	
TLR4-ex	F-up EGFR: Δ exceeds +10%	0,53	−0,010 to 1192	0,026

Table 6
Regression analysis (univariate and multivariate method) of factors influencing TLR4 expression.

All patients			Regression parameters								Regression summary			
Model Depend. var.	Continuous or categorical predictor	Level of Effect	B	B SE	p =	β	β 95% CI				R2	MS Res.	F (df;df)	p =
Univariate	Intercept		−0,96	0,610	0,12									
TLR4ex	Recipient Age		0,025	0,012	0,0419	0,180	0,006	to	0,353		0,032	2,89	4,22 (1:126)	0,0419
Univariate	Intercept		0,096	0,172	0,557									
TLR4ex	History of DGF (Yes/No)	No	0,336	0,172	0,053	0,171	−0,002	to	0,34		0,029	2,92	3,79 (1:125)	0,053
Univariate	Intercept		0,241	0,208	0,25									
TLR4ex	History of DGF (Yes/No)	No	0,457	0,208	0,031	0239	0,022	to	0,457		0,057	3,07	4,81 (1:79)	0,031
Tacrolimus group														
Univariate	Intercept		1,65	0,506	0,0016									
TLR4ex	sCr conc.		−0,75	0,281	0,0095	−0,28	−0,50	to	−0,071		0,081	2,95	7,05 (1:80)	0,0095
Univariate	Intercept		0,288	0,192	0,13									
TLR4ex	Renal failure sCr class	IRF	0397	0,254	0,12	0,193	−0,05	to	0,44					
		RF	−0,93	0,291	0,002	−0,40	−0,64	to	−0,15		0,114	2,87	5,12 (1:79)	0,008
Univariate	Intercept		1,15	0,34	0,001									
TLR4ex	Methyl-pred. iv total dose		−0,54	0,195	0,006	−0,31	−0,53	to	−0,088		0,096	2,97	7,72 (1:72)	0,0069
Univariate	Intercept		−1,11	0,449	0,0157									
TLR4ex	Tacrolimus concentr.		0,188	0,049	0,0003	0,392	0,185	to	0,600		0,154	2,76	14,23 (1:78)	0,0003
	Intercept		5785	2070	0,007									
	Serum creatinine (sCr)		−0,81	0,311	0,011	−0,28	−0,50	to	−0,06					
Multivariate	Total protein		−0,07	0,026	0,004	−0,38	−0,56	to	−0,11					
TLR4ex	Tacrolimus concentration		0,120	0,050	0,019	0,260	0,045	to	0,475					
	BMI Low/Nor/Overw/Obese	Low	−1,39	0,430	0,002	−0,38	−0,61	to	−0,145					
		Norm	0,363	0,298	0,228	0,134	−0,07	to	0,354					
		Overw.	0,664	0,350	0,063	0,220	−0,01	to	0,453		0,448	2,11	7,18 (6:53)	0,00001

Methyl-pred. = Methyl-prednisolone; iv = intravenous.

describing renal transplant patients, in our study we did not find a link between TLR4 expression and the age of the transplant donor. Our study involved peripheral blood monocytes of the recipient. We found a poor PBMC TLR4ex dependence on the TLR-day age of the recipient [14–16]. Low contribution of the recipient's age to TLR4 expression is due to the relative weakness of this factor compared to other assessed parameters. Delayed graft function in renal transplant recipients occurs in approximately 50% of cases. The emergence of DGF makes it difficult to diagnose acute rejection and to select appropriate immunosuppression, as well as it adversely affects the long-term survival of the transplanted kidney [10,17]. DGF can be predicted by observing TLR4 PBMC expression 24 h after renal transplantation. DGF is not just an irrelevant episode in the post-transplant period. We found that over a period of months the overall expression of PBMC4 TLR4 was reduced in patients who experienced DGF [4]. We proved this assumption to be correct stating that among renal recipients the prevalence of PBMC L-TLR4ex is significantly higher for those who experienced DGF. If DGF had already occurred there was no difference in length of DGF between L-TLR4ex and H-TLR4ex group.

In clinical practice the choice of IS regimens (e.g., use of Tac or CsA) results from the estimation of potential immunological reactivity and considers many parameters, including transplant history, age, course of therapy, presence of antibodies to the donor's antigens/epitopes and their mean fluorescence intensity (MFI) [18]. Immunosuppressive treatment guidelines recommend a gradual reduction in IS after transplantation [19,20]. Calcineurin inhibitors reduce the internalization of the TLR4-MD2-CD14 complex induced by ligand attachment, resulting in increased overall TLR4 expression [21], i.e. in renal epithelial cells [22]. We found no difference in the distribution of TLR4ex in PBMC between Tac and CsA groups but both the correlation analysis and the

Table 7
Regression analysis (univariate method) of factors influenced by TLR4 expression.

