

# Intercellular Adhesion Molecule-1 Deficiency Is Protective against Nephropathy in Type 2 Diabetic db/db Mice

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Diabetic nephropathy is a leading cause of end-stage renal failure and is a growing concern given the increasing incidence of type 2 diabetes. Diabetic nephropathy is associated with progressive kidney macrophage accumulation and experimental studies suggest that intercellular adhesion molecule (ICAM)-1 facilitates kidney macrophage recruitment during type 1 diabetes. To ascertain the importance of ICAM-1 in promoting type 2 diabetic nephropathy, the development of renal injury in ICAM-1 intact and deficient db/db mice with equivalent hyperglycemia and obesity between ages 2 and 8 mo was examined and compared with results with normal db/+ mice. Increases in albuminuria (11-fold), glomerular leukocytes (10-fold), and interstitial leukocytes (three-fold) consisting of predominantly CD68+ macrophages were identified at 8 mo in diabetic db/db mice compared with nondiabetic db/+ mice. In comparison to db/db mice, ICAM-1-deficient db/db mice had marked reductions in albuminuria at 6 mo (77% ↓) and 8 mo (85% ↓). There was also a significant decrease in glomerular (63% ↓) and interstitial (83% ↓) leukocytes in ICAM-1-deficient db/db mice, which were associated with reduced glomerular hypertrophy and hypercellularity and tubular damage. The development of renal fibrosis (expression of TGF- $\beta$ 1, collagen IV, and interstitial  $\alpha$ -smooth muscle actin) was also strikingly attenuated in the ICAM-1-deficient db/db mice. Additional *in vitro* studies showed that macrophage activation by high glucose or advanced glycation end products could promote ICAM-1 expression on tubular cells and macrophage production of active TGF- $\beta$ 1. Thus, ICAM-1 appears to be a critical promoter of nephropathy in mouse type 2 diabetes by facilitating kidney macrophage recruitment.

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**D**iabetic nephropathy is a major cause of end-stage renal failure worldwide, with a prevalence expected to double over the next decade (1). Type 2 diabetes accounts for >90% of patients with diabetic nephropathy. Despite conventional therapies of glycemic and BP control, many patients still have evidence of renal damage (2). A greater understanding of the pathogenesis of diabetic nephropathy, with the identification of appropriate therapeutic targets, is therefore of enormous importance.

Kidney macrophage accumulation is known to play a role in nondiabetic renal injury and the mechanisms of macrophage accrual are well-established in these diseases (3). However, diabetic nephropathy is not generally considered to be an inflammatory disease. This view is currently changing because recent studies of human biopsy samples and animal models have established kidney macrophage accumulation as a characteristic of diabetic nephropathy (4–7). Renal macrophage accumulation correlates with the severity of glomerular and tubulointerstitial injury in experimental diabetic nephropathy (7) and nondiabetic models of renal disease (8,9). Beneficial effects

of irradiation (6) and mycophenolate (10) in animal models of diabetic nephropathy have been attributed to inhibition of renal macrophage accumulation. Adoptive transfer studies have also demonstrated that macrophages can induce proteinuria and mesangial proliferation (11). This evidence strongly suggests that renal macrophage accumulation promotes the development of diabetic renal injury, although a functional role for leukocytes in the more prevalent type 2 disease has yet to be established.

The infiltration of leukocytes into sites of inflammation is mediated by sequential binding to specific cell adhesion molecules and chemokine release that together promote rolling, arrest, firm adhesion, and transmigration (12). Intercellular adhesion molecule (ICAM)-1 is a 90-kD cell surface glycoprotein of the Ig superfamily involved in the firm attachment of leukocytes to endothelium (13), which interacts with lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) that are present on most leukocytes. ICAM-1 expression is upregulated and associated with leukocyte infiltration in human and experimental glomerulonephritis (14–17). Furthermore, increased ICAM-1 expression is seen in models of type 1 (18) and type 2 diabetic nephropathy (19,20) in parallel with disease progression (19). ICAM-1 may be even more important in promoting nephropathy associated with type 2 diabetes because its expression is not only induced by factors common to both types of diabetes, such as hyperglycemia (21),

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advanced glycation end products (22), hyperfiltration (23), and oxidative stress (24), but it can also be increased by additional elements characteristic of type 2 diabetes, including hyperlipidemia (25), hyperinsulinemia (26), and elevated levels of circulating TNF- $\alpha$  (27), which are associated with obesity (28). If ICAM-1 is a critical mediator of macrophage accumulation in diabetic kidneys, then it may play a role in the progression of nephropathy, because recent evidence indicates that macrophages activated by the diabetic milieu stimulate fibroblast proliferation and may promote interstitial fibrosis (29).

Currently, there is controversy as to whether ICAM-1 has a role in diabetic nephropathy. Previous evidence from genetically deficient mice has indicated that ICAM-1 is important for kidney macrophage accumulation and nephropathy in a model of type 1 diabetes induced by a single high dose of streptozotocin (30). However, this study did not include an insulin-treatment control to demonstrate that blood sugar rather than streptozotocin toxicity to the kidney was the major contributor to the increased kidney ICAM-1 expression. Furthermore, an ICAM-1/LFA-1 antibody-blocking study performed in type 2 diabetic rats was unable to reduce nephropathy (19), casting doubt as to whether ICAM-1 promotes nephropathy in type 2 diabetes.

Our study aims to resolve this controversy by establishing whether ICAM-1 is required for nephropathy in type 2 diabetes. To achieve these aims, we created ICAM-1-deficient db/db mice that spontaneously develop type 2 diabetes and evaluated macrophage accumulation and nephropathy in these mice compared with wild-type db/db mice with equivalent obesity and hyperglycemia.

## Materials and Methods

### *Animal Model*

All type 2 diabetic obese mice (db/db) and lean heterozygote controls (db/+) were created from breeding pairs of C57BL/6 db/+ mice obtained from Jackson Laboratories (Bar Harbor, ME) and were identified by PCR tissue type analysis for the mutated leptin receptor. No phenotypic differences were found between age-matched db/+ and +/+ C57BL/6 mice that express the normal leptin receptor. ICAM-1<sup>-/-</sup> mice with a C57BL/6 background (31) were obtained from Jackson Laboratories and crossed with C57BL/6 db/+ mice to create db/+ ICAM-1<sup>-/-</sup> mice, which were validated by PCR-based genotyping. The latter were then interbred to create homozygous obese db/db ICAM-1<sup>-/-</sup> mice. Experimental db/db mice were maintained on a normal diet under standard animal house conditions and were examined for diabetes from age 2 mo. Blood glucose level was measured monthly *via* tail vein sampling using the diagnostic glucose oxidase enzymatic test (Medisense glucometer; Abbott Laboratories, Bedford, MA), and diabetes was defined as random morning blood glucose  $\geq$ 16 mmol/L (300 mg/dl).

Given the milder degree of diabetes in db/db mice on a C57BL/6 background as opposed to the C57BLKS background (32), we selected mice on the basis of persistently elevated blood glucose level. Groups of male diabetic db/db (n = 10) and ICAM-1<sup>-/-</sup> db/db mice (n = 10) with equivalent hyperglycemia and obesity during the period of investigation were killed at age 8 mo for analysis of renal tissue. A group of 8-mo-old male nondiabetic db/+ mice (n = 10) were also killed to obtain control tissue.

