Toll-like receptor 4 engagement contributes to expression of NKG2D ligands by renal tubular epithelial cells

Gang Eric Chen, Huiling Wu, Jin Ma, Steven J. Chadban and Alexandra Sharland

Collaborative Transplantation Research Group, Bosch Institute, The University of Sydney, Sydney, Australia

Correspondence and offprint requests to: Alexandra Sharland; E-mail: alexandra.sharland@sydney.edu.au

Abstract

Background. Engagement of Toll-like receptor (TLR) 4 on intrinsic kidney cells is critical for the full development of renal ischaemia–reperfusion injury (IRI). Effects of TLR signalling in renal parenchymal cells include the production of cytokines, chemokines and other soluble mediators which contribute to local inflammation and leucocyte accumulation. Whether engagement of TLR4 on kidney cells results in additional pro-inflammatory modifications of the renal microenvironment remains to be determined.

Methods. Renal IRI was induced by clamping of the renal pedicles, and expression of NKG2D ligands in mice deficient in TLR4 or its adaptor molecule MyD88, or else pretreated with blocking antibodies against the endogenous TLR4 ligand HMGB1, was compared to that in wild-type mice. Cultures of isolated renal tubular epithelial cells (TECs) from WT, TLR4−/− and MyD88−/− mice were stimulated with the TLR4 ligand lipopolysaccharide (LPS), or mineral oil occlusion was used to simulate IRI in vitro, prior to determination of NKG2D ligand expression. Chimeric mice lacking TLR4 in either the bone marrow derived or the parenchymal compartment were also subjected to IRI.

Results. In this study, we demonstrate a substantial increase in the expression of the NKG2D ligands retinoic acid early inducible-1 (RAE-1), murine ULBP-like transcript 1 (MULT-1) and histocompatibility-60 (H-60) in mouse kidneys during renal IRI. Expression of NKG2D ligands was attenuated in mice deficient in either TLR4 or the adaptor molecule MyD88. Antibody blockade of HMGB1 reduced NKG2D ligand expression by a comparable extent to TLR4 deficiency and did not result in further reduction of NKG2D ligand expression in TLR4−/− mice. Isolated TECs from normal mice but not those with defects in the TLR4–MyD88 signalling pathway expressed RAE-1 and MULT-1 upon exposure to LPS and after being subjected to in vitro conditions resembling ischaemia–reperfusion. TLR4 competence in the parenchymal but not the bone marrow-derived compartment was required for RAE-1 up-regulation in mouse kidneys after ischaemia, while TLR4 signalling in both compartments contributed to the intrarenal expression of MULT-1 during IRI.

Conclusion. Expression of the NKG2D ligands RAE-1 and MULT-1 on kidney cells in response to TLR4 engagement by HMGB1 represents another mechanism by which TLR4 signalling may participate in the pathogenesis of renal IRI.

Keywords: HMGB1; innate immunity; ischaemia–reperfusion injury; NKG2D; TLR4

Introduction

Engagement of Toll-like receptor (TLR) 4 on intrinsic kidney cells is critical for the full development of renal ischaemia–reperfusion injury (IRI) [1]. Endogenous TLR ligands
produced or released during IRI include heat shock proteins, biglycan, low-molecular weight forms of hyaluronan, heparan sulphate, nucleic acids and HMGB1 [2–5]. HMGB1 is a DNA-binding protein with an auxiliary function as an inflammatory mediator [5]. HMGB1 can be released from cells upon organ reperfusion, and HMGB1 binding to TLR4 is important in the pathogenesis of both liver and kidney IRI [6–8]. Recognized downstream effects of TLR signalling in renal parenchymal cells include the production of pro-inflammatory cytokines, chemokines and other soluble mediators, which contribute to local inflammation and leukocyte accumulation [1, 9]. Whether engagement of TLR4 on intrinsic kidney cells results in additional modifications of the renal microenvironment which further facilitate the development of kidney damage, remains to be determined.

