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Immature renal dendritic cells recruit regulatory CXCR6^{+} invariant Natural Killer T cells to attenuate crescentic GN

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Immature Renal Dendritic Cells Recruit Regulatory CXCR6$^+$ Invariant Natural Killer T Cells to Attenuate Crescentic GN

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ABSTRACT

Immature renal dendritic cells (DCs) are protective early in murine crescentic GN, but the mechanisms underlying this protection are unknown. Here, depletion of DCs reduced the recruitment of invariant natural killer T (iNKT) cells, which attenuate GN, into the kidney in the early stage of experimental crescentic GN. More than 90% of renal iNKT cells expressed the chemokine receptor CXCR6, and renal DCs produced high amounts of the cognate ligand CXCL16 early after induction of nephritis, suggesting that renal DC-derived CXCL16 might attract protective CXCR6$^+$ iNKT cells. Consistent with this finding, CXCR6-deficient mice exhibited less iNKT cell recruitment and developed nephritis that was more severe, similar to the aggravated nephritis observed in mice depleted of immature DCs. Finally, adoptive transfer of CXCR6-competent NKT cells ameliorated nephritis. Taken together, these results suggest an immunoprotective mechanism involving immature DCs, CXCL16, CXCR6, and regulatory iNKT cells, which might stimulate the development of new therapeutic strategies for GN.

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GN, as a disease category, is one of the leading causes of progressive renal failure leading to ESRD.[1] Among the different types of GN, crescentic GN is the most aggressive form with the worst prognosis. Crescentic GN represents a heterogeneous collection of disease entities, such as ANCA-associated GN and antiglomerular basement membrane (anti-GBM) nephritis.[2] Cell-mediated renal damage is, however, a fundamental characteristic of each form of crescentic GN.[3,4]

Nephrotoxic nephritis (NTN) is a well established murine model of crescentic GN.[5] In this model, a sheep antiserum raised against kidney cortical components is injected into mice, resulting in activation of Th1 and Th17 cells in lymphatic organs. Because of their specificity, sheep Igs are preferentially deposited in the kidney, inducing tissue destruction by infiltrating activated Th1 and Th17 cells.[6–8] These cells subsequently activate intrarenal macrophages and drive neutrophil recruitment, causing renal tissue injury.[9]

Dendritic cells (DCs) are professional antigen-presenting cells that play a pivotal role in the priming and activation of effector T cells, but under certain circumstances, they might also protect against an overwhelming inflammatory response by less well characterized mechanisms. Renal DCs form an

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extensive parenchymal network that spans the entire tubulointerstitium, ensuring complete surveillance of the kidney to protect it, for example, against infections ascending through the tubular system. Despite their absence from glomeruli, renal DCs are also involved in GN by capturing glomerular antigens or antigens filtered in glomeruli and presenting them to T effector cells, thereby triggering an inflammatory mononuclear infiltrate in the tubulointerstitium that drives renal disease.[10,11] It is important to note that renal DCs only exert this pathogenic effect when nephritis becomes chronic (DC maturation).[12] At steady state and in acute GN, they can also mediate anti-inflammatory and disease-attenuating effects.[13] Generally, immature DCs are thought to induce immune tolerance, and this finding is thought to result from the absence of costimulatory signals.[14,15] It is unclear whether this tolerance causes the protective function of immature kidney DCs.

Recent studies showed that renal DCs significantly contribute to the early production of chemokines and other inflammatory mediators, which regulates the recruitment of leukocytes into the inflamed kidney.[16] We were, therefore, interested in studying whether renal DCs promote the infiltration of potentially anti-inflammatory leukocyte subsets in the early course of experimental GN (NTN) and characterizing the underlying mechanisms.

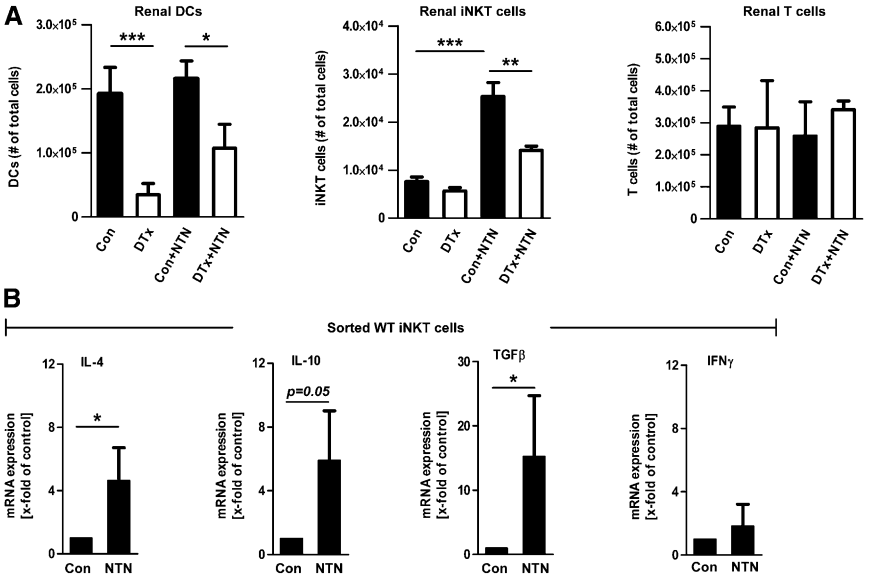

Figure 1. DC depletion reduces the recruitment of anti-inflammatory iNKT cells in experimental GN. (A) Quantification of FACS analyses of renal DCs ($CD45^+MHCII^+CD11c^+$), iNKT ($CD45^+TCR\beta^+CD1d\text{-}Tetramer^+$), and T cells ($CD45^+CD3^+$) at day 4 of NTN 1 day after DTx injection (numbers represent total cells/kidney; n=3–4/group). (B) Real-time RT-PCR analysis of FACS-sorted iNKT cells for IL-4, IL-10, TGFβ, and IFNγ isolated from kidneys of nephritic wild-type mice and wild-type controls (n=3–13/group). Bars represent means ± SD (*P<0.05, **P<0.01, ***P<0.001).

RESULTS

DC Depletion Reduced the Recruitment of Anti-Inflammatory Invariant Natural Killer T Cells in Experimental GN

To evaluate the protective mechanisms of DCs in early stages of NTN, $CD11c^+$ DCs were depleted on day 3 after induction of nephritis by injecting CD11c.LuciDTR[17] mice with diphtheria toxin (DTx). After 24 hours, renal leukocyte composition was analyzed. As expected, the number of renal DCs was reduced in the DTx group under both control and nephritic conditions ($1.9\times10^5\pm0.4\times10^5$ [Con] versus $3.5\times10^4\pm1.7\times10^4$ [DTx], $P<0.001$; $2.2\times10^5\pm0.3\times10^5$ [NTN] versus $1.1\times10^5\pm0.4\times10^5$ [DTx + NTN], $P<0.05$) (Figure 1A, left panel). Another interesting finding is that the recruitment of invariant natural killer T (iNKT) cells (defined as $CD45^+CD1d\text{-}Tetramer^+$ [loaded with αGalCer analog PBS-57] $TCR\beta^+$) into the inflamed kidney was significantly reduced after DC depletion (NTN: $2.5\times10^4\pm0.3\times10^4$ versus $1.4\times10^4\pm0.09\times10^4$, $P<0.01$) (Figure 1A, center panel), whereas the proportion of other immune cells, such as $CD3^+$ T cells (Figure 1A, right panel) and $CD11b^+$ macrophages (data not shown), remained unchanged. DTx-induced DC depletion had no effect on systemic numbers of iNKT cells in CD11cLuciDTR mice (Supplemental Figure 1).

A role for NKT and iNKT cells in the suppressive activity of immature DCs is supported by previous studies showing that these cells can ameliorate experimental GN.[18,19] In agreement with these results, real-time PCR analysis of FACS-sorted iNKT cells from the kidneys of nephritic wild-type mice showed increased expression of the anti-inflammatory mediators IL-4, IL-10, and TGFβ but not the proinflammatory mediator IFNγ compared with untreated control mice. Expression of IL-17 was not detectable (data not shown; IL-4: 4.6-fold versus controls; IL-10: 5.9-fold versus controls; TGFβ: 15.2-fold versus controls; IFNγ: 1.8-fold versus controls) (Figure 1B).

CXCR6 Is Highly Expressed on Renal iNKT Cells

The regulation of renal iNKT cell infiltration is largely unknown. Several chemokine receptors, including CXCR3, CCR5, CCR6, and CXCR6, have been reported to promote NKT and iNKT cell trafficking under inflammatory conditions.[20] We, therefore, analyzed the expression profile of these chemokine receptors in renal iNKT cells. More than 90% of renal iNKT cells during the early stages of NTN were CXCR6-positive, whereas CXCR3 (9%), CCR6 (19%), and CCR5 (11%) expression was much less frequent (Figure 2, A and B). By contrast, only about 40% of renal $CD4^+$ T cells and $CD8^+$ T cells were $CXCR6^+$, and less than 10% of $CD11b^+$ macrophages and

