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Analysis of TNF-mediated recruitment and activation of glomerular dendritic cells in mouse kidneys by compartment-specific flow cytometry

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Renal dendritic cells (DCs) form an interstitial network contributing to inflammatory and adaptive immune responses in the kidney. The presence and functional role of DC-like glomerular CD11c$^+$ mononuclear phagocytes is a matter of debate. Using compartment-specific flow cytometry we found that healthy mouse kidneys contained 1.3 CD11c$^+$ cells per 100 glomeruli and these increased by 4.6-fold and 13-fold after TNF stimulation and immune complex deposition, respectively. Compartment-specific mRNA expression revealed a predominantly glomerular expression of TNF receptors, chemokines, and adhesion molecules; all upregulated after TNF exposure. Intraperitoneal TNF injection induced influx of neutrophils and mononuclear phagocytes including DC-like CD11c$^+$ cells into both the glomerular and tubulointerstitial compartments, but reduced in TNF receptor (Tnfr) 1-deficient mice. Additionally, Tnfr2 deficiency impaired glomerular infiltration of CD11c$^+$ cells, but not neutrophils. Interstitial CD11c$^+$ cells infiltrated in the presence of Tnfr1 or Tnfr2. TNF exposure also induced similar maturation of glomerular and interstitial CD11c$^+$ cells as demonstrated by increased surface expression of MHC II, CD54, and costimulatory molecules CD40, CD80, and CD86. Thus, by compartment-specific flow cytometry we could demonstrate the constitutive presence of DC-like CD11c$^+$ mononuclear phagocytes in normal mouse glomeruli and their TNF-induced accumulation and activation.

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Dendritic cells (DCs) and macrophages are constitutively present in all tissues. In the kidney these cells form a mononuclear phagocytic system that coordinates innate and adaptive immune responses.[1] Early studies identified cells with DC-like characteristics in rodent kidneys by immunohistochemistry,[2] electron microscopy,[3,4] and flow cytometry.[5,6] Subsequently, it was demonstrated that these cells express the murine DC marker CD11c and possess functional properties of DCs, despite their expression of macrophage-associated molecules such as F4/80.[7,8] Indeed, mononuclear leukocytes present in healthy and diseased kidneys show a substantial phenotypic overlap in their expression of conventional DC and macrophage markers, and their functions.[7,9] These renal mononuclear phagocytes form a cellular system characterized by high plasticity and the capability of phenotypic reprogramming.[1]

CD11c$^+$ renal mononuclear phagocytes have been most frequently, but not exclusively, associated with typical DC-like markers and functions, including the expression of major histocompatibility complex (MHC) class II and costimulatory molecules, and the capacity to locally capture antigens and migrate to local renal lymph nodes, inducing T-cell responses.[10–12] Recent work in mice identified a network of DC-like resident mononuclear phagocytes within the renal interstitium,[8] where they may continuously probe the environment to alert the immune system against infectious or inflammatory injury.[9] Moreover, resident interstitial DC-like CD11c$^+$ cells are an important early source of proinflammatory mediators like tumor necrosis factor (TNF) after acute ischemia/reperfusion damage or obstructive nephropathy.[13,14]

The contribution of renal DCs to glomerular disease is less clear, although they have been shown to modulate disease in models of primary glomerular injury.[15,16] The presence of DC-like CD11c$^+$ mononuclear phagocytes in healthy or diseased glomeruli and their potential role in mediating glomerular injury locally is a matter of debate.[7,9] Several studies reported an occasional presence of DCs in inflamed mouse glomeruli [7,12,17] and in human glomeruli with lupus nephritis,[18] whereas other human renal biopsy studies could

not conclusively demonstrate the presence of glomerular DCs in a variety of glomerular diseases.[19,20]

Given the recently emerged role of renal CD11c$^+$ DCs in interstitial disease, glomerular CD11c$^+$ mononuclear phagocytes may contribute to glomerular inflammation, and their therapeutic modulation may be beneficial in glomerulonephritis (GN). For example, TNF, a potential therapeutic target, has been identified as a crucial factor in the recruitment and activation of DCs, thereby enhancing T-cell activation *in situ*.[21] In murine models of GN, TNF and TNF receptor 2 (Tnfr2) were described as proinflammatory mediators of disease.[22,23] Here, we demonstrate the constitutive presence of DC-like CD11c$^+$ mononuclear phagocytes in normal mouse glomeruli and their TNF-induced accumulation and activation by compartment-specific flow cytometry.

RESULTS

Separation of glomerular and tubulointerstitial tissue from mouse kidneys

We used a magnetic bead–based procedure to isolate mouse glomeruli after perfusion with paramagnetic Dynabeads that accumulate in glomerular capillaries.[24] Microscopically isolated glomeruli were lacking the Bowman's capsule (Figure 1a), as described.[24] The first supernatant obtained during the washing procedure was free of glomeruli, but contained tubular fragments, single tubular cells, and a variety of polymorphic interstitial cells. The second supernatant contained tubular fragments only (Figure 1b and c). This technique allowed a highly efficient separation of renal tissue into glomeruli and tubulointerstitial fractions.

To proof the glomerular and tubulointerstitial origin of separated tissue fractions, we examined mRNA expression of glomerular and tubular marker genes. The glomerular marker gene nephrin was prominently expressed in isolated glomeruli, but not in the tubulointerstitial or tubular fraction (Figure 1d). In contrast, expression of Fxyd2, the γ-subunit of the tubular Na,K-ATPase, was found in the tubulointerstitial and tubular tissue, but not in the glomerular preparation (Figure 1e).

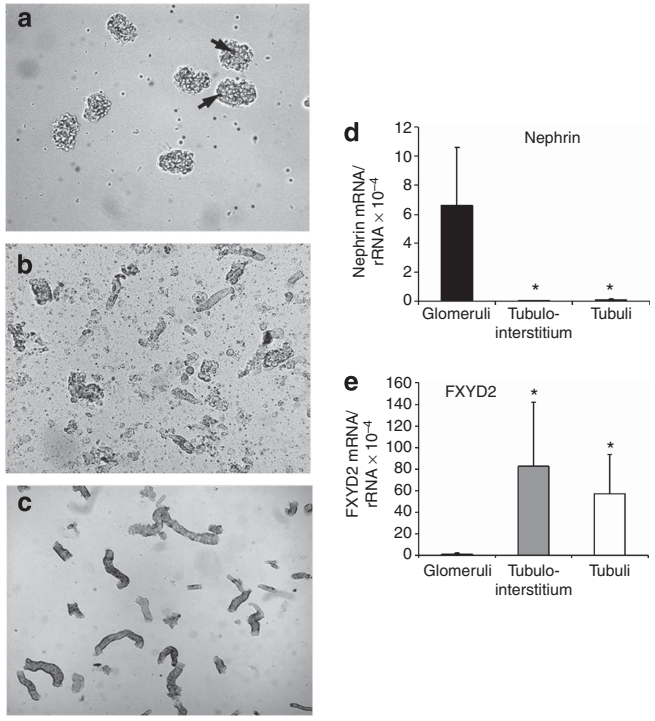

Figure 1 | Separation of glomerular, tubulointerstitial, and tubular tissue fractions from mouse kidneys. (**a**) Microscopic appearance of mouse glomeruli isolated after magnetic bead perfusion. Arrowheads indicate magnetic Dynabeads within glomerular capillaries. (**b**) Microscopic appearance of the tubulointerstitial tissue fraction obtained during the glomerular isolation procedure as first wash. This fraction contained tubular fragments, tubular cells, and polymorphic interstitial cells, but no glomeruli. (**c**) Microscopic examination of the tubular fraction obtained from the second wash. This fraction contained tubular fragments only, but no glomeruli or interstitial cells. (**a**–**c**) Original magnification ×100. Expression of glomerular (nephrin) and tubulointerstitial (FXYD2) marker genes was analyzed by quantitative real-time PCR. (**d**) Nephrin expression was detectable in the glomeruli but not tubulointerstitial and tubular fractions. (**e**) In contrast, mRNA expression of FXYD2, the γ-subunit of the tubular Na,K-ATPase, was barely present in the glomeruli, but was abundant in the tubulointerstitial and tubular tissue. Values were normalized to ribosomal RNA (rRNA) expression used as reference gene. Data represent mean ± s.d. of four mice. *$P<0.05$.

Quantification of glomerular and interstitial leukocyte populations in mice with unilateral ureteral obstruction (UUO) and nephrotoxic serum nephritis (NTN) by flow cytometry

Separated tissue fractions of individual mice were used to simultaneously quantify glomerular and tubulointerstitial leukocyte numbers by compartment-specific flow cytometry. Supplementary Figure S1 online illustrates the gating strategy for the different leukocyte subpopulations.

