Exclusive expression of transmembrane TNF aggravates acute glomerulonephritis despite reduced leukocyte infiltration and inflammation

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Tumor necrosis factor-α (TNF) is a cytokine mediating inflammatory kidney diseases such as immune complex glomerulonephritis. Its two receptors, TNFR1 and TNFR2, play distinct roles in this process, with TNFR2 strongly required for induction of disease. In contrast to soluble TNF (sTNF), transmembrane TNF robustly activates TNFR2. Thus, we examined the functional role of transmembrane TNF by inducing heterologous nephrotropic serum nephritis in wild-type and transgenic TNFΔ1-9.K11E knock-in mice expressing transmembrane TNF but no sTNF (memTNF mice). Compared to wild-type, nephritis was exacerbated in memTNF mice on day 5, indicated by increased albuminuria, higher serum urea levels, and more pronounced glomerular deposits, together with higher numbers of dying and proliferating glomerular cells. This was associated with greater loss of glomerular endothelial cells, increased podocyte stress, and signs of augmented necroptosis in memTNF kidneys. Aggravation of nephritis was dependent on transmembrane TNF expression in parenchymal cells, but not leukocytes. Surprisingly, increased kidney injury was associated with reduced renal leukocyte infiltration in memTNF mice, which correlated with decreased renal mRNA expression of pro-inflammatory mediators. This effect was also present in isolated memTNF glomeruli stimulated with interleukin-1β in vitro. Thus, uncleaved transmembrane TNF is an important mediator of renal tissue damage characterized by increased renal cell death and loss of glomerular endothelial cells in murine glomerulonephritis. In contrast, sTNF predominantly mediates renal leukocyte recruitment and inflammation. These findings highlight the importance of transmembrane TNF in inflammatory kidney disease as a possible therapeutic target.


KEYWORDS: cell death; cytokines; glomerulonephritis; inflammation

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Pro-inflammatory cytokines such as TNF play an important pathophysiologic role and contribute to inflammatory injury in glomerulonephritis (GN).1,2 TNF deficiency ameliorates murine nephrotic serum nephritis (NTN),3,4 a widely studied rodent model for immune complex-mediated GN.5 Interestingly, TNF expressed in intrinsic renal cells rather than infiltrating bone marrow-derived leukocytes promoted NTN.6 Consistently, therapy with TNF-blocking agents attenuated glomerular inflammation and crescent formation in rats with crescentic NTN.6,7 Apart from its pro-inflammatory and anti-infectious roles, TNF also possesses immunoregulatory functions, including suppression of autoimmunity by TNF-induced apoptosis of activated T-cells.8–10 The ability of TNF to limit autoinflammation is illustrated by the appearance of lupus-like symptoms and development of GN in patients with rheumatoid arthritis or inflammatory bowel disease treated with anti-TNF agents.11,12 Therefore, it is crucial to better understand the molecular basis of inflammatory tissue injury and immunoregulatory functions mediated by TNF in GN before TNF-targeting therapies can be implemented.

We have previously characterized distinct roles of the 2 TNF receptors (TNFRs), TNFR1 and TNFR2, in murine NTN, with TNFR2 strongly required for disease induction.13 TNFR2 was prominently expressed in glomerular endothelial cells in nephritic kidneys with NTN,13 and its expression was readily upregulated by inflammatory stimuli in mouse glomeruli in vitro.14 Furthermore, experiments with TNFR2-chimeric mice indicated that TNFR2 expressed by intrinsic renal cells is critical for the induction of NTN.13 TNF exists in 2 biologically active forms, both expressed on intrinsic renal cells upon stimulation.10 The membrane-bound precursor molecule (memTNF) can be released into circulating sTNF via cleavage of the ectodomain by the metalloproteinase TNF-α-converting enzyme (TACE/ADAM17).15,16 Both memTNF and preferably sTNF activate TNFR1 in the picomolar range, whereas TNFR2 is only robustly activated by memTNF.17,18 In line with this data, we have previously demonstrated in isolated mouse glomeruli in vitro that induced expression of inflammatory mediators upon stimulation with sTNF was mediated by TNFR1, but not TNFR2.14

Based on our previous findings identifying expression of TNFR2 in intrinsic renal cells as a critical step in inducing
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**a**

**b**

**c**

Human TNF

Normal kidney (living donor)  Technical control

WT NTN  memTNF NTN  WT control

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Figure 1 | Expression of tumor necrosis factor (TNF) in human and murine glomerulonephritis. (a) Gene expression data were obtained from micro-dissected glomeruli of human renal biopsies with IgA nephropathy (IgAN; n = 27), lupus nephritis (SLE; n = 32), rapidly progressive glomerulonephritis (RPGN; n = 23), minimal change disease (MCD; n = 14), focal segmental glomerular sclerosis (FSGS; n = 23), and diabetic nephropathy (DN; n = 14). Microarray expression experiments and analysis were performed as described in Materials and Methods. The heat map summarizes significantly regulated gene expression, with glomerular TNF and TNF receptor 2 (TNFR2) expression being most prominently upregulated in RPGN. Numbers represent fold changes compared with expression levels in healthy control glomeruli obtained from pre-transplantation biopsies of living donors (LD; n = 42). n.s., non-significant. (b) In human glomerulonephropathies including IgAN, SLE, granulomatosis with polyangiitis (GPA), MCD, FSGS, and membranous nephropathy (MN), immunohistochemistry demonstrated a prominent glomerular TNF expression. In the glomerulonephritides (IgAN, SLE, GPA) TNF localized to glomerular endothelial cells (black arrows), with additional expression in podocytes (black arrowheads). A prominent expression of TNF in podocytes was also present in nephrotic disease (MCD, FSGS, MN; black arrowheads), which lacked endothelial TNF (white arrows). Only background staining was present in healthy glomeruli of transplant biopsies obtained from living kidney donors. As a technical control for unspecific staining, tissue was incubated with the antibody diluent alone, but no primary antibody, followed by incubation with secondary antibody and detection reagents. Original magnification x400, bar = 50 μm. (c) A similar protein expression of TNF in glomerular endothelium (arrows) and podocytes (arrowheads) was found in murine kidneys at day 5 of heterologous nephrotoxic serum nephritis (NTN). TNF staining was more prominent in nephritic kidneys of mice with knock-in of 2 uncleavable Δ1-9,K11E TNF alleles (memTNF) compared to wild-type (WT), and absent in healthy control kidneys. Original magnification x400, bar = 50 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 2 | Increased mortality and aggravated renal functional impairment in mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF mice) with heterologous nephrotoxic serum nephritis (NTN). (a) Survival proportions in wild-type (WT) and memTNF mice after induction of NTN (n = 17–18 per group). (b) Serum interleukin-6 (IL-6) levels in WT and memTNF mice at day 5 of NTN measured by enzyme-linked immunosorbent assay (n = 4 per group). The dashed line indicates mean values in healthy WT mice. (c) Albuminuria was evaluated in spot urine samples at baseline and day 5 after injection of nephrotoxic serum and is expressed as urinary albumin-to-creatinine ratio (n = 13 per group). (d) Serum levels of urea were determined at day 5 of NTN. The dashed line indicates mean baseline values for normal WT mice (n = 7–8 mice per group). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01.

