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Down-regulation of inflammatory signaling pathways despite up-regulation of Toll-like receptors; the effects of corticosteroid therapy in brain-dead kidney donors, a double-blind, randomized, controlled trial

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ABSTRACT

Background: The brain death of a potential organ donor induces a systemic inflammatory response, resulting in inferior organ quality and function. Our study aimed to evaluate the effects of methylprednisolone (MPN) therapy on pattern recognition receptor (PRR) signaling in potential brain-dead (BD) kidney donors.

Material and methods: To evaluate the effects of MPN therapy on PRR signaling in BD kidney donors we performed a prospective randomized treatment-versus-control study. Fifty-one potential kidney donors were randomly divided into three groups: brain-dead donors (BDDs) who received 15 mg/kg/d of methylprednisolone (group T1, n = 17), BDDs who received 15 mg/kg/d of MPN at the time of filling consent for kidney donation and 100 mg/2 h until kidney harvest (group T2, n = 17), and normal donors as controls n = 17. Gene expression for Toll-like receptors (TLRs) 1–9 and their signaling pathway molecules including MYD88, TRIF, NF-KB1, IRAK, IRF3, and IRF7, as well as the inflammatory cytokines RANTES, IL-1β, TNF-α, IL-6, CXCL8, IL-18, IFN-α, and IFN-β was determined by PCR array. Due to the crucial role of TLRs 2 and 4 in pattern recognition, surface expression of these molecules was analyzed by flow cytometry. Plasma levels of inflammatory cytokines were measured by immunoassay. Finally, serum creatinine and cystatin C were measured in 100 kidney recipients one week and one, three, and six months after transplant.

Result: Polymerase chain reaction (PCR) array gene expression revealed greater expression of TLRs and signaling molecules in group T1 than in the controls. Surface expression of TLRs 2 and 4 were significantly greater in group T2 than in group T1 ($P < .05$). Plasma concentrations of inflammatory cytokines were significantly greater in group T1 than in controls ($P < .05$). The recipients that received kidneys from group T1 had significantly higher levels of creatinine and cystatin C than the recipients of kidneys from both group T1 and controls ($P < 0.05$).

Conclusion: Administration of MPN to BDDs at specified periods until kidney harvest resulted in less systemic inflammation in the BDDs and improved renal function in kidney graft recipients compared with common MPN therapy.

1. Introduction

Previous studies have suggested the brain death of potential organ donors induces an inflammatory response that significantly affects graft quality and function (Floerchinger et al., 2012; Pratschke et al., 2001; Pratschke et al., 2000; Wilhelm et al., 2000). Numerous molecular signaling pathways are involved in the generation of systemic inflammatory responses to danger signals (Castellheim et al., 2009). Among these, signaling pathways downstream of activation of members of the pattern-recognition receptor (PRR) family, which includes toll-like receptors (TLRs), play key roles (Lee and Kim, 2007). To date, 10 TLRs (TLRs 1 − 10) have been identified (Fernandes et al., 2016). Toll-like receptors 3, 7, 8, and 9 reside in intracellular organelles, while TLRs 1, 2, 4, 5, 6, and 10 are localized to the plasma membrane (Shah

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et al., 2014). Recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by PRRs leads to activation of downstream signaling molecules and transcription factors, which upregulate expression of genes involved in inflammatory responses (Venegas and Heneka, 2017). Pattern-recognition receptor ligation leads to recruitment of signaling pathways by using adaptor molecules including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). These pathways activate nuclear factor kappa B (NF-kB) and interferon regulatory factors (IRFs) 3 and 7, and subsequent expression and production of CD80, CD86, various inflammatory immune mediators including tumor necrosis factor α (TNFα), interleukins (ILs) 6 and 8, macrophage migration inhibitory factor (MIF), and interferons (IFNs) (Hoesel and Schmid, 2013; Kawasaki and Kawai, 2014).

