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Intrinsic Renal Cells Are the Major Source of Tumor Necrosis Factor Contributing to Renal Injury in Murine Crescentic Glomerulonephritis

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Abstract. Macrophages are prominent participants in crescentic glomerulonephritis (GN) and have been suggested to be the major source of TNF in this cell-mediated form of glomerular inflammation. Intrinsic renal cells also have the capacity to produce TNF. For dissecting the contribution of local *versus* bone marrow (BM)–derived TNF in inflammatory renal injury, TNF chimeric mice were created by transplanting normal wild-type (WT) BM into irradiated TNF-deficient recipients (WT→$TNF^{-/-}$ chimeras) and *vice versa* ($TNF^{-/-}$→WT chimeras). A model of crescentic GN induced by an intravenous injection of sheep anti-murine glomerular basement membrane antibody was studied in WT mice, mice with complete TNF deficiency (TNF−/−), and chimeric mice. Crescentic GN was attenuated in TNF−/− mice with fewer crescents (crescents, 13.7 ± 1.7% of glomeruli) and reduced functional indices of renal injury (serum creatinine, 15.2 ± 0.8 μmol/L). Similar protection (crescents, 14.3 ± 1.9% of glomeruli; serum creatinine, 18.9 ± 1.1 μmol/L) was observed in chimeric mice with intact BM but absent renal-derived TNF (WT→$TNF^{-/-}$ chimeras), suggesting a minor contribution of infiltrating leukocytes to TNF-mediated renal injury. Chimeric mice with TNF-deficient leukocytes but intact intrinsic renal cell–derived TNF (crescents, 20.5 ± 2.0% of glomeruli; serum creatinine, 21.6 ± 1.4 μmol/L) developed similar crescentic GN to WT mice (crescents, 22.3 ± 1.4% of glomeruli; serum creatinine, 24.8 ± 1.9 μmol/L). Cutaneous delayed-type hypersensitivity after subdermal challenge with the nephritogenic antigen was attenuated in the absence of BM cell–derived TNF but unaffected in WT→$TNF^{-/-}$ chimeric mice. These studies suggest that intrinsic renal cells are the major cellular source of TNF contributing to inflammatory injury in crescentic GN.

Crescentic glomerulonephritis (GN) is a severe and rapidly progressive form of renal inflammation that results from Th1-directed cell-mediated immune/inflammatory responses to antigens located in glomeruli. It is characterized by the recruitment of inflammatory cells into the glomerulus, the proliferation of glomerular cells, and accumulation of cells and fibrin in Bowman space to form the appearance of crescents surrounding the glomerular tuft. The net inflammatory injury represents the interactions between recruited bone marrow (BM)-derived inflammatory cells and resident cells within the kidney. The extent to which the responses of intrinsic renal cells may amplify or inhibit inflammatory injury is poorly understood.

TNF is a key participant in both innate and adaptive (Th1) immune responses. TNF is expressed in glomeruli in human crescentic GN (1) and in experimental models; the functional contribution of TNF to crescentic injury as been demonstrated (2–7). Various leukocytes, including macrophages isolated from nephritic glomeruli (8), are known to produce TNF. Intrinsic renal cells, including mesangial cells (9–11) and glomerular (12,13) and tubular epithelial cells (14,15), also have the capacity to produce TNF *in vitro*. Macrovascular endothelial cells also produce TNF (16,17), but TNF production by glomerular endothelial cells has not been reported.

TNF stimulates a variety of proinflammatory responses by intrinsic renal cells. In mesangial cells, TNF stimulates proliferation (18) and increases production of monocyte-colony stimulating factor (M-CSF) (19), monocyte chemoattractant protein (MCP-1) (20), reactive oxygen metabolites (21), and tissue factor expression (22). TNF also induces intercellular adhesion molecule-1 (ICAM-1) expression and enhances monocyte adhesion (23). TNF enhances IL-8 production by proximal tubular epithelial cells (24).

In experimental GN, administration of soluble TNF receptors to rats developing crescentic GN reduced glomerular injury and prevented crescent formation (4,5). Anti-TNF antibodies (Ab) attenuate acute heterologous phase anti–glomerular basement membrane (GBM) Ab–induced injury in rats (3). Systemic administration of TNF induces glomerular

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injury in rabbits (2). Studies in TNF-deficient mice have demonstrated that glomerular crescent formation, infiltration of T cells and neutrophils (but not macrophages), and expression of ICAM-1 and vascular cell adhesion molecule (VCAM-1) is partially dependent on TNF in murine anti-GBM GN (6,7).

The extent to which infiltrating leukocytes and intrinsic renal cells contribute to TNF-mediated renal injury in crescentic GN is unknown. Infiltrating macrophages have been considered to be a major source of TNF in crescentic glomeruli (8). Intrinsic renal cells are often considered as targets of leukocyte mediated injury rather than active contributors to glomerular inflammation. To address the relative contributions of TNF from BM-derived leukocytes and intrinsic renal cells to crescentic GN, we studied chimeric and TNF-deficient mice.

Materials and Methods

Mice

C57BL/6 TNF-deficient (TNF−/−) mice were generated by direct gene targeting of C57BL/6 embryonic stem cells (25). Mice were housed and bred under specific pathogen-free conditions at Monash University (Clayton, Victoria, Australia).

