The CD40–CD154 co-stimulation pathway mediates innate immune injury in adriamycin nephrosis

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Abstract

Background. Blockade of CD40–CD40 ligand (CD154) interactions protects against renal injury in adriamycin nephropathy (AN) in immunocompetent mice. To investigate whether this protection relied on adaptive or cognate immunity, we tested the effect of CD40–CD154 blockade in severe combined immunodeficient (SCID) mice.

Methods. SCID mice were divided into three groups: normal, AN + hamster IgG (ADR+IgG group) and AN + anti-CD154 antibody (MR1) (ADR+MR1 group). AN was induced by tail vein injection of 5.2 mg/kg of adriamycin (ADR). Hamster IgG (control Ab) or MR1 was administered intraperitoneally on days 5, 7, 9 and 11 after ADR injection. Histological and functional data were collected 4 weeks after ADR injection. In vitro experiments tested the effect of soluble and cell-bound CD154 co-cultured with CD40-expressing cells [macrophages, mesangial cells and renal tubular epithelial cells (RTEC)].

Results. All experimental animals developed nephropathy. Compared to the ADR+IgG group, ADR+MR1 animals had significantly less histological injury (glomerulosclerosis and tubular atrophy) and functional injury (creatinine clearance). Kidneys of ADR+MR1 animals had significantly less macrophage infiltration than those of ADR+IgG animals. Interestingly, expression of CD40 and CD41 (a platelet-specific marker) was significantly less in ADR+MR1 animals compared to ADR+IgG animals. In vitro, CD154 blockade significantly attenuated upregulation of CCL2 gene expression by RTEC stimulated by activated macrophage-conditioned medium. In contrast, platelet-induced upregulation of macrophage and mesangial cell proinflammatory cytokine gene expression were not CD154-dependent.

Conclusion. CD40–CD154 blockade has a significant innate renoprotective effect in ADR nephrosis. This is potentially due to inhibition of macrophage-derived soluble CD154.

Keywords: co-stimulation; focal sclerosing glomerulonephritis; macrophages; renal disease; tubulointerstitial injury

Introduction

The CD40–CD154 co-stimulatory pathway is an important regulator of adaptive immunity. Blockade of CD40–CD154 has been shown to significantly ameliorate organ injury in experimental models of islet xenotransplantation [1], allogeneic transplantation (liver [2], islet [3], heart [4] and kidney [5]), thyroiditis [6] and uveoretinitis [7]. In each of these situations, the protective mechanisms were shown or assumed to be lymphocyte-dependent.

CD154 (CD40 ligand) is primarily expressed on activated CD4+ T lymphocytes [8] but is also found in a soluble form [9]. Its expression has subsequently been found on a wide variety of cells, including platelets [10], mast cells and basophils [11], macrophages [12], NK cells, B lymphocytes, as well as non-haematopoietic cells (smooth muscle cells, endothelial cells and epithelial cells) [13]. Basophils, macrophages, smooth muscle cells, endothelial cells and
platelets all constitutively express CD154. Platelet CD154 translocates to the cell surface following stimulation by thrombin or other platelet agonists. CD40 is found constitutively on a large number of cells, including mononuclear, epithelial, endothelial and dendritic cells [14].

In addition to a role in T cell-dependent immunity, CD40–CD154 interactions participate in a vast array of pathophysiologic processes, including tumor surveillance and atherosclerosis. In particular, the innate arm of the immune system may under some circumstances depend on CD40–CD154 interaction. For example, platelets have been found to activate dendritic cells via this pathway [15].

Renal injury is mediated by a complex interplay of immunological and non-immunological pathways. Blockade of CD40 binding to CD154 has been shown to protect against renal injury in animal models of membranous nephritis [16] and lupus nephritis [17]. In murine adriamycin nephropathy (AN), blockade of the CD40–CD154 pathway by the monoclonal antibody (mAb) MR1 reduced renal injury [5].

The mechanism of protection against renal injury by CD40–CD154 blockade remains unclear. Our previous studies have shown that macrophage recruitment and infiltration are key components of renal injury. Both macrophage number and expression of the protein CCL2 (also known as macrophage chemoattractant peptide 1) correlate with the severity of renal injury and are significantly ameliorated by blockade of CD154 in vivo. CD40 is expressed on many cells within the kidney, including renal tubular epithelial cells (RTEC), macrophages and endothelial cells. CCL2 and other cytokines are produced by RTEC and mesangial cells [18,19] in response to various stimuli including CD40 ligation [20].