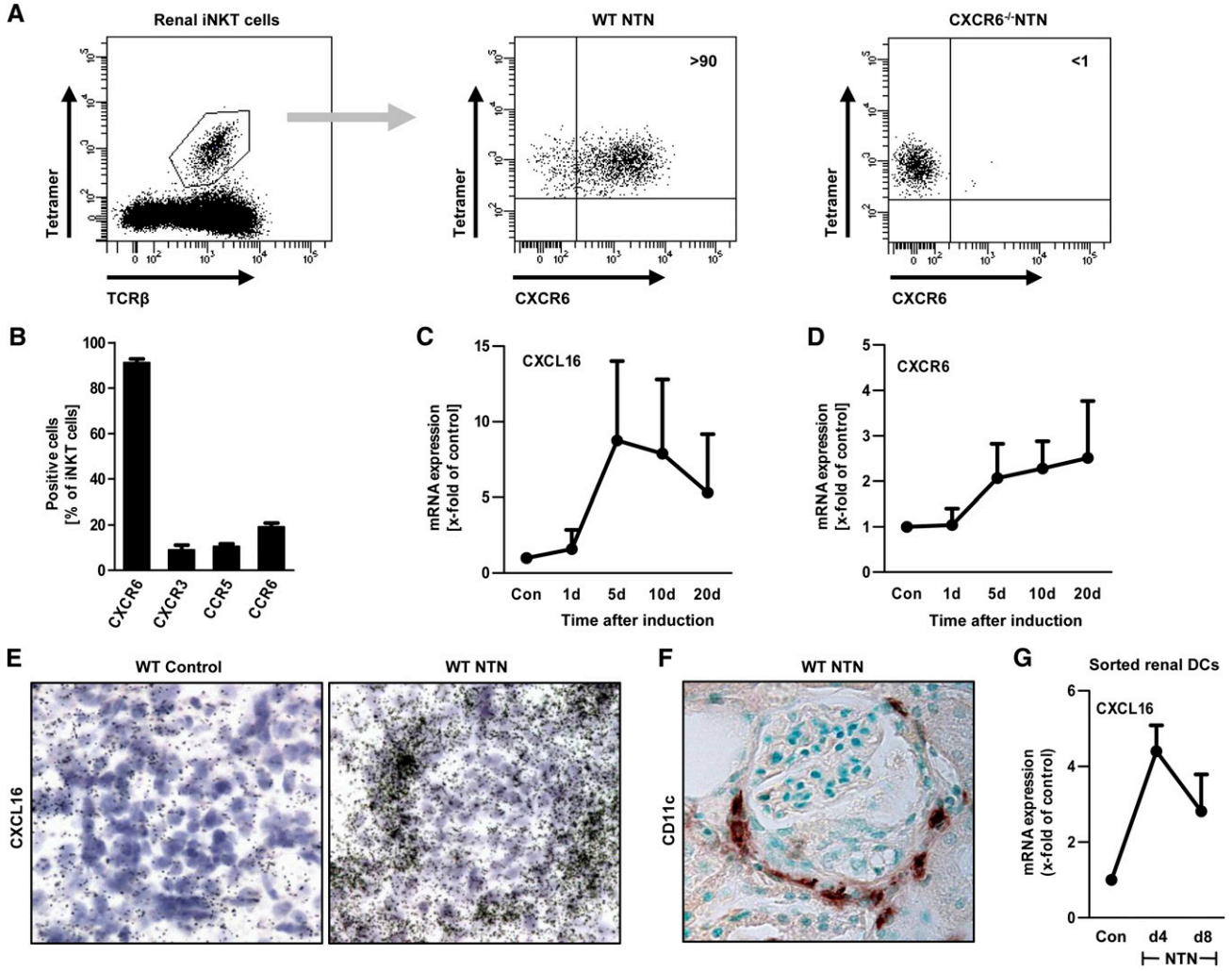

Figure 2. CXCR6 and CXCL16 expression in experimental GN. (A) Representative FACS analysis of CXCR6 expression on isolated renal iNKT cells, defined as CD1d-Tetramer$^+$TCRβ^+, gated for CD45$^+$ cells at day 8 of NTN. CXCR6-deficient mice were used as a specificity control. (B) Quantification of FACS analyses for different chemokine receptors on renal iNKT cells (n=6–9/group). (C and D) Real-time RT-PCR analysis of renal (C) CXCL16 and (D) CXCR6 expression in wild-type mice at different time points after induction of NTN (n=4–5/group). (E) Representative photographs of *in situ* hybridizations with a specific cRNA probe for CXCL16 of control and nephritic wild-type mice at day 6 of NTN. Original magnification, ×400. (F) Representative photograph of a kidney section from a nephritic wild-type mouse immunohistochemically stained for the DC marker CD11c 8 days after induction of NTN. Original magnification, ×400. (G) Real-time RT-PCR analysis of renal CXCL16 expression of DCs isolated from kidneys at days 4 and 8 after induction of nephritis (n=3/group). mRNA levels are expressed as x-fold of wild-type controls. Symbols represent means ± SD.

CD19$^+$ B cells showed CXCR6 expression (data not shown). The specificity of CXCR6 staining was confirmed by simultaneous analysis of renal leukocytes from nephritic CXCR6-deficient mice. These findings suggest a role for CXCR6 in iNKT cell recruitment to the kidney in early NTN.

Renal DCs Produce CXCL16 in the Early Stage of NTN

If CXCR6 is linked to the protective effect of iNKT cells in early NTN, its unique ligand CXCL16 should be expressed in the kidney. To test this hypothesis, we performed real-time PCR to quantify renal CXCL16 expression in nephritic mice at four different time points. mRNA expression of CXCL16 in the kidneys of nephritic wild-type mice peaked 5 days after induction of nephritis and remained elevated until the last time point analyzed (that is, at day 20 after induction of the disease) (Figure 2C). mRNA expression of CXCR6 in the kidneys of nephritic wild-type mice was also increased from day 5 on after induction of nephritis (Figure 2D).

To localize renal CXCL16 expression, we performed *in situ* hybridization experiments (Figure 2E) and found that mRNA expression of CXCL16 in nephritic mice was predominantly present in periglomerular and tubulointerstitial infiltrates, whereas intraglomerular CXCL16 signals were apparently restricted to cellular crescents and single infiltrating cells in the

glomerular tuft. The periglomerular expression pattern of CXCL16 (Figure 2E) was strikingly reminiscent of the location of renal DCs in NTN, which was revealed by immunohistochemical staining for the DC marker CD11c in nephritic mice (Figure 2F).

To test whether renal DCs might contribute to renal CXCL16 production, we analyzed the mRNA levels of this chemokine by quantitative RT-PCR in magnetic activated cell sorting (MACS)-sorted DCs isolated from kidneys at different time points after induction of nephritis. CXCL16 expression by renal DCs peaked at day 4 after induction of nephritis (4.8-fold higher than control) and subsequently declined at day 8 (2.8-fold higher than control) (Figure 2G). This finding suggests that the suppressive modes of action of immature renal DCs and iNKT cells are associated with the CXCL16–CXCR6 axis. These findings do not exclude a contribution of other cells to renal CXCL16 production.

Critical Role of the CXCL16–CXCR6 Axis in Renal iNKT Cell Localization and Function

To determine whether the CXCL16–CXCR6 axis is functionally relevant to NTN, we first analyzed whether CXCR6 deficiency affects the recruitment of iNKT cells to the inflamed kidney. We were able to detect iNKT cells in the kidneys of nephritic wild-type and CXCR6-deficient mice and found that the frequency (Figure 3A) and numbers (not shown) of iNKT cells were reduced in the kidneys of nephritic CXCR6-deficient mice. Quantification of their frequency showed one-half as many Tetramer^{+}TCRβ^{+} iNKT cells in CXCR6-deficient mice compared with wild-type mice (percent of CD3 cells; WT: 2.5±0.7%; CXCR6$^{-/-}$: 1.2±0.6%; $P<0.01$) (Figure 3B).

In addition to renal iNKT cell localization, the CXCL16–CXCR6 axis might also promote the production of anti- and proinflammatory cytokines, such as IL-4, IL-10, and IFNγ, by iNKT cells. This assumption was investigated by intracellular IL-4 and IFNγ staining of renal iNKT cells of nephritic wild-type and CXCR6-deficient mice. IL-4 expression was significantly reduced in iNKT cells from CXCR6-deficient mice (WT: 24.9±4.9%; CXCR6$^{-/-}$: 10.7±2.2%; $P<0.001$) (Figure 3, C and D), whereas IFNγ expression remained unchanged (WT: 57.6±5.5%; CXCR6$^{-/-}$: 52.3±6.0%) (Figure 3, E and F).

Next, we analyzed whether DC–NKT cell interaction/activation was CXCR6-dependent. We, therefore, purified hepatic NKT cells from wild-type and CXCR6-deficient mice by FACS sorting (purity>95%) followed by stimulation with α-GalCer using mouse myeloid DCs as antigen-presenting cells. The production of IL-4, IL-10, and IFNγ was strongly induced by α-GalCer in DC cocultures with wild-type NKT cells, whereas CXCR6-deficient NKT cells had a significantly reduced capacity to produce IL-4, IL-10, and IFNγ as measured in the supernatants by Cytometric Bead Assay (IL-4: wild-type NKT cells [88.1±18.6 pg/ml] versus CXCR6$^{-/-}$ NKT cells [23.8±2.2 pg/ml], $P<0.05$; IL-10: wild-type NKT cells [61.9±6.5 pg/ml] versus CXCR6$^{-/-}$ NKT cells [31.2±13.1 pg/ml], $P<0.05$; IFNγ: wild-type NKT cells [10.5±1.0 ng/ml] versus CXCR6$^{-/-}$ NKT cells [6.9±0.4 ng/ml], $P<0.05$) (Figure 3G). Furthermore, as determined by ELISA, DC/NKT cell stimulation with α-GalCer led to a high level of CXCL16 protein production (DC + wild-type NKT cells: 1925±126 pg/ml) and tended to be stronger combined with CXCR6-deficient NKT cells (2732±72 pg/ml). NKT cells alone did not produce detectable levels of CXCL16, confirming that DCs were required for CXCL16 formation (data not shown).

CXCR6 Deficiency Aggravates Renal Injury in NTN

To test whether the defect of renal iNKT recruitment and function in nephritic CXCR6$^{-/-}$ mice is of functional importance, we compared the clinical course of experimental GN in wild-type mice with the clinical course in CXCR6-deficient mice. Periodic acid–Schiff (PAS) -stained kidney sections of nephritic mice in the autologous phase at day 8 showed severe focal glomerular and tubular alterations with partial destruction of regular tissue structures (Figure 4A). Glomerular changes included hypercellularity and formation of cellular crescents, capillary aneurysms, and intraglomerular deposition of PAS-positive material. Serial fibrin staining showed the presence of fresh glomerular capillary necrosis (Figure 4A). The tubulointerstitial compartment showed tubular dilatation, necrosis, and atrophy along with protein casts and tubular protein reuptake caused by proteinuria. To quantify renal tissue damage, PAS-stained kidney sections were evaluated for the presence of crescents, glomerular sclerosis (deposition of PAS-positive material), and tubulointerstitial injury (Figure 4, B–D) as previously described.[21] The frequency of glomerular crescents at day 8 was significantly increased in nephritic CXCR6-deficient mice compared with the wild-type group (WT: 33.6±9.2%; CXCR6$^{-/-}$: 45.6±8.5%, $P<0.001$) (Figure 4B). Nephritic kidneys showed a higher frequency of glomerulosclerosis (WT: 50.3±21.9; CXCR6$^{-/-}$: 66.3±14.9, $P<0.01$) (Figure 4C) and considerable tubulointerstitial injury, which was reflected in a significant increase in the interstitial area (WT: 17.5±4.1%; CXCR6$^{-/-}$: 21.8±4.8%, $P<0.001$) (Figure 4D).

BUN and serum creatinine levels of nephritic CXCR6-deficient mice were significantly elevated at day 8 (0.41±0.12 and 173.7±58.4 mg/dl, respectively) compared with the nephritic wild-type group (0.32±0.08 and 132.0±64.2 mg/dl, $P<0.01$ and $P<0.05$, respectively) (Figure 4, E and F).

Nephritic wild-type and CXCR6-deficient mice showed a markedly increased albumin-to-creatinine ratio at day 8 after induction of nephritis compared with controls ($P<0.001$ versus controls). However, there was no statistically significant difference in albumin excretion between nephritic CXCR6-deficient and nephritic wild-type mice (Figure 4G).

Renal T Cell and Monocyte Immune Response in CXCR6-Deficient Mice

Because CXCR6 regulates the trafficking of other immune cell types, we determined the recruitment of leukocytes to the kidneys of nephritic CXCR6-deficient and wild-type mice.