All patients			Regression parameters								Regression summary			
Model Depend. var.	Categorical or Continuous predictor	level of Effect	B	B SE	p =	β	β 95% CI				R2	MS R	F (df;df)	p =
Univariate	Intercept		1997	0,138	0,0000									
F.-up: sCr	TLR4ex		−0,175	0,08	0,0297	−0,192	−0,365	to	−0,019		0,037	2,41	4,83 (1:126)	0,0297
Univariate	Intercept		46,7	1,67	0,0000									
F.-up: EGFR	TLR4ex		3,82	1,31	0,0355	0,186	0,012	to	0,359		0,063	350,8	4,51 (1:126)	0,0355
Univariate	Intercept		−2,04	2,28	0,37									
F.-up: ΔEGFR%	TLR4ex		3,81	1,31	0,0043	0,25	0,079	to	0,421		0,063	650,5	8,43 (1:126)	0,0043
Univariate	Intercept		1936	0,137	0,001									
F.-up: sCr	L-TLR4ex (No)/H-TLR4ex (Yes)	No	0,326	0,137	0,018	0,207	0,035	to	0,379		0,043	2,39	5,67 (1:126)	0,018
Univariate	Intercept		0,257	0,114	0,026									
F.-up: ΔsCr	L-TLR4ex (No)/H-TLR4ex (Yes)	No	0,260	0,114	0,024	0,198	0,025	to	0,371		0,039	1,67	5,16 (1:126)	0,024
Univariate	Intercept		−0,74	2258	0,74									
F.-up: ΔEGFR%	L-TLR4ex (No)/H-TLR4ex (Yes)	No	−6,50	2258	0,004	−0,248	−0,42	to	0,077		0,061	651,2	8,30 (1:126)	0,004
Tacrolimus group														
Univariate	Intercept		1709	0,074	0,0000									
TLR-Day: sCr	TLR4ex		−0,108	0,040	0,0095	−0,284	−0,498	to	−0,071		0,081	0,427	7,05 (1:80)	0,0095
Univariate	Intercept		1,88	0,174	0,0000									
F.-up: sCr	TLR4ex		0,33	0,096	0,0446	−0,222	−0,439	to	−0,006		0,049	2,37	4,17 (1:80)	0,0446
Univariate	Intercept		47,34	2,07	0,0000									
F.-up: EGFR	TLR4ex		3069	1,14	0,0086	0,288	0,075	to	0,501		0,082	335,3	7,23 (1:80)	0,0086

univariate regression analysis for tacrolimus only show a significant stimulatory effect of immunosuppressive intensity (concentration) on TLR4ex in spite of twice higher relative range of doses for CsA compared with Tac in the evaluated patient groups. This indirectly suggests the susceptibility of TLR4 expression to the regulatory effect of immunosuppressive drug concentration (different for both drugs but more linear for Tac) [21,23]. In PBMC high concentration of CsA directly stimulates an expression of TLR2 and triggers a release of exosomes containing putative endogenous ligand heat shock proteins (HSPs), i.e., HSP70 [24]. HSPs are ligands for TLR4. High release of HSP70 gives an opportunity to activate both primary and adaptive immune responses. In low to moderate concentrations of HSP70, for example, in mild ischemic reperfusion injury, it plays a protective role against stimulated PBMC [25].