To validate the compartment specificity of the flow cytometric technique, we isolated glomerular and tubulointerstitial tissue from obstructed kidneys of wild-type mice 5 days after UUO. Obstructed kidneys are characterized by interstitial nephritis, but lack glomerular pathology. UUO resulted in increased interstitial but not glomerular infiltration of CD45$^+$ leukocytes in obstructed kidneys at day 5 when compared with unobstructed contralateral kidneys of the same mice or untreated control kidneys (Supplementary Figure S2a online). Numbers of all tubulointerstitial leukocyte subsets significantly increased in UUO kidneys compared with contralateral kidneys (Supplementary Figure S2b–h online). In comparison with naive wild-type mice, contralateral unobstructed kidneys also showed a moderate increase in interstitial leukocytes, possibly because of a systemic inflammatory response in mice subjected to UUO (Supplementary Figure S2a–h online). Glomerular leukocyte infiltrates were not different between obstructed and contralateral unobstructed kidneys.

Immunohistochemistry confirmed the increased infiltration of neutrophils, F4/80$^+$ leukocytes, ER-HR3$^+$ macrophages, and CD3$^+$ T cells in the tubulointerstitial but not glomerular compartment of obstructed kidneys 5 days after UUO as compared with contralateral control kidneys (Supplementary Figure S3a–h online). It should be noted, however, that the F4/80 antibody has limited sensitivity in the detection of glomerular F4/80 in immunohistochemistry.[25] This may be because of a decreased F4/80 expression in leukocytes infiltrating the glomerulus, as suggested by our flow cytometry data that detected a reduced mean fluorescence intensity of F4/80 in glomerular versus tubulointerstitial leukocytes (Supplementary Figure S3i and j online).

To further proof that our technique is valid in detecting differential changes in glomerular and tubulointerstitial leukocyte accumulation, we analyzed mouse kidneys at day 7 after induction of NTN. In contrast to UUO kidneys, this model of immune complex–mediated GN is characterized by both glomerular and tubulointerstitial leukocyte infiltrates. Flow cytometric analysis confirmed the accumulation of all analyzed leukocyte subsets in both renal compartments, including a prominent glomerular infiltration of CD11c$^+$ cells (Supplementary Figure S4 online).

Importantly, knowing the number of glomeruli subjected to flow cytometric analysis, we quantified the absolute number of CD11c$^+$ cells present in the glomeruli. Although the frequency of CD11c$^+$ glomerular cells was low in normal kidney (1.3 CD11c$^+$ cells per 100 glomeruli) and obstructed kidneys with renal injury limited to the interstitium, it increased in conditions of glomerular injury because of immune-complex deposition (17.3 CD11c$^+$ cells per 100 glomeruli; Figure 2).

Taken together, these data proof the validity of compartment-specific flow cytometry as a technique to simultaneously characterize and quantitate glomerular and tubulointerstitial leukocyte infiltrates, including DC-like CD11c$^+$ mononuclear phagocytes.

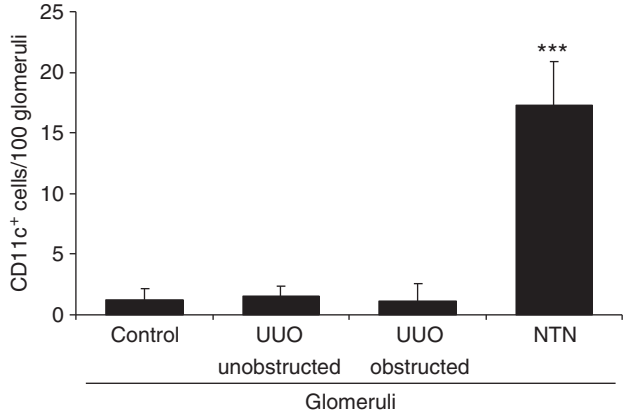

Figure 2 | Abundance of glomerular CD11c$^+$ mononuclear phagocytes. Glomeruli were isolated from untreated control kidneys, unobstructed contralateral and obstructed kidneys 5 days after unilateral ureteral obstruction (UUO), and nephritic kidneys 7 days after induction of nephrotoxic serum nephritis (NTN). The frequency of glomerular CD11c$^+$ cells was low in control kidneys (1.3 cells per 100 glomeruli). Cell numbers were similar in unobstructed and obstructed kidneys (1.5 and 1.1 cells per 100 glomeruli, respectively) lacking glomerular injury after UUO. In contrast, glomerular CD11c$^+$ cells significantly increased in NTN kidneys with immune complex–mediated injury (17.3 cells per 100 glomeruli). Data represent mean ± s.d. of four mice. ***$P < 0.001$ versus control.

Compartment-specific mRNA expression of TNF receptors, chemokines, and adhesion molecules and its TNF-mediated induction

Compartment-specific mRNA expression analysis revealed a preferential expression of the two TNF receptors Tnfr1 and Tnfr2 in the glomeruli of normal mouse kidneys as compared with the tubulointerstitial and tubular tissue preparations (Figure 3a and b). Tnfr1 mRNA was substantially more abundant than Tnfr2. The preferential glomerular expression of both TNF receptors was confirmed by immunohistochemistry that localized Tnfr1 and Tnfr2 protein expression mainly in the glomeruli (Figure 4a and b). Interestingly, glomerular Tnfr1 protein appeared to be expressed predominantly in a mesangial localization, whereas Tnfr2 expression localized mainly to glomerular capillaries. Glomerular expression of Tnfr1 and Tnfr2 could also be detected by western blot analysis of glomerular preparations obtained from normal and TNF-stimulated mice (Figure 4c). Following stimulation of mice with recombinant murine TNF, mRNA expression of Tnfr2 but not Tnfr1 significantly increased in the tubulointerstitial and tubular compartments (Figure 5a and b). In contrast, the glomerular mRNA expression of both TNF receptors was not significantly upregulated by TNF.

Interestingly, in naive kidneys constitutive mRNA expression of proinflammatory chemokines such as chemokine (C-C motif) ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1), chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1α (CCL3/MIP-1α), and chemokine (C-C motif) ligand 5/regulated and normal T cell expressed and secreted (CCL5/RANTES), and adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), E-selectin, and P-selectin was also more prominent in the glomeruli than tubulointerstitial and tubular tissue. For vascular cell adhesion molecule 1, an abundant expression could be detected in the glomerular and tubulointerstitial but not tubular compartment (Figure 3c–i). TNF exposure resulted in a dramatic induction of these mRNAs preferentially in the glomeruli (Figure 5c–j).

These data suggest that glomeruli constitutively express TNF receptors and an armament of molecules that are important for local leukocyte recruitment, with a capacity for rapid upregulation following TNF exposure. In contrast, mRNA expression of these molecules is substantially lower in the tubular and tubulointerstitial renal compartments, but also inducible by TNF.

