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**TLR2 and TLR4 initiate the innate immune response of the renal tubular epithelium to bacterial products**

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Abstract

Background: Renal tubular epithelial cells (TECs) respond diffusely to local infection, with the release of multiple cytokines, chemokines and other factors that are thought to orchestrate the cellular constituents of the innate immune response. We have investigated whether the Toll-like receptors TLR4 and TLR2, which are present on tubular epithelium and potentially detect a range of bacterial components, coordinate this inflammatory response acting through NF-\(\kappa\)B. Methods: Primary cultures of TECs were grown from C57BL/6, C3H/HeN, C3H/HeJ, TLR2 and TLR4 knockout mice. Cell monolayers were stimulated with lipopolysaccharide (LPS) and synthetic TLR2 and 4 agonists. The innate immune response was quantified by measurement of the cytokines TNF-\(\alpha\) and KC in cell supernatants by ELISA. Results: Cultured TECs grown from healthy mice produced the cytokines TNF-\(\alpha\) and KC in response to stimulation by LPS and synthetic TLR2 and TLR4 agonists. Cells lacking the respective TLRs had a reduced response to stimulation. The TLR2 and TLR4 mediated response to stimulation was dependent on NF-\(\kappa\)B signalling, as shown by curcumin pre-treatment of TECs. Finally apical stimulation of these TLRs elicited basal surface secretion of TNF-\(\alpha\) and KC (as well as the reverse), consistent with the biological response in vivo. Conclusions: Our data highlights the potential importance of TLR dependent mechanisms coordinating the innate immune response to upper urinary tract infection.
**Introduction**

Urinary tract infections are among the commonest bacterial infections affecting humans, and a major cause of morbidity and mortality. Chronic infection in children can also lead to renal scarring and subsequent end stage renal failure. During pyelonephritis, renal tubular epithelial cells (TECs) are one of the first host cells to come into contact with invading bacteria and influence the innate immune response via the production of cytokines and anti-microbial peptides[1]. This cytokine production is important in the recruitment and activation of inflammatory cells such as neutrophils, macrophages and lymphocytes. Cellular recruitment is a vital part of the innate immune response which plays a central role in maintaining sterility of the urinary tract. In particular, neutrophils have been shown to be of key importance in the process of bacterial clearance[2]. Neutrophils are attracted along a chemotactic gradient, and the chemokine interleukin-8 (IL-8) has been shown to play a critical role in neutrophil recruitment. TECs have been shown to secrete a number of cytokines including IL-8 during infection[3]. However, an excessive inflammatory response may lead to irreversible renal injury. Therefore the local response by tubular epithelial cells may have both beneficial and detrimental effects, the overall balance of which will determine the clinical outcome. Despite these important findings, our understanding of the mechanisms which initiate these local influences remains unclear. Toll-like receptors (TLRs) are a recently discovered group of pattern recognition receptors that recognise conserved molecular motifs found on a variety of organisms including bacteria, viruses and fungi[4]. Eleven mammalian TLRs have so far been described. TLR4 is the receptor which recognises lipopolysaccharide (LPS)[5;6], a constituent of the cell wall of gram negative bacteria such as *Escherichia coli*, which accounts for up to 80% of UTIs[7]. Meanwhile, TLR2 reacts with a wider spectrum of bacterial products found in both gram positive and negative bacteria including lipoproteins, peptidoglycans and lipoteichoic acid[8;9]. Constitutive expression of mRNA for TLR2 and TLR4 as well as associated molecules has been demonstrated on mouse tubular epithelial cells. Furthermore, C-C chemokine secretion was found to be strictly dependent on TLR2 and TLR4 [10]. Mice with defective TLR4 have previously been
shown to have reduced neutrophil recruitment and impaired bacterial clearance during experimental pyelonephritis [11]. More recently, using bone marrow chimeric mice, it has been shown that TLR4 is required on intrinsic renal cells as well as bone-marrow derived cells for effective control of ascending UTI [12]. The most recently described mammalian TLR, TLR11 has been noted to be present on uroepithelial cells and protects against infection from uropathogenic *E coli* in mice [13].

The interaction between TLRs and their specific ligands leads to activation of defined intracellular signalling pathways. The intracellular adaptor protein, myeloid differentiation factor 88 (MyD88) appears to be involved in signalling by all TLRs except TLR3. Activation of the MyD88 signalling pathway results in the translocation of nuclear factor NF-κB and activation of MAP kinases, leading to cytokine gene upregulation [14].