Ischemic brain damage in brain-dead donors (BDDs) induces the generation of free radicals of oxygen and nitrogen (Sanderson et al., 2013). These oxygen radicals cause biomolecular damage and the consequent increase of numerous DAMPs (Sangiuliano et al., 2014). The binding of damaged molecules, such as heat shock proteins (HSPs) and high mobility group box-1 (HMGB1), to PRRs induces dendritic cell maturation and innate immunity inflammatory responses2 (Tesniere et al., 2008; Škoberne et al., 2004). Many PRRs, such as TLRs 2, 3, 4, 7, and 9, and possibly others, recognize HMGB1 (Yanai et al., 2011; Apetoh et al., 2007; Tian et al., 2007; Vande Walle et al., 2011; Yu et al., 2006). Following ischemic insult, HMGB1 is released into the extracellular space and subsequently induces neuro-inflammation in the post-ischemic brain (Shukla et al., 2017). Ischemia-like conditions increase the levels of NLR family pyrin domain-containing (NLRP) 1 and 3, as well as IL-18 and IL-1β, in primary cortical neurons following induction of ischemic stroke in C57BL/6J mice (DY-W et al., 2013). After global cerebral ischemia in the hippocampus, NLRP3 inflammasome activation leads to an increase in proinflammatory cytokine production (Li et al., 2016). Ischemia reperfusion injury (IRI) may occur following ligation of TLRs 3, 7, and 9 by RNA and DNA in severely-damaged cells (Land, 2012). Signaling pathways mediated by TLR3 play a role in IRI and myocardial infarction (Lu et al., 2014); however, acute cerebral IRI in mice occurs in an independent TRIF-IRF3 signaling pathway manner (Hua et al., 2009). Gene expression of TNF-α, IL-6, IL-1β, and CCL2 was not significantly different between BDDs and normal healthy donors (Nijboer et al., 2004). Global gene expression assays in non-human primate BD kidney donors revealed up-regulation of JAK-STAT and apoptosis-promoting pathways as well as increased expression of TLRs and massive release of cytokines including G-CSF, IL-6, IL-9, IL-16, and MCP-1 immediately after transplantation compared to normal donors (Sperger et al., 2009). Furthermore, experimental genome-wide expression profiles for 20,550 genes suggested up-regulation of cytokines, chemokines, and adhesion molecules in BD kidney donors (Kusaka et al., 2007). A microarray gene expression study disclosed greater expression of JAK-STAT pathway signaling genes and cytokines IL-1β, MCP-1, ICAM-1, TGF-β, and TNF-α in deceased kidney donors than in normal donors (Guillén-Gómez et al., 2016). One study reported a negative correlation between renal function in kidney transplant recipients and increased IL-6 production in BDDs (Nijboer et al., 2009). The interaction between glucocorticoid and TLR signaling molecules resulted in direct repression of TLR-activated transcription factors and enhanced expression of the natural inhibitors of TLR pathways (Chinenov and Rogatsky, 2007). An increase in TLR2 expression was reported in human keratinocytes treated with glucocorticoids (Shibata et al., 2009). On the other hand, treatment with glucocorticoids led to decreased differentiation and antigen-presenting function of dendritic cells despite upregulation of TLRs (Rozkova et al., 2006). In a prospective randomized treatment study with brain-dead (BD) liver donors, plasma levels of ILs 2, 6, and 10, TNF-α, and MCP-1 were reduced after methylprednisolone (MPN) treatment (Kotsch et al., 2008).

Until recently, few gene expression studies in deceased donors focused on the effect of common MPN treatment on inflammatory genes or signaling pathways; therefore, more detailed evaluation of inflammatory factors in BDDs is needed. The present study aimed to determine the effects of different doses of MPN on PRRs and their associated signaling pathway molecules in BD kidney donors.

2. Material and methods

2.1. Patients

This randomized single-center study included 51 kidney donors: 17 BDDs who received common MPN therapy (15 mg/kg/d, T1 group), 17 BDDs who received MPN at 15 mg/kg/d after consent for kidney donation and an additional 100 mg/2 h until organ harvesting (T2 group), and 17 normal donors as controls. The subjects were enrolled over a 12-month period in the organ procurement area of Sina Hospital Organ Transplantation Research Center, Tehran University of Medical Sciences, Tehran, Iran. The study was performed under the guidance of the Ethics Committee for Human Studies at Sina General Hospital and Iranian Registry of Clinical Trials (IRCT2015071323192N1).

2.2. Donor's inclusion and exclusion criteria

Patients of either sex, aged 18–60 years, whose brain deaths were established according to standardized clinical criteria approved by the Ministry of Health and Medical Education of Iran as cadaver donors, and normal healthy related or unrelated donors who gave written informed consent were eligible to participate in the study. Negative HCG test for pregnancy was required for females of childbearing age. Patients were selected within 72–96 h post-brain death occurrence. Potential healthy control donors with human leukocyte antigen (HLA)-mismatched, cadaver donors with WBC cross match > 20%, and any potential donors with infections were excluded from the study (Table 1).

Table 1
Donor demographic characteristics The parameters used were age, gender, cause of death, and drug use.

Variable	[a]T 1 (n = 17)	[b]T 2 (n = 17)	Control (n = 17)
Gender (M/F)	13/4	12/5	14/3
Age (years ± SD)	34.5 ± 13.6	35 ± 8.7	28 ± 4
[1]Infection	NO	NO	NO
Cause of brain death			
Intracerebral hemorrhage (N, %)	3 (17.64%)	2 (11.76%)	0/17
Head trauma (N, %)	7 (41.17%)	8 (47.05%)	0/17
Subarachnoid hemorrhage (N, %)	2 (11.76%)	1 (5.88%)	0/17
Cardiac arrest (N, %)	3 (17.64%)	4 (23.52%)	0/17
Other (N, %)	2 (11.76%)	2 (11.76%)	0/17
Used drugs			
Pantoprazole	40 bd	40 bd	NO
Levothyroxine	N = 2TD	N = 2TD	NO
Methylprednisolone	15 mg/kg/d	15 mg/kg/d at first and 100 mg/2 h until organ recovery	NO
Vasopressin	1 mg/h	1 mg/h	NO
ICU/Hospitalization (h ± SD)	72.8 ± 19	74.5 ± 12	78 ± 12

Link text: No significant differences were found between groups T1 and T2 regarding donor age, gender, cause of brain death, or drugs administered during hospitalization.

[a] T1: treated with 15 mg/kg/d of methylprednisolone.

[b] T2: treated with 15 mg/kg/d and 100 mg/2 h from the time of consent for kidney donation until kidney harvest.

[1] Defined as infection of the donor requiring antibiotic treatment.

2.3. Recipient's inclusion and exclusion criteria

Potential recipients were excluded if they had previously received any transplanted organ. Those who had ABO incompatibility against the donor and positive WBC cross match or panel-reactive antibodies more than 50% before transplantation, tested positive for HIV or HBSAg, had uncontrolled diabetes mellitus, or any malignancies within the last 5 years, were also excluded.