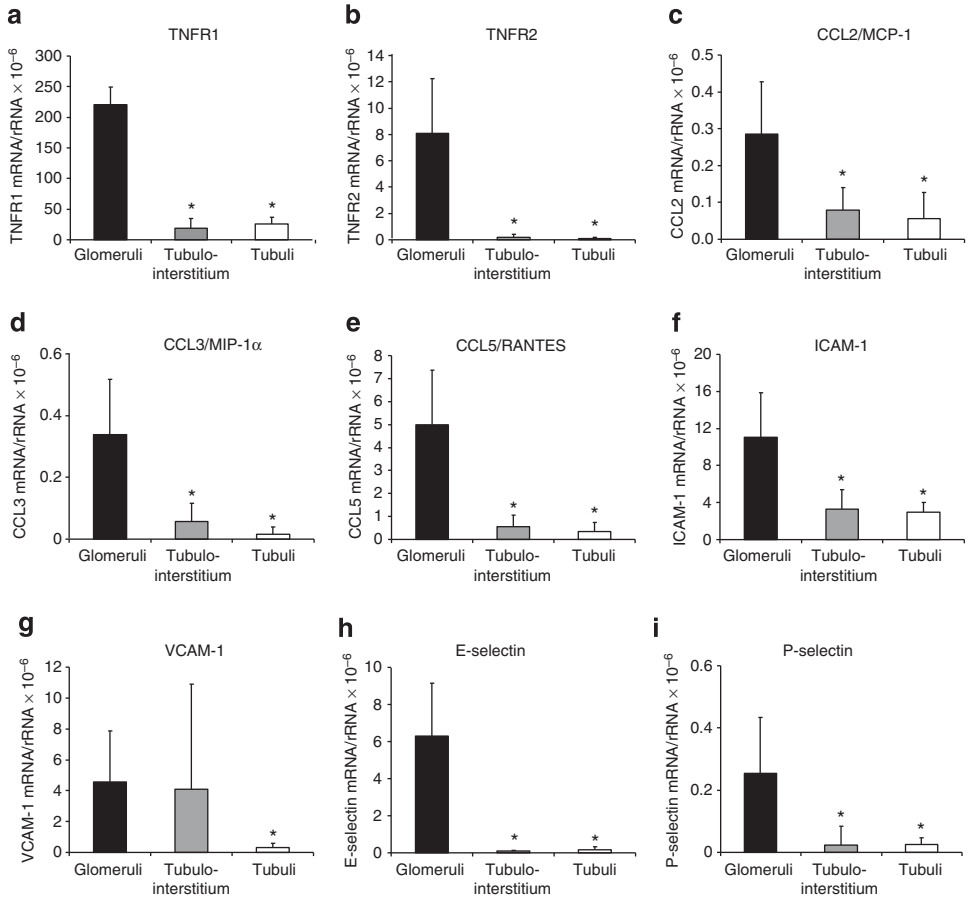

Figure 3 | Compartment-specific mRNA expression of tumor necrosis factor (TNF) receptors, chemokines, and adhesion molecules in normal mouse kidney. Quantitative real-time PCR demonstrates a preferential glomerular mRNA expression of (**a**) Tnfr1, (**b**) Tnfr2, (**c**) chemokine (C-C motif) ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1), (**d**) chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1α (CCL3/MIP-1α, (**e**) chemokine (C-C motif) ligand 5/regulated and normal T cell expressed and secreted (CCL5/RANTES), (**f**) intercellular adhesion molecule 1 (ICAM-1), (**g**) vascular cell adhesion molecule 1 (VCAM-1), (**h**) E-selectin, and (**i**) P-selectin in tissue fractions isolated from normal mouse kidney. Tnfr1 mRNA is ~30-fold more abundant than Tnfr2 mRNA. VCAM-1 is also expressed in the tubulointerstitial but not tubular compartment. Values were normalized to ribosomal RNA (rRNA) expression used as reference gene. Data represent mean ± s.d. of 5–6 mice. *$P < 0.05$.

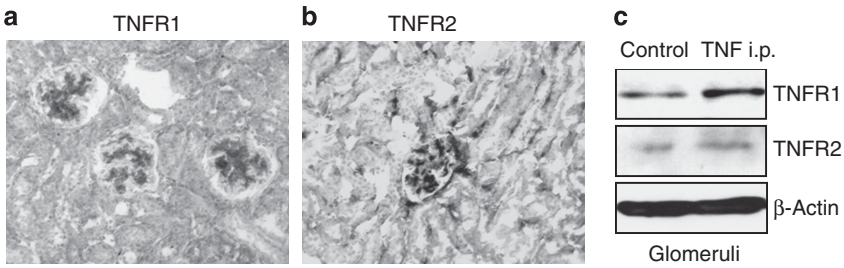

Figure 4 | Renal expression of tumor necrosis factor receptors 1 and 2 (Tnfr1 and Tnfr2). Immunohistochemical localization of (**a**) Tnfr1 and (**b**) Tnfr2 in 4 μm cryostat sections of normal mouse kidney. Both receptors are predominantly expressed in the glomeruli but not the tubulointerstitial compartment. (**a, b**) Original magnification ×400. (**c**) Western blot analysis of pooled glomerular preparations from three phosphate-buffered saline (PBS)-injected and three TNF-exposed mice 8 h after intraperitoneal (i.p.) injection of 5 μg TNF confirms the glomerular expression of Tnfr1 and Tnfr2 in both groups of mice.

Compartment-specific quantification of glomerular and interstitial leukocyte populations in normal and TNF-stimulated mouse kidneys by flow cytometry

Consistent with the prominent glomerular expression of TNF receptors, chemokines, and adhesion molecules, transient renal exposure to TNF following intravenous bolus injection resulted in an accumulation of $CD45^+$ leukocytes in the glomeruli, but not in the tubulointerstitium, after 6 h (Figure 6a). In both compartments, the numbers of $Ly6G^+$ neutrophils remained unchanged (Figure 6b). However, there

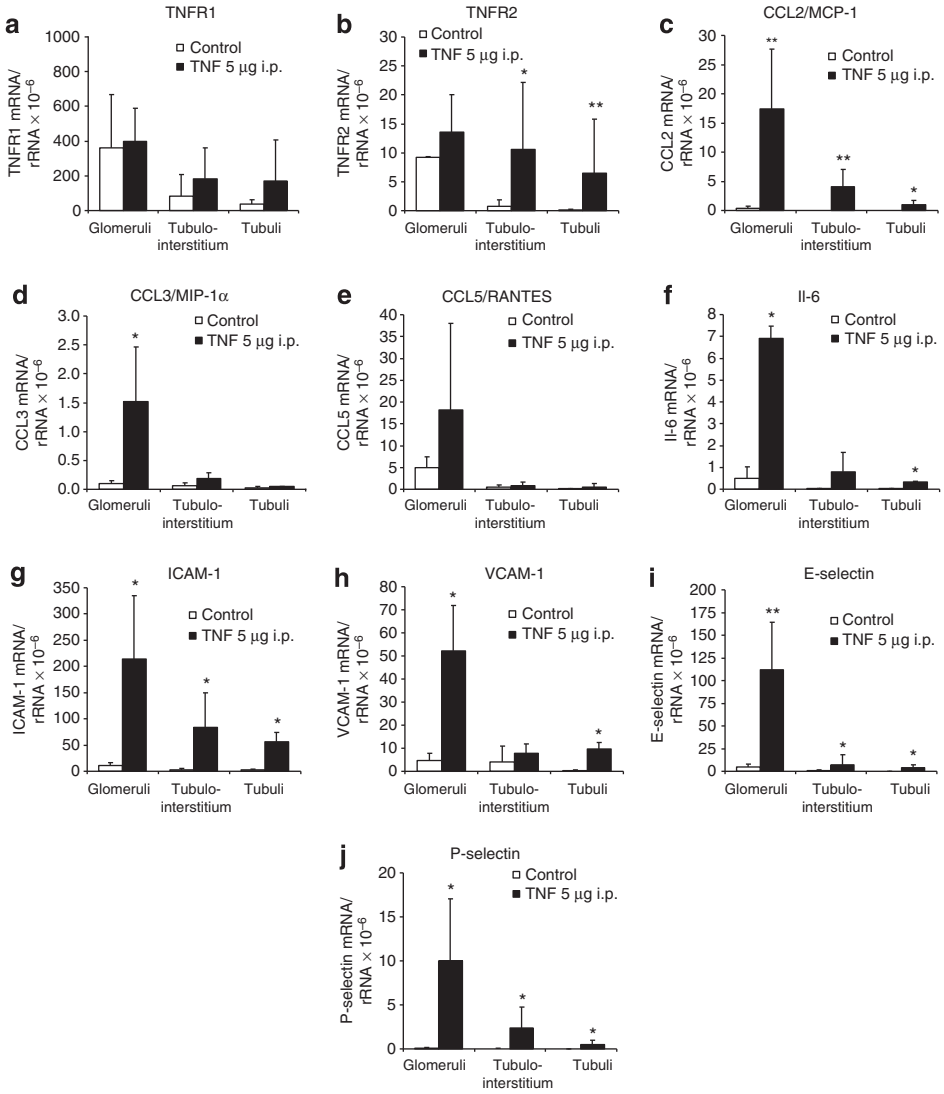

Figure 5 | Quantitative real-time PCR of mRNA expression of tumor necrosis factor (Tnf) receptors, chemokines, cytokines, and adhesion molecules 8 h after injection of TNF (5 µg intraperitoneal (i.p.)). (**a**) TNF resulted in a trend toward induced mRNA expression of Tnfr1 in the tubulointerstitial and tubular but not glomerular tissue. (**b**) Tnfr2 expression was significantly upregulated in the tubulointerstitial and tubular compartment. Expression of inflammatory chemokines such as (**c**) chemokine (C-C motif) ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1), (**d**) chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1α (CCL3/MIP-1α, and (**e**) chemokine (C-C motif) ligand 5/ regulated and normal T cell expressed and secreted (CCL5/RANTES), cytokines such as (**f**) interleukin-6 (Il-6), and adhesion molecules such as (**g**) intercellular adhesion molecule 1 (ICAM-1), (**h**) vascular cell adhesion molecule 1 (VCAM-1), (**i**) E-selectin, and (**j**) P-selectin was more prominently induced in the glomeruli than tubulointerstitial or tubular tissue after TNF injection. Quantitative real-time PCR results were normalized to ribosomal RNA (rRNA) expression used as reference gene. Data represent mean ± s.d. of 5–6 mice. $^{*}P<0.05$, $^{**}P<0.01$.