The aim of our study was to determine the role of TLR2 and TLR4 in the generation of TNF-α and KC by renal tubular cells during pyelonephritis. TNF-α has a variety of pro-inflammatory effects but overproduction can lead to septic shock following gram negative infection [15]. KC is a murine homologue of IL-8, and an important neutrophil chemotactant [16]. Furthermore, epithelial production of IL-8 plays a vital role in the transmigration of neutrophils across the epithelial cell layer and into the urinary tract lumen [17]. We further investigated whether the TLR dependent production of these cytokines by TECs required activation of NF-κB. Although the vast majority of cases of pyelonephritis occur as a result of ascending infection, some may occur following haematogenous spread of bacteria via the bloodstream. We therefore examined whether cytokine production by TECs could occur as a result of either apical or basolateral exposure to bacterial products. An improved understanding of these molecular events may allow separation of the host response into those which are of benefit and those which have potentially detrimental effects.
**Materials and Methods**

*Antibodies and reagents*

DMEM-F12 culture medium was purchased from Gibco (Paisley, UK). All other cell culture reagents were obtained from Sigma (Poole, UK) except where otherwise stated. LPS from *E coli* (serotype 0111:B4) was obtained from Sigma, purified LPS from *E coli* (serotype R515 Re) was obtained from Alexis Biochemicals (Lausen, Switzerland), the synthetic lipoprotein Palm\textsubscript{3}Cys-SKKK x 3HCl was obtained from EMC Microcollections (Tuebingen Germany) and the synthetic Lipid A analogue (ONO 4007) was a kind gift from ONO Pharmaceuticals (Osaka, Japan). Cyclohexamide and curcumin were purchased from Sigma. Rat anti-mouse CD68 and rat IgG2a isotype control were purchased from Serotec (Oxford, UK). Mouse anti-rat FITC-conjugated secondary was purchased from Jackson ImmunoResearch Europe Ltd (Soham, UK). FITC-conjugated mouse anti-pan cytokeratin monoclonal antibody was purchased from Sigma. Reagents for RT-PCR were purchased from Promega (Southampton, UK) and primers were constructed by MWG-Biotech (Ebersberg, Germany).

*Cell culture and stimulation assays*

C57/BL6, C3H/HeN and C3H/HeJ mice were purchased from Harlan, (Oxon, UK). TLR2 and TLR4 knockout mice were a kind gift from Professor Akira (Osaka, Japan). Kidneys from 6-8 week old mice were used to grow primary cultures of proximal tubular cells (TECs) as previously described [18]. Kidneys were bisected and outer cortical tissue was separated. Tissue was then minced and digested in collagenase II (Worthington, Lakewood, USA) for 20 minutes at 37\(^0\)C. The digest was then passed through graduated sieves (250, 106, 75 and 40nm). Cells trapped in the 40nm sieve were collected by washing, centrifuged to obtain a pellet and re-suspended in culture medium (DMEM-F12 supplemented with 2\%FCS, ITS, T3 (10^{-12}M), hydrocortisone (40ng/ml) and penicillin/streptomycin (100\,\mu\text{g/ml penicillin}, 100\,\mu\text{g/ml streptomycin}). The cell suspension was added to 1\% gelatin coated culture plates and incubated at 37\(^0\)C, 5\%CO\textsubscript{2}.
TECs were grown until confluent under direct light microscopy. **Cell phenotype was confirmed by positive staining for cytokeratin and brush border alkaline phosphatase together with demonstration of apical surface microvilli under electron microscopy. Absence of contamination with macrophages was confirmed by negative staining for CD68. Thioglycolate induced peritoneal macrophages were used as a positive control. (This portion of text has been moved to results section.)**

Culture medium was changed to serum free conditions 24 hours prior to stimulation with bacterial products. Experiments were performed in triplicate using three consecutive wells of 6 well plates.

**Polarity**

For polarity experiments, TECs were grown on 30mm polycarbonate cell culture plate inserts (Millipore, Billerica, USA) with a pore size of 0.4µm. Inserts were coated with fibrinogen prior to seeding with cells. Cell growth was monitored by measuring trans-epithelial cell resistance (TER) using Millicell-ERS micro-voltmeter (Millipore). TER was found to plateau at values between 550-600 Ω/cm², which was taken as indicating formation of a confluent monolayer. This corresponded to values obtained using the MDCK cell line, with which this technique has been validated in the literature[19]. TECs grew with the apical surface uppermost, therefore isolated stimulation of the apical surface was achieved by addition of LPS to the upper chamber. Similarly, stimulation of the basolateral cell surface was performed by addition of LPS to the lower chamber. Monitoring of TER following stimulation demonstrated that integrity of the monolayer was maintained.