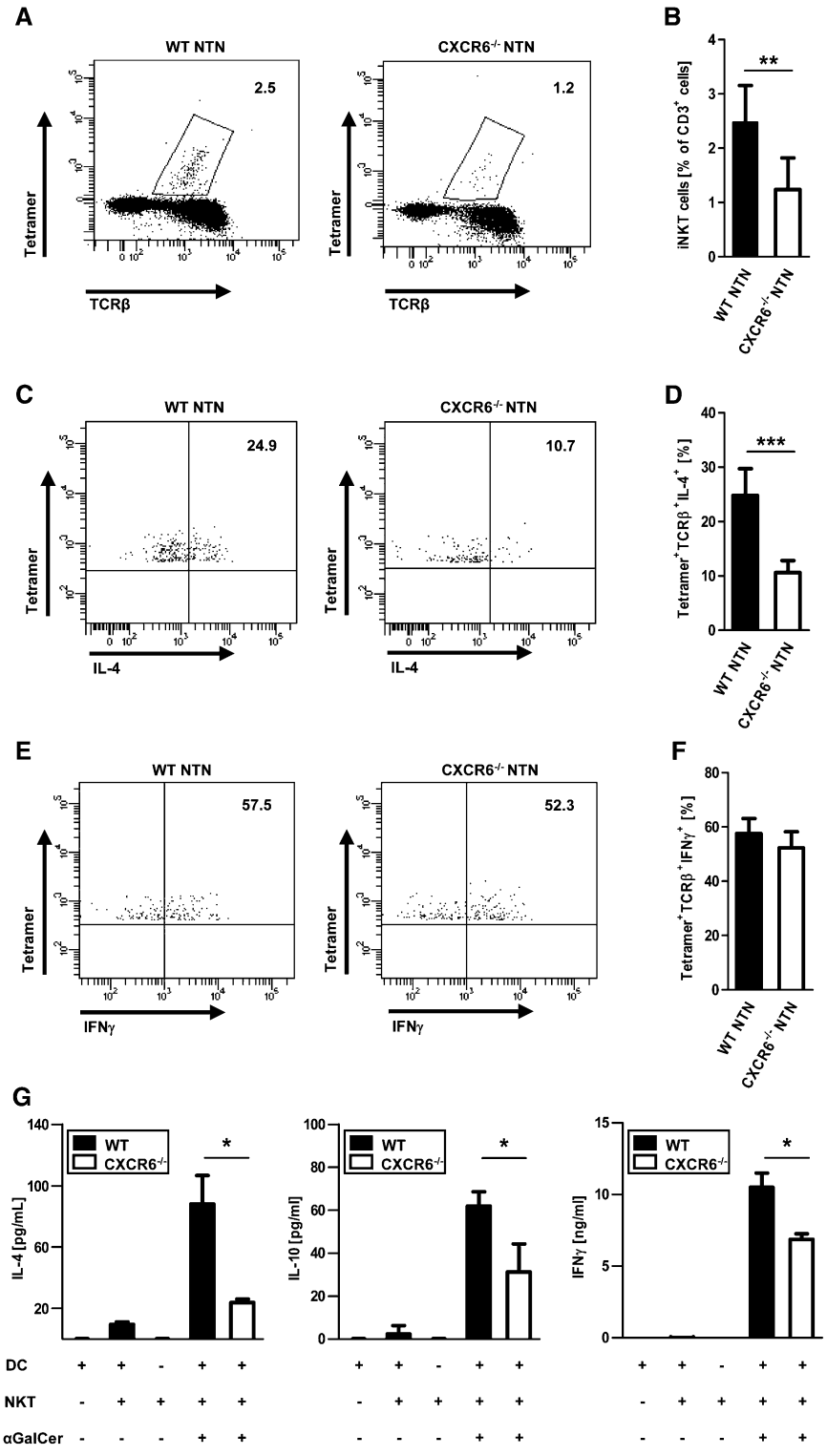

Figure 3. Critical role of the CXCL16–CXCR6 axis for renal iNKT cell localization and function. Representative FACS (A) analysis and (B) quantification of Tetramer$^+$TCRβ^+ iNKT cell frequency at day 8 of NTN in wild-type and CXCR6-deficient mice. Results are representative of four independent experiments (n=9/group). (C–F) Representative intracellular IL-4 FACS (C) analysis and (D) quantification as well as IFNγ FACS (E) analysis and (F) quantification of isolated renal leukocytes gated for CD45$^+$TCRβ^+Tetramer$^+$ iNKT cells at day 8 of NTN (n=6/group). (G) Cytometric bead assay analysis of IL-4, IL-10, and IFNγ levels of supernatants of 72-hour DC/NKT cell co-cultures with or without the addition of a-GalCer (n=3/group). Symbols represent means ± SD (*P<0.05, **P<0.01, ***P<0.001).

In contrast to the reduced frequency of renal iNKT cells, tubulointerstitial CD3 T cell infiltration was significantly increased in nephritic CXCR6-deficient mice (WT: 26.8±9.9/high-power field [hpf]; CXCR6$^{-/-}$: 33.4±10.6/hpf, P<0.05) (Figure 5, A and B). Infiltration of glomerular T cells, tubulointerstitial F4/80$^+$ macrophages/DCs, and glomerular Mac2$^+$ macrophages did not differ between wild-type and CXCR6-deficient mice (Figure 5C and Supplemental Figure 2, A and B). In addition, infiltrating renal macrophages and DCs in nephritic wild-type and CXCR6-deficient mice were analyzed in more detail by FACS, but no major phenotypical differences in terms of MHCII expression, a marker for antigen presentation, and Ly6C, an activation marker, between these two groups could be observed (Supplemental Figure 2, C and D). The mRNA level of the macrophage and T cell-attracting chemokine CCL2 was significantly upregulated in nephritic CXCR6-deficient mice, whereas no significant differences in renal CCL5, iNOS, IL-12p40, and IL-1β expression could be found (Supplemental Figure 2E).

To characterize the T cell subtypes in the kidneys of nephritic animals, we performed intracellular flow cytometric analysis for IFNγ^+/Th1 and IL-17A$^+$/Th17 cells (Figure 5D). In both nephritic groups, we detected IFNγ^+ and IL-17A$^+$ production by renal CD4$^+$ T helper cells but no significant difference between CXCR6-deficient and wild-type mice (Figures 5, D–F).

The renal mRNA levels of the proinflammatory mediators TNF-α and (to a lesser degree) CXCL10 were upregulated in nephritic CXCR6-deficient mice, whereas the anti-inflammatory mediators IL-4, IL-10, and TGFβ were slightly decreased compared with the nephritic wild-type group (Figure 5G).

Next, we were interested to study whether CXCR6 deficiency might result in a defect of renal Treg cell trafficking and

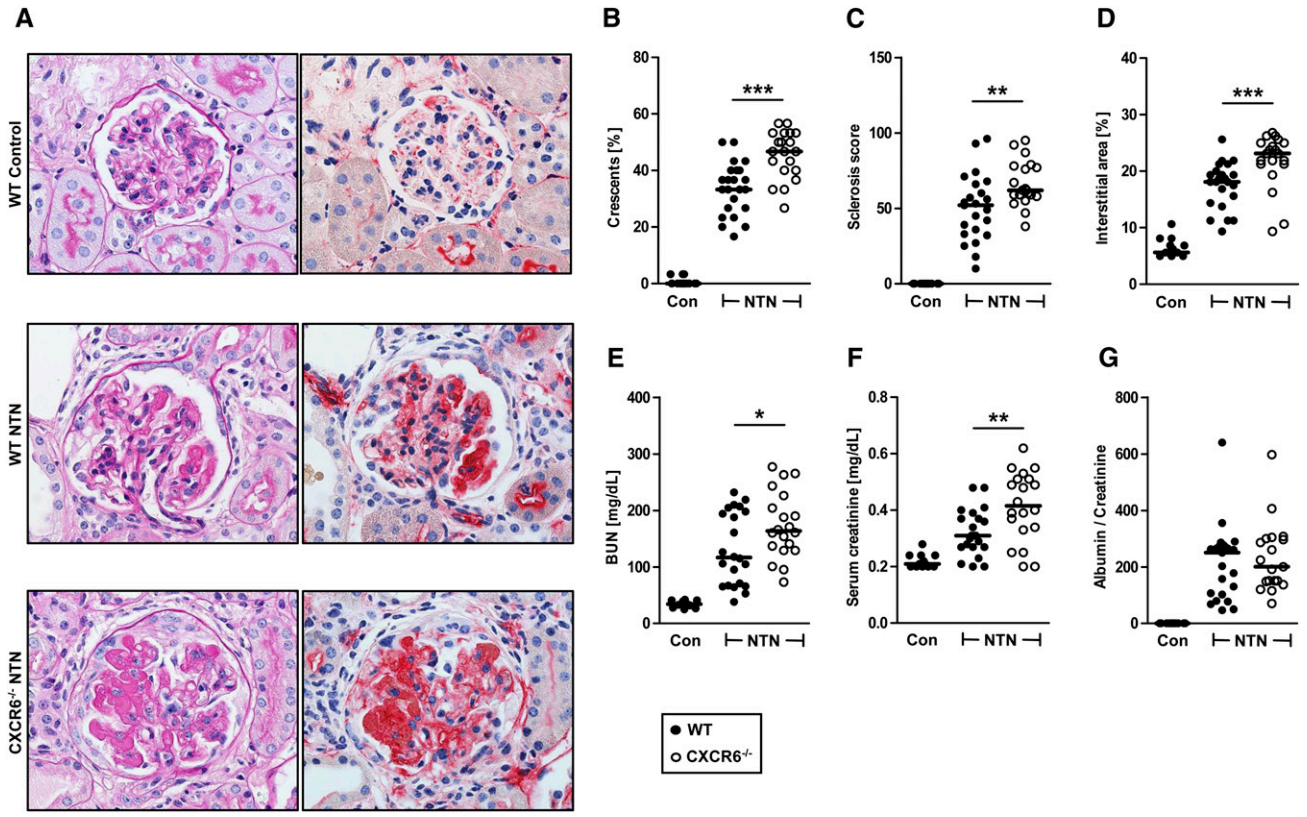

Figure 4. CXCR6 deficiency aggravated renal injury in experimental GN. (A) Representative serial photographs of PAS- and fibrin-stained kidney sections of control, nephritic wild-type, and nephritic CXCR6-deficient mice at day 8 of NTN show glomerular crescent formation, glomerular deposition of PAS-positive material, abundant tubulointerstitial leukocyte infiltrates, and fresh necrosis. Original magnification, ×400. (B–D) Quantification of (B) glomerular crescent formation, (C) glomerular sclerosis, and (D) interstitial area in controls (n=13), nephritic wild-type mice (n=23), and nephritic CXCR6-deficient mice (n=20) 8 days after induction of nephritis. (E) BUN levels, (F) serum creatinine, and (G) albumin-to-creatinine ratio of controls (n=13), nephritic wild-type mice (n=23), and nephritic CXCR6-deficient mice (n=20) 8 days after induction of nephritis. Symbols represent individual data points with median values as horizontal lines (*P<0.05, **P<0.01, ***P<0.001).

thus, an aggravated course of nephritis. FACS analysis revealed that up to 20% of renal Tregs were CXCR6-positive (Figure 6A). However, quantification of tubulointerstitial infiltration of $FoxP3^+$ cells revealed no differences between the wild-type and knockout groups, arguing against a unique role for CXCR6 in renal Treg cell trafficking (Figure 6, B and C). In line with the protein data, we found similar expression of renal FoxP3 mRNA in both groups (Figure 6D).

Humoral and Cellular Nephritogenic Immune Responses

Semiquantitative scoring of glomerular sheep and mouse IgG deposition revealed no differences between nephritic wild-type and knockout mice (Figure 7, A and B). In line with this finding, wild-type and $CXCR6^{-/-}$ mice had similar serum titers of anti-sheep IgG, IgG1, and IgG2a antibodies directed against the nephritogenic antigen, which was assessed by ELISA (Figure 7C).

To address the question of whether CXCR6-deficient mice have an altered systemic cellular immune response, we analyzed splenocytes from nephritic wild-type and CXCR6-deficient mice for the production of IFNγ and IL-17 after restimulation with the nephritogenic antigen. ELISA analyses of the cytokine production from cultured splenocytes showed no significant differences in the Th1-associated IFNγ and Th17-associated IL-17 levels between the two groups (Figure 7D). Furthermore, the frequencies and numbers of $CD4^+$ T cells, $CD8^+$ T cells, $CD11b^+$ macrophages, and most importantly, iNKT cells in the spleens (Figure 7E) and renal lymph nodes (Figure 7F) of nephritic wild-type and CXCR6-deficient mice were almost identical. Taken together, these data strongly argue against an accelerated humoral or cellular systemic nephritogenic immune response or a lymphocyte survival function of the CXCR6–CXCL16 axis as a cause of aggravated nephritis in CXCR6-deficient mice.