was a significant glomerular accumulation of $CD11c^+$ mononuclear cells, whereas the numbers of tubulointerstitial $CD11c^+$ cells were not significantly different compared with control (Figure 6c). Similarly, the numbers of all $F4/80^+$ leukocytes and $F4/80^+CD11c^-$ mononuclear phagocytes increased in the glomeruli, but not in the tubulointerstitium (Figure 6d and e). Profiling the accumulating mononuclear phagocytes according to CD11c and F4/80 expression revealed an enrichment of glomerular macrophage-like $F4/80^+$ $CD11c^-$ leukocytes (Figure 7a and b). In contrast, the relative fraction of glomerular $CD11c^+F4/80^+$ and $CD11c^+F4/80^-$ DC-like mononuclear cells decreased after intravenous TNF administration (Figure 7a and b). These data indicate a preferential accumulation of $F4/80^+CD11c^-$ mononuclear phagocytes in TNF-exposed glomeruli, with the numbers of DC-like $CD11c^+$ cells increasing less rapidly. Interestingly, the predominant phenotype of $CD11c^+$ cells present in glomeruli was $F4/80^-$, whereas in the tubulointerstitium $CD11c^+$ cells were mostly $F4/80^+$. Tubulointerstitial fractions of mononuclear phagocytes remained unchanged following intravenous TNF injection (Figure 7a and b).

To investigate the effects of a more prolonged TNF exposure, we analyzed leukocyte infiltrates 8 h after

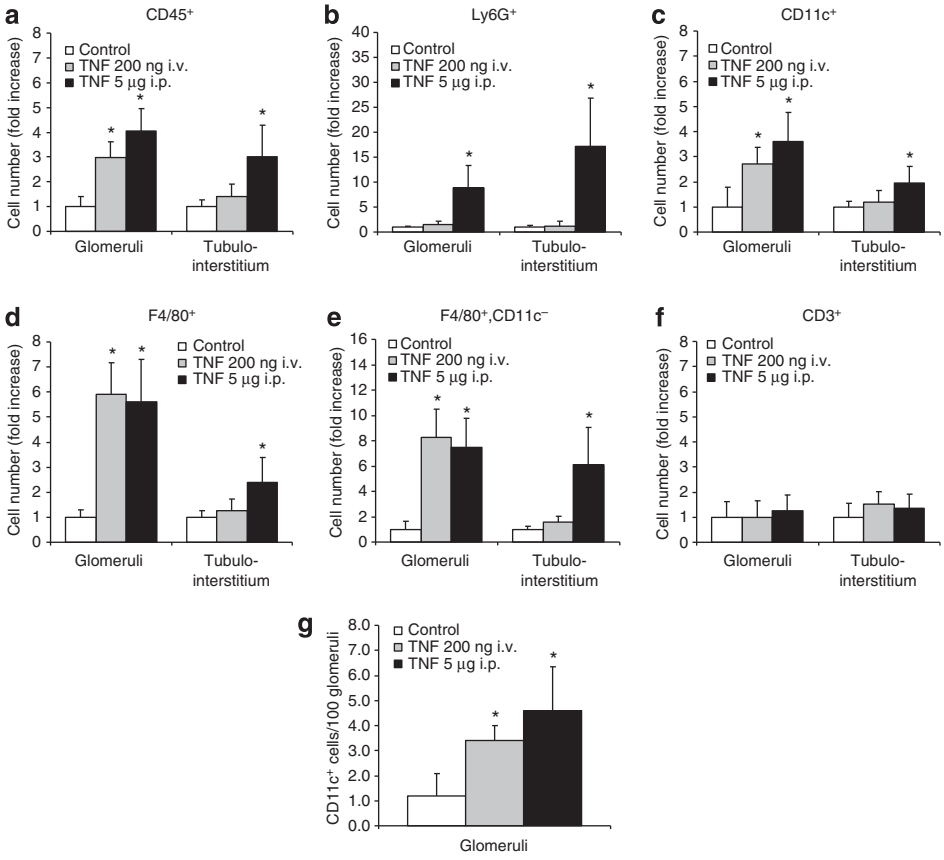

Figure 6 | Quantification of glomerular and interstitial leukocyte populations in normal and tumor necrosis factor (TNF)-stimulated mouse kidneys by compartment-specific flow cytometry. (**a**) Intravenous (i.v.) injection of 200 ng TNF (gray bars) resulted in a glomerular but not tubulointerstitial accumulation of $CD45^+$ leukocytes by 3.0-fold after 6 h as compared with phosphate-buffered saline (PBS)-treated control kidneys (white bars). Prolonged exposure following intraperitoneal (i.p.) injection of 5 µg TNF (black bars) increased both glomerular and tubulointerstitial numbers of $CD45^+$ leukocytes after 8 h by 4.1- and 3.0-fold, respectively. (**b**) The numbers of $Ly6G^+$ neutrophils were not altered by i.v. TNF in both compartments, but increased after i.p. TNF injection in the glomerulus (8.9-fold) and tubulointerstitium (17.1-fold). Following i.v. TNF, glomerular (**c**) $CD11c^+$, (**d**) $F4/80^+$, and (**e**) $F4/80^+$ $CD11c^-$ mononuclear phagocytes increased by 2.7-fold, 5.9-fold, and 8.3-fold, respectively, without significant changes in the tubulointerstitium. (**c**–**e**) After i.p. TNF administration, these leukocyte populations accumulated in both compartments. $CD11c^+$ cells increased in the glomeruli and tubulointerstitium by 3.6-fold and 1.9-fold, respectively. The numbers of $F4/80^+$ leukocytes increased by 5.6-fold in the glomeruli and 2.4-fold in the tubulointerstitial compartment. $F4/80^+CD11c^-$ mononuclear phagocytes accumulated in the glomeruli by 7.5-fold and the tubulointerstitium by 6.1-fold. (**f**) The numbers of $CD3^+$ T cells were not significantly changed by stimulation with i.v. or i.p. TNF. Leukocyte accumulation was analyzed as a percentage of stained cells in relation to total glomerular or tubulointerstitial cells, respectively. Results are expressed as fold increase relative to control kidneys to facilitate direct comparison of the glomerular and tubulointerstitial effects of TNF stimulation. (**g**) Absolute numbers of glomerular $CD11c^+$ cells increased from 1.2 to 3.4 and 4.6 per 100 glomeruli following i.v. or i.p. TNF injection, respectively. Data represent mean ± s.d. of 4–6 mice $^*P < 0.05$ versus control.

intraperitoneal injection of a larger TNF dose. We found increased numbers of $CD45^+$ leukocytes not only in the glomeruli, but also in the tubulointerstitium (Figure 6a). In contrast to intravenous TNF, intraperitoneal TNF led to a significant accumulation of $Ly6G^+$ neutrophils in both glomeruli and tubulointerstitium (Figure 6b). Similarly, $CD11c^+$, $F4/80^+$, and $F4/80^+CD11c^-$ mononuclear phagocytes accumulated in the glomeruli and tubulointerstitium (Figure 6c–e). Profiling of $CD11c^+$ and $F4/80^+$ mononuclear phagocytes after intraperitoneal TNF injection demonstrated a similar glomerular enrichment of $F4/80^+$ $CD11c^-$ macrophages as in intravenously injected mice (Figure 7c). Additionally, in the tubulointerstitium, intraperitoneal TNF resulted in an enrichment of $F4/80^+CD11c^-$ macrophages and DC-like $CD11c^+$ $F4/80^-$ cells, reducing the relative abundance of the constitutively high prevalent $CD11c^+F4/80^+$ cells (Figure 7a and c). Low numbers of $CD3^+$ T lymphocytes were detectable in the glomeruli and tubulointerstitial tissue of control mice and were not altered after TNF injections (Figure 6f).

