Renal expression of Toll-like receptor 2 and 4: Dynamics in human allograft injury and comparison to rodents

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**A R T I C L E   I N F O**

Article history:
Received 31 October 2014
Accepted 3 November 2014
Available online 29 November 2014

Keywords:
- Toll-like receptor 2
- Toll-like receptor 4
- Kidney
- Transplantation
- Inflammation

**A B S T R A C T**

Activation of the innate immunity through Toll-like receptors (TLRs) has been postulated to play an important role in the pathophysiology of renal allograft dysfunction. TLR2 and TLR4 dynamics in different human post-transplant pathological entities has never been studied. Therefore, we evaluated pre- and post-transplantation protein expression of TLR2 and TLR4 in human kidney biopsies.

Human kidney biopsies obtained from living kidney donors and patients with acute tubular necrosis, acute cellular and vascular rejection and interstitial fibrosis/tubular atrophy (IF/TA) were used. Translating results from animal studies to the clinical situation is highly important considering the upcoming clinical studies with TLR inhibitors in human renal transplantation. Hence, the TLR2 and TLR4 expression in healthy mouse and rat kidneys was analyzed and compared with human kidneys. In healthy human kidneys, TLR2 is expressed on the endothelium and Bowman’s capsule, while TLR4 is expressed on the endothelium only. No tubular staining was found for both receptors in human kidneys. In contrast to human biopsies, TLR2 and TLR4 expression in rodents was observed on tubular epithelial cells. In all acute rejection human biopsies, increased infiltration of TLR4+ leukocytes was observed. In conclusion, a discrepancy exists between human and rodent renal TLR expression, which suggests careful attention when translating results from rodent studies to the human situation. Additionally, this study revealed human TLR2 and TLR4 expression dynamics in human biopsies pre- and post-transplantation.

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**1. Introduction**

Renal transplantation currently represents the preferred treatment for the majority of patients with end-stage renal disease (ESRD). The number of patients with ESRD has doubled during the last decade in Europe and the United States (Grassmann et al., 2005). Advancements in renal transplantation immunology has led to a success rate of >90% for the first year, a result of the decreased incidence of acute rejection (Kasiske et al., 2005).

Unfortunately, long-term renal allograft survival has not improved significantly over the past decade (Meier-Kriesche et al., 2004). Mechanisms responsible for long-term renal allograft loss are both immune and non-immune mediated (Nankivell and Kuypers, 2011). Early pre- and post-transplantation events such as donor brain death, tubular injury caused by ischemia-reperfusion injury (IRI) and episodes of acute rejection have an important impact on late chronic kidney injury and subsequent allograft dysfunction (Nankivell and Chapman, 2006). Recently, innate immune activation through Toll-like receptors (TLRs) has been demonstrated to be an important driver in the pathogenesis of renal IRI and acute rejection (Dessing et al., 2010; Kruger et al., 2009; McDaniel et al., 2013; Naensens et al., 2009). At this moment, 10 human and 12 murine TLRs have been identified and each receptor recognizes a multitude of pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPs) (Kawai and Akira, 2010; Sloane et al., 2010). Upon activation, TLR signaling causes release of cytokines and chemokines resulting in cell apoptosis,
bacterial death, activation of adaptive immunity and inflammation (Yamamoto and Takeda, 2010).

TLR2 and TLR4 have found to be expressed on renal tubular epithelial cells on RNA level (Tsboi et al., 2002) and after IRI in mice their expression is increased (Tsboi et al., 2002; Wolfs et al., 2002). Importantly, it was demonstrated that TLR2−/− or TLR4−/− mice are protected from renal IRI, supporting the hypothesis of a key role for both receptors in mediating IRI (Leemans et al., 2009; Wu et al., 2007). No such data is available for human IRI. It has not been fully established whether TLR expression in renal cells or TLR expression on infiltrating cells influences renal allograft injury during acute rejection. The expression pattern of TLR2 and TLR4 in normal human kidneys compared to IRI and acute rejection induced renal changes is unknown. With regards to the introduction of TLR inhibitors in human transplantation clinical studies to prevent IRI (Reilly et al., 2013), the abovementioned issues need to be addressed. Therefore, it is important to understand the expression pattern of TLR2 and TLR4 in healthy human and diseased conditions in humans as well as in rodents.

We hypothesize that TLRs play a role in renal allograft injury. To assess this, renal protein expression of TLR2 and TLR4 will be assessed in different patterns of renal injury after transplantation: acute tubular necrosis (ATN), acute allograft rejection (Banff grade Ia, Ib, Ila, Ilb) and interstitial fibrosis/tubular atrophy (IF/TA). Renal biopsies taken from healthy human kidneys were used as basal expression of TLR2 and TLR4. Furthermore, mouse and rat TLR expression will be analyzed and compared to the human TLR expression in order to determine whether renal allograft injury in animal models can be translated to the clinical situation.

