Expression and regulation of Toll-like receptors in lupus-like immune complex glomerulonephritis of MRL-Fas(lpr) mice


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Abstract

Background. How microbial infections exacerbate immune complex glomerulonephritis remains speculative. Toll-like receptors (TLRs) may be involved in this phenomenon, because TLRs have potent immunostimulatory functions when exposed to selected pathogen-associated molecules.

Methods. We addressed this issue by characterizing the expression of TLR1–9 in MRLlpr/lpr mice that spontaneously develop immune complex glomerulonephritis as part of a systemic lupus-like autoimmune syndrome.

Results. Five-week-old healthy MRL lpr/lpr mice expressed TLR3 mRNA in kidneys at comparable levels as in the spleen, while all other TLRs were expressed at low levels in the kidney. In 20-week-old nephritic MRLlpr/lpr mice, renal mRNA levels had increased for TLR1–9. Renal TLR mRNA originated at least in part from glomeruli as evidenced by real-time RT-PCR from laser capture microdissected glomeruli. Immunostaining for TLR3, TLR7 and TLR9 revealed their expression by F4/80-positive infiltrating macrophages in 20-week-old nephritic MRLlpr/lpr mice. In addition, TLR3 localized to glomerular mesangial cells. Cultured mesangial cells expressed TLR1–4 and TLR6, while murine macrophages expressed TLR1–9. TNF-α and IFN-γ induced TLR2, TLR3 and TLR6 mRNA in mesangial cells, while they down-regulated TLR1–9 mRNA in macrophages. Stimulation of both cell types with ligands for TLR1–4, TLR5, TLR7 and TLR9 induced IL-6 production consistent with their respective TLR expression patterns. TNF-α and IFN-γ enhanced ligand-induced IL-6 production in both cell types irrespective of their modulatory effect on respective TLR mRNA levels.

Conclusion. Thus, cell-type-specific expression and regulation of TLRs may be involved in infection-associated exacerbation of immune complex glomerulonephritis of MRLlpr/lpr mice.

Keywords: autoimmune diseases; innate immunity; kidney; lupus; Toll-like receptor

Introduction

Immune complex glomerulonephritis develops from immune complex deposits that activate renal cells to produce pro-inflammatory mediators, e.g. cytokines and chemokines. Subsequent macrophage recruitment contributes to glomerular injury through additional production of pro-inflammatory factors [1]. Microbial infections can exacerbate chronic immune complex glomerulonephritis, e.g. in IgA nephropathy or lupus nephritis, but the molecular mechanisms of this phenomenon are poorly understood.

The discovery of the Toll-like receptors (TLRs) as human homologues to the Drosophila Toll gene product has offered new perspectives for the understanding of immune modulation induced by microbial infections [2,3]. TLR are a group of innate pattern recognition receptors that recognize multiple classes of microbes through conserved molecular patterns [4]. For example, TLR1, TLR2 and TLR6 heterodimerize for the recognition of lipoproteins and peptidoglycans present in viral envelopes and Gram-positive bacteria [5–7]. Gram-negative bacteria are mostly recognized through TLR4, a critical component of the lipopolysaccharide (LPS) receptor complex [8]. TLR3, TLR7/8 and TLR9 form a subgroup of nucleic acid-specific TLRs located in intracellular endosomes [9,10]. TLR3 is specific for viral double-stranded (ds) RNA [11], marine (m) TLR7 and human (h) TLR8 recognize viral single-stranded (ss) RNA [12,13], and bacterial or viral CpG-deoxyribonucleic acid (DNA) ligates TLR9 [14]. All these TLRs induce innate and adaptive antimicrobial immunity by activating immune and non-immune cell types [15–17].

*The authors wish it to be known that, in their opinion, the first two authors contributed equally to this work.

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For example, microbial CpG-DNA is a potent B-cell mitogen and inducer of Th1 responses through TLR9 [18] so as to control microbial infection [19,20]. However, CpG-DNA has been shown to trigger immune complex glomerulonephritis. Two injections with synthetic CpG-oligodeoxynucleotides aggravated apoferritin-induced glomerulonephritis in Balb/c mice [21]. Furthermore, a course of intraperitoneal injections of either bacterial DNA or synthetic CpG-DNA aggravated the lupus-like immune complex glomerulonephritis of MRL[pr/lpr] mice [22]. In both models, injected CpG-DNA localized to TLR9-positive intrarenal macrophages and dendritic cells, and induced local chemokine production associated with additional inflammatory cell infiltrates. In addition, TLR9 activation on B cells stimulated antigen-specific IgG2a production and subsequent glomerular immune complex deposition [22]. In another study, autoimmune MRL[pr/lpr] mice were exposed to viral dsRNA, a ligand for TLR3. Interestingly, we found that injected viral dsRNA localized to TLR3-positive glomerular mesangial cells [23]. A course of dsRNA injections caused crescentic glomerulonephritis, but did not affect dsDNA autoantibody production or renal immune complex deposition, consistent with the lack of TLR3 on B cells. Obviously, microbial components can trigger immune complex glomerulonephritis through different immune mechanisms that relate to cell-type-specific expression patterns of TLRs.