**Cytokine gene expression and protein biosynthesis**

Total RNA was extracted from cells using a commercially available kit (Qiagen, Crawley, UK) according to manufacturer’s instructions. RNA concentration was determined by spectrometer absorbance at 260nm. 5 µg of RNA was reverse transcribed in a reaction together with 160ng
oligo(dT)$_{15}$, 500µM of each dNTP and 200 U Moloney murine leukaemia virus reverse transcriptase in 20µl solution for 90 minutes at 37°C.

PCR was carried out using the primer sequences shown in table 1 according to published conditions in a 25µl reaction with 12.5 pmol each of forward and reverse primers, 200µM of each dNTP and 3 U Taq polymerase. Products were stained on a 1.2% agarose gel and stained with ethidium bromide. Unconverted RNA for each sample was also run to demonstrate the absence of any genomic contamination.

Concentrations of TNF-α and KC were determined in culture supernatants using commercially available ELISA kits (R & D Systems, Oxon, UK) according to manufacturer’s instructions. All samples were assayed in duplicate and data presented as the mean.

**Statistics**

Experiments were performed in triplicate with data shown as the mean with error bars depicting the standard deviation. P values were calculated using the unpaired t test or Mann-Whitney where there was a significant difference in variance. P values of less than 0.05 were regarded as significant.
**Table 1**- Primer sequences used in RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Ref</th>
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<td>GGA TGC CAC AGG ATT CCA TAC C-3’</td>
<td></td>
</tr>
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<td>CAT CGG CTG GCA CCA CTA GGT-3’</td>
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<tr>
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<td>CCT TGA CCC TGA AGC TCC CTT GGT TC-3’</td>
<td>CGT GCG TGT TGA CCA TAC AAT ATG-3’</td>
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</tr>
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<td>AGA GTC AGG TGA TGG ATG TCG -3’</td>
<td>[22]</td>
</tr>
<tr>
<td>TLR4</td>
<td>CAA GGG ATA AGA ACG CTG AGA- 3’</td>
<td>CGA ATG TCT CTG GCA GGT GTA- 3’</td>
<td>[22]</td>
</tr>
<tr>
<td>CD14</td>
<td>CTA GTC GGA TTC TAT TCG GAG C-3’</td>
<td>AGA CAG GTC TAA GGT GGA GAG G-3’</td>
<td>[23]</td>
</tr>
<tr>
<td>MD-2</td>
<td>CTG AAT CTG AGA AGC AAC AGT GG 3’</td>
<td>CAG TCT CTC CTT TCA GAG CTC TGC 3’</td>
<td>[24]</td>
</tr>
<tr>
<td>MyD88</td>
<td>ATC CGA GAG CTG GAA ACG- 3’</td>
<td>GCA AGG GTT GGT TA ATC- 3’</td>
<td>[25]</td>
</tr>
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</table>
Results

Cell phenotype was confirmed by positive staining for cytokeratin (figure 1a) and brush border alkaline phosphatase together with demonstration of apical surface microvilli under electron microscopy. Absence of contamination with macrophages was confirmed by negative staining for CD68. Thioglycolate induced peritoneal macrophages were used as a positive control (figure 1b).

Gene expression of TLR2, TLR4, CD14, MD-2 and MyD88 in TECs

The presence of mRNA for TLR2 and TLR4 was verified in primary cultures of TECs from C57/BL6 mice by RT-PCR (figure 2). The presence of message for CD14 and MD-2, which are both required in TLR4 activation by LPS were also confirmed, along with that for MyD88, the adaptor molecule involved in the intracellular signalling pathway common to all TLRs and interleukin 1 and 18. RT-PCR was performed on RNA extracted from thioglycolate induced peritoneal macrophages as a positive control. Lack of corresponding bands in unconverted mRNA was demonstrated to show absence of contamination with genomic DNA.

