Adoptive transfer studies demonstrate that macrophages can induce proteinuria and mesangial cell proliferation

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Background. Glomerular macrophage accumulation is a feature of proliferative human and experimental glomerulonephritis. However, our understanding of the role of macrophages in the induction of renal injury is based upon indirect evidence from depletion studies, most of which lack specificity for this cell type. Therefore, an adoptive transfer approach was used to directly assess the potential of macrophages to induce renal injury.

Methods. Accelerated anti-glomerular basement membrane (anti-GBM) disease was induced in rats by immunization with sheep IgG (day −5), followed by administration of sheep anti-rat GBM serum (day 0), with animals killed on day 2. To facilitate the adoptive transfer studies, immunized animals were made leukopenic by cyclophosphamide (CyPh) given on day −2. Bone marrow-derived (BM) or NR8383 macrophages were transferred by tail vein injection 24 hours after injection of anti-GBM serum, with animals killed 3 or 24 hours after transfer.

Results. Pretreatment with CyPh prevented glomerular leukocyte accumulation and completely inhibited proteinuria, glomerular cell proliferation and hypercellularity in accelerated anti-GBM disease. Adoptive transfer led to significant glomerular accumulation of BM or NR8383 macrophages within 3 hours of injection, and this was still evident 24 hours later. Adoptive transfer of BM or NR8383 macrophages induced proteinuria (63 ± 16 BM vs. 5 ± 2 mg/24 h CyPh control; \( P < 0.001 \)), glomerular cell proliferation (5.1 ± 1.2 BM vs. 0.5 ± 0.1 PCNA+ cells/gcs CyPh; \( P < 0.001 \)) and glomerular hypercellularity (51.2 ± 2.0 BM vs. 41.9 ± 0.9 nuclei/gcs CyPh; \( P < 0.001 \)). The degree of renal injury correlated with the number of transferred glomerular macrophages. Two-color immunostaining demonstrated that most glomerular proliferative cell nuclear antigen+ (PCNA+) proliferating cells were OX-7+ mesangial cells. CyPh treatment did not prevent up-regulation of glomerular intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) expression or an increase in urinary monocyte chemoattractant protein-1 (MCP-1) excretion.

Conclusion. This study provides the first direct evidence that macrophages can induce renal injury in terms of proteinuria and mesangial cell proliferation.

Glomerular macrophage accumulation, first identified in crescentic glomerulonephritis [1], is a common feature in proliferative forms of human glomerulonephritis [2–4]. The ability of macrophages to secrete a wide range of factors that can cause tissue injury has led to the proposal that these cells actively participate in mediating renal injury, rather than simply being a response to tissue damage [5]. Analyses of renal biopsies have produced conflicting results as to whether glomerular macrophage accumulation does [3, 6, 7] or does not [4, 8, 9] correlate with the degree of proteinuria in glomerulonephritis. However, there is general agreement that glomerular macrophages are associated with glomerular lesions, such as mesangial cell activation, mesangial expansion, hypercellularity and glomerulosclerosis [3, 7, 9–11].

The pathogenic role of macrophages in renal injury has been examined in animal models of kidney disease, particularly experimental anti-glomerular basement membrane (anti-GBM) disease. Time-course studies have shown a temporal association between glomerular macrophage accumulation and the induction of renal injury in rat anti-GBM disease [12]. A variety of depletion studies have shown that a reduction in glomerular macrophage accumulation is associated with inhibition of proteinuria [13–21]. However, caution must be used in interpreting these studies since the methodologies employed have broader effects than just depleting macrophages.

An alternative to depletion strategies and their inherent limitations is the use of adoptive transfer to provide direct evidence that macrophages can induce renal injury. A number of studies have shown that macrophages transferred into animals can enter and accumulate within the inflamed glomerulus [22–25]. However, the key issue of whether transferred macrophages directly cause renal...
injury, such as significant proteinuria and glomerular cell proliferation, remains to be established.

Therefore, the aim of this study was to determine whether macrophages directly induce renal injury. To this end, we used adoptive transfer of macrophages in a model of rat anti-GBM disease and quantified renal injury in terms of proteinuria, glomerular cell proliferation and hypercellularity.