BM Transplantation

Irradiation and BM reconstitution were carried out as described previously (26,27). At 8 wk after transplantation, circulating leukocyte numbers, lymphocyte subsets (assessed by flow cytometry), and splenic distribution of T cells and macrophages (assessed by immunohistology) were normal, as previously reported (26,27). The efficiency of BM replacement using this protocol has been previously assessed by using CD45 congenic mice, in which 98 ± 1% of BM cells, 92 ± 1% of circulating leukocytes, and 90 ± 2% of splenic leukocytes were of donor origin (26). The capacity of BM-derived cells to repopulate the glomerulus was studied by transplanting BM from C57BL/6 mice transgenic for green fluorescence protein (GFP) cDNA under a chicken β-actin promoter with a cytomegalovirus enhancer (C57BL/6-TgN(ACTbEGFP)1Osb; Jackson Laboratories, Bar Harbor, ME). Wild-type (WT) recipients were studied 8 wk after transplantation, immediately before induction of GN. GFP-positive cells were counted in 6-μm cryostat cut tissue sections of kidneys from four mice that received a transplant. The mean number of GFP-positive cells was 1.6 ± 0.3 cells per glomerular cross-section (c/gcs). The total glomerular cell count in periodic acid–Schiff (PAS)-stained paraffin-embedded section of similar thickness was 31 ± 1 c/gcs, indicating that BM-derived cells represent approximately 5% of cells in normal mouse glomeruli at this stage after BM transplantation. Some of these cells may be passenger leukocytes.

Induction of Crescentic GN

Crescentic anti-GBM GN was induced in mice by intravenous injection of a total of 14.4 mg of sheep anti-mouse GBM globulin (in 900 μl of PBS) divided equally into two doses given 3 h apart. The development of crescentic GN and cutaneous delayed-type hypersensitivity (DTH) was assessed in WT and TNF−/− mice and two groups of irradiated BM transplanted chimeric mice. All mice were 13 to 14 wk of age at the time of anti-GBM globulin administration. Renal injury and cutaneous DTH was assessed 21 d later.

Histological Assessment of Glomerular Injury

Glomerular Crescent Formation. Kidney tissue was fixed in Bouin fixative and embedded in paraffin, and 3-μm sections were stained with PAS reagent. Glomeruli were considered to exhibit crescent formation when two or more layers of cells were observed in Bowman space. A minimum of 50 glomeruli were assessed to determine the crescent score for each animal.

Glomerular CD4$^+$ Cell and Macrophage Accumulation. Kidney tissue and spleen were fixed in periodate/lysine/paraformaldehyde. Six-micrometer cryostat-cut sections were stained to demonstrate CD4$^+$ cells and macrophages with monoclonal Ab GK1.5 and M/170, respectively, using a three-layer immunoperoxidase technique (28). A minimum of 20 equatorially sectioned glomeruli were assessed per animal, and results are expressed as cells/gcs.

Tubulointerstitial Infiltration. The number of interstitial cells in PAS-stained renal tissue sections was counted using a 10-mm^2 graticule fitted in the eyepiece of the microscope. Five randomly selected cortical areas, which excluded glomeruli, were counted for each animal. Each high-powered field represented an area of 1 mm^2. Data are expressed as cells/mm^2 and represent the mean ± SEM for four animals in each group.

Functional Assessment of Glomerular Injury

Proteinuria. Mice were housed individually in cages to collect urine before administration of anti-GBM globulin and during the final 24 h of the experiment. Urinary protein concentrations were determined by a modified Bradford method. Before induction of GN, all groups of mice had 24-h urine protein excretion in the normal range (0.5 to 2.0 mg/24 h).

Serum Creatinine. Serum creatinine was measured by an enzymatic creatininase method (CREA Plus, Cat. No. 1775685; Roche, Basel, Switzerland) with a lower limit of detection of 2.7 μmol/L using a Roche Cobas Bio analyzer.

Demonstration of Renal Co-localization of TNF with Macrophages, Mesangial Cells, and Endothelial Cells by Confocal Microscopy

Expression of TNF and its co-localization with macrophages, mesangial cells, and endothelial cells was demonstrated in renal tissues by confocal microscopy. The following directly conjugated monoclonal Ab were used: PE-conjugated rat anti-mouse TNF (clone MP6-XT22; PharMingen, San Diego, CA), Alexa Fluor 594 (Molecular Probes, Eugene, OR), conjugated protein G–purified monoclonal rat–anti-mouse macrophage Ab (clone M170; American Type Culture Collection, Rockville, MD), FITC-conjugated anti-mouse α-smooth muscle actin (clone 1A4; Sigma Aldrich, Castle Hill, NSW, Australia) as a marker of activated mesangial cells, and Alexa Fluor 488 (Molecular Probes) conjugated anti-mouse von Willebrand factor (Cedarlane, Ontario, Canada) as an endothelial cell marker. Cryostat-cut snap-frozen kidney tissue sections (6 μm) were blocked with 10% normal rat serum in 5% BSA/PBS and then incubated with both Ab at a final dilution of 1 in 40 for 60 min at room temperature. Confocal images were collected using a Bio-Rad (Hampstead, UK) confocal inverted Nikon microscope equipped with an air cooled 25-mWatt argon/krypton laser, as described previously (27). Digital images were collected using BioRad laser sharp 2000 version 4.1 software.

Demonstration of Renal MHC II and E-Selectin Expression by Confocal Microscopy

Macrophage MHC II expression and endothelial cell E-selectin expression in renal tissues were assessed by confocal microscopy using directly conjugated monoclonal Ab. These included protein G–purified monoclonal rat–anti-mouse MHC class II Ab (clone Y3P, a gift from Prof. K. Shortman) conjugated to Alexa Fluor dye 488

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(Molecular Probes), Alexa Fluor 594–conjugated protein G–purified monoclonal rat–anti-mouse macrophage Ab, anti-mouse E-selectin (clone RME-1, a gift from Dr. A. Issekutz) conjugated to Alexa Fluor dye 594 (Molecular Probes), and Alexa Fluor 488–conjugated anti-mouse von Willebrand factor. Staining was performed on frozen renal tissue sections as described above.

Demonstration of Renal ICAM-1 Expression and Fibrin Deposition

Evaluation of ICAM-1 expression was performed on 6-μm cryostat-cut sections of periodate/lysine/paraformaldehyde-fixed kidney tissue. Sections were stained with a monoclonal hamster–anti-mouse ICAM-1 (clone 3E2; PharMingen) Ab, using a three-layer immunoperoxidase technique (28) and using biotinylated mouse–anti-hamster IgG (clone G94-56, PharMingen) as the secondary Ab.