AN is a robust rodent model of toxin-induced renal injury which can be induced in immunocompetent and lymphocyte-deficient severe combined immunodeficient (SCID) mice [21]. We hypothesized that the protective effect of CD40–CD154 blockade in AN involves the innate arm of the immune response. We found that MR1 protects against renal injury in SCID mice and identified a mechanism involving activation of RTEC by soluble macrophage-derived CD154.

Materials and methods

General reagents

Blocking mAb specific for murine CD154 (clone MR1, Armenian hamster IgG) was purchased from Bioexpress Cell Culture Services (West Lebanon, New Hampshire, USA). This antibody was purified by protein G chromatography from the culture supernatant of an immunoglobulin-producing hybridoma [22]. This antibody neutralizes binding of CD154 to its ligand CD40 in BALB/c and C57BL/6 mice [23]. The control hamster IgG was purified by protein G (Amersham Pharmacia Biotech, NJ, USA) affinity chromatography according to the manufacturer's instructions from the hamster serum obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Antibodies

Antibodies against macrophages (clone F4/80). CD40 (clone 3/23) and goat anti-Armenian hamster immunoglobulin were purchased from eBioscience (CA, USA). Antibodies against CD41 (clone MW30reg) and CD154 (MR1 antibody), PE-labelled anti-CD40, PE-labelled anti-CD154, fluorescein isothiocyanate (FITC)-labelled anti-CD62P and FITC-conjugated goat anti-rat polyclonal IgG were purchased from BD Biosciences (NJ, USA). Polyclonal biotinylated rabbit anti-rat immunoglobulin was purchased from DakoCytomation, Denmark. Goat anti-mouse CD40 and FITC donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (CA, USA). Rhodamine rabbit anti-rat IgG was purchased from Rockland (PA, USA).

Animals

All animal experiments were approved by the Animal Ethics Committee of the Sydney West Area Health Service. SCID mice (BALB/c) were divided into three groups: normal (n = 3), AN + hamster IgG (ADR+IgG) (n = 6) and AN + MR1 antibody (ADR+MR1) (n = 5). AN was induced by a single tail vein injection of 5.2 mg/kg of doxorubicin [Adriamycin® (ADR)]. Hamster IgG (control Ab) or MR1 was administered intraperitoneally on days 5, 7, 9 and 11 after ADR injection. A dose of 0.4 mg of antibody was administered on each occasion. In this model, renal injury was established by day 3 post-ADR injection, as assessed by dipstick proteinuria.

Functional parameters of renal injury

Mice were placed in metabolic cages for 12 h before sacrifice to collect urine for determination of urinary protein and creatinine. Urinary volume was measured. Blood samples for serum creatinine were collected at the time of sacrifice. Serum and urinary creatinine were measured by an automated analyser (BM/Hitachi 747) using a method based on the Jaffé reaction. Serum albumin was measured using the dye-binding bromcresol green procedure, whilst urinary protein was measured using the Biuret method. These assays were performed on an automated analyzer (BM/Hitachi 747) within the Institute of Clinical Pathology and Medical Research, Westmead. The ratio of urine protein to creatinine was determined.

Histological assessment of renal injury

Kidney slices were fixed in 10% neutral-buffered formalin for 24 h and then dehydrated in graded alcohols and embedded in paraffin. Tissues were cut at 5 μm and stained with periodic acid-Schiff (PAS).

Quantitative analysis of glomerulosclerosis was performed using a modification of a technique described by Saito et al. [24]. The degree of sclerosis was scored from 0 to 4 in each of 20 glomeruli from PAS-

### Table 1. Primers used in real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primer</th>
<th>Size of product (bp)</th>
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<tr>
<td>CCL2 (MCP-1)</td>
<td>Forward 5′-GCTGGAGAAGCTACAAGAGATCA-3′</td>
<td>79</td>
</tr>
<tr>
<td>CD54 (ICAM-1)</td>
<td>Forward, 5′-GCTACCTCTGCTCTCCTG-3′</td>
<td>115bp</td>
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<td>iNOS</td>
<td>Forward, 5′-TGGTGTTGACAAAGACAT-3′</td>
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<tr>
<td>CD40</td>
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<td>128bp</td>
</tr>
<tr>
<td>CD154</td>
<td>Proprietary (SABiosciences)</td>
<td>191bp</td>
</tr>
</tbody>
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**References:**