METHODS

Antibodies

The following mouse monoclonal antibodies (mAb) were used in this study: ED1, anti-rat CD68 that labels monocytes and macrophages [26]; OX-42, anti-rat CD11b that labels monocytes, most macrophage populations and some dendritic cells [27]; OX-1, anti-rat CD45 that labels all leukocytes [28]; OX-7, anti-Thy-1 antigen (CD90) that labels glomerular mesangial cells [29]; IA29, anti-rat intracellular adhesion molecule-1 (ICAM-1; CD54) [30]; SFI0, anti-vascular cell adhesion molecule-1 (VCAM-1; CD106) [31]; R73, anti-rat αβ T-cell receptor non-polymorphic determinant [32]; PC-10, anti-proliferating cell antigen (PCNA) that labels cells in the G1, S, and G2 phases of the cell cycle (Dako Ltd, Glostrup, Denmark); and M744, anti-β2 T-cell receptor non-polymorphic determinant [32].

Macrophage culture

NR8383 is a well characterized, semi-adherent macrophage cell line derived from Sprague-Dawley rat lung [33]. The NR8383 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in culture in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM-F12; Gibco BRL, Gaithersburg, MD, USA) with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS; Trace Biosciences, Melbourne, VIC, Australia).

Bone marrow-derived (BM) macrophages were prepared by flushing dissected femurs and tibias of male inbred Sprague-Dawley rats using 0.2% ethylenediaminetetraacetic acid (EDTA) in sterile phosphate-buffered saline (PBS). Cells were teased apart, washed, and resuspended in DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FCS. The adherent stromal cell population was removed by a two hour culture in petri dishes, and the non-adherent fraction was recovered and then cultured for five days in the presence of 20% L-cell conditioned medium as a source of macrophage colony-stimulating factor. At the end of the culture period, cells were at least 90% macrophages as judged by flow cytometric analysis and immunoperoxidase using CD68 (ED1) and CD11b (OX-42) monoclonal antibodies.

Macrophages were incubated with 50 μg/mL BrdU during the last 24 hours of culture, washed three times in sterile saline, and then transferred to recipient animals. Immunohistochemistry staining showed that BrdU was incorporated into DNA in approximately 70% of the cells and this label was used to identify cells after transfer into recipient animals.

Accelerated anti-GBM disease

The experimental strategy is outlined in Figure 1. Accelerated anti-GBM disease was induced in inbred male Sprague-Dawley rats (150 to 180 g) by subcutaneous immunization with 5 mg sheep IgG in Freund’s complete adjuvant followed five days later by intravenous injection of sheep anti-GBM serum (day 0). Animals were placed in metabolic cages on day 1 for a 24-hour urine collection and then killed. To deplete circulating leukocytes, animals were given an intraperitoneal injection of 165 mg/kg cyclophosphamide (CyPh) on day –2. This caused >98% depletion of circulating leukocytes within 48 hours (that is, at the time of anti-GBM serum injection), and this was maintained for three further days. Macrophage transfer was performed in CyPh-treated rats by an intravenous injection of NR8383 or BM macrophages 24 hours after administration of sheep anti-rat
GBM serum. Animals were either killed three hours after the transfer, or were placed in metabolic cages after the transfer for 24 hours and killed afterwards. In some studies, animals received an intraperitoneal injection of rat anti-sheep IgG serum (1 mL of pooled serum from rats that had been immunized twice with 5 mg of sheep IgG) six hours after administration of sheep anti-rat GBM serum. Animals were kept in autoclaved cages to prevent infection.

**Heterologous phase of rat anti GBM disease**

Heterologous phase anti-GBM disease was induced in inbred male Sprague-Dawley rats by intravenous injection of sheep anti-rat GBM serum (day 0). Animals were placed in metabolic cages on day 1 for a 24-hour urine collection and then killed. Leukocyte depletion via CyPh treatment and macrophage transfer was performed the same way as for accelerated anti-GBM disease.

**Biochemical analysis**

Protein excretion in 24-hour urine collections was determined using the benzethonium chloride method. Concentrations of plasma and urine creatinine were measured using the standard Jaffe rate reaction (alkaline picrate method). Whole blood cell counts were performed on a Cell-Dyn 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL, USA) using heparinized blood collected from tail veins. All analyses were performed by the Department of Biochemistry, Monash Medical Centre.