Reconstitution of NKT Cells Attenuates Nephritis in CXCR6-Deficient Mice

The aggravation of GN in CXCR6-deficient mice might be caused by the impaired trafficking and function of potentially protective iNKT cells. To prove this hypothesis, we transferred

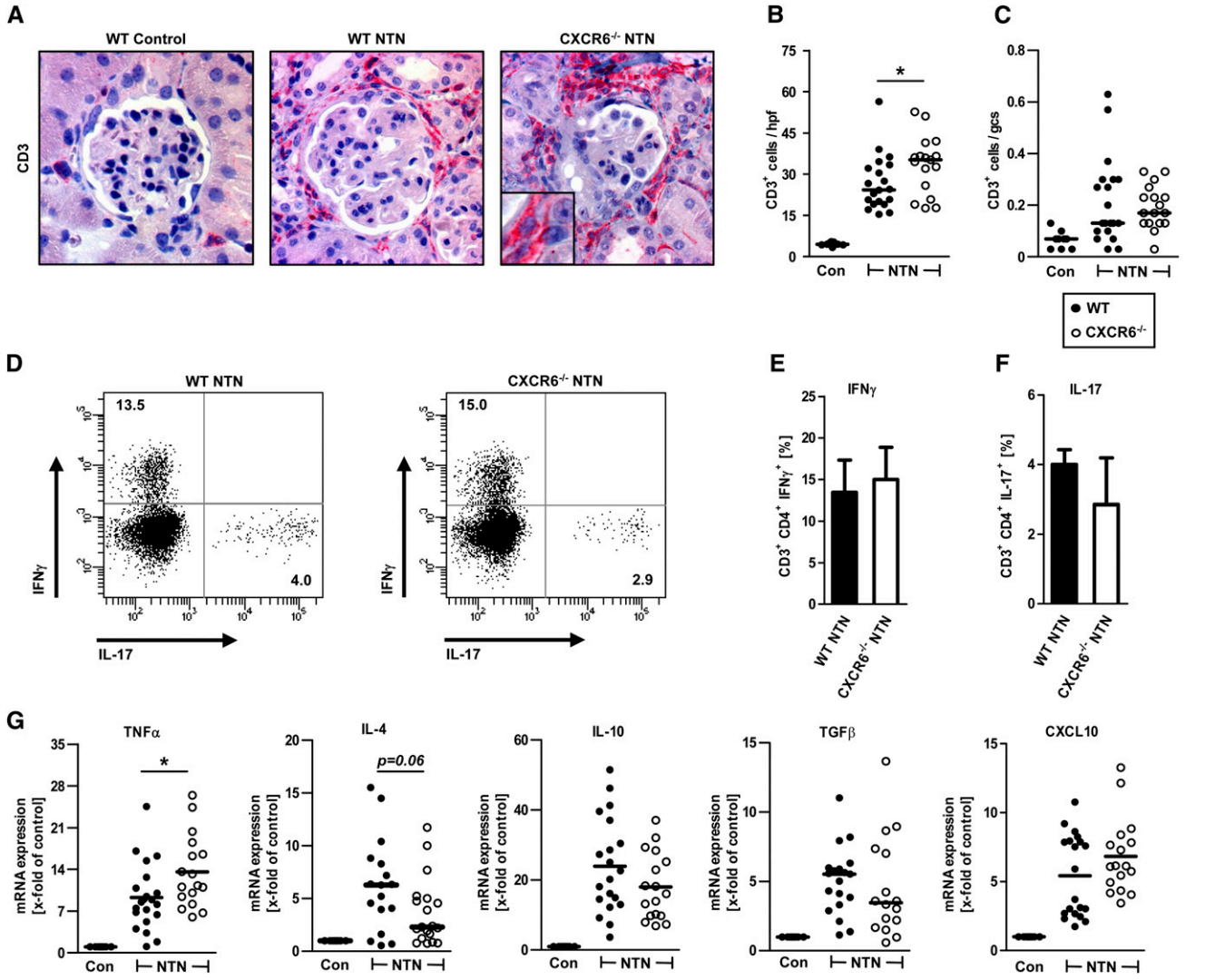

Figure 5. Renal T cell immune response. (A) Representative photographs of kidney sections from control, nephritic wild-type, and CXCR6-deficient mice immunohistochemically stained for the T cell marker CD3 8 days after induction of NTN. Original magnification, ×400. (B and C) Quantification of (B) tubulointerstitial and (C) glomerular $CD3^+$ T cells in nephritic wild-type (n=20), $CXCR6^{-/-}$ mice (n=17), and wild-type controls (n=9). (D) Representative intracellular IFNγ and IL-17 FACS analysis of isolated renal leukocytes, gated for $CD4^+$ T cells, at day 8 of NTN and quantification of (E) Th1 and (F) Th17 cell frequencies (n=4/group). (G) Real-time RT-PCR analysis of renal cytokine expression in nephritic wild-type, $CXCR6^{-/-}$ mice, and wild-type controls (n=9–20 per group) for TNFα, IL-4, IL-10, TGFβ, and CXCL10. mRNA levels are expressed as x-fold of controls. Symbols represent individual data points with median values as horizontal lines (*P<0.05).

wild-type NKT cells, isolated by FACS sorting from the livers of naive wild-type mice, to wild-type and CXCR6-deficient mice 12 hours before induction of NTN. We isolated hepatic NKT cells for these transfer experiments, because the liver contains the highest rate of NKT cells in the body. To avoid extensive activation of the isolated cells through the tetramer-bound α-GalCer analog PBS57, we used TCRβ and NK1.1 as NKT-defining markers for FACS sorting experiments. FACS analysis of isolated NKT cells before transfer revealed that 95% of the isolated cells were $TCR\beta^+NK1.1^+$, and over 75% coexpressed an invariant glycolipid antigen-recognizing T cell receptor (Supplemental Figure 3, A and B). In addition, over 80% of the isolated cells were CXCR6-expressing NKT cells (Supplemental Figure 3C). This result was further confirmed by analysis of enhanced green fluorescent protein (eGFP) expression on hepatic NKT cells isolated from $CXCR6^+$/$eGFP^+$ heterozygous mice (Supplemental Figure 3D).

Transfer of 1×10^6 naive wild-type NKT cells to wild-type and CXCR6-deficient mice 12 hours before induction of nephritis resulted in almost identical histologic tissue injury in both groups at day 8 of nephritis (Figure 8, A and B). In contrast,

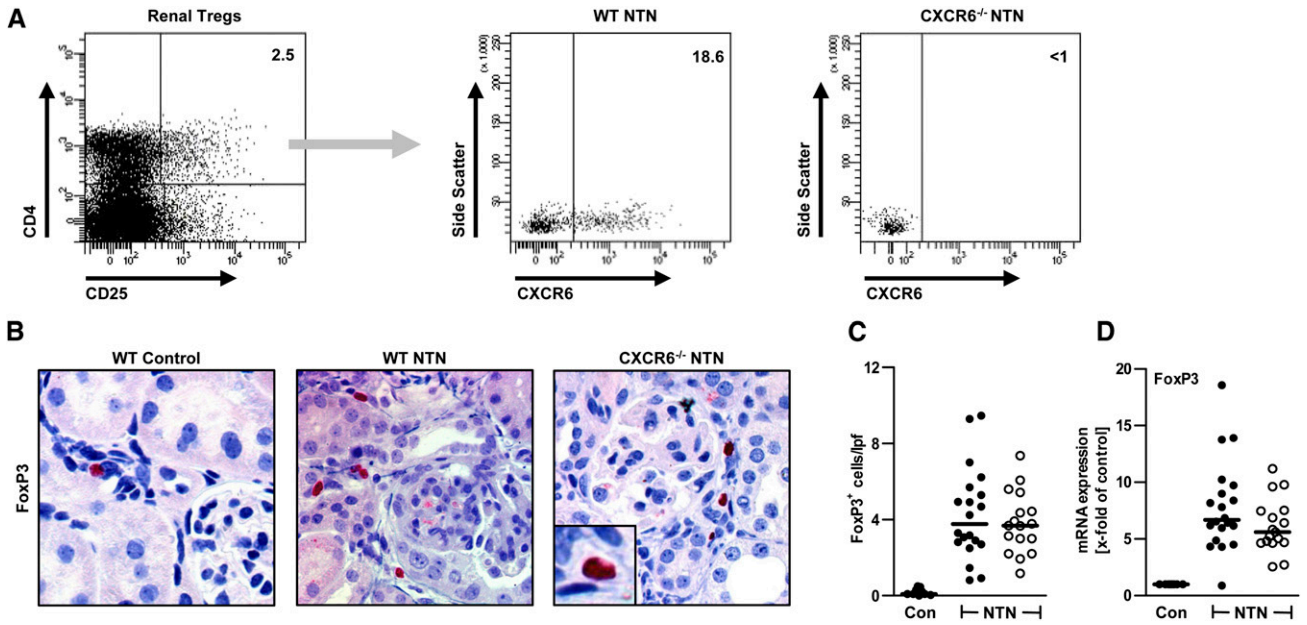

Figure 6. Renal regulatory T cell characteristics. (A) Representative FACS analysis of CXCR6 expression on renal Tregs, defined as $CD4^+CD25^+$ cells, gated for $CD45^+$ cells at day 8 of NTN. CXCR6-deficient mice were used as a specificity control (n=5/group). (B) FoxP3 immunohistochemistry of kidney sections from control, nephritic wild-type, and CXCR6-deficient mice at day 8 of NTN. Original magnification, ×400. (C) Quantification of tubulointerstitial $FoxP3^+$ cells in nephritic wild-type (n=20), $CXCR6^{-/-}$ mice (n=17), and wild-type controls (n=9). (D) Real-time RT-PCR of FoxP3 expression in nephritic wild-type (n=20) and $CXCR6^{-/-}$ mice (n=17) as well as wild-type controls (n=9). mRNA levels are expressed as x-fold of controls. Symbols represent individual data points with median values as horizontal lines.

without NKT cell transfer, $CXCR6^{-/-}$ nephritic mice developed more severe nephritis than wild-type animals (Figure 8, A–C). Most important, renal tissue injury in terms of glomerular crescent formation, glomerular sclerosis, and tubulointerstitial damage and functional parameter (serum creatinine level) was significantly reduced in nephritic $CXCR6^{-/-}$ after the cell transfer compared with CXCR6-deficient animals that received vehicle treatment (Figure 8, A–C), thus showing the protective effect of CXCR6-bearing NKT cells in crescentic GN.

Tubulointerstitial CD3 T cell infiltration, infiltration of glomerular T cells, and infiltration of tubulointerstitial $F4/80^+$ macrophages/DCs did not significantly differ between nephritic wild-type and nephritic CXCR6-deficient mice receiving vehicle treatment or NKT cells (Figure 8D).

DISCUSSION

In experimental crescentic GN, immature kidney DCs are protective[13] until they mature, when inflammation becomes chronic.[12] The underlying mechanisms remain to be elucidated. We speculated that, in this early phase, DCs might suppress harmful immune responses by recruiting anti-inflammatory leukocytes, and we addressed this hypothesis in NTN, a model of crescentic GN. When we depleted DCs in CD11c.LuciDTRmice with NTN, we noted that iNKT cells but not proinflammatory leukocytes were markedly reduced within the kidney. This finding sparked our interest, because two recent studies had shown a protective role for NKT cells in renal inflammation. Anti-GBM GN was aggravated in NKT cell-deficient CD1d knockout mice, and adoptively transferred NKT cells localized to the inflamed kidney and prevented this phenotype, which was astonishing, because NKT cells cannot recognize antigen in CD1d-deficient mice.[18] Nevertheless, Mesnard *et al.*[19] performed this experiment 1 year later in Jα18 knockout mice, which are also deficient in NKT cells but possess CD1d that allows transferred NKT cells to recognize antigen.[22] Also, these animals developed more severe nephritis than wild-type mice, verifying the protective role of iNKT cells.[19] These findings led us to hypothesize that the protective capacity of immature renal DCs might be mediated through the recruitment of iNKT cells into the kidney.