Fibrin deposition was assessed by direct immunofluorescence on 4-μm cryostat-cut sections of snap-frozen kidney. Sections were blocked with 15% normal goat serum in 5% BSA and then incubated with FITC-conjugated goat–anti-mouse fibrin/fibrinogen serum (Nordic Immunological Laboratories, Tilburg, The Netherlands) at a dilution of 1 in 20 for 60 min at room temperature.

In a blinded protocol, ICAM-1 and fibrin expression was scored semiquantitatively from 0 to 3 as follows: 0, background staining; 1, the lowest clearly positive staining; 2, moderate staining; and 3, intense deposition. ICAM-1 was assessed in 10 randomly selected cortical areas, whereas 20 glomeruli were assessed for fibrin; five animals in each experimental group were scored.

Induction of Cutaneous DTH to Sheep Globulin

Mice that developed GN were challenged 24 h before the end of the experiment by intradermal injection of 500 μg of sheep globulin in 50 μl of PBS into one hind footpad. A similar dose of an irrelevant antigen (horse globulin) was injected into the other footpad as a control. Footpad swelling was quantified 24 h later using a micrometer (Mitutoyo Corporation, Kawasaki, Japan). Antigen-specific DTH was taken as the difference in skin swelling between sheep globulin– and horse globulin–injected footpads and expressed as Δ footpad thickness (mm).

Humoral Immune Responses to Sheep Globulin

Mouse anti-sheep globulin Ab titers were measured by ELISA on serum collected at the end of each experiment. Assays were performed using microtiter plates coated with sheep globulin at a concentration of 10 μg/ml, and bound mouse Ig was detected using horseradish peroxidase–conjugated sheep–anti-mouse Ig (Amersham, Little Chalfont, UK) as the detecting Ab, as described previously (29).

Statistical Analyses

The development of GN was studied in a total of 24 WT mice, 11 TNF−/− mice, 16 WT→$TNF^{-/-}$chimeric mice, and 9 $TNF^{-/-}$→WT chimeric mice. These were "WT→$TNF^{-/-}$ chimeras" in which WT BM was transplanted into TNF−/− mice and "$TNF^{-/-}$→WT chimeras" in which TNF−/− BM was transplanted into WT mice. Results are expressed as the mean ± SEM. The statistical significance was determined by one-way ANOVA, followed by Newman-Keuls *post hoc* test.

Results

TNF Deficiency Provides Significant Protection from Crescentic GN

WT mice developed proliferative GN with crescent formation (Figure 1A), glomerular T-cell and macrophage accumulation, and functional renal injury. Significant protection against the development of crescentic GN was observed in TNF−/− mice (Figure 1B). Glomerular crescent formation (13.1 ± 1.7% of glomeruli) was reduced compared with WT mice (22.3 ± 1.4% of glomeruli; $P < 0.01$; Figure 2), as was glomerular infiltration of $CD4^+$ cells (TNF−/−, 0.15 ± 0.03 c/gcs; WT, 1.06 ± 0.13 c/gcs; $P < 0.001$), macrophages (TNF−/−, 0.56 ± 0.11c/gcs; WT, 2.37 ± 0.12 c/gcs; $P < 0.001$), and the interstitial cell infiltrate (TNF−/−, 109 ± 4 cells/mm^2; WT, 137 ± 4 cells/mm^2; $P < 0.001$; Figure 3). Reduction in the histologic injury was associated with significant protection of renal function, indicated by reduction in proteinuria (TNF−/−, 2.6 ± 0.5 mg/24 h; WT, 9.6 ± 1.4 mg/24 h; $P < 0.001$) and serum creatinine (TNF−/−, 15.2 ± 0.8 μmol/L; WT, 24.8 ± 1.9 μmol/L; $P < 0.01$) in TNF−/− mice compared with WT mice (Figure 2).

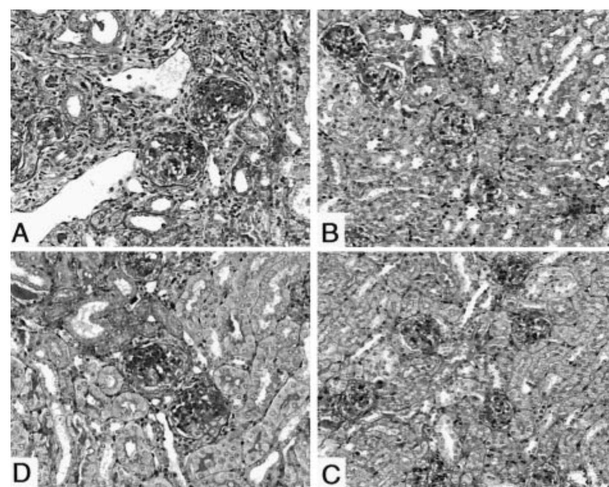

Figure 1. Histological appearances of glomerulonephritis (GN), 21 d after administration of sheep anti-mouse GBM globulin. WT mice (A) developed proliferative GN with glomerular crescents (*). Proliferative changes and crescents were attenuated in TNF−/− mice (B) and WT→$TNF^{-/-}$ chimeric mice (D). However, $TNF^{-/-}$→WT chimeric mice (C) developed similar crescent formation to that observed in WT mice. Magnification, ×200, periodic acid–Schiff (PAS)-stained sections.

TNF Production by Intrinsic Renal Cells Is Required for Full Expression of Crescentic GN

Chimeric mice with WT BM and absent renal TNF (WT→$TNF^{-/-}$ chimeras) showed similar protection from crescentic GN to mice with complete TNF deficiency (Figure 1D). In contrast, mice with absent BM-derived TNF but intact renal TNF ($TNF^{-/-}$→WT chimeras) did not show significant protection from crescent formation (Figure 1C) compared with WT mice. Crescent formation was significantly reduced in WT→$TNF^{-/-}$ chimeras (14.3 ± 1.9% of glomeruli) compared with $TNF^{-/-}$→WT chimeras (20.5 ± 2.0% of glomeruli; $P < 0.05$; Figure 2).

