Impact of Toll-like receptor 2 expression in renal allograft rejection

Ute Hoffmann1,*, Tobias Bergler1,*, Munhie Rihm1, Claudia Pace1, Bernd Krüger2, Bettina Jung1, Stephan W. Reinhold1, Stefan Farkas3, Petra Rümmele4, Bernhard K. Krämer2 and Bernhard Banas1

1Department of Internal Medicine II, University Medical Center Regensburg, Regensburg, Germany, 2Department of Internal Medicine V, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68135 Mannheim, Germany, 3Department of Surgery, University Medical Center Regensburg, Regensburg, Germany and 4Department of Pathology, University Medical Center Regensburg, Regensburg, Germany

Correspondence and offprint requests to: Ute Hoffmann; E-mail: ute.hoffmann@klinik.uni-regensburg.de

*These authors contributed equally to this study.

Abstract

Background. An important role of TLR2 has been shown in various experimental models of renal ischaemia/reperfusion injury. To study the expression of TLR2 in renal allograft rejection systematically, we established an experimental rat transplantation model.

Methods. TLR2 expression was analysed in 99 human renal allograft biopsies, and in rat allografts at Day 6 and 28 after experimental renal transplantation. To discriminate whether regulation of TLR2 was following immunological processes after allogeneic transplantation or was a consequence from ischaemia/reperfusion injury, control animals subjected to syngeneic transplantation or to ischaemia/reperfusion damage were also investigated.

Results. TLR2 mRNA was significantly elevated in rat allografts with acute rejection on Day 6 and decreased spontaneously towards Day 28. TLR2 induction correlated with renal function and TLR2 excretion in the urine of transplanted rats. TLR2 staining was also significantly increased in human allografts with acute rejection. TLR2 protein could be localized in tubular epithelial cells and vascular endothelial cells, and in CD68- and CD4-positive infiltrating cells.

Conclusions. TLR2 is markedly up-regulated in both experimental and human acute renal allograft rejection. Our data suggest a role for TLR2 during allogen-dependent graft damage after renal transplantation.

Keywords: experimental rat model; human allograft biopsies; renal transplantation; TLR2

Introduction

Acute allograft rejection has long been thought to be mediated solely by components of the adaptive immune system. Thus current immunosuppressive drug therapies have been focused on addressing this arm of the immune response [1]. However, the innate immune response is now being recognized as important in initiating acute inflammatory responses to provide early defences to non-specific injury, such as ischaemia/reperfusion (I/R) damage prior to, during and after organ transplantation [2,3]. Toll-like receptors (TLR) have been identified to augment innate immune defence mechanisms and furthermore play an important role in initiation and modulation of adaptive immune responses, T-helper subset differentiation, and immune tolerance [4,5].

After stimulation of TLR with their specific exogenous as well as endogenous ligands, both immune and non-immune cells release inflammatory cytokines and chemokines [6–8], which are of main importance in allograft rejection.

TLR2 has been demonstrated to be induced in I/R models [9], whereas TLR2-deficient mice were protected from I/R injury [10]. However, the role of TLR2 in allograft rejection is still inconsistent. In a rat heart transplantation model [11], extensive expression of TLR2 protein was detected in heart allografts of untreated or only cyclosporine-treated animals, whereas in human renal allograft biopsies, TLR2 expression in tubuli was recently found to be associated with better kidney function [12].

As baseline characteristics, treatment modalities and comorbidity of patients in biopsy studies differ significantly, we established an experimental rat transplantation model with a 6-day and a 28-day protocol to study the TLR2 mRNA and protein expression in renal allografts under standardized conditions. Control animals allowed us to discriminate whether regulation of TLR2 was following immunological processes after allogeneic transplantation or was a consequence from ischaemia/reperfusion injury. These data were subsequently compared to a large number of human renal allograft biopsies with different types of allograft damage and correlated with clinical outcome. Double-labelling immunofluorescence was furthermore performed to define the TLR2-positive cell population.
TLR2 in experimental and human renal allograft rejection

Protocol biopsies 2 weeks/3 0, 7, 14, 21 and 28. The histopathological diagnoses according to the cages on Day 0, 3 and 6, and in the 28-day experimental protocol on Day 28 were evaluated. Tissues were fixed in paraffin or snap-frozen in N2, and stored at −80°C.