The *in vivo* mechanisms of iNKT cell trafficking to the kidney are unknown. iNKT cells are known to express high amounts of CXCR6,[23–26] and indeed, over 90% of iNKT cells in the inflamed kidney expressed the chemokine receptor CXCR6. We detected message for its unique ligand CXCL16 in immature DCs isolated from the kidney early after induction of nephritis. This finding identified a new cellular source for this chemokine in addition to human tubular cells and podocytes, which had been previously shown to produce the unique CXCR6 ligand, CXCL16.[27,28] Moreover, we noted periglomerular CXCL16 expression resembling the periglomerular pattern of renal DCs in both human and experimental

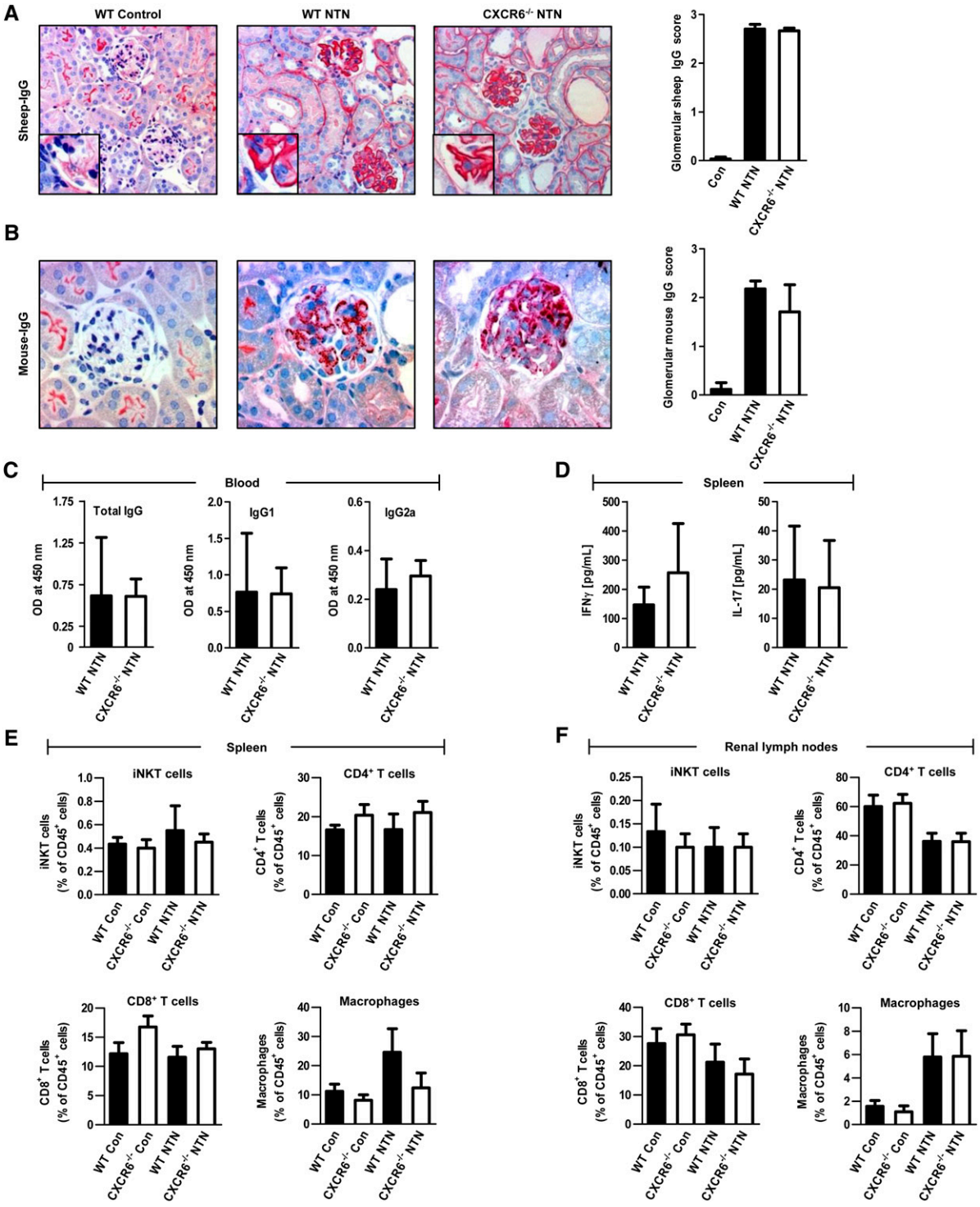

Figure 7. Humoral and cellular nephritogenic immune responses. (A and B) Representative photographs and quantification of glomerular sheep IgG deposition at day 2 after induction of NTN and glomerular mouse IgG deposition at day 6 after induction of the nephritis (n=4/group). Original magnification A, ×200; B, ×400. ELISA analysis of (C) circulating serum total IgG, IgG1, and IgG2a levels of wild-type and CXCR6-deficient mice (n=9/group) and (D) IFNγ and IL-17 levels of supernatants of sIgG-stimulated splenocytes at day 8 of NTN (n=6/group). (E and F) FACS quantification of iNKT cells, $CD4^+$ T cells, $CD8^+$ T cells, and macrophages isolated from (E) spleens and (F) lymph nodes of wild-type and CXCR6-deficient mice under control and nephritic conditions (n=3/group). Symbols represent means ± SD.

GN.[11,29] Indeed, iNKT cells failed to accumulate in the kidney of nephritic CXCR6-deficient mice. Interestingly, most of the remaining iNKT cells in the kidneys of nephritic CXCR6-deficient mice are NK1.1$^+$ (Supplement Figure 4), whereas the work by Germanov *et al.*[26] reported that CXCL16 neutralization reduced the accumulation of NK1.1$^+$ but not NK.1.1$^-$ iNKT cells in the liver. Most importantly, nephritic CXCR6-deficient mice developed more severe disease. Causality between the reduced iNKT cell recruitment and the aggravated disease in nephritic CXCR6$^{-/-}$ mice was supported by the ability of adoptively transferred CXCR6-competent NKT cells to attenuate disease. Developmental defects in the kidney architecture of CXCR6-deficient mice (Supplemental Figure 5) and an overwhelming humoral or cellular nephritogenic immune response as alternative explanations were ruled out. Thus, our findings reveal a new mechanism by which immature DCs attenuate immune-mediated disease by harnessing iNKT cells with a regulatory functionality.

In experimental GN, two groups independently reported that (i)NKT cell deficiency exacerbates disease.[18,19] This finding indicates that (i)NKT cells play a role in suppressing renal autoimmunity, which is in line with our findings. The mechanisms by which regulatory CXCR6$^+$ iNKT cells protect against autoimmune diseases, such as GN, remain to be fully elucidated but likely involve the production of immunomodulatory cytokines, such as IL-4, IL-10, and TGFβ. All of these cytokines have been shown to suppress the model of NTN.[13,30,31] The work by Mesnard *et al.*[19] reported that mRNA expression of TGFβ was higher in nephritic wild-type compared with iNKT cell-deficient mouse kidneys and that this reduction contributes to disease exacerbation. In line with these findings, we found increased mRNA expression of IL-4, IL-10, and TGFβ (but not IFNγ) in sorted iNKT cells from nephritic mice. In nephritic CXCR6$^{-/-}$ mice, the number of renal iNKT cells was reduced, and the capacity of CXCR6$^{-/-}$ iNKT cells to produce IL-4 and IL-10 was disturbed. In accordance, the renal expression of IL-4, IL-10, and TGFβ was reduced in nephritic knockout mice, whereas important proinflammatory mediators, such as TNF-α and the chemokines CCL2 and CXCL10, are upregulated and thus, might directly contribute to exacerbation of renal tissue injury in CXCR6$^{-/-}$ mice. However, the exact suppressive mechanism of CXCR6$^+$ iNKT cells may be pinpointed in future studies by, for example, adoptively transferring iNKT cells from cytokine-deficient donor mice or generating iNKT cell-specific CXCR6 knockout mice, which are not yet available. So far, we cannot rule out that CXCR6 deficiency on immune cells other than iNKT cells might contribute to the observed effects in nephritic CXCR6$^{-/-}$ mice. Moreover, it would be interesting to study whether the adoptively transferred NKT cells migrate to the nephritic kidney, which was previously shown in the work by Yang *et al.*,[18] and locally suppress immune-mediated renal inflammation or if the trafficking of NKT cells to secondary lymphatic organs might also be important for their anti-inflammatory properties.

The CXCR6–CXCL16 axis has previously been shown to recruit proinflammatory CD4$^+$ and CD8$^+$ T cells to the inflamed allografts, vessels, and human joints, resulting in tissue damage.[32–34] In anti-GBM nephritis, blocking CXCL16 with the use of a polyclonal antiserum attenuated the disease, mainly by hindering macrophage influx.[35] Furthermore, fibroblasts can express CXCR6, and CXCL16 knockout mice showed reduced fibroblast accumulation in unilateral ureteral obstruction, leading to their protection from renal fibrosis.[36] However, recent studies showed that this axis may also exert anti-inflammatory functions. For example, in a murine heart transplant model, the CXCL16–CXCR6 axis plays a pivotal role for the maintenance of transplant tolerance mediated by infiltrating NKT cells.[25] Our present study supports this notion by showing aggravation of crescentic GN in CXCR6 knockout mice. It is possible that the CXCL16–CXCR6 axis attracts iNKT cells to maintain immune homeostasis in acute inflammatory conditions but drives proinflammatory effector T cell infiltration in chronic inflammatory reactions. This proinflammatory NKT cell function might synergize with the function of matured DCs in chronic inflammation at aggravating GN.[12]

Regulatory T cells are widely recognized as being protective in various immune-mediated diseases.[37] In NTN, these cells, under the control of CCR6 and CCR7,[38,39] were also found to attenuate disease.[40–42] Our present study highlights iNKT cells as a second regulatory lymphocyte subset that can protect against renal tissue injury in experimental GN and also shows that it is under control of a different chemokine system, the CXCR6–CXCL16 axis. However, the extrapolation of findings from murine crescentic GN to the immunopathogenesis of human rapidly progressive glomerulonephritis is restricted, because human iNKT cells present phenotypic differences relative to their murine counterpart. A better understanding of human iNKT cell biology in autoimmune disease, such as the different forms of crescentic GN, is needed before the manipulation of iNKT cells might represent a new therapeutic target in humans.

CONCISE METHODS

Animals

CXCR6-deficient mice (C57BL/6 background)[43] were purchased from The Jackson Laboratory (Bar Harbor, ME), and the CXCR6$^{-/-}$ genotype was confirmed by PCR analysis in each animal before experimental use. DC depletion in CD11c.LuciDTR mice has been described recently.[17] Knockout mice underwent embryo transfer to meet the general standards of our institution. Age-matched C57BL/6 wild-type controls also derived from the strain were bred in our animal facility, and all animals were raised in specific pathogen-free conditions. All animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by the local committees.

Induction of NTN and Functional Studies

Nephrotoxic serum nephritis was induced in 8- to 12-week-old male CXCR6-deficient and wild-type mice by intraperitoneal injection of

0.5 ml NTN serum/mouse as previously described.[40] Controls were injected intraperitoneally with an equal amount of nonspecific sheep IgG. For urine sample collection, mice were housed in metabolic cages for 6 hours. Urinary albumin excretion was determined by standard ELISA analysis (Mice Albumin Kit, Bethyl), whereas urinary creatinine, BUN, and serum creatinine were measured using standard laboratory methods.

Real-Time RT-PCR Analysis

Total RNA of the renal cortex was prepared from murine kidneys according to standard laboratory methods. RNA from FACS-sorted iNKT cells was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed for 40 cycles (primer sequences are available on request) in a Step One Plus RT-PCR System (Applied Biosystems, Foster City, CA) as previously described.[44] All samples were run in duplicate and normalized to 18S rRNA.