2.4. Sample preparation

Peripheral blood samples were collected from donors immediately before organ harvesting. Following centrifugation, plasma samples were aliquoted into cryogenic vials and immediately stored at < −80 °C until analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples for flow cytometry and RNA extraction by Ficoll/Paque density gradient centrifugation.

2.5. RNA extraction from PBMCs

RNA was extracted using TRIzol (Lot 94402, Sigma Aldrich) according to the manufacturer's protocols. In brief, the cells were pelleted, washed in Hank's solution, and resuspended in TRIzol at a concentration of 5 × 10^6 cells/mL TRIzol. Two hundred ul of chloroform/mL of TRIzol was added and the solution was mixed vigorously before centrifuging at 12,000 × g for 10 min at 4 °C. The aqueous phase was then transferred to a fresh tube, mixed with 500 ul of isopropanol/mL of TRIzol, and maintained at −20 °C for 1 h. The samples were centrifuged at 12,000*g* for 10 min at 4 °C, after which the supernatant was discarded, and the RNA pellet was washed with 1 ml of 70% EtOH per1 ml of TRIzol. The samples were centrifuged at 7500 × g for 10 min at 4 °C, and the supernatant was removed. After air drying, the pellet was resuspended in 20 ul of molecular-grade water. Finally, the RNA samples were purified using the RNeasy mini kit (Cat No 74104 QIAGEN), according to the manufacturer's protocol.

2.6. First-strand cDNA synthesis and PCR array

Expression of TLRs 1, 2, 3, 4, 5, 6, 7, 8, and 9, NLRP3, MYD88, IRAK1, NF-kB1, NF-kB1A, caspase-1, TICAM1/TRIF, IRF7, IRF3, NOD1, NOD2, RIG-1, ICAM1, RANTES, IL-1β, TNF-α, IL-6, CXCL8, IL-18, IFN-α, IFN-β, CD80, and CD86 was evaluated by real-time PCR array.

Total RNA from each group was pooled and used for cDNA synthesis according to the RT2 First Strand Kit protocol (QIAGEN, Germany). In brief, 3 µl of control P2, 12 µl of 5 x buffer BC3, 6 µl of RE3 reverse transcriptase, and 9 µl of RNase-free water were combined for a final volume of 30 µl. Ten µl of RT mix was added to each tube containing 10 µl of genomic DNA elimination mix and incubated at 42 °C for 15 min, after which the reaction was stopped by immediately incubating at 95 °C for 5 min. A total of 166.5 µl of RNase-free water was added to each cDNA reaction, the reactions in triplicate were loaded onto RT2 Profiler 96-well PCR array plates (330231 PAHS-052ZA), and amplified using the Step One Plus Real-time PCR system (Applied Biosystems) for 35 cycles. GAPDH and β-actin were used as housekeeping controls. Furthermore, reverse transcriptases control (RTC) and a positive PCR control (PPC) were used according to the RT2 profiler PCR Array protocol. The results from threshold cycle values (Ct) were uploaded onto the SABioscience website (http://www.sabiosciences.com/pcr/arrayanalysis.php), and $2^{-\Delta\Delta Ct}$ was used to calculate relative gene expression.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of cytokines and chemokines were measured using TNF-α (Cat. No 430207, Biolegend), IL-1β (Cat. No 437007, Biolegend), IL-6 (Cat. No 430507, Biolegend), and CCL2 (Cat. No 438807, Biolegend) ELISA kits according to the manufacturer's instructions. Briefly, the samples and diluted standards were added to the wells and incubated at room temperature (RT, 25 °C) on a plate shaker for all incubation steps. The detection antibody solutions were added to the plates and incubated at RT. Avidin-HRP solution was added and incubated at RT. The substrate solution was added to the sample wells and plates were incubated in the dark. Stop solution was added and absorbances were read at 450 nm and a secondary wavelength on a MultisKan Spectrum, according to the manufacturer's instructions.

2.8. Flow cytometry

The surface expression of TLR2 and TLR4 on the monocytes was determined with anti-human TLR4-PE (eBioscience, Cat No 12-9917), anti-human TLR2-FITC (eBioscience, Cat No 11-9922), mouse IgG2a K isotype control-FITC (eBioscience, Cat. No 11-4724), and mouse IgG2a K isotype control-PE (eBioscience, Cat. No 12-4724) according to the following protocol: 5 µl of antibody solution was incubated with 95 µl of freshly isolated PBMCs in Hank's solution with 2% FBS for 1 h at 4 °C. The stained samples were washed three times with Hank's solution and sorted on a BD FACS Calibur. Data were analyzed using FlowJo software version 7.6.1.

2.9. Creatinine and cystatin C production

To evaluate of effects of MPN therapy on the donors we analyzed the filtration markers creatinine and cystatin C, which are associated with outcomes, in renal graft recipients at one week and one, three, and six months post-transplantation. We measured the serum concentrations of these proteins in graft recipients' sera using ELISA as described above.

2.10. Statistical analysis

Normally and non-normally –distributed data were analyzed using one-way ANOVA and Kruskal–Wallis tests, respectively. Data in the text, tables, and figures are reported as means ± SDs or means ± SEMs. All data were analyzed using Prism 7 software (GraphPad Software) and $P < 0.05$ was considered statistically significant. False discovery rate (FDR) was used for multi-comparison analysis (Figs. 1–4).