WT→$TNF^{-/-}$ chimeras also showed similar protection to TNF−/− mice with regard to other parameters of severity of GN. Glomerular accumulation of macrophages (WT→$TNF^{-/-}$ chi-

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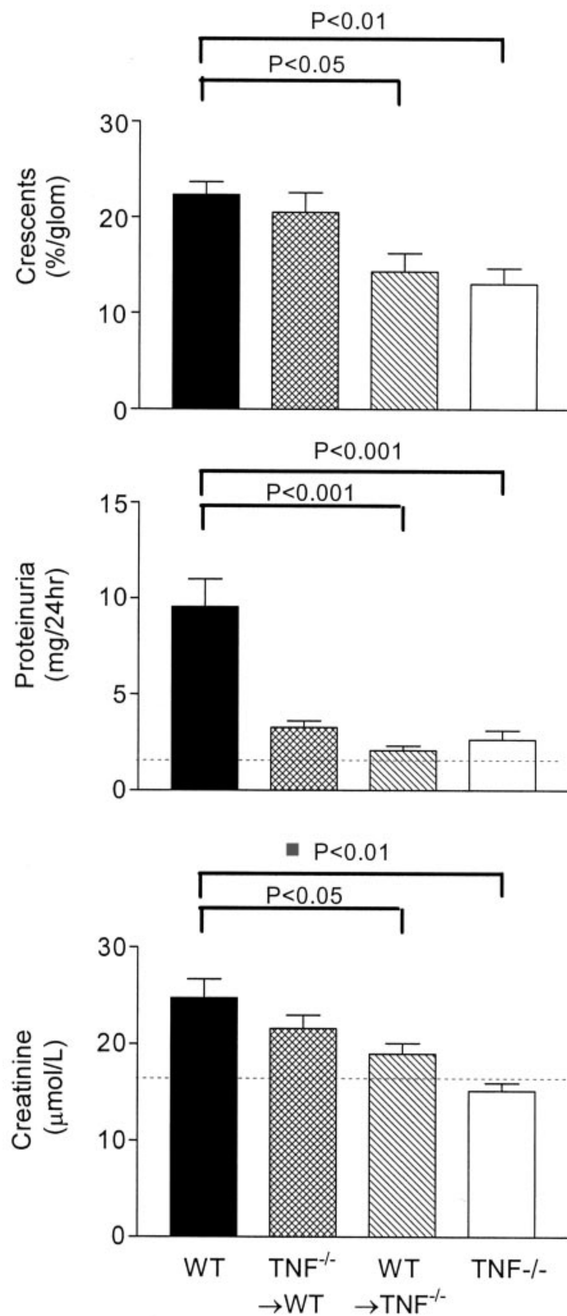

Figure 2. The incidence of crescent formation (% glomeruli affected), proteinuria (mg/24 h), and serum creatinine (μmol/L) in WT, $TNF^{-/-}$→WT chimeric, WT→$TNF^{-/-}$ chimeric, and TNF−/− mice developing crescentic GN. The dotted line represents the baseline value observed in normal WT mice.

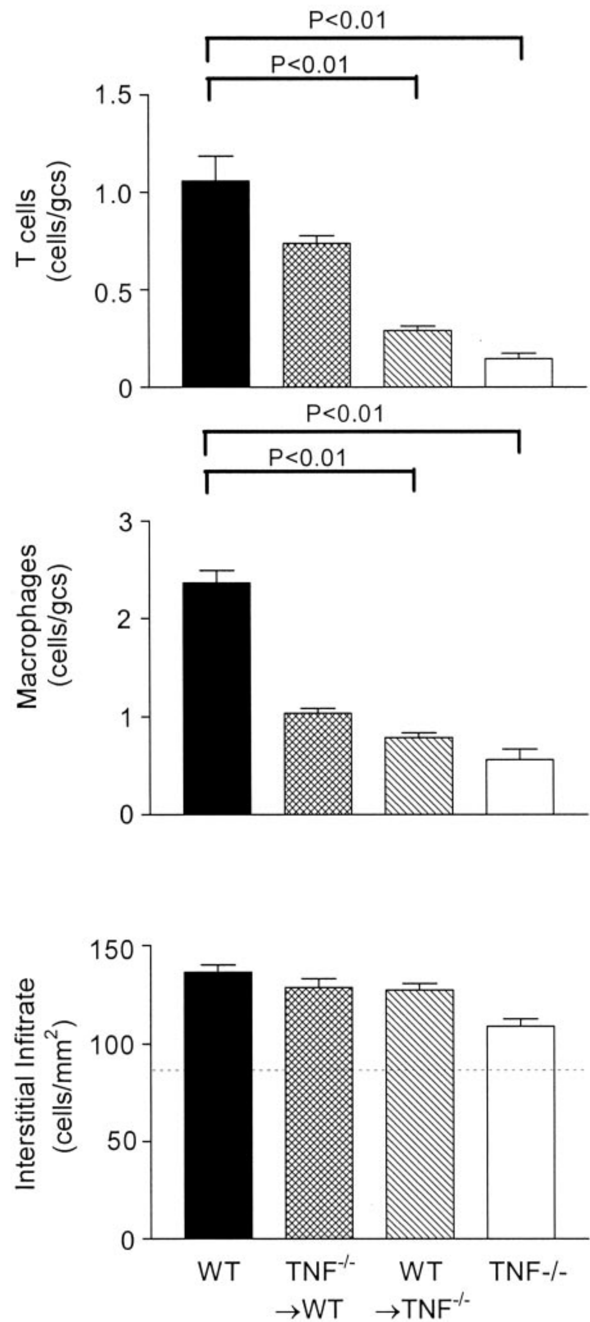

Figure 3. Glomerular infiltration of $CD4^+$ cells and macrophages (cells/glomerular cross-section [c/gcs]) and the interstitial cell counts (cells/mm^2) of WT, $TNF^{-/-}$→WT chimeric, WT→TNF^- chimeric, and TNF−/− mice developing crescentic GN. The dotted line represents the baseline value observed in normal WT mice.