Intrigued by these findings, we intended to characterize the expression of TLR1–9 in immune complex glomerulonephritis of MRL[pr/lpr] mice. We hypothesized that immune cells and glomerular mesangial cells would express distinct patterns of TLRs. In fact, we found that macrophages express TLR1–9, whereas mesangial cells express TLR1–4 and TLR6. Furthermore, these cell types regulate TLR1–9 differentially upon exposure to TNF-α and IFN-γ. Nevertheless, when exposed to these pro-inflammatory cytokines, both, macrophages as well as mesangial cells, enhance TLR-induced IL-6 production. These data support the concept that microbial products can trigger flares of immune complex glomerulonephritis of MRL[pr/lpr] mice through specific interactions with TLRs on immune and non-immune cells in the nephritic kidney.

Material and methods

Animal studies

MRL and MRL[pr/lpr] mice were obtained from Harlan Winkelmann (Borchen, Germany) and housed in groups of five mice in filter top cages with a 12 hr dark/light cycle and unlimited access to food and water. Cages, nestlets, food and water were sterilized by autoclaving before use. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities. Blood samples were collected by bleeding from the retro-orbital venous plexus under general anesthesia with inhaled ether. After centrifugation, all serum samples were stored at –80°C until analysis. Mice were sacrificed by cervical dislocation. Serum dsDNA autoantibody levels specific for IgG2a were determined using a modified enzyme-linked immunosassay (ELISA), in which murine dsDNA coated plates were used and remaining steps were carried out as per the manufacturers instructions (Bethyl labs, Montgomery, TX, USA). From each mouse the left kidney was fixed in 10% buffered formalin and embedded in paraffin. Sections for periodic acid Schiff stains were prepared as described [24].

Immunostaining

Paraffin-embedded sections were prepared as described [1]. In brief, sections of formalin-fixed and paraffin-embedded tissues were de-waxed and re-hydrated. Endogenous peroxidase was blocked by 3% hydrogen peroxide and antigen retrieval was performed in Antigen Retrieval Solution (Vector, Burlingame, CA) in an autoclave oven. Biotin was blocked using the primary antibodies for 1 hr, followed by biotinylated secondary antibodies (anti-rat IgG, Vector) and the ABC reagent (Vector). Slides were washed in phosphate-buffered saline between the incubation steps. 3’/3′Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement was used as detection system, resulting in a black-coloured product. Methyl green was used as a counter-stain, and slides were dehydrated and mounted in Histomount (Zymed Laboratories, San Francisco, CA). The following primary antibodies were used: anti-mF4/80 (1:50, monocytes/macrophages, 1:50; Serotec Ltd, Oxford, UK), and anti-mCD3 (1:100, T lymphocytes, clone CD3-12, Serotec, Raleigh, NC), rabbit anti-mIgG (1:50, Dianova). Azetone-fixed frozen sections were prepared for staining with the following TLR antibodies as described [1]: anti-mTLR3 (1:50, IMG516, Imgenex, San Diego, CA), anti-mTLR7 (1:50, IMG581, Imgenex) and anti-mTLR9 (1:10, IMG431, Imgenex). An anti-rabbit IgG secondary antibody (1:100, Jackson Immunoresearch Laboratories, West Grove, PA) was used for detection. A rabbit IgG isotype antibody (Imgenex, no 20304) was used instead of the TLR-specific antibodies to control for the specificity of TLR immunostaining. To detect TLR on macrophages, a F4/80 antibody (1:50, Serotec) was used for sequential co-staining. For quantitative analysis, positive cells were counted in 15 high-power fields (n = 5).