Together, these data show a glomerular but not tubulointerstitial infiltration of leukocytes following intravenous bolus injection of TNF, with a significant accumulation of $F4/80^+CD11c^-$ and $CD11c^+$ mononuclear phagocytes into glomeruli. In contrast, prolonged exposure to TNF after intraperitoneal administration resulted in leukocyte infiltrates in both the glomerular and tubulointerstitial compartments, mainly by neutrophils, $F4/80^+CD11c^-$, and DC-like

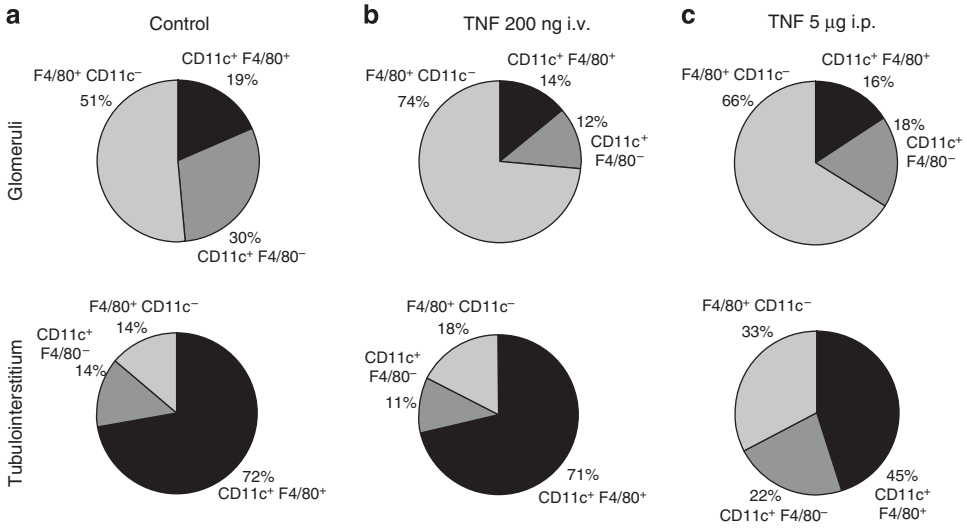

Figure 7 | Renal leukocyte populations expressing CD11c and F4/80 following tumor necrosis factor (TNF) exposure. Pie graphs depicting the plasticity of CD11c and F4/80 expression on glomerular and tubulointerstitial mononuclear phagocytes present in untreated control kidneys, and kidneys following intravenous (i.v.) or intraperitoneal (i.p.) TNF injection. (**a**) In control glomeruli, a large fraction of F4/80$^+$CD11c$^-$ mononuclear phagocytes was present. Glomerular CD11c$^+$ cells were mostly CD11c$^+$F4/80$^-$, with a smaller fraction being CD11c$^+$F4/80$^+$ dendritic cell (DC)-like cells. In contrast, CD11c$^+$F4/80$^+$ cells were the most abundant cell type in the interstitium. (**b**) After i.v. or (**c**) i.p. TNF, the relative abundance of glomerular F4/80$^+$CD11c$^-$ mononuclear phagocytes increased, whereas glomerular fractions of both CD11c$^+$ populations decreased. Intravenous TNF injection did not alter interstitial proportions of these cell populations. After i.p. TNF, interstitial F4/80$^+$CD11c$^-$ and CD11c$^+$F4/80$^-$ mononuclear phagocytes enriched, with a concomitant relative reduction of the double-positive CD11c$^+$F4/80$^+$ fraction. Results shown are representative data obtained from 3–4 mice per group.

CD11c$^+$ mononuclear phagocytes. Compared with control mice, intravenous or intraperitoneal TNF injection increased absolute numbers of glomerular CD11c$^+$ cells from 1.2 to 3.4 and 4.6 cells per 100 glomeruli, respectively (Figure 6g). Interestingly, the predominant phenotype of CD11c$^+$ cells constitutively present in normal glomeruli is F4/80$^-$, whereas in the tubulointerstitium CD11c$^+$ cells are mostly F4/80$^+$.

Contribution of TNF receptor 1 and 2 signaling to TNF-mediated infiltration of glomerular and interstitial leukocytes

To delineate the contribution of Tnfr1 and Tnfr2 to glomerular and tubulointerstitial leukocyte accumulation, we performed compartment-specific flow cytometry on isolated renal tissue from Tnfr1$-/-$, Tnfr2$-/-$ and Tnfr1,2$-/-$ mice 8 h after intraperitoneal TNF injection. As shown in Figure 8a, Tnfr1 deficiency almost abrogated TNF-mediated infiltration of CD45$^+$ leukocytes in glomeruli. When analyzing leukocyte subtypes, we found a significant reduction of Ly6G$^+$ neutrophils, CD11c$^+$, F4/80$^+$, and F4/80$^+$CD11c$^-$ mononuclear phagocytes in Tnfr1$-/-$ glomeruli (Figure 8b–e). In the tubulointerstitium, Tnfr1 deficiency abrogated the TNF-induced influx of Ly6G$^+$ neutrophils and F4/80$^+$ CD11c$^-$ mononuclear phagocytes, but did not significantly alter the numbers of CD45$^+$ leukocytes and CD11c$^+$ and F4/80$^+$ cells (Figure 8a–d). In contrast to Tnfr1$-/-$ mice, lack of Tnfr2 did not affect the overall numbers of glomerular CD45$^+$ leukocytes and Ly6G$^+$ neutrophils, but significantly reduced CD11c$^+$, F4/80$^+$, and F4/80$^+$CD11c$^-$ mononuclear phagocytes in glomeruli but not tubulointerstitial tissue (Figure 8a–e). Glomerular and tubulointerstitial leukocyte infiltrates were grossly reduced in Tnfr1,2 double-deficient mice following TNF injection (Figure 8a–e), consistent with a lack of TNF-mediated signaling. The numbers of CD3$^+$ T cells and CD4$^+$ and CD8$^+$ subsets were not significantly different in the four genotypes (Figure 8f–h).

These data demonstrate a predominant role of Tnfr1 in the recruitment of glomerular leukocytes after TNF exposure. In addition, glomerular infiltration of CD11c$^+$, F4/80$^+$, and F4/80$^+$CD11c$^-$ mononuclear phagocytes but not neutrophils is also dependent on the presence of Tnfr2. The absolute numbers of glomerular CD11c$^+$ cells decreased from 4.6 cells per 100 glomeruli in TNF-injected wild-type mice to 2.3 to 2.4 cells per 100 glomeruli in the Tnfr1 and 2 single- or double-deficient mice (Figure 8i). In the tubulointerstitial compartment, expression of Tnfr1, but not of Tnfr2, is essential for the TNF-induced influx of neutrophils and F4/80$^+$CD11c$^-$ mononuclear phagocytes. Instead, both TNF receptors redundantly mediate accumulation of interstitial F4/80$^+$ and CD11c$^+$ leukocytes (being mainly F4/80$^+$), as only combined deficiency of Tnfr1 and Tnfr2 significantly ameliorated TNF-induced interstitial infiltration of these leukocyte populations.

Characterization of glomerular and interstitial CD11c$^+$ cells following TNF exposure

We characterized the phenotype of glomerular and tubulointerstitial CD11c$^+$ cells following intraperitoneal