2. Materials and methods

2.1. Immunohistochemistry

For the detection of TLR2 and TLR4, frozen kidney sections (4 μm, with the exception of 2 μm for human biopsies) were first dried by airflow, fixed in cold acetone and blocked with 0.09% H2O2 in PBS. The sections were stained with a specific antibody to TLR2 or TLR4 for human, mouse and rat (Table 1). A number of primary antibodies were kindly provided by Hycult (Uden, The Netherlands). Horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Glostrup, Denmark) were used. As negative control, the primary antibody was replaced by isotype controls. All antibodies were diluted in 1% BSA/PBS and if needed, 1% normal human serum was added (Sigma–Aldrich, St. Louis, MO, USA) or rat/mouse normal serum. The antibodies were finally stained using 3-amino-9-ethylcarbazole (AEC) with 0.03% H2O2. The sections were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany) and analyzed by light microscopy. For the anti-TLR4 antibody sc-12511, the slides were blocked with normal donkey serum to eliminate background staining.

For the staining with anti-mouse TLR2 monoclonal antibodies clone 2.5 and 2.7, biotin labeling was used. The sections were prepared as previously described and blocked with a Biotin Blocking system (X0590, Dako) before applying the primary antibody. The biopsies were then stained with a specific primary antibody to TLR2 for mouse and rat sections. As secondary antibody, Streptavidin horseradish peroxidase (P0397, Dako) was used and then exposed to AEC.

All antibodies were also tested with the same concentration on paraffin sections (human, mouse and rat healthy kidney sections were used). After deparaffinisation, all different antigen retrieval methods were tested (glycine, Tris/EDTA, citrate, pepsin and protease). After these steps, the same protocol as frozen sections staining was used.

2.2. Kidney biopsies

In order to compare the expression of TLR2 and TLR4 in different types of human kidney injury after transplantation, human kidney biopsies were stained. The analyzed renal injury patterns (minimum n = 6 per group) were acute tubular necrosis (ATN), acute rejection (Banff Ia, Ib, Ila, Ilb) and interstitial fibrosis and tubular atrophy (IF/TA, at least 30%). Living donor renal biopsies were used as healthy controls; the biopsy was taken before kidney retrieval and before renal blood vessels were clamped. A 16-gauge needle (Accut®, TSK Laboratory, Japan) was used to obtain kidney transplant biopsies. Frozen sections were incubated with anti–TLR2 monoclonal antibody (clone 2.3) and anti–TLR4 monoclonal antibody (76B357.1).

2.3. Scoring of the kidney biopsies

The biopsies were scored by two independent observers in a blinded fashion. For every section, the presence of TLR2+ and TLR4+ inflammatory cells, glomeruli, interstitial space, endothelium and tubuli was scored independently. They were given a score ranging from 0 to 4. Score zero meaning there was no TLR2 or TLR4 positive staining and the scores ranging from 1 to 4 showing the intensity and quantity of TLR positive cells.

2.4. Tissue specimen

For TLR expression analysis in mice and rat, kidneys from healthy animals were used (n = 5).

2.5. Western blot

Aliquots (20 and 40 μg) of peripheral blood mononuclear cells with 5× sample buffer were heated up to 95 °C. The samples were then immediately cooled on ice. Gel electrophoresis was performed at 110V. The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS. Subsequently, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 350 mA. The blot buffer contained 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were blocked in 5% skim milk in TBS-0.1% Tween and then incubated with the primary antibody overnight at 4 °C in 5% skim milk in TBS-0.1% Tween. As control, the primary antibody was omitted. Subsequently, the membranes were incubated with the appropriate HRP-labeled secondary antibody (refer to Table 1), Chemiluminescence technology (Supersignal, Pierce, Rockford, IL) was used for detection.