Laser capture microdissection of paraffin-embedded renal tissue

A Robot MicroBeam system (P.A.L.M, Wolfratshausen, Germany) was used to isolate glomeruli from formaldehyde-fixed and paraffin-embedded renal sections. Under direct visual control, ~100 glomeruli per animal were isolated by the focused nitrogen laser beam from the surrounding tissue, as recently described [25]. For harvesting the sample, the energy of the laser was increased and the microdissected glomerulus was catapulted with a single laser shot. The detached glomeruli were collected in a microforge cap coated with mineral oil (Fluka Sigma-Aldrich, Deisenhofen, Germany). Samples were stored in liquid nitrogen until being further processed.
Probes were from ABI Biosystems, Weiterstadt, Germany. Oligonucleotide primer (300 nM) and housekeeper genes. Oligonucleotide primer (300 nM) and housekeeper genes. Oligonucleotide primer (300 nM) and housekeeper genes. Oligonucleotide primer (300 nM) and housekeeper genes. Controls consisting of ddH2O were negative for target glomerular RNA were performed as described [24,25]. Glomerular RNA was dissolved in 10 mM chloroform-based RNA extraction was performed. RNA isolation from organs of MRLlpr/lpr mice was performed as described [26]. The following primary antibodies were used to detect TLRs on mesangial cells and macrophages: rabbit IgG (Immuntools, Gruenberg, Germany) or TLR ligands as follows: TLR1/2: 1 μg/ml Pam3Cys (Alexis Biochemicals, Grunberg, Germany), TLR3: 25 μg/ml plC RNA (Sigma-Aldrich, Taufkirchen, Germany), TLR4: 1 μg/ml ultrapure LPS (Invivogen, San Diego, CA), TLR5: 100 ng/ml flagellin (Alexis Biochemicals), TLR7: 100 μg/ml RNA40 (IBA GmbH, Goettingen, Germany) and TLR9: 1 μM CpG-DNA 1668 (TIBMolbiol, Berlin, Germany). Before use all TLR ligands (except LPS) were pre-incubated with polymixin-B (Invivogen) to block residual LPS contamination. After a period of 24 h culture supernatants were collected and IL-6 levels were determined using a commercial ELISA kit (OptEIA, BD Biosciences).

Flow cytometry

Flow cytometry of cultured cells was performed as previously described [23]. The following primary antibodies were used to detect TLRs on mesangial cells and macrophages: anti-mTLR2 (1:50, clone 6C2, Serotec, Oxford, UK), anti-mTLR3 (1:50) and anti-mTLR4-MD2 (1:100, MBL, Nagoya, Japan), anti-mTLR7 (1:50), anti-mTLR9 (1:50). A rabbit IgG (Imgenex, no 20304) was used as isotype control.

Statistical analysis

Data are presented as mean ± SEM. Comparison of groups was performed using unpaired Student’s t-test. A value of P < 0.05 was considered to indicate statistical significance.

Table 1. Probes used for real-time RT-PCR

<table>
<thead>
<tr>
<th>TLR</th>
<th>Accession-number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>AF316985</td>
<td>Forward primer: 5'-GTCAAAGCTTGAGAATCTGAAAG-3'</td>
</tr>
<tr>
<td>TLR2</td>
<td>AF124741</td>
<td>Forward primer: 5'-CAGGCGTCAAGAAAACACTTAC-3'</td>
</tr>
<tr>
<td>TLR3</td>
<td>AF355152</td>
<td>Forward primer: 5'-CTGGCCACAGTACTTCTGTA-3'</td>
</tr>
<tr>
<td>TLR4</td>
<td>AF110133</td>
<td>Forward primer: 5'-TTCAGAATCGTGGCTGGATGT-3'</td>
</tr>
<tr>
<td>TLR5</td>
<td>AF186107</td>
<td>Forward primer: 5'-CCCAGCTTCTTCTTGAAACACTA-3'</td>
</tr>
<tr>
<td>TLR6</td>
<td>AB020808</td>
<td>Forward primer: 5'-GGGTCAATTCTAATAGTGCCTGG-3'</td>
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<td>AY035889</td>
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<tr>
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<td>Forward primer: 5'-AAAAGCAGTCGAATCCATGTA-3'</td>
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<td>TLR9</td>
<td>NM 031178</td>
<td>Forward primer: 5'-CAATCTGACCTCCTCCTCAGTACT-3'</td>
</tr>
<tr>
<td>18srRNA</td>
<td></td>
<td>Pre-developed TaqMan assay reagents</td>
</tr>
</tbody>
</table>

RNA preparation and real-time quantitative (TaqMan) RT-PCR

Glomerular RNA was isolated from paraffin-embedded sections and incubated successively in Xylol, 100, 90 and 70% ethanol. Deparaffinized glomerular RNA were incubated in lysis buffer (10 mM TrisHCl, 0.1 mM EDTA, 2% SDS and 20 μg/ml ProteinaseK) for 16 h at 60°C before phenol-chloroform-based RNA extraction was performed. Glomerular RNA was dissolved in 10 μl RNase free water. RNA isolation from organs of MRLlpr/lpr mice was performed using standard methods as described [25]. Reverse transcription and real-time RT-PCR from total organ and glomerular RNA were performed as described [24,25]. Controls consisting of ddH2O were negative for target and housekeeper genes. Oligonucleotide primer (300 nM) and probes (100 nM) were used as listed in Table 1. Primers and probes were from ABI Biosystems, Weiterstadt, Germany.