In addition to the immunosuppressive properties that are shared with Tac, high concentrations of CsA induce a nuclear-cytoplasmic translocation and facilitate the release of high-mobility group box 1 (HMGB1), whose nephrotoxicity is partly dependent on the TLR4 signaling pathway. A high concentration of CsA in the cytoplasm induces the stress of the endoplasmic reticulum, which releases the unfolded protein response (UPR). In macrophages UPR promotes inflammatory cytokines following TLR4 activation [22]. CsA also has a non-linear, concentration-dependent vasoconstrictive effect that is responsible for the nephrotoxicity of the drug [19]. The correlation analysis reveals that the relationship between TLR4 expression and parameters of renal efficiency is inverse in the CsA and Tac groups. Positive correlation between sCr and TLR4ex in the CsA group may indicate a disproportionate effect of CsA-dependent toxicity and secondary inflammation (both described for high concentrations of CsA) compared to its immunosuppressive effects [24]. In the case of Tac, the immunosuppressive effect appears to be more dominant and within the range of therapeutic concentrations is closer to the linear one. This leads to the conclusion that in the case of PBMC, appropriately selected immunosuppression effectively uses diverse inhibition of transmission from many receptors, increases TLR4ex and, at the same time, minimizes the adverse effects of calcineurin inhibitors [1,21,23]. According to our results, the higher Tac concentrations, the greater occurrence of high expression TLR4 in PBMCs. If the immunosuppressive effect is insufficient, neutrophils and monocytes that express TLR4 are usually attracted to inflammatory sites [1]. In this way the proportion of cells with low expression of TLR4 and Myeloid differentiation primary response 88 (MyD88) may rise among monocytes that remained in circulation [10]. This observation led us to treat patients with low (L-TLR4ex) or high (H-TLR4ex) overall TLR4 PBMC expression as patients with important pathophysiologic differences. In the L-TLR4ex group there was a higher proportion of patients treated with CsA, while for both CsA and Tac a higher proportion of L-TLR4ex patients had concentrations and doses of drugs per kilogram of body weight below the median for the general population. Thus, to put it simply, on TLR4-day the effect of immunosuppression on PBMC in L-TLR4ex was lower than in H-TLR4ex. Higher TLR4ex may be an indirect effect of the inhibition of its internalization induced by the action of a calcineurin inhibitor and the shift of the phenotypic proportions of cells towards the anti-inflammatory phenotype [21].

Experimental and clinical studies have shown that PBMCs/macrophages are actively involved in acute rejection [26]. But in the experimental model of early rejection after transplantation in biopsy specimens, most CD11b + and dendritic cells (DC) cells exhibited donor mean histocompatibility complex 2 (MHC2), but not recipient one. Most DC are formed from precursors that reside in the kidney, whereas the few DC from the recipient precursors are recruited from the blood upon local up-regulation of, e.g. macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) [27,28]. In the period between renal transplant surgery and TLR-day between L-TLR4ex and H-TLR4ex there was no significant difference in the occurrence of histologically confirmed acute rejection episodes. This partly explains why acute rejection experienced by the transplant recipient is not seen as a more permanent change in TLR4ex of PBMC expression on TLR-day. In our material all renal biopsies were done before TLR-day. A significant proportion of the biopsies were performed in the post-transplantation period, where the incidence of full-blown acute rejections is already reduced. We found that both positive BANFF intimal arteritis score and histological diagnosis of chronic graft nephropathy were more frequent in the L-TLR4ex group. It is difficult to resolve whether monocytes are the cause of an inflammatory reaction that damages the arterioles or are attracted to the vascular wall following its antibody dependent damage. Both stressed or damaged epithelial and endothelial cells [12] and steady, moderate secretion of PAMPs/DAMPs (i.e., HMGB1, hyaluronan, uric acid – ligands of TLR4) by the chronically damaged kidney attract new monocytes secreting proinflammatory cytokines [1] and chemokines (e.g., MCP-1, tumor growth factor β- TGFβ) [12]. An accumulation of patrolling monocytes to endothelia depends on the dominating stimulation of particular TLR type, including TLR4 [26]. In chronic allograft injury monocytes pass from the blood through the endothelium to the interstitial space of the kidney with intensity depending on the type of graft pathology and the time from the kidney transplant [1,4]. The donor/recipient ratio of monocytes (macrophages) in the transplant interstitium changes slowly along the time after transplantation [27,28]. This means that there is a dynamic relationship between these two populations associated with the immunogenicity of the transplant and the immunological response of the recipient [4]. In the kidney monocytes can be transformed into macrophages or DC [29]. Stimulation of TLR2 and TLR4 with HMGB1 and hyaluronan fragments promotes the maturation of DC [30]. Functional deficiency of TLR4 in the recipient (e.g., inactivating polymorphism) reduces the abundance of tissue macrophages, DC and primary T cells, leading to reduced infiltration of fibroblasts. In this way, TLR4 inhibition can slow down fibrosis [12,31]. This indicates the participation of TLR4-dependent signaling cascade of both types of monocytes/macrophages residing in the kidney in the development of chronic damage [2,9].