3. Results

3.1. Donor and recipient characteristics

3.1.1. Donor characteristics

No significant differences were found between groups T1 and T2 regarding donor age, gender, cause of brain death, or drugs administered during hospitalization (Table 1). The preservation times, including cold and warm ischemia times, were not significantly different between the groups (634 ± 121 min in T1, 630 ± 118 min in T2, and 601 ± 153 min in controls).

3.1.2. Recipient characteristics

No significant differences were seen with regard to gender, age, or status of end-stage renal disease (ESRD) in recipients who received kidneys from either BD or normal donors (Table 2). All recipients received Valganciclovir (450 mg), prednisolone (5–50 mg), Sandimmune (10–25 mg), Prograf (0.5–1 mg), rapamycin (1 mg), azathioprine (50 mg), and CellCept (250–500 mg).

3.2. PCR array data analysis

Profiler PCR Array data analysis identified significantly greater expression of TLRs 1, 2, 4, 5, 6, 7, and 8, and NLRP3, NOD1, and RIG-

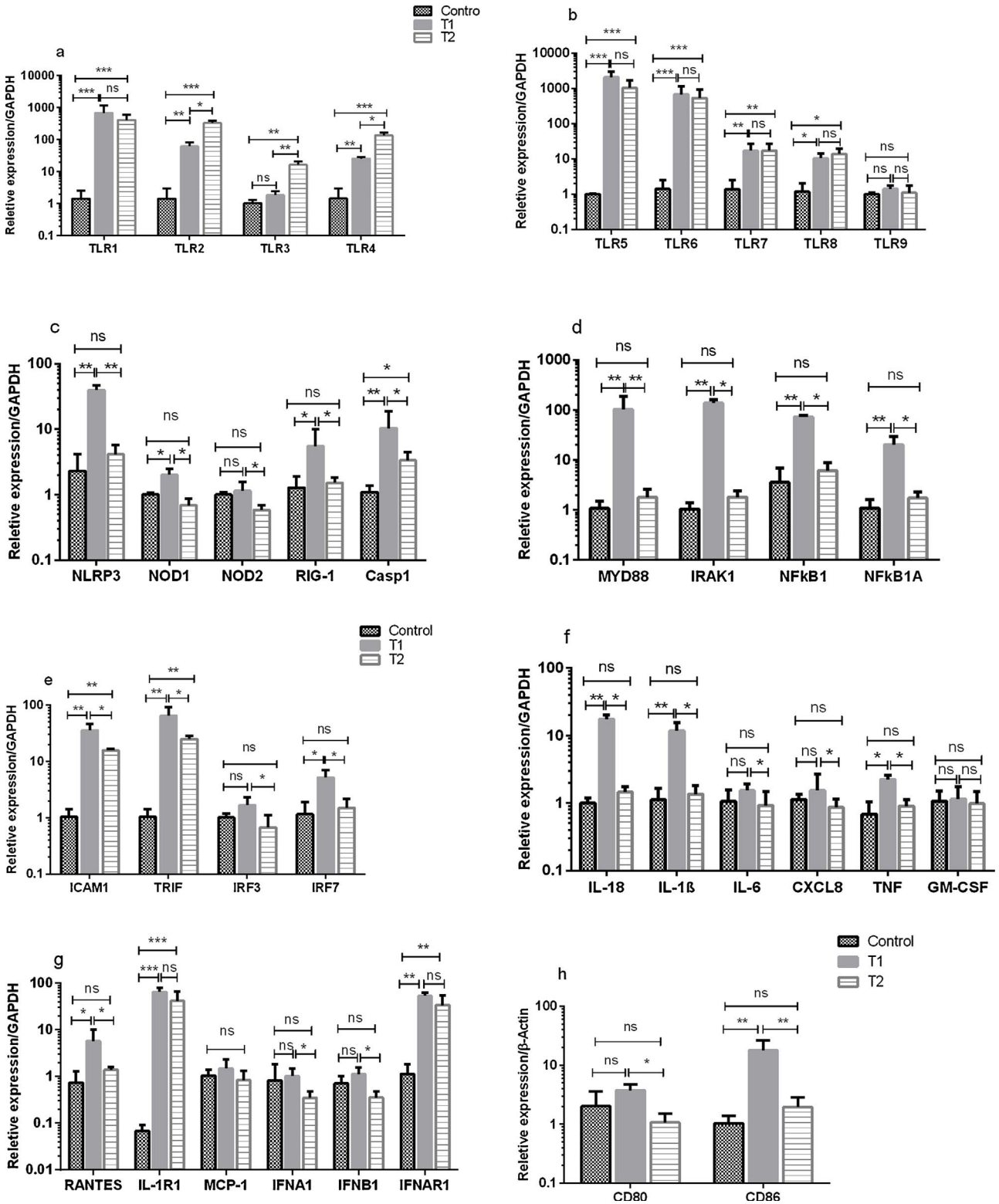

Fig. 1. Expression of pattern recognition receptors in the studied groups. Gene expression was evaluated using the RT2 Profiler PCR Array (Biosciences, QIAGEN). Expression is shown relative to GAPDH (a-g) or β-actin (h). Data are presented as means ± SEMs, and significant values are represented as * P < .05, ** P < .01 and *** P < .001.
Link Text: Profiler PCR Array data analysis identified significantly greater expression of TLRs 1, 2, 4, 5, 6, 7, and 8, and NLRP3, NOD1, and RIG-1,but not of TLRs 3 or 9, or NOD2 in group T1 than in controls (P < .05).