Cell culture conditions

J774 murine macrophages (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. A murine mesangial cell line [26] was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with 5% fetal bovine serum (Serum Supreme, BioWhittaker, Walkersville, MD) and 100 units/ml penicillin and streptomycin 100 μg/ml. Cells were treated with medium control or 200 U/ml IFN-γ (PeproTech, Rocky Hill, NJ) + 500 U/ml TNF-α (ImmunTools, Firenysythe, Germany) or TLR ligands as follows: TLR1/2: 1 μg/ml Pam3Cys (Alexis Biochemicals, Grunberg, Germany), TLR3: 25 μg/ml plC RNA (Sigma-Aldrich, Taufkirchen, Germany), TLR4: 1 μg/ml ultrapure LPS (Invivogen, San Diego, CA), TLR5: 100 ng/ml flagellin (Alexis Biochemicals), TLR7: 1 μg/ml RNA40 (IBA GmbH, Gottingen, Germany) and TLR9: 1 μM CpG-DNA 1668 (TIBMolbiol, Berlin, Germany). Before use all TLR ligands (except LPS) were pre-incubated with polymixin-B (Invivogen) to block residual LPS contamination. After a period of 24 h culture supernatants were collected and IL-6 levels were determined using a commercial ELISA kit (OptEIA, BD Biosciences).
Results

dsDNA autoantibody production and renal disease in MRL<sup>lpr/lpr</sup> mice

MRL<sup>lpr/lpr</sup> mice are a suitable model for human lupus nephritis because renal disease in MRL<sup>lpr/lpr</sup> mice develops secondary to dsDNA autoantibody production, immune complex formation and subsequent renal immune complex deposition. In female MRL<sup>lpr/lpr</sup> mice, serum levels of dsDNA antibodies progressively increased from 8 weeks of age (Figure 1A). Increasing levels of serum dsDNA autoantibodies were associated with immune complex deposits in glomeruli, as shown for 18-week-old MRL<sup>lpr/lpr</sup> mice (Figure 1B). IgG deposits distribute in a granular pattern in the mesangium and along glomerular capillaries. In contrast, age-matched MRL wild-type mice lack dsDNA autoantibody production and glomerular IgG deposits.

These abnormalities were associated with typical histopathological changes of proliferative lupus nephritis. In kidneys of 8-week-old MRL<sup>lpr/lpr</sup> mice, histomorphological abnormalities were not detected (Figure 2). Eighteen-week-old MRL<sup>lpr/lpr</sup> mice showed mesangioproliferative glomerulonephritis. Twenty-four-week-old MRL<sup>lpr/lpr</sup> mice revealed diffuse global proliferative lupus nephritis and crescentic lesions in 25–50% of glomeruli associated with marked periglomerular and interstitial infiltrates of CD3-positive lymphocytes and F4/80-positive macrophages (Figure 2). In contrast, MRL wild-type mice had a normal renal histomorphology at 24 weeks of age.

Total-organ TLR mRNA expression in 5- and 20-week-old MRL<sup>lpr/lpr</sup> mice

First, we examined the expression of TLR1–9 mRNA in solid organs of MRL<sup>lpr/lpr</sup> mice. We studied 5-week-old healthy MRL<sup>lpr/lpr</sup> mice as well as 20-week-old MRL<sup>lpr/lpr</sup> mice with advanced autoimmune tissue injury in selected organs (Figure 3). Because immune cells express many TLR, we hypothesized that in young MRL<sup>lpr/lpr</sup> mouse mRNAs of TLR1–9 would be expressed at much higher levels in the spleen, compared with non-lymphoid solid organs. In fact, mRNAs of TLR1–9 were expressed in the spleen. In contrast, in the kidney all TLR mRNAs were found at much lower levels than in the spleen except for TLR3 mRNA. TLR3 mRNA levels comparable with those in the spleen were also found in other non-lymphoid solid organs, i.e. brain, heart, lung and liver. Heart, lung and liver of 5-week-old MRL<sup>lpr/lpr</sup> mice expressed various levels of the other TLR mRNAs, but TLR1 and TLR9 mRNA were low in all non-lymphoid organs.

