Toll-Like Receptor 4 Ligation on Intrinsic Renal Cells Contributes to the Induction of Antibody-Mediated Glomerulonephritis *via* CXCL1 and CXCL2

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Autoimmune diseases such as glomerulonephritis are exacerbated by infection. This study examined the effect of the Toll-like receptor 4 (TLR4) ligand lipid A on the development of heterologous nephrotoxic nephritis. Administration of nephrotoxic antibody resulted in significant glomerular neutrophil infiltration and albuminuria only when a TLR4 ligand was administered simultaneously. The contribution of TLR4 on renal cells and circulating leukocytes was assessed. Bone marrow chimeras were constructed with TLR4 only on renal cells or bone marrow–derived cells. The administration of nephrotoxic serum and lipid A caused a neutrophil influx in both chimeric groups greater than in sham chimeras that were totally TLR4 deficient but significantly less than in sham chimeras that were totally TLR4 sufficient. Both chimeric groups had greater albuminuria than totally TLR4-deficient sham chimeras; however, the chimeras with TLR4 only on intrinsic renal cells had significantly less than the sham positive group. *In situ* hybridization showed expression of TLR4 mRNA in mesangial cells and glomerular epithelial cells. For investigation of the potential mechanism by which renal cells could contribute to disease exacerbation, mesangial cells were cultured and found to express mRNA for TLR4, and stimulation of wild-type and TLR4-deficient mesangial cells with LPS caused production of CXC chemokines by wild-type cells only. Treatment of chimeras with TLR4 present only on intrinsic renal cells with anti-CXCL1 and anti-CXCL2 antibody before disease induction significantly reduced renal neutrophil infiltration. These results show that TLR4 on both circulating leukocytes and intrinsic renal cells contributes to the inflammatory effects of antibody deposition within the glomerulus, which depends at least in part on the production of CXC chemokines by intrinsic renal cells.

Autoimmune diseases including glomerulonephritis can be exacerbated by systemic infection. In IgA nephropathy, an upper respiratory tract infection commonly precedes disease presentation (1); relapses of ANCA-associated vasculitis are associated with nasal carriage of *Staphylococcus aureus* (2,3); and in anti–glomerular basement membrane disease, exacerbations during treatment are often related to infection (4). Toll-like receptors (TLR) are a family of receptors that recognize a variety of ligands that are derived from pathogens. They influence both the inflammatory and the adaptive immune response to invading pathogens and are likely to be important in the exacerbation of autoimmune disease by infection.

TLRs are widely distributed on professional immune cells but are also found on non–bone marrow (BM)-derived cells (5).

In the mouse, TLR4 expression in the kidney has been shown to be predominantly tubular with sparse glomerular expression (6). TLR4 has also been shown to be expressed in a mesangial cell line (7). Infection may exacerbate glomerulonephritis in a number of ways by activating TLR. The adaptive immune response may be influenced to promote renal tissue injury. TLR agonists could act on neutrophils, macrophages, or other cells of the innate immune system to increase glomerular inflammation and exacerbate disease. Alternatively, inflammation could be mediated by TLRs that are present on intrinsic renal cells such as mesangial cells.

In this study, we used heterologous nephrotoxic nephritis, a passive model of antibody-mediated glomerular inflammation (8,9). Disease is characterized by a glomerular neutrophil influx, which peaks at approximately 2 h and is largely resolved by 24 h with accompanying proteinuria. Previous studies have shown a role for LPS in this model in both the rat and the mouse (10,11). Endotoxin preparations that were used previously were crude, and repurification has been shown to reduce signaling by TLR2 (12). The precise role for pure TLR4 ligands in disease exacerbation therefore cannot be established from these studies. We used a synthetic analogue of the active part of

endotoxin called lipid A and highly purified LPS derived from *Escherichia coli*, both of which are known to ligate TLR4 and contain undetectable levels of other TLR ligands. Heterologous nephrotoxic nephritis is ideally suited to investigate mechanisms independent of any effects of TLR stimulation on the adaptive immune response. We show that for glomerular inflammation to occur in response to antibody deposition, TLR4 ligation is also required. By the production of BM chimeras, we show that TLR4 on both intrinsic renal cells and circulating leukocytes contribute toward the production of disease. We show by *in situ* hybridization that TLR4 mRNA is expressed in mesangial and epithelial cells within the glomerulus. We also show that cultured mesangial cells express TLR4 and can produce the CXC chemokines that are responsible for neutrophil chemotaxis in response to TLR4 ligation, and we show that blocking these chemokines *in vivo* reduces disease severity in chimeric mice expressing TLR4 only on intrinsic renal cells.