TECs produce the cytokines TNF-α and KC in a dose and time dependent manner following stimulation with LPS

Both TNF-α and KC production was seen clearly with the highest dose (10 µg/ml) at 2 hours post stimulation (fig 3). At 24 hours, there was no significant difference between the responses to 100ng/ml or 10µg doses, suggesting that this represented a maximal response.

LPS and synthetic lipid A stimulate cytokine production in TECs via a TLR4 dependent mechanism

In order to assess the role of TLR4 signalling in TNF-α and KC production by TECs, we stimulated primary cultures of TECs from C3H/HeJ strain mice with LPS (fig 4c and d). Although these mice have TLR4, they have a point mutation in the tlr4 gene which results in substitution of histidine for proline resulting in defective signalling[26]. C3H/HeN mice without this defect were used as a
positive control (fig4a and b). Interestingly, the response to LPS was not absent in TECs from C3H/HeJ mice, although the magnitude of the response was reduced in comparison with control cells from C3H/HeN mice. A problem that has previously been highlighted in using LPS preparations derived from bacteria is that of purity, and it has been shown that contaminants can lead to stimulation via other TLRs. To overcome this, we also stimulated cells concurrently with a highly purified source of LPS, and with a synthetic lipid A analogue. Both purified LPS and synthetic lipid A did not induce production of either TNF-α or KC in TECs from C3H/HeJ mice, indicating that the response seen with standard LPS formulation was a result of contaminants. In assessing the TNF-α response in C3H/HeN cells, it was found that the standard preparation of LPS was able to induce a greater response in comparison with lipid A. Higher doses of lipid A did not produce a greater response than that shown (data not shown).

We further assessed the roles of both TLR2 and TLR4 in cytokine production following stimulation with LPS using TECs grown from TLR2 and TLR4 knockout mice which had been backcrossed onto a C57/BL6 background (fig 4g-j). Wild type C57/BL6 mice were used to grow control cells (fig 4e and f). TECs from TLR4 knockouts failed to respond to either purified LPS or synthetic lipid A, confirming that the response to LPS is dependent on TLR4. A response to crude LPS was seen in TLR4 knockout TECs, presumably due to contamination. Interestingly, the response to both LPS preparations and lipid A in cells grown from TLR2 knockout mice, although present, was significantly reduced in magnitude compared with that seen from wild type cells (for stimulation with purified LPS, TNF-α production C57BL/6 v TLR2 knockout p<0.0001, KC production p<0.005). This suggests TLR2 might help augment the response to LPS by TLR4, independent of stimulation by contaminants.

**Synthetic lipoprotein stimulates cytokine production in TECs via TLR2**

To assess the role of TLR2, we grew primary cultures of TECS from wild type C57/BL6, TLR2 knockout and TLR4 knockout strain mice. These were then stimulated with a synthetic lipopeptide, Palm₃Cys-SKKKK (fig 5). In wild type cells, a response to stimulation was seen, both in terms of
TNF-α and KC secretion into the culture medium. This response was absent in TLR2 knockout cells, indicating that TLR2 is required for the response to lipopeptides. Although TLR4 knockout cells demonstrated a response, the magnitude was reduced in comparison with that seen in wild type cells (for stimulation with 10mcg lipoprotein, TNF-α secretion wild type v TLR4 knockout p<0.005, KC p< 0.05). This further supports our theory of co-operation between TLR2 and TLR4.

**TNF-α and KC production in response to LPS follows gene upregulation which does not require new synthesis of protein intermediates but is NF-κB dependent**

In macrophages, TLR4 activation has been shown to lead to translocation of the nuclear factor NF-κB which is leads to cytokine production. This pathway requires recruitment of the intracellular adaptor protein MyD88, gene expression of which was demonstrated in figure 1. To examine whether NF-κB activation was required for the TNF-α and KC production seen, we pre-treated cells with curcumin prior to stimulation. It has previously been reported that pre-treatment with 5µM curcumin inhibits JNK activation, while concentrations of 50µM inhibit NF-κB activation[27]. Pre-treatment of TECs with 50µM curcumin was found to completely abolish any increase in TNF-α and KC in culture supernatants following stimulation with LPS (fig 6a). Analysis of RNA extracted from cells revealed that this was a consequence of failure of gene upregulation (fig 6b). Therefore activation of NF-κB was critical for TNF-α and KC production by TECs in response to LPS. 5µM curcumin did not abolish production of either cytokine, however the magnitude of response was reduced in both, suggesting JNK activation may be required for a maximal response. In order to assess if this was a direct effect or required the new synthesis of intermediate cytokines, cells were also pre-treated with cyclohexamide prior to stimulation. Cyclohexamide inhibits any new protein synthesis, and failed to inhibit gene upregulation (fig 6b).
**TECs express immunological polarity**