In Situ Hybridization

In situ hybridization procedures were performed as previously described.[44] In brief, the CXCL16 cRNA probe was labeled by *in vitro* transcription (Maxiscript Ambion) with $\alpha[^{35}S]$-UTP (1250 Ci/mmol; PerkinElmer) of subcloned cDNA corresponding to nucleotides 503–820 of cDNA sequence NM_023158. *In situ* hybridization was performed on 16-μm cryosections using 1×10^6 cpm/slide of the ^{35}S-labeled antisense or sense RNA probes. After washing, sections were dipped into Kodak NTB nuclear track emulsion and stained with Mayer's Haemalaun.

Morphologic Examinations

Crescent formation and glomerular sclerosis (deposition of PAS-positive material) were assessed in 50 glomeruli per mouse in a blinded fashion in PAS-stained paraffin sections. As a measure of tubulointerstitial injury, the interstitial area was estimated by point counting four independent areas of renal cortex per mouse in low magnification fields (200×) as previously described.[21] Paraffin-embedded sections (2 μm) were stained with an antibody directed against CD3 (A0452; Dako, Germany), FoxP3 (FJK-16s; eBiosciences, San Diego, CA), F4/80 (BM8; BMA, Germany), MAC-2 (M3/38; Cedarlane, Canada), CD11c (M17/4; BD Pharmingen, Germany), and sheep IgG or mouse IgG (Jackson Immunoresearch Laboratories). Tissue sections were developed using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA). For FoxP3 staining, tissue sections were incubated with a polyclonal rabbit anti-rat secondary antibody (Dako, Germany) and developed using the ZytoChem Plus (AP) Polymer Kit (Zytomed). MAC-2^+ and $CD3^+$ cells in 30 glomerular cross-sections and F4/80^+ and $CD3^+$ cells in 30 tubulointerstitial hpf (magnification=400×) per kidney were counted by light microscopy in a blinded fashion. For the quantification of $FoxP3^+$ cells, at least 10 low power fields (magnification=200×) were counted.[40] Glomerular mouse IgG deposition was scored from zero to three in 30 glomeruli per mouse as previously described.[38]

Assessment of the Humoral and Cellular Nephritogenic Immune Responses

Mouse anti-sheep IgG antibody titers were measured by ELISA using sera collected 8 days after induction of nephritis as previously described.[40] To analyze the cellular immune response, splenocytes (4×10^6 cells/ml) were cultured in RPMI/10% FCS with sheep IgG (10 μg/ml) at 37°C for 72 hours. IFNγ and IL-17A concentrations were measured in the supernatants by ELISA as previously described.[40]

Renal Leukocyte Isolation

Previously described methods for leukocyte isolation from murine kidneys were used.[45] In brief, kidneys were finely minced and digested for 45 minutes at 37°C with 0.4 mg/ml collagenase D (Roche, Mannheim, Germany) and 0.01 mg/ml DNAse I in RPMI 1640 (Roche) supplemented with 10% heat-inactivated FCS (Invitrogen). Cell suspensions were sequentially filtered through 70- and 40-μm nylon meshes and washed with HBSS without Ca^{2+} and Mg^{2+} (Invitrogen). Single-cell suspensions were separated using Percoll density gradient (70% and 40%) centrifugation. The leukocyte-enriched cell suspension was aspirated from the Percoll interface. Cell viability was assessed by trypan blue staining before flow cytometry.

Isolation of Hepatic Leukocytes and Splenocytes and Renal DC Isolation

To isolate hepatic leukocytes, livers were passed through 100-μm nylon meshes, and hepatocytes were removed by centrifugation (800×*g* for 20 minutes) in 37% Percoll solution (Amersham-Biosciences, Freiburg, Germany) containing 100 U/ml heparin at room temperature. For the isolation of splenocytes, spleens were minced and sequentially passed through 70- and 40-μm nylon meshes on ice. After removing the erythrocytes from both cell suspensions using lysis solution (139 mM NH_4Cl, 19 mM Tris), cells were washed with HBSS and resuspended in FCS-containing buffer. For the isolation of DCs, tissue was digested, and renal single-cell suspensions were resuspended in MACS buffer (500 ml PBS, 0.1% BSA, 2 mM EDTA) and coincubated with mouse-CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, DCs were positively separated using MACS LS Separation Columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Renal DCs were resuspended in PBS for additional analysis.

Flow Cytometry

For FACS analysis, renal leukocyte suspensions were stained at 4°C for 20 minutes using the following fluorochrome-conjugated antibodies: CD45 (30-F11), CD3 (17A2), CD4 (GK1.5), CD8 (53–6.7), CD25 (PC61.5), CD11b (M1/70), CD19 (1D3), TCRβ (H57–597), CD11c(HL3), and NK1.1 (PK136; BD Biosciences, Franklin Lakes, NJ or eBioscience, San Diego, CA). Staining of renal leukocytes using commercially available CXCR6 antibodies resulted in unacceptably high background signals in CXCR6-deficient mice (data not shown); CXCR6 receptor expression was, therefore, detected by using a well characterized CXCL16-Fc fusion protein[23] and a phycoerythrin-labeled $F(ab')_2$ goat anti-human Fcγ polyclonal Ab (Jackson Immuno-Research Europe, Suffolk, United Kingdom). For the staining of iNKT cells, antigen-presenting cell-labeled CD1d tetramers loaded with the α-Gal-Cer analog PBS57 were obtained from the National Institutes of Health Tetramer Core Facility (Emory Vaccine Center

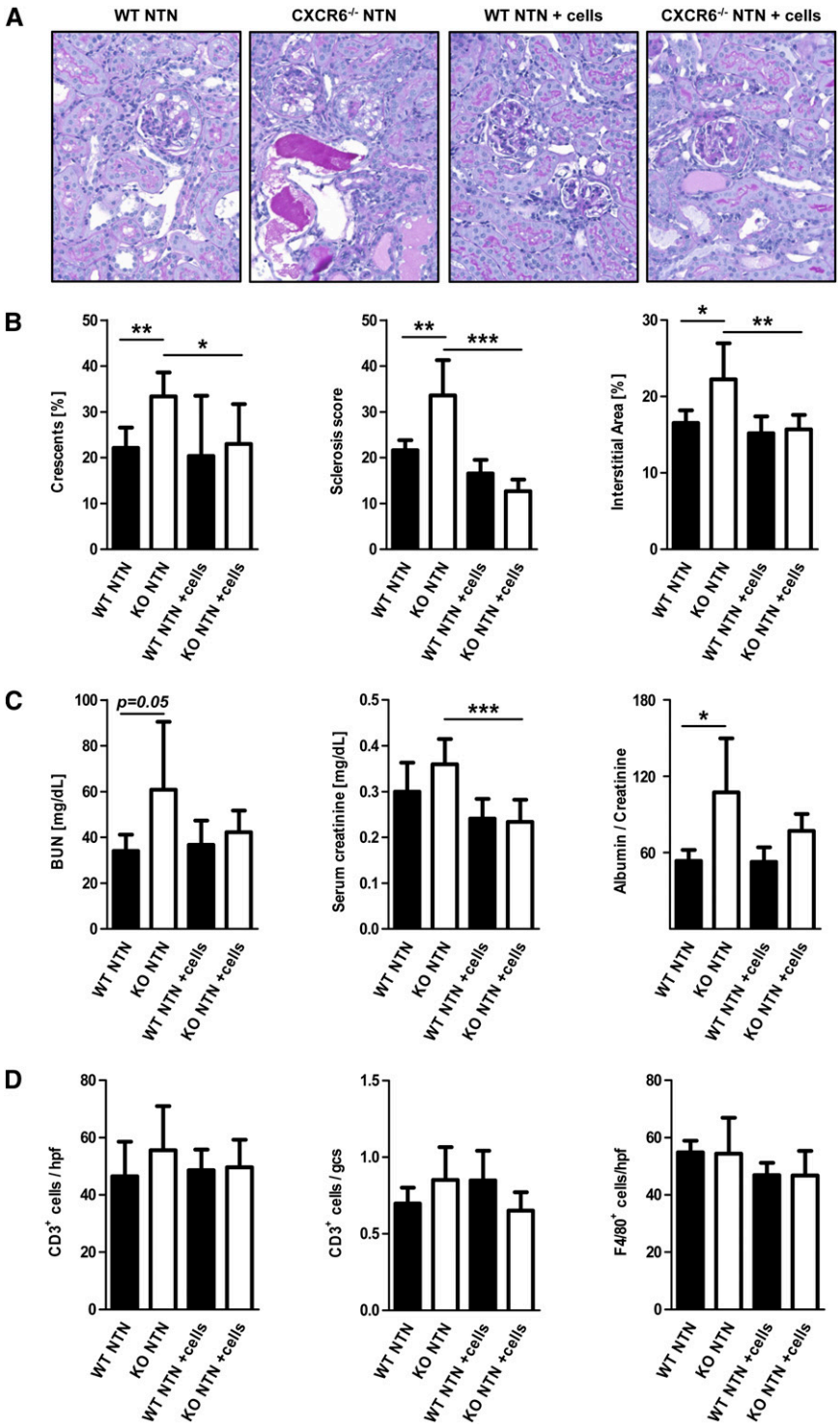

Figure 8. Transfer of NKT cells ameliorates nephritis in CXCR6-deficient mice. (A) Representative photographs of PAS-stained kidney sections, (B) quantification of glomerular crescent formation, glomerular sclerosis, and interstitial area, and (C) BUN levels, serum creatinine, and albumin-to-creatinine ratio. (D) Quantification of tubulointerstitial and glomerular $CD3^+$ T cells as well as interstitial $F4/80^+$ macrophages/DCs 8 days after induction of nephritis of wild-type mice ($n=6$) and nephritic CXCR6-deficient mice ($n=6$) receiving vehicle treatment as well as wild-type mice ($n=9$) and CXCR6-deficient mice ($n=9$) repopulated with 1×10^6 hepatic wild-type NKT cells 12 hours before the induction of NTN. Symbols represent means ± SD (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

at Yerkes, Emory University, Atlanta, GA). Unloaded tetramers were used as a control. Before antibody incubation, unspecific staining was blocked with normal mouse serum (Sigma). Staining of intracellular IFNγ (XMG1.2), IL-4 (11B11), and IL-17 (TC11–18H10) was performed as recently described.[38] Analyses were performed on a Becton & Dickinson LSRII System.

NKT Cell Transfer Experiments

Liver $CD45^+TCR\beta^+NK1.1^+CD19^-$ NKT cells were isolated using a FACS Aria sorter as previously described.[46] Wild-type and CXCR6-deficient mice were intravenously injected with 1×10^6 isolated cells in 250 μl PBS per animal followed by induction of nephritis after 12 hours.

In Vitro NKT Cell Stimulation Assays

NKT cells were isolated as described above, seeded in 96-well cell culture plates (0.8×10^5 cells/well) together with bone marrow-derived DCs (0.4×10^5 cells/well), which were used as α-GalCer–presenting cells, and stimulated with 15 ng/ml α-GalCer. After 72 hours, the cytokine concentrations in the supernatant were determined by Cytometric Bead Array according to the manufacturer's instructions (Becton & Dickinson).

Statistical Analyses

The results are shown as the mean ± SD when presented as a bar graph and the mean with the median when presented as single data points in a scatter dot plot. The results reported in the text are the mean ± SD. Differences between two individual experimental groups were compared using a two-tailed t test. For multiple comparisons, the one-way ANOVA with Bonferroni multiple comparisons test was used. Experiments that did not yield enough independent data for statistical analysis because of the experimental setup were repeated at least three times.

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DISCLOSURES

None.