2.6. Statistical analysis

Data analysis was performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the mean ± SEM. A student’s t-test was used to compare TLR2 and TLR4 expression within one biopsy type. Statistical differences between the different groups were analyzed by using one-way ANOVA followed by post hoc Tukey’s Multiple comparison test. For the demographic characteristics, the Kruskall–Wallis test was used for continuous variables and the Chi-square test for categorical variables. All p-values ≤0.05 were considered significant.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Isotype</th>
<th>Primary antibody against TLR2 or TLR4</th>
<th>Company</th>
<th>Secondary and tertiary antibodya</th>
<th>Human cryo renal tissue</th>
<th>Human paraffine renal tissue</th>
<th>Mouse cryo renal tissue</th>
<th>Rat cryo renal tissue</th>
<th>Remark</th>
</tr>
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<td>TLR2</td>
<td>Mouse IgG2a</td>
<td>Human anti-TLR2 monoclonal (clone 2.3)</td>
<td>Hycult Biotech Inc.</td>
<td>Rabbit anti-mouse, goat anti-rabbit anti-chicken</td>
<td>+(1:100)</td>
<td>–</td>
<td>–</td>
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<td>Rat IgG2b</td>
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<td>TLR4</td>
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<td>Mouse anti-TLR4 monoclonal (768357.1)</td>
<td>Abcam</td>
<td>Rabbit anti-mouse, goat anti-rabbit anti-rabbit</td>
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<td></td>
<td>Goat IgG</td>
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<td>–</td>
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</table>

a All antibodies are HRP labeled and concentration used is 1:100.

b All the following antigen retrieval methods were tested: Tris/HCl, glycine, citrate, pepsin, protease.

c Biotin labeled antibody.
3. Results

3.1. Patient demographics

A total of 47 different renal biopsies were included in this study. Patients from different post-transplant injury (ATN, Banff la, Banff lb, Banff lla, Banff llb and IF/TA) displayed similar donor and recipient baseline characteristics (Table 2).

3.2. Antibody screening

For TLR2, out of the four antibodies tested on cryo sections, the monoclonal anti-human TLR2 antibody (clone 2.3) was chosen for further experiments. For TLR4, monoclonal anti-human TLR4 antibody (clone 76B357.1) for frozen sections was chosen out of the three TLR4 antibodies. Therefore TLR2 antibody clone 2.3 and TLR4 antibody clone 76B356.1 were selected for further experiments. Specificity of these two antibodies was verified by Western blotting. A clear detection band was seen for both TLR2 and TLR4 at approximately 90 kDa (Fig. 1). A specific staining for TLR2 and TLR4 was not achieved on paraffin sections with any of the tested antibodies.

### Table 2

<table>
<thead>
<tr>
<th>Characteristics of recipient, donor and transplantation procedure</th>
<th>ATN</th>
<th>Banff la</th>
<th>Banff lb</th>
<th>Banff lla</th>
<th>Banff llb</th>
<th>IF/TA</th>
<th>p-Value</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>50</td>
<td>33</td>
<td>20</td>
<td>80</td>
<td>50</td>
<td>20</td>
<td>0.30</td>
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<tr>
<td>Age at transplant</td>
<td>53.2 ± 10.4</td>
<td>50.2 ± 13.1</td>
<td>38.2 ± 12.5</td>
<td>40.2 ± 19.8</td>
<td>50.8 ± 9.6</td>
<td>39.8 ± 12.1</td>
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<td>Previous transplantation</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<td>0.44</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
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<td>Glomerulopathy/Interst. nephritis</td>
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<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
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<tr>
<td>Chronic renal failure</td>
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<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Other (incl. diabetes mellitus, hypertension)</td>
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<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
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<td><strong>Donor characteristics</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Gender (% female)</td>
<td>67</td>
<td>33</td>
<td>50</td>
<td>40</td>
<td>50</td>
<td>80</td>
<td>0.91</td>
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<tr>
<td>Age</td>
<td>53.0 ± 17.9</td>
<td>31.3 ± 19.0</td>
<td>33.5 ± 11.1</td>
<td>30.9 ± 20.1</td>
<td>51.6 ± 15.4</td>
<td>55.0 ± 8.5</td>
<td>0.18</td>
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<tr>
<td>Donor type</td>
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<td>Living</td>
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<td>2</td>
<td>2</td>
<td>3</td>
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<td>Deceased HB donor</td>
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<td>1</td>
<td>2</td>
<td>3</td>
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<td>Deceased NHB donor</td>
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<td>3</td>
<td>1</td>
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<td>HLA mismatches</td>
<td>2.2 ± 2.2</td>
<td>2.0 ± 1.4</td>
<td>2.7 ± 1.4</td>
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<td>2.8 ± 1.6</td>
<td>2.6 ± 0.9</td>
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<td>1st warm ischemia time</td>
<td>10.8 ± 10.9</td>
<td>4.7 ± 8.5</td>
<td>9.3 ± 6.8</td>
<td>9.9 ± 15.4</td>
<td>4.2 ± 8.0</td>
<td>11.4 ± 12.3</td>
<td>0.50</td>
</tr>
<tr>
<td>Cold ischemia time</td>
<td>20.5 ± 5.5</td>
<td>13.8 ± 9.4</td>
<td>10.0 ± 6.6</td>
<td>10.0 ± 7.5</td>
<td>11.5 ± 10.1</td>
<td>6.4 ± 6.9</td>
<td>0.10</td>
</tr>
<tr>
<td>2nd warm ischemia time</td>
<td>42.3 ± 12.3</td>
<td>40.0 ± 8.5</td>
<td>35.2 ± 11.3</td>
<td>38.1 ± 12.9</td>
<td>35.0 ± 7.9</td>
<td>42.2 ± 14.5</td>
<td>0.57</td>
</tr>
</tbody>
</table>