### *Biochemical Analysis*

Urine was collected every 2 mo from mice housed in metabolic cages for 18 h. At the end of experimentation, heparinized whole blood and serum were collected from anesthetized mice *via* cardiac puncture. HbA1c levels, urine creatinine and plasma levels of creatinine, triglycerides, and cholesterol were analyzed by the Department of Biochemistry at the Monash Medical Centre. ELISA kits were used to measure plasma insulin (Mercodia, Uppsala, Sweden) and urine albumin (Bethyl Laboratories, Montgomery, TX).

### *Antibodies*

Antibodies used in this study were rat anti-mouse ICAM-1 (MALA-2); rat anti-mouse CD45 (M1/9.3.4); rat anti-mouse CD68 (FA-11, Serotec, Oxford, UK); rat anti-mouse F4/80 (Serotec); rat anti-mouse CD4 (GK1.5); rat anti-mouse CD8 (YTS169.4); rabbit anti-TGF- $\beta$ 1 (Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-mouse collagen IV (Santa Cruz); fluorescein-conjugated anti- $\alpha$ -smooth muscle actin (1A4; Sigma, St. Louis, MO); fluorescein-conjugated sheep anti-mouse Ig (Silenus, Melbourne, Australia); and mouse anti-rat ICAM-1 (1A29; Serotec, Oxford, UK). Isotype-matched irrelevant IgG 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 (ATCC, Manassas, VA).

### *Renal Pathology*

Formalin-fixed kidney sections (2  $\mu$ m) were stained with periodic acid-Schiff reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of glomeruli and interstitial areas were obtained from microscopy (magnification  $\times$ 400). Glomerular cellularity was determined by counting the number of nuclei in 20 hilar glomerular tuft cross-sections per animal. Glomerular volume was assessed by measuring the glomerular tuft area with computer image analysis. The percentage of atrophic tubules (dilation, cell loss, necrosis) was assessed by scoring 400 renal cortical tubules per kidney in randomly selected microscopic fields (magnification  $\times$ 250). All scoring was performed on blinded slides.

### *Immunohistochemistry Staining*

Immunoperoxidase staining for ICAM-1, leukocytes (CD45, CD68, CD4, CD8) and collagen IV was performed on 2% paraformaldehyde-lysine-periodate-fixed kidney cryostat sections (5  $\mu$ m). Immunostaining for  $\alpha$ -smooth muscle actin, F4/80, and TGF- $\beta$ 1 was performed on 10% formalin-fixed sections (4 or 5  $\mu$ m). For TGF- $\beta$ 1 detection, tissue sections were microwave-treated (1000 W) for 12 min in 400 ml sodium citrate buffer pH 6.0 before immunostaining. For immunostaining, tissue sections were incubated with 20% normal rabbit or sheep serum for 30 min and then overnight at 4°C with 2 to 5  $\mu$ g/ml of primary antibody in 1% BSA. Sections labeled with nonconjugated antibodies were then incubated for 20 min each with 0.6% hydrogen peroxide, followed by avidin and biotin block (Vector Laboratories, Burlingame, CA). After washing in PBS, sections were incubated with biotinylated goat antibody (anti-rat IgG, 1:200; Vector), biotinylated goat antibody (anti-rabbit IgG, 1:200; Zymed, San Francisco, CA), or biotinylated rabbit antibody (anti-goat IgG, 1:200; Zymed) for 1 h, followed by ABC peroxidase solution (Vector) for 1 h and developed with 3,3'-diaminobenzidine (DAB; Sigma, St Louis, MO) to produce a brown color. Sections labeled with fluorescein-conjugated antibodies were incubated for 20 min each with 0.6% hydrogen peroxide and 20% normal sheep serum. These sections were then incubated for 1 h with peroxidase-conjugated sheep anti-fluorescein Fab fragments (1:300; Roche Biochemicals, Mannheim, Germany) and developed with DAB (Sigma).

To identify interstitial macrophages expressing TGF- $\beta$ 1, formalin-fixed tissue sections (5  $\mu$ m) were first labeled with F4/80 antibody and detected by immunoperoxidase staining with DAB. Sections were then microwave-treated and labeled with TGF- $\beta$ 1 antibody, which was detected by incubation with ABC alkaline phosphatase solution (Vector) for 1 h and development with Fast Blue BB salt (Sigma) to produce a blue color.

### *Kidney Ig Deposition*

Kidney Ig deposits were assessed in 4- $\mu$ m snap-frozen tissue sections fixed in 100% ethanol for 10 min at 4°C. Tissue sections were rehydrated in PBS and incubated in 20% normal sheep serum for 30 min, followed by fluorescein-conjugated antibody detecting mouse Ig for 30 min, washing, and mounting in anti-fade medium containing 5% DABCO (1,4-diazabicyclo[2.2.2] octane; Sigma) and 70% glycerin in PBS (pH8.5). Immunofluorescence staining was assessed by titrating the antibody on serial tissue sections, using two-fold dilution steps (1:200 to 1:6400), and determining the dilution at which specific staining disappears by fluorescence microscopy.

### *Quantitation of Immunohistochemistry*

Immunostained glomerular leukocytes were counted at high magnification ( $\times$ 400) in 20 hilar glomerular tuft cross-sections per animal. Immunostained interstitial leukocytes were counted in 25 consecutive high-magnification ( $\times$ 400) interstitial fields (representing 30 to 40% of kidney cortex in the cross-section) by means of a 0.02 mm<sup>2</sup> graticule fitted in the eyepiece of the microscope and expressed as cells/mm<sup>2</sup>. Glomerular expression of TGF- $\beta$ 1 and collagen IV were assessed as percent area stained within the glomerular tuft by computer image analysis software (Image Pro Plus, Media Cybernetics, CA). Interstitial expression of  $\alpha$ -smooth muscle actin and collagen IV were scored as percent area within the renal cortex excluding glomeruli and vessels. All scoring was performed on blinded slides.

### *Preparation of Advanced Glycation End Products and Macrophage-Conditioned Medium*

N<sup>ε</sup>-(carboxymethyl)lysine (CML) modifications of proteins are the predominant advanced glycation end products (AGE) that accumulate *in vivo*. CML-BSA was produced by incubating 2 mmol BSA with 0.15 mol glyoxylic acid and 0.45 mol sodium cyanoborohydride in phosphate buffer (pH 7.8) for 1 wk at 37°C. Control BSA was prepared by incubation in the absence of reactants. Preparations of CML-BSA and control BSA were extensively dialyzed against PBS, purified on a Detoxigel column (Pierce, Rockford, IL) to remove endotoxin, and sterilely filtered (0.2  $\mu$ m). Both substances were determined to have insignificant levels of endotoxin (<0.1 ng/mg) by limulus amebocyte lysate assay (E-toxate; Sigma).