1,but not of TLRs 3 or 9, or NOD2 in group T1 than in controls ($P < 0.05$). In addition, expression of the downstream signaling pathway molecules MYD88, IRAK1, NF-kB1, NF-kB1A TRIF, caspase-1, TICAM1/TRIF, and IRF7 was significantly greater in group T1 than in controls (Fig. 1). However, expression of NLRP3, NOD1, NOD2, RIG-1, MYD88, NF-κB1, NF-κB1A,IRAK1, ICAM1, Caspase 1, RANTES, TRIF, IL-1β, TNF-α, IL-6, CXCL8, IL-18, IFN-α, IFN-β, CD80, and CD86 in was significantly less in group T2 than in group T1. Moreover, expression of IL-1β, IL-18, IL-1R1, IFNR1, RANTES, CD86 and TNF-α was greater in group T1 than in group T2. No significant differences in IL-8, IL-6, IFN-

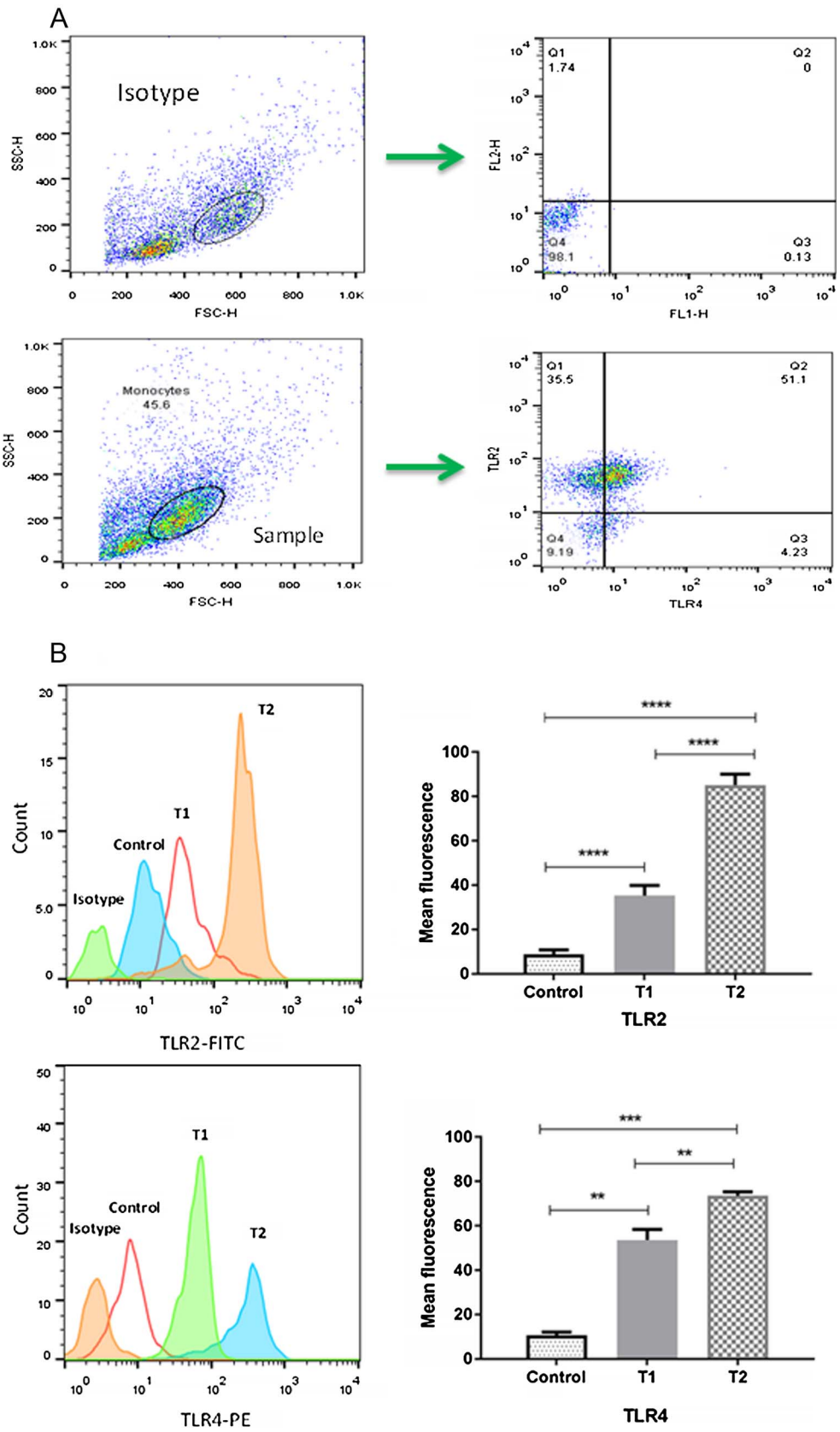

Fig. 2. Flow cytometry mean fluorescence intency (MFI) for surface TLR2 and TLR4 PBMC samples were stained with isotype control antibodies (Abs) or Abs to TLR2 (IgG2a) and TLR4 (IgG2a). Monocytes were separated by FSC/SSC gating (A) and MFI (B) was determined for surface expression of TLR2 and TLR4. Histograms represented the MFI of TLR2and TLR4 expression in the three studied groups. Data analysis showed significantly greater TLR2 and TLR4 expression on monocytes from groups T1 and T2 than from controls (c and d). Data are presented as means ± SEMs, and significant values are represented as *** $P < .001$, **** $p < .0001$.