*Clinical information from two patients could not be retrieved (one with Banff lla and one with IF/TA).*

3.3. Renal TLR2 and TLR4 expression in healthy human renal tissue

Native expression for TLR2 and TLR4 was analyzed in renal biopsy sections from healthy living donors (Fig. 2). TLR2 and TLR4 expression in healthy renal tissue was found on the vascular cell wall. As shown in Figs. 2 and 4, TLR2 was also present in Bowman’s capsule and rarely in the interstitium. TLR2 and TLR4 expression

![Fig. 1. Verification of antibody specificity by Western blot for the two antibodies used to stain the human renal biopsy sections (TLR2 clone 2.3 and TLR4 clone 76B357.1). Lanes 1 and 2 showing the tested antibody and lane 3 being the negative control.](image)

![Fig. 2. Representative pictures from human living donor biopsies showing the native expression of (A) TLR2 and (B) TLR4. The inlet shows the vascular cell wall. Magnification 200×.](image)
3.4. Expression of TLR2 and TLR4 in human renal transplantation biopsies

Expression of TLR2 and TLR4 after transplantation was analyzed in post-transplant biopsies diagnosed with ATN, acute rejection (Banff Ia, Ib, Ila, IIb) or IF/TA. The TLR4 stained human biopsies are shown in Fig. 3. Some biopsies had to be excluded due to poor quality of the biopsy.

In renal transplant biopsies from patients with the clinical diagnosis of ATN, no enhanced expression of TLR2 or TLR4 compared to healthy kidney biopsies could be demonstrated. In all kidney injury biopsies, a diffuse interstitial TLR2 staining was present (Fig. 4). Expression of TLR4 and occasionally for TLR2, was found on infiltrating cells in the biopsies (Fig. 4). Interestingly, biopsies taken during acute interstitial and vascular rejection episodes (type I and II respectively) showed a significant increase of TLR4+ infiltrating cells (Fig. 3) compared to healthy renal tissue ($p < 0.001$). No difference between the different types of rejection was observed in TLR4+ infiltrating cells. In renal biopsies with IF/TA (chronic allograft damage) no TLR2 or TLR4 expression was found on infiltrating cells or on tubular epithelial cells. Similar to the results found in healthy renal tissue, no tubular staining for TLR2 or TLR4 was observed in any of the human transplantation biopsies.

3.5. Renal TLR2 and TLR4 expression in rodent kidney

Rodent models have been used to study the role of TLR mediated renal allograft injury. Therefore, normal expression patterns of TLR2 and TLR4 in mouse and rat renal tissue were analyzed to compare with human expression patterns. Out of the four antibodies tested on mouse and rat frozen renal tissue (Table 1), we were able to have a good and specific staining for TLR2 in rat renal tissue and TLR4 in mouse renal tissue. A TLR2 staining in mouse and TLR4 staining in rat was not successful.

In rat renal frozen tissue, we demonstrated TLR2 expression using the polyclonal anti-TLR2 antibody (Fig. 5). In line with human findings, rat kidney demonstrated a TLR2+ staining on the vascular
cell wall. In contrast to the human situation, a tubular epithelial staining was observed in rat. Also, the epithelial brush border stained positive for TLR2.

In mouse frozen tissue, expression of renal TLR4 was demonstrated with the anti-TLR4 polyclonal antibody (sc-12511). As seen in the TLR2 staining in mouse, we observed a TLR4+ staining in tubular epithelial cells, in the brush border and in the endothelium. For both TLR2 and TLR4, the tubular staining was predominantly located on the apical membrane and in some tubuli also at the basolateral side.

4. Discussion

Upon activation by DAMPs and PAMPs, TLRs lead to an inflammatory response and are implicated in allograft injury after renal transplantation. TLR antagonists might be useful to attenuate injury in IRI or acute rejection. The aims of this study were to provide insight into the possible role of human renal TLR2 and TLR4 expression in different pre- and post-transplant injury biopsies. As studies reporting positive effects of TLR blocking antibodies were performed in rodents, we also analyzed TLR expression in rodents.