In 20-week-old MRL<sup>lpr/lpr</sup> mice, the splenic follicular structure was replaced by large disorganized collections of monocytes and lymphocytes (Figure 3). In spleen TLR2, TLR5 and TLR8 mRNA levels were increased, when compared with 5-week-old MRL<sup>lpr/lpr</sup> mice. Advanced immune complex glomerulonephritis of 20-week-old MRL<sup>lpr/lpr</sup> mice was associated with an increase of TLR mRNA levels as follows: TLR1, 3-fold; TLR2, 5-fold; TLR3, 3-fold; TLR4, 4-fold; TLR5, 5-fold; TLR6, 7-fold; TLR7, 6-fold; TLR8, 4-fold; and TLR9, 7-fold; respectively. Autoimmune tissue injury of the other solid organs was as follows: lung—peribronchial and perivascular infiltrates, severe; liver—moderate periportal infiltrates; heart—mild perivascular infiltrates; brain—none. In the brain, no significant changes in TLR mRNA levels were detected between 5- and 20-week-old MRL<sup>lpr/lpr</sup> mice. In the heart, TLR2 and TLR5–8 mRNA levels were found to be reduced at 20 weeks, while TLR3 mRNA levels were increased. In lungs, most TLR mRNA were found to be increased at 20 weeks except for TLR3 and TLR4 mRNA. In liver, TLR3, TLR8 and TLR9 levels were increased at 20 weeks compared with 5 weeks.

Together, these data show that mRNA for TLR1–9 is present in most organs of autoimmune MRL<sup>lpr/lpr</sup> mice, but TLR1–9 mRNAs are differentially expressed throughout disease progression. Kidneys and lungs, which were affected by severe autoimmune tissue injury, mostly increased TLR mRNA expression levels. In contrast, in brain, heart and liver, expression levels of most TLRs remained unchanged or even reduced. Thus, TLR mRNA expression in MRL<sup>lpr/lpr</sup> mice may either relate to the number of infiltrating immune cells or to expression and regulation of TLRs.
on immune cell infiltrates or intrinsic parenchymal cells.

**TLR mRNA expression in glomeruli of 5- and 20-week-old MRL\(^{lpr/lpr}\) mice**

Next, we examined whether glomeruli contribute to renal TLR mRNA levels of MRL\(^{lpr/lpr}\) mice. We used laser capture microscopy for microdissecting paraffin-embedded renal tissue samples and subsequent gene expression analysis by real-time RT-PCR [25]. Laser capture microdissection allowed us to obtain glomerular tissue samples of high purity, which were not contaminated by extraglomerular cells. Real-time RT-PCR detected TLR3, TLR4, TLR7 and TLR9 mRNA in glomeruli at low levels in 5-week-old MRL\(^{lpr/lpr}\) mice (Figure 4A–D). The glomerular expression of these TLRs was increased in 20-week-old MRL\(^{lpr/lpr}\) mice. These findings confirm the increasing glomerular expression of TLRs, e.g. TLR3, TLR4, TLR7 and TLR9, during the progression of immune complex glomerulonephritis of MRL\(^{lpr/lpr}\) mice. However, whether glomerular TLR mRNA expression relates to infiltrating immune cells or to intrinsic renal cells requires immunostaining.

**TLR immunostaining in kidneys of 18-week-old MRL\(^{lpr/lpr}\) mice**

Thus, immunostaining was performed on frozen sections for those TLRs, for which appropriate antibodies are available, i.e. TLR3, TLR7 and TLR9. Immunostaining for either TLR3, TLR7 or TLR9 was positive for single cells in periglomerular fields (Figure 5). Co-staining for F4/80 identified 87±7% of the F4/80-positive cells to stain positive for TLR3, 80±10% for TLR7 and 89±3% for TLR9. Thus, the majority of the F4/80-positive renal cell population expressed TLR3, TLR7 and TLR9. In addition, glomeruli stained positive for TLR3 in a mesangial cell staining pattern. Together, these data show that TLR3, TLR7 and TLR9 are expressed by renal macrophages. Glomerular mesangial cells express TLR3, which is consistent with high TLR3 mRNA expression levels in healthy and diseased MRL\(^{lpr/lpr}\) mice. In contrast, TLR7 and TLR9 are absent on intrinsic renal cells. Unfortunately, we were unsuccessful in staining primary cells prepared from MRL\(^{lpr/lpr}\) mice.
Fig. 3. Total organ TLR mRNA expression in MRL<sup>lpr/lpr</sup> mice. mRNA was extracted from organs of 5- and 20-week-old MRL<sup>lpr/lpr</sup> mice as indicated. TLR mRNA expression levels were determined from pooled samples (n = 4) by real-time RT–PCR as described in the Material and methods section. TLR mRNA levels are expressed as ratio to the 18s-rRNA expression. Expression of TLR mRNA in spleens of 5-week-old MRL<sup>lpr/lpr</sup> mice is set as 1. Images below indicate periodic acid Schiff staining of the respective organs of 5- and 20-week-old MRL<sup>lpr/lpr</sup> mice (original magnification 200×).
mice for TLR3, TLR7 and TLR9 with commercially available TLR antibodies. Thus, we next studied TLR expression and regulation in vitro with established cell lines of murine macrophages and mesangial cells.