Materials and Methods

Preparation of Nephrotoxic Serum and TLR Agonists

A mouse glomerular extract was made and nephrotoxic serum (NTS) was prepared as described previously (13). The endotoxin content of the serum was measured using a chromogenic kinetic Limulus Amoebocyte Lysate assay (Cambrex Bioscience, Walkersville, MO). The amount of endotoxin received by each mouse was <0.1 EU. Synthetic lipid A ONO-4007 (Ono Pharmaceutical Co., Ltd., Osaka, Japan) was used as a pure TLR4 ligand. It was dissolved in 50% ethanol. The final concentration of ethanol in injected NTS was 0.5%. The synthetic lipopeptide Pam_3CysSK_4 (EMC Microcollections, Tübingen, Germany) was used as a pure TLR2 ligand. This was dissolved in DMSO. The final concentration of DMSO in injected NTS was 0.1%. For mesangial cell stimulation experiments, the highly purified TLR4 agonist LPS from *E. coli* R515 was used (581-007-L002; Alexis, San Diego, CA). Synthetic lipopeptide was used as a positive control to show that TLR4-deficient animals and TLR4-deficient mesangial cells do not have an inherent inability to recruit neutrophils or secrete chemokines, respectively.

Induction of Glomerulonephritis

TLR4-deficient mice (backcrossed seven generations to C57BL/6) were obtained from S. Akira (14), and wild-type C57BL/6 mice were from B&K Universal Ltd (Hull, UK). Animal experiments were performed according to Home Office regulations. Glomerulonephritis was induced by giving a single injection of 200 μl of NTS with or without TLR agonist (10 μg/mouse) or vehicle, or the TLR agonist was given alone.

Histologic Assessment of Glomerular Inflammation

The glomerular histology was assessed at 2 h after disease induction with NTS. Kidneys were fixed in Bouin's solution and stained with periodic acid-Schiff reagent. At 2 h, the number of neutrophils (identified by their characteristic nuclear morphology) per 50 glomerular cross-sections was counted. All sections scored were done so blindly. We compared this method with quantitative immunofluorescence. Frozen sections were incubated with rat anti-mouse neutrophil antibody (MCA771G; Serotec Ltd., Oxford, UK) and then with FITC-conjugated mouse anti-rat antibody (Jackson Immunoresearch Laboratories, West Grove, PA) Mean glomerular immunofluorescence scores were calculated and correlated with scores that were obtained when neutrophils were counted on periodic acid-Schiff–stained sections. The correlation coefficient was 0.78.

Urinary Albumin Measurement

Mice were housed singly in glass metabolic cages for 24 h immediately after disease induction with NTS to measure the 24-h albuminuria. The urinary albumin concentration was measured by radial immunodiffusion as described previously (13). The sensitivity of the radial immunodiffusion assay for albumin was 0.05 mg/ml.

Peripheral Neutrophil Counts and Measurement of Glomerular Sheep IgG Deposition

The total white cell number was counted on tail-vein blood after lysing red cells in 2% acetic acid, and a blood film was made to assess the percentage of neutrophils. Frozen sections were stained with FITC-conjugated donkey anti-sheep IgG (Jackson Immunoresearch Laboratories), and images of 20 glomeruli for each sample were saved. The mean fluorescence intensity was then measured using Adobe Photoshop software version 8.0 (Adobe Systems, San Jose, CA).

Construction of BM Chimeras

Mice at 6 to 10 wk of age were used to construct chimeric mice by irradiation with a dosage of 9 Gy and reconstitution with 5×10^6 donor BM cells. We previously established that this method gives >95% reconstitution with donor BM cells (15).

In Situ Hybridization

In situ hybridization was carried out on 3-μm paraffin-embedded kidney sections as described previously (6). Control samples of normal kidney from this previous study on interstitial disease were used and reexamined for glomerular expression.

Mesangial Cell Culture

Wild-type and TLR4-deficient mesangial cells were isolated and cultured as described previously(15) and used after four passages. Cells were grown in 24-well plates to generate supernatants for ELISA. All cultures were performed in triplicate. Cells were stimulated with TLR agonists (prepared as in Preparation of Nephrotoxic Serum and TLR Agonists) for 24 h, after which supernatants were taken for ELISA for CXCL1 and CXCL2 (R&D Systems, Minneapolis, MN). The sensitivity of the assays were 156 and 31 pg/ml for CXCL1 and for CXCL2, respectively. For reverse transcription–PCR, the primer sequences and conditions were as described previously (16).