complement-dependent kidney injury,13 we hypothesized that TNFR2 activation via memTNF mediates murine NTN. As a specific role for memTNF in inflammatory kidney disease has not been investigated, we induced heterologous NTN in C57BL/6 wild-type mice and mice with knock-in of 2 uncleavable Δ1-9,K11E TNF alleles (memTNF mice). memTNF mice can only express the transmembrane form of TNF (i.e., memTNF due to a modification on the cleavage site for TACE) and lack secreted sTNF.19 Importantly, memTNF is functional, normally regulated and expressed in these mice.19

Our results demonstrate that memTNF is an important mediator of renal tissue damage in NTN by induction of glomerular and tubulointerstitial cell death, and loss of glomerular endothelial cells and podocyte integrity. In
contrast, NFκB-mediated expression of pro-inflammatory mediators and recruitment of infiltrating leukocytes into nephritic kidney is dependent on the presence of sTNF, as these were significantly reduced in memTNF mice despite increased renal injury. These data indicate that memTNF and sTNF play distinct roles in mediating inflammatory kidney disease and have important implications for designing TNF targeting therapies in GN.

RESULTS
Induced expression of TNF in human glomerulonephritis
We first investigated glomerular expression levels of TNF analyzing mRNA expression data from human biopsy samples of the European Renal cDNA Bank - Kröner-Fresenius Biopsy Bank. Compared with healthy controls, glomerular expression of TNF and predominantly its receptor TNFR2 was significantly induced in various glomerular diseases, including glomerulonephritides like IgA nephropathy, lupus nephritis, and most prominently rapidly progressive GN (Figure 1a). Within glomeruli immunohistochemistry localized TNF protein expression to endothelial cells and podocytes, with a staining pattern consistent with a cell membrane-associated localization particularly in glomerular endothelial cells. Interestingly, nephrotic disease with predominant podocyte injury lacked endothelial TNF expression (Figure 1b), indicating that TNF expression associates with the primarily injured cell type. We found a similar expression in murine kidneys with heterologous NTN (Figure 1c). These findings suggested that memTNF may contribute to glomerular injury in GN of humans and mice.

Increased mortality despite reduced systemic inflammation in memTNF mice
Following induction of heterologous NTN, memTNF mice displayed increased mortality in the early phase of the disease compared with wild-type controls. At day 1 and 2 after injection of nephrotoxic serum, 35.3% of memTNF mice required killing due to moribund appearance, whereas mortality was only 5.6% in wild-type mice (Figure 2a). To investigate whether an increased systemic inflammatory response toward the injected nephrotoxic serum could explain excessive mortality in memTNF mice, we measured interleukin (IL)-6 serum levels in nephritic mice at day 5 of NTN. In both genotypes levels of serum IL-6 increased in NTN mice, but despite higher mortality IL-6 serum concentrations were significantly lower in nephritic memTNF mice (Figure 2b). In contrast, 50.0% of memTNF mice compared with 22.2% of wild-type mice developed hematuria, and moribund mice manifested ascites, which suggested that acute kidney failure was a potential cause of increased mortality in memTNF mice.

Exclusive expression of memTNF worsens renal functional parameters in acute NTN
To investigate a potential role of memTNF in mediating renal injury in NTN, we first analyzed albuminuria and serum urea levels in nephritic wild-type and memTNF mice. At day 5 of NTN, albuminuria was significantly worse in memTNF mice (Figure 2c). Consistently, renal function deteriorated more significantly in memTNF mice, as revealed by higher serum urea levels compared with wild-type at day 5 of NTN (Figure 2d). Thus, exclusive expression of memTNF significantly aggravated functional parameters of renal injury in acute NTN.

Expression of memTNF aggravates glomerular injury and cell death in acute NTN
Increased albuminuria suggested more severe glomerular injury in memTNF mice. Accordingly, glomeruli of memTNF mice showed increased mesangial matrix expansion and fibrinoid necrosis at day 5 of NTN as revealed by semi-quantitative analysis of intraglomerular deposition of periodic acid–Schiff-positive material (Figure 3a). In addition, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining demonstrated significantly increased cell death in memTNF glomeruli (Figure 3b), which was associated with increased numbers of proliferating cells in injured glomeruli (Figure 3c). To analyze further which cell type of the glomerular filtration barrier was affected by memTNF-mediated injury, podocyte and endothelial cell markers were evaluated. Whereas podocyte loss was comparable in nephritic wild-type and memTNF mice, reduced

Figure 3 | Increased glomerular injury and cell death in mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF mice) with heterologous nephrotoxic serum nephritis (NTN). (a) Representative photomicrographs of periodic acid-Schiff (PAS)-stained kidney sections from wild-type (WT) and memTNF mice illustrating glomerular matrix deposition at day 5 of NTN. Semi-quantitative scoring revealed more extensive glomerular injury in memTNF mice. (b) Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining demonstrated increased glomerular cell death in memTNF mice compared with WT mice. (c) This was associated with increased numbers of proliferating cell nuclear antigen (PCNA)-positive cells in memTNF glomeruli indicating enhanced regenerative cell proliferation. (d) Podocytes were identified by immunofluorescence double-staining for nuclear Wilms tumor antigen (WT)-1 and nephrin. Podocyte numbers similarly decreased in WT and memTNF mice. The dashed line indicates mean podocyte numbers in healthy WT mice. Instead, renal nephrin mRNA expression was significantly reduced in memTNF mice compared with WT mice at day 5 of NTN, indicating increased podocyte stress. (e) Immunofluorescence staining for CD31 was reduced in memTNF glomeruli, demonstrating aggravated glomerular endothelial cell injury compared with WT glomeruli. (f) Glomerular deposition of injected heterologous sheep antibodies and (g) complement C3d was comparable in both genotypes, consistent with similar immune-mediated injury that triggered NTN. Images show representative glomeruli from each group; original magnification ×400, bar = 50 μm. Semiquantitative and morphometric analysis was performed as described in Materials and Methods. Data are expressed as mean ± SEM of 6 mice per group and are representative for 2 independently performed experiments. *P < 0.05; n.s., not significant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
renal mRNA expression of nephrin indicated greater podocyte stress in memTNF mice (Figure 3d). Moreover, reduced glomerular staining of CD31 revealed more severe glomerular endothelial cell injury in memTNF mice, whereas CD31 expression in nephritic wild-type mice was comparable to that of healthy controls (Figure 3e). Importantly, glomerular deposition of injected heterologous sheep antibodies and complement C3d was comparable in both genotypes (Figure 3f and g), suggesting that initial immune-mediated injury was similar.