Animals/experimental renal transplantation

Animal experiments were performed following the German law of animal protection and the NIH’s principles of laboratory animal care. Male Brown Norway rats (BN) serving as donors and Lewis rats (LEW) serving as recipients (Charles River Laboratories, Sulzfeld, Germany) (200–250 g) were kept under a conventional housing and diet. The number and treatment regimen of the different groups are listed in Table 1.

Detailed protocols for performance of renal transplantation have been previously published by our group [13]. In brief, male BN rats served as donors, and LEW rats served as recipients. Left kidneys were explanted, flushed with cold saline and transplanted orthotopically in LEW recipients by end-to-end anastomosis of the vessels and, subsequently, the ureter. Recipient rats were sacrificed either 6 or 28 days after transplantation. To compare allograft-dependent damage (BN rats as donors and LEW rats as recipients) with allograft independent damage, changes after syngeneic transplantation (LEW rats as donors and LEW rats as recipients) as well as I/R were also studied in the 6-day protocol.

Detailed protocols for performance of renal transplantation have been previously published by our group [13]. In brief, male BN rats served as donors, and LEW rats served as recipients. Left kidneys were explanted, flushed with cold saline and transplanted orthotopically in LEW recipients by end-to-end anastomosis of the vessels and, subsequently, the ureter of the donor and recipient. Cold and warm ischaemia times were 35 and 30 min, respectively. Nephrectomy of the right kidney was performed at the end of surgery. In the I/R group, after removing the contralateral right kidney, the left kidney was mobilized, and the renal artery and vein were dissected carefully. Renal ischaemia was induced by placing straumatic microvascular clamps on both vessels for 30 min. Afterwards, the clamps were removed, and reperfusion was confirmed by macroscopic appearance. Before removing the clamp, 100 IU of heparin was administered to all patients for at least 3 months after transplantation, tacrolimus or cyclosporine A, and mycophenolate mofetil or mycophenolic acid. Human tissue was used following the guidelines of the Ethics Committee of the Medical Faculty of the University of Regensburg, Germany.

RNA isolation, reverse transcription and real-time PCR in rat specimens

After homogenization of frozen tissue sections in peqGOLD TriFast (Peqlab, Erlangen, Germany), total RNA was extracted and reverse transcribed into cDNA as described before. The sequences of the primers were GAPDH: 5′-gtcgttgtagcagttgc-3′ (forward), 5′-gactgacttgtcaccacctt-3′ (reverse), rTLR2: 5′-aagagggcagcaaaagag-3′(forward) and 5′-tgcaggggctccggc-3′ (reverse).

Quantification of TLR2 protein in urine

Twenty-four-hour urine samples of naive LEW rats (control, n = 6) and transplanted rats with cyclosporine (KTx + CsA, n = 6) and without immunosuppression (KTx, n = 6) on Day 3 and 6 were desalted using Amicon Ultra-4 centrifugal filter units (Millipore Corp., Billerica, MA, USA). On Day 28, control animals ± CsA (n = 6) were compared with KTx + CsA (n = 6). Protein concentrations were determined with the method of Pierce using BCA Protein Assay Kit (Thermo Scientific). The concentration of TLR2 was determined using the human TLR2 ELISA Kit (R&D Systems, Minneapolis, USA).

Table 1. Experimental renal transplantation model in rats: abbreviations, cohort sizes and renal function within the different groups

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Abbreviation</th>
<th>Number of rats, Day 6</th>
<th>Number of rats, Day 28</th>
<th>Serum creatinine, Day 6 (mg/dL ± SD)</th>
<th>Serum creatinine, Day 28 (mg/dL ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control without CsA</td>
<td>Control</td>
<td>7</td>
<td>7</td>
<td>0.34 ± 0.07</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Control with CsA</td>
<td>Control + CsA</td>
<td>7</td>
<td>12</td>
<td>0.31 ± 0.08</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>Unilateral nephrectomy without CsA</td>
<td>UNx</td>
<td>8</td>
<td>12</td>
<td>0.35 ± 0.07</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Unilateral nephrectomy with CsA</td>
<td>UNx + CsA</td>
<td>6</td>
<td>12</td>
<td>0.40 ± 0.06</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Ischaemia–reperfusion</td>
<td>I/R</td>
<td>6</td>
<td>0</td>
<td>0.29 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Syngeneic renal transplant</td>
<td>synKTx</td>
<td>7</td>
<td>0</td>
<td>0.48 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Allogeneic renal transplant without CsA</td>
<td>KTx</td>
<td>10</td>
<td>0</td>
<td>3.15 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>Allogeneic renal transplant with CsA</td>
<td>KTx + CsA</td>
<td>10</td>
<td>12</td>
<td>0.62 ± 0.21</td>
<td>0.37 ± 0.04</td>
</tr>
</tbody>
</table>

CsA, cyclosporine A.