CXCL1 and CXCL2 Blocking Studies

Monoclonal neutralizing antibodies to CXCL1 and CXCL2 (MAB453 and MAB452, respectively) were purchased from R&D Systems, along with rat IgG2a and IgG2b isotype controls. The antibodies and isotypes were diluted in sterile endotoxin-free PBS. One hour before disease induction with NTS and lipid A, each mouse was given a single intravenous injection that contained a combination of 100 μg of anti-CXCL1 and 100 μg of anti-CXCL2 or a single intravenous injection that contained a combination of 100 μg of rat IgG2a and 100 μg of rat IgG2b isotype controls.

Statistical Analyses

The groups of data were compared using the unpaired *t* test and GraphPad Prism software (GraphPad Software, San Diego, CA); when there were more than two groups of data, a one-way ANOVA was used. When the *F* test suggested that variances were significantly different, data were analyzed after a logarithmic transformation.

Results

Lipid A Exacerbates Disease in Wild-Type But Not TLR4-Deficient Mice

The role of lipid A in glomerulonephritis was assessed by giving NTS with or without 10 μg of lipid A, or lipid A was given alone, to wild-type and TLR4-deficient mice. In each case, the components were given together as a single intravenous injection. At 2 h, a significant glomerular neutrophil infiltrate was seen only in wild-type mice that were given lipid A and NTS together ($P < 0.001$ *versus* any other group). Data are shown graphically in Figure 1A, with representative histology in Figure 1C. To establish that the TLR4-deficient mice were able to mount a neutrophil influx, their response to the TLR2 agonist lipopeptide when given with NTS was assessed as a control. Nephritis was induced in TLR4-deficient mice by giv-ing the lipopeptide (10 μg per mouse) and NTS together. The TLR4-deficient mice developed a significant glomerular neu-trophil influx at 2 h in response to NTS and lipopeptide (Figure 1A). At 24 h after injection of lipid A and NTS, the renal neutrophil influx had virtually completely resolved, and there was no significant difference between TLR4-deficient and wild-type groups at this point (Figure 1B). The 24-h albuminuria was measured after disease induction in wild-type and TLR4-deficient mice that were given both lipid A and NTS together. Only wild-type mice produced significant albuminuria, with TLR4-deficient mice being protected against disease ($P < 0.0001$; Figure 1D). These data show that both antibody deposition and lipid A are necessary for the neutrophil influx in this model. When both NTS and lipid A are given together, there is a strong synergistic effect through a mechanism that is entirely depen-dent on TLR4.
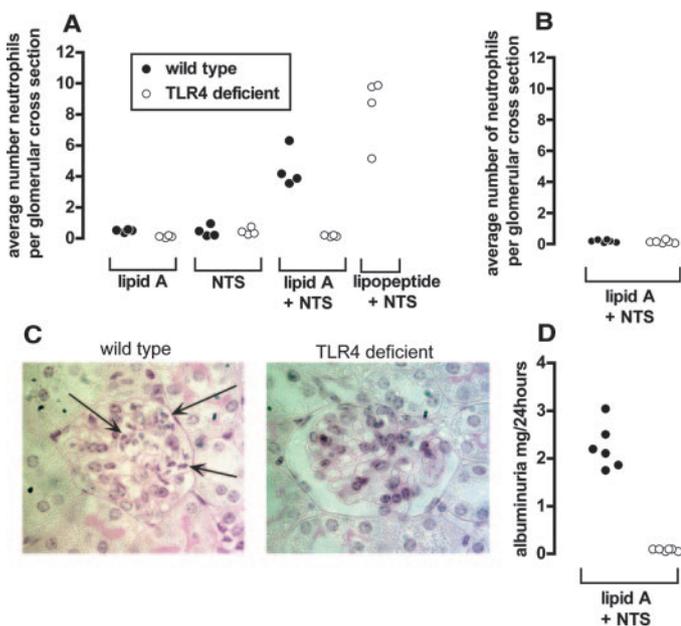
Differences in Disease Severity between Wild-Type and TLR4-Deficient Mice Cannot Be Explained by Differences in Circulating Numbers of Neutrophils

For investigation of whether the observed differences in glo-merular neutrophil influx were related to changes in circulating numbers, peripheral blood neutrophil counts were taken at baseline and 2 h after disease induction. There was no signifi-cant difference in neutrophil numbers at baseline between wild-type and TLR4-deficient mice. The TLR4-deficient group had a greater circulating neutrophil count than wild-type mice at 2 h after disease induction ($P < 0.05$). The circulating neu-trophil count remained unchanged in the wild-type group that was given NTS and lipid A when baseline numbers were compared with those seen at 2 h. These data are shown in Figure 2A. Therefore, TLR4-deficient mice had less severe dis-ease despite having a larger number of circulating peripheral blood neutrophils.