Depending on the quantitative or qualitative classification criteria, we found the bi-directional relationship between PBMC TLR4ex and the transplanted kidney function. Our results do not allow to evaluate its symmetry. Initially, on the TLR-day on which TLR4-ex was evaluated, there was no difference between the L-TLR4ex and H-TLR4ex populations in terms of current renal graft function (sCr and EGFR), but a greater proportion of patients with renal failure (sCr $>$ 2 mg/dL) was found in the L-TLR4ex group. This association becomes clearer after reducing the study population to only those treated with tacrolimus due to its less nephrotoxic properties compared to cyclosporin A. The regression analyses (several uni- and one multivariable) reinforce this hypothesis, suggesting the overall effect of kidney efficiency on TLR4ex. In our data it is reflected in continuous parameters by sCr, uric acid and total protein levels. In qualitative parameters, this is reflected by being included in the group of renal failure (RF, sCr $>$ 2 mg/dL) or not lower than 3-rd EGFR level. The single- and multivariate regression analysis, in addition to Tac and sCr/EGFR, indicated two metabolic parameters: total protein and low BMI as influencing TLR4ex [32,33]. The distinction of total protein may mean, for example, indirect evidence of the effect of acute phase proteins or immunoglobulins [34]. Poor kidney function - low EGFR $<$ 45 mL/min (and its metabolic complications related to protein malnutrition) can lead to acquired immunodeficiency, increasing the risk of late, low level viral replication, rising overall immunogenicity and accelerating the progression of chronic graft damage [35].

We also found evidence of a link between current TLR4ex and transplanted kidney function in the future. In All pts., between the TLR-day and F-up points, in the L-TLR4ex and H-TLR4ex groups the opposite direction of graft function changes (gains significance only in H-TLR4ex) is visible. The results show also the stabilizing effect of two factors on renal function: treatment with tacrolimus (sCr and eGFR) and

high TLR4 expression. Tacrolimus freezes - stabilizes creatinine levels in L-TLR4ex and in H-LTR4ex (Tables 4,5). The CsA group is not subject to the action of tacrolimus and the CsA concentration differences between L-TLR4ex and H-TLR4ex do not reach statistical significance. Among CsA + L-TLR4ex patients the results of the comparisons show a tendency to deteriorate the kidney function. In the CsA + H-TLR4ex group there is a tendency to improve the kidney function. We emphasize that this shows shifts in opposite directions. We also think that the TLR4ex median we calculated (different for all pts., Tac and CsA) is rather close to the hypothetical point of return between the tendency to deteriorate or to improve the function of graphene, rather than designate it. Excessive reduction in immunosuppression intensity results in a decrease in the mean TLR4 PBMC expression and future deterioration in renal function. Our data show that the baseline TLR4ex was lower in patients with a future deterioration of the graft function [10]. In CAI, depending on the degree and duration of the injury, the kidney transplant releases DAMPs favoring PBMC recruitment and graft infiltration [1]. There is a balance between infiltrating cells and those that remain in peripheral blood [4]. Transplant infiltrating monocytes are considered to be an important element of CAI pathophysiology [12] demonstrating the functional association of the expression of its own TLR4 with chronic endothelial dysfunction [6,10]. The overall analysis indicates that the improvement in renal transplant function assessed by various parameters was associated with TLR4ex already higher on TLR-day.

6. Summary

Our study indicates that adverse events of early post-transplantation period have long-term effects on the recipient's immunity, including the averaged PBMC TLR4 expression. The prevalence of L-TLR4ex is higher for those with the history of DGF. Tacrolimus in the range of therapeutic concentrations has only approximately linear, concentration-dependent and strong stabilizing effect on TLR4ex. Appropriately selected type (i.e., tacrolimus-based) and dose of IS suppresses the mechanisms that reduce the PBMC TLR4ex [1,23]. Both factors are associated with an improvement in the graft function [4]. L-TLR4ex group indicates higher frequency of both positive BANFF intimal arteritis score and histological diagnosis of chronic graft nephropathy. There is a multidirectional relationship between PBMC TLR4ex, the concentration of calcineurin inhibitor and the transplanted kidney function [10]. Excessive reduction in immunosuppression intensity results in a decrease in the mean TLR4 PBMC expression and future deterioration in renal function. Evaluation of the averaged TLR4ex can be used to assess the efficacy of immunosuppression, including personalized verification of recommended concentration, especially in the treatment with tacrolimus and to estimate the likelihood of deterioration in renal function.

Limitations of the study:

The results may be influenced by some heterogeneity in the clinical practice of CsA treatment compared with Tac. Only in the CsA group did we find a negative correlation between the cumulative intravenous dose of methylprednisolone and the concentration of the calcineurin inhibitor. Clinical practice indicates that intravenous glucocorticoids are usually used early after renal transplantation. A high cumulative dose of methyl prednisolone indicates prolonged therapy due to DGF or possible acute rejection. We had no data on serum MMF concentration. The factor interfering with the clarity of the conclusions is the unexplored degree of non-linearity of the effect of changes in CsA/Tac concentrations on PBMC metabolism.

Conflict of interest and funding

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