© 2010 International Society of Nephrology

Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis

Shaun A. Summers[1], Betty S. van der Veen[2], Kim M. O'Sullivan[1], Poh-Yi Gan[1], Joshua D. Ooi[1], Peter Heeringa[2], Simon C. Satchell[3], Peter W. Mathieson[3], Moin A. Saleem[4], Kumar Visvanathan[1], Stephen R. Holdsworth[1,5] and A. Richard Kitching[1,5]

[1]*Department of Medicine, Centre for Inflammatory Diseases, Monash University, Clayton, Victoria, Australia;* [2]*Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;* [3]*Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK;* [4]*Children's Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK and* [5]*Department of Nephrology, Monash Medical Centre, Clayton, Victoria, Australia*

Antimyeloperoxidase antibodies can cause crescentic glomerulonephritis and pulmonary hemorrhage. Toll-like receptors (TLRs) respond to infectious agents activating host defenses, whereas infections potentially initiate disease and provoke relapses. Neutrophils were found to be key effector cells of injury in experimental models, as disease does not occur in their absence and injury is enhanced by lipopolysaccharide (LPS). In this study, highly purified LPS (a pure TLR4 ligand) acted with antimyeloperoxidase antibodies to synergistically increase kidney and lung neutrophil recruitment and functional injury; effects abrogated in TLR4-deficient mice. Increased kidney TLR4 expression after stimulation predominantly occurred in glomerular endothelial cells. Enhanced glomerular neutrophil recruitment correlated with increased kidney mRNA expression of CXCL1 and CXCL2 (homologs of human CXCL8), whereas their preemptive neutralization decreased neutrophil recruitment. Disease induction in bone marrow chimeric mice showed that TLR4 in both bone marrow and renal parenchymal cells is required for maximal neutrophil recruitment and glomerular injury. Further studies in human glomerular cell lines stimulated with LPS found that glomerular endothelial cells were the prominent sources of CXCL8. Thus, our results define a role for TLR4 expression in bone marrow–derived and glomerular endothelial cells in neutrophil recruitment and subsequent functional and histological renal injury in experimental antimyeloperoxidase glomerulonephritis.

Kidney International advance online publication, 15 September 2010; doi:10.1038/ki.2010.327

KEYWORDS: anti-MPO antibody; chemokine; endothelial cell; neutrophil

Correspondence: *A. Richard Kitching, Department of Medicine, Monash Medical Centre, Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia. E-mail: richard.kitching@med.monash.edu.au*

Parts of these studies were published in abstract form (*Nephrology* **13** (Suppl 3): A140, *J Am Soc Nephrol* 2008 Nov(S) A659).

Received 7 December 2009; revised 25 June 2010; accepted 20 July 2010

Small vessel vasculitis and pauci-immune necrotizing glomerulonephritis (GN) induced by antineutrophil cytoplasmic antibodies (ANCAs) target specific neutrophil cytoplasmic antigens, myeloperoxidase (MPO), and proteinase 3.[1,2] Combined renal and pulmonary disease is common in ANCA vasculitis and has considerable morbidity and mortality. Experimental animal studies have shown that ANCA are pathogenic. Passive transfer of ANCA can induce necrotizing GN and/or pulmonary capillaritis.[3–7] Links between infection and ANCA vasculitis are well established. Seasonal variation in patients presenting with the disease suggests a correlation with microbial infection,[8] infection may predate disease initiation and/or relapse[9–12] and prophylactic antibiotic therapy has also been shown to successfully decrease disease relapses in ANCA vasculitis.[13] Infection is likely to be important in experimental ANCA models and lipopolysaccharide (LPS) dose dependently increases renal injury after the passive transfer of MPO–ANCA.[6]

Neutrophils are amongst the first immune cells to traffic to inflamed sites. In experimental ANCA-induced GN neutrophils are the primary effector cells and neutrophil depletion protects mice from renal injury.[4] In humans, the chemokine CXCL8 (interleukin-8) is a potent neutrophil chemoattractant.[14] Renal biopsies from patients with ANCA disease demonstrated positive CXCL8 immunostaining in crescentic glomerular lesions, suggesting that CXCL8 contributes to glomerular injury seen in ANCA-associated GN.[15] The murine chemokines CXCL1 (KC) and CXCL2 (MIP-2), that bind to CXCR2, are homologs of human CXCL8[16] and serve as major chemoattractants for neutrophils in mice.[17]

Toll-like receptors (TLRs) recognize pathogen associated molecular patterns from infectious agents and after ligation activate immune cells. TLR4 is expressed on neutrophils[18] and augments their migratory responses.[19] Previously, TLR4 has been demonstrated in the kidney, in the glomerulus on mesangial cells, epithelial cells,[20] and also in proximal and distal tubular epithelial cells,[21] but not in glomerular endothelial cells (GEnCs).

Starting from the known capacity of LPS to enhance the activity of anti-MPO antibodies in experimental systems,[6,22] we aimed to define and explore a pathogenic and mechanistic role for TLR4 in experimental ANCA-induced glomerular neutrophil recruitment. We studied the effect of LPS and anti-MPO antibodies on glomerular and pulmonary neutrophil recruitment, which develops early in the diseases process and is TLR4 dependent. Functional and histological renal injury, which develops later, is also TLR4 dependent. TLR4 expression in the glomerulus increases after LPS and anti-MPO antibody stimulation. TLR4 is produced by GEnCs, which are also positive for CXCL1 and CXCL2. *In vitro* LPS stimulation of human glomerular cell lines implicates GEnC as a major source of TLR4. We demonstrate a functional relationship between CXCL1 and CXCL2, glomerular neutrophil recruitment and subsequent renal injury, and demonstrate that full expression of these chemokines is TLR4 dependant. Furthermore, we demonstrate in human cell lines that CXCL8 mRNA and protein production increases considerably after LPS stimulation, predominantly mediated through GEnC. Finally using bone marrow (BM) chimeric mice we identify the individual contributions of BM and tissue cell (TC) TLR4 to neutrophil recruitment and glomerular injury.

RESULTS

ANCA/LPS-induced glomerular neutrophil recruitment and lung MPO activity

Glomerular and pulmonary neutrophil recruitment was induced by administering either highly purified LPS (hpLPS, which specifically engages TLR4) with anti-MPO antibodies, anti-MPO antibodies alone, or hpLPS and control (anti-ovalbumin (OVA)) antibodies to genetically intact wild-type (WT) C57BL/6 mice. WT mice were injected with hpLPS, then anti-MPO or anti-OVA antibodies at 2 h and killed after a further 3 h. LPS, anti-MPO antibodies, or both induced glomerular leukocyte recruitment (Figure 1a). Control anti-OVA antibodies alone had no effect on glomerular neutrophil recruitment compared with untreated mice (untreated mice 0.25 ± 0.01 neutrophils/glomerular cross-section (n/gcs), mice given anti-OVA antibodies 0.40 ± 0.02 n/gcs). Mice injected with anti-MPO antibodies alone exhibited a significant glomerular neutrophil influx, similar to that observed in mice treated with hpLPS and anti-OVA antibodies. Together, hpLPS and anti-MPO antibodies synergistically increased glomerular neutrophil recruitment. Representative photomicrographs of glomerular neutrophil recruitment are shown (Figure 1c–e). In the same studies, neutrophil accumulation in lung tissue was assessed by measuring pulmonary MPO activity (Figure 1b). Untreated WT mice had 0.62 ± 0.03 U of MPO activity per gram of lung tissue. Administration of either hpLPS and anti-OVA antibodies, or anti-MPO antibodies alone increased MPO activity to a similar degree. Administration of hpLPS and anti-MPO antibodies led to a further increase in lung MPO activity. Anti-OVA antibodies alone had minimal effect (1.04 ± 0.29 U/g).

The requirement for TLR4 in maximal neutrophil recruitment was confirmed by comparing TLR4$^{-/-}$ mice with WT mice (Figure 1f and g). Glomerular neutrophil recruitment in untreated TLR4$^{-/-}$ mice (0.23 ± 0.06n/gcs) was similar to untreated WT controls. Neutrophil recruitment in TLR4$^{-/-}$ mice given hpLPS (with anti-OVA antibodies) was similar to untreated TLR4$^{-/-}$ mice. TLR4$^{-/-}$ mice given anti-MPO antibodies had similar glomerular neutrophil numbers to WT mice given anti-MPO antibodies, but TLR4$^{-/-}$ mice given hpLPS and anti-MPO antibodies recruited fewer neutrophils to glomeruli compared with WT given hpLPS and anti-MPO antibodies. Similar TLR4-dependent patterns were present in the pulmonary leukocyte recruitment, although in the absence of TLR4 (baseline activity in untreated TLR4$^{-/-}$ mice 0.61 ± 0.07 U/g), pulmonary MPO activity was reduced in all three groups of mice: those given hpLPS and anti-OVA antibodies, hpLPS and anti-MPO antibodies, as well as those injected with anti-MPO antibodies alone.