Materials and methods

Patients/human renal allograft biopsies

In our centre, protocol biopsies are routinely performed 2 weeks and 3 months after transplantation. Additional indication biopsies are taken when allograft dysfunction is apparent. A total of 99 formalin-fixed, paraffin-embedded renal specimens were included in the analysis (Table 2). Each biopsy was graded according to the Banff 97 classification [14] by experienced pathologists. Clinical data related to the kidney biopsy samples were obtained from all renal transplant recipients of our centre including age and gender of the patient, time point of biopsy after transplantation, concentration of serum creatinine, and tacrolimus/cyclosporine trough levels on the day of biopsy. Nearly all renal transplant recipients were treated with triple immunosuppressive therapy including prednisolone, which was administered to all patients for at least 3 months after transplantation, tacrolimus or cyclosporine A, and mycophenolate mofetil or mycophenolic acid. Human tissue was used following the guidelines of the Ethics Committee of the Medical Faculty of the University of Regensburg, Germany.

Banff 97 classification [14] of the kidney specimens were made by experienced pathologists.

Table 2. Human renal allograft biopsies: patient demographics according to histopathological diagnosis

<table>
<thead>
<tr>
<th>Normal</th>
<th>Antibody-mediated acute rejection</th>
<th>Acute tubulointerstitial and vascular rejection</th>
<th>Chronic allograft lesions</th>
<th>Acute tubular necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of biopsies</td>
<td>20</td>
<td>8</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>16/4</td>
<td>2/6</td>
<td>16/8</td>
<td>19/1</td>
</tr>
<tr>
<td>Recipient age (years ± SD)</td>
<td>51 ± 14</td>
<td>57 ± 11</td>
<td>49 ± 16</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>Biopsies from deceased/living donors</td>
<td>15/5</td>
<td>6/2</td>
<td>17/7</td>
<td>17/3</td>
</tr>
<tr>
<td>Protocol biopsies 2 weeks/3 months after transplantation</td>
<td>20/0</td>
<td>6/0</td>
<td>9/5</td>
<td>5/1</td>
</tr>
<tr>
<td>Indication biopsies</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Time point of renal biopsy after transplantation (days ± SD)</td>
<td>14 ± 1</td>
<td>12 ± 4</td>
<td>43 ± 40</td>
<td>66 ± 60</td>
</tr>
</tbody>
</table>
Sigma reagents. Total protein (25 μg) was denatured (95°C for 5 min), subjected to 12% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany). Membranes were blocked and incubated with the chicken anti-rat TLR2 primary antibody (ProSci Incorporated, Poway, CA, USA) overnight at 4°C. Binding of the primary antibody was visualized with a peroxidase-conjugated goat anti-rat IgG-HRP secondary antibody (1:5000, Santa Cruz, Heidelberg, Germany) and enhanced detection system (ECL, Amersham-Bioscience, Buckinghamshire, UK). Membranes were then exposed to Hyperfilm (Amersham-Bioscience).

Afterwards, the nitrocellulose membranes were stripped (30 min at 60°C, stripping buffer: 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 7.6), washed and blocked with TBST containing 5% non-fat dry milk for 90 min before the detection was performed with a goat anti-β-actin antibody (1:1000, Santa Cruz Biotechnology; 1 h at room temperature) and a donkey anti-goat secondary antibody (1:2000, Santa Cruz; 1 h at room temperature) coupled to peroxidase and ECL.

**Immunohistochemistry**

Immunohistochemistry was performed on 3-μm formalin-fixed, paraffin-embedded sections as previously described [15]. A polyclonal chicken anti-human, anti-rat TLR2 antibody (ProSci Incorporated, Poway, CA, USA) was applied as primary antibody.