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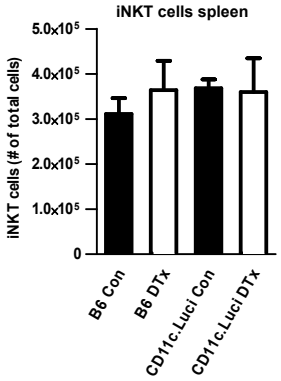
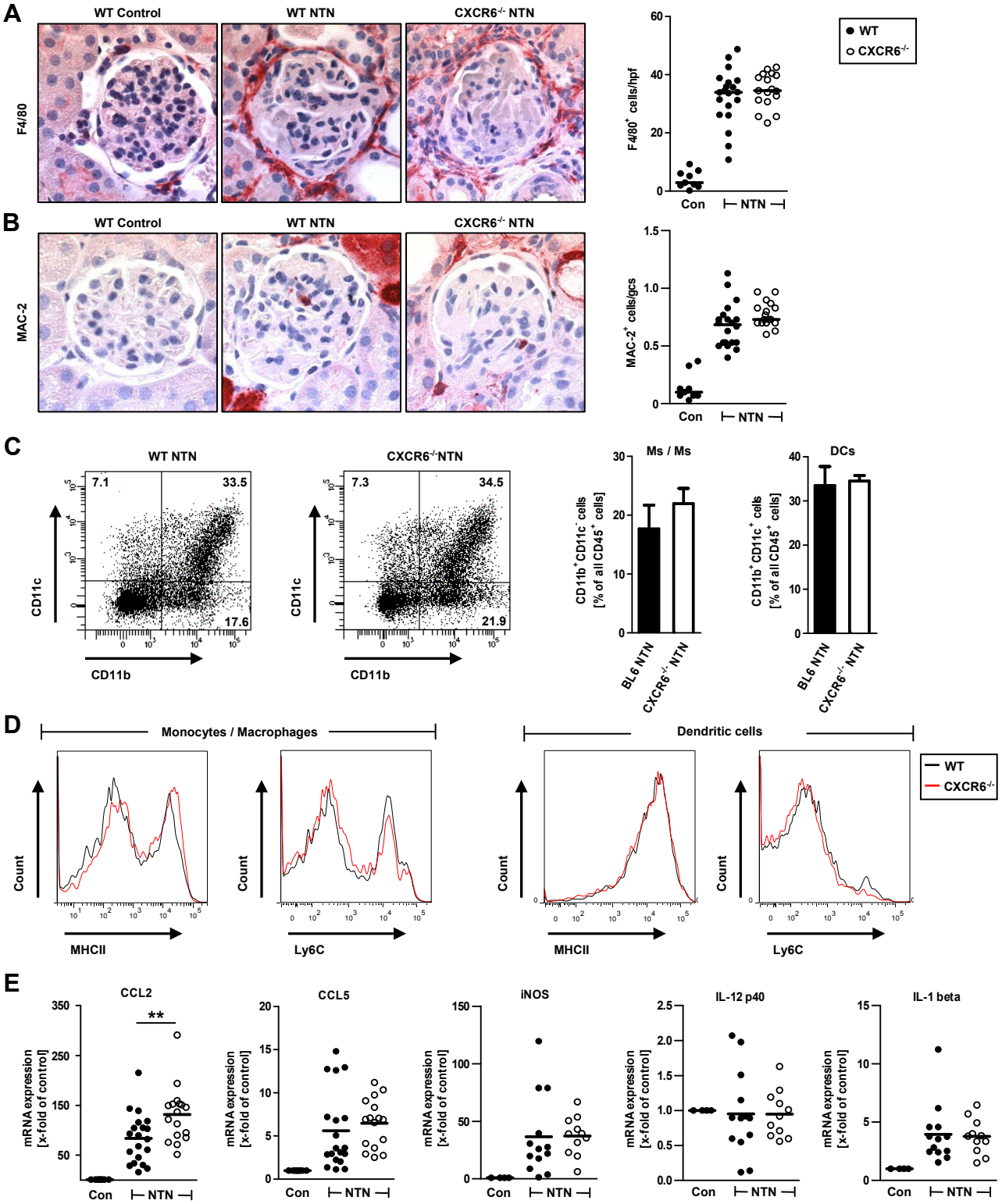
Supplement 1: Effect of diphteria toxin induced DC depletion on systemic iNKT cells in CD11c.LuciDTR mice



Supplement 1
Effect of diphteria toxin induced DC depletion on systemic iNKT cells in CD11c.LuciDTR mice. Quantification of FACS analysis of splenic iNKT cells ($CD45^+TCR\beta^+CD1d\text{-}Tetramer^+$) at day 4 of NTN one day after DTx injection (*n = 3/group*). Symbols represent means ± SD.

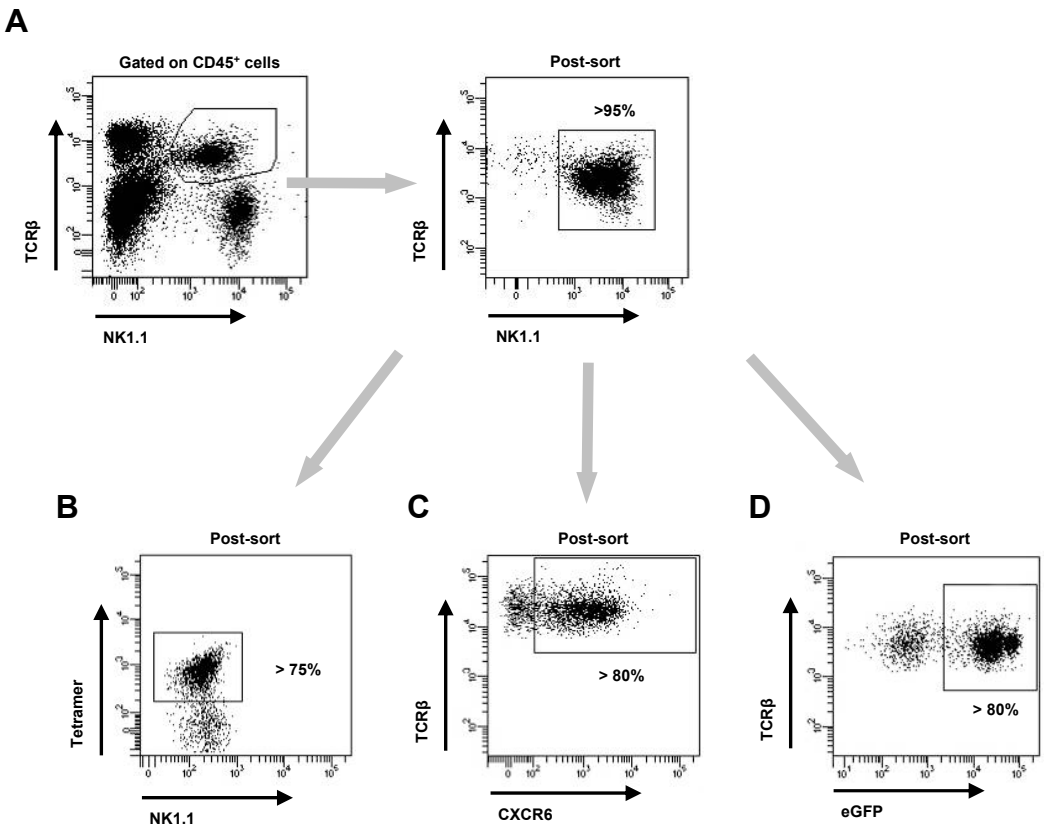
Supplement 2: Renal macrophage/DC infiltration



Supplement 2

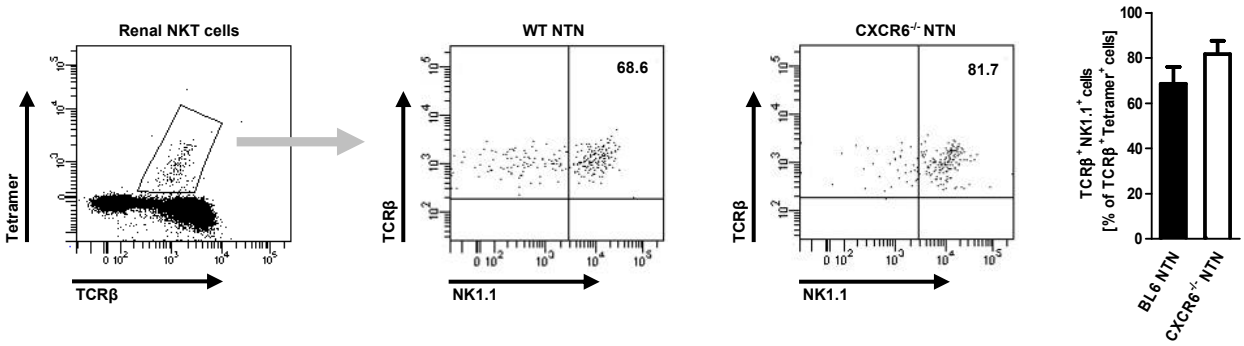
Renal macrophage/DC infiltration. Representative photographs and quantification of kidney sections from controls, nephritic wild-type, and CXCR6-deficient mice immunohistochemically stained for (A) the interstitial macrophage/DC marker F4/80 (*n= 9-20/group*) and (B) the glomerular monocyte/macrophage marker MAC-2 (*n = 9-20/group*) 8 days after induction of NTN (magnification 400x). (C, D) FACS analysis at day 8 of NTN of $CD11c^{-}CD11b^{+}$ positive cells, representing monocytes/macrophages, and $CD11c^{+}CD11b^{+}$ double-positive cells, representing renal dendritic cells, revealed no difference in the infiltration of these cell subtypes between wild-type and $CXCR6^{-/-}$ mice. Flow cytometry for MHCII, a marker for antigen presentation, demonstrated that monocytes/macrophages showed an intermediate MHCII expression, whereas DCs contained high percentage MHCII positive cells. Inversely, Ly6C, an activation marker of monocytes, was highly expressed on a subset of $CD11c^{-}CD11b^{+}$ cells, while the $CD11c^{+}CD11b^{+}$ subtype showed low expression of Ly6C. The lack of CXCR6, however, did not selectively affect the infiltration of the myeloid cell subsets defined by the above-mentioned markers (*n = 5/group*). (E) Real-time RT-PCR analysis of renal cytokine expression in nephritic wild-type, $CXCR6^{-/-}$ mice, and wild-type controls (*n = 9–20/group*) for CCL2, CCL5, iNOS, IL-12p40 and IL-1beta. mRNA levels are expressed as x-fold of controls. Symbols represent individual data points with median values as horizontal lines or means ± SD when presented as bar graphs. (** *p < 0.01)*

Supplement 3: NKT-cell sorting



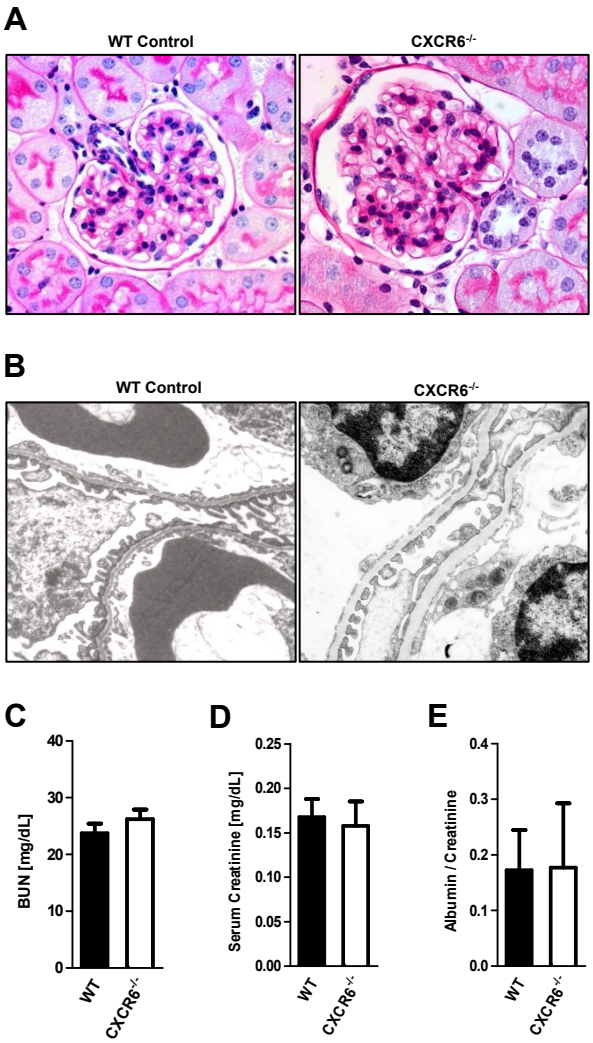
Supplement 3
NKT-cell sorting. (A) Representative FACS plots of NKT cell sorting strategy and post-sort purity analysis of sorted cells. (B, C) Post-sort analysis of $CD45^+TCR\beta^+NK1.1^+$ hepatic NKT cells for glycolipid antigen-recognizing T-cell receptor (B) and CXCR6 (C) co-expression. (D) Representative FACS analysis of eGFP expression on $CD45^+TCR\beta^+NK1.1^+$ hepatic NKT cells sorted from $CXCR6^+/eGFP^+$ heterozygous mice.