### *Preparation and Assessment of Macrophage-Conditioned Medium*

Rat bone marrow macrophages were isolated from dissected femurs and tibias of Sprague-Dawley rats and maintained in culture as described previously (11). After 8 d of growth in 20% L cell-conditioned medium and 10% FCS in low-glucose DMEM, macrophages were seeded into 24-well tissue culture plates at a density of  $1 \times 10^6$  cells/ml in the same media and allowed to adhere overnight. The next day, the medium was replaced with 10% FCS without L cell-conditioned medium and cells cultured for 24 h. Macrophages were then incubated in serum-free low-glucose DMEM containing recombinant murine macrophage colony-stimulating factor at 50 ng/ml (R&D Systems, Minneapolis, MN), insulin-transferrin-selenium supplement (Invitrogen,

Carlsbad, CA) and one of the following additives: high-glucose (25 mmol), carboxymethyl lysine bovine serum albumin (CML-BSA, 200  $\mu$ g/ml) or control BSA (200  $\mu$ g/ml). Macrophage-conditioned medium was then harvested after a 72-h incubation and assessed by ELISA for levels of active TGF- $\beta$ 1 and total (active and latent) TGF- $\beta$ 1 (Promega, Madison, WI). For determining total TGF- $\beta$ 1, the pH of supernatants was lowered to pH 2 to 3 with acid (1 mol/L HCl) for 20 min to make latent TGF- $\beta$ 1 become active, and then samples were neutralized before ELISA analysis.

### *Analysis of ICAM-1 Expression on Tubular Epithelial Cells*

Rat proximal tubular epithelial cells (NRK52E) were seeded into 6-well tissue culture plates at a density of  $0.5 \times 10^6$  cells/well in low-glucose DMEM containing 10% FCS and allowed to adhere overnight. The next day, the medium was replaced with 1% FCS and cells cultured for 24 h. The next day, media was replaced with serum-free media containing insulin-transferrin-selenium supplement and either low glucose (5 mmol/L), high glucose (25 mmol/L), control BSA (200  $\mu$ g/ml), or CML-BSA (200  $\mu$ g/ml), or in conditioned medium from macrophages exposed to the same stimuli.

After incubation for 24 h, cells were treated with 0.25% trypsin for 3 min, washed in ice-cold PBS, and fixed in 2% paraformaldehyde on ice for 30 min. All experimental cells were found to have >95% viability before fixation based on lactate dehydrogenase release cytotoxicity assay (Roche). The fixed cells were then washed and incubated for 30 min with ICAM-1 mAb (2  $\mu$ g/ml) and then for 30 min with FITC-conjugated sheep anti-mouse IgG Fab (1:50). After washing, the cells were assessed for ICAM-1 expression on a flow cytometer connected to a data acquisition system (Cytomation, Fort Collins, CO). Each experiment was repeated at least three times.

### *Statistical Analyses*

Statistical differences between two groups were analyzed by *t* test and differences between multiple groups of data were assessed by one-way ANOVA with Bonferroni multiple comparison test for groups. Data were recorded as the mean  $\pm$  standard error, and values of *P* < 0.05 were considered significant. All analyses were accomplished using the software in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

## **Results**

### *Metabolic Data in the Experimental Mice*

Obesity and hyperglycemia rapidly developed in db/db and ICAM-1<sup>-/-</sup> db/db mice, and groups of 10 animals were selected at age 2 mo with equivalent body weight and blood glucose (Figure 1). The progression of type 2 diabetes in these groups was well-matched over the 2- to 8-mo assessment period (Figure 1). Body weight, blood glucose, glycated hemoglobin (HbA1c), plasma lipids, and plasma insulin were equivalent in db/db and ICAM-1-deficient db/db mice at age 8 mo (Table 1). In comparison, db/+ mice remained lean and normoglycemic at age 8 mo (Table 1).

### *Renal Expression of ICAM-1 Is Chronically Increased in Diabetes*

Immunostaining revealed that ICAM-1 was weakly expressed in glomerular and peritubular capillaries and the tubular brush border in nondiabetic db/+ mice at age 8 mo (Figure 2A). Expression of ICAM-1 was increased in glomeruli and the interstitium in diabetic db/db mice at ages 2 and 8 mo, indicating persistent upregulation in the diabetic state (Figure 2, A and B). As



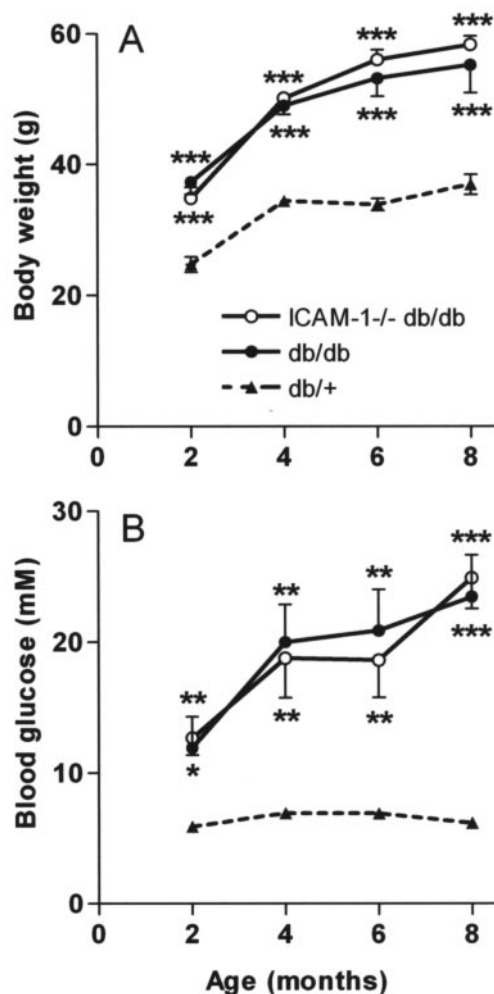


Figure 1. Chronic obesity and hyperglycemia in diabetic db/db mice. Body weight (A) and blood glucose levels (B) were assessed at 2-mo intervals from age 2 to 8 mo in db/+, db/db, and ICAM-1-deficient db/db mice. Data = mean  $\pm$  SEM; n = 10; \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 versus db/+.

expected, ICAM-1 staining was absent from the kidneys of diabetic ICAM-1<sup>-/-</sup> db/db mice at age 8 mo (Figure 2D).

#### ICAM-1 Deficiency Reduces Kidney Macrophage Accumulation in Diabetic db/db Mice

Increased numbers of kidney leukocytes (CD45+, CD68+, CD4+, but not CD8+) were identified in diabetic db/db mice at age 8 mo (Table 2, Figure 3). Comparison of total kidney leukocytes (CD45+) with the numbers of macrophages (CD68+) and T cells (CD4+, CD8+) at 8 mo indicated that almost all glomerular leukocytes (97%) and interstitial leukocytes (85%) in diabetic kidneys were macrophages. Diabetic ICAM-1<sup>-/-</sup> db/db mice showed a marked reduction in glomerular (63%  $\downarrow$ ) and interstitial (83%  $\downarrow$ ) CD45+ leukocytes compared with diabetic db/db mice at 8 mo (Table 2).

#### ICAM-1 Deficiency Reduces Renal Injury and Fibrosis in Diabetic db/db Mice

Histologic assessment identified glomerular hypertrophy, glomerular hypercellularity, and tubular atrophy/dilation in

diabetic wild-type db/db mice at age 8 mo (Figure 3, Table 3). These pathologic changes were ameliorated significantly in ICAM-1<sup>-/-</sup> db/db mice.

Urine albumin excretion increased progressively in diabetic db/db mice and was 11-fold greater than normal db/+ mice at 8 mo (Figure 4, Table 3). In comparison, albuminuria increased at a slower rate in diabetic ICAM-1<sup>-/-</sup> db/db mice and was substantially reduced at 6 mo (77%  $\downarrow$ ,  $P$  < 0.05) and 8 mo (85%  $\downarrow$ ,  $P$  < 0.01) compared with diabetic db/db mice. Creatinine clearance was reduced in db/db mice at 8 mo, and there was a trend toward improvement in the ICAM-1-deficient db/db mice at the same age, but this did not reach statistical significance (Table 3).

Assessment of immunofluorescence staining indicated that glomerular deposition of Ig was similar in db/db (fluorescence titer,  $6.9 \pm 0.6 \times 10^{-4}$ ) and ICAM-1-deficient db/db (fluorescence titer,  $6.6 \pm 0.7 \times 10^{-4}$ ) diabetic mice.

Immunostaining analysis found increased glomerular expression of TGF- $\beta$ 1 and collagen IV and increased interstitial expression of  $\alpha$ -smooth muscle actin and collagen IV in diabetic db/db mice compared with nondiabetic db/+ mice, whereas diabetic ICAM-1<sup>-/-</sup> db/db mice were markedly protected from these changes (Figure 5, Table 3). TGF- $\beta$ 1 was also detected in some interstitial cells in diabetic mice, including peritubular macrophages (Figure 6). Glomerular expression of  $\alpha$ -smooth muscle actin and tubular expression of TGF- $\beta$ 1 were not significantly increased in diabetic mice by immunostaining (data not shown).

#### Macrophages Stimulated by the Diabetic Milieu Secrete More Active TGF- $\beta$ 1 and Promote ICAM-1 Expression on Tubular Cells

Serum-free medium collected from bone marrow-derived macrophages cultured in the presence of high glucose (25 mmol/L) was found to have a six-fold greater level of active TGF- $\beta$ 1 compared with macrophages maintained in physiologic levels of glucose (5 mmol/L) (Figure 7a). Similarly, macrophages cultured with CML-BSA secreted a two-fold increase in active TGF- $\beta$ 1 compared with those cultured with control BSA. In contrast, the total combined levels of active and latent TGF- $\beta$ 1 released by macrophages were not different between cells exposed to the diabetic milieu (high glucose, CML-BSA) and controls (low glucose, control BSA) (Figure 7b).

A 24-h exposure of NRK52E tubular cells to media containing high glucose or CML-BSA resulted in no significant changes to the basal level of ICAM-1 expression seen with low glucose or control BSA, respectively (Figures 8, a and b). Similarly, conditioned media from macrophages cultured with high glucose did not increase ICAM-1 on tubular cells compared with conditioned media from macrophages cultured with low glucose (Figure 8c). In contrast, conditioned media from macrophages cultured with CML-BSA stimulated a significant increase in tubular ICAM-1 expression compared with conditioned media from macrophages cultured with control-BSA (linear mean channel fluorescence: CML-BSA  $86.9 \pm 12.6$  versus control BSA  $23.4 \pm 2.7$ ,  $P$  < 0.01, n = 4) (Figure 8d).

Table 1. Mouse characteristics at age 8 mo

	db/+ (n = 10)	db/db (n = 10)	ICAM-1 <sup>-/-</sup> db/db (n = 10)
Body weight, g	37.0 ± 1.5	55.3 ± 4.3 <sup>c</sup>	58.4 ± 1.3 <sup>c</sup>
Blood glucose, mmol/L	6.2 ± 0.3	23.5 ± 3.2 <sup>c</sup>	24.9 ± 2.4 <sup>c</sup>
HbA1c, %	2.9 ± 0.08	7.1 ± 0.5 <sup>c</sup>	7.0 ± 0.5 <sup>c</sup>
Plasma insulin, ng/ml	0.15 ± 0.04	70.4 ± 9.4 <sup>c</sup>	58.8 ± 14.8 <sup>c</sup>
Plasma cholesterol, mmol/L	2.7 ± 0.3	2.7 ± 0.9	2.1 ± 0.3
Plasma triglyceride, mmol/L	1.0 ± 0.09	1.7 ± 0.3	1.2 ± 0.2

Data = mean ± standard error.

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 versus db/+ mice.

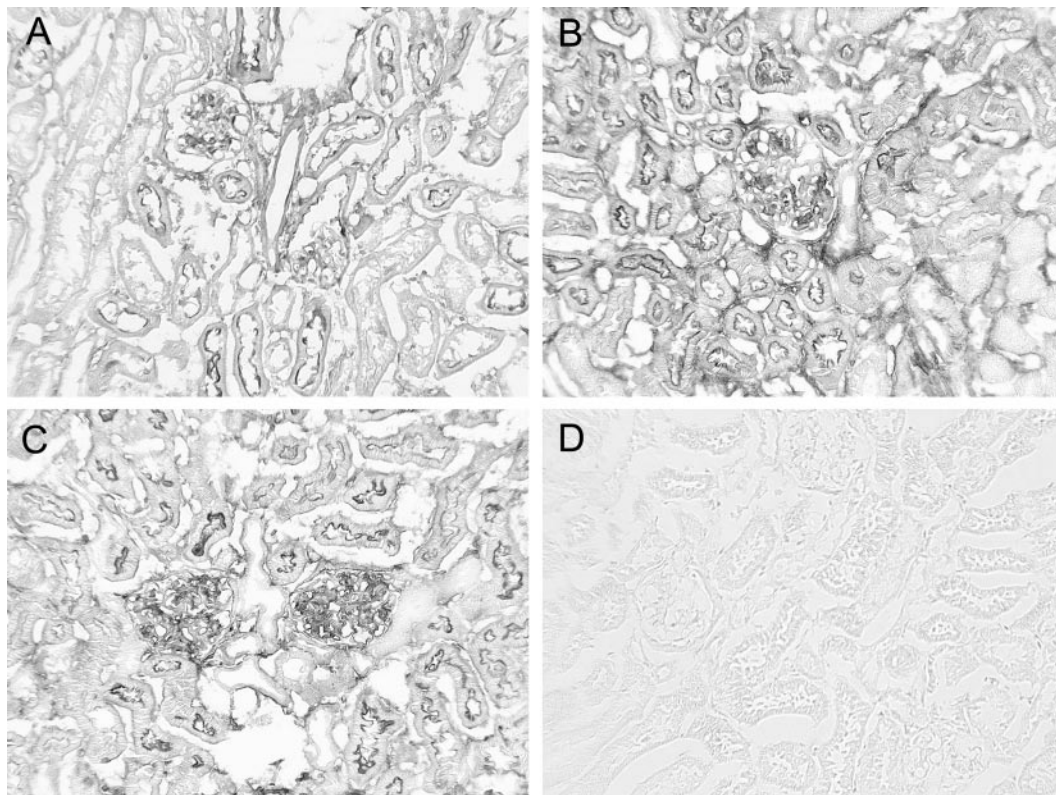


Figure 2. Kidney intercellular adhesion molecule-1 (ICAM-1) expression in diabetic db/db mice. Immunoperoxidase staining identified weak expression of ICAM-1 in glomerular and peritubular capillaries and on the tubular brush border of (A) a nondiabetic db/+ mouse. In comparison, ICAM-1 expression was increased in glomeruli and on the tubular brush border in diabetic db/db mice at age (B) 2 mo and (C) 8 mo. As expected, ICAM-1 was absent in a diabetic kidney from (D) an 8-mo-old ICAM-1<sup>-/-</sup> db/db mouse. Magnification: ×400.

## Discussion

Our study demonstrates, for the first time to our knowledge, that deficiency of ICAM-1 in the db/db mouse model of type 2 diabetic nephropathy results in a marked reduction in the accumulation of glomerular and interstitial macrophages, in parallel with significant reductions in renal injury (albuminuria), glomerular damage (hypertrophy, hypercellularity, fibrosis), tubular atrophy, and interstitial fibrosis. These findings indicate that macrophages are likely to be key mediators of renal damage in type 2 diabetic nephropathy, and that ICAM-1

plays a critical role in facilitating renal macrophage influx in the type 2 diabetic state.

The onset of type 2 diabetes increased ICAM-1 expression in the glomerular capillaries and tubular brush border of diabetic db/db mice. This is similar to what has been described for inflammatory models of glomerulonephritis (16,33) and type 1 diabetic nephropathy (18). Cellular activation by the diabetic milieu provides a number of mechanisms (oxidative and mechanical stress, cytokine induction), which can promote renal ICAM-1 expression, and studies suggest that activation of the



Table 2. Kidney leukocytes in experimental mice at age 8 mo

Kidney Leukocytes	db/+ (n = 10)	db/db (n = 10)	ICAM-1 <sup>-/-</sup> db/db (n = 10)
<b>Glomerular</b>			
CD45 + cells/gcs	1.23 ± 0.05	12.43 ± 2.2 <sup>b</sup>	5.37 ± 1.08 <sup>d</sup>
CD68 + cells/gcs	1.14 ± 0.03	12.18 ± 2.4 <sup>b</sup>	4.96 ± 1.35 <sup>c</sup>
CD4 + cells/gcs	0.15 ± 0.02	0.32 ± 0.05 <sup>a</sup>	0.15 ± 0.03 <sup>e</sup>
CD8 + cells/gcs	0.01 ± 0.007	0.015 ± 0.001	0.01 ± 0.007
<b>Interstitial</b>			
CD45 + cells/mm <sup>2</sup>	156.2 ± 6.1	432.2 ± 53.3 <sup>b</sup>	233.8 ± 19.1 <sup>e</sup>
CD68 + cells/mm <sup>2</sup>	146.8 ± 5.8	397.4 ± 41.6 <sup>b</sup>	195.4 ± 9.3 <sup>e</sup>
CD4 + cells/mm <sup>2</sup>	13.0 ± 1.2	15.8 ± 5.2	6.8 ± 0.6
CD8 + cells/mm <sup>2</sup>	1.2 ± 0.7	0.8 ± 0.3	0.6 ± 0.3

Data = mean ± SE.

<sup>a</sup>*P* < 0.01, <sup>b</sup>*P* < 0.001 versus db/+ mice; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01, <sup>e</sup>*P* < 0.001 versus db/db mice.  
gcs indicates glomerular cross-section.

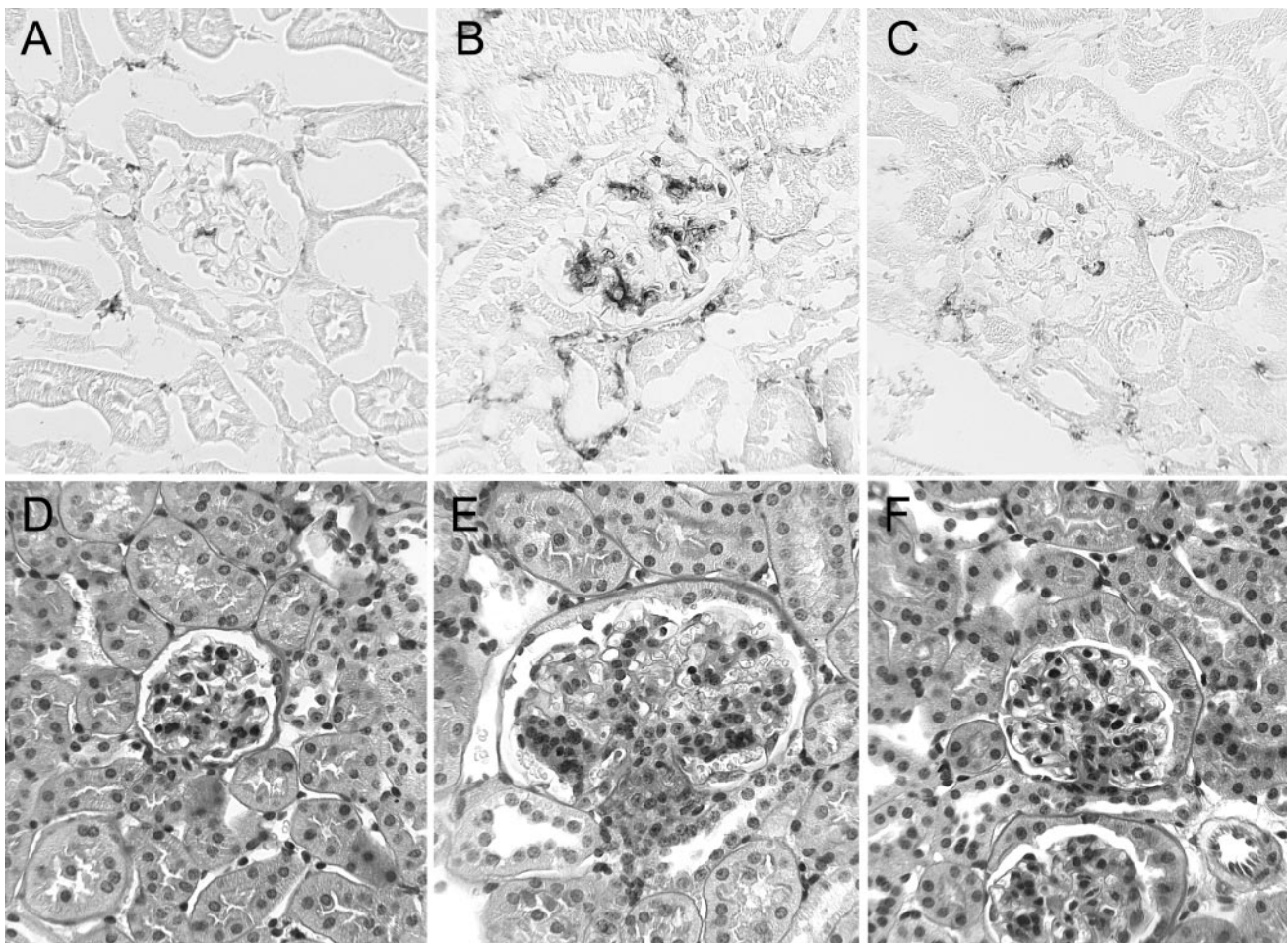


Figure 3. ICAM-1 deficiency reduces kidney macrophage accumulation and histologic damage in diabetic db/db mice. Immunostaining for CD68 in 8-mo-old mice shows very few macrophages in (A) a normal db/+ mouse kidney, which is markedly increased in the glomeruli and interstitium of (B) a diabetic db/db mouse kidney. (C) An ICAM-1<sup>-/-</sup> db/db mouse with equivalent diabetes has a reduced accumulation of kidney macrophages. Histologic staining with periodic acid-Schiff (PAS) and hematoxylin shows the normal kidney structure of (D) a nondiabetic db/+ mouse at 8 mo. In comparison, there is significant damage to glomeruli (hypertrophy, hypercellularity, mesangial PAS deposits) and tubules (dilation, atrophy) in (E) a diabetic db/db mouse at 8 mo, which is attenuated in (F) an equally diabetic ICAM-1<sup>-/-</sup> db/db mouse at the same age. Magnification: ×400.

Table 3. Renal injury in experimental mice at age 8 mo

	db/+ (n = 10)	db/db (n = 10)	ICAM-1 <sup>-/-</sup> db/db (n = 10)
Renal function			
UAER, $\mu\text{g}/18\text{ h}$	7.8 $\pm$ 1.1	89.3 $\pm$ 22.8 <sup>b</sup>	19.9 $\pm$ 8.5 <sup>b,f</sup>
plasma creatinine, $\mu\text{mol}/\text{L}$	23.4 $\pm$ 1.0	35.0 $\pm$ 2.9 <sup>b</sup>	32 $\pm$ 2.4 <sup>a</sup>
CrCl, $\mu\text{l}/\text{min}$	156.9 $\pm$ 26.0	55.3 $\pm$ 7.4 <sup>c</sup>	75.4 $\pm$ 10.6 <sup>b</sup>
Glomerular damage			
volume, $\mu\text{m}^3 \times 10^4$	2.95 $\pm$ 0.14	8.67 $\pm$ 2.2 <sup>a</sup>	4.77 $\pm$ 0.45
cellularity, cells/gcs	34.5 $\pm$ 0.8	56.7 $\pm$ 2.9 <sup>c</sup>	42.7 $\pm$ 4.2 <sup>a,f</sup>
TGF- $\beta$ 1, % area	4.3 $\pm$ 0.6	13.9 $\pm$ 1.7 <sup>c</sup>	8.5 $\pm$ 3.8 <sup>d</sup>
collagen IV, % area	17.5 $\pm$ 0.4	21.5 $\pm$ 1.0 <sup>c</sup>	17.5 $\pm$ 0.5 <sup>c,f</sup>
Tubular damage			
atrophic tubules, %	0.05 $\pm$ 0.01	9.7 $\pm$ 1.2 <sup>c</sup>	4.2 $\pm$ 1.2 <sup>a,e</sup>
Tubulointerstitial fibrosis			
$\alpha$ -SMA, % area	1.3 $\pm$ 0.2	4.4 $\pm$ 0.7 <sup>c</sup>	1.8 $\pm$ 0.1 <sup>f</sup>
collagen IV, % area	17.2 $\pm$ 0.5	25.3 $\pm$ 0.5 <sup>c</sup>	20.2 $\pm$ 0.3 <sup>c,f</sup>

Data = mean  $\pm$  SE.

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  versus db/+; <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$  for db/db ICAM-1<sup>-/-</sup> versus db/db. CrCl indicates creatinine clearance; SMA, smooth muscle actin; UAER, urine albumin excretion rate.

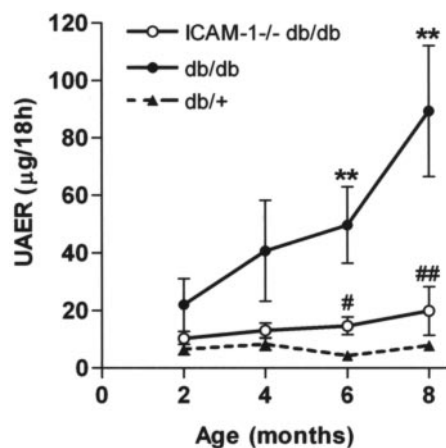


Figure 4. ICAM-1 deficiency reduces albuminuria in diabetic db/db mice. Urinary albumin excretion rate (UAER) was evaluated at 2-mo intervals from ages 2 to 8 mo in db/+, db/db, and ICAM-1<sup>-/-</sup> db/db mice. From age 6 mo, ICAM-1<sup>-/-</sup> db/db mice have significantly less albuminuria than db/db mice. Data = mean  $\pm$  SEM; n = 10; \*\* $P < 0.01$  versus db/+; # $P < 0.05$ , ## $P < 0.01$  versus db/db.

protein kinase C–nuclear factor- $\kappa$ B pathway is an important part of this process (21,34). Thus, upregulation of kidney ICAM-1 appears to be a common response to diabetic and nondiabetic renal injury.

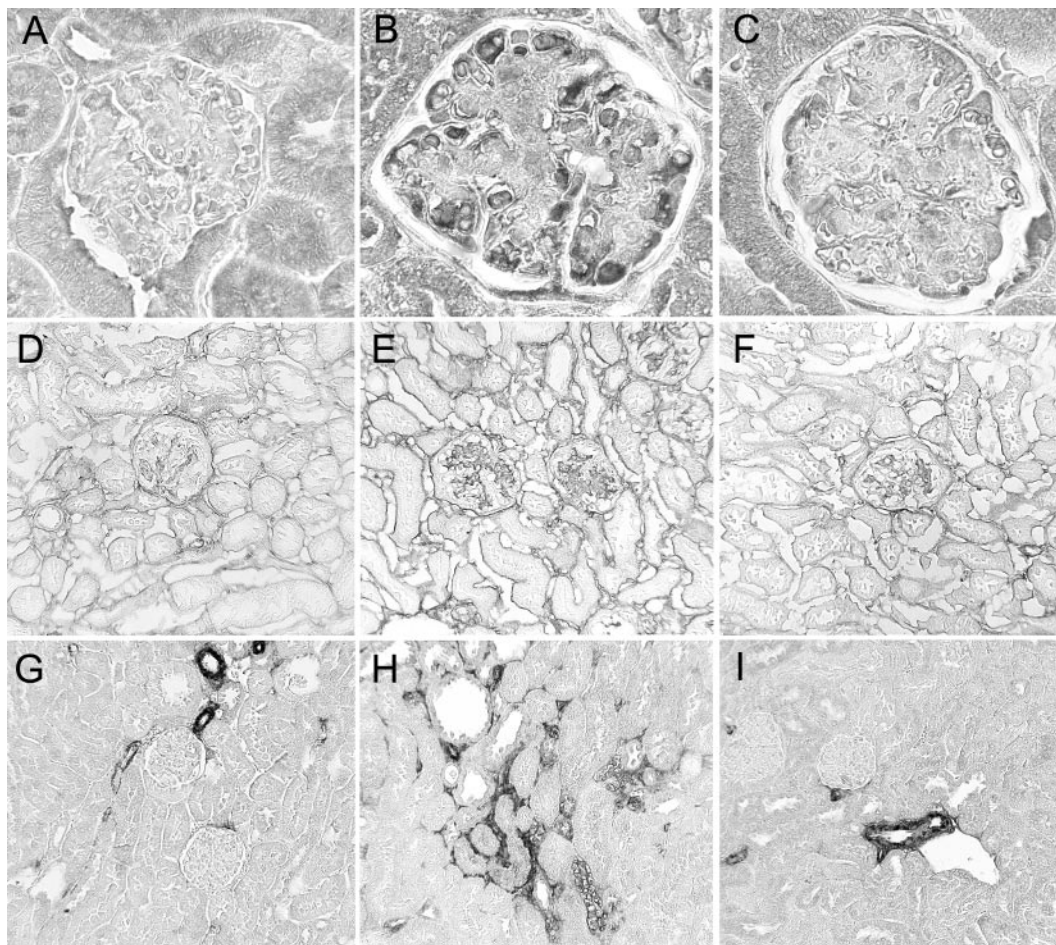
Our study shows that ICAM-1 promotes macrophage accumulation in diabetic kidneys. ICAM-1 deficiency reduced glomerular and interstitial macrophages by >60% in diabetic db/db mouse kidneys, indicating that ICAM-1 is critical for recruitment of kidney macrophages in this model. A similar reduction in kidney macrophages has been seen in studies of streptozotocin-induced type 1 diabetes in which mice were

either ICAM-1-deficient or were treated with a neutralizing ICAM-1 antibody (30,18). This suggests that hyperglycemia, rather than obesity, is likely to be the main factor responsible for ICAM-1-mediated macrophage accumulation in diabetic kidneys, a concept that is supported by previous analysis of diabetic db/db mice, showing kidney macrophages correlate with hyperglycemia but not with obesity or plasma lipids (7). Furthermore, because glomerular Ig deposition in diabetic db/db mice is unaffected by ICAM-1 deficiency, we can exclude the possibility that part of ICAM-1-mediated macrophage recruitment is through altering glomerular immune complex accumulation.