TLR2 in human living donors was expressed in the vascular cell wall, in Bowman’s capsule and rarely in the interstitium, but never on the tubular epithelium. Under all post-transplant conditions, an additional diffuse interstitial TLR2 expression was found compared to the TLR2 interstitial expression in healthy subjects. Our results are in agreement with Leemans et al. (2009) who showed a similar interstitial staining for TLR2 using frozen sections. Even more importantly, as far as we know this is the first report on biopsies from healthy individuals stained for TLRs. Other studies for example used unaffected parts of kidneys with a renal cell carcinoma (RCC). It is well known, for instance, that seemingly healthy tissue from kidneys with renal cell carcinoma is activated by cytokines released by these tumors (Blay et al., 1997; Tsukamoto et al., 1992). Moreover, our group has shown that RCC can influence receptor localization (van Werkhoven et al., 2013) and therefore living donor biopsies should be used as baseline expression.

TLR4, in human living donors, was mainly expressed in the vascular cell wall. In concordance with our findings, an interstitial staining has not been reported for TLR4 (Batsford et al., 2011; de Groot et al., 2008; Kruger et al., 2009; Lim et al., 2009; Shah et al., 2013). When comparing the healthy situation to the post-transplant biopsies, we observed that all acute rejection biopsies (Banff Ia, Ib, Ila and Iib) were characterized by a TLR4+ inflammatory cell influx. We speculate that macrophages mainly constitute this influx; further research with double staining is needed to substantiate this finding. This suggests that TLR4+ lymphocytes and not renal TLR4 activation mediate the process of acute rejection. We speculate that DAMPs released during tissue injury attract and activate dendritic cells and macrophages via TLRs,
which subsequently leads to bridging the adaptive immunity by involving T cells. In this model, both innate and adaptive immunity play an important role in renal allograft rejection, which would be compatible with studies on skin allografts (Goldstein et al., 2003).

Noteworthy, no TLR2 or TLR4 baseline tubular staining was found in human frozen biopsies (healthy and post-transplant injury). This is in concordance with others who also reported the absence of a baseline tubular staining in renal frozen tissue (Batsford et al., 2011; Leemans et al., 2009). These results have great implications on clinical trials using TLR blocking antibodies. Phase I clinical studies using antibodies blocking TLR2 or TLR4 have already been realized (Reilly et al., 2013; Rossignol et al., 2004; Wong et al., 2003). We speculate that TLR blocking antibodies could have a beneficial effect on pathophysiological processes at the level of vascular cell wall, interstitial and inflammatory cells. However we would not expect protective effects in humans through tubular cells as one would hope for in case of tubular damage such as ATN.

Remarkably, studies using paraffin sections reported both TLRs to be present in tubular cells (de Groot et al., 2008; Hoffmann et al., 2011; Lim et al., 2009; Lin et al., 2012; Shah et al., 2013). This might be related to the fact that formaldehyde-fixed paraffin-embedded sections often show nonspecific background staining. Tissue antigenicity is better preserved in frozen sections as it is minimally exposed to fixatives.

Based on the beneficial effect of TLR blockade in animal transplant models, these agents are under development for clinical use (Farrar et al., 2012; Liu et al., 2010). However, it remains to be investigated whether the expression pattern of TLR in rodents can be compared to the human situation. Therefore, we compared localization of TLR2 and TLR4 in kidney from healthy mice and rats with healthy human kidneys. We and others found a staining in tubuli for rat TLR2 and mouse TLR4 on frozen sections. The advantageous effect of TLR blockade in animals on early allograft outcome might be partially explained by the tubular presence of TLRs. We observed a difference in tubular TLR expression between humans and rodents. Therefore, if protective effects of TLR blockade will be observed in humans, the underlying mechanisms might be different when compared with rodents. Therefore, caution is needed when translating results from animal studies to the human situation.

In conclusion, a discrepancy exists between rodent and human renal TLR expression. TLR2 and TLR4 expression was not found on tubular epithelial cells humans. These results can have great implications for clinical trials with TLR antagonists as their positive effect have only been tested in rodents. Acute rejection is characterized by TLR4* infiltrating cells. Therefore, due to the TLR4* infiltrating cells, TLR blocking agents might have an indication in renal allograft transplantation.

Conflict of interest
All authors declared that there are no conflicts of interest.

Authors contribution
ES, MW, FP, HG and JD performed research. ES, MW and MS wrote the paper. HG, PO, WS and MS conceived, supervised and critically reviewed the paper.

Acknowledgements
We wish to thank Anita Meter-Arkema for her expert technical support.

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