Link Text: Expression of both TLR2 and TLR4 proteins was significantly greater in group T2 than in controls ($P < .0001$ and $P < .001$, respectively).

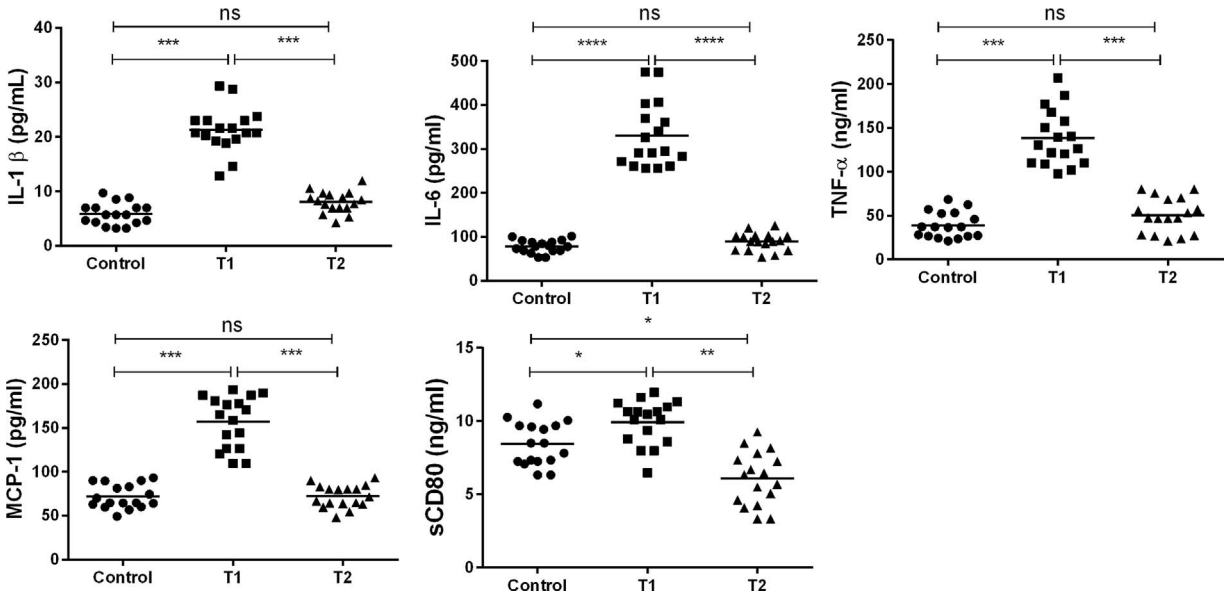

Fig. 3. Plasma levels of inflammatory mediators The immunoassays showed significantly greater concentrations of IL-1β, IL-6, TNF-α, MCP-1, and sCD80 in sera from group T1 than from sera from group T2 or control donors (Mann-Whitney test). Data are presented as means ± SEMs and significant values are represented as * $p < .05$, ** $P < .01$, ***$P < .001$ and **** $P < .0001$. Protein concentrations were determined by ELISA.

Link Text: Plasma samples from the three donor groups were analyzed by ELISA as described above. Protein expression of IL-1β, IL-6, TNF-*α,* MCP-1, and sCD80, was significantly greater in group T1 than in group T2 or controls ($P < .05$).