Differences in Disease Severity between Wild-Type and TLR4-Deficient Mice Cannot Be Explained by Differences in Glomerular Sheep IgG Binding

For examination of whether the decreased neutrophil influx in TLR4-deficient mice was related to less glomerular antibody
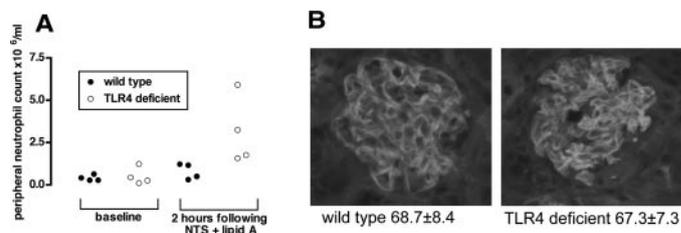

Figure 1. (A) Glomerular neutrophil influx in wild-type and Toll-like receptor 4 (TLR4)-deficient mice at 2 h after intrave-nous lipid A (10 μg) and nephrotoxic serum (NTS) given alone or together. There is a significantly greater neutrophil influx in the wild-type group that was given lipid A and NTS compared with any other group that was given either NTS alone, lipid A alone, or NTS and lipid A together ($P < 0.001$ for all compar-isons). In addition, to show that TLR4-deficient mice can mount a neutrophil influx in response to stimulation with a different TLR ligand, the glomerular neutrophil influx at 2 h is shown after administration of synthetic lipopeptide (TLR2 ligand) and NTS in a TLR4-deficient group. (B) Glomerular neutrophil in-flux at 24 h in wild-type and TLR4-deficient mice after intra-venous lipid A (10 μg/mouse) and NTS. (C) Representative glomerular histology at 2 h after the intravenous injection of NTS and lipid A in a wild-type and TLR4-deficient mice. At 2 h, there is a significant glomerular neutrophil influx in the wild-type group (neutrophil influx is highlighted by arrows) that is not seen in the TLR4-deficient group. (D) Twenty-four-hour albuminuria in wild-type and TLR4-deficient mice after lipid A and NTS given together. There was a significant difference between the two groups ($P < 0.0001$).

Figure 2. (A) Peripheral neutrophil counts at baseline and 2 h after intravenous lipid A and NTS in wild-type and TLR4-deficient mice. $P < 0.05$ for the TLR4-deficient mice compared with the wild-type mice at 2 h. (B) Immunofluorescence for glomerular deposition of sheep IgG at 2 h after NTS and lipid A in a wild-type mouse (left) and a TLR4-deficient mouse (right). The number given is the mean fluorescence $\pm$ SEM for each group of mice.

binding, the amount of sheep IgG deposited within the glomerulus was quantified. Kidney sections were taken at 2 h after disease induction with NTS and lipid A in wild-type and TLR4-deficient mice. There was no difference between the two groups, as shown in Figure 2B. TLR4-deficient mice therefore had less severe disease, even though they had a similar amount of sheep IgG in their glomeruli at 2 h.

TLR4 on Both BM-Derived and Non–BM-Derived Cells Is Required for Maximal Neutrophil Influx

For investigation of the relative contribution of TLR4 on leukocytes and non–BM-derived cells to the neutrophil influx, BM chimeras were constructed. Mice with TLR4 present on leukocytes but absent on non–BM-derived cells or with TLR4 present on non-BM cells but absent on leukocytes (wild-type BM into TLR4-deficient host [WT→TLR4$^{-/-}$] and TLR4-deficient BM into wild-type host [TLR4$^{-/-}$→WT]) were constructed. Two sham chimeric groups were also produced with TLR4 present on all cells (WT→WT) or with a complete absence of TLR4 (TLR4$^{-/-}$→TLR4$^{-/-}$). These sham chimeras were made using identical methods to those used for the chimeric mice. At 8 wk after reconstitution, the chimeric and sham chimeric groups were given NTS and lipid A (10 μg per mouse). A significant glomerular neutrophil influx at 2 h was seen in the WT→WT group compared with the TLR4$^{-/-}$→TLR4$^{-/-}$ group ($P < 0.001$), consistent with the results that we had previously seen in nonchimeric mice as shown in Figure 1. In addition, mice from both chimeric groups (WT→TLR4$^{-/-}$ and TLR4$^{-/-}$→WT) developed significant glomerular neutrophil influx when compared with the sham chimeric TLR4$^{-/-}$→TLR4$^{-/-}$ group ($P < 0.001$ for both comparisons) but significantly less disease than that seen in the WT→WT sham group ($P < 0.05$ and $P < 0.001$, respectively). Data are shown in Figure 3A, with representative histology shown in Figure 3C.