**Histopathology**

Tissues were fixed in 4% buffered formalin and then embedded in paraffin. Sections (2 μm) were stained with periodic acid-Schiff (PAS) reagent and hematoxylin. Glomerular hypercellularity was determined by counting the number of nuclei in 30 full-sized glomerular cross-sections (gcs) per animal in PAS-stained sections.

**Immunohistochemistry**

Three layer immunoperoxidase staining was used to detect macrophages (ED1), T-cells (R73), total leukocytes (OX-1), ICAM-1 and VCAM-1 in cryostat sections (5 μm) of tissue fixed in 2% paraformaldehyde-lysine-periodate, as previously described [34]. Antigen retrieval using 10 minutes of microwave oven heating in 0.01 mol/L sodium citrate, pH 6.0 was performed on sections prior to ED1 immunostaining. Sections were then blocked with 10% FCS and 10% normal sheep serum in PBS for 20 minutes, drained, incubated with the appropriate mouse mAb for 60 minutes, washed in PBS, and then endogenous peroxidase inactivated by incubation in 0.3% H2O2 in methanol. Sections then were washed in PBS, incubated with peroxidase-conjugated goat anti-mouse IgG, washed in PBS, incubated with mouse PAP complexes, and developed with 3,3-diaminobenzidine to produce a brown color. Immunoperoxidase staining for PCNA (PC10) and BrdU (M744) was performed in 4 μm paraffin sections of formalin-fixed tissues using the same method with microwave-based antigen retrieval.

Two-color immunostaining was used to detect BrdU within the transferred macrophages. After staining for BrdU using the M744 mAb in 4 μm paraffin tissue sections as described earlier, sections were given a second round of microwave oven heating to block antibody cross-reactivity, inactivate endogenous alkaline phosphatase and enhance detection of the CD68 antigen [34]. Sections then were blocked as above, drained, incubated with the ED1 mAb for 60 minutes, washed, and incubated sequentially with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse APAAP complexes, and then developed with Fast Blue BB Salt (Ajax Chemicals, Melbourne, Australia).

The same methodology was used in two-color staining to examine mesangial cell proliferation. Paraffin sections (4 μm) of methylcarn-fixed tissue were stained with the OX-7 mAb using a three-layer immunoperoxidase method, microwaved and then stained for BrdU using the M744 mAb using a three-layer immunoalkaline phosphatase method.

The number of cells stained using ED1, R73 and OX-1 mAb were counted in at least 50 glomerular cross-sections (gcs) per animal on coded slides. Data are expressed as the mean count ± SD.

**MCP-1 ELISA**

Twenty-four-hour urine samples were centrifuged, aliquoted and then stored at –20°C. The level of monocyte chemoattractant protein-1 (MCP-1) in urine samples was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Pharmingen, San Diego, CA, USA). Rat urine samples (100 μL) were assayed in a dilution range of 1:2 to 1:64.

**Serum rat anti-sheep IgG antibody response**

Circulating levels of rat IgG reactive with rabbit immunoglobulin were quantified by a sandwich ELISA as described previously [35]. Briefly, 96-well plates were coated overnight with normal sheep IgG, washed with 0.05% Tween 20 in PBS, blocked with 1% bovine serum albumin (BSA), washed, incubated with varying dilutions of rat serum for two hours, washed, incubated with peroxidase-conjugated rabbit anti-rat IgG and color development using the 3,3',5,5'-tetramethyl benzidine (TMB) substrate. The optical density was read at 490 nm on a Dynatec MR 5000 ELISA plate reader.

**Immunofluorescence**

Kidney deposition of sheep Ig, rat IgG and rat complement component C3 was determined by direct immunofluorescence staining. Sections (4 μm) of snap frozen tissue were blocked in 10% FCS and 10% rabbit serum
Table 1. Effects of cyclophosphamide (CyPh) treatment on rat accelerated anti-GBM disease