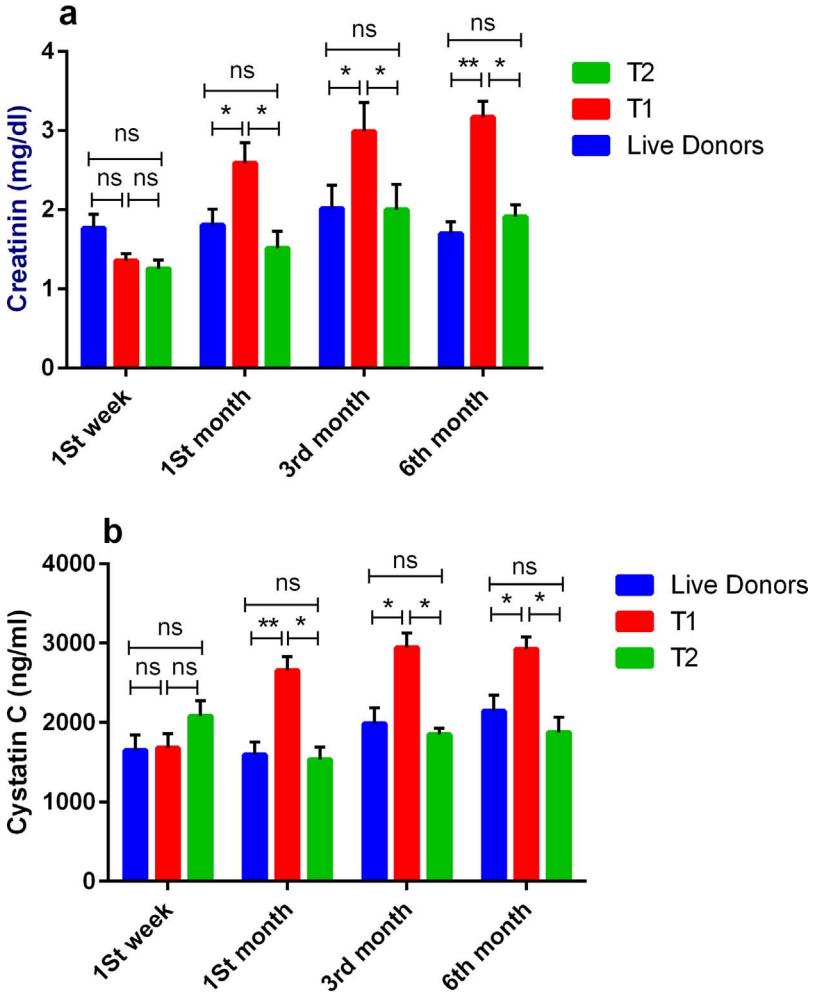

Fig. 4. Creatinine and cystatin C serum levels in recipients. Serum concentrations of creatinine and cystatin C were significantly greater in recipients who received grafts from group T1 than recipients who received grafts from group T2 or controls at one, three, and six months post-transplant ($P < .05$). Protein concentrations were determined by ELISA.

Link test: Serum creatinine and cystatin C in the three groups were measured at one week and one, three, and six months after transplantation. At one, three, and six months both proteins were expressed at significantly greater levels in the group T1 recipients than in the T2 or control recipients. ($P < .05$).

Table 2
Recipient demographic characteristics.

Variable	[a]T 1 (n = 34)	[b]T2 (n = 34)	[c]Control (n = 17)
Gender (M/F)	20/14	22/12	10/7
Age (years ± SD)	45 ± 11	48 ± 9	43 ± 16
Cold ischemia time (min)	520 ± 80	518 ± 90	490 ± 60
Etiology end-stage kidney disease			
Diabetes	7 (20.58%)	6 (17.64%)	6 (35.29%)
Hypertension	10 (29.41%)	11 (32.35%)	5 (29.41%)
[1]SLE	2 (5.88%)	3 (8.82%)	0
Kidney stone	3 (8.82%)	2 (5.88%)	2 (11.76%)
Congenital	3 (8.82%)	3 (8.82%)	0
Alport syndrome	2 (5.88%)	3 (8.82%)	0
Polycystic	2 (5.88%)	2 (5.88%)	1 (5.88%)
Unknown	2 (5.88%)	3 (8.82%)	1 (5.88%)
Other	3 (8.82%)	1 (2.94%)	2 (11.76%)

Link text: No significant differences were seen with regard to gender, age, or status of end-stage renal disease (ESRD) in recipients who received kidneys from either BD or normal donors.

[a] Recipients who received graft from group T1.

[b] Recipients who received graft from group T2.

[c] Recipients who received graft from healthy controls.

[1] Systemic lupus erythematosus.

α, IFN-β, or GM-CSF expression were seen between group T1 and controls

3.3. Increased surface expression of TLR2 and TLR4

Specific anti-human TLR antibodies were used for PBMC staining and analysis by FACSCalibur. Side- and forward-scatter flow cytometry profiles were used to gate monocytes according to TLR2 and TLR4 expression. Cell surface TLR expression was determined using mean fluorescence intensity (MFI). Expression of both TLR2 and TLR4 proteins was significantly greater in groups T1 and T2 than in controls ($P < 0.001$). Also we detected significantly greater TLR2 (c) and TLR4 (d) protein expression in group T2 than in group T1 (Fig. 2).

3.4. Decreased levels of soluble inflammatory mediators

Plasma samples from the three donor groups were analyzed by ELISA as described above (Fig. 3). Protein expression of IL-1β, IL-6, TNF-α (File1.CSV), sCD80 and MCP-1 (File2.CSV) was significantly greater in group T1 than in group T2 or controls ($P < .05$)

3.5. Creatinine and cystatin C serum levels in graft recipients

Serum creatinine and cystatin C in the three groups were measured at one week and one, three, and six months after transplantation. At one, three, and six months both proteins were expressed at significantly greater levels in the group T1 recipients than in the T2 or control recipients ($P < 0.05$). No differences were found for either protein between the T2 and control group recipients (Fig. 4).

4. Discussion

To our knowledge, this study is the first to evaluate the PRR signaling pathway in BDDs treated with varying doses of MPN. Also, this is the first study to report increased expression of TLRs 2, 3, and 4 following MPN therapy in BDDs. Because of limitations that we had in the clinical situation we were not allowed to apply placebo medicine or lowering the corticosteroids levels in a separate group of brain-dead donors. Therefore, we considered conventionally treated brain-dead donors and living donors as two reference groups and compered the results of interventional study groups with them.

The innate immune system defends itself from invading pathogens using a variety of PRRs (Kawai and Akira, 2010; Janeway and Medzhitov, 2002); it has been established that TLRs play a pivotal role in the activation of innate immunity by recognizing specific patterns of microbial components (Takeda and Akira, 2004).

An increase was reported in the levels of the NLRP inflammasome proteins IL-18 and IL-1β in cortical neurons in ischemia-like conditions (DY-W et al., 2013). The pro-inflammatory cytokines IL-1β and IL-18 are produced by caspase-1 activation following the detection of cellular stress molecules by NLRP3. Our study showed that not only were expression of NOD1, NLRP3, TLRs 1, 2, 4, 5, 6, 7, and 8, caspase-1, IL-18, IL-1β, and TNF-α significantly greater in group T1 than in controls, but also plasma levels of IL-1β, IL-6, TNF-α, MCP-1, and sCD80 were greater in group T1 than in controls. In addition, expression of these genes and plasma levels of the inflammatory cytokines were less in group T2 than in group T1. Therefore, treatment of BDDs with MPN before organ transplantation reduces both the mRNA expression and protein levels of inflammatory cytokines in PBMCs. It may do this by suppressing gene expression of these pro-inflammatory cytokines; alternatively, treatment with glucocorticoids, which include MPN, leads to decreased differentiation and antigen presentation by dendritic cells, which may inhibit inflammatory responses in cadaver donors (Rozkova et al., 2006).