1. et al.
Fig. 1. Quantitative analysis of renal injury. PAS-stained kidney sections (10–20 per mouse) were photographed and analysed by a blinded observer. Kidneys of AN+MR1 mice (C) had less glomerulosclerosis (D) and tubular atrophy (E) than kidneys of AN+IgG animals (B). Both AN groups had significantly more injury than normal controls (A). Histological analysis was performed as described in Materials and methods. *P < 0.05 vs IgG group, ANOVA, P < 0.01.
each section. Spleens from BALB/c mice with or without ADR were used or five (CD40 and CD41) non-overlapping cortical fields (×400 magnification) of cells positive for F4/80, CD40 and CD41 was counted in eight (F4/80) mice. Slides were counterstained with haematoxylin (Sigma), aldehyde dehydrochloride. Stain kit; Vectra Laboratories, UK). After washing, the reaction was visualized by the addition of freshly prepared 3,3-diaminobenzidine tetrahydrochloride. Slides were counterstained with haematoxylin (Sigma), dehydrated, cover-slipped and examined by light microscopy. The number of cells positive for F4/80, CD40 and CD41 was counted in eight (F4/80 or five (CD40 and CD41) non-overlapping cortical fields (×400 magnification). The mean number of positive cells per field was calculated for each section. Spleens from BALB/c mice with or without ADR were used as positive controls for macrophage, CD154, CD40 and platelet staining.

**Immunohistochemical staining**

Coronal slices of kidneys were embedded in Optimum Cutting Temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen and stored at −80°C. Frozen sections were cut and stained as previously described [26]. Sections were incubated with primary antibodies to F4/80 (1:200), CD40 (1:100), CD41 (1:200) or CD154 (1:100), then incubated with secondary antibody (polyclonal biotinylated rabbit anti-rat immunoglobulin or goat anti-Armenian hamster immunoglobulin) (1:300) and streptavidin/peroxidase complex (Vectra Laboratories, UK). After washing, the reaction was visualized by the addition of freshly prepared 3,3-diaminobenzidine tetrahydrochloride. Slides were counterstained with haematoxylin (Sigma), dehydrated, cover-slipped and examined by light microscopy. The number of cells positive for F4/80, CD40 and CD41 was counted in eight (F4/80 or five (CD40 and CD41) non-overlapping cortical fields (×400 magnification). The mean number of positive cells per field was calculated for each section. Spleens from BALB/c mice with or without ADR were used as positive controls for macrophage, CD154, CD40 and platelet staining.

**Immunofluorescence staining of kidney**

Acetone-fixed frozen kidney sections were incubated, after blocking with Background Terminator, overnight at 4°C with either goat anti-mouse CD40 (Santa Cruz Biotechnology) and/or rat anti-mouse F4/80 (eBioscience), washed and then stained with FITC donkey anti-goat IgG (Santa Cruz Biotechnology) and/or rhodamine rabbit anti-rat IgG (Roelkland) for 1 h at room temperature and then visualized under fluorescence microscopy.

**Cell lines**

The SV40-transformed murine RTE cell line C1.1 was a kind gift of Drs Rudolf Wüthrich and Patricia Wahl (University of Zurich, Switzerland). The C1.1 cell line was grown in modified K1 medium with 5% fetal calf serum (FCS). A murine macrophage cell line (J774.1, American Type Culture Collection (ATCC), H-2b) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, cat no 11995) with 10% FCS. A murine mesangial cell line (CRL-1927, ATCC, H-2d) was maintained in DMEM, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and FCS. Cells were incubated at 37°C in a 5% CO2 humidified environment.

**Primary RTEC**

Primary proximal RTEC were isolated from the cortex of mouse kidneys using a method adapted from Doctor et al. [27]. Cells were cultured in serum-free K1 medium until needed. These cells (~90%) stained positive for cytokeratin and negative for vimentin. RTEC were prepared for flow cytometry [fluorescent-activated cell sorting (FACS)] by light trypsination, washed in phosphate-buffered saline (PBS) and resuspended in FACS staining solution (BD Biosciences).

**Isolation of murine platelets**

All isolation steps were performed at room temperature. Mouse (BALB/c or C57BL/6) were anaesthetised, and blood was taken by subternal puncture and collected into citrate tubes. Blood tubes were centrifuged for 20 min at 600 r.p.m. (75 g) at room temperature. The platelet-rich plasma was collected and recentrifuged for 10 min at 2400 r.p.m. (1500 g). The supernatant was discarded, and the platelet pellet was resuspended in PBS. Purity was assessed by FACS by staining for CD41 and found to be >98% (Figure 8). Platelet stimulation was performed with 0.5 units/mL human thrombin (Sigma) for 20 min at room temperature at a concentration of 8 × 10^6 cells/mL PBS.