NKG2D (NK group 2, member D) is an activating receptor expressed by a range of effector cell types, including natural killer (NK) cells, macrophages, γδ T cells and CD8+ T cells. For CD8+ cells, NKG2D engagement triggers cytotoxicity and cytokine production [10–12]. Two families of NKG2D ligands have been described in humans; MIC (MHC class-I chain related) A and B and ULBP (cytogalovirus UL16-binding protein) 1–5. Murine NKG2D ligands comprise RAE-1 (retinoic acid early inducible-1), MULT-1 (murine ULBP-like transcript-1) and H60 (histocompatibility 60). They are expressed at low levels in normal adult tissues, but are inductively expressed, primarily in tissues of epithelial origin, in response to cellular stress [13–16]. RAE-1 induction on renal tubular epithelial cells (TECs) has been reported during IRI [17], and the presence of RAE-1 on the cell surface rendered these cells susceptible to NKG2D-mediated lysis by syngeneic NK cells [18]. The relevance of this finding to in vivo IRI was underscored by the evidence that NK cell depletion protected against kidney damage following ischaemia [18]. Heat shock elements are present in the promoter regions of the MIC family of human ligands, but neither the human ULBP ligand family nor the known mouse NKG2D ligands possess these motifs, and the pathways by which cellular stress leads to their expression in epithelial cells are poorly understood [19]. A recent study in mouse peritoneal macrophages described up-regulation of cell surface RAE-1 in response to stimulation through TLR4 by lipopolysaccharide (LPS) [20]. On this basis, we hypothesized that TLR4 engagement on intrinsic kidney cells during renal IRI may stimulate expression of the NKG2D ligands RAE-1 and MULT-1 thus potentiating further tissue damage.

Materials and methods

Animals

Male Balb/c, C57BL/6, TLR4−/− and MyD88−/− mice weighing 23–28 g (10–12 weeks old) were used in all experiments. Balb/c and C57BL/6 mice were obtained from the Animal Resources Centre (Perth, Australia), while TLR4−/− and MyD88−/− mice were kindly provided by Prof D. Hume (University of Queensland, Brisbane, Australia) and Dr W. Health (The Walter and Eliza Hall Institute, Melbourne, Australia) with permission from Prof S. Akira (Osaka University, Osaka, Japan) and were back-crossed more than nine times onto a C57BL/6 background. The mice were housed under specific pathogen-free conditions in the University of Sydney’s animal facility. Experiments were conducted following established guidelines for animal care and were approved by the Animal Care and Ethics Committee of the University of Sydney.

Induction of renal IRI in vivo

Mice were anesthetized using isoflurane (Abbott Australasia Pty Ltd, Kurnell, Australia). Following a midline abdominal incision, renal pedicles were clamped with microaneurysm clamps. For the initial experiments in Balb/c mice depicted in Figure 1, the period of unilateral ischaemia was 45 min. In subsequent experiments using C57BL/6 wild-type (WT), TLR4−/− and MyD88−/− mice, the duration of bilateral ischaemia was 22 min. During the period of ischaemia, body temperature was maintained by placing the mice on a 37°C heat pad. After removal of the clamps, the kidneys were inspected for 1 min for prompt restoration of blood flow, indicated by a return to their original colour. The abdomen was then closed. Each experimental group included 6–8 animals. Sham-operated mice (n = 5 per group) underwent identical surgical procedures except that microaneurysm clamps were not applied and were sacrificed 1 day after operation. To maintain fluid balance, all mice received 1 mL of saline subcutaneously. Mice were sacrificed at intervals between 1 and 28 days after reperfusion (n = 6–8 per group). Part of each C57BL/6 WT kidney sample collected on Day 1 was reserved for isolation and staining of mouse TECs. The remaining kidney samples were snap frozen for subsequent messenger RNA (mRNA) extraction.