meras, 0.8 ± 0.05 c/gcs; $P < 0.001$ cf. WT) were not significantly different from TNF−/− mice. Glomerular $CD4^+$ cells (WT→$TNF^{-/-}$ chimeras, 0.3 ± 0.02 c/gcs) were significantly reduced compared with WT mice ($P < 0.001$ cf. WT) and $TNF^{-/-}$→WT chimeras ($P < 0.001$) but were also significantly greater than in TNF−/− mice ($P < 0.05$; Figure 3). Proteinuria (2.1 ± 0.2 mg/24 h; $P < 0.001$ cf. WT) and serum creatinine (18.9 ± 1.1 μmol/L; $P < 0.05$ cf. WT) were reduced to a similar extent in WT→$TNF^{-/-}$ chimeras and TNF−/− mice (Figure 2).

In comparison, $TNF^{-/-}$→WT chimeras demonstrated less protection from glomerular leukocyte accumulation and functional renal injury than WT→$TNF^{-/-}$ chimeras. Glomerular accumulation of T cells (0.7 ± 0.04 c/gcs; $P < 0.001$ cf. WT) and macrophages (1.1 ± 0.05 c/gcs; $P < 0.001$ cf. WT) was significantly less than in WT mice but significantly greater

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than in TNF−/− mice ($P < 0.01$ in both cases). Serum creatinine (21.6 ± 1.4 μmol/L) was not reduced compared with WT mice, but proteinuria (3.3 ± 0.3 mg/24 h; $P < 0.05$ cf. WT) was decreased to a similar extent as in TNF−/− mice (Figure 2).

The interstitial inflammatory infiltrate in both chimeras (WT→TNF$^{-/-}$ chimeras, 127 ± 3 cells/mm^2; TNF$^{-/-}$→WT chimeras, 129 ± 4 cells/mm^2) was not significantly different from WT mice but was significantly greater than in TNF−/− mice, indicating that both leukocyte and intrinsic renal cell TNF production contributes to interstitial inflammation and that different mechanisms of inflammatory cell recruitment exist in the glomerulus and interstitial compartments (Figure 3).

TNF Expression in Chimeric Mice

TNF (Figure 4,red) was expressed in the spleen and kidneys of WT mice with GN. Prominent TNF expression was observed in glomeruli, tubules, and interstitial areas (Figure 4A). Co-localization (yellow) with α-smooth muscle actin demonstrated expression of TNF by activated mesangial cells in glomeruli, although some mesangial cells (green) did not seem to express TNF. Co-localization with von Willebrand factor was not observed, suggesting that glomerular endothelial cells do not express TNF. TNF was also expressed by some periglomerular macrophages but rarely by intraglomerular macrophages (not shown). In TNF−/− mice, the absence of staining with the TNF Ab confirms the specificity of this Ab (Figure 4B). Mesangial cells demonstrated prominent α-smooth muscle cell actin expression (green) in the absence of TNF (Figure 4B). In TNF$^{-/-}$→WT chimeric mice, TNF expression in glomeruli showed a similar intensity to that in WT mice, with co-localization of TNF with mesangial cells observed (Figure 4C). TNF expression was detected in tubular cells but not in periglomerular macrophages. In WT→TNF$^{-/-}$ chimeras (Figure 4D), TNF was rarely detected within glomeruli and did not co-localize with mesangial cells. Occasional periglomerular macrophages and interstitial cells were TNF positive, but expression by tubular cells was not detected.

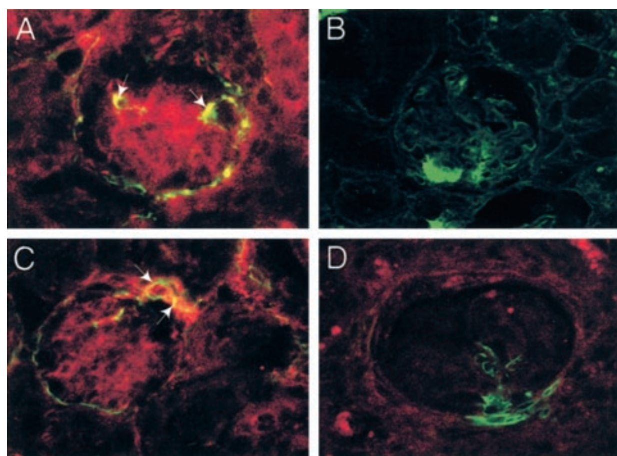

Figure 4. Confocal images demonstrating TNF expression (red), anti–α-smooth muscle actin expression (green), and co-localization of TNF with anti–α-smooth muscle actin (yellow) in kidneys of mice with GN. In the kidneys of WT mice (A), coexpression (yellow, arrow) of TNF with anti–α-smooth muscle actin (a marker of activated mesangial) was observed in intraglomerular cells, particularly in the glomerular stalk. TNF expression was also observed on tubular cells. Staining with the TNF antibodies (Ab) was absent in TNF−/− mice (B), demonstrating the specificity of this Ab. Anti–α-smooth muscle cell actin (green) was expressed by mesangial cells in the absence of TNF. In TNF$^{-/-}$→WT chimeric mice (C), TNF expression by mesangial cells was evident (arrow). In WT→TNF$^{-/-}$ chimeras (D), TNF was rarely detected within glomeruli and did not co-localize with mesangial cells (green). Magnification, ×600, confocal immunofluorescence, under oil immersion.