**Platelet-macrophage co-culture**

Platelets were co-cultured with macrophages. Platelets (BALB/c) were prepared and resuspended in PBS. Macrophages (J774) were harvested, and adherent macrophages were washed once with PBS. Subsequently, prepared platelets were added to macrophages in the presence of IFN-γ, in a ratio of 1 macrophage:100 platelets. Co-culture was maintained for 2 h at 37°C with 5% CO2. mRNA from adherent macrophages was extracted at the end of culture using the RNase mini kit (Qiagen). The role of CD40–CD154 pathway was tested in similar experiments where MR1 (100 μg/mL) was added to the platelet suspension prior to thrombin stimulation.

**Platelet-RTEC co-culture**

RTBC (BALB/c) were isolated by primary isolation and plated onto either 6-well or 24-well tissue culture plates in K1 medium with 5% fetal calf serum (FCS). A macrophage number correlated with tubular cell height (E), glomerulosclerosis (F) and normalized total kidney CCL2 mRNA expression (G). *P < 0.01.
CD40 expression in mouse kidney. Immunohistochemical staining of frozen kidney sections demonstrated marked upregulation of CD40 expression in ADR+IgG mice (B) compared with ADR+MR1 (C) and normal mice (A). Quantitation was performed by a blinded observer by counting the number of stained cells per high power field in at least five sections per mouse (D). To identify which cells express CD40, kidney sections were stained for F4/80 (primary ab: rat anti-mouse F4/80, secondary ab: rhodamine conjugated rabbit anti-rat IgG) and CD40 (primary ab: goat anti-mouse CD40, secondary ab: FTTC-conjugated donkey anti-goat IgG). Whole kidney CD40 mRNA expression correlated with interstitial volume (E) but not with glomerulosclerosis or tubular cell height (data not shown). Some sections as illustrated in F showed lack of co-localization of staining, whilst there was co-localization of staining (yellow colour) (G) in other parts of the kidney. Therefore, CD40 is expressed on both F4/80 positive and negative cells in vivo. F4/80 staining alone (H) is shown for comparison. CD40 was not expressed in glomeruli (I). White arrows—glomeruli, blue arrows—double stained cells (yellow) positive for CD40 and F4/80.
1:100–1:500) (BALB/c) were added. At the end of 4 h of co-culture, mRNA was extracted from the RTEC.

**Platelet-mesangial cell co-culture**
Mesangial cells from a murine cell line CRL-1927 (H-2 b) were cultured in DMEM:F12 (3:1) supplemented with HEPES and 5% FCS. A total of 5 × 10^5 mesangial cells/well was co-cultured with 5 × 10^7 platelets (C57BL/6, H-2 b) for 6 to 24 h with and without MR1 antibody (100 μg/mL), and mRNA was collected like the other co-culture experiments.

**Activated macrophage-conditioned medium (AMCM) cultured with RTEC**
Primary RTEC were co-cultured with AMCM. To make AMCM, J774 macrophages were stimulated for 24 h in DMEM supplemented with 10% FCS and 10 μg/mL of lipopolysaccharide (LPS), washed twice with PBS, then incubated for a further 48 h in serum-free DMEM. The culture medium was collected and filtered through a 0.22-μm syringe filter to obtain AMCM and stored at 4°C. Primary RTEC were cultured with AMCM for 4 and 24 h with MR1 antibody (20 μg/mL), isotype control (20 μg/mL) or no antibody. Cells were harvested for RNA, and the supernatant was stored at −20°C until further use. All in vitro experiments were performed at least three times.

**Reverse-transcription and real-time (quantitative) polymerase chain reaction**
Total kidney RNA was extracted using TRIZOL (Gibco BRL, NY, USA). Frozen kidney tissue was homogenized in a volume of 0.7 mL of TRIZOL, and the extracted RNA was precipitated according to the manufacturer's instructions and suspended in 30 μl of RNase-free water. Cell culture RNA was extracted using the Qiagen RNAse mini kit as per the manufacturer's instructions. First strand cDNA was transcribed from total RNA using a Supertranscript RT–PCR kit (Invitrogen, NJ, USA). Real-time amplification of cDNA was carried out in a Corbett Rotorgene 3000 or 6000 real-time PCR machine using a 20-μL reaction mixture containing cDNA, primer and SYBR green PCR mixture (Invitrogen). For in vitro experiments involving RTEC and mesangial cells, CCL2 (a macrophage chemokine) was chosen as the outcome endpoint due to its importance in macrophage recruitment, whereas inducible nitric oxide synthase (iNOS) mRNA was chosen as the marker of macrophage activation because of its role as an important proinflammatory cytokine.

**Primers**
Primers used are detailed in Table 1.

**FACS analysis**
RTEC from the C1.1 cell line and primary RTEC were stimulated with IFN-γ (100 units/mL) for 4 h and stained with PE-labelled rat anti-mouse CD40. Platelets were stained with rat anti-mouse CD41 and FITC-conjugated goat anti-rat polyclonal IgG, PE-labelled CD154 and FITC-labelled CD62P. J774.1 macrophages were stimulated with IFNγ for 24 h and then stained for CD40 expression using rat anti-mouse CD40. Isotype control antibodies were used in all experiments. Data collection was performed using a BD FACScan and analysed with WinMDI software.
Fig. 5. CD40 expression in vitro. Single cell suspensions of primary renal tubular epithelial cells (RTEC), macrophages (J774 cell line) and mesangial cells (CRL-1927 cell line) were stained for PE-CD40 or isotype control and analysed by flow cytometry. RTEC constitutively expressed high levels of CD40 (A), whereas macrophages (B) and mesangial cells (C) expressed only low amounts of CD40. Interferon-γ stimulation upregulated CD40 on macrophages (strongly) and mesangial cells (weakly) but not RTEC. Data show percentage of cells staining for PE-CD40.
Enzyme-linked immunosorbent assay (ELISA)

Soluble CD154 levels were measured in culture supernatants using the soluble CD40 ligand ELISA kit (R&D systems, MN, USA) following the manufacturer's instructions. Soluble CCL2 levels were measured in culture supernatants (BD Biosciences) following the manufacturer's instructions. CCL2 protein levels were normalized against total protein. Total protein was measured using the BioRad protein assay system based on the method of Bradford (BioRad, CA, USA).

Statistical analysis

All data are represented as mean ± SEM. Two group differences were analysed by Student's t test, whilst differences among more than two groups were analysed by analysis of variance (ANOVA). Non-parametric data were compared using Wilcoxon Rank Sums test. Correlation studies were performed using simple linear regression. JMP software (SAS Institute, NC, USA) and Microsoft Excel were used for statistical analyses. A P value of <0.05 was considered statistically significant.

Results

Renal injury

In mice with AN, there was a marked reduction in renal injury in those treated with MR1 (ADR+MR1) in comparison to those treated with isotype control antibody (ADR+IgG). Glomerulosclerosis and tubular atrophy were reduced significantly by MR1 (Figure 1). Creatinine clearance was significantly better in ADR+MR1 (50.4 ± 10.3 μL/min) compared to ADR+IgG mice (22.6 ± 8.8 μL/min, P < 0.05). Serum albumin levels and urine protein-to-creatinine ratios were not significantly different (ADR+MR1 vs ADR+IgG: serum albumin 20.4 ± 1.5 vs 20.0 ± 1.9g/L; urine protein-to-creatinine ratio 8.15 ± 3.0 vs 10.06 ± 6.62 g/mmol) (Figure 2).

Macrophage infiltration

The number of macrophages was markedly decreased in kidneys of ADR+MR1 mice compared with those of
treated with soluble mouse CD154 (10 μM). Macrophages accumulated within the tubulointerstitial compartment, whilst very few macrophages were found in glomeruli. The reduction in interstitial macrophages was not accompanied by a significant change in interstitial volume (ADR+MR1 34.0 ± 2.0, ADR+IgG 35.0 ± 2.4%, P = 0.76). Macrophage numbers correlated with tubular atrophy, glomerulosclerosis and whole kidney CCL2 mRNA expression (Figure 3).

**Confirmation of SCID phenotype**

As not all SCID mice lack functioning lymphocytes, confirmation of their lymphocyte-deplete phenotype was sought by examining for T lymphocytes (CD3) and B lymphocytes (B220) in spleen from experimental mice, using wild type BALB/c mouse spleen as a positive control. As expected, no CD3+ or B220+ cells were visualized.

**CD40 expression**

CD40 staining on kidney frozen sections was markedly upregulated in ADR+IgG mice, but this effect was diminished in mice treated with MR1 antibody (Figure 4). CD40 staining was prominent in RTEC and interstitial cells and not in glomeruli. CD40 was expressed on F4/80 positive cells (macrophages) and F4/80 negative cells lining the tubules (RTEC). CD40 mRNA was expressed in kidney cortical extracts and correlated significantly with interstitial volume (P = 0.04) (Figure 4) but not with glomerulosclerosis, tubular cell height or macrophage number (data not shown). In *vitro*, resting primary RTEC expressed CD40 (60–70%) and this was not enhanced by a 4-h stimulation with IFN-γ (200 units/mL). In contrast, CD40 expression was minimal on RTEC from the C1.1 cell line (<10%), even after 24-h stimulation with IFN-γ. Therefore, primary RTEC were chosen in preference to the C1.1. cell line for experiments investigating cell-bound and soluble CD154 effects. Macrophages derived from the mouse J774 cell line stained for CD40 only upon priming with IFN-γ. Murine mesangial cells expressed only low levels of CD40 even upon stimulation with IFN-γ (Figure 5), a finding corroborated by the lack of *in vivo* CD40 staining in glomeruli of ADR-treated mice. No CD154 staining was detected within kidneys of normal or AN mice. CD154 mRNA was expressed in kidney cortical extracts, but its expression did not correlate with glomerulosclerosis, tubular cell height, interstitial volume, macrophage number or platelet number (data not shown).

**Soluble CD154-induced effects on cytokine production identifies potential target cells**

Macrophages, primary RTEC and mesangial cells were treated with soluble mouse CD154 (10 μg/mL). Recombinant soluble CD154 upregulated cytokine gene expression of macrophages and primary RTEC, effects inhibited by MR1 antibody (20 μg/mL). Soluble CD154 mildly upregulated mesangial cell cytokine gene expression, an effect not significantly inhibited by MR1, and was consistent with the low level of mesangial CD40 expression (Figure 6).

**AMMC-induced upregulation of CCL2 gene expression in primary RTEC**

Macrophage (J774) surface CD154 expression was found on only 5% of cells stimulated with LPS. However, soluble CD154 was detected using ELISA in conditioned medium from activated J774 cells (58.1 ± 2.4 pg/mL). AMMC upregulated mouse primary RTEC mRNA expression of CCL2. Addition of anti-CD154 antibody (MR1) resulted in a 30% reduction in CCL2 gene expression in RTEC cultured with AMMC. Measurement of CCL2 protein in supernatant of co-culture experiments showed that upregulation of its synthesis by AMMC was also CD154 dependent. Therefore, AMMC stimulation of primary RTEC was partially dependent on CD154 (Figure 7).

**Platelet accumulation in ADR-induced renal injury**

Numerous platelets were found throughout the kidneys of AN mice, significantly more than in kidneys of normal mice. Interestingly, in AN platelet, staining was most intense within glomeruli and at sites of more severe tubular dilatation and/or atrophy. Administration of MR1 restored platelet accumulation within glomerular and tubulointerstitial compartments to normal (Figure 8).

**Platelet-induced cytokine expression by RTEC, macrophages and mesangial cells**

*Ex vivo*, resting platelets did not express significant amounts of membrane-bound CD154, however, after a 20-min stimulation with 0.5 units/mL human thrombin, up to 14% of platelets expressed CD154 as assessed by FACS analysis. Using ELISA, soluble CD154 was detected in the supernatant of both resting and activated platelets (43.6 ± 2.1 pg/mL). Fifty to sixty percent of thrombin-stimulated platelets expressed CD62P (P-selectin), an activation marker for platelets (Figure 8).

Upon co-culture with RTEC, platelets did not upregulate RTEC cytokine expression (data not shown). IFN-γ primed J774 macrophages co-cultured with stimulated platelets expressed significantly more iNOS mRNA than did primed macrophages alone, but this was not dependent on CD154. Both unstimulated and thrombin-stimulated platelets upregulated mesangial cell CCL2 mRNA and protein production, but these effects were not inhibited by MR1 antibody (Figure 8).

**Fig. 8.** Platelets in renal injury. Frozen sections of kidneys from normal (A), ADR−IgG (B) and ADR−MR1 (C) mice stained with MWReg30 (CD41), a platelet-specific marker. Platelet number was significantly greater in ADR−IgG-treated animals compared to ADR−MR1 and normal mice (D). In *vitro*, murine platelets (purity >98% as assessed by MWReg30 staining) were stimulated with thrombin and expressed CD62P (P-selectin) and CD154 (E). Co-culture of stimulated platelets (PLT) with interferon-γ-primed macrophages resulted in upregulation of macrophage iNOS in a non-CD154 dependent manner (F). Stimulated platelets upregulated mesangial cell CCL2 gene (G) and protein production (H) in a non-CD154 dependent manner. Platelets did not upregulate CCL2 gene expression on RTEC (data not shown). Thrombin did not affect gene expression on any of the three cell types tested. *P equal or <0.05, NS = no significant difference.
Discussion

By blockade of CD154 in immunodeficient mice with AN, the current study showed for the first time the involvement of CD40–CD154 in lymphocyte-independent renal injury. Since lymphocytes are not present in SCID mice, the interaction between CD40 and CD154 must involve cell types other than or in addition to lymphocytes. These data demonstrate that macrophages are the likely source of CD154 to promote renal injury in AN.

Administration of a neutralizing antibody to CD154 markedly reduced the severity of functional and histological renal injury in SCID mice with AN. Moreover, MR1 was administered 5 days after ADR, therefore it was effective in treating, rather than just preventing renal injury. Other studies have also demonstrated a protective effect of CD40–CD154 blockade in renal injury [5,16,17], but all of those studies were performed in immunocompetent mice. Modulation of T lymphocyte interactions with antigen-presenting cells may have explained those findings, but cannot explain the findings in the current study.

Macrophage accumulation was found to correlate with the severity of renal injury, and CD40–CD154 blockade was associated with a marked reduction in macrophage infiltration. Macrophage number was also associated with total renal CCL2 gene expression. These findings suggest that MR1 directly or indirectly inhibited macrophage recruitment into the kidney. Macrophages are essential to and potent in the modulation of renal injury, as shown by previous depletion [28,29] and reconstitution [30] studies.

Expression of CD40 was found most prominently in RTEC and macrophages rather than endothelial cells, in accordance with previous findings in the AN model [5]. CD40 was expressed on both F4/80 positive cells (macrophages) and F4/80 negative cells lining the tubular lumen (RTEC). Administration of MR1 reduced the expression of CD40 within the kidney. This is not unexpected as it parallels the overall reduction of renal injury and number of infiltrating macrophages. AN enhanced expression of CD40, which might render these cells more sensitive to the effects of cell-bound and soluble CD154 and thereby promote renal injury. Therefore, this inhibition of CD40 expression may be involved in the attenuation of renal injury by MR1.

The effect of CD154 was tested on three distinct varieties of CD40-expressing renal cells. Soluble mouse CD154 activated cytokine production on both macrophages and RTEC, and these effects were inhibited by MR1 antibody. RTEC are an important source of proinflammatory signals within the kidney. Binding of CD154 to RTEC CD40 induced a range of chemokines (IL-8, CCL2 and CCL5) [31,32], IL-6 [33], complement C3 [34] and reactive oxygen species [35]. Upregulation of the adhesion molecule CD54 (intercellular adhesion molecule 1 or ICAM-1) on the surface of RTEC by soluble CD154 resulted in increased adhesion of mononuclear cells [36]. Inhibition of soluble CD154-dependent proinflammatory cytokine upregulation by MR1 may explain its protective effect against renal injury. However, mesangial cells did not express significant amounts of CD40 in vivo or in vitro and MR1 did not significantly inhibit CD154-induced CCL2 upregulation. These data suggest that CD40–CD154 co-stimulation within the glomerular compartment is unlikely to be important in the pathogenesis of AN. This study does not directly address whether or not the expression of cytokines and chemokines on RTEC and macrophages decreases following CD154 blockade in vivo, nor if the improvement of renal injury is dependent on the number of infiltrating macrophages.

Such a question would need to be addressed by examining the cytokine/chemokine expression of macrophages isolated from the kidneys from mice from each of the three experimental groups. Studies in the AN model suggest that renal macrophage phenotype is capable of changing in vivo in response to intervention [30].

Macrophages are known to produce a soluble form of CD154 [37] and are found adjacent to areas of severe renal injury. We found that conditioned medium from stimulated mouse macrophages upregulated CCL2 gene expression of RTEC in a CD154-dependent manner. Macrophages are known to induce cell death [38] and epithelial–mesenchymal transition (TK Tan & DCH Harris, unpublished observations) in RTEC. No study, to our knowledge, has shown a role for CD154 in macrophage-mediated RTEC CCL2 expression. These data suggest that MR1 antibody exerts its protective effect on ADR-induced renal injury by blocking macrophage-mediated RTEC CCL2 expression, hence reducing macrophage recruitment to areas of renal injury. The reduction in macrophage accumulation and RTEC atrophy in MR1-treated animals is consistent with these findings.

Platelets were examined as a possible source of cell-bound or soluble CD154 and for their possible role in the pathogenesis of AN. Platelets bearing CD154 are active participants in innate immune responses [15]. Platelet aggregation was significantly greater in kidneys of mice with AN compared to normal controls and was significantly reduced in AN mice treated with MR1. Co-culture of platelets activated both macrophages and mesangial cells in vitro, but this activation was independent of CD40–CD154. Platelets are known to activate macrophages (e.g. in atherosclerotic plaque formation [39–41]), but no data implicate a role of platelet-derived CD154 in such interactions. In contrast, previous in vitro human data have shown that platelets are capable of upregulating human mesangial cell cytokine (CCL2 and TGF-β) production in a CD154-dependent manner [19,42]. However, our studies show that it is unlikely that CD40–CD154 interactions play a significant role in mouse platelet-mesangial cell interactions, given the lack of CD40–CD154 effect in the co-culture experiments of platelet-mesangial cells and the relative lack of CD40 staining on murine mesangial cells in vivo and in vitro.

The inability of platelet-derived CD154, in contrast to soluble CD154, to activate macrophages, RTEC and mesangial cells is likely due to their different molecular configurations. Transmembrane CD154 expressed by activated platelets is cleaved to generate a soluble hydrolytic fragment known as soluble CD154. Transmembrane CD154 may differ in its biological properties to soluble...
CD154. As has been shown recently [43], soluble CD154 may be able to evoke pathological responses independently of cell-bound CD154. This may reflect the differences in their distribution. Platelet CD154 is most important in activating endothelial expressed CD40 where it is bound in clot formation. In AN, the major expression of CD40 was in the macrophages and RTECs.

Platelets expressing membrane-bound CD154 did not upregulate macrophage iNOS gene expression in a CD154-dependent manner. Moreover, platelets did not upregulate RTEC cytokine gene expression. These findings may be explained by the specificity of MR1 for soluble, rather than membrane-bound CD154.

For reasons outlined above, platelets and macrophages were believed to be the most likely source of CD154 in AN in SCID mice. However, other potential sources of CD154 such as vascular endothelial cells and tubular epithelial cells [13] were not examined in this study.

Although the CD40–CD154 pathway is commonly described as a co-stimulatory pathway necessary for cognate immunity, these data demonstrate a major effect on innate immunity. Macrophages are the major players in the innate immune response in AN. In this model of AN, previous studies have shown that depletion of macrophages [28] or down-regulation of macrophages [44] reduces renal injury. In this study, the potent renoprotective effect of CD40–CD154 blockade in SCID mice reproduces that is found in immunocompetent mice [5]. NK cells are unlikely to be involved in the renoprotective effect of CD40–CD154 blockade as a previous study has shown that NK cells do not participate in renal injury in AN [45]. These data show that the innate arm of the immune response is as important as the cognate immune response in AN.

In summary, our studies have shown for the first time that blockade of the CD40–CD154 co-stimulatory pathway can ameliorate renal injury by a lymphocyte-independent mechanism. Our studies implicate soluble CD154 released from macrophages as a pathogenic factor in renal injury. Treatments targeting the CD40–CD154 pathway may reduce renal injury in patients with proteinuric renal diseases through the inhibition of pathways other than those of T cell activation.

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Conflict of interest statement. None to declare. The results presented in this paper have not been published previously in whole or part, except in abstract format.

References

Protection from sepsis-induced acute renal failure by adeno viral-mediated gene transfer of β2-adrenoceptor

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Abstract

Background. Sepsis is a common cause of acute renal failure (ARF) and results in a high mortality rate. The objective of the present study was to evaluate adeno viral transgenes containing the human β2-adrenoceptor (aden o-β2-AR) as a possible therapy for subjects at high risk for developing sepsis-induced ARF.

Methods. An endotoxaemic rat model of ARF was induced by renal artery occlusion plus subcutaneous injections of Escherichia coli in 4-week-old Wistar rats. A subset of rats was given intraperitoneal injection of the adeno-β2-AR gene.

Results. Sepsis produced a depression in glomerular filtration rate and in the renal β2-AR signalling system, which were both reversed by delivery of the β2-AR gene. While delivery of the adeno-β2-AR gene had no effect on recovery of cytokines and C-reactive protein in the systemic circulation, it did significantly depress (P < 0.01) the expression of the renal cannabinoid-1 (CB-1) receptor.