TLR4 on Both BM-Derived and Non–BM-Derived Cells Contributes to Albuminuria

Similar to the experiments outlined in the previous section, chimeric and sham chimeric mice were constructed to assess the relative importance of TLR4 on BM-derived and non–BM-derived cells toward the development of tissue injury as measured by albuminuria. At 10 wk after reconstitution, the chimeric and sham chimeric mice were given NTS and lipid A (10 μg per mouse). Significant albuminuria developed in the WT→WT group compared with the TLR4$^{-/-}$→TLR4$^{-/-}$ group ($P < 0.001$). Both chimeric groups developed a greater albuminuria than the sham TLR4$^{-/-}$→TLR4$^{-/-}$ group ($P < 0.05$ for TLR4$^{-/-}$→WT and $P < 0.001$ for WT→TLR4$^{-/-}$). There was significantly less albuminuria in the TLR4$^{-/-}$→WT group than in the WT→WT group ($P < 0.001$) but no significant difference in albuminuria between the WT→TLR4$^{-/-}$ and WT→WT groups. These data are shown in Figure 3B.

TLR4 mRNA Is Expressed within the Glomerulus

To investigate the glomerular expression of TLR4, *in situ* hybridization was carried out on normal murine kidney sec-
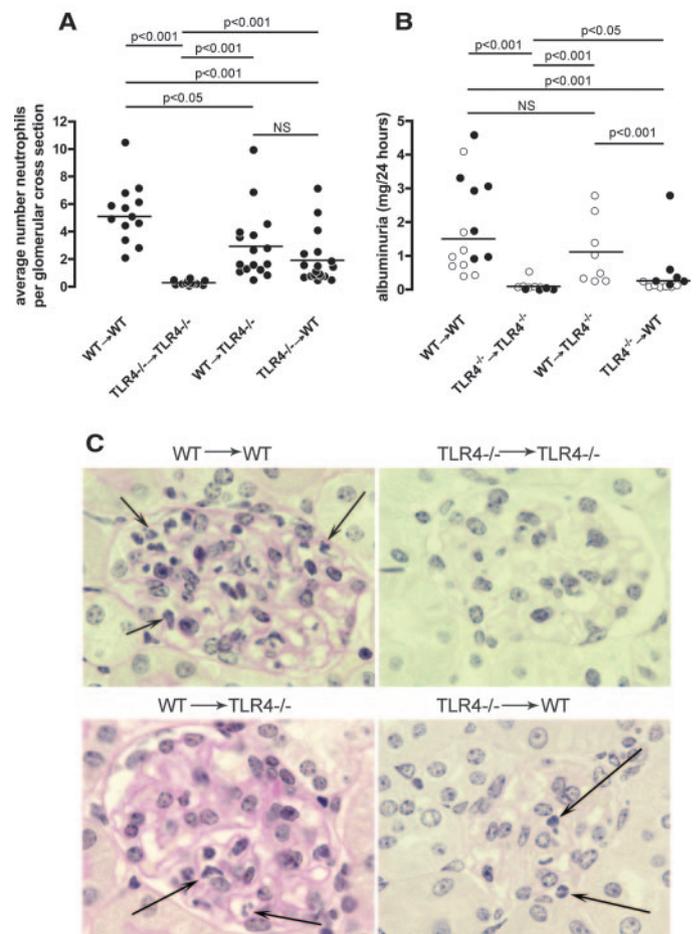

Figure 3. (A) Glomerular neutrophil influx at 2 h after intravenous lipid A and NTS in chimeric mice with TLR4 present on intrinsic renal cells but absent on BM-derived cells (TLR4$^{-/-}$ →WT) and *vice versa* (WT→TLR4$^{-/-}$) and sham chimeric mice. (B) Albuminuria at 24 h after intravenous lipid A and NTS in sham WT→TLR4$^{-/-}$ and TLR4$^{-/-}$→WT chimeric mice. The data illustrated are from two independent experiments indicated by ○ and ●. Statistics given for the WT→TLR4$^{-/-}$ group compare only with data from the same experiment. (C) Representative histology taken at 2 h after NTS and lipid A given together. Neutrophil influx is highlighted by arrows.

tions. Sections were incubated with the antisense riboprobe to assess the expression of TLR4 mRNA, and as a control, they were incubated with a sense riboprobe. Results show that TLR4 mRNA is expressed within the glomerulus in mesangial cells and epithelial cells (both podocytes and Bowman's capsule). In total, kidneys from four mice were examined, and all showed an identical distribution and intensity of staining. Representative pictures from one of these four mice are shown in Figure 4.

Mesangial Cells Express TLR4 and Produce CXC Chemokines in Response to Lipid A

Mesangial cells could potentially be one cell type within the kidney that contributes to disease exacerbation. Therefore, the expression of TLR4 by mesangial cells and their ability to respond to TLR4 ligands was assessed. Primary cultures of
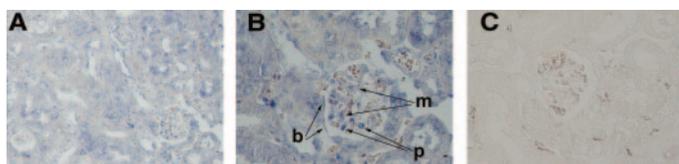
Figure 4. Localization of TLR4 mRNA in the glomerulus of normal mice by *in situ* hybridization. In total, kidneys from four mice were examined, and all showed an identical distribution and intensity of staining. Representative pictures from one of these four mice are shown. TLR4 localization is shown by incubation with the antisense riboprobe at low power (A) and high power (B). As a negative control, sections were incubated with the sense riboprobe (C). Positive staining is shown within the mesangium (m) and also in podocytes (p) and Bowman's capsule (b).

mouse mesangial cells were used. First we examined the expression of TLR4 on mesangial cells by reverse transcription–PCR. Figure 5A shows that mRNA for TLR4 is expressed by mesangial cells. We then stimulated mesangial cells with LPS or vehicle and measured levels of CXCL1 and CXCL2 in the supernatant by ELISA. These chemokines are known to be important for neutrophil chemotaxis. We established a dosage response for CXCL1 as shown in Figure 5B. A dosage of 1 μg/ml LPS was then used in subsequent studies. To show that

the response was mediated by TLR4, we compared the response of wild-type and TLR4-deficient mesangial cells. Figure 5C shows that wild-type but not TLR4-deficient mesangial cells produced CXCL1 in response to LPS. Figure 5D shows that wild-type but not TLR4-deficient mesangial cells produced CXCL2 protein in response to LPS. To establish that there was no intrinsic inability of TLR4-deficient mesangial cells to produce CXCL1 and CXCL2, these cells were also stimulated with the synthetic lipopeptide and mounted a good response. These data showed that mesangial cells produced CXCL1 and CXCL2 protein in response to LPS, an effect entirely mediated by TLR4.

CXCL1 and CXCL2 Blocking Studies

To investigate the importance of CXCL1 and CXCL2 production by intrinsic renal cells in neutrophil recruitment in this model, we carried out blocking studies. Chimeric mice that had TLR4 only on intrinsic renal cells but not on BM-derived cells were constructed. Mice were given 100 μg of anti-CXCL1 and anti-CXCL2 antibodies or the equivalent-dosage of isotype control. After 1 h, disease was induced by the administration of 0.2 ml of NTS with 10 μg of lipid A. Assessment of disease severity at 2 h is shown in Figure 6. There was significantly less glomerular neutrophil influx in the group that was given anti-CXCL1 and anti-CXCL2 antibodies than in the group that was given isotype control ($P < 0.0001$), showing that the glomerular neutrophil influx in this model depends on the production of CXC chemokines in response to TLR4 ligation on intrinsic renal cells.

Discussion

We have shown that the pure TLR4 agonist lipid A is required in addition to glomerular antibody deposition to cause disease. Using TLR4-deficient mice, we showed that this is through a mechanism that depends on the presence of TLR4. We showed that the TLR4-deficient mice are able to produce a
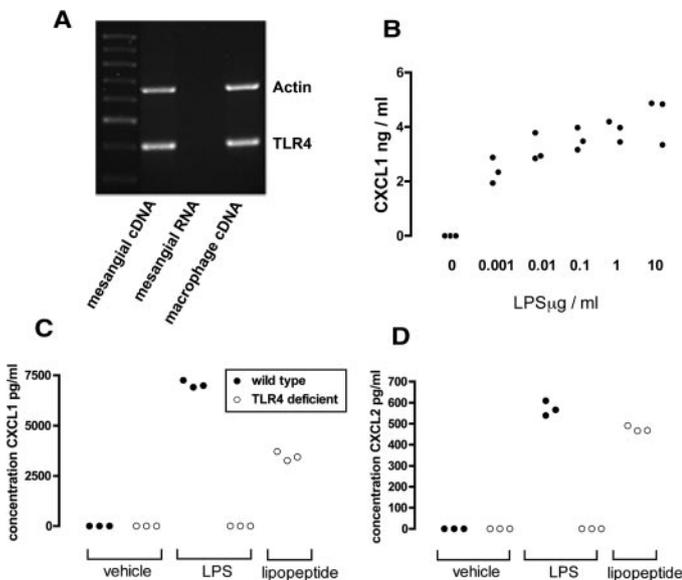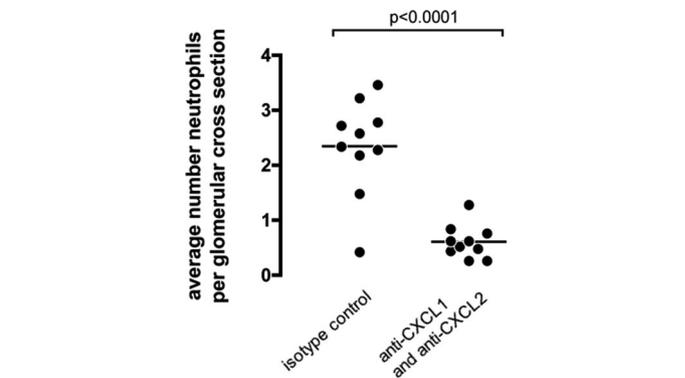

Figure 5. (A) Reverse transcription–PCR for TLR4 on mRNA that was extracted from mesangial cells using actin as a reference gene. Mesangial cell RNA and macrophage cDNA are included as negative and positive controls. (B) Dosage-response curve showing the concentration of CXCL1 in supernatant taken from mesangial cells that were stimulated with increasing concentrations of LPS. (C) CXCL1 concentration measured by ELISA in supernatant of wild-type and TLR4-deficient mesangial cells that were stimulated with LPS or lipopeptide. (D) CXCL2 concentration measured by ELISA in supernatant of wild-type and TLR4-deficient mesangial cells that were stimulated with LPS or lipopeptide.

Figure 6. Glomerular neutrophil influx at 2 h after disease induction in chimeric mice with TLR present only on intrinsic renal cells (TLR4$^{-/-}$→WT), which were given anti-CXCL1 and anti-CXCL2 antibody or isotype control at 1 h before disease induction with NTS and lipid A. Administration of anti-CXCL1 and anti-CXCL2 antibody before disease induction significantly reduced the renal neutrophil influx at 2 h ($P < 0.001$) when compared with the isotype control group.

glomerular neutrophil infiltration in response to antibody deposition and stimulation with a TLR2 ligand, demonstrating that the TLR4-deficient mice do not have an intrinsic inability to mount a neutrophil response.

The absence of significant glomerular inflammation in TLR4-deficient mice when compared with the wild-type group may have been due to differences in the circulating leukocyte counts. At 2 h after the administration of lipid A and serum, the TLR4-deficient group had a significantly greater peripheral neutrophil count than the wild-type group. This is probably due to the release of neutrophils from the BM in response to the presence of foreign serum, but they are unable to migrate to the kidney because of the lack of either tissue or neutrophil activation. We also showed by quantitative immunofluorescence that the differences in disease severity that were seen in the two groups cannot be attributed to differences in sheep IgG binding within the glomerulus, because at 2 h, both groups had indistinguishable amounts.