Effect of TNF Chimerism on MHC II and E-Selectin Expression

In WT mice with GN (Figure 5A), MHC II expression (green) was prominent on tubular cells and co-localization (yellow) with periglomerular macrophages was observed. In TNF$^{-/-}$→WT chimeric mice (Figure 5C), MHC II expression on tubular cells was reduced but co-localization on periglomerular macrophages remained prominent. In contrast, renal MHC II expression was markedly reduced in WT→TNF$^{-/-}$ chimeric mice (Figure 5D) and TNF−/− mice (Figure 5B), in which MHC II was restricted to macrophages in periglomerular areas (yellow).

E-selectin (red) was prominent in the glomerular tufts of WT (Figure 5E) and TNF$^{-/-}$→WT chimeric mice (Figure 5G). Co-localization (yellow) with von-Willebrand factor (green) on endothelial cells was clearly observed. However, E-selectin expression on glomerular endothelial cells (green) was absent in WT→TNF$^{-/-}$ chimeras (Figure 5H) and TNF−/− mice (Figure 5F).

Effect of TNF Chimerism on Glomerular Fibrin Deposition and Renal ICAM-1 Expression

Fibrin is an important mediator of crescentic anti-GBM GN (30). Fibrin deposition was prominent in the majority of glomeruli of WT mice with GN (glomerular fibrin score, 2.6 ± 0.3) and in TNF$^{-/-}$→WT chimeras (score, 2.3 ± 0.3) but was significantly reduced in WT→TNF$^{-/-}$ chimeras (score, 1.3 ± 0.3; $P < 0.001$ cf. WT) and in TNF−/− mice (score, 0.7 ± 0.3, $P < 0.001$ cf. WT). Glomerular fibrin deposition in TNF−/− mice was not significantly less than in WT→TNF$^{-/-}$ chimeras (Figure 6).

Expression of ICAM-1 was prominent in interstitial and periglomerular areas, on tubular cells, and in glomeruli of WT mice with GN, with a mean score of 2.5 ± 0.4. ICAM-1 expression was significantly reduced in TNF−/− mice (score, 0.9 ± 0.2, cf. WT; $P < 0.001$). ICAM-1 expression in TNF$^{-/-}$→WT chimeras (1.9 ± 0.4) and WT→TNF$^{-/-}$ chimeric mice (2.5 ± 0.4) was not significantly different from WT mice. Thus, the expression of ICAM-1 corresponds with the observed pattern of interstitial inflammation.

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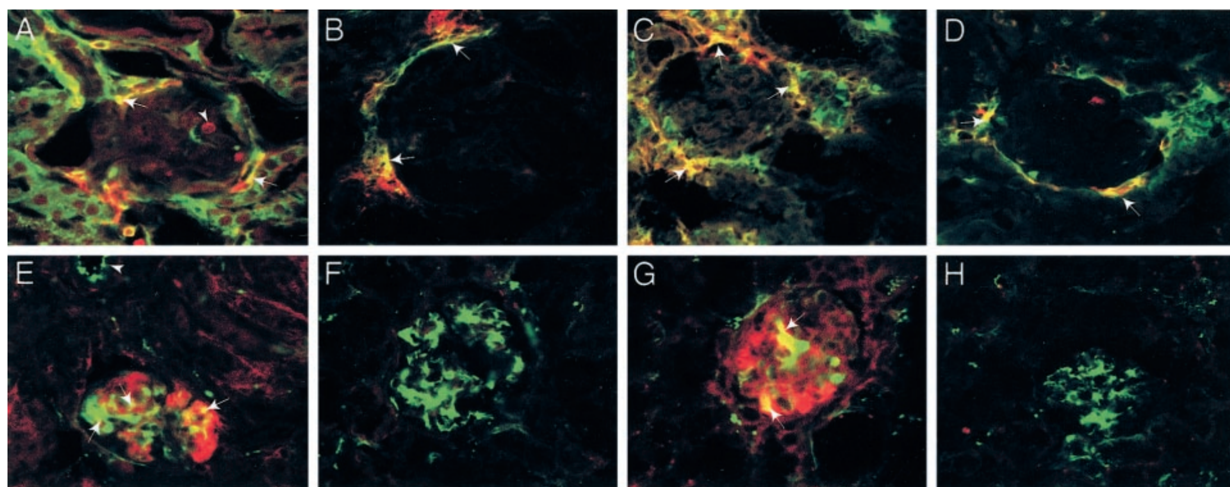

Figure 5. Confocal images demonstrating MHC II (A–D) and E-selectin (E–H) expression in the kidneys of mice with GN. In WT mice (A), MHC II expression (green) was prominent on tubular cells, and co-localization (yellow, arrow) with periglomerular macrophages was observed. Intraglomerular macrophages (red, arrowhead) did not express MHC II. In TNF−/− mice (B), MHC II expression was markedly reduced and restricted to periglomerular areas, mostly in association with macrophages (yellow, arrow). The expression of MHC II in $TNF^{-/-}$→WT chimeric mice (C) was similar to that observed in WT mice, with co-localization on periglomerular macrophages (arrow). Renal MHC II was markedly reduced in WT→$TNF^{-/-}$ chimeric mice (C) and showed a similar pattern to expression to TNF−/− mice. E-selectin (red) was expressed in glomeruli of WT mice with GN (E) and co-localized (yellow, arrow) with von Willebrand factor (green) on endothelial cells. E-selectin was not observed on interstitial vessels (arrowhead). E-selectin expression was absent in TNF−/− mice (F) and in WT→$TNF^{-/-}$ chimeras (G). In $TNF^{-/-}$→WT chimeras (H), expression of E-selectin was similar to WT mice. Magnification, ×400, confocal immunofluorescence, under oil immersion.