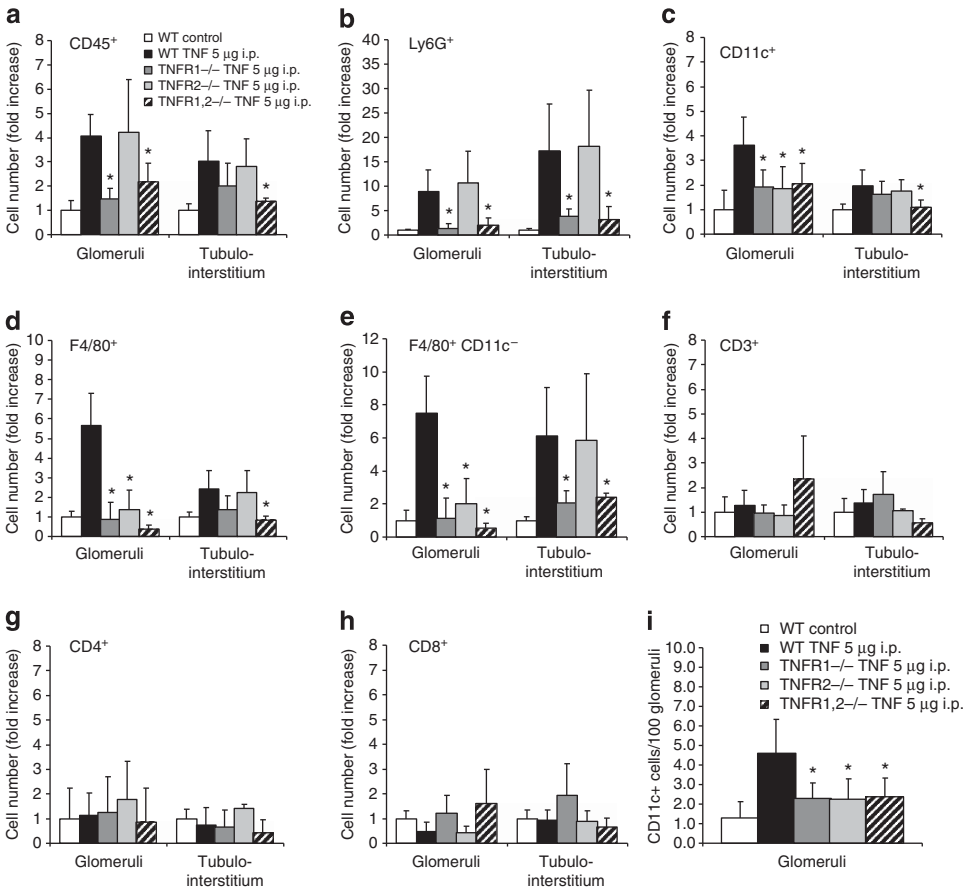

Figure 8 | Contribution of tumor necrosis factor (TNF) receptor 1 and 2 signaling to TNF-mediated infiltration of glomerular and interstitial leukocytes. Compartment-specific flow cytometry was performed on glomerular and tubulointerstitial tissue prepared from wild-type (black bars), Tnfr1 − / − (dark gray bars), Tnfr2 − / − (bright gray bars), and Tnfr1,2 − / − mice (shaded bars) 8 h after intraperitoneal (i.p.) injection of 5 µg TNF. Results are expressed as fold increase relative to phosphate-buffered saline (PBS)-treated control kidneys (white bars). (**a**) In Tnfr1-deficient mice, TNF-induced accumulation of CD45$^+$ leukocytes was significantly reduced in glomeruli but not tubulointerstitial tissue. Tnfr2 deficiency did not affect C45$^+$ leukocyte numbers in both compartments. (**b**) In the absence of Tnfr1, glomerular and tubulointerstitial infiltration of Ly6G$^+$ neutrophils decreased, whereas Tnfr2 deficiency did not inhibit infiltration. In contrast, glomerular accumulation of (**c**) CD11c$^+$, (**d**) F4/80$^+$, and (**e**) F4/80$^+$CD11c$^-$ mononuclear phagocytes was reduced in Tnfr1 − / − and Tnfr2 − / − mice. In the tubulointerstitium only infiltration of F4/80$^+$CD11c$^-$ cells decreased in Tnfr1 − / −, but not Tnfr2 − / − mice, whereas the numbers of interstitial CD11c$^+$ and F4/80$^+$ cells were not affected by either Tnfr1 or Tnfr2 deficiency. In Tnfr1,2 double-deficient mice, TNF-induced infiltration of all leukocyte subpopulations was abrogated. Glomerular and interstitial numbers of (**f**) CD3$^+$ T cells, (**g**) CD4$^+$ and (**h**) CD8$^+$ T cells did not significantly change in TNF-treated mice of all genotypes. (**i**) Absolute numbers of glomerular CD11c$^+$ cells decreased from 4.6 cells per 100 glomeruli in TNF-injected wild-type mice to 2.3 cells in Tnfr1 − / − and Tnfr2 − / − mice, and 2.4 cells per 100 glomeruli in Tnfr1,2 double-deficient mice. Data represent mean ± s.d. of 4–6 mice. * $P<0.05$ compared with TNF-injected wild-type mice.

phosphate-buffered saline or TNF injection. In phosphate-buffered saline–treated control kidneys, 50% of glomerular and 95% of tubulointerstitial CD11c$^+$ cells expressed MHC class II (that is, I-A^b) molecules. After TNF exposure, the percentage of MHC II$^+$ CD11c$^+$ cells decreased slightly in both compartments (Figure 9a). However, the mean fluorescence intensity values of MHC II increased after TNF stimulation, indicating an induced MHC II surface expression in MHC II$^+$ CD11c$^+$ cells in both compartments (Figure 9a). The lower fraction of MHC II$^+$ CD11c$^+$ cells most likely reflects a preferential recruitment and accumulation of immature versus mature CD11c$^+$ cells into the kidney. No relevant changes in CD11b expression were present (Figure 9b). However, the intensity of CD11c expression decreased in glomerular CD11c$^+$ cells following TNF exposure (Figure 9c). The fraction of CD54/ICAM-1$^+$ CD11c$^+$ cells and corresponding mean fluorescence intensity values increased in glomeruli as well as tubulointerstitial tissue after TNF injection (Figure 9d). TNF stimulation also induced the expression of costimulatory molecules in renal CD11c$^+$ cells. The number of CD11c$^+$ cells with detectable surface expression of CD40, CD80, and CD86 as well as corresponding mean fluorescence intensity values increased in the glomeruli and tubulointerstitium following TNF exposure (Figure 9e–g). Only a small fraction of B220$^+$ plasmacytoid DCs could be detected in both renal compartments, which was not altered by TNF (Figure 9h). Additional staining of splenic CD11c$^+$ DCs isolated from phosphate-buffered saline- and TNF-injected mice revealed a similar response to TNF (Supplementary Figure S5 online).

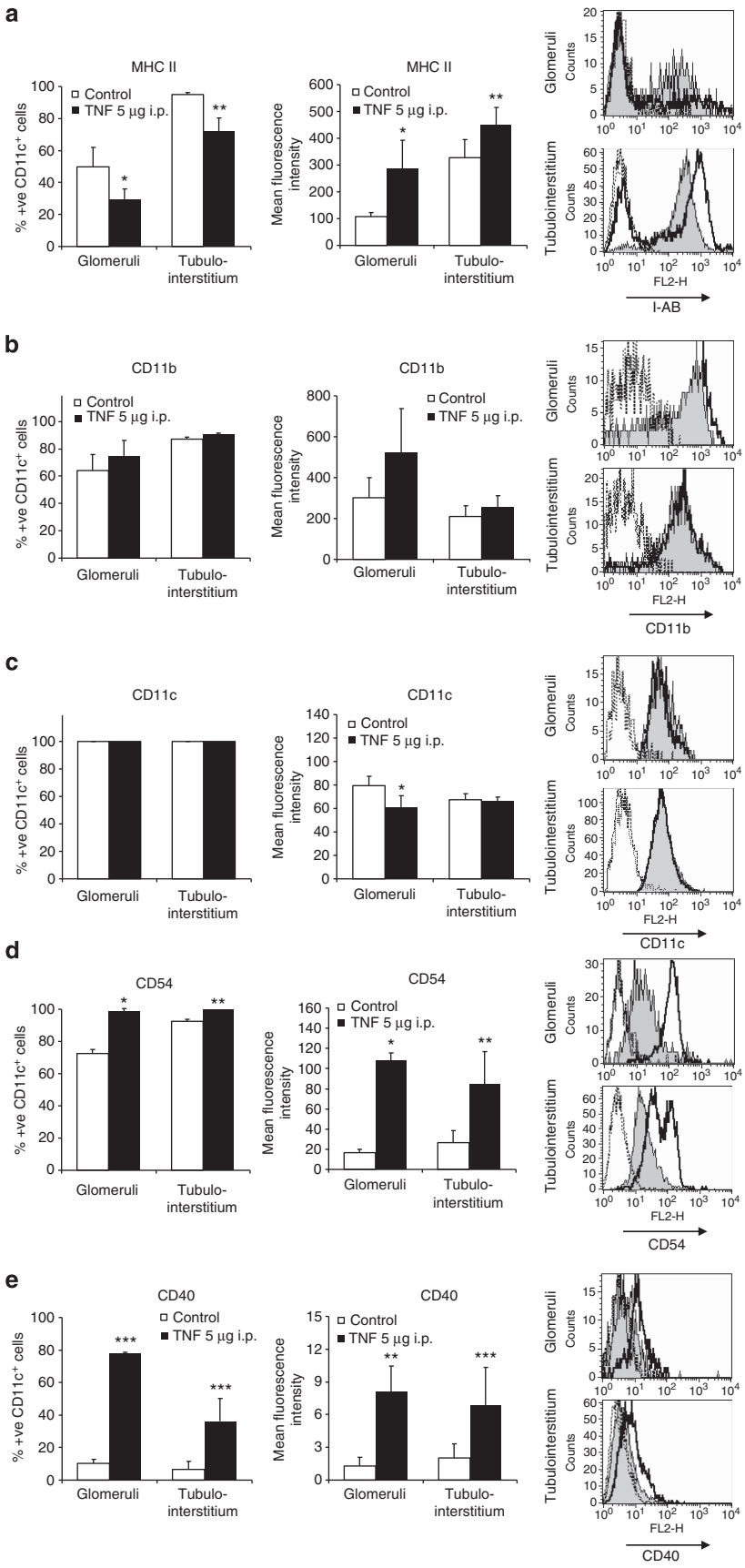

Figure 9 | Continued on following page.