While histopathology demonstrated more severe glomerular damage in memTNF mice, tubulointerstitial injury analyzed in periodic acid–Schiff-stained sections was not significantly different in both genotypes at day 5 of NTN, including the extent of tubular dilation, intratubular cast formation, tubular cell damage, and tubular vacuolar degeneration (Supplementary Figure S1). However, mRNA expression of the 2 tubular injury markers liver-type fatty acid binding protein (L-FABP) and α-glutathione S-transferase (α-GST) significantly increased in memTNF kidneys as an early sign of aggravated tubular injury (Figure 4a). Moreover, similar to the glomerular compartment, memTNF kidneys showed increased cell death and a trend toward enhanced cell proliferation in the tubulointerstitium (Figure 4b). Taken together, memTNF expression in the absence of sTNF aggravates functional and structural renal damage in acute NTN, and particularly leads to increased cell death and glomerular endothelial cell injury.

Mediators of regulated necrosis are more abundantly expressed in nephritic memTNF kidneys

To explore mechanisms of increased cell death in nephritic memTNF kidneys we analyzed markers of known TNF-induced cell death pathways. Similar to other models of

![Figure 4](link)
acute kidney injury, in neither wild-type nor memTNF mice could cleaved caspase 3 as an indicator of ongoing apoptosis be detected in nephritic kidneys (Figure 5a). As an alternative mechanism of regulated cell death particularly associated with inflammatory tissue injury, necroptosis has been identified as an important mediator of renal pathology in models of acute kidney injury. We therefore investigated protein expression of core components of the necroptotic pathway, receptor interacting protein 1 (RIP1) and RIP3, in kidneys at day 5 of NTN. RIP1 protein levels significantly increased in nephritic kidneys of memTNF mice compared with wild-type, and a similar trend was noted for RIP3 (Figure 5a). As increased expression of RIP1 and RIP3 correlates with the extent of necroptotic cell death in tissue, we next determined whether necroptosis could be induced by TNF in glomerular endothelial cells, which were more

**Figure 5 | Role for regulated necrosis but not apoptosis in mediating renal injury of mice with knock-in of 2 uncleavable Δ1-9.K11E tumor necrosis factor (TNF) alleles (memTNF mice).** (a) Protein expression levels of cleaved caspase 3, RIP1, and RIP3 were analyzed in nephritic kidneys of wild-type (WT) and memTNF mice at day 5 of nephrotoxic serum nephritis (NTN) by immunoblotting. Representative Western blot images are shown. β-Actin served as a loading control. Data are expressed as mean ± SEM, n = 4. (b) Detection of necroptosis in a murine glomerular endothelial cell line after treatment with 300 ng/ml TNF and 10 μM ZVAD-FMK (ZVAD) for 6 hours (MTT assay). Protection from cell death by necrostatin-1s (Nec-1s) treatment (100 μM) confirmed induced necroptosis in treated cells. Data are expressed as mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
**basic research**

**MB Müller et al.: Transmembrane TNF in GN**

**Figure a**

- **WT** vs **memTNF**
  - CD45+ cells
  - CD11c+ cells
  - F4/80+ cells (glomerulus)
  - CD11c+ F4/80+ cells (glomerulus)
  - F4/80+ CD11c- cells (glomerulus)
  - F4/80+ area / hpf (%)
  - Ly6G+ cells / glomerulus
  - CD3+ cells / glomerulus
  - CD3+ cells / total renal cells (%)
  - Mac2+ cells / glomerulus

**Figure b**

**Wild-type** vs **memTNF**

- Ly6G+ neutrophils (glomerulus)
- Ly6G+ neutrophils (tubulointerstitium)
- Mac2+ macrophages (glomerulus)
- F4/80+ phagocytes (tubulointerstitium)
- CD3+ T cells (glomerulus)
- CD3+ T cells (tubulointerstitium)

**Graphs**

- Bar charts showing comparison between **WT** and **memTNF**
- **p**-values indicated: n.s., *, **, ***
severely injured in nephritic memTNF mice. In a murine glomerular endothelial cell line, exposure to high doses of TNF in combination with the pan-caspase inhibitor Z-VAD-FMK resulted in substantial caspase-independent non-apoptotic cell death. Cell death was significantly attenuated by co-incubation with the necroptosis inhibitor Nec-1s (Figure 5b), indicating that TNF exposure under caspase-inhibiting conditions causes necroptosis in intrinsic glomerular cells. Together, these in vivo and in vitro data suggest that increased necroptosis contributes to more abundant renal cell death and kidney injury in memTNF mice with acute NTN.

Renal leukocyte accumulation decreases in memTNF mice with acute NTN despite aggravated renal injury

Cell death and tissue injury elicits a necroinflammatory response characterized by leukocyte infiltration and expression of inflammatory mediators, which leads to further tissue damage. In acute kidney injury, the severity of tissue damage usually correlates with the extent of renal leukocyte infiltrates. Surprisingly, flow cytometric analysis revealed a highly significant reduction of renal leukocyte numbers in memTNF kidneys at day 5 of NTN compared with wild-type (Figure 6a). We noted significantly reduced infiltrates of mononuclear phagocyte populations, reduced CD3+ T cells, and a trend toward reduced neutrophil numbers in nephritic mTNF kidneys (Figure 6a). Compartment-specific evaluation of renal leukocyte infiltration by immunohistochemistry confirmed reduced glomerular and tubulointerstitial infiltration of neutrophils and macrophages into nephritic mTNF kidneys, with similar trends seen for CD3+ T cells (Figure 6b). Thus, memTNF expression causes exacerbated renal injury independent of renal leukocyte infiltration through direct effects on renal parenchymal cells.