Pretreatment with blocking antibodies against HMGB1

Anti-HMGB1 and isotype control antibodies were sourced from Shino-Test Corporation Sagamihara-shi, Kanagawa, Japan. Mice received polyclonal chicken IgY chicken anti-HMGB1 antibody (300 μg/mouse) or isotype control IgY by intra-peritoneal injection 1 h before the induction of ischaemia. The blocking antibody used in these studies has been demonstrated in vitro to bind specifically to HMGB1 and not to other related members of the HMGB family [21]. The ability of comparable doses of this antibody to bind to and neutralize extracellular HMGB1 in vivo has been previously verified in murine models [22–25].

Isolation and primary culture of mouse renal TECs

Primary mouse renal TECs were generated following the method described by Wuthrich et al. [26]. Briefly, kidneys were flushed with saline in vivo to remove blood cells, then removed. The kidney cortices from WT, TLR4−/− and MyD88−/− mice were cut into pieces of ~1 mm3 and then digested in Hanks’ buffered saline solution containing 3 mg/mL of collagenase at 37°C for 25 min and washed in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Invitrogen Corp. Grand island, NY). The kidney digest was washed through a series of sieves (mesh diameters of 250, 150, 75 and 40 μm). After digestion and sieving, cells to be used for flow cytometry were collected by centrifugation, resuspended in 40% Percoll (Sigma) in RPMI1640 medium and overlayed on 66.7% Percoll solution. Gradient separation was carried out at 716 g for 20 min at room temperature. Separation produced a top layer containing tubular cells, a layer of mononuclear cells at the interface and a bottom layer of debris and red blood cells. Tubular cells were washed before antibody staining and flow cytometry.

Cortical tubular cells to be established in primary culture were spun down at 300 g for 5 min and further washed. The cell pellet was resuspended in defined K1 medium [26] comprising DMEM/F12 medium supplemented with 25 ng/mL epidermal growth factor, 1 ng/mL PGE1, 5 × 10−11 M tri-iodothyronine, 5 × 10−6M hydrocortisone (Sigma-Aldrich Inc, St. Louis, MO), insulin-transferrin-sodium selenite media supplement, 1% penicillin/streptomycin, 25 mM HEPES and 5% fetal calf serum (FCS) (Invitrogen). The cell suspension was then placed in cell culture petri dishes and incubated at 37°C for 2–3 h to facilitate adherence of contaminating glomeruli. The non-adherent tubules were then collected and cultured on collagen-coated petri dishes (BD Biosciences, Bedford, MA) in K1 medium until epithelial colonies were established. Expression of an epithelial cell marker was verified by immunofluorescent staining with an anti-cytokeratin antibody (Sigma-Aldrich). Cells were 96–100% cytokeratin positive. Experiments were commenced after the cells had reached 80–90% confluence, which was usually between 5 and 7 days after the isolation procedure.
TLR4-dependent renal expression of NKG2D ligands

Fig. 1. Expression of NKG2D ligands in murine renal IRI. Balb/c mice were subjected to a brief period of renal ischaemia, followed by reperfusion (n = 6–8 per group). Gene expression of RAE-1, MULT-1 and H60 was measured by real-time reverse transcription–PCR. The time course of gene expression for NKG2D ligands in the kidneys is shown in panels (a–c). Compared with sham-operated controls (n = 5), substantial up-regulation of mRNA levels for RAE-1 (a), MULT-1 (b) and H60 (c) is evident at all time points between D1 and D28 (P < 0.05). (d) Renal TECs isolated from sham-operated or ischaemic C57BL/6 mouse kidneys 24 h after reperfusion were stained with a pan-RAE-1 antibody (red histograms). Isotype control staining is shown in green. Surface expression of RAE-1 protein was demonstrated on the cells isolated from ischaemic, but not control kidneys.

Flow cytometric analysis
The renal tubular cells were stained with goat anti-mouse RAE-1γ antibody (clone: AF1136), which has been shown to recognize RAE-1γ as well as RAE-1γ. Goat IgG was used as an isotype control (both antibodies from R&D Systems, Minneapolis, MN). Following secondary incubation with anti-goat IgG conjugated with fluorescein isothiocyanate (Zymed, San Francisco, CA), all samples were analysed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson, Mountain View, CA).