TECs grown in culture grow with their apical surfaces uppermost. During ascending UTI, bacteria will initially interact with the apical surface of epithelial cells. Renal epithelial cells are polarised cells with many surface proteins expressed either on the apical or basolateral cell surface. Similarly, any epithelial response to exogenous stimuli may be polarised. Addition of LPS to either the upper or lower chambers did not affect TER suggesting the integrity of the monolayer was unaffected (data not shown). Stimulation of the apical surface led to TNF-α secretion as would be expected from previous experiments. However, independent sampling of the upper and lower chambers revealed that this secretion was polarised, occurring predominantly apically (fig 7a). Stimulation via the basolateral surface also led to a predominantly apical TNF-α response. In contrast, the KC response was non-polarised, with both apical and basolateral secretion occurring regardless of whether LPS was applied to the apical or basolateral cell surface (figure 7b).
Discussion

The findings presented in this paper support the hypothesis that TECs play a vital role in initiating the innate immune response to ascending urinary tract infection. Innate immunity is critical for the clearance of bacteria, and epithelial cells, the first host cells to interact with invading bacteria, are in a key position to initiate this response. They are capable of synthesising chemotactic and inflammatory cytokines in response to bacterial products through engagement of TLRs expressed on their surface.

Previous studies have shown that mouse TECs are able to synthesise C-C chemokines via engagement of TLRs and therefore promote T cell infiltration[28]. However in the context of acute UTI and pyelonephritis, the function of T cells is uncertain. The predominant infiltrating cell in the early stages of infection is the neutrophil and neutrophil depletion renders normal mice susceptible to UTI[29]. Chemokines are the main neutrophil chemoattractants and are vital for migration into the inflamed kidney. Previous studies have shown that human TECs produce IL-8 in response to infection with *E coli*[30], however their ability to do so in response to isolated bacterial products is unclear. It has been shown that human TECs, despite possessing TLR4, respond poorly to LPS[31]. This may be due to lack of CD14 expression[32]. It is clear from the work presented here that murine cells *express both TLR4 and the required accessory molecules*, and can produce KC in response to purified LPS. In addition, murine tubular epithelial cells produce TNF-α. This cytokine has a wide variety of pro-inflammatory effects including stimulating local cells to up-regulate cytokine and adhesion molecule expression, which will further assist in the recruitment and activation of neutrophils into the kidney[33].

Although it is clear that TLR4 is an absolute requirement for the response to LPS, our studies using standard LPS preparations demonstrate that the response to Gram negative organisms is not wholly dependent on TLR4. A response to crude LPS is seen in TLR4 defective or knockout cells. This response was lost when using either highly a highly purified LPS or a synthetic lipid A analogue. This confirms that contaminants in the standard LPS preparation were able to stimulate cytokine
production, possibly acting via other TLRs. A previous study has demonstrated that contaminants in commercial LPS preparations can signal through TLR2[34]. Furthermore, dual stimulation of TLR2 and TLR4 on macrophages has been shown to have synergistic effects[35]. This would explain why our standard preparation of LPS was able to elicit a greater maximal response than the pure agonists on their own.

Although purified LPS and lipid A were able to elicit a response in TLR2 knockout cells, the magnitude of the both TNF-α and KC secretion was significantly reduced in comparison with wild type cells suggesting that TLR2 may co-operate with TLR4 in order to achieve a maximal response to LPS. The response to TLR2 ligands in TLR4 knockout cells was also reduced. Furthermore, the response to the impurities in standard LPS was greater in C3H/HeJ cells than in cells grown from TLR4 knockouts. Although C3H/HeJ mice have defective TLR4 signalling, TLR4 is still present and can therefore augment signalling via other TLRs such as TLR2. Previous studies have highlighted co-operation between TLRs. For example, TLR2 has been shown to co-operate with TLR1 and TLR6[36], however as yet TLR4 has not been shown to co-operate with any other TLR. TLR4 however requires a number of accessory molecules in order to respond to LPS, including CD14 and MD-2[37;38]. Our findings suggest that in order to achieve a maximal response to pure agonists, co-operation between TLR2 and TLR4 is required. This adds a further facet to the synergy previously described when both TLRs are stimulated simultaneously.

By growing cells on inserts, we were able to stimulate the apical and basolateral cell surfaces separately. Both cell surfaces of TECs responded to LPS, suggesting that TLR4 is present on both the apical and basolateral sides. However another possibility is that this represents the ability of LPS to internalise from either cell surface, with TLR4 located within the cell, as has been demonstrated in intestinal epithelial cells[39]. It has been postulated that the reason for this is that the gut is normally a non-sterile environment and that if TLR4 were located on the cell surface, this would lead to an unwanted host response to commensal bacteria. By locating TLR4 within the cell, the response can be limited to those bacteria that are invading the epithelium. However unlike the
gut, the urinary tract is normally a sterile environment, and any bacteria within the tubular lumen would constitute infection. In this situation it would be beneficial for TLR4 to be located on the apical cell surface where it can sample luminal contents.

Isolated stimulation of apical and basolateral surfaces did however reveal the fascinating discovery that the cytokine responses can be polarised. It is already established that TECs are polarised with respect to their ability to transport ions and proteins across their membranes. It appears that this polarisation extends to their immunological response. Stimulation of either the apical or basolateral cell membrane with purified LPS resulted in predominantly apical TNF-α secretion. KC secretion however did not demonstrate any polarity. Given the functions and effects of these cytokines, this might be expected. TNF-α is a potent inflammatory cytokine, and its systemic effects include the vascular collapse seen in patients with septic shock[40]. Such a response would not be of benefit to the host in the case of a localised infection. This may explain why there is little basolateral secretion of TNF-α which would then enter the systemic circulation. Conversely, TNF-α is needed for neutrophil activation, hence apical secretion into the lumen would be beneficial during infection of the upper urinary tract. KC on the other hand is needed for neutrophil chemotaxis from the vascular compartment into the tubular lumen, hence the need for basolateral secretion. Studies have also demonstrated a key role for IL-8 and its receptor in trans-epithelial migration of neutrophils into the tubular during UTI[41]. Failure of epithelial trans-migration leads to defective bacterial clearance and accumulation of neutrophils in the sub-epithelial space resulting in greater tissue injury. Therefore both apical and basolateral secretion may be needed, initially to recruit cells from the circulation, and subsequently to allow their passage across the epithelial membrane and into the tubular lumen where the bacteria are resident.

In terms of the pathways involved in cytokine production, our data confirms a critical role for NF-κB activation leading to cytokine gene upregulation. **Cytokine production was also found not to require the synthesis of any protein intermediates, however the involvement of pre-formed intermediates cannot be ruled out.** Whether TECs utilise exactly the same signalling pathways as
macrophages remains to be determined, although the presence of genetic message for MyD88 raises this as a possibility.

Our study confirms previous findings that TLR2 and TLR4 are present on renal epithelial cells and demonstrates a role in the innate immune response to bacteria during ascending urinary tract infection. However we also highlight that although TLRs have specific ligands, co-operation may be required between these TLRs and maybe other molecules in order to achieve a maximal response. This supports previous findings that rather than acting alone, TLRs might form receptor complexes within lipid rafts on the cell surface[42]. This might explain how using a relatively small number of TLRs, the innate system can provide tailored responses to a wide variety of molecules. Further complexity is added when we consider that in the clinical setting, cells are exposed to whole organisms rather than their individual components. Therefore it is likely that a number of TLRs are stimulated simultaneously on each cell, and that the overall response will be the sum of a number of individual interactions. Endogenous ligands have been identified for both TLR2 and TLR4, suggesting they potentially have a role in a variety of inflammatory renal conditions (ref).

Acknowledgements:

We would like to thank Professor Akira for kindly allowing us to use the TLR2 and TLR4 mice generated by his group. We also thank ONO Pharmaceuticals for providing the synthetic lipid A ONO-4007. The work presented here was supported by a grant from the Guy’s and St Thomas’ Charitable Foundation.
Figure 1

Figure 1a

Figure 1b

Figure 1c
Figure 2

TLR4  macro  ptec

CD14  macro  ptec

TLR2

MD-2

MyD88

β actin

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**Figure 3**

**a)** TNF-\(\alpha\)

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**b)** KC

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Figure 4

**Figure 4**

(a) TNF-α

(b) KC

(c) TNF-α

(d) KC

(e) TNF-α

(f) KC

(g) TNF-α

(h) KC

(i) TNF-α

(j) KC

**C3/HeN**

**C3H/HeJ**

**C57/BL6**

**TLR2 KO**

**TLR4 KO**
Figure 5

a) TNF-α

C57/BL6

b) KC

TLR2 KO

c) d)

e) f)