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<th>Normal</th>
<th>Anti-GBM disease</th>
<th>Anti-GBM disease + CyPh treatment</th>
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<td>Renal injury</td>
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<tr>
<td>Proteinuria mg/24 h</td>
<td>4.2 ± 1.1</td>
<td>177.8 ± 78.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Renal function</td>
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<td>Serum creatinine μmol/L</td>
<td>60.7 ± 7.0</td>
<td>59.8 ± 4.6</td>
<td>54.7 ± 6.0</td>
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<td>Creatinine clearance mL/min</td>
<td>1.04 ± 0.06</td>
<td>0.94 ± 0.19</td>
<td>0.87 ± 0.16</td>
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<td>Glomerular histology</td>
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<tr>
<td>Proliferation PCNA+ cells/gcs</td>
<td>0.2 ± 0.1</td>
<td>10.3 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Cellularity nuclei/gcs</td>
<td>40.8 ± 1.5</td>
<td>63.8 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.8 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Glomerular leukocytes</td>
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<td>Leukocytes OX-1+ cells/gcs</td>
<td>0.50 ± 0.14</td>
<td>15.59 ± 1.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Macrophages ED1+ cells/gcs</td>
<td>0.43 ± 0.09</td>
<td>13.27 ± 2.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>T cells R73+ cells/gcs</td>
<td>0.14 ± 0.04</td>
<td>0.55 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Blood leukocyte counts</td>
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<tr>
<td>Total white blood cells 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>16.9 ± 2.9</td>
<td>12.3 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.078 ± 0.057&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Neutrophils 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>1.3 ± 0.2</td>
<td>3.5 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.005 ± 0.004&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Lymphocytes 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>14.1 ± 2.7</td>
<td>7.8 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.061 ± 0.052&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Monocytes 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>0.49 ± 0.12</td>
<td>0.53 ± 0.25</td>
<td>0.009 ± 0.022&lt;sup&gt;b&lt;/sup&gt;</td>
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Abbreviations are: GBM, glomerular basement membrane; PCNA, proliferating cell nuclear antigen; gcs, glomerular cross-section.  
<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.001 vs. normal group  
<sup>c</sup>P < 0.001 vs. disease group, by ANOVA with the Bonferroni’s post-test

Fig. 2. Glomerular histology of macrophage adoptive transfer in rat anti-GBM disease. Histology was assessed on PAS stained 2 μm formalin-fixed, paraffin sections. (a) Normal rat kidney. (b) Control anti-GBM disease showing glomerular hypercellularity and glomerular deposition of PAS stained material. (c) Cyclophosphamide treatment of rat anti-GBM disease prevents glomerular damage. (d) Adoptive transfer of 7 × 10<sup>7</sup> bone marrow-derived macrophages into a cyclophosphamide treated rat with anti-GBM disease results in glomerular hypercellularity and mild PAS stained deposits. Original magnification, ×400.

for 30 minutes, drained, and incubated with appropriate FITC-conjugated rabbit polyclonal antibody. After washing, sections were mounted in 5% diazabicyclo[2.2.2]octane (Sigma-Aldrich) and examined under a fluorescence microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Comparison between groups of animals was performed by one-way analysis of variance (ANOVA) with individual group means, and then compared with the Bonferroni multiple comparison test. Correlation analysis was performed using the Pearson single correlation coefficient. All values were expressed as mean ± SD.

