Brief Report

Heterogeneity of antigen expression explains controversy over glomerular macrophage accumulation in mouse glomerulonephritis

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Abstract

Background. Many antibody labelling studies have suggested that there are few or no glomerular macrophages in mouse models of glomerulonephritis, despite the presence of a prominent interstitial macrophage infiltrate. These findings conflict with studies of human and rat glomerulonephritis. Therefore, we examined whether heterogeneity of macrophage antigen expression could explain this apparent discrepancy.

Methods. Kidneys were collected from normal mice and mice killed at 2 and 10 days after induction of accelerated anti-glomerular basement membrane (GBM) glomerulonephritis. Following fixation, macrophages were detected by immunoperoxidase staining in serial kidney sections using antibodies recognising CD11b, F4/80 and CD68.

Results. Induction of anti-GBM nephritis caused a progressive increase in glomerular and interstitial leukocytes. At days 2 and 10, there were more CD68+ macrophages in glomeruli than macrophages expressing CD11b or F4/80. At day 10, CD68+ macrophages accounted for almost all glomerular CD45+ leukocytes. In contrast, CD11b+ and F4/80+ macrophages at day 10 accounted for only 65 and 13% of glomerular leukocytes, respectively. However, in the interstitium the number of macrophages expressing CD68, CD11b and F4/80 were not different.

Conclusion. Antibody detection of mouse CD68 identifies all glomerular macrophages in mouse anti-GBM nephritis, indicating a similar infiltrate to that seen in human and rat anti-GBM nephritis. Our finding of substantial heterogeneity in glomerular macrophage antigen expression in this model suggests that previous studies of mouse glomerulonephritis may have underestimated glomerular macrophages and their role in glomerular injury.

Keywords: antigen expression; interstitial macrophage infiltrate; glomerular injury; mouse glomerular macrophages; mouse glomerulonephritis

Introduction

Macrophages are implicated in causing renal injury in glomerulonephritis [1]. Glomerular macrophage accumulation is a prominent feature in human and rat experimental glomerulonephritis and is associated with the development of glomerulosclerosis, glomerular hypertrophy and crescent formation [1]. These findings have relied mostly on immunodetection of the CD68 antigen as a pan macrophage marker [1]. In contrast, studies in mouse models of anti-glomerular basement membrane (GBM) glomerulonephritis and lupus nephritis have reported little or no glomerular macrophage accumulation [2–8]. The main macrophage markers used in mouse studies are antibodies recognizing the F4/80 antigen and CD11b [2–8], which are known to detect blood monocytes [9]. However, detection of CD68 has not been reported in mouse kidney disease. Therefore, the differences in detection of glomerular macrophages between mice and rats or humans in glomerulonephritis may be due primarily to the heterogeneity of antigens expressed by macrophages in kidney disease.

The aim of the current study was to evaluate expression of CD68 against other macrophage antigens (CD11b and F4/80) in order to investigate the heterogeneity of glomerular macrophage antigens in mouse anti-GBM nephritis.

Materials and methods

Materials

Antibodies used in this study were: M1/9.3.4, anti-mouse CD45 (5 μg/ml); FA/11, anti-mouse CD68 (1:50 hybridoma...
supernatant; Serotec, Oxford, UK); Cl-A3-1, anti-mouse F4/80 antigen (1:20 hybridoma supernatant; Serotec); M1/70, anti-mouse CD11b (5 µg/ml; BD Pharmingen, San Diego, CA); 5C6, anti-mouse CD11b (5 µg/ml); 7/4, anti-mouse neutrophils (5 µg/ml; Serotec); KT3, anti-mouse CD3 (5 µg/ml). Isotype-matched irrelevant rat IgGs were used as negative controls. Antibodies not purchased from commercial sources were produced by cell culture of hybridomas obtained from the American Tissue Culture Collection.

**Animal model**

Male inbred C57BL/6 mice were obtained from Monash Animal Services, Melbourne, Australia. Anti-GBM disease was induced in groups of eight C57BL/6 mice (20–25 g) using an antiseraum prepared in sheep. Briefly, mice were immunized with 1 mg of normal sheep IgG in Freund’s complete adjuvant and injected intravenously with 0.5 mg/g sheep anti-mouse GBM serum 10 days later. Mice were killed on days 2 and 10 after administration of anti-GBM serum and kidneys were collected. A group of four normal C57BL/6 mice (20–25 g) was used as a control.

**Renal function analysis**

Urine was collected from mice housed in metabolic cages for 24 h. Serum was collected from whole blood via cardiac puncture of anaesthetized mice. Urine protein, urine creatinine and serum creatinine were analysed by the Department of Biochemistry at the Monash Medical Centre.

**Immunohistochemistry**

Immunostaining for kidney leukocytes was performed on 5 µm cryostat sections of tissue fixed in 2% paraformaldehyde-lysine-peroxide (PLP). All antibodies were used at their optimal labelling concentration. The antibodies M1/70 and 5C6 were combined to achieve maximum CD11b labelling. For single antibody labelling, tissue sections were preincubated with 20% normal rabbit serum for 30 min. Primary antibodies were incubated with tissue sections overnight at 4°C in 5% normal rabbit serum. Endogenous peroxidase activity was then blocked by incubation in 0.6% hydrogen peroxide in methanol for 20 min. Sections were then incubated with 2 µg/ml biotin-conjugated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) in 5% normal rabbit serum and 2% normal mouse serum for 45 min. Sections were incubated for a further 45 min with ABC-peroxidase complex (Vectastain; Vector Laboratories) and developed with diaminobenzidine (DAB, Sigma, St Louis, MI). For double labelling of neutrophils and CD11b+ cells, sections were incubated sequentially with 7/4 mAb, FITC-conjugated rabbit anti-rat IgG (Sigma) and alkaline phosphatase-conjugated anti-FITC (Roche Diagnostics GmbH, Mannheim, Germany), and then developed with fast blue BB salt (Sigma) to detect neutrophils. Sections were then incubated sequentially with 20% rat serum, biotinylated anti-CD11b mAbs and ABC-peroxidase complex, and developed with DAB to detect CD11b+ cells. Immunostained sections were counterstained with periodic-acid Schiffs (PAS) reagent to distinguish kidney structure and haematoxylin to identify cell nuclei. Glomerular leukocytes were counted under high power (×400) in 20 glomerular tuft cross-sections per animal. The number of cortical interstitial leukocytes was assessed by counting the number of stained cells in 20 consecutive high-power fields (×250) using a 0.02 mm² graticule fitted in the eyepiece of the microscope. These fields progressed from the outer to the inner cortex, avoiding only large vessels and glomeruli. No adjustment of the cell count was made for tubules or the luminal space. All cell counting was performed on blinded slides.

**Statistical analysis**

Statistical differences between comparable groups were analysed by one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test using the software in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Data were recorded as mean ± SD.

**Results**

**Pathology of mouse anti-GBM nephritis**

Induction of anti-GBM disease resulted in transient proteinuria (normal, 4.6 ± 1.5 mg/24 h; day 2, 18.4 ± 6.8 mg/24 h; day 10, 6.0 ± 5.4 mg/24 h) and a loss of renal function indicated by a 4-fold increase in serum creatinine at day 10 of anti-GBM disease (normal, 14 ± 3 mM vs day 10, 58 ± 21 mM, P < 0.0001). Anti-GBM disease was characterized by distinct pathological changes in glomeruli (hypercellularity, glomerulosclerosis and crescent formation) and in the interstitium (cell infiltrate, tubular dilatation and atrophy, and fibrosis) (Figure 1A and B).

**Detection of glomerular macrophages in normal and diseased kidneys**

A small number of resident glomerular CD45+ leukocytes (0.9 ± 0.3 cells/gcs) were detected in normal mouse kidneys (Figure 2A). Approximately half of these normal glomerular leukocytes (54%) expressed CD68, while only 32% were CD11b+ 7/4+ macrophages and 18% F4/80+ macrophages (Figure 2A). In comparison, 14% of normal glomerular leukocytes expressed CD3 and 22% were CD11b+ 7/4+ neutrophils.

At day 2 of anti-GBM disease, there was a 2–3-fold increase in glomerular CD45+ leukocytes (2.3 ± 0.9 cells/gcs) compared to normal kidneys. This early glomerular infiltrate consisted of equal numbers of neutrophils (1.2 ± 0.3 cells/gcs) and CD68+ macrophages (1.3 ± 0.4 cells/gcs). In comparison, glomerular macrophages expressing CD11b (1.1 ± 0.5 cells/gcs) were similar in number to those expressing CD68 and glomerular F4/80+ macrophages were unchanged compared to normal (Figure 2A).

At day 10 of disease, glomerular CD45+ leukocytes were increased 8-fold compared to normal (Figure 2A). In these severely injured kidneys, the number of glomerular cells expressing CD68 (6.9 ± 1.2 cells/gcs) was similar to the total number of glomerular
CD45 + leukocytes (7.3 ± 1.5 cells/gcs) and relatively few glomerular CD3 + cells (0.5 ± 0.2 cells/gcs) and neutrophils (0.2 ± 0.1 cells/gcs) were detected. Macrophages expressing CD68 were present in both the glomerular tuft (Figure 1C) and in developing crescents (data not shown). In comparison, 65% of glomerular leukocytes were CD11b+/F4/80− macrophages and 13% expressed F4/80, which were both significantly less than the number of CD68 + glomerular macrophages detected (Figures 1C–F and 2A).

Detection of interstitial macrophages in normal and diseased kidneys

In the interstitium of normal mouse kidneys, macrophages expressing CD68 or CD11b accounted for 30% of the total CD45 + leukocytes detected while only 15% expressed F4/80 (Figure 2B). At day 2 of anti-GBM disease, numbers of interstitial leukocytes were similar to normal (data not shown). In contrast, at day 10 of disease, there was a 5-fold increase in interstitial leukocytes that were mainly macrophages (Figure 2B). The number of interstitial macrophages identified by CD68, CD11b and F4/80 were similar in these diseased kidneys.

Discussion

Our study indicates that the mouse glycoprotein CD68 is expressed by all macrophages that accumulate in glomeruli during anti-GBM nephritis. In comparison, CD11b and F4/80 appear to be expressed by a subset of these glomerular macrophages. This contrasts with macrophages accumulating in the interstitium, which express each of these antigens. The difference in antigen expression between glomerular and interstitial macrophages suggests that a major portion of macrophages accumulating in glomeruli have a different activation or differentiation status to those found in the interstitium. This finding may reflect a difference in the rates in which macrophages traffic through glomeruli and the interstitium, or alternatively, may be due to the different signals that macrophages receive in the different compartments of the injured kidney.

The discovery that F4/80 is expressed by a subset of CD68 + macrophages is not limited to glomeruli. In normal mice, macrophages localized to the T-cell areas of the spleen (white pulp) and lymph nodes (paracortex) express CD68 but not F4/80 [10]. Similarly, expression of CD11b is heterogeneous on normal murine tissue macrophages with high levels present on resident peritoneal macrophages and splenic marginal zone macrophages, low levels on Kupffer cells and none detected on resident alveolar macrophages [9].

Our current findings suggest that previous studies using F4/80 and CD11b to identify glomerular macrophages in mouse glomerulonephritis have underscored glomerular macrophage accumulation and possibly the importance of macrophages in the development of glomerular injury and crescent formation. In this study, we were able to identify underscoring of...
glomerular macrophages, particularly by F4/80 antibody, in PLP-fixed normal and diseased mouse kidneys by direct comparison with immunostaining of total CD45+ leukocytes, CD68+ macrophages, F4/80+ macrophages, CD11b+ macrophages (CD11b+7/4−), CD11b+ neutrophils (CD11b+7/4−) and CD3+ T cells were assessed in (A) glomeruli and (B) the interstitium in the kidneys of normal mice and mice at days 2 and 10 of anti-GBM glomerulonephritis (GN). Data = mean±SD, *P<0.05, **P<0.001 and NS (not significant) by ANOVA.

Fig. 2. Comparison of antibodies detecting macrophages in mouse kidneys. Kidney accumulation of CD45+ total leukocytes, CD68+ macrophages, F4/80+ macrophages, CD11b+ macrophages (CD11b+7/4−), CD11b+ neutrophils (CD11b+7/4−) and CD3+ T cells were assessed in (A) glomeruli and (B) the interstitium in the kidneys of normal mice and mice at days 2 and 10 of anti-GBM glomerulonephritis (GN). Data = mean±SD, *P<0.05, **P<0.001 and NS (not significant) by ANOVA.

In conclusion, our study demonstrates that there is substantial heterogeneity of antigen expression by glomerular macrophages in mouse anti-GBM glomerulonephritis. Furthermore, detection of CD68 in mice allows accurate identification of glomerular macrophages in mouse glomerulonephritis and indicates that macrophages may play an important role in glomerular injury and crescent formation, as previously suggested by human and rat studies. This information will help researchers in future studies to better understand the role of macrophages in glomerular injury.

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References

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