As rapid infiltration of neutrophils occurs within hours after induction of NTN, we further analyzed the relationship between renal injury and leukocyte infiltration in wild-type and memTNF mice during this early induction phase of the disease. Two hours after injection of the nephrotoxic serum mice of both genotypes developed substantial but comparable albuminuria. Instead, serum urea levels and cell death of tubulointerstitial cells significantly increased in memTNF mice compared with wild-type mice, indicating more severe acute kidney injury in memTNF mice early in NTN (Figure 7a and b). Moreover, renal infiltration of leukocytes and particularly Ly6G+ neutrophils was significantly reduced in memTNF kidneys at this time point (Figure 7c). Taken together, these data indicate that memTNF expression aggravates renal injury despite early and sustained reductions in leukocyte infiltration in NTN.

Exclusive expression of memTNF in parenchymal cells, but not leukocytes aggravates renal injury in acute NTN

Prominent glomerular expression of membrane-associated TNF in human and murine GN as well as aggravated renal injury in memTNF mice despite reduced renal leukocyte infiltration suggested a predominant role of parenchymal cell-expressed memTNF in mediating renal damage. To determine the extent to which intrinsic cell- versus leukocyte-expressed memTNF exacerbates disease, we analyzed mice expressing memTNF only in bone marrow (BM)-derived cells or intrinsic cells that were generated by BM transplantation between wild-type (WT) and memTNF-deficient animals. Chimeric mice with wild-type BM transplanted into irradiated wild-type recipients and memTNF BM transplanted into memTNF recipients served as controls. As expected, serum levels of sTNF were barely detectable in memTNF→memTNF chimeras, and greatly reduced in memTNF→WT and WT→memTNF chimeras compared with WT→WT controls (Figure 8a). Consistent with the phenotypes of wild-type and memTNF mice, memTNF→memTNF chimeras showed increased albuminuria, more severe hypoalbuminemia, hyperlipidemia, renal functional impairment, and glomerular injury compared with WT→WT chimeras (Figure 8), but reduced renal leukocyte infiltrates (Supplementary Figure S2). A similar aggravation of disease was present in WT→memTNF chimeras, but not in memTNF→WT chimeras (Figure 8). These results clearly indicate a role for renal cell-expressed but not leukocyte memTNF in aggravating heterologous NTN.

Of note, our chimera experiments further support a function for sTNF in mediating renal leukocyte infiltration, as only memTNF→memTNF chimeras, which completely lack sTNF, showed reduced leukocyte numbers in nephritic kidneys (Supplementary Figure S2). Interestingly, we noted increased accumulation of glomerular and interstitial neutrophils in kidneys of memTNF→WT and WT→memTNF chimeras, respectively. This indicates that in the presence of residual sTNF production, memTNF expression in leukocytes or parenchymal cells enhances renal leukocyte infiltration.

Figure 6 | Decreased leukocyte infiltration in kidneys of mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF mice) despite aggravated renal injury. (a) Flow cytometric analysis of renal single cell suspensions prepared from nephritic kidneys at day 5 of nephrotoxic serum nephritis (NTN) demonstrated reduced leukocyte infiltrates in memTNF mice compared with wild-type (WT) mice. Cells were stained for CD45 (pan leukocyte marker), CD11c (DC like mononuclear phagocytes), F4/80 (mononuclear phagocytes), Ly6G (neutrophils), CD3 (T-cells), CD3/CD4 (CD4+ T helper cells), and CD3/CD8 (CD8+ cytotoxic T cells). Dashed lines indicate mean baseline values for healthy WT mice. Data are expressed as mean ± SEM of 11 to 13 mice per group. (b) Representative renal sections of WT and memTNF mice stained for Ly6G+ neutrophils, Mac2+ glomerular macrophages, F4/80+ tubulointerstitial phagocytes, and CD3+ T cells.

Original magnification 400x, bar = 50 μm. Glomerular and tubulointerstitial leukocyte infiltrates were quantified as described in Materials and Methods. Data are expressed as mean ± SEM of 6 to 8 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
**Expression of proinflammatory mediators and NFκB activation decreases in memTNF kidneys**

Our results suggested that increased renal cell death due to regulated necrosis mediates exacerbated renal injury in memTNF kidneys. However, mechanisms of impaired renal leukocyte infiltration despite increased tissue injury in memTNF mice remained unclear. As expression of pro-inflammatory cytokines, chemokines, and adhesion molecules mediates leukocyte recruitment into diseased kidneys, we compared renal mRNA expression of these inflammatory mediators in wild-type and memTNF mice. This analysis revealed decreased mRNA expression of the cytokines IL-1β, IL-6, IL-10, and interferon (IFN)-β, the chemokines CCL2 and CXCL10, and the adhesion molecule E-selectin in nephritic memTNF kidneys, whereas CCL5 expression was similar to wild-type (Figure 9a). Interestingly, memTNF
kidneys expressed significantly more TNF mRNA, whereas expression of TNFR1 and TNFR2 was comparable to wild-type (Figure 9b).

As expression of inflammatory mediators is induced by the transcription factor NFκB, we analyzed NFκB activation in kidneys of wild-type and memTNF mice at day 5 of NTN. Significantly decreased protein levels of phosphorylated inhibitor of NFκB subunit α (IκB-α), the abundance of which correlates with NFκB pathway activation, confirmed reduced NFκB activation in memTNF kidneys (Figure 10a). A significantly reduced NFκB p52-to-p100 ratio additionally suggested reduced activity of the alternative NFκB pathway in memTNF kidneys (Figure 10a). Moreover, mRNA expression levels of the NFκB inhibitors IκB-α and TNF-α-induced protein 3 (TNFAIP3/A20) as well as the NFκB-associated anti-apoptotic proteins cIAP1 and XIAP significantly decreased in memTNF kidneys (Figure 10c). As transcription of these negative regulators is induced upon NFκB activation,
Figure 9 | Decreased expression of proinflammatory mediators in nephritic kidneys of mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF kidneys). (a) Induced mRNA expression of pro-inflammatory cytokines, chemokines, and adhesion molecules in nephritic wild-type (WT) kidneys was significantly attenuated in memTNF kidneys at day 5 of nephrotoxic serum nephritis (NTN). (b) TNF mRNA expression increased in nephritic memTNF kidneys, and expression of TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) was not different in both genotypes. Polymerase chain reaction results were normalized to 18S rRNA as a housekeeping gene. Dashed lines indicate mean baseline expression in healthy WT kidneys, with similar expression in healthy memTNF kidneys (data not shown). Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; n.s., not significant; VCAM, vascular cell adhesion molecule.
Figure 10 | Reduced NF-κB activation in nephritic kidneys of mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF kidneys). (a) Western blot analysis of IκB phosphorylation and NF-κB2 degradation in nephritic wild-type (WT) and memTNF kidneys at day 5 of nephrotoxic serum nephritis (NTN) demonstrating reduced canonical and noncanonical NF-κB activation in memTNF mice. β-actin is shown as loading control. Data are expressed as mean ± SEM; n = 3. (b) Decreased mRNA expression levels of the NF-κB inhibitors IκB-α and TNFAIP3/A20, and of NF-κB-associated anti-apoptotic proteins cIAP1 and XIAP in memTNF kidneys. Polymerase chain reaction results were normalized to 18S rRNA as a housekeeping gene. Dashed lines indicate mean baseline expression in healthy WT kidneys. Data are expressed as mean ± SEM of 6 mice per group. *P < 0.05. cIAP, cellular inhibitor of apoptosis protein; IκB, inhibitor of NF-κB; TNFAIP, TNF-α-induced protein; XIAP, X-linked inhibitor of apoptosis protein. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Expression of inflammatory mediators decreases in memTNF glomeruli in vitro