RESULTS

Cyclophosphamide pretreatment suppresses accelerated anti-GBM disease

In order to examine macrophage adoptive transfer in anti-GBM disease, it was necessary to remove the contri-
Fig. 3. Immunohistochemistry assessment of macrophage adoptive transfer in rat accelerated anti-GBM disease. (a) Immunoperoxidase staining fails to detect glomerular ED1+ macrophages in anti-GBM disease with cyclophosphamide (CyPh) treatment. (b) Several ED1+ macrophages (brown) are present within a glomerulus 3 hours after adoptive transfer of $7 \times 10^7$ bone marrow-derived (BM) macrophages into a CyPh treated rat with anti-GBM disease. (c) Glomerular ED1+ macrophages (brown) are still evident 24 hours after adoptive transfer of BM macrophages into CyPh treated rats with anti-GBM disease. (d) Double immunostaining for ED1 (blue) and BrdU (brown nuclei; arrowheads) confirms the donor origin of glomerular macrophages seen 3 hours after adoptive transfer of BM macrophages. Note that not all ED1+ macrophages (blue) are sectioned through the nucleus. (e) Control accelerated anti-GBM disease showing many proliferating glomerular cells as identified by staining for the proliferating cell nuclear antigen (PCNA, brown nuclei). (f) CyPh treatment of anti-GBM disease prevents the appearance of glomerular PCNA+ cells. (g) Adoptive transfer of $7 \times 10^7$ BM macrophages into CyPh treated anti-GBM disease, induces the appearance of glomerular PCNA+ cells (brown), and (h) two-color staining identifies the PCNA+ cells (gray-blue nuclei in this case) as mesangial cells on the basis of OX-7 staining (brown) around the PCNA+ nuclei (arrows). Sections in panels a, b and d were counterstained with hematoxylin. Original magnification, $\times 400$. 
bution of endogenous leukocytes. This was achieved by treatment with a single dose of 165 mg/kg cyclophosphamide (CyPh) two days before administration anti-GBM serum (Fig. 1). This resulted in $>98\%$ reduction in white blood cells at the time of anti-GBM serum administration (day 0), which was maintained until animals were killed on day 2 (Table 1). CyPh treatment abolished glomerular leukocyte accumulation and prevented renal injury in accelerated anti-GBM disease in terms of proteinuria, glomerular cell proliferation and glomerular hypercellularity (Table 1). Glomerular pathology is mild at this early stage of accelerated anti-GBM disease [12]. However, CyPh treatment completely prevented glomerular hypercellularity and glomerular deposition of PAS stained material (Fig. 2). Renal function was unchanged at day 2 in this disease model (Table 1).

Glomerular accumulation of transferred macrophages

Macrophages from two independent sources were used in transfer studies; bone marrow-derived (BM) and NR8383 macrophages. Significant numbers of BM or NR8383 macrophages were evident within the glomerulus three hours after tail vein injection into CyPh treated rats with anti-GBM disease (Fig. 3). Quantitative analysis found that administration of $5 \times 10^7$ BM macrophages into a group of five animals with CyPh treated accelerated anti-GBM disease resulted in $2.6 \pm 0.5$ ED1+ macrophages/gcs at three hours after transfer, a 20-fold increase compared to CyPh treated accelerated anti-GBM disease without transfer ($0.12 \pm 0.07$ ED1+ macrophages/gcs; $P < 0.001$). There was a reduction in the number of macrophages remaining in glomeruli by 24 hours after transfer ($0.9 \pm 0.2$ ED1+ macrophages/gcs), although this still represents a four- to fivefold increase over animals without transfer ($P < 0.01$). To confirm the donor origin of glomerular macrophages, BM and NR8383 macrophages were labeled in vitro with BrdU prior to adoptive transfer. Double staining with ED1 and BrdU antibodies confirmed that glomerular macrophages were of donor origin (Fig. 3d).

Adoptive transfer of macrophage-mediated renal injury

Glomerular accumulation of BM or NR8383 macrophages in CyPh treated anti-GBM disease was dependent upon the number of macrophages transferred (Fig. 4A). Adoptive transfer of BM or NR8383 macrophages caused significant proteinuria and glomerular cell proliferation in a dose-dependent fashion (Figs. 3 and 4). The injury assessed by 24-hour urinary protein excretion. (C) Glomerular cell proliferation assessed by PCNA+ cells/gcs. (D) Glomerular cellularity assessed by the number of nuclei/gcs. Data are expressed as the mean of groups of 5 animals ± SD. Groups were compared by ANOVA using the Bonferroni post-test analysis.
number of proliferating glomerular cells was significantly greater than the number of transferred macrophages (Fig. 4); therefore, we examined whether this could be due to proliferation of intrinsic cells. Double immunostaining demonstrated that mesangial cells accounted for most of the proliferating glomerular cells (Fig. 3h). Significant glomerular hypercellularity was seen with transfer of the highest dose of BM macrophages, but hypercellularity was not evident using adoptive transfer with NR8383 macrophages (Figs. 2 and 4D). There was a significant correlation between the number of transferred BM or NR8383 macrophages and the degree of proteinuria and glomerular cell proliferation, however; only transfer of BM macrophages correlated with glomerular hypercellularity (Fig. 5).

As a control, the highest number of NR8383 or BM macrophages was transferred into normal rats. Transfer did not result in glomerular macrophage accumulation and did not cause renal injury in normal rats (proteinuria, 3.9 ± 1.4 mg/24 h; glomerular cell proliferation, 0.35 ± 0.08 PCNA+ cells/gcs; and glomerular cellularity, 41.7 ± 2.1 cells/gcs; all $P = 0$ not significant compared with the normal group; Table 1).