TLR4 is expressed in murine glomeruli and produced by murine GEnCs and human intrinsic glomerular cells

We then examined TLR4 production in murine kidneys and human glomerular cells. TLR4 protein was readily detected in glomeruli of WT mice treated with LPS and anti-MPO antibodies. TLR4$^{-/-}$ mice treated with anti-MPO antibodies were a negative control. Using confocal microscopy TLR4 was colocalized with GEnCs. Other glomerular cell types, probably podocytes and possibly mesangial cells also expressed TLR4. Illustrative photomicrographs are shown in Figure 2a–d. TLR4 mRNA expression from glomerular and tubular interstitial compartments was assessed using laser capture microdissection (Figure 2e; the mean value for the tubulointerstitium was assigned a value of 1). Baseline TLR4 mRNA expression in glomeruli was 20-fold higher than the tubulointerstitium and increased further 24 h after LPS and anti-MPO antibodies. There was no change in tubulointerstitial TLR4 expression. We then analyzed TLR4 mRNA expression in human conditionally immortalized GEnC (ciGEnC), podocytes, and mesangial cell lines after stimulation with LPS (Figure 2f; the mean value for podocyte basal TLR4 mRNA expression was assigned a value of 1). Basal TLR4 mRNA expression was highest in ciGEnC and not detected in mesangial cells. Flow cytometric analysis of ciGEnC for TLR4 protein confirmed the expression of TLR4 protein (Figure 2g). After LPS, TLR4 mRNA expression was increased at 24 h in ciGEnC, but podocyte and mesangial cell TLR4 mRNA expression was unchanged.

CXCL1 and CXCL2 is induced in kidney tissue after hpLPS and anti-MPO antibodies

To investigate mechanisms of glomerular neutrophil recruitment we studied the neutrophil chemoattractants CXCL1 and CXCL2 in renal tissue. CXCL1 and CXCL2 expression was assessed 5 h after both hpLPS and anti-MPO antibodies. Compared with untreated WT mice (means for untreated WT CXCL1 or CXCL2 mRNA expression were assigned a value of 1), CXCL1 mRNA expression increased 5 h after

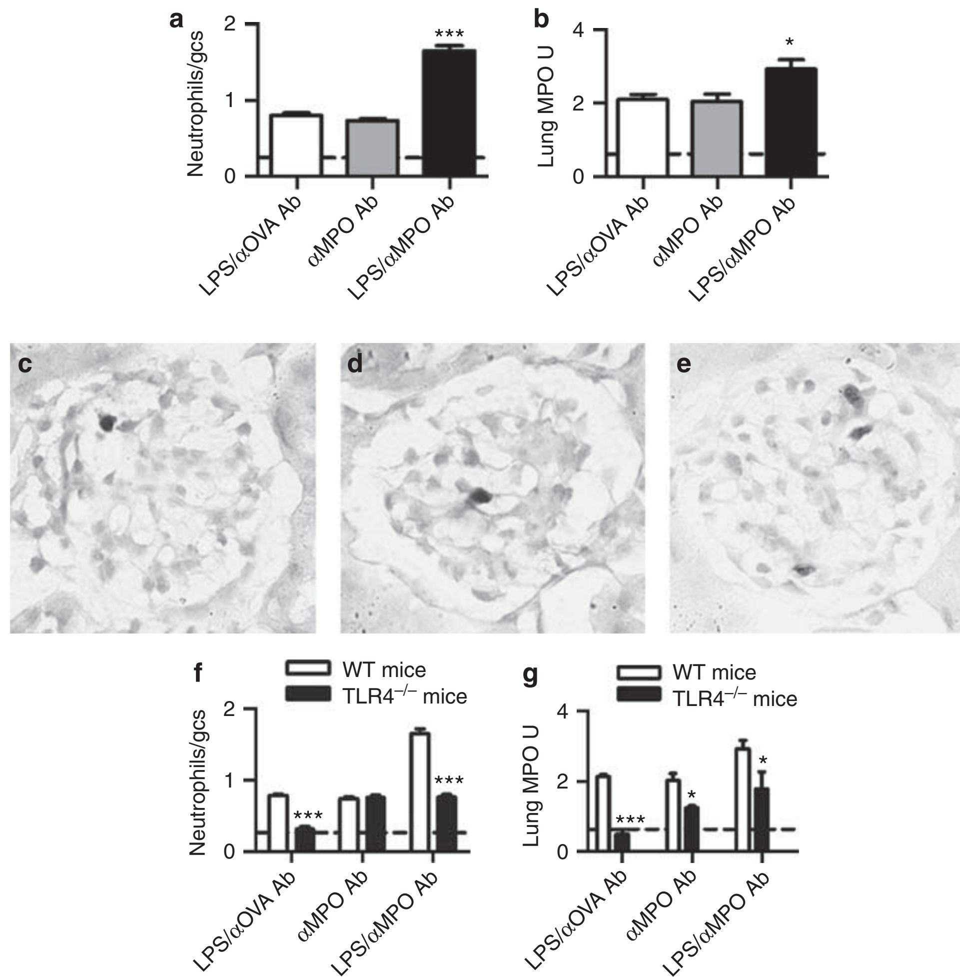

Figure 1 | Leukocyte recruitment after anti-MPO antibody administration. (**a**) Glomerular neutrophil recruitment to C57BL/6 wild-type (WT, $n=6$) mice. Highly purified lipopolysaccharide (hpLPS) and anti-OVA (αOVA) antibodies ($n=6$) or anti-MPO (αMPO) antibodies alone ($n=6$) increased glomerular neutrophil recruitment compared with untreated WT animals ($n=4$), represented as a dotted line ($P<0.001$). Co-administration of hpLPS and αMPO antibodies ($n=6$) induced more neutrophil recruitment compared with hpLPS and αOVA antibodies or αMPO antibodies alone. (**b**) Pulmonary MPO activity in WT mice. Untreated WT mice had 0.62 U of MPO activity/g (dotted line); MPO activity was increased in all antibody injected groups, compared with untreated mice. Significantly more pulmonary MPO activity was seen in the WT mice treated with hpLPS and αMPO antibodies compared with other treatment groups. Photomicrographs representative of glomerular neutrophil recruitment, with one glomerular neutrophil in WT mice treated with hpLPS and αOVA antibodies (**c**) or αMPO antibodies alone (**d**), or three neutrophils in mice treated with LPS and αMPO antibodies (**e**) are demonstrated. In panels **f** and **g** glomerular and lung neutrophil recruitment in WT mice are compared with recruitment in TLR4$^{-/-}$ mice ($n=4$). Neutrophil recruitment in untreated in TLR4$^{-/-}$ mice ($n=5$) did not differ from untreated WT mice (dotted line). Glomerular neutrophil recruitment (**f**) and lung MPO activity (**g**) decreased in TLR4$^{-/-}$ mice compared with WT mice. *$P<0.05$, ***$P<0.001$. Original magnification x400. gcs, glomerular cross-section; MPO, myeloperoxidase; OVA, ovalbumin; TLR, Toll-like receptor.

hpLPS and anti-MPO antibodies, in a partly TLR4-dependent manner (Figure 3a). A similar pattern was seen with CXCL2 mRNA expression, but the reduction in gene expression in the absence of TLR4 was more profound (Figure 3b). Immunohistochemical examination of glomeruli for CXCL1 and CXCL2 demonstrated minimal signal in untreated WT mice, with increased expression in all experimental groups (data not shown). WT mice given hpLPS and anti-MPO antibodies exhibited increased glomerular (with surrounding tubular) staining of CXCL1 and CXCL2 when compared with mice given either hpLPS with anti-OVA antibodies, or anti-MPO antibodies alone. As hypothesized, compared with WT mice, CXCL1 and CXCL2 staining was decreased in TLR4$^{-/-}$ mice given hpLPS and anti-MPO antibodies (Figure 3c and d). Representative kidney sections of CXCL1 (Figure 3e and f) and CXCL2 (Figure 3g and h) immunostaining in WT and TLR4$^{-/-}$ mice treated with hpLPS and anti-MPO antibodies are shown.

CXCL1 and CXCL2 colocalize with GEnCs, which also express TLR4

To determine whether murine GEnCs are a source of CXCL1 and CXCL2 production, we immunostained kidneys from WT mice treated with LPS and anti-MPO antibodies for an endothelial marker (CD31), TLR4, CXCL1, and CXCL2 using confocal microscopy CXCL1 colocalized to GEnC and to