Reduced NFkB-mediated expression of inflammatory mediators in memTNF kidneys could result in reduced renal leukocyte infiltration, but may also be the consequence of decreased intrarenal accumulation of inflammatory leukocytes. Therefore, we analyzed inflammatory responses in intact glomeruli isolated from healthy wild-type and memTNF mice in vitro, independently of infiltrating leukocytes. Isolated tissue was stimulated with IL-1β, which in vivo upon secretion by leukocytes induces TNF expression in intrinsic renal cells, which are a major source of TNF contributing to glomerular injury in NTN.4,29 As expected, glomeruli of memTNF mice did not secret TNF upon IL-1β stimulation, which readily induced TNF secretion in wild-type glomeruli. IL-1β stimulation also induced secretion of IL-6 and CCL2 in both wild-type and memTNF glomeruli, but IL-6 and CCL2 levels were significantly lower in stimulated memTNF glomeruli lacking sTNF (Figure 11a). Moreover, whereas IL-1β induced mRNA expression of TNF similarly in both genotypes, induced IL-6 and CCL2 mRNA levels were again significantly lower in memTNF glomeruli upon IL-1β stimulation (Figure 11b). In summary, these results indicate that decreased renal inflammation in nephritic memTNF mice is caused by reduced expression of inflammatory mediators by intrinsic renal cells, which subsequently results in reduced renal leukocyte recruitment into damaged renal tissue. Moreover, these data suggest that secreted sTNF, which is absent in memTNF tissue, contributes to NFkB-dependent expression of inflammatory mediators and subsequent leukocyte recruitment into nephritic kidneys.

DISCUSSION

TNF is an important mediator of GN, and TNF-targeting therapies have been successfully applied in rodent models.3,4,6,7,30 However, next to its classic pro-inflammatory functions TNF also possesses immunoregulatory activity, which can limit autoimmune responses and explains occurrence of autoimmune disease and even GN in patients treated with TNF-blocking agents.1 The 2 TNF receptors, TNFR1 and TNFR2, may differentially mediate these TNF functions, with TNFR2 being robustly activated by memTNF only, but not

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**Figure 11** | Reduced expression of inflammatory mediators in isolated glomeruli of mice with knock-in of 2 uncleavable Δ1-9,K11E tumor necrosis factor (TNF) alleles (memTNF glomeruli) in vitro. Glomeruli were isolated from healthy wild-type (WT) and memTNF mice as described in Materials and Methods and stimulated with 10 ng/ml interleukin (IL)-1β for 24 hours. (a) Levels of TNF, IL-6, and C-C motif chemokine ligand (CCL2) in the supernatant were measured by enzyme-linked immunosorbent assay. As expected, TNF secretion was absent in memTNF glomeruli. Production of IL-6 and CCL2 was significantly reduced in memTNF glomeruli. (b) Analysis of mRNA expression levels revealed comparable levels of TNF transcripts in WT and memTNF glomeruli, but reduced IL-6 and CCL2 expression in memTNF glomeruli. Polymerase chain reaction results were normalized to 18S rRNA as a housekeeping gene. Data are expressed as mean ± SEM of 4 independently performed experiments. *P < 0.05, **P < 0.01, ***P < 0.001. n.d., not detected.
sTNF. Thus, more specific inhibition of TNF signaling pathways targeting 1 individual TNF receptor or memTNF versus sTNF may maintain anti-inflammatory efficacy while reducing adverse immuno-suppressive and autoimmune effects. Here, we hypothesized that memTNF plays a distinct pathophysiologic role in GN, as development of murine NTN, a model of immune complex-mediated GN, depends on the presence of the memTNF receptor TNFR2 expressed by intrinsic renal cells, but not on TNFR1 expression. Our studies using transgenic TNFΔ1-9, K11E knock-in mice with exclusive expression of functional memTNF in the absence of sTNF confirm this concept and further demonstrate that memTNF aggravates NTN by enhancing renal cell death, whereas sTNF contributes to intrarenal and systemic expression of inflammatory mediators and renal leukocyte infiltration. The fact that renal functional deterioration and glomerular injury increased in memTNF mice despite reduced leukocyte infiltrates and renal inflammation underlines the importance of memTNF as a mediator of renal tissue damage and identifies the memTNF/TNFR2 axis as a preferential therapeutic target in GN.

Consistent with our results, memTNF-induced tissue injury has been reported in other disease models. For example, memTNF mediates chronic inflammatory arthritis and concanavalin A-induced experimental hepatitis. Moreover, inhibition of TNF processing by melphalan was shown to elicit memTNF on Kupffer cells, which triggered cell death of hepatocytes. In experimental colitis blocking memTNF and sTNF with a TNF-inhibiting antibody, but not treatment with a dominant negative TNF mutant exclusively neutralizing sTNF induced remission in T-cell-mediated colitis, suggesting that memTNF is mediating inflammatory bowel disease. In experimental autoimmune uveitis signaling via memTNF was sufficient to mediate tissue damage, although leukocyte infiltration into injured organs was dependent on sTNF, which is consistent with our findings in the NTN model.