Serum Ab Titers to Sheep Globulin and Cutaneous DTH

Serum mouse anti-sheep globulin Ab titers were equivalent in all groups and were unaffected by complete or partial TNF deficiency. Subdermal challenge with sheep globulin produced prominent antigen-specific footpad swelling (0.45 ± 0.08 mm) in WT mice with GN, indicative of a cutaneous DTH response. Cutaneous DTH was significantly reduced in the complete absence of TNF (TNF−/− mice, 0.25 ± 0.08 mm; $P < 0.001$ cf. WT). In contrast to crescentic GN, cutaneous DTH was significantly reduced in chimeras that lacked TNF production by BM-derived cells ($TNF^{-/-}$→WT chimeras, 0.28 ± 0.08 mm; $P < 0.05$, cf. WT) but not in chimeras whose BM-derived cells were capable of producing TNF (WT→$TNF^{-/-}$ chimeric 0.35 ± 0.08 mm; Figure 7).

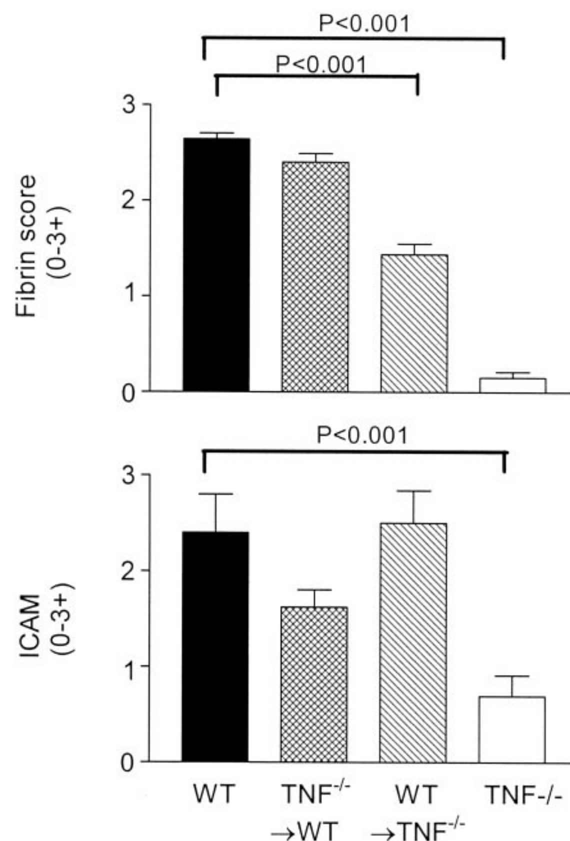

Figure 6. Renal fibrin deposition and intercellular adhesion molecule expression in WT, $TNF^{-/-}$→WT chimeric, WT→$TNF^{-/-}$ chimeric, and TNF−/− mice developing crescentic GN.

Discussion

Parenchymal cells in organ-specific inflammatory diseases are frequently considered to be passive targets for mediators of inflammation. However, in the present study, we demonstrated that rather than being passive targets of injury, intrinsic renal cells via their capacity to produce TNF make a significant contribution to crescent formation and renal injury in a Th1-dependent model of cell-mediated renal inflammation.

For addressing the relative contribution of TNF from intrinsic renal cells and infiltrating (BM-derived) leukocytes to TNF-dependent inflammatory renal injury, GN was induced in TNF chimeric mice by a planted glomerular antigen (heterologous anti-GBM Ab). Recently, the potential of BM-derived cells to differentiate into glomerular mesangial cells has been

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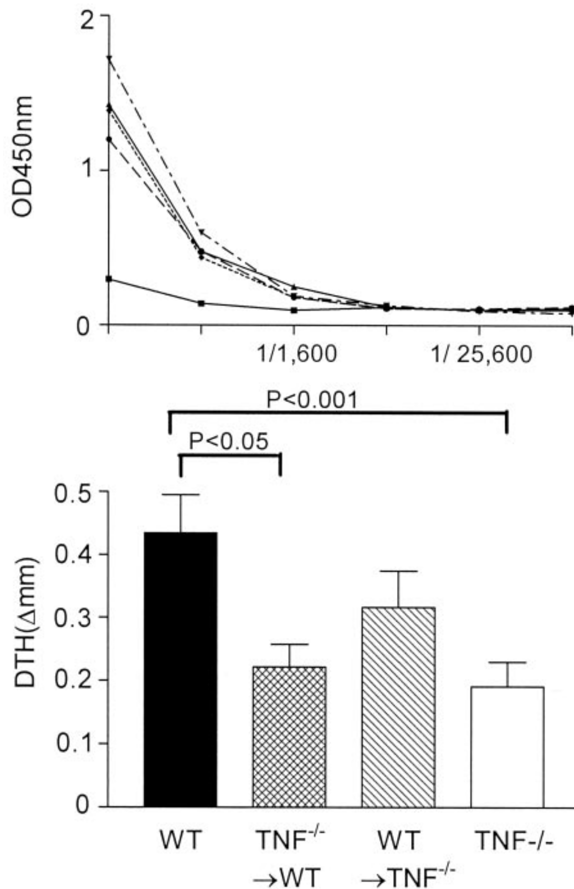

Figure 7. Serum titers of mouse anti-sheep globulin in WT (●), $TNF^{-/-}$→WT chimeric (▲), WT→$TNF^{-/-}$ chimeric (▼), and TNF−/− (◇) mice developing crescentic GN. ELISA reactivity of normal mouse serum is shown (■). Ab titers were equivalent in all groups with GN and were unaffected by complete or partial TNF deficiency. Cutaneous DTH produced prominent antigen specific footpad swelling in WT mice with GN. Cutaneous delayed-type hypersensitivity (DTH) was significantly reduced in the TNF−/− mice and was also reduced in chimeras lacking TNF production by BM-derived cells. However, cutaneous DTH was not reduced in chimeras with intact TNF production by BM-derived cells.

demonstrated in mice (31) and rats (32). Furthermore, the transferal of mesangial cells with a sclerotic phenotype has been achieved by BM transplantation (33). In the current studies, GFP-positive cells were infrequently observed in normal glomeruli before induction of GN, with approximately 5% of glomerular cells expressing GFP. Studies of transplantation of GFP transgenic BM in SD rats demonstrated that intraglomerular cells rarely expressed GFP before initiation of anti–Thy-1 disease. After mesangiolysis and mesangial cell proliferation after administration of anti–Thy-1 Ab, approximately 8% regenerating mesangial cells seemed to be of BM origin (32). We were unable to confirm the recent observation that approximately 20% of glomerular cells are donor BM-derived mesangial cells 8 wk after BM transplantation in mice (31).