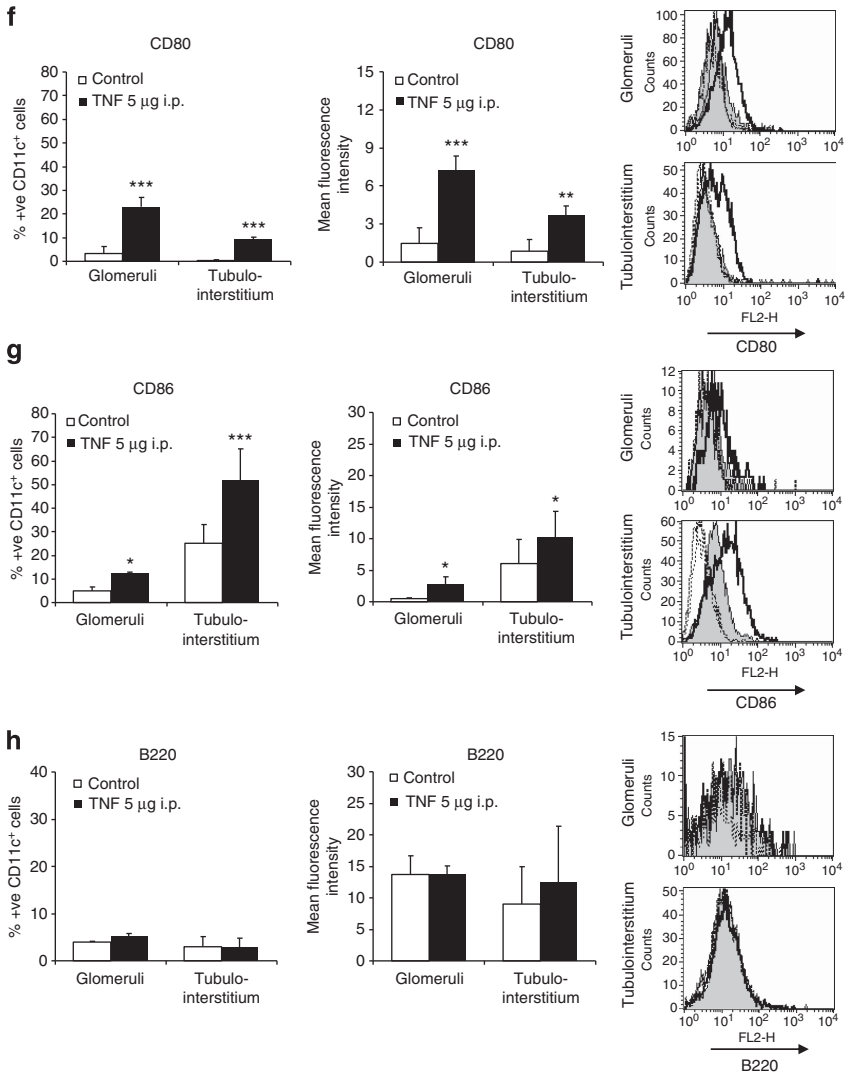

Figure 9 | Characterization of glomerular and tubulointerstitial CD11c$^+$ cells following tumor necrosis factor (TNF) exposure by compartment-specific flow cytometry. Dendritic cell (DC)-like mononuclear phagocytes were identified as CD45$^+$ CD11c$^+$ cells. In addition, the expression of a series of surface markers was analyzed, including (**a**) major histocompatibility complex II (MHC II), (**b**) CD11b, (**c**) CD11c, (**d**) CD54, the costimulatory molecules (**e**) CD40, (**f**) CD80, and (**g**) CD86, and the plasmacytoid DC marker (**h**) B220, or respective isotype controls. Surface expression of these molecules was compared between renal CD11c$^+$ cells of TNF-stimulated (5 μg intraperitoneal (i.p.), 8 h) mice (black bars) and phosphate-buffered saline (PBS)-treated controls (white bars). For each surface protein, the fraction of positive CD11c$^+$ cells and the mean fluorescence intensity (MFI) is shown. Representative histogram blots illustrate shifts of the MFIs in glomerular and tubulointerstitial DC preparations (gray-shaded histogram: control kidney, black-lined histogram: TNF-stimulated kidney, dotted-lined histograms: isotype controls). Data represent mean ± s.d. of 3–8 mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control.

These data demonstrate that both glomerular and tubulointerstitial DC-like CD11c$^+$ cells acquire an activated phenotype after TNF exposure. Thus, glomerular CD11c$^+$ cells can be matured by inflammatory stimuli like TNF in a comparable way as in the tubulointerstitium.

DISCUSSION

An extensive network of resident DC-like CD11c$^+$ mononuclear phagocytes is present in the kidney tubulointerstitium. As nonmigratory DCs, they are important contributors to local innate immunity[13,14] and may activate infiltrating T cells to modulate adaptive immune responses within the kidney.[14–16] In addition, there is growing experimental evidence that interstitial CD11c$^+$ cells capture endogenous or exogenous antigens and migrate to local renal lymph nodes, where they can either tolerize or activate T cells, the latter leading to nephritogenic T-cell responses.[10–12] It is thought that filtered or shed glomerular antigens reach the tubular lumen, where they are either reabsorbed and processed by tubular epithelial cells before activation of local DCs, or may be directly sampled by interstitial DCs.[26,27] However, this scenario cannot be easily applied to nephritogenic T-cell responses induced by antigens exclusively present in the glomeruli, such as immune complexes that are deposited or formed *in situ* during GN.

As such, the presence of glomerular DC-like CD11c$^+$ cells has been postulated. Stellate-shaped cells with dendrite extensions have not been described within the glomerulus by electron microscopy.[3,4] However, several reports suggest the glomerular presence of DC-like cells in the rat, intermingling with mesangial cells in low density.[28–31] By compartment-specific flow cytometry we confirmed the presence of CD11c$^+$ mononuclear phagocytes in the glomeruli of normal, nephritic, and TNF-stimulated mice, and were able to simultaneously quantitate and characterize these cells and other leukocyte populations from glomerular and tubulointerstitial tissue preparations of individual mice.

In healthy glomeruli, CD11c$^+$ mononuclear phagocytes were present at very low frequency, that is, 1.3 cells per 100 glomeruli. Although being phenotypically similar to their interstitial counterparts in the expression of DC surface markers, their low abundance in glomeruli does not support an important role of glomerular DC-like CD11c$^+$ cells in immune surveillance within glomeruli at steady state, a function that was demonstrated for CD11c$^+$ cells in the interstitium.[1,11,26,32] However, after proinflammatory stimulation with TNF or glomerular immune complex formation in the NTN model, the numbers of glomerular CD11c$^+$ cells significantly increased by 4.6-fold and 13-fold, respectively. Consistently, in lupus nephritis of NZM 2328 mice, a disease induced by glomerular immune complex deposition, the developing proliferative GN was associated with the detection of CD11c$^+$ DC-like cells and T cells in affected glomeruli.[17] Glomerular infiltration by DC-like mononuclear phagocytes has also been described in human lupus nephritis.[18] These data suggest a potential role of intraglomerular CD11c$^+$ mononuclear phagocytes in innate and adaptive immune responses during glomerular inflammation.

As reported for murine interstitial CD11c$^+$ leukocytes,[7,8,13,14] we detected a subpopulation of glomerular CD11c$^+$ mononuclear phagocytes in normal mouse kidney expressing the macrophage marker F4/80. Interestingly, this fraction of F4/80$^+$ CD11c$^+$ cells was substantially smaller in the glomeruli than the tubulointerstitium, suggesting a compartment-specific heterogeneity of resident renal CD11c$^+$ mononuclear phagocytes. On the other side, compartment-specific flow cytometry demonstrated that glomerular CD11c$^+$ mononuclear phagocytes were activated by TNF in a similar way as were CD11c$^+$ cells in the tubulointerstitium and spleen. Maturation of glomerular CD11c$^+$ cells was indicated by TNF-induced upregulation of DC activation markers like MHC II and CD54/ICAM, a downregulated surface expression of CD11c,[33] and an increased costimulatory capacity through induced expression of CD40, CD80, and CD86.