Adoptive transfer led to the accumulation of many BM and NR8383 macrophages in the lung at 3 and 24 hours after injection. This occurred in both CyPh-treated anti-GBM disease and in normal rats, indicating that pulmonary macrophage accumulation is not related to anti-GBM disease. In addition, small numbers of transferred BM and NR8383 macrophages were evident in the spleen. These macrophages were seen in the red pulp, located around the lymphoid areas that showed marked involution due to cyclophosphamide treatment.

**Mechanisms of glomerular accumulation in the adoptive transfer model**

The number of glomerular macrophages obtained after adoptive transfer into CyPh treated accelerated anti-GBM disease was well below that seen in disease without CyPh treatment. This simply may reflect the difference between a single injection of cells in the transfer model versus the constant supply of blood monocytes in the control disease group. However, we investigated whether known mechanisms of glomerular macrophage accumulation in rat anti-GBM disease, such as adhesion

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**Fig. 5. Correlation between the glomerular accumulation of transferred macrophages and the severity of renal injury in rat anti-GBM disease.** The number of glomerular ED1+ macrophages in animals given adoptive transfer of NR8383 or bone marrow-derived macrophages was correlated with: (A, D) proteinuria; (B, E) PCNA+ cells per glomerular cross-section (gcs); and (C, F) glomerular cellularity (nuclei/gcs) using the Pearson single correlation coefficient ($N = 10$).
molecule expression and chemokine production [18, 19, 36, 37], were affected by CyPh treatment.

Immunostaining of tissue sections showed up-regulation of glomerular intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular adhesion molecule-1 (VCAM-1, CD106) expression in control anti-GBM disease compared to normal rat kidney (Fig. 6). Up-regulation of both ICAM-1 and VCAM-1 was still evident in CyPh treated anti-GBM disease (Fig. 6). A marked increase in urine levels of the chemokine monocyte chemotactic protein-1 (MCP-1) was evident in control anti-GBM disease (36.2 ± 17.7 vs. 6.7 ± 1.2 ng/mL in normal rats; \( P < 0.01 \)), and this was unchanged with CyPh treatment of anti-GBM disease (34.1 ± 5.0 ng/mL).

Accelerated anti-GBM disease in control rats exhibited a humoral immune response to the immunizing antigen (sheep IgG) as shown by glomerular deposition of rat IgG and C3 (Fig. 7 a-c), and high levels of rat antibodies against sheep IgG in the serum, which were readily detected at a 1 in 10,000 dilution. In contrast, CyPh treatment of accelerated anti-GBM disease prevented glomerular deposition of rat IgG and C3, but had no effect upon the deposition of sheep IgG (Fig. 7 d-f). There was also a lack of detectable serum antibodies against sheep IgG in CyPh-treated rats. CyPh treatment also inhibited the cellular immune response as shown by abrogation of glomerular T-cell accumulation (Table 1).

Administration of rat anti-sheep IgG serum to CyPh-treated anti-GBM disease reconstituted the humoral immune response as shown by glomerular deposition of rat IgG and C3 (Fig. 7). Under these conditions, there was a twofold increase in glomerular accumulation of transferred BM macrophages and a twofold increase in the severity of proteinuria, glomerular cell proliferation and hypercellularity (Fig. 8).

**Macrophage adoptive transfer reconstitutes renal injury in the heterologous phase of anti-GBM disease**

Suppression of the humoral and cellular immune response by CyPh treatment effectively changed this model of accelerated anti-GBM disease from one of autologous phase injury to one of heterologous phase injury. However, renal injury in the heterologous phase of anti-GBM disease is thought to be mediated by neutrophils rather than by macrophages. To examine this important point, we performed a separate study of macrophage adoptive transfer in the heterologous phase of anti-GBM disease. As shown in Figure 8, CyPh treatment of the heterologous phase of anti-GBM disease abrogated glomerular macrophage accumulation and prevented proteinuria, glomerular cell proliferation and glomerular hypercellu-
Fig. 7. Immunofluorescence staining of glomerular deposition of humoral immune reactants in rat anti-GBM disease. Anti-GBM disease induced in a control animal shows (a) strong linear deposition of sheep anti-GBM antibody, (b) linear deposition of rat IgG, and (c) patchy linear deposition of rat C3. Anti-GBM disease induced in a cyclophosphamide (CyPh) treated animal shows (d) strong linear deposition of sheep anti-GBM antibody, (e) absence of rat IgG, and (f) absence of rat C3. Anti-GBM disease induced in a CyPh treated animal given passive transfer of rat anti-sheep IgG serum shows (g) strong linear deposition of sheep anti-GBM antibody, (h) linear deposition of rat IgG, and (i) patchy linear deposition of rat C3. Original magnification, ×400.