**Expression, regulation and function of TLR in murine macrophages**

TLR mRNA expression was determined in J774 macrophages cultured under basal culture conditions. J774 macrophages expressed TLR1–9 mRNA, of which TLR2, TLR4 and TLR9 were expressed at high levels (Figure 6A). Protein expression was confirmed by flow cytometry for those TLR for which appropriate antibodies are available. J774 macrophages revealed surface as well as intracellular expression of TLR2 and TLR4 (Figure 6B). In contrast, TLR3, TLR7 and TLR9 were absent on the cell surface but found to be expressed intracellularly after permeabilization (Figure 6B). To answer the question how macrophages regulate TLR mRNA expression in inflammatory environments, we compared TLR mRNA expression either under normal culture conditions or after pre-stimulation with IFN-γ and TNF-α for 24 h. IFN-γ and TNF-α down-regulated the expression of virtually all TLRs (Figure 6C). Next, we questioned whether macrophages respond to TLR ligands as predicted from their respective TLR expression profile. We stimulated J774 macrophages with TLR ligands either under normal culture conditions or after pre-stimulation with IFN-γ and TNF-α. IL-6 production was analysed as a marker for macrophage activation. Stimulation with either Pam3Cys (TLR1/2), pI:C RNA (TLR3), LPS (TLR4), flagellin (TLR5), RNA40 (TLR7) or CpG-DNA (TLR9) induced IL-6 production (Figure 6D). Pre-incubation with IFN-γ and TNF-α increased macrophage IL-6 production by direct and

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**Fig. 4.** Glomerular TLR mRNA expression in MRL<sup>lpr/lpr</sup> mice. Glomerular samples were microdissected from paraffin-embedded renal sections using a laser capture microdissection system by a laser beam and laser pressure catapulting as described in the Material and methods section. An average amount of 100 glomeruli was harvested from renal sections of each MRL<sup>lpr/lpr</sup> mouse of either 5 or 20 weeks of age. mRNA expression levels for TLR3 (A), TLR4 (B), TLR7 (C) and TLR9 (D) were determined from ~600 pooled glomeruli from 6 MRL<sup>lpr/lpr</sup> mice of each age group. TLR mRNA levels are expressed as ratio to the respective 18s-rRNA expression and the mRNA levels at week 5 were set as 1.

**Fig. 5.** Immunostaining for TLR in kidneys of MRL<sup>lpr/lpr</sup> mice. TLR specific antibodies together with an appropriate PE labelled secondary antibody were used on renal sections of 20-week-old nephritic MRL<sup>lpr/lpr</sup> mice. An FITC-labelled F4/80-specific antibody was used to assess colocalization with renal macrophages (original magnification 400×).
TLR-independent effects. However, IL-6 production was increased in all groups that were exposed to TLR ligands except for Pam3Cys (Figure 6D). These data further show that cultured murine macrophages express and produce IL-6 upon ligation of TLR1–9. Down-regulation of TLR1–9 seen with exposure to IFN-γ and TNF-α, however, does not reduce but rather enhances IL-6 production.

Expression, regulation and function of TLR in murine mesangial cells

Next, we determined TLR mRNA expression in cultured murine mesangial cells. Mesangial cells expressed TLR1–4 and TLR6 under basal culture conditions, of which TLR3 was expressed at high levels (Figure 7A). mRNA levels for TLR5 and TLR7–9 were not detected. TLR protein expression was confirmed by flow cytometry for TLR2–4. Mesangial cells revealed positive staining signals for cell surface expression of TLR2 and TLR4 (Figure 7B). TLR3 was expressed in an intracellular compartment (Figure 7B). Next, we compared TLR mRNA expression in mesangial cells with or without pre-stimulation with IFN-γ and TNF-α. Both cytokines markedly up-regulated TLR2, TLR3 and TLR6 mRNA levels, while TLR1 mRNA was down-regulated. TLR4 mRNA levels were not affected. TLR5 and TLR7–9 mRNA remained undetectable (Figure 7C). In order to test whether mesangial cells respond to TLR ligands as predicted from their respective TLR expression profile, mesangial cells were stimulated with TLR ligands either under normal culture conditions or after pre-stimulation with IFN-γ and TNF-α. Again IL-6 production was analysed as a marker for mesangial cell activation. Unstimulated mesangial cells produced IL-6 only after exposure to pI:C RNA (TLR3) and LPS (TLR4) (Figure 7D). Pre-incubation with IFN-γ and TNF-α increased mesangial cell IL-6 production due to direct and TLR-independent effects. However, pre-stimulated mesangial cells produced higher amounts of IL-6 when exposed to Pam3Cys (TLR1/2), pI:C RNA (TLR3) and LPS (TLR4) (Figure 7D). In contrast, flagellin (TLR5), RNA40 (TLR7) and CpG-DNA (TLR9) did not increase IL-6 production consistent with the lack of mRNA expression of the respective TLR. These data demonstrate that a murine mesangial cell line constitutively expresses TLR1–4, and TLR6 and produces IL-6 in response to dsRNA and LPS. In cultured mesangial cells IFN-γ and TNF-α induce the expression of TLR2, TLR3, TLR4 and TLR6 and promote IL-6 production upon exposure to bacterial lipoprotein, LPS and viral dsRNA.

Discussion

Intrigued by our recent observation that dsRNA and CpG-DNA modulate immune complex glomerulonephritis through cell-type-specific interactions with TLR3 and TLR9 [22,23], we intended to characterize the expression of TLR1–9 mRNA in kidneys of young and adult MRL<br><br>lpr/lpr mice. This allowed a comparison between TLR expression in healthy and nephritic kidneys affected by immune complex glomerulonephritis. We found that TLR1–9 mRNA levels were all increased in advanced immune complex glomerulonephritis. In addition, we found that cultured mesangial and macrophage cell lines express distinct patterns of TLR. Interestingly, TNF-α and IFN-γ enhance TLR-induced IL-6 production in both cell types despite discordant effects of these cytokines on TLR mRNA expression in cultured macrophages and mesangial cells.

TLR1–9 mRNA levels increase with progression of lupus nephritis in MRL<br><br>lpr/lpr mice

No inflammatory cell infiltrates were present in the kidneys of 5-week-old MRL<br><br>lpr/lpr mice. Thus, any TLR mRNA expressed in these kidneys should originate from intrinsic renal cells. At this time point, only TLR3 was found to be expressed at comparable levels as in the spleen. TLR1, TLR2, TLR4 and TLR6 showed some intermediate mRNA expression levels when compared with those in the spleen. TLR5, TLR7, TLR8 and TLR9 were virtually absent in kidneys of 5-week-old MRL<br><br>lpr/lpr mice. This renal TLR mRNA expression profile was consistent with the profile found in a cultured mesangial cell line, which express TLR3 at high and TLR1, TLR2, TLR4 and TLR6 mRNA at moderate levels. Tubular epithelial cells isolated from the mouse kidney have been reported to express the identical TLR pattern [27]. Thus, we conclude that mesangial cells and tubular epithelial cells express TLR1–4 and TLR6 in the healthy kidney.

After the onset of immune complex glomerulonephritis, renal mRNA levels of TLR1–9 were up-regulated. The same was shown for TLR3, TLR4, TLR7 and TLR9 mRNA in glomerular isolates. This could either relate to significant numbers of immune cell infiltrates or to induction of TLR mRNA in intrinsic renal cells. In fact, proliferative immune complex glomerulonephritis was associated with significant numbers of glomerular, periglomerular and interstitial macrophage and T-cell infiltrates, the latter known to express negligible amounts of TLR [28]. A cultured macrophage cell line expressed TLR1–9 but down-regulated TLR mRNA expression upon stimulation with IFN-γ and TNF-α. This finding suggests that macrophages suppress TLR mRNA expression in a pro-inflammatory cytokine environment. In contrast, IFN-γ and TNF-α induced the mRNA expression of TLR2–4 and TLR6 in mesangial cells. We therefore conclude that, both increasing numbers of immune cell infiltrates as well as induction of TLR expression by intrinsic renal cells, e.g. mesangial cells, contribute to induction of renal TLR1–9 mRNA levels in nephritic kidneys of MRL<br><br>lpr/lpr mice.
Macrophages and mesangial cells have distinct TLR expression profiles