Immunohistochemistry
Formalin-fixed sections of 5 μm thickness were deparaffinized and boiled for 10 min in 10 mM sodium citrate buffer (pH 6.0). Sections were stained with goat anti-RAE-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or isotype control (goat IgG) at room temperature for 60 min. Following washing, the sections were exposed to 3% H2O2 in methanol for 5 min and then incubated with the biotinylated secondary antibody, anti-goat IgG (Vector Laboratories, Inc.). ABC reagent from a Vectastain ABC peroxidase kit (Vector Laboratories Inc.) was applied to the tissue followed by 3,3’-diaminobenzidine substrate–chromogen solution (DAKO, Carpinteria, CA). Slides were counterstained with Harris’ haematoxylin. Ten high-power fields were assessed per slide for the extent of tubular staining. Representative images are shown in Figure 2.

Stimulation of renal TECs with LPS in vitro
When TECs reached 80% confluence, they were placed in serum-free K1 medium for 24 h, washed twice with PBS and immersed in mineral oil (Sigma-Aldrich) for 60 min at 37°C. After extensive washing with PBS, cells were incubated in K1 medium and then collected for RNA extraction at 2 h after medium replacement. Control cells did not undergo mineral oil occlusion and were collected at the same time as treated cells.

Generation of bone marrow chimeric mice
Bone marrow cells were collected from WT or TLR4−/− mice by flushing femurs and tibiae with RPMI 1640 containing 5% FCS. Chimeric mice were produced by transfer of donor bone marrow cells into irradiated recipient animals using the following recipient/donor combinations of WT and TLR4−/− mice (n = 8–10 per group): WT/WTBM, WT/TLR4−/−BM and TLR4−/−/WTBM. Recipient mice had been lethally irradiated with a signal dose of 9.5 Gy from a caesium source using a 137Cs irradiator (Nordion International, CIS bio international; SCHERING S.A.). Six hours after irradiation, recipient irradiated mice received 8 × 10^6 bone marrow cells via the tail vein. The animals were allowed to recover for 8–10 weeks to ensure stable engraftment, before being subjected to 22 min of kidney ischaemia and 24 h reperfusion. Full chimerism of each mouse was confirmed by genotyping of DNA from peripheral blood by using REDExtract-N-AmpTM Blood PCR kits (Sigma-Aldrich) following a standard protocol (data not shown).

Extraction of RNA and cDNA synthesis
Total RNA was extracted from renal tissue and TECs using TRIZol (Invitrogen) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using oligo (dT)16 (Applied Biosystems, Foster City, CA) and the SuperScript III Reverse Transcriptase kit (Invitrogen) following a standard protocol [1].

Real-Time PCR
Primers and probes for RAE-1 (forward 5’-atgattttcagcatccagga-3’, reverse 5’-ggaaggtgtagttaagttgata-3’; probe 6FAM-agccaagatcaacctc-
H60 (forward 5'-attgcctcgaggtgatacactctaaagt-3'; reverse 5'-tatcctcgagcagaccctggttgtcaattatgtc-3') and MULT1 (forward 5'-tttgactgacatcgcactggagtttcctggctctggcgt-3'; reverse 5'-aaggggctgcaagtgatacagctctctggt-3') were obtained from Applied Biosystems. Primers and a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene control were used as previously described [28]. cDNA was amplified in 1× Universal Master Mix (Applied Biosystems) with gene-specific primers and probe on the
TLR4-dependent renal expression of NKG2D ligands

ABI Prism 7700 Sequence Detection System (Applied Biosystems), according to the manufacturer’s instructions. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data were analysed using the Sequence Detector VI.9 Analysis Software (Applied Biosystems). Expression of the gene was normalized against mRNA expression of the housekeeping gene GAPDH, as previously outlined [28]. Real-time polymerase chain reaction (PCR) experiments for each gene were performed on three separate occasions.

**Statistics**

Results are expressed as mean ± SD. Multiple groups were compared using either one-way or two-way analysis of variance, as appropriate, with post hoc Bonferroni’s correction (Graph Pad Prism 4.0 software). Two groups were compared using an unpaired t-test (two tailed). A value of P < 0.05 was considered significant.

**Results**

**NKG2D ligand expression is up-regulated in the kidney during IRI**

To determine whether kidney IRI induces expression of mouse NKG2D ligands in addition to RAE-1, we examined the mRNA expression of RAE-1, MULT-1 and H-60 in ischaemic Balb/c kidney by real-time PCR. Balb/c mice were used for these exploratory studies, as this strain expresses all known murine NKG2D ligands, in contrast to C57BL/6 mice, which lack H60. Sham-operated kidney tissue expressed these ligands at a basal level. mRNA expression for all three ligands was significantly increased from Day 1 to Day 28 in kidneys subjected to IRI compared with sham controls (P < 0.05) (Figure 1a–c). RAE-1 protein was detected on tubular cells isolated from ischaemic kidney on Day 1 after ischaemia, but not on those isolated from sham-operated control (Figure 1d).

**TLR4/MyD88 signalling contributes to induction of RAE-1 and MULT-1 expression in vivo**

Next, to assess the contribution of TLR4 signalling to mouse NKG2D ligand induction, we examined C57/BL6 WT, TLR4−/− and MyD88−/− animals in the IRI model at Day 1, Day 3 and Day 5. H60 is expressed in Balb/c, but not in C57/B6 mice [17], and so this ligand was not evaluated in further studies. Baseline expression of both NKG2D ligands in the kidneys of WT mice was low, but increased >50-fold after ischaemia, peaking on D3 (P < 0.01) (Figure 2a–b). An initial increase in NKG2DL mRNA expression was noted in all groups, whereas further up-regulation of RAE-1 and MULT-1 at later time points was restricted in both the TLR4−/− and MyD88−/− mice (P < 0.01 for WT versus TLR4−/− and WT versus MyD88−/− at each of D3 and D5). While additional pathways may be involved in the increased transcription of NKG2D ligands which follows renal ischaemia, TLR4/MyD88 signalling appears to be necessary for achieving the sustained high levels of RAE-1 and MULT-1 expression seen on Days 3 and 5. In situ expression of RAE-1 protein was evaluated by immunohistochemistry (Figure 2c–g). Tubular expression of RAE-1 was evident in sections from WT kidneys collected at D1 following renal ischaemia (Figure 2c) while staining of sections from the same kidneys with the isotype control was negative (Figure 2d). Only minimal tubular staining could be detected in the kidneys of sham-operated WT mice (Figure 2e) or TLR4−/− or MyD88−/− mice following ischaemia (Figure 2f and g, respectively). Intensity of tubular staining for RAE-1 protein did not increase further at later time points (data not shown).

**HMGB1 blockade reduces up-regulation of NKG2D ligands after renal ischaemia**

In this model, increased levels of HMGB1 protein can be detected in kidney homogenates from D1 and peaking on D5 after ischaemia [8]. To determine whether HMGB1 is playing a role in up-regulation of NKG2D ligand expression during IRI, we pretreated mice with a blocking antibody against HMGB1 or with an irrelevant isotype control antibody. Kidneys were harvested on Days 1 and 5 post-ischaemia. RAE-1 expression in the control mice reached a peak on D5, while the maximum level of MULT-1 expression was attained on D1. Whereas some up-regulation of both ligands was evident in the presence of anti-HMGB1, peak expression of these ligands was reduced substantially by antibody blockade (P < 0.001; Figure 3a–b), HMGB1 blockade administered to TLR4−/− animals did not reduce expression of NKG2D ligands to a significantly greater degree than that seen with TLR4 deficiency alone (Figure 3c–d), suggesting that the effects of HMGB1 on NKG2D ligand expression in this model are mostly mediated through TLR4.

**NKG2D ligand expression is induced by LPS in tubular cells isolated from WT but not TLR4−/− mice**

As well as parenchymal cells, intact mouse kidneys contain a network of resident Dendritic cells and macrophages. TLR4 engagement on cells of similar myeloid lineage has been shown to induce expression of RAE-1 [20]. To establish whether NKG2D ligand up-regulation could result from TLR4 engagement on TECs, isolated TECs were stimulated with the known TLR4 ligand, LPS. After 8 h of stimulation, a substantial increase in RAE-1 and MULT-1, mRNA expression was induced in TECs isolated from WT mice (P < 0.05), whereas there was no significant increase in expression in cells isolated from TLR4−/− mice (Figure 4c–d).

**NKG2D mRNA expression induced by ischaemia is attenuated in TLR4−/− and MyD88−/− TECs**

*In vitro* IRI also led to up-regulation of NKG2D ligand expression in TECs isolated from WT mice (RAE-1: P < 0.01 and MULT-1: P < 0.01). Expression of MULT-1 by TECs increased considerably, whereas up-regulation of RAE-1 expression following *in vitro* IRI was modest. There was no increase in mRNA expression for either RAE-1 or MULT-1 in TECs originating from TLR4−/− or MyD88−/− mice, further supporting the dependence of ischaemia-induced NKG2D ligand expression by renal tubular cells upon an intact TLR4–MyD88 signalling pathway (Figure 4e–f).
Parenchymal cell TLR4 signalling is both necessary and sufficient to cause NKG2D ligand expression in intact kidneys during IRI

Having first determined that expression of NKG2D ligands during IRI is attenuated in mice deficient in either TLR4 or MyD88, and that TLR4 stimulation of isolated renal TECs could result in induction of RAE-1 and MULT-1, we proceeded to examine the effect of TLR4 deficiency in either the bone marrow-derived resident leucocyte or parenchymal cell compartment upon NKG2D ligand expression, using bone marrow chimeric mice. As expected, expression of both RAE-1 and MULT-1 at 24 h after an ischaemic insult was substantially reduced in TLR4−/− mice reconstituted with TLR4+/+ bone marrow, compared with WT recipients of WT bone marrow (P < 0.001) (Figure 5 a–b). Deficiency of TLR4 in the parenchymal compartment alone constrained RAE-1 expression to the same extent as global deficiency (P < 0.001) (Figure 5 a), whereas RAE-1 levels in mice lacking TLR4 in bone marrow-derived cells alone were equivalent to those in WT/WT mice (Figure 5 a), implying that intrinsic kidney cells are principally responsible for RAE-1 expression early during IRI. In contrast, MULT-1 expression was reduced similarly by deficiency in either the bone marrow derived or parenchymal compartments (Figure 5 b), suggesting that both parenchymal cells and resident cells of haematopoietic origin contribute to the renal expression of this ligand early during IRI.

Discussion

NKG2D ligands are distant MHC class I homologues, which include MICA, MICB and the ULBPs in humans and RAE-1, H60 and MULT1 in mice. Expression of these ligands can be up-regulated in response to a variety of stimuli including viral infection, malignant transformation, tissue ischaemia and classical heat shock [13–18,29,30]. In this study, expression of the NKG2D ligands RAE-1, MULT-1 and H60 was up-regulated in the kidney in vivo and (for RAE-1 and MULT-1) in renal TECs in vitro following ischaemia. Increased expression of all three ligands was evident from D1 post-ischaemia, with a further increase in the second week. Late inflammatory sequelae of renal ischaemia are well described [31], and in this situation, the late increase in NKG2D ligand expression may reflect the presence of a macrophage or lymphocytic infiltrates [1] while the likely cellular sources of the up-regulation seen at earlier time points are renal tubular cells and resident leucocytes. The signalling pathways
responsible for up-regulation of these ligands following renal IRI are unknown. A recent study in mouse peritoneal macrophages demonstrated induction of RAE-1 expression through the MyD88 signalling pathway upon engagement of TLR4 [20]. Given that mouse renal tubular cells constitutively express TLR4, we tested the hypothesis that engagement of TLR4 on renal TECs by endogenous ligands may also induce the expression of RAE-1. As predicted, we demonstrated that TLR4/MyD88 signalling is involved in up-regulation of RAE-1 expression on TECs as well as that of MULT-1. TLR2 is another receptor present on renal tubular epithelium [32], and signalling pathways downstream of TLR engagement converge [33], making it likely that more than one species of TLR could contribute to the up-regulation of NKG2D ligands in ischaemic kidneys. At early time points post-transplantation, it appears that NKG2DL up-regulation can be initiated via additional pathways. However, at Days 3 and 5, peak expression levels are only reached in TLR4-competent mice.

Ischaemic injury to the kidney results in the generation or release of various endogenous ligands able to bind to TLRs. These ligands include HMGB1, biglycan, heat shock proteins, low molecular weight hyaluronan and heparan sulphate. Considerable receptor–ligand promiscuity exists,
such that many ligands which bind TLR4 may also bind other receptors such as TLR2 and The receptor for advanced glycation end-products (RAGE), and multiple potential ligands exist for each receptor [33]. Moreover, the relative expression of different endogenous ligands could vary, depending upon the severity of the ischaemic insult, and the duration post-ischaemia. HMGB1 is an endogenous ligand released from cells upon organ reperfusion. Engagement of TLR4 by HMGB1 has been demonstrated to play an important role in the pathogenesis of liver and kidney IRI [7,8]. In these studies, administration of blocking antibodies against HMGB1 attenuated expression of NK2D ligands after renal ischaemia. The effect of HMGB1 blockade was not additive to that of TLR4 deficiency, implying that the major effect of HMGB1 in this setting was mediated by TLR4, rather than by ligation of other receptors including TLR2 and RAG.

Intact kidneys contain cell populations of haematopoietic origin in addition to parenchymal cells. Experiments in isolated TECs showed that a variable degree of NKG2DL expression resulted from TLR4 engagement after in vitro ischaemia. Strong up-regulation was demonstrated for MULT-1, whereas increases in RAε-1 expression were modest. This in vitro model differs from in vivo ischaemia in a number of ways, including the absence of the haematopoietic compartment, but also of the extracellular matrix, which is the source of some endogenous ligands and which modulates inflammation after ischaemia by binding various pro-inflammatory cytokines and chemokines [34]. It is possible that the more limited up-regulation of RAε-1 in this situation compared with that observed in vivo is due in part to the lack of matrix components.

In order to delineate TLR4-mediated expression of NKG2DL by parenchymal cells from that by renal-resident leucocytes in vivo, we established bone marrow-chimeric mice, competent or deficient in TLR4 signalling in either the parenchymal or haematopoietic compartments. While we found that TLR4 signalling in intrinsic kidney cells was largely responsible for RAε-1 expression early during IRI, MULT-1 expression was reduced to a similar extent by the absence of TLR4 in either compartment, implying that MULT-1 was being expressed on both resident leucocytes and parenchymal cells. The reasons for differential expression of MULT-1 and RAε-1 in the two compartments are not known, but it is well-established that different cell types may have divergent expression profiles for the various NK2D ligands [15]. NK2D is a C-type lectin-like activating receptor expressed on NK cells, macrophages, γδ T cells and CD8αβ T cells. Ligation of NK2D provides a co-stimulatory signal to enhance TCR-mediated antigen specific CD8 T cell activation, proliferation and granule exocytosis [12,29], and directly activates the effector functions of other leukocytes: NK cells release both granules and cytokines (IFN-γ), while macrophages produce the inflammatory mediators nitric oxide and TNF-α [30]. A recent report indicates that RAε-1 expression on renal TECs during IRI renders them susceptible to NK2D-dependent lysis by syngeneic natural killer cells, and that depletion of NK cells reduces the severity of functional impairment after renal ischaemia [18]. Up-regulation of RAε-1 and MULT-1 on TECs resulting in NK cell-mediated attack upon tubules is thus another mechanism by which TLR4 signalling could contribute to the development of kidney IRI.

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