larity. Adoptive transfer of BM macrophages to CyPh treated, heterologous phase anti-GBM disease resulted in glomerular macrophage accumulation and the induction of proteinuria and glomerular cell proliferation that was equivalent to that seen in control heterologous phase anti-GBM disease without CyPh treatment (Fig. 9).

DISCUSSION

To our knowledge, this study provides the first direct evidence that macrophages can induce renal injury. Adoptive transfer using two independent macrophage populations directly demonstrated macrophage-mediated proteinuria, mesangial cell proliferation and hypercellularity in rat anti-GBM disease.

A body of evidence supports the postulate that macrophages can induce renal injury such as proteinuria, but direct proof has been lacking. Studies of human glomerulonephritis have produced conflicting results as to whether glomerular macrophage accumulation correlates with proteinuria [3, 4, 6–9]. This is not surprising given the inherent difficulties of these studies, such as being limited to analysis at a single time-point, and variation in the patient populations examined. Time-course studies in animal models of glomerulonephritis have shown clear associations between macrophage accumulation and induction of proteinuria [12]. In addition, macrophage depletion using X-irradiation [13, 14], anti-macrophage sera [15, 16], lipid-encapsulated drugs [17], and blockade of chemokines such as MCP-1 and osteopontin [18–21] can prevent the induction of proteinuria in models of anti-GBM disease, puromycin aminonucleoside nephrosis and
Heymann nephritis. However, these studies have inherent problems in verifying that inhibition of proteinuria is solely due to macrophage depletion. For example, X-irradiation affects all proliferating cells, the specificity of polyclonal anti-macrophage sera is difficult to characterize, lipid-encapsulated drugs can deplete complement and inhibit glomerular neutrophil accumulation [38, 39], and MCP-1 and osteopontin also are involved in T-cell recruitment and activation [19, 20, 40]. In contrast, the current study used macrophage adoptive transfer to induce renal injury.

Two different macrophage populations were used in the transfer studies. BM macrophages are a heterogeneous population that contains a minor component (<10%) of other cell types. We don’t believe that these other cell types contributed to renal injury in the transfer model, since virtually all BrdU-labeled cells in glomeruli were double stained with the macrophage marker ED1. In addition, the BM macrophage culture was not exposed to the sheep IgG antigen. Importantly, renal injury was induced by the NR8383 macrophage cell line in a manner very similar to that seen with BM macrophages. With the transfer of similar cell numbers, both BM and NR8383 macrophages induced proteinuria and glomerular cell proliferation. Indeed, the degree of proteinuria and glomerular cell proliferation was directly proportional to the number of transferred glomerular macrophages. The main difference between the two cell types is in the efficiency in glomerular accumulation. Transfer of BM macrophages consistently resulted in a greater number of glomerular macrophages compared to NR8383 cells. This was reflected in the higher levels of proteinuria and glomerular cell proliferation. However, when compared to the actual number of transferred glomerular macrophages, the degree of renal injury was very similar for both macrophage types. The only other difference between the two macrophage populations was that BM macrophages, but not NR8383 macrophages, induced glomerular hypercellularity. There was a trend toward glomerular hypercellularity with transfer of the highest number of NR8383 macrophages, suggesting that transfer of greater numbers of NR8383 macrophages into the glomerulus may result in hypercellularity.