We detected glomerular accumulation of both DC-like CD11c$^+$ F4/80$^+$ and CD11c$^+$ F4/80$^-$ mononuclear cells, and macrophage-like F4/80$^+$ CD11c$^-$ leukocytes after stimulation with TNF or glomerular immune complex formation. Influx and expansion of activated mononuclear phagocytes is well described in glomerular inflammation.[1,34,35] Activated glomerular phagocytes produce reactive oxygen species[36,37] and proinflammatory cytokines including interleukin IL-1β and TNF in experimental GN, which substantially contribute to renal injury.[38–40] Glomerular phagocytes infiltrating nephritic glomeruli also upregulate MHC II expression.[36] Interestingly, labeled macrophages that were injected into rats with NTN and localized into inflamed glomeruli disappeared from glomeruli again with a half-life of ~48 h.[41,42] These data suggest that intraglomerular mononuclear phagocytes not only contribute to local innate immune mechanisms, but continually traffic through nephritic glomeruli and potentially induce adaptive immune responses in secondary lymphatic tissue. However, afferent lymphatic vessels draining to local lymph nodes have not been demonstrated in glomeruli, and thus it is unclear to which extent activated glomerular DC-like CD11c$^+$ cells migrate to secondary lymphoid tissue to induce T-cell immunity, or generate local proinflammatory mediators and modulate glomerular immune responses similar to nonmigratory DCs of the interstitium. In either case, DC-like glomerular CD11c$^+$ mononuclear phagocytes will possess phenotypes and functions previously assigned exclusively to activated glomerular macrophages.

TNF and TNF receptors were identified as proinflammatory mediators and potential therapeutic targets in glomerular diseases.[43] Modulation of renal DC infiltration and function may be one underlying mechanism. We demonstrate that intravenous TNF injection induced a significant glomerular accumulation of leukocytes, mainly of macrophage-like F4/80$^+$CD11c$^-$ and DC-like CD11c$^+$ mononuclear phagocytes, but not neutrophils. In contrast, no significant tubulointerstitial leukocyte infiltration could be detected. However, prolonged TNF exposure after intraperitoneal injection of a larger TNF dose induced interstitial Tnfr2 expression and increased leukocyte numbers in glomeruli and tubulointerstitium, including CD11c$^+$ and F4/80$^+$CD11c$^-$ mononuclear cells and most prominently neutrophils. TNF injection led to a relative enrichment of F4/80$^+$CD11c$^-$ phagocytes, which was paralleled by a reduced relative fraction of CD11c$^+$F4/80$^-$ cells in the glomeruli and CD11c$^+$F4/80$^+$ cells in the tubulointerstitium. This phenotypic switch among mononuclear phagocytic cells may reflect a predominant influx of bone marrow—derived macrophages compared with dendritic-like cells during TNF-induced inflammation in the kidney. In glomeruli, we identified Tnfr1 as an essential mediator of TNF-induced leukocyte infiltration. Moreover, glomerular infiltration of CD11c$^+$ and F4/80$^+$CD11c$^-$ mononuclear phagocytes, but not neutrophils, was also dependent on Tnfr2. These data indicate that both TNF receptors contribute to glomerular infiltration of CD11c$^+$ and F4/80$^+$CD11c$^-$ mononuclear phagocytes, whereas glomerular neutrophils are recruited solely via Tnfr1-mediated mechanisms. In the tubulointerstitium neutrophils and F4/80$^+$CD11c$^-$ phagocytes were substantially diminished in Tnfr1$-/-$ mice. In contrast, overall

numbers of interstitial CD45$^+$ leukocytes, F4/80$^+$ cells, and CD11c$^+$ cells (being the largest F4/80$^+$ interstitial leukocyte population) were significantly reduced in Tnfr1,2 $-/-$ mice only, indicating a redundant role of both TNF receptors for accumulation in this compartment. Compared with the tubulointerstitium, it appears that TNF-induced recruitment of DC-like CD11c$^+$ cells into the glomerulus is more tightly controlled with its dependence on both TNF receptors. Interestingly, experimental treatment of patients with type V lupus nephritis (that is, membranous GN due to local immune complex deposits) with the TNF-neutralizing antibody infliximab resulted in a long-standing remission of nephritis.[44] Although the underlying mechanisms are not known, one could speculate that, among other effects, TNF blockade may reduce glomerular accumulation and activation of DCs and macrophages, and subsequent inflammatory damage. To our knowledge, glomerular leukocyte infiltrates have not been studied in renal biopsies of these patients.

In summary, we demonstrate the constitutive presence of DC-like CD11c$^+$ mononuclear phagocytes in normal mouse glomeruli, as well as the accumulation, activation, and maturation of glomerular CD11c$^+$ cells upon TNF exposure. These data suggest that glomeruli possess the intrinsic capacity to recognize both pathogen and danger-associated molecular patterns through resident and especially infiltrating DC-like cells in inflamed glomeruli. These processes may be therapeutically targetable by TNF or TNF receptor blocking therapies. The contribution of glomerular DCs to the glomerular immune response, for example, by local production of proinflammatory chemokines and cytokines, and their role in mediating nephritogenic T-cell responses to pathogens or glomerular autoantigens remain to be elucidated.

MATERIALS AND METHODS

Animal models

Genetically deficient mice in Tnfr 1 (Tnfr1 $-/-$, B6.129-*Tnfrsf1a*^{tm1Mak}) and Tnfr2 (Tnfr2 $-/-$, B6.129-*Tnfrsf1b*^{tm1Mwm}) were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in the Central Animal Facility (University Hospital Innenstadt, Ludwig-Maximilians-University Munich, Munich, Germany) under specific pathogen-free conditions. Tnfr1 and 2 double-deficient mice (Tnfr1,2 $-/-$) and C57Bl/6J wild-type controls were generated by cross-breeding Tnfr1 $-/-$ and Tnfr2 $-/-$ mice.

To induce renal leukocyte infiltration, male mice aged 6 to 8 weeks were treated with murine TNF (Invitrogen, Karlsruhe, Germany). Either 200 ng of TNF was injected intravenously into the tail vein or 5 μg TNF was injected intraperitoneally. Both regimens reportedly result in an induced expression of renal adhesion molecules and leukocyte accumulation.[45,46] Perfused kidneys were harvested 6 or 8 h after TNF injection, respectively. Interstitial nephritis due to obstructive nephropathy was induced by UUO in female C57Bl/6J mice aged 8–10 weeks as described.[47,48] Accelerated NTN was induced in male C57Bl/6J mice between 7 and 9 weeks essentially as previously described,[48] injecting 50 μl of a commercially available sheep nephrotoxic serum (Probetex, San Antonio, TX) in the tail vein of presensitized mice. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Paramagnetic separation of glomeruli and tubulointerstitial tissue

The procedure to separate glomeruli and tubulointerstitial tissue from mouse kidneys was adapted from a magnetic bead-based isolation method described by Takemoto *et al.*[24] A detailed protocol of the procedure is provided in the Supplementary Methods online. The final magnetically labeled fraction contained highly purified glomeruli with a yield of ~10,000 glomeruli per mouse kidney and a purity of >97% (intact glomeruli vs. tubular fragments or single cells). The first supernatant obtained in the washing steps was free of glomeruli, but contained tubular fragments, single tubular cells, and a variety of polymorphic interstitial cells. The second supernatant contained tubular fragments only.

Quantification of glomerular and interstitial leukocyte subsets by flow cytometry

Antibody staining of single-cell suspensions prepared from glomeruli and tubulointerstitial tissue (first supernatant) of individual mice was performed as previously described.[48,49] Antibodies and gating strategies are detailed in the Supplementary Methods online. Stained cells were analyzed with a FACS Calibur flow cytometer and Cellquest software (BD Biosciences, Heidelberg, Germany). The number of stained leukocytes was expressed as a percentage of total glomerular or tubulointerstitial cells, respectively. Absolute numbers of glomerular CD11c$^+$ cells detected were expressed per 100 glomeruli.

Phenotypic characterization of glomerular and interstitial DCs by flow cytometry

The activation status of DCs in glomerular or tubulointerstitial cell preparations was analyzed by flow cytometry as described in the Supplementary Methods online.

Statistical analysis

Numerical results of each experimental group were expressed as mean ± s.d. and were compared using two-sided nonparametric Mann–Whitney *U*-test. When more than two experimental groups were compared, Kruskal–Wallis test was applied, followed by pairwise Mann–Whitney *U*-tests. Significance was assigned to $P<0.05$. Experiments were performed between three and eight times to ensure reproducibility.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods.
Table S1. Primers and probes used for real-time RT-PCR.
Figure S1. Gating strategy for quantification of glomerular and interstitial leukocyte populations.
Figure S2. Quantification of glomerular and tubulointerstitial leukocyte populations in obstructed mouse kidneys by compartment-specific flow cytometry.
Figure S3. Immunohistochemical localization of leukocyte populations in contralateral and obstructed kidneys five days after UUO.
Figure S4. Quantification of glomerular and tubulointerstitial leukocyte populations in NTN kidneys by compartment-specific flow cytometry.
Figure S5. Characterization of splenic CD11c+ cells following TNF exposure.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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