The slides were evaluated by light microscopy. In human biopsies, only areas in the cortex were analysed. Subcapsular infiltrates or unspecified staining at the edges of the biopsies was excluded from the analyses. In the remaining tissue, the positive TLR2 staining of tubules, glomeruli, vessels and interstitium was analysed in neighbouring high-power fields (original × 400, covering an area of 296 μm × 222 μm). In rat specimens, the TLR2 positive staining of tubules, glomeruli, vessels and interstitium was analysed in five neighbouring high-power fields of two different areas. The two areas were chosen in the middle of the cortex in the opposite poles and marked on the slides by a thin marker. For tubules, scores were attributed as percentage positive staining of tubular cross sections compared with all tubular cross sections in the cortex. For glomeruli, vessels and the interstitium, scores were attributed as percentage TLR2-positive cells compared with all cells within these compartments. Score 0 was attributed for positive staining <5% (‘basically no staining’), score 1 for 5–25% positive (‘weak’ staining), score 2 for 26–50% positive (‘moderate’ staining) and score 3 for >50% positive (‘strong’ staining). Mean values were calculated and used for comparison of the different entities.

**Immunofluorescence**

Double-labelling immunofluorescence was performed on selected human biopsies and in explanted human allografts to define the TLR2-positive cell population on formalin-fixed, paraffin-embedded materials as previously described [13,15]. The biotinylated secondary antibody of TLR2 was detected by streptavidin–Alexa Fluor 546 conjugate (Invitrogen, Karlsruhe, Germany). As marker for T cells, a CD4 antibody (polyclonal goat anti-human, R&D Systems, Minneapolis, USA) was used and detected by AlexaFluor 594 donkey anti-goat (Invitrogen), and a CD8 antibody (monoclonal mouse anti-human, Ventana, Illkirch, France) was used and detected by AlexaFluor 594 goat anti-mouse (Invitrogen). A CD68 antibody (monoclonal mouse anti-human, DAKO, Glostrup, Denmark) was marker for monocytes/macrophages, a CD79 antibody (monoclonal mouse anti-human, DAKO, Glostrup, Denmark) as marker for B cells, and a CD209/DC-SIGN antibody (monoclonal mouse anti-human, BD Biosciences, Franklin Lakes, USA) as marker for dendritic cells were used and detected by AlexaFluor 594 goat anti-mouse (Invitrogen).

**Statistical analysis**

Values are provided as mean ± SE. Statistical analysis was performed by the Student’s t-test, the non-parametric Mann–Whitney U-test and univariate ANOVA. P < 0.05 was considered to be statistically significant.

**Results**

**Experimental renal transplantation model in rats**

An allogeneic renal transplantation model in rats treated with and without cyclosporine with a test duration of 6 or 28 days was established. Cohort sizes, treatment regimens and renal function of the different groups are listed in Table 1. Allografts of all control groups showed normal histology. After allogeneic renal transplantation of rats without immunosuppression, the histopathological diagnosis of severe tubulointerstitial and vascular rejection was made at Day 6. In rats treated with cyclosporine, allografts had signs of tubulointerstitial rejection at Day 6. At Day 28, they had either normal histology or tubulointerstitial rejection.

**TLR2 mRNA expression.** Compared with control specimens, 6 days after allogeneic kidney transplantation, a significantly up-regulated TLR2 mRNA expression was seen in renal allografts of rats treated with (KTx + CsA, P = 6 × 10^{-14}) or without cyclosporine (KTx, P = 6 × 10^{-10}, Figure 1). TLR2 mRNA expression was significantly higher
in KTx than in KTx + CsA rats (P = 0.02). All other groups showed no significantly altered TLR2 expression compared with controls on Day 6, and the treatment with CsA also caused no difference in TLR2 mRNA expression. TLR2 mRNA expression of KTx + CsA rats was still significantly elevated on Day 28 compared with control specimens (P = 1 × 10^{-16}), whereas all other groups were not significantly different from baseline level. However, TLR2 expression of KTx + CsA rats on Day 28 was significantly lower than TLR2 expression of KTx + CsA rats on Day 6 (P = 6 × 10^{-6}).

**Immunohistochemistry for TLR2 in the different groups.**

The polyclonal TLR2 antibody used for immunohistochemistry resulted in a very reliable staining pattern with very low background (Figure 2). Negative controls replacing the primary antibody with irrelevant IgG did not demonstrate positive staining (Figure 2A). Mean staining scores ± SEM of the different groups are shown in Figure 3. In renal specimens of control groups (control), of the unilateral nephrectomized animals (UNx) and of the I/R group, focal weak TLR2 positive granular cytoplasmatic staining was found in proximal and distal tubuli (Figure 2B). Compared with control specimens, renal allografts without immunosuppression (KTx) demonstrated an up-regulation of TLR2 in tubuli on Day 6 (Figure 2C). A positive staining was furthermore observed in vessels and in infiltrating cells in this group (Figure 2D). In transplanted rats treated with cyclosporine (KTx + CsA), the same expression pattern was observed on Day 6 but with significantly lower interstitial expression due to less infiltrating cells (Figure 2E). Compared with KTx + CsA on Day 6, a still prominent tubular and vascular TLR2 positive staining could be detected in KTx + CsA on Day 28, whereas the amount of interstitial TLR2-positive cells was significantly lower, due to the smaller amount or the absence of infiltrating cells on Day 28 (Figure 2F).

![Fig. 2. Immunohistochemistry for TLR2 in the experimental rat transplantation model. Arrows indicate TLR2 positive staining. (A) Negative control. (B) In controls Day 6, focal weak TLR2 positive granular cytoplasmatic staining was found in proximal and distal tubules. (C) In KTx Day 6, an up-regulation of TLR2 in tubules and, furthermore, in infiltrating glomerular (C) and interstitial cells (D) could be detected. In KTx + CsA Day 6 (E) and Day 28 (F), an elevated TLR2-positive tubular expression was found.](https://academic.oup.com/ndt/article-abstract/26/3/1080/1834701)

![Fig. 3. Western blot analysis of TLR2 urinary protein on Day 3 (A) and Day 6 (B) of control rats without cyclosporine (ctrl) and transplanted rats + cyclosporine (KTx + CsA) (n = 6). (C) Western blot analysis of TLR2 urinary protein on Day 28 of control rats ± cyclosporine (ctrl ± CsA) and transplanted rats + cyclosporine (KTx + CsA). TLR2 expression is shown in comparison with actin (loading control). Representative results of three independently repeated experiments are shown.](https://academic.oup.com/ndt/article-abstract/26/3/1080/1834701)
Urine TLR2 protein. In comparison with untreated controls, we detected a marked increase of TLR2 protein in urine of transplanted rats already on Day 3 (Figure 3). Urinary TLR2 protein level of transplanted rats without immunosuppression (KTx) was markedly higher than in KTx + CsA. On Day 6, the differences between these three groups (control, KTx and KTx + CsA) were even more pronounced with the highest urinary TLR2 protein level in KTx rats. On Day 28, we compared control specimens with KTx + CsA rats and detected only a slight increase in urinary TLR2 protein level in KTx + CsA.

Correlation of TLR2 with renal function in the experimental protocol. To assess the role of TLR2 concerning the clinical outcome, we compared the mean mRNA expression of TLR2 with serum creatinine concentrations at the end of the experimental protocol on Day 6 or Day 28 after renal transplantation (Figure 4). The level of TLR2 mRNA was divided into three equal groups (Group 1: <1, Group 2: 1–1.4, and Group 3: >1.4×-fold renal TLR2 mRNA-expression). Enhanced mRNA levels were significantly associated with higher serum creatinine concentrations (Group 1: 0.31 mg/dL, Group 2: 0.39 mg/dL, and Group 3: 1.19 mg/dL).

Correlation of TLR2 with cyclosporine levels. Cyclosporine levels were divided into three nearly equal groups (<100 μg/L, 100–250 μg/L and >250 μg/L). There was no association between cyclosporine concentrations and TLR2 mRNA level or immunohistochemical staining scores.

Human renal allograft biopsies

The classification of the human renal allograft biopsies and the corresponding clinical information are summarized in Table 2.

Immunohistochemistry for TLR2 in different disease entities. Negative controls omitting the primary antibody did not demonstrate positive staining (Figure 5A). Renal allograft biopsies without signs of rejection showed a weak TLR2 positive staining in Bowman’s capsule, in proximal and distal tubuli, and in endothelial cells of vessels (Figure 5B).

In biopsies with acute antibody-mediated rejection (Figure 5C) with acute tubulointerstitial (Figure 5D and E) and with combined acute tubulointerstitial/vascular rejection, we observed an up-regulation of TLR2 in tubuli and in inflammatory interstitial cells compared with normal biopsies. In biopsies with chronic allograft lesions,
tubular, vascular and interstitial TLR2 staining scores were not higher compared with normal biopsies. In biopsies with acute tubular necrosis, tubular TLR2 positive staining was higher than in normal biopsies, almost comparable to biopsies with acute rejection (Figure 5F).

**Double immunofluorescence with subpopulations of infiltrating cells.** To further define the TLR2-positive cell population, selected human biopsies and explanted human allografts were used for double immunofluorescence with CD4 and CD8 as marker for T cells, CD79 as marker for B cells, CD68 as marker for monocytes/macrophages and CD209/DC-SIGN as marker for dendritic cells.

A significant number of TLR2-positive infiltrating cells were also CD68-positive (Figure 6A–C), whereas a smaller percentage of TLR2-positive cells were CD4-positive (Figure 6D–F). Neither CD8-, CD79- nor DC-SIGN-positive cells were TLR2-positive (data not shown).

**Correlation with therapeutic regimens.** TLR2 expression scores for the different renal compartments did not correlate with different therapeutic regimens, and neither with tacrolimus nor cyclosporine whole-blood trough levels.

**Correlation with donor data.** Donor characteristics as donor age, donor sex, cold ischaemia time, warm ischaemia time or number of mismatches did not influence the expression of TLR2.

**Discussion**

This is the first study demonstrating the time course of TLR2 expression after kidney transplantation under standardized conditions in an experimental rat model. These results were then compared to TLR2 expression in human renal allograft biopsies.

Compared with control rats, we detected an induction of TLR2 mRNA in transplanted rats with acute rejection on Day 6, which significantly decreased towards Day 28. TLR2 mRNA induction was only found after allogeneic transplantation and was most prominent in rats without CsA. These novel findings suggest a role for TLR2 in the early time course of acute rejection. The cellular localization of TLR2 in the different renal compartments was analysed in detail by immunohistochemistry. Our TLR2 positive staining data in uninjured renal tissue of control rats are in accordance with other studies [10,16,17] demonstrating only focal weak TLR2 positive granular cytoplasmatic staining in proximal and distal tubuli. In renal allografts with acute rejection on Day 6, increased TLR2 protein expression was found in tubuli, vessels and infiltrating cells.

A detailed immunohistochemical staining pattern of TLR2 in different compartments of human renal allograft biopsies with a wide range of pathological diagnoses has been described in our study for the first time.

The experimental protocol was also designed to discriminate whether induction of TLR2 is a consequence of I/R injury alone or associated with the alloimmune response. In our study set-up, a non-transplanted rat group underwent clamping of the renal artery lasting 30 min analogous to the cold ischaemia time of the allografts. TLR2 mRNA and protein expression within this I/R group 6 days later were not increased compared with naïve control rats. An ischaemia-reperfusion independent but alloimmune-mediated induction of TLR2 in the experimental rat model has therefore to be assumed. However, up-regulation of TLR2 mRNA and protein expression in tubuli of ischaemic rat and mice kidneys has previously been described.
In the set-up of these studies, examination of renal tissue was performed earlier after I/R, so that besides an increase of TLR2, histopathological signs of tubular damage as well as a decline in renal function were more pronounced. The different findings between these studies and our study might well be due to the almost normal renal function and the absence of histopathological signs of acute tubular damage in our experimental study. Interestingly, the tubular and interstitial TLR2 protein expression observed by immunohistochemistry in human biopsies with ATN was elevated and reached the same or an even higher level as was detected in human biopsies with acute allograft rejection. The explanation for this discrepancy between species might be once more that renal rat specimens 6 days after the I/R injury showed normal morphology and kidney function, whereas human renal biopsies had definite histomorphologic signs of ATN and markedly reduced kidney function.

In our study, tubular TLR2 staining scores were not associated with tacrolimus or cyclosporine whole-blood trough concentration levels, neither in the experimental model nor in human transplant recipients. TLR2 up-regulation seems thus not to be an effect of a potential calcineurin inhibitor-mediated tubular damage.

The role of TLR2 on different cell types is not yet entirely understood. Activation of TLR2 in tubular epithelial cells has been demonstrated to mediate induction of chemokines, which initiates phagocyte influx and immune activation during acute tubular necrosis [16,17,19], but which also plays a pivotal role for leucocyte recruitment in renal allograft rejection [20]. In response to prolonged ischaemia, the absence of TLR2 has been beneficial for reduced influx of leucocytes, reduced cytokine and chemokine induction, and thus avoidance of severe renal damage and dysfunction [18]. I/R injury in the early transplant period has been associated with late allograft failure [21]. The activation of TLR2, e.g. by ligands released during I/R injury and allograft transplantation, might be an important link between an immune response in the kidney and the subsequent damage of this organ.

A description of TLR2 expression on various subtypes of infiltrating cells in the kidney has not been reported before. In our study, TLR2-positive infiltrating cells could be identified as CD68-positive monocytes/macrophages and to a lesser degree as CD4-positive T cells in human renal specimens by double immunofluorescence. In tumour immunity, TLR2 has recently been shown to be capable of inhibiting regulatory T-cell function [8]. The presence of TLR2 on monocytes, which can differentiate into either macrophages or dendritic cells in inflamed tissue, is in accordance with previous reports [5] but has not been reported in kidney transplantation. Persistent macrophage infiltration, both in glomeruli or in the interstitium, is known to be profibrotic and thus is a risk factor for the development of chronic allograft nephropathy [22,23].

Further analysis of TLR2 induction with renal function demonstrated that higher TLR2 mRNA levels were associated with higher serum creatinine levels in the experimental protocol under standardized conditions. TLR2 was not only induced within the kidney but was also excreted in the urine of transplanted rats in correlation with the severity of allograft rejection and renal function. We conclude that TLR2 could contribute to mechanisms triggering the induction and severity of acute rejection episodes. Furthermore, we hypothesize that activation of innate immunity in kidney transplantation through TLR2 contributes to acute allograft rejection.

Innate immune responses activated not only by transplant antigens but also by antigen-independent factors including brain death and ischaemia/reperfusion initiate vascular damage and ultimately lead to acute rejection [9,24,25]. These innate responses can be suppressed only partially by the conventional combination treatment of corticosteroids, mycophenolate mofetil and calcineurin inhibitors [26], where significant vasculopathy, nephrotoxicity and chronic rejection are observed [3,27,28]. Thus, new therapeutic options aimed at diminishing innate immunity to prevent graft rejection are warranted [29,30]. TLR2 antisense oligonucleotide treatment reduced renal dysfunction after I/R injury in an experimental I/R model in mice compared with nonsense oligonucleotide treatment [18].

In summary, this is the first systematic description of the expression and detailed localization of TLR2 in both intrinsic and extrinsic cells in renal allograft rejection. Our data suggest an important role of TLR2 as part of the innate immunity in the early time course of acute rejection. Under strictly standardized conditions, our experimental rat transplantation model allowed us to demonstrate an I/R injury-independent up-regulation of TLR2 in renal allografts with acute rejection and, furthermore, an association of TLR2 expression with renal function.

Acknowledgements. We thank Mrs Stefanie Ellmann and Mrs Alexandra Wilhelm for their excellent technical assistance and Mrs Lydia Walkowski for the collection of patient data. This work was supported by the Else Kröner-Fresenius-Stiftung, the Regensburger Forschungsförderung in der Medizin (ReForM A/B-project) and the Deutsche Forschungsgemeinschaft (BA 2137).

Conflict of interest statement. None declared.

References

Conflict of interest statement. None declared.
T-cell phenotype in protocol renal biopsy from transplant recipients treated with belatacept-mediated co-stimulatory blockade

Philippe Grimbert1, Vincent Audard1, Carine Diet1, Marie Matignon1, Anne Plonquet2, Hicham Mansour1, Dominique Desvaux1, Antoine Durrbach3, José Laurent Cohen4,* and Philippe Lang1,*

1Department of Nephrology and Transplantation, Institut Mondor de Recherche Biomédicale (IMRB) INSERM U955, CHU Henri Mondor and Université Paris XII, Association pour l’Utilisation du Rein Artificiel (AURA), Créteil, France, 2Department of Immunobiology, CHU Henri Mondor and Université Paris XII, Créteil, France, 3Department of Nephrology and Transplantation, CHU Kremlin-Bicêtre and Paris XI University, Le Kremlin-Bicêtre, France and 4Centre d’Investigation Clinique-Biothérapies (CIC-BT), CHU Henri Mondor and Université Paris XII, Créteil, France

Correspondence and offprint requests to: Philippe Grimbert; E-mail: philippe.grimbert@hmn.aphp.fr

J.L.C. and P.L. contributed equally to this work.

Abstract

Background. Belatacept is thought to disrupt the interaction between CD80/86 and CD28, thus preventing T-cell activation by blocking the co-stimulatory second signal. However, the consequences on the T-cell profile in human renal transplant cases have not been determined.