The role of macrophages as a cause or effect in the development of mesangial cell proliferation and glomerulosclerosis has yet to be established [41]. Macrophages may directly stimulate mesangial cell proliferation through the production of mesangial cell growth factors such as...
platelet-derived growth factor, interleukin-1, and basic fibroblast growth factor [42–44]. Studies in human glomerulonephritis support a role for macrophages in promoting mesangial hypercellularity and the development of glomerulosclerosis [10, 45]. In addition, the appearance of glomerular macrophages precedes mesangial hypercellularity and glomerulosclerosis in experimental models of glomerulonephritis [7, 46, 47]. The use of X-irradiation has suggested that macrophages promote mesangial cell proliferation and the development of glomerulosclerosis in puromycin aminonucleoside nephrosis and renal ablation [7, 48]. In contrast, macrophage depletion in rat anti-Thy-1 mesangial proliferative nephritis did not affect mesangial cell proliferation [38], arguing against a direct role for macrophages in this process. However, mesangial cell proliferation following severe mesangial lysis—a situation rarely seen in human glomerulonephritis—may operate via different mechanisms compared to mesangial cell proliferation induced in an intact glomerulus. The current studies using adoptive transfer demonstrate that mesangial proliferation in rat anti-GBM disease is macrophage-dependent. However, we cannot determine whether this mesangial proliferation operates directly via macrophage-derived growth factors, or if it is an indirect response to glomerular injury. Further studies are required to address this important question.

Glomerular accumulation of transferred macrophages was dependent upon deposition of sheep anti-GBM antibodies, since no accumulation or renal injury was evident with macrophage transfer into normal animals. However, macrophage accumulation was independent of a cellular or humoral immune response, both of which were abolished by CyPh treatment. Up-regulation of molecules involved in glomerular macrophage recruitment (ICAM-1, VCAM-1, MCP-1) also was dependent upon glomerular deposition of sheep IgG and was not significantly affected by CyPh treatment, suggesting a role for these molecules in glomerular accumulation of transferred macrophages. However, an additional role for the humoral immune response in glomerular macrophage recruitment was clearly demonstrated. Reconstitution of the autologous humoral immune response in CyPh-treated animals by passive administration of rat anti-sheep IgG serum caused a twofold increase in the glomerular accumulation of transferred macrophages, with a proportional increase in the severity of proteinuria, glomerular cell proliferation and hypercellularity.

An interesting aspect of the study was the rapid disappearance of macrophages from the glomerulus between 3 and 24 hours after transfer. This is presumably the
result of rapid loss of glomerular macrophages through apoptosis or perhaps migration into the urinary space, in the absence of continued recruitment of monocytes from the circulation. This is consistent with previous studies in this model supporting a rapid turnover of glomerular macrophages through recruitment, local proliferation and apoptosis [49, 50]. However, this transient macrophage transfer was able to induce mild glomerular lesions (hypercellularity, mesangial cell proliferation and PAS deposits) characteristic of this early stage of rat anti-GBM disease [12]. The short-term nature of this model does not allow study of severe glomerular lesions, such as crescent formation, necrosis and glomerulosclerosis, which develop later in the course of anti-GBM glomerulonephritis [12]. We do not know the mechanism(s) by which glomerular macrophage transfer induces proteinuria. However, the potential for genetic manipulation of the transferred macrophages may provide a means to address this important question.

An unexpected finding was that adoptive transfer of macrophages reconstituted renal injury in the heterologous phase of rat anti-GBM disease. Previous studies in the heterologous phase of rabbit anti-GBM disease have shown that the induction of proteinuria during the first four hours following administration of anti-GBM antibody is neutrophil-mediated [51]. Also, administration of anti-macrophage serum did not modulate renal injury in the heterologous phase of rabbit or rat models of anti-GBM disease [15, 16]. An explanation for these apparently contradictory results is that renal injury during the heterologous phase may have two components: an initial period of neutrophil-mediated injury followed by macrophage-mediated injury. Previous studies of the heterologous phase have focused on the first 3 to 12 hours after injection of anti-GBM sera, which is the period during which transient glomerular neutrophil influx occurs. In contrast, our study examined renal injury during 24 to 48 hours after administration of anti-GBM sera, which is when glomerular macrophage accumulation is prominent. Glomerular accumulation of transferred macrophages at this time was independent of an autologous immune response, and was presumably mediated by the up-regulation of adhesion molecule and chemokine production caused by deposition of the heterologous anti-GBM antibody.

In summary, this study has used adoptive transfer to demonstrate that macrophages can directly induce renal injury in terms of significant proteinuria and mesangial cell proliferation. The rigor of this approach is shown by the highly significant correlation between the number of transferred glomerular macrophages and the severity of renal injury.

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