TLR4 KO

***       ***

***        ***

**         ***

**          **
Figure 6

(a) TNF-α conc pg/ml

(b) KC conc pg/ml

(c) LPS - + + - + - +
CHX - - +
Curcumin - - - 5µM 5µM 50µM 50µM

B actin

TNF-α

B actin

KC
Figure 7

a) 

**

**

b) 

ns

ns

Supernatant
Legends for figures

Figure 1a) shows positive staining for cytokeratin in tubular cell cultures. Figure 1b) shows positive staining of peritoneal macrophages with anti-CD68 antibody. Simultaneous staining of tubular cell cultures for CD68 was negative as shown in figure 1c).

Figure 2- Expression of TLR2, TLR4 and related molecules in TECs from C57/BL6 mice.
Thioglycolate induced peritoneal macrophages from the same strain were used as a positive control. Cells were cultured under basal conditions. mRNA was extracted and RT-PCR was performed as described.

Figure 3- Dose response and time course for TNF-α and KC secretion by TECs from C57/BL6 mice in response to stimulation with LPS. TECs were stimulated with 1, 100 and 10000ng/ml of LPS and cell supernatant sampled over a period of 24 hours. TNF-α (figure 3a) and KC (figure 3b) protein concentration in samples as determined by ELISA. Data shown is representative of three separate experiments.

Figure 4- TNF-α and KC production by TECs in response to LPS is dependent on the presence of a functional TLR4. TECs were cultured from C3H/HeJ, C3H/HeN, TLR2 knockout, TLR4 knockout and C57/BL6 mice. Cells were stimulated with LPS, a highly purified LPS preparation or synthetic lipid A (ONO 4007) diluted to a concentration of 1µg/ml in sterile water. Culture supernatants were collected at 24 hours post stimulation, and TNF-α (figures 4a, c, e, g and i) and KC concentrations (figures 4b, d, f, h and j) determined by ELISA. Data shown representative of two separate experiments

* (p<0.05), ** (p<0.005) and *** (p<0.0005) highlight responses which are statistically significant when compared that seen from cells stimulated with an equivalent volume of sterile water alone.
Figure 5- TNF-α and KC production by PTECs in response to lipoprotein is dependent on TLR2. TECs from C57/BL6, TLR2 knockout and TLR4 knockout mice were grown as described and stimulated with 1µg/ml and 10µg/ml of Palm3Cys-SKKKK, a synthetic lipoprotein dissolved in DMSO. Cytokine concentration in the culture supernatant was determined at 24 hours by ELISA. Data shown representative of two separate experiments. * (p<0.05), ** (p<0.005) and *** (p<0.0005) highlight responses which are statistically significant when compared with that seen from cells stimulated with an equivalent volume of DMSO alone.

Figure 6- TNF-α and KC gene upregulation in response to LPS does not require new protein synthesis and is dependent on NF-κB activation. TECs were pre-treated with either 100µM cyclohexamide (CHX) or stated concentrations of curcumin for 1 hour prior to stimulation with 1µg/ml of LPS. Samples of culture supernatant were collected at 4 hours post-stimulation and RNA extracted from cells. TNF-α (figure 6a) and KC (figure 6b) concentrations were determined by ELISA on supernatants from curcumin and control groups. RT-PCR was performed on extracted RNA as described. β actin was used as a housekeeping gene to ensure that pre-treatment did not adversely affect cell viability (figure 6c).

Figure 7- Stimulation via the apical cell or basolateral cell surface results in polarised responses. TECs from C57/BL6 mice were grown on inserts within 6 well plates as described. Trans-epithelial resistance (TER) was used to confirm confluency of the cell monolayer as described. Cells were then stimulated via either their apical surface, by addition of 1µg/ml of purified LPS to the upper chamber, or via the basolateral surface by addition of LPS to the lower chamber. Monitoring of TER demonstrated that the monolayer remained intact following stimulation. Supernatant samples were obtained from upper and lower chambers and TNF-α (figure 7a) and KC (figure 7b)
concentrations determined via ELISA as previously described. Data shown is representative of three separate experiments.

** (p<0.005)  ns= non significant difference
Reference List