We identified increased renal cell death as an underlying mechanism for aggravated injury in nephritic memTNF kidneys. memTNF-induced cell death and regulated necrosis are well described, and involve TNFR2 activation and TNFR2-dependent enhancement of TNFR1-mediated cytotoxicity. Moreover, reduced NFκB activation in memTNF kidneys was associated with decreased expression of the intracellular survival factors cLAP1 and XIAP. Depletion of these proteins facilitates TNF-induced apoptosis and regulated necrosis. Similar to other models of acute kidney injury, we did not detect ongoing apoptosis in NTN kidneys. Instead, levels of RIP1 and RIP3 proteins were increased in nephritic kidneys of memTNF mice compared with wild-type. In vivo, more abundant expression of these molecules is associated with induction of regulated necrosis, particularly necroptosis, which has an important pathogenic role in acute kidney injury. This suggested enhanced necroptotic cell death in memTNF kidneys as a potential mechanism of aggravated renal injury, which included more severe damage to the glomerular endothelium as demonstrated by significantly reduced CD31 expression. Consistently, our in vitro data confirmed that TNF-exposed glomerular endothelial cells in the presence of caspase inhibition can undergo necroptosis. Moreover, glomerular endothelial cells may be particularly vulnerable to memTNF/TNF2-mediated cell death, potentially in an autostimulatory fashion, as we showed that these cells prominently express memTNF in GN and readily induce expression of TNFR2 upon inflammatory stimulation in vitro, and during NTN in vivo. Importantly, endothelial dysfunction has been recently recognized to play a critical role in the development and progression of glomerular disease. Thus, memTNF/TNFR2-mediated glomerular endothelial cell injury may be an important determinant of glomerular pathology in NTN, leading to podocyte stress, damage of the glomerular filtration barrier, albuminuria and glomerular scarring, which was more prominent in memTNF kidneys with NTN.

In general, necrotic cell death and tissue damage release damage-associated molecular patterns (DAMPs) and trigger leukocyte influx and sterile inflammation leading to the self-perpetuating process of necroinflammation. Leukocyte accumulation and inflammation usually correlate with the extent of tissue injury in affected organs including nephritic kidneys with NTN. Paradoxically, memTNF kidneys with NTN displayed exacerbated tissue injury but significantly reduced renal leukocyte infiltration and inflammation. Firstly, this suggested that aggravated memTNF-driven tissue injury is independent of infiltrating leukocytes and increased local inflammation and is mediated through mechanisms involving intrinsic renal cells. Our studies in BM-chimeric mice confirmed that parenchymal cell- but not leukocyte-expressed memTNF aggravates renal injury. Moreover, it has been shown that expression of TNF as well as TNFR2 in parenchymal renal cells predominantly contributes to NTN. Secondly, these data indicate that sTNF, which is absent in memTNF mice, mediates leukocyte infiltration and inflammatory responses in nephritic kidneys. sTNF binding to TNFR1 induces classic pro-inflammatory TNF effects via NFκB activation, including expression of cytokines, chemokines and adhesion molecule that facilitate leukocyte infiltration. Consistently, we demonstrated impaired activation of NFκB in nephritic memTNF kidneys, which explains reduced renal expression of inflammatory mediators. Importantly, our in vitro experiments prove that absent sTNF secretion in memTNF glomeruli reduced expression of chemokines and cytokines, independently of altered numbers of infiltrating leukocytes in vivo. Lastly, reduced NFκB-mediated expression of cellular survival factors may enhance memTNF-induced cell death as discussed above. Of note, next to impaired TNFR1 signaling due to absent sTNF, binding of memTNF to TNFR2 may directly contribute to inhibition of pro-inflammatory NFκB activity in memTNF kidneys as TNFR2 activation can lead to degradation of the intracellular adapter molecule TRAF2, which is essential in
inducing canonical NFκB responses upon TNF receptor ligation.41

In conclusion, we show that parenchymal expression of memTNF increases renal cell death and aggravates renal injury in NTN, despite reduced renal leukocyte influx and inflammation due to absent sTNF. Our results indicate that uncleaved transmembrane TNF plays an important pathophysiologic role in GN. Therapeutic targeting of specifically the memTNF/TNFR2 axis,49 may be more beneficial than unspecific TNF blockade preserving TNF-mediated immunoregulatory effects and antimicrobial functions.

MATERIALS AND METHODS

Expression of TNF mRNA and protein in human glomerulopathies

Human renal biopsies from patients and controls were collected within the framework of the European Renal cDNA Bank - Kröner-Fresenius Biopsy Bank.20 Diagnostic renal biopsies were obtained from patients after informed consent and with approval of the local ethics committees. Glomeruli were microdissected, total RNA isolated, linearly amplified, and hybridized to Affymetrix HG-U133A and HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA) as reported previously.50 Pre-transplantation kidney biopsies from living donors were used as control tissue. Fragmentation, hybridization, linearization, staining, and imaging were performed according to the Affymetrix expression analysis technical manual (Affymetrix). The raw data were normalized using robust multi-array average and annotated by Human Entrez Gene custom CDF annotation version 18 (http://brainarray.mbnl.med.umich.edu/Brainarray/default.asp). The log-transformed dataset was corrected for batch effect using ComBat from the GenePattern pipeline (http://www.broadinstitute.org/cancer/software/genepattern/).51 To identify differentially expressed genes the SAM (significance analysis of microarrays) method was applied using TiGR (MeV, Version 4.8.1).52 A q-value below 5% was considered to be statistically significant. Paraffin sections of human kidney biopsies were stained with 1:120 rabbit anti-memTNF antibody (ab183896; Abcam, Cambridge, MA). WT and memTNF BM into lethally irradiated wild-type recipients were performed as previously19 and were originally provided by Bernhard Ryffel (University of Orléans, France). All experiments were performed according to the German animal care and ethics legislation and were approved by the local government authorities.