In the current studies, TNF expression was significantly upregulated in the kidneys of WT mice with crescentic GN. TNF expression was prominent in glomerular tufts and on tubular cells as well on occasional interstitial macrophages but not by intraglomerular macrophages. Renal TNF expression in glomeruli and tubules was equivalent in chimeric mice that lacked the capacity for leukocytes TNF production. It was demonstrated that TNF expression did not co-localize with von Willebrand factor, indicating that endothelial cells are not the main cellular source of TNF. Thus, the abundant TNF observed in the glomerulus may be derived form either mesangial cells or podocytes that have been demonstrated to express TNF in human membranous GN (12). However, renal TNF expression was substantially reduced in the mice that lacked the capacity for TNF production by intrinsic renal cells, despite normal leukocyte TNF production. TNF expression by intrinsic renal cells could not be detected in WT→$TNF^{-/-}$ chimeric mice, indicating that these cells maintained their original phenotype.

Deficiency of TNF in intrinsic renal cells in the presence of normal leukocyte TNF production provided equivalent protection from the development of crescentic GN to complete TNF deficiency. Crescent formation, glomerular macrophage accumulation, MHC II expression, serum creatinine, and proteinuria all were reduced to the same extent in WT→$TNF^{-/-}$ chimeras as in TNF−/− mice. Only the recruitment of glomerular $CD4^+$ T cells in WT→$TNF^{-/-}$ chimeras was not reduced to the same extent as in TNF−/− mice.

Glomerular fibrin deposition was also reduced to a similar extent in WT→$TNF^{-/-}$ chimeras and TNF−/− mice. Fibrin deposition in crescentic GN is initiated by local upregulation of tissue factor (34,35) and is dependent on glomerular macrophage infiltration (36). Tissue factor is expressed by glomerular macrophages (37), and its expression on glomerular epithelial cells (38), mesangial cells (22), and endothelial cells (39) is induced by TNF. TNF from intrinsic renal cells thus may augment glomerular fibrin deposition both through its role in facilitating macrophage recruitment and by inducing tissue factor on intrinsic renal cells.

E-selectin is expressed on endothelial cells after activation with TNF (40) and other proinflammatory cytokines. It plays an important role in recruitment of T lymphocytes and macrophages into inflammatory lesions (41). In the current study, E-selectin expression was widely observed on glomerular endothelial cells of WT and $TNF^{-/-}$→WT chimeric mice with GN but was not observed on renal interstitial vessels. In TNF−/− mice and chimeric mice with absent intrinsic renal cell TNF production, E-selectin was not observed on glomerular endothelial cells, indicating an autocrine or paracrine effect of intrinsic renal-derived TNF in E-selectin upregulation. Glomerular T-cell and macrophage accumulation was also similarly reduced in both of these groups of mice.

TNF induces ICAM-1 expression on tubular epithelial cells (42), mesangial cells (43), and glomerular epithelial cells (44). ICAM-1 expression is induced by INF-γ (45), reactive oxygen species (ROS) (46), and IL-1 (47). ICAM-1 is widely expressed in glomerular and interstitial areas in GN (48,49) and was also prominently expressed in this murine model of GN. Induction of ICAM-1 expression in GN was significantly decreased in TNF−/− mice, this corresponding with a significant reduction of glomerular and interstitial inflammatory cells.

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ICAM-1 was not reduced in chimeric mice that lacked either leukocyte or intrinsic renal cell TNF. Therefore, the absence of TNF from either compartment does not affect the upregulation of ICAM-1, and this accompanied with the observation that the interstitial infiltrate was also unaffected suggests that this inflammatory cell infiltration depends on other pathways than does glomerular inflammation, as E-selectin seems to play a more critical role for glomerular leukocyte recruitment than interstitial infiltration.

The development of crescents was unaffected in the absence of leukocyte-derived TNF. These $TNF^{-/-} \rightarrow WT$ chimeric mice also showed less protection in other indices of inflammatory glomerular injury, indicating a minor role for leukocyte-derived TNF in this model of inflammatory renal injury. This is in contrast to their dominant role in cutaneous and CNS inflammation. In experimental encephalomyelitis, mice that lacked TNF from leukocytes but not TNF from parenchymal CNS cells showed delayed onset of disease and reduced movement of leukocytes (particularly macrophages) from the perivascular space to deeper CNS areas (50,51). This suggests that intrinsic renal cells have a greater capacity to produce TNF in response to inflammatory stimuli than do parenchymal cells in the CNS. Similarly in the current study, DTH induced by subdermal challenge with the same antigen used to initiate crescentic GN was dependent on TNF from leukocytes and possibly BM-derived Langerhans cells but not TNF from keratinocytes or other non–BM-derived dermal cells.

In summary, these studies show an important role for intrinsic renal cell–derived TNF in crescentic GN. They demonstrate that in the kidney, parenchymal cells produce proinflammatory cytokines that significantly contribute to renal inflammation and are not merely targets for proinflammatory cytokines. With regard to their ability to produce TNF, parenchymal renal cells seem to play a more dominant role than infiltrating leukocytes in immune-initiated crescentic GN. This contrasts directly with immune cutaneous and CNS inflammation, where TNF from parenchymal cells plays only a minor role in organ-specific inflammatory injury.

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