A murine macrophage cell line expressed TLR1–9 mRNA and responded to all TLR ligands tested consistent with previous observations [29]. Macrophage TLR1–9 expression is consistent with their important roles in innate immunity. Thus, the expression of TLR on intrinsic renal cells would not generally be suspected. However, it has long been known that mesangial cells respond to LPS and lipoproteins [30], which have now been identified as ligands for TLR1/2 and TLR4 [5,8]. In fact, LPS triggers successive waves of mesangial cell gene expression, but the relevance of this finding to disease
remains to be established [31]. Here, we show that glomerular mesangial cells express a subset of TLR, which are TLR1–4 and TLR6. The TLR expression profile data detected in a murine mesangial cell line are consistent with that of primary mesangial cells prepared from mice glomeruli as well as with that of human mesangial cells [32]. This repertoire of TLR allows the recognition of pathogen-associated molecular patterns that occur in a wide range of microbes. For example, TLR4 recognizes LPS, which originates from gram-negative bacteria. TLR1, TLR2 and TLR6 recognize lipoproteins from gram-positive...
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bacteria and zymosan from yeast [6,33,34]. Most interestingly, the mesangial cell line expressed high levels of TLR3 in an intracellular compartment. TLR3 belongs to the subgroup of nucleic acid-specific TLR, but mesangial cells neither express the other members of this subgroup, namely TLR7–9, nor do they respond to their ligands, i.e. ssRNA40 or CpG-DNA [12,18]. The natural ligand for TLR3 is viral dsRNA [11], but TLR3-deficient mice also lack appropriate immune responses to infection with ssRNA viruses [35,36]. RNA viruses release RNA during infection, which can persist RNAse degradation when the RNA is complexed to other viral proteins. For example, hepatitis-C virus (HCV) replication in infected livers releases HCV RNA, which can be amplified from serum cyroprecipitates and renal infected livers releases HCV RNA, which can be amplified from serum cyroprecipitates and renal biopsies, e.g. in HCV-associated membranoproliferative glomerulonephritis [37,38]. In fact, injections with viral dsRNA aggravate immune complex glomerulonephritis in MRL<i>lpr</i/lpr mice through TLR3 on mesangial cells and infiltrating antigen-presenting cells [23]. Based on these findings, we conclude that multiple microbial components can activate macrophages as well as glomerular mesangial cells. However, the distinct TLR expression profile of cultured mesangial cells restricts their response to ligands that ligate TLR1–4 and TLR6, e.g. LPS, lipoproteins or viral RNA.

**IFN-γ and TNF-α increase TLR signaling irrespective of TLR mRNA expression**

Our data show that both mesangial cells and macrophages increase TLR-mediated IL-6 production after exposure to IFN-γ and TNF-α. This is a surprising finding because these cytokines down-regulate TLR mRNA expression in a macrophage cell line. In fact, cytokine-induced down-regulation of TLR expression has been recently described as one of several counter-regulatory mechanisms of TLR activation in immune cells [39]. Here, we first describe that TLR mRNA expression is regulated differently in glomerular mesangial cells. IFNγ and TNF-α induced the expression of mRNA for TLR2, TLR3 and TLR6. The up-regulation of TLR3 mRNA was most prominent, irrespective of its comparable high basal expression level. Similar data have been reported from colonic epithelial cells [40], lung fibroblasts [35], lung epithelial cells [41] and astrocytes [42]. Regulation of TLRs can relate either to specific promoter-binding elements [43] or to cell type-specific expression of additional regulatory factors [39]. These data support the hypothesis that activated immune and renal cells, both present in immune complex glomerulonephritis, are highly susceptible to stimulation with microbial components that ligate TLR. The cell type-specific response will depend on the distinct TLR expression profile of each cell type.

Cultured murine macrophages as well as mesangial cells express TLR in distinct expression profiles, which can both modulate immune complex glomerulonephritis of MRL<i>lpr</i/lpr mice. Macrophages express TLR1–9 and mesangial cells express TLR1–4 and TLR6. Both cell types produce IL-6 in response to respective TLR ligands that can originate from Gram-positive bacteria, Gram-negative bacteria, yeast and RNA viruses. These data may add to our understanding how different microbial components can trigger flares of immune complex glomerulonephritis of MRL<i>lpr</i/lpr mice.

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**References**

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