Toll-like receptors 3, 7, and 9 may play roles in IRI by recognizing RNA and DNA in severely damaged cells (Land, 2012). The TLR3-dependent signaling pathway plays a role in IRI and myocardial infarction (Lu et al., 2014). However, Hua et al. reported that the TRIF-IRF3-dependent signaling pathway is not necessary for acute cerebral IRI in mice (Hua et al., 2009). Greater expression of the signaling genes JAK-STAT, TLRs, IL-6, and MCP-1 was seen in experimental non-human primate BD kidney donors than in normal donors after transplantation (Sperger et al., 2009), which agrees with our PCR array findings; albeit, we found no statistically significant differences between group T1 and controls in expression of TLRs 3 or 9 or IL-6. Likely IL-6 expression is more affected by MPN treatment than other inflammatory cytokines. HMBG1 mediates IRI via TRIF-adaptor-independent TLR4 (Yang et al., 2011). Our PCR array gene profile analysis showed that ischemic brain death may promote upregulation of TRIF and IRF7, but not IFNα and IFN-β, in group T1. In addition, we detected significantly greater expression of TLRs 2, 3, and 4 in group T2 group than in group T1. These findings agree with those of other studies (Chinenov and Rogatsky, 2007; Shibata et al., 2009; Rozkova et al., 2006), and show that despite an impairment in the TLR signaling pathways and reduction of inflammatory mediators, treatment with MPN resulted in increased expression of TLRs 2, 3, and 4.

mRNA expression of TNF-α, IL-6, IL-1β, and CCL2 was reportedly not significantly different between BD and normal donors (Nijboer et al., 2004); however, we found a significant increase in both mRNA expression and plasma levels of IL-1β and TNF-α in the T1 group. Despite a significant increase in CCL2 and IL-6 concentrations in the T1 group, no significant differences were seen between the three groups in relative gene expression of GM-CSF and a difference in IL-6 expression was only seen between groups T1 and T2. Microarray analysis in a previous study suggested a significant difference in expression of some signaling pathway proteins as well as some cytokines including IL-1β, MCP-1/CCL2, ICAM-1, TGF-β and TNF-α in BD kidney donors compared to healthy donors (Guillén-Gómez et al., 2016). Our study showed significantly greater expression and plasma levels of CD80 in group T1 than in groupT2 and greater plasma level than in controls, and greater CD86 expression in group T1 than in either T2 or controls. Methylprednisolone and other glucocorticoids used as anti-inflammatories and immunosuppressants have both positive and negative effects. Glucocorticoids induce the expression of complement system components, chemokines, cytokines, and TLRs. Moreover, simultaneous inhibition and stimulation have been demonstrated in inflammatory T helper subsets treated with glucocorticoids (GALON et al., 2002). In addition, glucocorticoids induce major histocompatibility complex class II co-stimulatory molecules and cytokines, including IL-1β, IL-6, and IL-

12 in tolerogenic dendritic cells (Zen et al., 2011). These findings represent potential new mechanisms by which glucocorticoids exert both beneficial and deleterious effects.

Several experimental and clinical studies have reported donor pretreatment with steroids had beneficial effects on renal graft function and survival2 (Pakferat et al., 2011; Almasi-Hashiani et al., 2011). Serum creatinine acts as a marker for renal function and glomerular filtration rate in kidney allograft recipients (Kim et al., 2006). Cystatin C is a filtration marker associated with inferior outcomes in renal graft recipients and may be superior to creatinine-based estimates in predicting outcomes (Okonkwo, 2017). Similar to these studies we detected higher levels of serum creatinine and cystatin C in the recipients who received grafts from the T1 group than in those who received grafts from normal donors. However, lower serum levels of creatinine and cystatin C were found in recipients who received grafts from BDDs treated with MPN at 100 mg/2 h than in those who received grafts from BDDs treated with common MPN therapy. Also our results indicated that recipients of kidney grafts from group T2 with decreased levels of IL-1β, IL-6, and TNF-α had lower levels of serum creatinine and cysteine and superior graft quality and function than recipients who received grafts from the other groups (data not shown). Moreover, fewer infections were seen in the recipients who received grafts from group 2 (6/34) and had lower levels of IL-1β, IL-6, and TNF-α than were seen in those who received grafts from the T1 group (11/34). Also, fewer recipients who received grafts from group T2 died than those who received grafts from the T1 group (1 vs. 4). These findings show that inhibition of innate alloimmunity in BDDs may lead to decreased inflammatory responses in the recipients resulting in improvement of kidney transplantation outcome.

5. Conclusion

Our findings suggest that activation of downstream signaling pathways of PRRs may generate a systemic inflammation response that is not significantly affected by conventional treatment with MPN. Therefore, MPN may undesirably increase the antigenicity of the organs prior to transplantation and negatively affect the quality and function of organs harvested from BDDs. Overall, our study indicates that treatment of BDDs with MPN at specified periods may improve graft function in kidney transplant recipients.

Disclosure

The authors declare that there are no conflicts of interest regarding this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.molimm.2017.12.012.

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