In contrast to our findings, a previous study examining the Wistar fatty rat model of type 2 diabetes showed that administration of monoclonal antibodies against ICAM-1 or LFA-1 for 3 mo was unable to prevent the development of nephropathy (19). There are many reasons why ICAM-1/LFA-1 antibody blockade may not have worked successfully in this study. ICAM-1 has several integrin-binding sites (35) that may not be all neutralized by antibody treatment, and it may be difficult to achieve effective antibody levels *in vivo*. In addition, the rats may have developed an immune response against the administered antibodies, which would reduce the effectiveness of antibody treatment over time. In our current study, we have overcome these difficulties by using a gene knockout strategy for functional blockade of ICAM-1.

Our findings indicate that macrophages are key mediators of renal damage and fibrosis in type 2 diabetic nephropathy. Previous work in our laboratory has demonstrated that the adoptive transfer of macrophages into leukocyte-depleted animals can lead to glomerular damage and contribute to proteinuria and mesangial proliferation (11). Macrophages are capable of secreting a variety of factors that promote renal injury (nitric oxide, reactive oxygen species, IL-1,





**Figure 5.** ICAM-1 deficiency decreases renal fibrosis in diabetic db/db mice. Kidney immunostaining in 8-mo-old mice identified weak glomerular and tubular expression of TGF- $\beta$ 1 in (a) a nondiabetic db/+ mouse. Increased levels of glomerular, tubular, and interstitial TGF- $\beta$ 1 were observed in the diabetic kidney of (b) a db/db mouse, but were noticeably reduced in (c) an ICAM-1<sup>-/-</sup> db/db mouse. Collagen IV immunostaining was present in the mesangium and basement membranes of (d) a nondiabetic db/+ mouse. Increased glomerular and interstitial collagen IV were detected in the diabetic kidney of (e) a db/db mouse, but were diminished in (f) an ICAM-1<sup>-/-</sup> db/db mouse. Immunostaining of  $\alpha$ -smooth muscle actin was detected in kidney vessels in (g) a nondiabetic db/+ mouse. Many interstitial cells expressing  $\alpha$ -smooth muscle actin were identified in the diabetic kidneys of (h) a db/db mouse, and these cells were markedly reduced in (i) an ICAM-1<sup>-/-</sup> db/db mouse. Magnification: a to c,  $\times$ 400; d to i,  $\times$ 250.

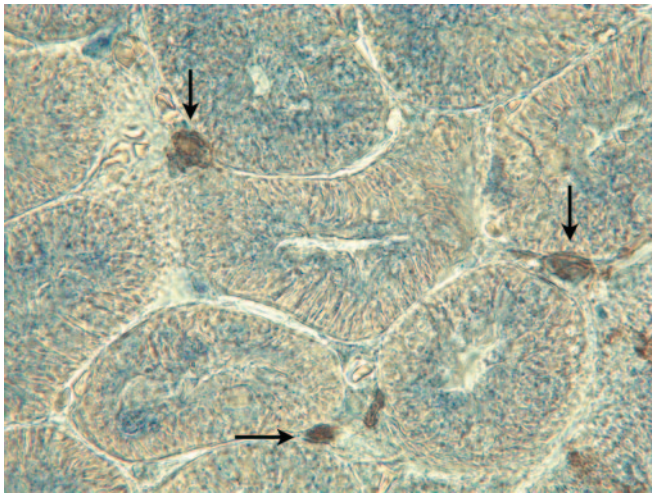
TNF- $\alpha$ , metalloproteinases) (3), and their production can be induced by elements of the diabetic milieu (36–38). In addition to causing injury, macrophage activation by the diabetic milieu may also promote renal fibrosis through the secretion of factors that promote fibroblast proliferation (IL-1, PDGF) (29) and TGF- $\beta$  production (IL-1) (39). In this study, we demonstrated that macrophages in diabetic kidneys express TGF- $\beta$ 1 and that macrophages cultured with high glucose or AGE produce increased amounts of active, but not latent, TGF- $\beta$ 1, which would be expected to enhance matrix production (39). This may result, in part, from the diabetic milieu stimulating increased macrophage production of factors that activate latent TGF- $\beta$ 1 (40). Therefore, macrophages may promote diabetic nephropathy by inducing tissue injury and fibrosis.

Our study showed that macrophages stimulated by an ad-

vanced glycation end product (CML-BSA) secreted factors that increase ICAM-1 expression on tubular cells. One of the secreted factors responsible for inducing tubular ICAM-1 expression may be IL-1 $\beta$ , because AGE are known to induce macrophage IL-1 $\beta$  production (29), and IL-1 is a potent stimulator of ICAM-1 on renal cells (33). This effect could lead to increased interaction between infiltrating macrophages and tubular cells that may promote injury, further inflammation and perhaps fibrosis. A recent *in vitro* study has shown that tubular expression of ICAM-1 mediates an interaction with monocytes that results in increased TGF- $\beta$ 1 mRNA and protein synthesis by the tubular cells (41). Thus, kidney macrophages activated by the diabetic milieu may also be able to promote renal fibrosis by their interactions with other renal cells.

It is possible that some protective effects of ICAM-1 deficiency in db/db mice may be independent of its role in

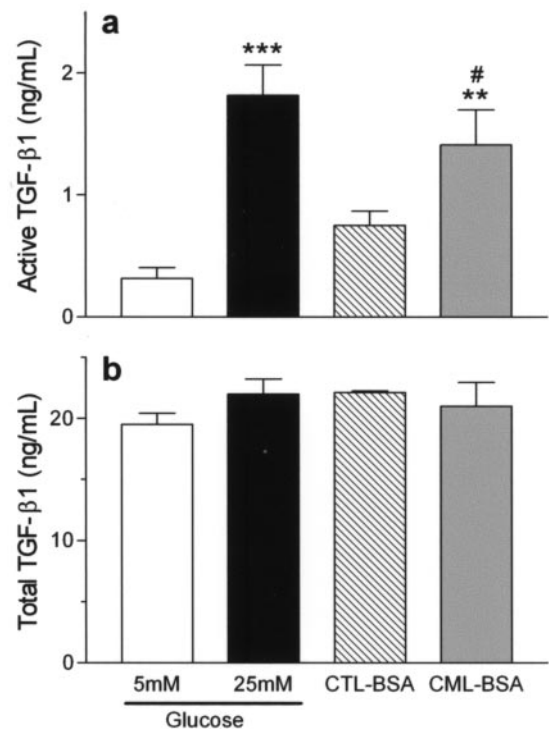




**Figure 6.** Interstitial macrophages in diabetic kidneys express TGF- $\beta$ 1. Double immunostaining shows three peritubular F4/80+ macrophages (brown) expressing TGF- $\beta$ 1 (blue) in a diabetic db/db mouse at age 8 mo. Magnification:  $\times 1000$ .

kidney macrophage accumulation. The presence of ICAM-1 on kidney cells (endothelial, epithelial, mesangial, and antigen presenting cells) also promotes the firm adhesion of T cells and can stimulate T cell activation *via* interaction with LFA-1 (42). In addition, advanced glycation end products can induce CD4+ and CD8+ T cells to produce IFN- $\gamma$  (43) which can then promote further inflammation and oxidative stress *via* interaction with other kidney cells. Our study showed that CD4+ T cells were increased in the glomeruli but not the interstitium of diabetic db/db mouse kidneys. This glomerular increase in CD4+ T cells was not detected in the diabetic kidneys of ICAM-1-deficient db/db mice. Therefore, because ICAM-1 facilitates an increase in glomerular CD4+ T cells in diabetic db/db mice, it is possible that this effect, although small compared with ICAM-1-mediated macrophage recruitment, may contribute to the inflammatory process and injury in diabetic kidneys.

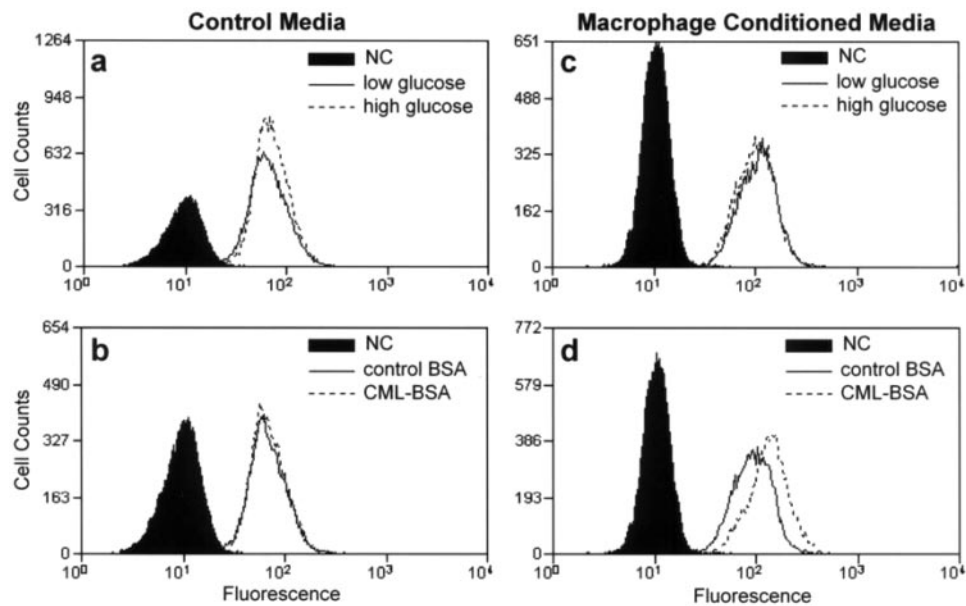
Despite the substantial improvements to albuminuria, renal inflammation, and histologic damage, ICAM-1 deficiency in diabetic db/db mice did not significantly improve renal function as measured by creatinine clearance. However, it is possible that our determination of declining renal function by assessment of creatinine clearance may be confounded by ongoing muscle wastage occurring in the obese db/db mice caused by lack of exercise. Diminished amounts of skeletal muscle in obese diabetic db/db mice were observed during necropsy at 8 mo compared with lean db/+ mice at the same age, and because creatinine is produced by muscle, a reduced muscle mass in db/db mice would indicate that our measurements of creatinine clearance are probably underestimating the real loss of renal function in these animals. In addition, type 2 diabetes may also affect creatinine clearance by the formation of advanced glycation end products that reduce expression of organic cation transporters that promote tubular secretion of creatinine (44). Therefore, it is possible that ICAM-1 deficiency



**Figure 7.** Macrophages stimulated with high glucose or advanced glycation end products (AGE) produce more active TGF- $\beta$ 1. Cell supernatant was collected from  $1 \times 10^6$  bone marrow-derived macrophages cultured for 72 h in serum-free medium containing low glucose (5 mmol/L), high glucose (25 mmol/L), control BSA (CTL-BSA, 200  $\mu$ g/ml) or carboxymethyllysine BSA (CML-BSA, 200  $\mu$ g/ml). Supernatants were assessed by ELISA for levels of (a) active TGF- $\beta$ 1 and (b) total (active and latent) TGF- $\beta$ 1 after acid treatment. Data = mean  $\pm$  SD; n = 3; \*\*P < 0.01, \*\*\*P < 0.001 *versus* 5 mmol/L glucose; #P < 0.05 *versus* CTL-BSA.

may actually be providing significant protection to renal function in diabetic db/db mice; however, determination of this will require either significant modification to our assessment of creatinine clearance allowing for changes to muscle mass and tubular secretion of creatinine or a different method of evaluation.

Reducing ICAM-1 may be an important therapeutic goal for treating diabetic nephropathy. The pathologic role of ICAM-1 could be inhibited by reducing ICAM-1 expression on the cell surface of endothelium and other renal cells, competitive blockade, or interference with receptor activation (45). Hyperglycemia, advanced glycation end products, and oxidative stress are elements of the diabetic state that are known to induce ICAM-1 upregulation (21,22,24) and decreasing any of these factors is likely to reduce renal injury, in part, through diminishing ICAM-1 expression. Signaling pathways stimulated by the diabetic milieu, including protein kinase C, NF- $\kappa$ B, and mitogen-activated protein kinases, are known to induce ICAM-1 expression and inhibitors of these pathways provide protection (46,21,26). In addition, small molecule antagonists of ICAM-1



**Figure 8.** AGE-stimulated macrophages promote ICAM-1 expression on tubular cells. ICAM-1 is normally expressed on NRK52E tubular cells cultured in (a) low glucose (5 mmol/L) or (b) control BSA (200  $\mu$ g/ml). Direct stimulation of NRK52E cells with high glucose (25 mmol/L) or CML-BSA (200  $\mu$ g/ml) had no effect on the level of ICAM-1 detected (a, b). NRK52E cells incubated with supernatant from macrophages cultured in the presence of low glucose, high glucose, or control BSA had similar levels of ICAM-1 expression (c, d), which were slightly greater than that observed for cells incubated directly with each stimulant. In comparison, supernatant from macrophages cultured in the presence of CML-BSA significantly increased ICAM-1 expression on NRK52E cells relative to control BSA supernatant (d). Each experiment was performed at least three times and produced similar results.

are currently in development (42) and hold promise for the management of inflammatory diseases, including diabetic nephropathy, through specific targeting of ICAM-1. Therefore, targeting of ICAM-1 either directly or indirectly is a realistic therapeutic goal for the management of renal disease in diabetic patients.

In summary, our study demonstrates that macrophage accumulation in type 2 diabetic kidneys can be markedly diminished by preventing ICAM-1-mediated interactions between macrophages and renal cells, resulting in reduced renal injury. Therefore, strategies that suppress ICAM-1 expression in diabetic kidneys are likely to retard the progression of diabetic nephropathy by reducing the level of macrophage-mediated damage.

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See related editorial, "Linking Metabolism and Immunology: Diabetic Nephropathy Is an Inflammatory Disease," on pages 1537–1538.