BM transplantation

We generated memTNF BM chimeric mice by transplanting memTNF BM into lethally irradiated wild-type recipients (memTNF→WT chimeras) or vice versa (WT→memTNF chimeras). WT→WT and memTNF→memTNF chimeras served as controls. Irradiation and BM reconstitution was carried out as described.53 To assess the efficiency of BM replacement, CD45.1 congenic C57BL/6J wild-type mice were used to distinguish

### Table 1 | Primers used for real-time quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrin</td>
<td>5’-ACCTCCCGATTACCTGCTTTG-3’ (forward), 5’-ATCCAGGAGGACCTTGTA-3’ (reverse)</td>
</tr>
<tr>
<td>L-FABP</td>
<td>5’-AGAGTGGCCTGGAGGACCTAACAT-3’ (forward), 5’-GCTTTCTGGATGTCACAGGCGA-3’ (reverse)</td>
</tr>
<tr>
<td>α-GST</td>
<td>5’-GGGACACGGACGGAGGACCTAACAT-3’ (forward), 5’-ACACCGGCTGAACTGGGAGA-3’ (reverse)</td>
</tr>
<tr>
<td>CCL2</td>
<td>5’-CTCCTGTGTCACAGTTCG-3’ (forward), 5’-ATGTGGGATGTCACAGGCGA-3’ (reverse)</td>
</tr>
<tr>
<td>CCL5</td>
<td>5’-CCACCTTCTCTCTGTTGGG-3’ (forward), 5’-GTGCCCCAGCTCAGGAGATAGT-3’ (reverse)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5’-GGCTGTGACCTCTTCAAGAAG-3’ (forward), 5’-ATGGATGAGCAGAAGAGACG-3’ (reverse)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-TTCCCTGGTCAAGGATGTCAGA-3’ (forward), 5’-CATTGTCAAAAGGGTGAGGTT-3’ (reverse)</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-ACCAAGGAAATTTAACCTAGGCGC-3’ (forward), 5’-AGGTTTGCAGAGAAGAGACG-3’ (reverse)</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’-ATCGGTTTTCCCCCTGTAAGA-3’ (forward), 5’-TGTCAAAATCTCAGGCGC-3’ (reverse)</td>
</tr>
<tr>
<td>IFN-β</td>
<td>5’-CTCAGGGTGCAGATGAGGTC-3’ (forward), 5’-CCTAAGTGGTTTCTCTCAGG-3’ (reverse)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5’-ACAGAAAGGCAGAAAAAGTATG-3’ (forward), 5’-TACAGAATGTCGCAAGAGAG-3’ (reverse)</td>
</tr>
<tr>
<td>TNF</td>
<td>5’-CACCAAGCTCTTCTGCTTAC-3’ (forward), 5’-AGGTTTGGCCATGTAAG-3’ (reverse)</td>
</tr>
<tr>
<td>TNFR1</td>
<td>5’-GCAACAGCACAGCGAGAGCTGA-3’ (forward), 5’-GTGCTGCGCTCTGACACCT-3’ (reverse)</td>
</tr>
<tr>
<td>TNFR2</td>
<td>5’-CAAGACGACACTGGGTCGG-3’ (forward), 5’-CTGTTGGCGGCGTGGTCG-3’ (reverse)</td>
</tr>
<tr>
<td>E-selectin</td>
<td>5’-CACTATGGCCTTTCTACCAAG-3’ (forward), 5’-ATTAAGCAAGAAAGGAAAC-3’ (reverse)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5’-GTACGGCTGGTATGACCTTC-3’ (forward), 5’-AACAGTGCTACTGACGACG-3’ (reverse)</td>
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<tr>
<td>VCAM-1</td>
<td>5’-GCTATGAGGTGGAGAAGACTCTGG-3’ (forward), 5’-ACTTGGTGGCCACCTCAGGATC-3’ (reverse)</td>
</tr>
<tr>
<td>IkB-α</td>
<td>5’-CAAGAGCTCAGCAGAGAGCTG-3’ (forward), 5’-GCTTCCCTCCATCGACAA-3’ (reverse)</td>
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<tr>
<td>TNFAIP3/A20</td>
<td>5’-CAAGGAGGACTGTCGTCG-3’ (forward), 5’-TGACATCCGGTACCTCTC-3’ (reverse)</td>
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<tr>
<td>cIAP1</td>
<td>5’-TATGGGAGGTCCTTCTCAGG-3’ (forward), 5’-CCCTTATCACGTCATAG-3’ (reverse)</td>
</tr>
<tr>
<td>XIAP</td>
<td>5’-CATCGGGAGACGACTTACTA-3’ (forward), 5’-TCTTGGAGGAGCTTACTG-3’ (reverse)</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5’-GCAAATTTCCCCATGAGAAC-3’ (forward), 5’-AGGCGCTCCTAAACATCC-3’ (reverse)</td>
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</table>

α-GST, α-glutathione 5-transferase; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; cIAP, cellular inhibitor of apoptosis protein; ICAM, intercellular adhesion molecule; IFN, interferon; IkB, inhibitor of NFκB; IL, interleukin; L-FABP, liver-type fatty acid binding protein; qRT-PCR, quantitative reverse transcriptase-PCR; TNF, tumor necrosis factor-α; TNFAIP, TNF-α-induced protein; TNFR, tumor necrosis factor receptor; VCAM, vascular cell adhesion molecule; XIAP, X-linked inhibitor of apoptosis protein.

CD45.2+ memTNF leukocytes from CD45.1+ wild-type leukocytes by flow cytometry. Flow cytometry of peripheral blood samples, spleens, and kidneys at the end of the experiment revealed that more than 95% of leukocytes in the chimeras were of donor origin.

Induction of heterologous NTN

Heterologous NTN was induced in male mice by i.v. tail vein injection of 100 μl of sheep nephrotoxic serum (Probetex, San Antonio, TX).3 Spot urine samples were taken at days 0 and 5 of the experiment. At day 5 mice were killed, peripheral blood was collected by retro-orbital bleeding and phosphate-buffered saline-perfused...
kidneys were harvested for further analysis. Four mice of each group were analyzed 2 hours after injection of nephrotoxic serum.

**Functional assessment of renal injury**

Albuminuria in spot urine samples was determined as described. Serum urea levels were measured with an Olympus AU-640 autoanalyzer at Synlab.vet (Augsburg, Germany).

**Histological evaluation of renal injury**

Renal injury was assessed on 2-μm formalin-fixed, paraffin-embedded renal sections stained with periodic acid–Schiff reagent. Fifty glomeruli per mouse were analyzed, applying a semi-quantitative score for glomerular matrix deposition and sclerosis as described. Tubulointerstitial injury was quantified by superposing a grid containing 100 sampling points on images of 20 periodic acid–Schiff–stained cortical high power fields (400x) as described. Points overlaying tubular spaces, casts, flattened and necrotic tubular cells, and vacuolar degenerated tubuli were counted. Renal cell death was detected with an in situ cell death detection (TUNEL) kit (Roche, Mannheim, Germany). For immunostaining applying standard techniques the following primary antibodies were used: monoclonal mouse anti-PCNA (clone PC-10; Cell Signaling Technology, Danvers, MA), polyclonal rat anti-WT1 (Santa Cruz Biotechnology, Dallas, TX), polyclonal hamster anti-neprhin (Acris Antibodies, Herford, Germany), monoclonal rat anti-CD31 (clone RM0032-1D12; Abcam), polyclonal goat-anti sheep IgG (Vector Laboratories, Burlingame, CA), and polyclonal goat anti-C3d (Cederlane, Ontario, Canada). Stained cells were quantified in 20 glomeruli and 20 high-power fields (×400) per mouse. Glomerular deposition of sheep IgG and complement C3d was quantified as the fraction of stained glomerular area using ImageJ (National Institutes of Health, Bethesda, MD). All assessments were performed in a blinded protocol.