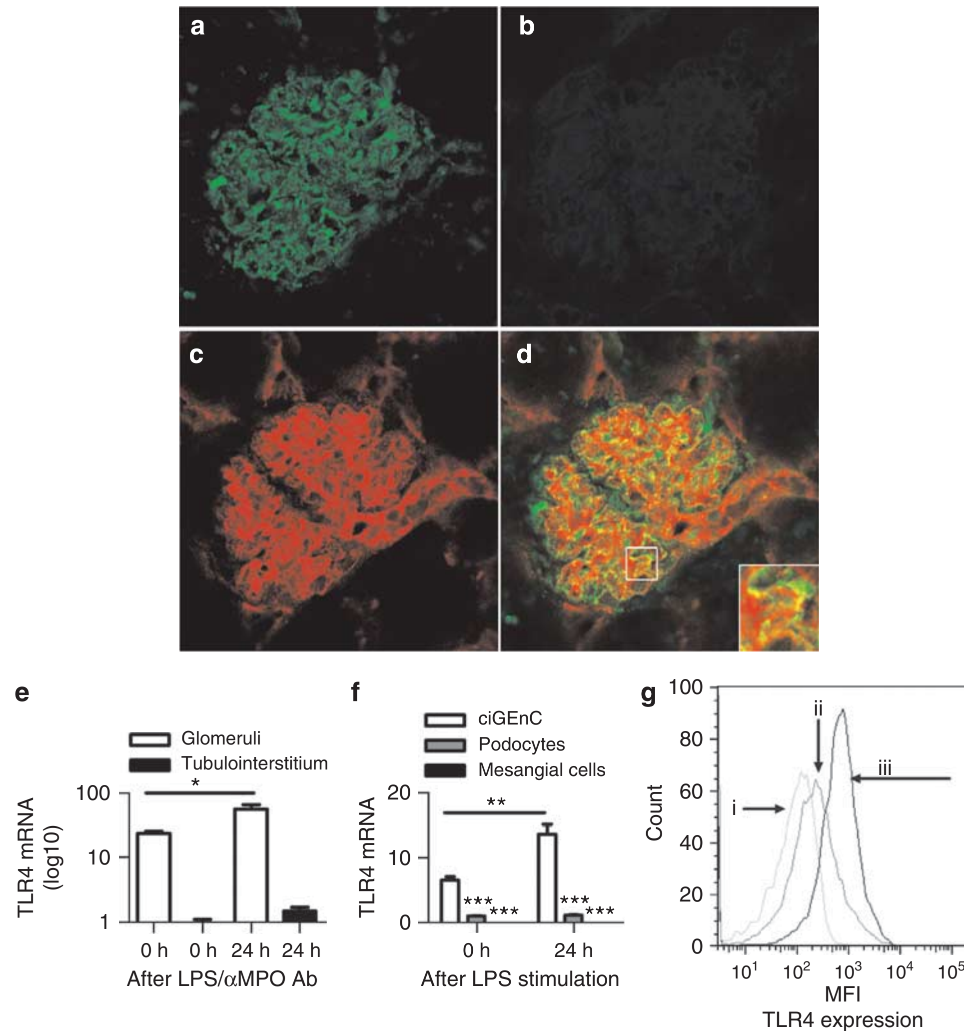

Figure 2 | TLR4 staining and expression in the kidney. Positive TLR4 staining (green) was detectable in glomeruli of WT mice treated with LPS and anti-MPO antibodies (**a**). No TLR4 staining was visible in $TLR4^{-/-}$ mice treated with anti-MPO antibodies (**b**). Glomeruli from WT mice were stained with anti-CD31 antibodies to identify endothelial cells (red staining) (**c**). Merged image of TLR4 and endothelial cell staining that identifies endothelial cell TLR4 production (yellow) (**d**). After stimulation with LPS and anti-MPO (αMPO) antibodies there was an increase in TLR4 mRNA expression in microdissected murine glomeruli, and little change in TLR4 expression was seen in the tubulointerstitium ($n=4$) (**e**). In human conditionally immortalized ciGEnC lines, baseline TLR4 expression was increased compared with both podocytes and mesangial cells (which did not express TLR4), $n=6$ for all experimental groups (**f**). After LPS stimulation TLR4 expression increased only in endothelial cells. (**g**) TLR4 protein expression by ciGEnC. Flow cytometric analysis of cultured ciGEnC incubated with no antibody (i), isotype control antibody (ii), and anti-human TLR4 antibody (iii) demonstrated TLR4 protein expression. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Original magnification x800. Ab, antibody; ciGEnC, conditionally immortalized glomerular endothelial cell, LPS, lipopolysaccharide; MFI, mean florescence intensity; MPO, myeloperoxidase; TLR, Toll-like receptor; WT, wild type.

glomerular cells producing TLR4. GEnC produce both TLR4 and CXCL1 (Figure 4a–f). Similarly, CXCL2 colocalized with GEnC and cells producing TLR4. Furthermore, we demonstrated that GEnC can produce CXCL2 and TLR4 (Figure 4g–l). Relevant negative controls are shown in Supplementary Figure S1.

Neutrophil recruitment is CXCL1 and CXCL2 dependent

Given the enhanced expression of CXCL1 and CXCL2 in experimental anti-MPO antibody-induced glomerular neutrophil recruitment, we neutralized either protein by administering a monoclonal anti-CXCL1 antibody, an anti-CXCL2 antibody, or isotype control 1 h before hpLPS and anti-MPO antibodies and assessed neutrophil recruitment. Compared with isotype control antibody, mice given either anti-CXCL1 or anti-CXCL2 antibody before hpLPS and anti-MPO antibodies showed significant decreases in glomerular neutrophil recruitment and lung MPO activity (Figure 5a–b), confirming a functional role for both CXCL1 and CXCL2 in glomerular and pulmonary neutrophil recruitment.

TC TLR4 contributes to neutrophil recruitment

Given the expression of TLR4 in glomeruli, we defined the contributions of BM and TC TLR4 (glomerular and lung) expression in neutrophil recruitment by injecting LPS and anti-MPO antibodies into TLR4 BM chimeric mice. Chimeric mice were generated by injecting intact or deficient

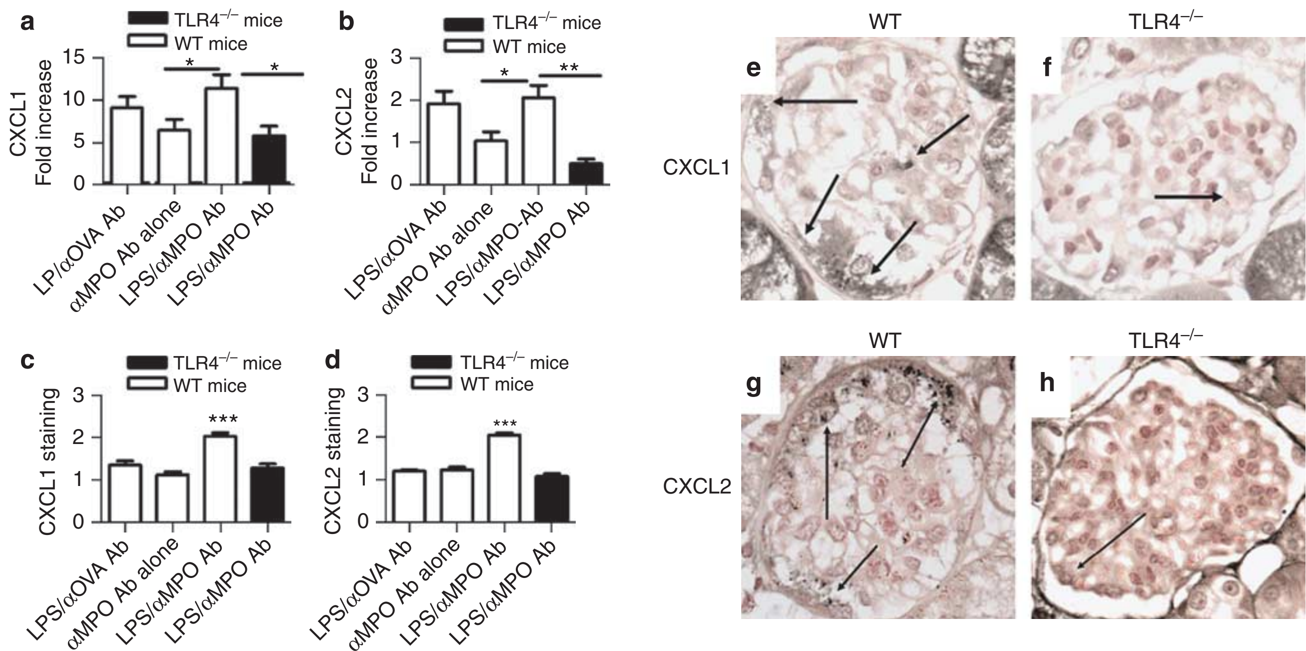

Figure 3 | CXCL1 and CXCL2 kidney mRNA expression and immunostaining. Using tissues from experiments detailed in Figure 1, kidney CXCL1 mRNA expression was increased in WT mice after hpLPS and anti-OVA (αOVA) antibodies ($P<0.001$), $n=6$, αMPO antibodies alone ($P<0.05$), $n=6$, and hpLPS and αMPO antibodies ($P<0.001$), $n=6$. Compared with WT mice given hpLPS and αMPO antibodies, TLR4$^{-/-}$ mice (closed bars) treated with hpLPS and αMPO antibodies expressed less CXCL1 (**a**), $n=4$. Kidney CXCL2 mRNA expression in WT mice increased after treatment with hpLPS and αMPO antibodies ($P<0.05$). Compared with WT mice treated with hpLPS and αMPO antibodies, TLR4$^{-/-}$ mice treated with hpLPS and αMPO antibodies expressed less CXCL2 (**b**). Immunostaining showed that CXCL1 (**c**) and CXCL2 (**d**) were significantly increased in WT mice treated with hpLPS and αMPO antibodies compared with all other groups. Representative glomerular sections from (**e**) WT mice and (**f**) TLR4$^{-/-}$ mice treated with hpLPS and αMPO antibodies and immunostained for CXCL1 are shown. Similarly treated (**g**) WT and (**h**) TLR4$^{-/-}$ glomeruli immunostained for CXCL2 are shown. Black arrowheads represent areas where staining intensity was most pronounced. Increased staining was seen in WT mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Original magnification ×400. hpLPS, highly purified lipopolysaccharide; MPO, myeloperoxidase; OVA, ovalbumin; WT, wild type.

BM into irradiated mice. WT BM transplanted into WT mice (BM+TC+; 'sham' chimeras) were a positive control, and TLR4 TC intact, BM TLR4 deficient (BM–C+) and TC TLR4 deficient, BM intact (BM+TC–) chimeras were studied. Both BM and TC TLR4 are required for maximal neutrophil recruitment. Compared with BM+TC+ chimeras, glomerular neutrophil recruitment was reduced in either TLR4 BM–TC+ chimeras or TLR4 BM+TC– mice (Figure 6a), but BM-derived TLR4 has a more prominent role. Both BM and TC TLR4 are required for maximum pulmonary neutrophil recruitment (Figure 6b).

Glomerular CXCL1 and CXCL2 production was assessed in kidneys of chimeric mice. Compared with 'sham' chimeras (BM+TC+), semiquantitative assessment of CXCL1 and CXCL2 showed a reduction in both chemokines in TLR4 BM–TC+ mice and BM+TC– mice (Figure 6c and d), corresponding with the reduction in neutrophil recruitment. The trend towards decreased CXCL1 and CXCL2 mRNA expression in kidneys of BM–TC+ and BM+TC– chimeric mice did not reach statistical significance (data not shown).

CXCL8 is produced by human GEnCs after LPS stimulation

Having established a role for TLR4 on intrinsic renal cells in inducing CXCL1 and CXCL2 dependant experimental glomerular neutrophil recruitment, we analyzed human glomerular cells for CXCL8 production (CXCL1 and CXCL2 are the murine homologs of CXCL8). Baseline mean ciGEnC mRNA expression was assigned a value of 1. Although CXCL8 mRNA expression was increased in ciGEnC, podocyte, and mesangial cells (Table 1), the relative increase was most pronounced in ciGEnC (Figure 7a). Basal CXCL8 production was highest in podocytes (Table 1), however, after stimulation the increase is most pronounced in ciGEnC, with significant increases at 2, 4, and 24 h (Figure 7b). These increases in both mRNA expression and protein production demonstrate that GEnC are the cell type most responsible for enhanced CXCL8 production.

The contribution of TLR4, CXCL1, CXCL2, and BM and TC TLR4 to renal injury

Administration of hpLPS and anti-MPO antibodies (100 μg/g) induced functional renal injury (albuminuria and hematuria), and glomerular hypercellularity with focal and segmental lesions, including fibrin deposition in WT mice at day 6 (Figure 8a–d), significantly decreased in TLR4$^{-/-}$ mice. Representative photomicrographs of renal injury showing glomerular hypercellularity and glomerular fibrin staining in WT, reduced in TLR4$^{-/-}$ mice are shown in Figure 8e–h.

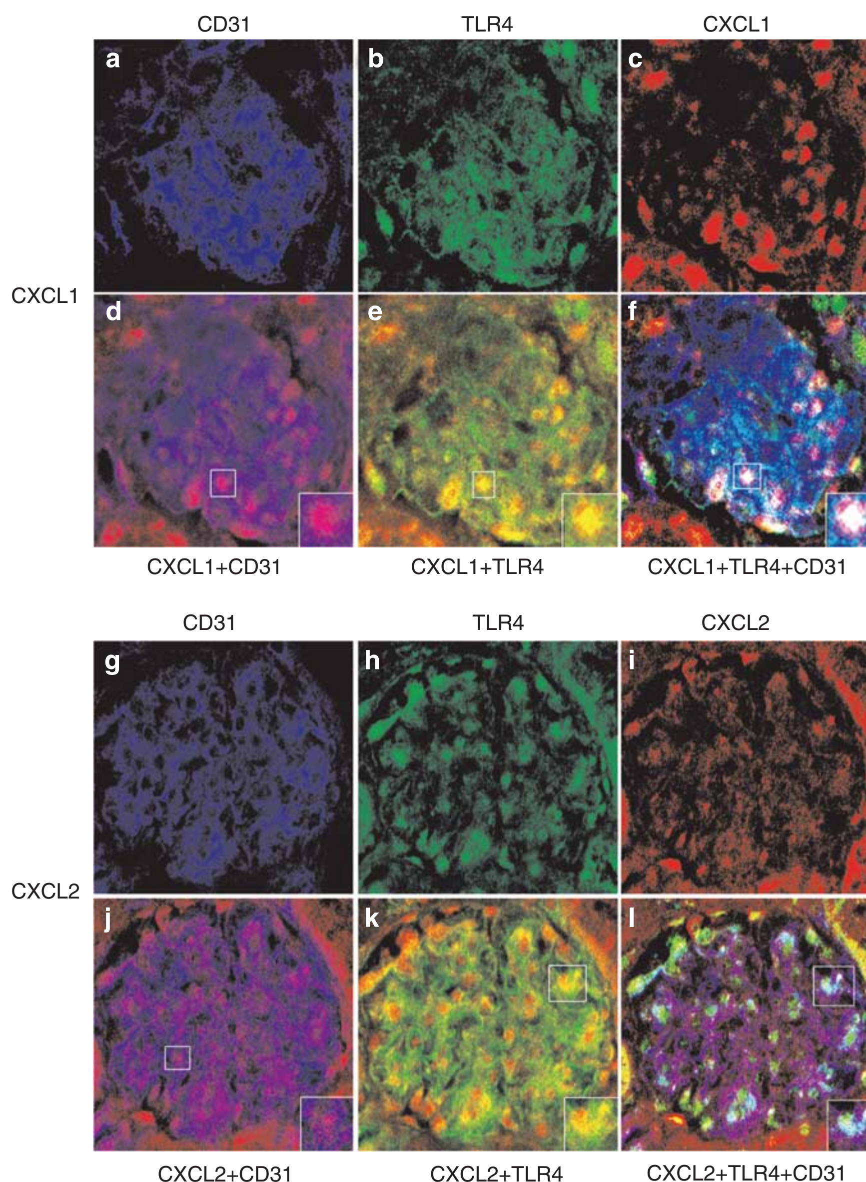

Figure 4 | Glomerular endothelial cell, CXCL1, CXCL2, and TLR4 colocalization. Kidneys from WT mice given LPS and anti-MPO antibodies were stained for (**a**) CD31 (blue), (**b**) TLR4 (green), and (**c**) CXCL1 (red). Endothelial cells and CXCL1 colocalized (magenta) (**d**). CXCL1 and TLR4 also colocalized (yellow) (**e**), and a merged three-color image showed that some endothelial cells were positive for both CXCL1 and TLR4 (white) (**f**). To assess CXCL2 production kidneys were stained for (**g**) CD31 (blue), (**h**) TLR4 (green), and (**i**) CXCL2 (red). CXCL2 colocalized with glomerular endothelial cells (magenta) (**j**) and TLR4 (yellow) (**k**). Merged three-color image showing that some endothelial cells were positive for both CXCL2 and TLR4 (white) (**l**). Original magnification x800. LPS, lipopolysaccharide; MPO, myeloperoxidase; TLR, Toll-like receptor; WT, wild type.

As CXCL1 and CXCL2 were important in glomerular neutrophil recruitment, we defined their role in the development of histological and functional renal injury by extending studies to day 6. Compared with mice given ANCA/LPS and isotope control antibodies, albuminuria and hematuria were decreased after the administration of anti-CXCL2 antibody and ANCA/LPS, whereas a trend to decreased injury was seen after the administration of anti-CXCL1 antibody (Figure 9a and b). Albuminuria, measured in the initial 24 h in mice administered control antibody and ANCA/LPS ($170 \pm 39\,\mu g/24\,h$) was decreased in mice given anti-CXCL1 antibody and ANCA/LPS ($55 \pm 14\,\mu g/24\,h$, $P < 0.001$) and anti-CXCL2 and ANCA/LPS ($87 \pm 10\,\mu g/24\,h$, $P < 0.01$). Glomerular histological injury (Figure 9c and d) was attenuated after the administration of CXCL1 or CXCL2 neutralizing antibodies, though reduced hypercellularity after anti-CXCL1 antibody did not reach statistical significance.

Having demonstrated a role for both BM and glomerular TLR4 in neutrophil recruitment, we confirmed that both are required for maximal renal injury. Compared with BM+TC+ 'sham' chimeras, albuminuria was decreased

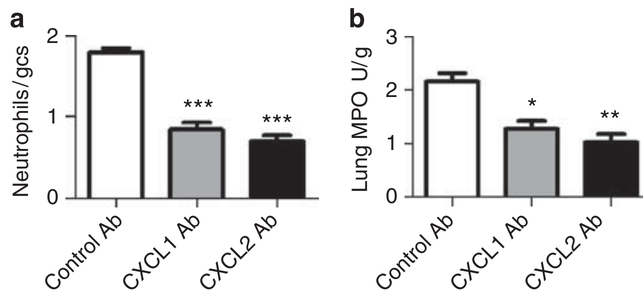

Figure 5 | Neutrophil recruitment after neutralization of CXCL1 or CXCL2. Administering CXCL1 ($n=4$) or CXCL2 ($n=4$) neutralizing antibodies prior to the administration of hpLPS and anti-MPO (αMPO) antibodies decreased glomerular neutrophil recruitment (**a**) and lung MPO activity (**b**) relative to control-treated mice ($n=8$). *$P<0.05$, **$P<0.01$, ***$P<0.001$. Ab, antibody; gcs, glomerular cross-section; MPO, myeloperoxidase.