We showed a role for TLR4 on both BM-derived and non–BM-derived cells in this model by constructing BM chimeras. In the chimeric mice, neutrophil influx was shown to be more variable than in preliminary experiments using normal wild-type mice. Despite this, sham chimeras that were totally TLR4 sufficient demonstrated significant glomerular neutrophil influx and albuminuria when compared with those that were totally TLR4 deficient when exposed to NTS and lipid A, as we expected. Both chimeric groups demonstrated significant glomerular neutrophil infiltrate at 2 h after the administration of NTS and lipid A when compared the sham-negative group but significantly less than the sham-positive group, showing that TLR4 on both circulating leukocytes and intrinsic renal cells is important. With respect to albuminuria, both chimeric groups developed significantly greater albuminuria than the sham-negative group. However, only the chimeric group with TLR4 present on intrinsic renal cells had significantly less albuminuria than the sham group that was totally TLR4 sufficient. The chimeric group with TLR4 present on neutrophils had similar albuminuria to the sham group that was totally TLR4 sufficient, despite lower neutrophil numbers at 2 h. Therefore, although TLR4 on both intrinsic renal cells and circulating leukocytes contributed equally to glomerular neutrophil influx, it is TLR4 on circulating leukocytes that contributed greatest to tissue injury as assessed by albuminuria.

The response of neutrophils to TLR4 ligation has been previously investigated. TLR4 ligation on human neutrophils causes changes in adhesion molecule expression, superoxide generation, and release of proinflammatory cytokines and augments neutrophil migration and survival (17–19). Studies in the mouse have shown that endotoxin causes L selectin shedding and CD11b upregulation, which is known to be essential for leukocyte adhesion and transmigration (20). In this study, we focused on the role of local renal cells in disease exacerbation.

Three studies have highlighted the potential importance of TLR4 on local tissue cells in disease. TLR4 on lung endothelial cells and not circulating leukocytes was important in the sequestration of neutrophils in a murine model of septic lung injury (21). Resistance to uropathogenic *E. coli* in the bladder

and *E. coli* pyelonephritis requires the presence of TLR4 on both stromal cells and circulating leukocytes (22,23). These studies show an important role for TLR4 in the control of infection, and our study differs from these because we show a role for TLR4 stimulation of intrinsic renal cells in the expression of antibody-mediated inflammation and tissue damage.

In situ hybridization showed expression of TLR4 in mesangial cells and epithelial cells within the glomerulus. Although both cell types could contribute to the observed effects of ligation of TLR4 on intrinsic renal cells, mesangial cells were a particularly likely candidate. They are immunologically active cells that are known to be capable of producing a number of chemokines and cytokines and have also previously been noted to respond to stimulation by endotoxin (24–26). No previous study has shown the expression of TLR4 in primary cultures of mesangial cells, and we have shown this. Furthermore, in response to stimulation with pure LPS, wild-type but not TLR4-deficient mesangial cells produce CXC chemokines that are responsible for neutrophil chemotaxis (27,28). This provides a biologically plausible mechanism whereby TLR4 agonists, acting on renal cells, could exacerbate disease. To support these findings, we carried out *in vivo* studies. We showed using chimeras that had TLR4 only on intrinsic renal cells that blocking CXCL1 and CXCL2 reduces the glomerular neutrophil influx that is seen at 2 h. This demonstrates that the production of CXC chemokines in response to ligation of TLR4 on intrinsic renal cells contributes to glomerular neutrophil influx in this model.

It has been shown that TLR3 ligands exacerbate lupus nephritis. Although in this study TLR3 was expressed on mesangial cells, there was no direct demonstration of a contribution to disease by mesangial cell TLR3 (29). The same group investigated the role of TLR7 and TLR9 ligands in lupus nephritis, and both exacerbated disease. Neither TLR7 nor TLR9 was expressed on intrinsic renal cells, and the mechanisms in these studies involved effects on the adaptive immune response (30,31). Taken together, these studies highlight the individual roles for each specific TLR; although they share the same common structure and signaling pathways, each has a unique distribution and function in relation to ligand stimulation. In the autologous phase of nephrotoxic nephritis, nephrotoxic antibody and LPS were required to produce disease at time points beyond 2 wk (32). It is possible that differences in disease were related to an effect on the adaptive immune response in this study.

Conclusion

We have shown that TLR4 ligands promote renal injury by increasing inflammation, independent of any effects from the adaptive immune response. Renal autoimmune disease is frequently worsened by infection, in the absence of any measurable effect on the serum levels of pathogenic autoantibodies, such as anti-GBM or ANCA (4), and we provide a molecular explanation for this clinical observation. Our data suggest that specific targeting of mesangial cell TLR4 may be a useful therapeutic strategy for preventing exacerbations of glomerulonephritis, because disease is significantly less in chimeric mice

with TLR4 absent on intrinsic renal cells. These findings contribute to our understanding of the diverse mechanisms by which TLR may contribute to the exacerbation of autoimmune disease and in particular glomerulonephritis.

Acknowledgments

Disclosures

None.

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