**Analysis of renal leukocyte infiltration by flow cytometry and immunohistochemistry**

Quantitative assessment of renal leukocyte infiltration by flow cytometry was performed as described. For immunohistochemistry, 2-μm paraffin-embedded renal sections were stained with the following primary antibodies: rat anti-CD3 (clone CD3-12), rat anti-F4/80 (clone CL:A3-1), and rat anti-Mac 2 (clone M3/38; all from AbD Serotec, Oxford, UK). Leukocyte infiltration was analyzed in 50 glomeruli and 20 cortical high-power fields (×400) per mouse.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from whole kidneys using the Purelink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA). SYBRGreen master mix (Thermo Fisher Scientific) were used to perform quantitative real-time polymerase chain reaction on a Light Cycler 480 (Roche, Mannheim, Germany). Primers used for amplification (Metabion, Martinsried, Germany) are listed in Table 1. All samples were run in duplicates and normalized to 18s rRNA.

**Immunoblotting**

For Western blotting the following antibodies were used: rabbit polyclonal anti-cleaved caspase 3 (Cell Signaling Technology), mouse anti-RIPl (clone 7H10, Abcam), polyclonal rabbit anti-RIPl (Abcam), rabbit anti-phospho-IκB-α (clone 14D4, Cell Signaling Technology), and rabbit anti-NF-kB2 p100/p52 (clone 18D10, Cell Signaling Technology). β-actin (rabbit polyclonal serum; Cell Signaling Technology) levels were measured as loading control. Protein bands were visualized using a chemiluminescence kit (Amersham ECL Prime; GE Healthcare, Little Chalfont, UK). Densitometry analysis was performed with ImageJ software.

**Cell culture studies**

A murine glomerular endothelial cell line was grown in Dulbecco’s modified Eagle’s medium with GlutaMax (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (PAA Laboratories, Pasching, Austria). After transfer to 96-well plates, 10,000 cells/well were incubated for 6 hours in vehicle control (dimethylsulfoxide) or 300 ng/ml recombinant murine TNF (Thermo Fisher Scientific) and 10 μM Z-VAD-FMK (InvivoGen, San Diego, CA) to induce cell death in the presence or absence of 100 μM of the necroptosis inhibitor necrostatin-1s (BioVision, Milpitas, CA) as indicated. The CellTiter 96 nonradioactive cell proliferation assay (MTT) kit (Promega, Mannheim, Germany) was used to evaluate cell survival following the manufacturer’s instructions. Results were expressed as percentage of the vehicle control.

**Isolation and stimulation of glomerular cells**

Paramagnetic isolation of glomeruli from healthy wild-type and memTNF mice was performed as described previously. Then 5000 intact glomeruli were resuspended in 3 ml Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 15% FCS and were incubated in 6-well plates at 37 °C for 24 hours. After serum starving for an additional 24 hours, glomeruli were stimulated with 10 ng/ml IL-1β (Thermo Fisher Scientific) in RPMI 1640 for 24 hours. Supernatant was collected for protein measurements using commercially available enzyme-linked immunosorbent assay kits for TNF (BioLegend, San Diego, CA), CCL2 (R&D Systems), and IL-6 (R&D Systems). Cells were lysed and used for RNA extraction as described above.

**Statistical analysis**

Results are presented as mean ± SEM as indicated. Differences between 2 individual experimental groups were compared using 2-tailed Student’s t-test. Comparisons of multiple groups were performed with Kruskal-Wallis test followed by pairwise Mann-Whitney U tests. Survival curves were compared by log-rank test. P < 0.05 was considered to be statistically significant.

**DISCLOSURE**

All the authors declared no competing interests.

**ACKNOWLEDGMENTS**

The expert technical assistance of Dan Draganovici and Jana Mandelbaum is gratefully acknowledged. This study was supported by grants from the Faculty of Medicine, Ludwig-Maximilians-Universität München to MBM and VV. Portions of this work were prepared by MBM as part of his doctoral thesis at the Faculty of Medicine, Ludwig-Maximilians-Universität München.

**SUPPLEMENTARY MATERIAL**

Figure S1. Tubulointerstitial injury in wild-type (WT) and memTNF mice with heterologous nephrotoxic serum nephritis (NTN). Representative photomicrographs of periodic acid–Schiff (PAS)–stained kidney sections from wild-type and memTNF mice illustrating tubulointerstitial injury at day 5 of NTN. Semiquantitative scoring of tubular dilation, intratubular casts, flattened and necrotic tubular cells, and tubuli with vacuolar degeneration was performed as...
described in Material and Methods. Arrows indicate respective pathological lesions. Original magnification x400, bar = 50 μm. Data represent mean ± SEM of 6 mice per group; n.s., not significant.

Figure S2. Renal leukocyte infiltration in wild-type (WT) and memTNF bone marrow (BM)-chimeric mice with heterologous nephrotoxic serum nephritis (NTN). Representative renal sections of chimeric mice with memTNF BM (memTNF→WT) or WT mice expressing parenchymal cells (WT→memTNF) at day 5 of NTN. WT mice transplanted with WT BM (WT→WT chimeras) and memTNF mice transplanted with memTNF BM (memTNF→memTNF) served as controls. Tissue was stained for Ly6G+ neutrophils, Mac2+ glomerular macrophages, F4/80+ tubulointerstitial phagocytes, and CD3+ T cells. Compared with WT→WT chimeras, renal accumulation of neutrophils, glomerular macrophages, interstitial phagocytes, and interstitial T cells was significantly reduced in memTNF→memTNF chimeras only. In contrast, glomerular and interstitial neutrophil numbers increased in memTNF→WT and WT→memTNF chimeras, respectively. Original magnification x400, bar = 50 μm. Data represent mean ± SEM of 5 mice per group. *P < 0.05, **P < 0.01.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES