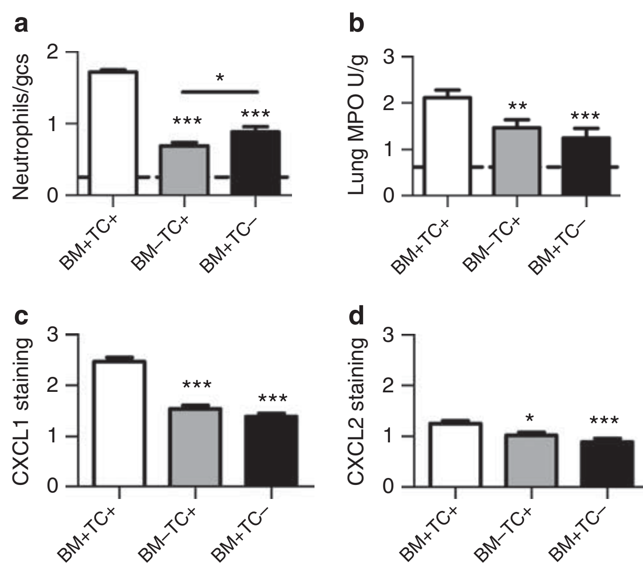

Figure 6 | Assessment of glomerular leukocyte recruitment, lung MPO activity, and glomerular chemokine staining in BM chimeric wild type (WT)→WT mice (bone marrow (BM)+, tissue cell (TC)+, $n=8$), TLR4$^{-/-}$→WT mice (BM–TC+, $n=8$), and WT→TLR4$^{-/-}$ mice (BM+ TC–, $n=8$) injected with hpLPS and anti-MPO (αMPO) antibodies. Glomerular neutrophil recruitment (**a**) was decreased in both BM–TC+ and BM+TC– compared with BM+TC+ mice. There was decreased glomerular neutrophil recruitment in BM–TC+ mice compared with BM+TC– mice. Lung MPO activity was decreased in both BM–TC+ and BM+TC– mice compared with BM+TC+ mice (**b**). Kidney CXCL1 (**c**) and CXCL2 (**d**) immunostaining was decreased in BM–TC+ and BM+TC– mice compared with BM+TC+ mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$. gcs, glomerular cross-section; MPO, myeloperoxidase.

in both BM–TC+ and BM+TC– mice, (Figure 10a and b; trends to reduction in hematuria did not reach significance). Histological injury was reduced in BM–TC+ and BM+TC– mice with less glomerular fibrin deposition and hypercellularity (Figure 10c and d).

Table 1 | CXCL8 mRNA and protein production in human glomerular cell lines at 0, 2, 4, and 24 h after lipopolysaccharide stimulation

CXCL8 mRNA	0 h	2 h	4 h	24 h
ciGEnC	1.0 ± 0.1	46.2 ± 2.0##	33.4 ± 2.6##	15.5 ± 1.8##
Podocytes	2.3 ± 0.2	28.0 ± 1.9##	19.8 ± 0.8##	17.9 ± 0.9##
Mesangial cells	0.0 ± 0.0	0.4 ± 0.0##	0.2 ± 0.0#	0.1 ± 0.0
CXCL8 protein (ng/ml)	**0 h**	**2 h**	**4 h**	**24 h**
ciGEnC	2.3 ± 0.1	3.0 ± 0.1	9.0 ± 0.5##	35.8 ± 1.9##
Podocytes	27.1 ± 1.3	24.5 ± 1.8	28.5 ± 1.7	40.8 ± 4.3#
Mesangial cells	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0##

Abbreviation: ciGEnC, conditionally immortalized glomerular endothelial cells. Baseline mRNA expression in ciGEnC was assigned a value of 1. Increased expression is seen in all cell lines, most pronounced at 2 h. CXCL8 protein production also increases in each cell type, most pronounced after 24 h. #$P<0.01$ ##$P<0.001$ compared with t=0 h.

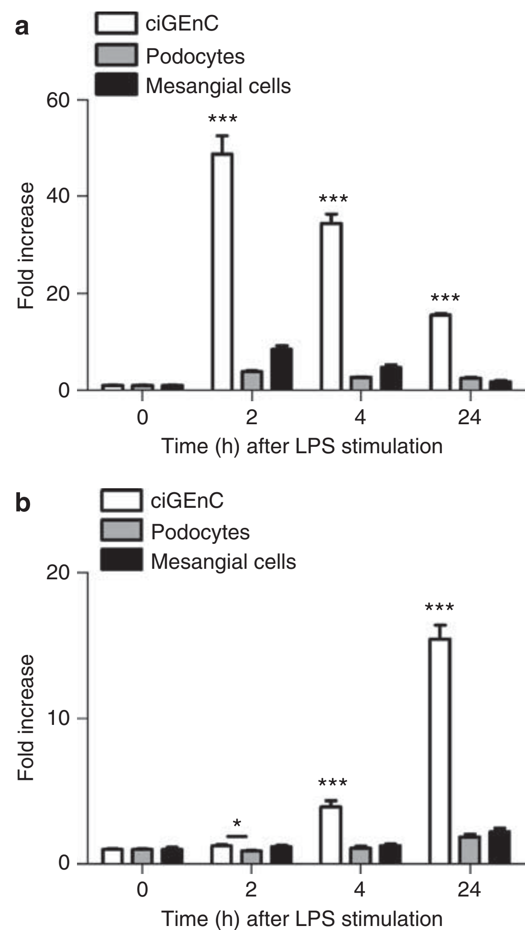

Figure 7 | CXCL8 mRNA and protein production in human glomerular cell lines. After lipopolysaccharide (LPS) stimulation, CXCL8 mRNA expression was increased in all three cell types, most pronounced in conditionally immortalized glomerular endothelial cells (ciGEnC) (**a**). Similarly, ciGEnC produced the greatest proportional increase in CXCL8 protein production (**b**), $n=6$ for all experimental groups. Absolute values (in pg/ml) of CXCL8 measurements are shown in Table 1. *$P<0.05$, ***$P<0.001$.

DISCUSSION

These studies define roles for both BM cell and TC-derived TLR4 in the pathogenesis of neutrophil recruitment in LPS/anti-MPO antibody renal and lung injury, and the roles of

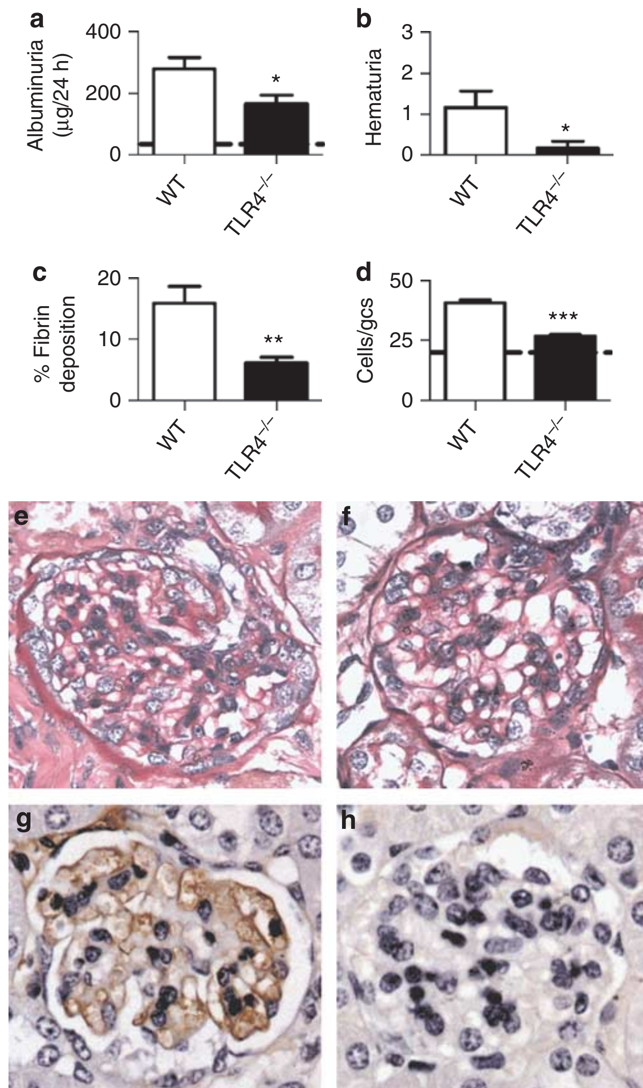

Figure 8 | Functional and histological renal injury in WT and TLR4$^{-/-}$ mice treated with LPS and anti-MPO antibodies. At 6 days after the administration of αMPO antibody/LPS 24 h albuminuria (**a**), dipstick hematuria (**b**), glomerular fibrin deposition (**c**), and glomerular hypercellularity (**d**) was attenuated in TLR4$^{-/-}$ mice ($n=6$) compared with WT controls ($n=6$). The dotted line in (**a**) and (**d**) represents mean values in untreated WT mice. Representative figures are shown demonstrating glomerular injury in WT mice (**e**) compared with TLR4$^{-/-}$ mice (**f**). More glomerular fibrin deposition was seen in WT mice (**g**) compared with TLR4$^{-/-}$ mice (**h**). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Original magnification x400. gcs, glomerular cross-section; LPS, lipopolysaccharide; MPO, myeloperoxidase; TLR, Toll-like receptor; WT, wild type.

CXCL1 and CXCL2. After stimulation, the glomerulus is the major site of TLR4 expression in the kidney, whereas GEnC are the cell type most responsible. Both BM and TC TLR4 are required for full expression of CXCL1 and CXCL2, and maximal neutrophil recruitment. Murine GEnC are a source of TLR4, CXCL1, and CXCL2, with some GEnC producing both TLR4 and neutrophil chemoattractants. Using human glomerular cells, we demonstrated that after stimulation

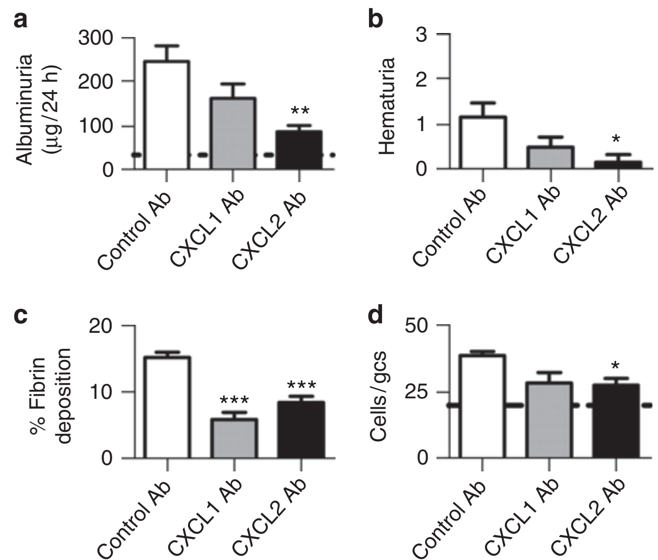

Figure 9 | Functional and histological renal injury in WT mice treated with LPS and anti-MPO antibodies with previous neutralization of CXCL1 ($n=6$) and CXCL2 ($n=6$). Compared with control antibody–treated mice ($n=6$), 24 h albuminuria (**a**) and dipstick hematuria (**b**) were significantly decreased after CXCL2 neutralization; trends after administration of CXCL1 neutralizing antibody did not reach significance. Less glomerular fibrin deposition (**c**) was evident with neutralization of either CXCL1 or CXCL2, whereas compared with control antibody–treated mice, hypercellularity was decreased with neutralization of CXCL2 (**d**). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Ab, antibody; LPS, lipopolysaccharide; MPO, myeloperoxidase; WT, wild type.

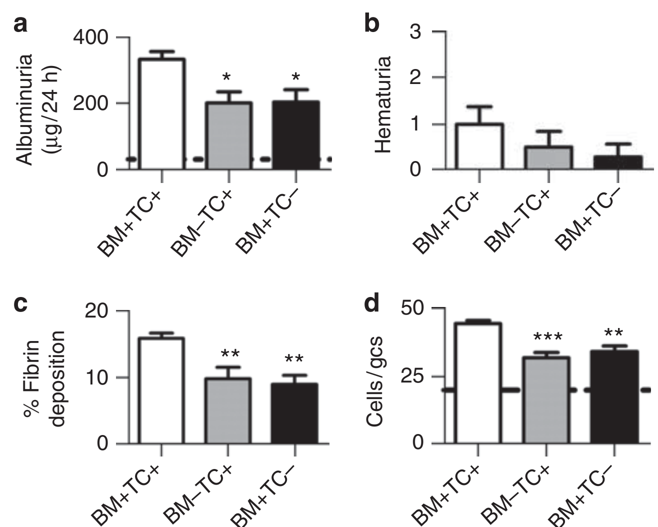

Figure 10 | Functional and histological renal injury in BM chimeric mice wild type (WT)→WT mice (bone marrow (BM)+, tissue cell (TC)+, $n=7$), Toll-like receptor (TLR4$^{-/-}$)→WT mice (BM–TC+, $n=6$), and WT→TLR4$^{-/-}$ mice (BM+ TC–, $n=7$) injected with hpLPS and αMPO antibodies. Compared with 'sham chimeras' (BM+TC+) 24 h albuminuria (**a**) was decreased in BM–TC+ and BM+TC– chimeras. There was a non-significant trend to decrease in dipstick hematuria (**b**). Glomerular fibrin deposition (**c**) and glomerular hypercellularity (**d**) was decreased in BM–TC+ and BM–TC+ chimeric mice compared with sham chimeras BM+TC+. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

GEnC are largely responsible for the increase in CXCL8 expression and protein production. *In vivo* studies at a later time point confirmed the functional relevance of both immune cell- and TC-derived TLR4, and of neutrophil chemoattractants, especially CXCL2.

Experimental anti-MPO antibody-induced GN is neutrophil dependent.[4] Neutrophils express TLR4, and LPS that engages TLR4, is a potent stimulus for neutrophil activation. TLR4 ligation has pleiotropic effects on neutrophils including neutrophil adhesion,[23,24] delayed apoptosis, enhanced chemokine production and increased superoxide generation.[25] In the current studies, the first series of experiments showed that administering both hpLPS and anti-MPO antibodies led to increased glomerular neutrophil recruitment and lung MPO activity. The effects of hpLPS on neutrophil recruitment were not seen in TLR4$^{-/-}$ mice. These studies are in accordance with previous work showing that LPS had the capacity to markedly increase anti-MPO antibody-induced injury in mice.[6,26]

In the current studies, we used three separate techniques to determine the glomerular cell types responsible for TLR4 production. First, using confocal microscopy, we demonstrated that TLR4 is present in murine glomeruli and colocalizes with GEnC. Other glomerular cells also express TLR4 in this model. Second, using microdissected glomeruli, TLR4 mRNA expression was quantitated in glomeruli and the tubulointerstitium. After the administration of LPS and anti-MPO antibodies glomerular, but not tubulointerstitial TLR4 expression was increased. Third, using isolated human glomerular cells, we demonstrated that GEnC express significant amounts of TLR4, and that enhanced mRNA expression after stimulation is attributable to GEnC.

Therefore, GEnC are significant sources of glomerular TLR4 expression after the administration of LPS and anti-MPO antibodies. The sites of intrarenal TLR4 production, both in the glomerulus and the tubulointerstitium have been addressed in several studies in other experimental models. TLR4 has been localized to the glomerulus in other models of renal disease.[20,27,28] *In situ* hybridization showed that mesangial and epithelial cells can express TLR4,[27] whereas in experimental cryoglobulinemic GN podocytes express TLR4.[20] Studies assessing TLR4 in murine models of tubulointerstitial injury have demonstrated TLR4 mRNA production from primary renal tubular epithelial cells,[29] whereas confocal microscopy has suggested TLR4 is present in proximal collecting tubules.[30] From the existing literature and the current studies, it is clear that TLR4 expression from intrinsic renal cells can vary according to the nature of the injurious stimulus. In the current studies, the results of a combination of *in vivo* and *in vitro* studies imply a role for the glomerular endothelium in TLR4 responses in the context of LPS and anti-MPO antibodies as initiators of injury.

In vivo murine models have shown that the chemokines CXCL1 and CXCL2 directs neutrophil recruitment to the cornea,[31] peritoneum,[32,33] and the joint.[34] The addition of LPS to antiglomerular basement membrane (GBM) globulin enhances heterologous renal injury.[27,35] Furthermore, Brown *et al.*,[27] demonstrated that neutralizing CXCL1 and CXCL2, which was TLR4 mediated and produced by renal cells, resulted in decreased glomerular injury. We have demonstrated that renal CXCL1 and CXCL2 expression (mRNA and protein) increases in a TLR4-dependent manner, both of which are produced by GEnC. The receptor for CXCL1 and CXCL2, CXCR2, is present on neutrophils, but LPS does not induce its expression or alter migration induced by neutrophil chemoattractants.[23,36] The current studies show that CXCL1 and CXCL2 direct anti-MPO antibody glomerular and pulmonary neutrophil recruitment, as neutralizing CXCL1 and CXCL2 decreased glomerular neutrophil recruitment and lung MPO activity early, and functional and histological renal injury later in the disease.

Both BM-derived cells[37] and an activated glomerular endothelium[38,39] are thought to be important in glomerular neutrophil recruitment in GN induced by anti-MPO antibodies. The current studies demonstrate that both BM and TC TLR4 are required for maximal glomerular and lung neutrophil requirement, underlying separate roles in the disease process. These effects within the kidney extended out to at least 6 days, wherein mice deficient in either BM cell or TC TLR4 exhibited less injury, even in the face of more profound initial decrease in glomerular neutrophil recruitment in BM–TC+ mice. As CXCL1 and CXCL2 staining was decreased in BM–TC+ mice and BM+TC– mice, both BM and TC-derived TLR4 are important in the renal production of CXCL1 and CXCL2, required for glomerular neutrophil recruitment. Neutralizing CXCL2 at the induction of injury resulted in attenuated functional and histological glomerular injury after 6 days; the effects of CXCL1 blockade were less prolonged. Although the current studies did not assess the cell type in the lung that produces neutrophil chemoattractants, previous studies have demonstrated that CXCL1 and CXCL2 are produced by Clara cells (non-ciliated bronchoalveolar epithelial cells in the distal airways).[40] Previous studies analyzing lung MPO activity to quantitate neutrophil recruitment in TLR4 chimeric mice have yielded conflicting results, with one study implicating TCs,[41] whereas another showed BM cell TLR4 to be important.[42] In experimental anti-MPO antibody-induced neutrophil recruitment MPO activity is decreased in both BM–TC+ and BM+TC– chimeric mice.

Observations in human renal biopsies suggest a pathogenic role for CXCL8, the key neutrophil-attracting chemokine in ANCA GN.[15] Previous studies have suggested that CXCL8 can be produced by 'generic' macrovascular endothelial cells (HUVECs),[43] and cultured human mesangial cells.[44] We compared CXCL8 production by different human glomerular cells, including ciGEnC, concurrently, in a single study. Although baseline expression of CXCL8 mRNA and protein production was higher in podocytes; ciGEnC showed the most significant increase in expression and production after stimulation, suggesting that during inflammation GEnC produce CXCL8, which is responsible for neutrophil recruitment.

We have demonstrated a pivotal role for both BM and intrinsic renal cell TLR4 in glomerular and lung neutrophil recruitment and injury in experimental ANCA disease. Maximal neutrophil recruitment is dependant on CXCL1 and CXCL2, TC expression, which is TLR4 dependant. Therefore, in addition to immune cell TLR4-mediated activation and recruitment, the current studies demonstrate a role for the glomerular endothelium, which involves GEnC TLR4 expression, and CXC chemokine production, that enhances neutrophil recruitment. Results from these studies add to evidence linking infection to autoimmune GN and provide evidence for possible benefits of TLR inhibition in immune glomerular disease.

MATERIALS AND METHODS

Generation of mouse anti-MPO immunoglobulin G (IgG), control mouse anti-OVA IgG, and hpLPS

Murine MPO (mMPO) was generated as described previously.[45] Globulin was precipitated (50% ammonium sulfate) and IgG affinity purified by fast protein liquid chromatography and dialyzed against PBS. For anti-OVA antibodies, $Mpo^{-/-}$ mice were immunized with OVA using the same protocol. Endotoxin concentration measured <0.01 ng/ml, Limulus Amebocyte Lysate E-TOXATE (Sigma-Aldrich, St Louis, MO). For *in vivo* experiments LPS-L2654 (Sigma-Aldrich) was repurified to ensure TLR4 specificity.[46]

Experimental design and statistical analyses

Eight week old male WT (C57BL/6 (CD45.2) and for some chimeric studies congenic CD45.1 mice) and $TLR4^{-/-}$ mice were used. Mice were from Monash University Animal Services (Melbourne, Australia), $TLR4^{-/-}$ mice originally from Professor S Akira.[47] Studies adhered to the National Health and Medical Research Council of Australia guidelines for animal experimentation. For neutrophil accumulation, mice were injected intraperitonally with hpLPS (10 μg), followed 2 h later by intravenous injection of IgG (anti-MPO antibodies 50 μg/anti-OVA (control) antibodies 50 μg), 3 h later animals were killed. For studies analyzing functional and histological renal injury, mice were injected with hpLPS 10 μg followed by anti-MPO antibodies (100 μg/g) and killed on day 6. Neutralizing monoclonal antibodies directed against CXCL1 or CXCL2 (MAB 453 and MAB 452 respectively; R&D systems, Minneapolis, MN, USA) were used with appropriate control antibodies, (rat IgG2a and IgG2b). At 1 h before the disease induction, mice were administered; anti-CXCL1-Ab (100 μg), anti-CXCL2-Ab (100 μg), or control Ab (100 μg) intravenous. BM (BM) chimeric mice were generated as previously described.[48] Flow cytometry demonstrated $>92\%$ reconstitution. Data are expressed as mean ± s.e.m. Groups of data were analyzed using student's *t*-test for analysis of two groups and one-way ANOVA (Tukey's post test) for more than two groups of data. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used to analyze the data. A *P*-value of <0.05 was considered significant.

Assessment of glomerular neutrophil accumulation, CXCL1 and CXCL2, histological and functional renal injury and pulmonary MPO

Neutrophils were demonstrated by immunoperoxidase staining of periodate lysine paraformaldehyde fixed tissue as previously described,[49] a minimum of 50 glomeruli were assessed. Results are expressed as neutrophils per glomerular cross-section (n/gcs). CXCL1 and CXCL2 immunohistochemical staining was performed as previously described.[50] To assess the intensity of glomerular and juxtaposed tubular CXCL1 and CXCL2 staining a semiquantative intensity staining scale was used, previously described and published.[50] Values are expressed as numbers ranging from 0–4, where 0: no staining seen; 1: staining $<25\%$; 2: 25–50%; 3: 51–75%; and 4: $>75\%$ of the glomerulus and adjacent tubules.

For histological assessment, 40 consecutive glomeruli/mouse were examined. Kidneys were fixed with Bouin's fixative, embedded in paraffin and stained with Periodic Acid-Schiff reagent. Fibrin staining was performed as previously described.[51] and results expressed as percentage of glomeruli with fibrin deposition. Albuminuria was measured on 24 h urine collections using a Mouse Albumin ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX, USA). Hematuria was measured using urinary dipstix (Combur Tests, Roche, Basel, Switzerland). Lung MPO was used to assess pulmonary neutrophil accumulation as previously described.[52] One unit of MPO activity was defined as a change in Δ_{460} of 1.0 after 2 min; results are expressed as U of MPO activity/g of lung tissue (U/g).

For colocalization experiments using confocal microscopy, kidneys were perfused and fixed with zinc fixative, paraffin embedded and sectioned (3 μm). For endothelial cell/TLR4 colocalization, sections were blocked (10% chicken and donkey serum in 5% bovine serum albumin), then incubated with rabbit anti-mouse TLR4 (10 μg/ml, Invitrogen, Carlsbad, CA, USA) and rat anti-mouse CD31 (1:50, BD Biosciences, San Jose, CA, USA), followed by chicken anti-rabbit Alexa Fluor 488 and donkey anti-rat Alexa Fluor 594 (both 1:200, Molecular Probes, Invitrogen, Carlsbad, CA, USA). For colocalization of neutrophil chemokines, TLR4 and endothelial cells, anti-CD31 antibodies were followed by donkey anti-rat Alexa Fluor 647 (1:200, Molecular Probes). For CXCL1 colocalization, antibodies were rabbit anti-mouse TLR4 (20 μg/ml), and goat anti-mouse CXCL1 (20 μg/ml, R&D Systems), then chicken anti-rabbit Alexa Fluor 488 and chicken anti-goat Alexa Fluor 594 (1:200, Molecular Probes). For CXCL2 co-localization, antibodies were goat anti-mouse TLR4 (20 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), with chicken anti-goat Alexa Fluor 488, and rabbit anti-mouse CXCL2 (10 μg/ml, R&D Systems). CXCL2 signal was amplified with swine anti-rabbit HRP conjugated antibody (1:100, DAKO, Glostrup, Denmark) with a Cyanine-3-Tyramide Signal Amplification kit (PerkinElmer, Waltham, MA, USA). For all samples concurrent negative controls included substituting the primary antibodies for non-immune goat, rat or rabbit IgG, and additionally for TLR4, using $TLR4^{-/-}$ mice receiving hpLPS and anti-MPO antibodies. Images were acquired using a Nikon C1 confocal laser scan head attached to a Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan) using 488, 561, and 637 nm lasers. Single plane $512 \times 512 \times 12$-bit images were captured in a line sequential manner (three line averaging). Confocal images were converted using Macbiophotonics Image J software (NIH, Bethesda, MD, USA).

Culture of human glomerular cells and assessment of TLR4, interleukin-8, CXCL1 and CXCL2 mRNA and protein

Human ciGEnC,[53] podocytes[54,55] and human immortalized glomerular mesangial cells, kindly provided by Dr Banas (Ludwig-Maximilians University, Munich, Germany),[56] were cultured. Cells were treated with 1 μg/ml LPS (*Escherichia Coli*, serotype O26:B6; Sigma-Aldrich) for 2, 4, or 24 h, medium, collected and

cells lysed for RNA isolation. For laser microdissection, glomeruli ($2.72 \pm 0.29 \times 10^6\,\mu m^2$) and surrounding tubulointerstitial tissue ($2.88 \pm 0.25 \times 10^6\,\mu m^2$) were dissected from renal cryosections of anti-MPO IgG/LPS treated mice from a previous study[26] using the Laser Robot Microbeam System (P.A.L.M. Micro laser Technology, Bernried, Germany) described previously.[57]

Real-time PCR analysis on complementary DNA was carried out using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using assay on demand primer-probe sets for TLR4 (Hs00152939_m1), interleukin-8 (Hs00174103_m1), and *GAPDH* (Hs99999905_m1; glyceraldehyde 3-phosphate dehydrogenase) as the house keeping gene. For measurement of CXCL1 and CXCL2 real-time PCR was as previously described, standardized to 18S, expressed as a fold increase relative to untreated WT mice.[58]

For fluorescence-activated cell sorting analysis of TLR4 protein expression on ciGEnC 2.3×10^5 cells were cultured for 5 days, subsequently, TLR4 protein was detected using PE-Cy7 anti-human TLR4 antibody (eBioscience, San Diego, CA, USA). Mean fluorescence intensity of PE-Cy7 was measured using a LSR-II flow cytometer. CXCL8 was measured in culture medium from ciGEnC, podocytes and mesangial cells using ELISA (R&D systems).

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

Shizuo Akira is thanked for the TLR4$^{-/-}$ mice. Alice Wright and Sophia Ling are thanked for technical assistance. Bernhard Banas is thanked for the immortalized human mesangial cells. Camden Lo, Monash Microimaging, is thanked for help with confocal microscopy. These studies were supported by a Program Grant (334067) and a Postgraduate Research Scholarship (SAS 519426) from the National Health and Medical Research Council of Australia, and a grant from the Genzyme Renal Innovations Program. PH is supported by a grant from the Dutch Organization for Scientific Research (NWO VIDI 917.66.341).

SUPPLEMENTARY MATERIAL

Figure S1. Further negative controls for immunofluorescence, showing substitution of primary antibodies with appropriate non-immune IgG.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Jennette JC, Xiao H, Falk RJ. Pathogenesis of vascular inflammation by anti-neutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 2006; **17**: 1235–1242.
2. Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 1988; **318**: 1651–1657.
3. Xiao H, Heeringa P, Hu P *et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; **110**: 955–963.
4. Xiao H, Heeringa P, Liu Z *et al.* The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol* 2005; **167**: 39–45.
5. Xiao H, Schreiber A, Heeringa P *et al.* Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol* 2007; **170**: 52–64.
6. Huugen D, Xiao H, van Esch A *et al.* Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. *Am J Pathol* 2005; **167**: 47–58.
7. Little MA, Smyth CL, Yadav R *et al.* Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. *Blood* 2005; **106**: 2050–2058.
8. Tidman M, Olander R, Svalander C *et al.* Patients hospitalized because of small vessel vasculitides with renal involvement in the period 1975-95: organ involvement, anti-neutrophil cytoplasmic antibodies patterns, seasonal attack rates and fluctuation of annual frequencies. *J Intern Med* 1998; **244**: 133–141.
9. Stegeman CA, Tervaert JW, Sluiter WJ *et al.* Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann Intern Med* 1994; **120**: 12–17.
10. Pinching AJ, Rees AJ, Pussell BA *et al.* Relapses in Wegener's granulomatosis: the role of infection. *Br Med J* 1980; **281**: 836–838.
11. Capizzi SA, Specks U. Does infection play a role in the pathogenesis of pulmonary vasculitis? *Semin Respir Infect* 2003; **18**: 17–22.
12. Arimura Y, Minoshima S, Kamiya Y *et al.* Serum myeloperoxidase and serum cytokines in anti-myeloperoxidase antibody-associated glomerulonephritis. *Clin Nephrol* 1993; **40**: 256–264.
13. Stegeman CA, Tervaert JW, de Jong PE *et al.* Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med* 1996; **335**: 16–20.
14. Yoshimura T, Matsushima K, Tanaka S *et al.* Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci USA* 1987; **84**: 9233–9237.
15. Cockwell P, Brooks CJ, Adu D *et al.* Interleukin-8: a pathogenetic role in antineutrophil cytoplasmic autoantibody-associated glomerulonephritis. *Kidney Int* 1999; **55**: 852–863.
16. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000; **12**: 121–127.
17. Lee J, Cacalano G, Camerato T *et al.* Chemokine binding and activities mediated by the mouse IL-8 receptor. *J Immunol* 1995; **155**: 2158–2164.
18. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood* 2003; **102**: 2660–2669.
19. Fan J, Malik AB. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. *Nat Med* 2003; **9**: 315–321.
20. Banas MC, Banas B, Hudkins KL *et al.* TLR4 links podocytes with the innate immune system to mediate glomerular injury. *J Am Soc Nephrol* 2008; **19**: 704–713.
21. Shirali AC, Goldstein DR. Tracking the toll of kidney disease. *J Am Soc Nephrol* 2008; **19**: 1444–1450.
22. Kuligowski MP, Kwan RY, Lo C *et al.* Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood* 2009; **113**: 6485–6494.
23. Sabroe I, Prince LR, Jones EC *et al.* Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J Immunol* 2003; **170**: 5268–5275.
24. Sabroe I, Jones EC, Usher LR *et al.* Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol* 2002; **168**: 4701–4710.
25. Parker LC, Whyte MK, Dower SK *et al.* The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol* 2005; **77**: 886–892.
26. Huugen D, van Esch A, Xiao H *et al.* Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int* 2007; **71**: 646–654.
27. Brown HJ, Lock HR, Wolfs TG *et al.* Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody-mediated glomerulonephritis via CXCL1 and CXCL2. *J Am Soc Nephrol* 2007; **18**: 1732–1739.
28. Patole PS, Pawar RD, Lech M *et al.* Expression and regulation of Toll-like receptors in lupus-like immune complex glomerulonephritis of MRL-Fas(lpr) mice. *Nephrol Dial Transplant* 2006; **21**: 3062–3073.
29. Tsuboi N, Yoshikai Y, Matsuo S *et al.* Roles of toll-like receptors in C-C chemokine production by renal tubular epithelial cells. *J Immunol* 2002; **169**: 2026–2033.
30. Zhang B, Ramesh G, Uematsu S *et al.* TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity. *J Am Soc Nephrol* 2008; **19**: 923–932.
31. Lin M, Carlson E, Diaconu E *et al.* CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J Leukoc Biol* 2007; **81**: 786–792.

32. De Filippo K, Henderson RB, Laschinger M *et al.* Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J Immunol* 2008; **180**: 4308–4315.
33. Wengner AM, Pitchford SC, Furze RC *et al.* The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood* 2008; **111**: 42–49.
34. Coelho FM, Pinho V, Amaral FA *et al.* The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature. *Arthritis Rheum* 2008; **58**: 2329–2337.
35. Fu Y, Xie C, Chen J *et al.* Innate stimuli accentuate end-organ damage by nephrotoxic antibodies via Fc receptor and TLR stimulation and IL-1/TNF-alpha production. *J Immunol* 2006; **176**: 632–639.
36. Sabroe I, Jones EC, Whyte MK *et al.* Regulation of human neutrophil chemokine receptor expression and function by activation of Toll-like receptors 2 and 4. *Immunology* 2005; **115**: 90–98.
37. Schreiber A, Xiao H, Falk RJ *et al.* Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. *J Am Soc Nephrol* 2006; **17**: 3355–3364.
38. Calderwood JW, Williams JM, Morgan MD *et al.* ANCA induces beta2 integrin and CXC chemokine-dependent neutrophil-endothelial cell interactions that mimic those of highly cytokine-activated endothelium. *J Leukoc Biol* 2005; **77**: 33–43.
39. Nolan SL, Kalia N, Nash GB *et al.* Mechanisms of ANCA-mediated leukocyte-endothelial cell interactions *in vivo*. *J Am Soc Nephrol* 2008; **19**: 973–984.
40. Elizur A, Adair-Kirk TL, Kelley DG *et al.* Clara cells impact the pulmonary innate immune response to LPS. *Am J Physiol Lung Cell Mol Physiol* 2007; **293**: L383–L392.
41. Andonegui G, Bonder CS, Green F *et al.* Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 2003; **111**: 1011–1020.
42. Hollingsworth JW, Chen BJ, Brass DM *et al.* The critical role of hematopoietic cells in lipopolysaccharide-induced airway inflammation. *Am J Respir Crit Care Med* 2005; **171**: 806–813.
43. Strieter RM, Kunkel SL, Showell HJ *et al.* Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. *Science* 1989; **243**: 1467–1469.
44. Brown Z, Strieter RM, Chensue SW *et al.* Cytokine-activated human mesangial cells generate the neutrophil chemoattractant, interleukin 8. *Kidney Int* 1991; **40**: 86–90.
45. Apostolopoulos J, Ooi JD, Odobasic D *et al.* The isolation and purification of biologically active recombinant and native autoantigens for the study of autoimmune disease. *J Immunol Methods* 2006; **308**: 167–178.
46. Hirschfeld M, Ma Y, Weis JH *et al.* Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 2000; **165**: 618–622.
47. Takeuchi O, Hoshino K, Kawai T *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999; **11**: 443–451.
48. Timoshanko JR, Sedgwick JD, Holdsworth SR *et al.* Intrinsic renal cells are the major source of tumor necrosis factor contributing to renal injury in murine crescentic glomerulonephritis. *J Am Soc Nephrol* 2003; **14**: 1785–1793.
49. Huang XR, Holdsworth SR, Tipping PG. Evidence for delayed-type hypersensitivity mechanisms in glomerular crescent formation. *Kidney Int* 1994; **46**: 69–78.
50. Roche JK, Keepers TR, Gross LK *et al.* CXCL1/KC and CXCL2/MIP-2 are critical effectors and potential targets for therapy of Escherichia coli O157:H7-associated renal inflammation. *Am J Pathol* 2007; **170**: 526–537.
51. Drew AF, Tucker HL, Liu H *et al.* Crescentic glomerulonephritis is diminished in fibrinogen-deficient mice. *Am J Physiol Renal Physiol* 2001; **281**: F1157–F1163.
52. Odobasic D, Kitching AR, Semple TJ *et al.* Endogenous myeloperoxidase promotes neutrophil-mediated renal injury, but attenuates T cell immunity inducing crescentic glomerulonephritis. *J Am Soc Nephrol* 2007; **18**: 760–770.
53. Satchell SC, Tasman CH, Singh A *et al.* Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney Int* 2006; **69**: 1633–1640.
54. Saleem MA, O'Hare MJ, Reiser J *et al.* A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002; **13**: 630–638.
55. Coward RJ, Welsh GI, Koziell A *et al.* Nephrin is critical for the action of insulin on human glomerular podocytes. *Diabetes* 2007; **56**: 1127–1135.
56. Banas B, Luckow B, Moller M *et al.* Chemokine and chemokine receptor expression in a novel human mesangial cell line. *J Am Soc Nephrol* 1999; **10**: 2314–2322.
57. Asgeirsdottir SA, Kamps JA, Bakker HI *et al.* Site-specific inhibition of glomerulonephritis progression by targeted delivery of dexamethasone to glomerular endothelium. *Mol Pharmacol* 2007; **72**: 121–131.
58. Phoon RK, Kitching AR, Odobasic D *et al.* T-bet deficiency attenuates renal injury in experimental crescentic glomerulonephritis. *J Am Soc Nephrol* 2008; **19**: 477–485.