Supplement 4 : Renal iNKT cell characteristics



Supplement 4
Renal iNKT cell characteristics. Representative FACS plots and quantification of NK1.1 expression on renal iNKT cells from nephritic wildtype and nephritic CXCR6-deficient mice on day 8 of NTN (*n = 6/group*). Symbols represent means ± SD.

Supplement 5: Renal phenotype of CXCR6-deficient mice



Supplement 5
Renal phenotype of CXCR6-deficient mice. Representative renal PAS staining (magnification 400x) (A) and electron microscopic analysis (original magnification 7000x) (B) of 45-week-old wild-type and CXCR6 knockout mice revealed no histological differences between the knockout and wild-type group. (C–E) Functional analysis of CXCR6-deficient and wild-type mice demonstrated identical serum BUN (C) and creatinine levels (D). Moreover, CXCR6-deficient mice and wild-type animals had comparable urinary albumin/creatinine ratios (E). (*n = 4/group*). Symbols represent means ± SD.

2 Zusammenfassende Darstellung der Publikation

Glomerulonephritiden sind entzündliche Nierenerkrankungen, die nach der diabetischen Nephropathie und der hypertensiven/ischämischen Nephropathie die häufigste Ursache für die Entwicklung einer terminalen Niereninsuffizienz in der westlichen Welt darstellen (Chadban und Atkins 2005). Bei ca. 10-15% der Patienten mit terminaler Niereninsuffizienz liegt als Grunderkrankung eine Glomerulonephritis vor. Die klinisch schwerwiegendste Variante eines nephritischen Verlaufes ist die rasch progrediente Glomerulonephritis (RPGN) unterschiedlicher Ätiologie und Pathogenese, der jedoch der zellvermittelte renale Schaden als charakteristisches Merkmal gemein ist (Bolton 1996; Couser 2012). Sie stellt einen nephrologischen Notfall dar, der ohne Therapie zu einem raschen Nierenfunktionsverlust mit der Entwicklung einer terminalen Niereninsuffizienz innerhalb von Tagen bis Wochen führt. Schätzungen liegen bei sieben Fällen pro eine Million Einwohner/Jahr (Couser 1988). Die RPGN hat also eine schlechte Prognose spricht aber oft auf eine frühe und aggressive Therapie an, die jedoch komplex und oft mit schweren Nebenwirkungen verbunden ist. Für die Simulation der humanen RPGN existieren verschiedene Tiermodelle, unter diesen stellt das Modell der Nephrotoxischen Nephritis (NTN) eines der am besten charakterisierten Modelle der murinen, iatrogen erzeugten RPGN dar, welche eine humane RPGN imitiert und durch das Einbringen von Immunglobulinen gegen Antigene der murinen Nierenrinde in Mäuse erzeugt wird. Diese Antikörper werden aus dem Blut von mit Bestandteilen des murinen Nierencortex zuvor immunisierten Schafen gewonnen (Tipping, Huang et al. 1998). Das Modell zeigt sowohl Eigenschaften einer Immunkomplexnephritis als auch die spezifische, lineare Ablagerung von Immunglobulinen entlang der renalen Basalmembran, was zu Chemokin vermittelter Einwanderung zellulärer Effektoren, wie z.B. zuvor in lymphatischen Organen aktivierten Th1- und Th17-Zellen, führt (Turner, Paust et al. 2010; Kitching und Holdsworth 2011). Diese induzieren eine Gewebezerstörung im Sinne eines klassischen Typ IV Hypersensitivitätssyndrom. Heute gilt es als gesichert, dass T-Zellen als Effektoren an Autoimmunerkrankungen und der Pathogenese von Glomerulonephritiden wesentlich beteiligt sind. Einige Subtypen von T-Zellen stellen anti-inflammatorische Gegenspieler der erwähnten Effektor-T-Zellen dar, von denen v.a. die regulatorischen T-Zellen am besten charakterisiert sind (Feuerer, Hill et al. 2009). Relativ neu in dem Konzept von pro- und antiinflammatorischen T-Zellen sind

die invarianten Natürlichen Killer T Zellen (iNKT-Zellen), dies sind CD1d-limitierte, Lipid-Antigen reaktive, immunregulatorische T-Lymphozyten, welche eine zellvermittelte Immunität gegenüber Tumoren und Infektionserregern, Bakterien und Viren fördern, aber paradoxerweise gleichzeitig zellvermittelte Immunität unterdrücken können, welche mit Autoimmunerkrankungen und Transplantatabstoßung assoziiert ist (Godfrey, Stankovic et al. 2010). Darüber hinaus gibt es Hinweise darauf, dass NKT Zellen in allergischen Erkrankungen und Arteriosklerose eine schädigende Wirkung haben (Galkina, Harry et al. 2007). Während die genauen Mechanismen, mit denen diese Zellen solche teilweise gegensätzlich erscheinenden Funktionen ausüben, noch nicht aufgeklärt werden konnten, hoben die Studien hervor, dass verschiedene Subtypen von NKT Zellen mit jeweils verschiedenen Eigenschaften existieren (Bendelac, Savage et al. 2007). Da die Anzahl dieser Zellen zwischen einzelnen Individuen starken Schwankungen unterliegt, ist es wesentlich, dass die Mechanismen verstanden werden, welche Entwicklung und Aufrechterhaltung von NKT Zellen und deren Subtypen regulieren.

Zelluläre Effektoren wie T-Zellen sind darauf angewiesen, dass sie durch Dendritische Zellen (DC) lizenziert und aktiviert werden. Dazu nehmen dendritische Zellen Antigen auf, prozessieren es und präsentieren es T-Zellen in den sekundären lymphatischen Geweben (Bretscher und Cohn 1970; Steinman, Hawiger et al. 2003). Doch es gibt auch ortsständige dendritische Zellen in nicht-lymphatischen Geweben wie der Niere. Dort sind sie vor allem im Tubulointerstitium zu finden, wo sie im Entzündungszustand akkumulieren und dort die T-Zellaktivierung, z.B. über IL-10, modifizieren können (Scholz, Lukacs-Kornek et al. 2008). Depletionsexperimente zeigen, dass, abhängig vom Zeitpunkt der Depletion der dendritischen Zellen, protektive und aggravierende Effekte im NTN Modell erzielt werden können (Heymann, Meyer-Schwesinger et al. 2009; Hochheiser, Engel et al. 2011). Die Rolle der dendritischen Zellen ist weiterhin unklar, in menschlichen GNs konnte ihre Anwesenheit, ähnlich den murinen Modellen, in den periglomerulären Infiltraten nachgewiesen werden.

Die Migration von Leukozyten, und damit wesentlich auch T-Zellen, zwischen den verschiedenen Kompartimenten (Gefäßsystem, Endorgane, lymphatische Organe) wird überwiegend durch Chemokine (Chemotaktische Zytokine) gesteuert, ein weiterer wesentlicher Bestandteil des humanen und murinen Immunsystems (Baggiolini 1998; Sallusto und Baggiolini 2008). Es handelt sich um 8 bis 12 kDalton große Proteine, die eine zentrale Rolle beim zielgerichteten Trafficking und der Aktivierung von

Leukozyten unter homeostatischen und inflammatorischen Bedingungen übernehmen. Derzeit sind mehr als 50 Chemokine und 20 korrespondierende Chemokinrezeptoren bekannt, die nach ihrer Struktur in vier Familien unterteilt werden. Chemokinrezeptoren sind die Zellmembran heptahelikal durchspannende Proteine, die an ein heterotrimeres G Protein gekoppelt sind. Nach Bindung der entsprechenden Chemokine dissoziieren alpha- und beta-gamma-Untereinheit und durch Calciuminflux werden PI3-Kinasen und Rho-GTPasen Signalwege angeschaltet (Thelen und Stein 2008). Mit spezifischen Chemokinrezeptoren ausgestattete Zellen haben, nach Aufbau eines Konzentrationsgradienten der korrespondierenden Chemokine, welche von ortsständigen oder ihrerseits eingewanderten Zellen sezerniert werden, die Möglichkeit, Gefäßwände zu durchwandern und das Zielgewebe zu erreichen. Dieses Prinzip gilt so auch für die Niere und ihr komplexes System aus Blutgefäßen und dem von diesen versorgten Parenchym (Segerer 2003).
Die Experimente der vorliegenden Arbeit hatten den Zweck, ein besseres Verständnis der Interaktionen zwischen den oben beschriebenen, zugrunde liegenden Mechanismen und Faktoren in Glomerulonephritiden zu erzielen, um langfristig neue therapeutische Optionen für deren Therapie zu generieren. Die komplexen immunologischen Entzündungsmechanismen werden durch die immunpathologische Grundlagenforschung schrittweise entschlüsselt und um neu hinzu gewonnene Erkenntnisse ergänzt. Dies ist von besonderem Interesse, da aktuell die pharmakologische Blockade einzelner Chemokinrezeptoren, wie z.B. des CCR5, erstmalig klinisch angewendet werden.

Im Modell der experimentellen murinen Glomerulonephritis konnte gezeigt werden, dass unreife Dendritische Zellen protektiv wirken, aber im Rahmen der Chronifizierung der Entzündungsreaktion einen Reifungsprozess durchlaufen und diese aufrechterhalten (Scholz, Lukacs-Kornek et al. 2008; Hochheiser, Engel et al. 2011). Die Zugrunde liegenden Mechanismen konnten jedoch bisher nicht in vollem Umfang aufgeklärt werden. Unter der Annahme, dass unreife DCs in der Frühphase der Entzündungsreaktion Zytokine und Chemokine produzieren, um antiinflammatorische Zellen zu rekrutieren, wurden in der vorliegenden Arbeit im Modell der NTN in CD11c.LuciDTR Mäusen die DCs in dieser Phase depletiert (Tittel, Heuser et al. 2012). Die Analysen der renalen Zellpopulationen in den nephritischen Tieren zeigte eine signifikante Verminderung der iNKT Zellen, während sich die Anzahl

proinflammatorischer Zellen nicht veränderte. Vor dem Hintergrund, dass zwei Studien kürzlich die protektive Rolle von NKT Zellen in renalen Entzündungsmodellen demonstrieren konnten, in denen jeweils der adoptive Transfer dieser Zellen die vorher beobachtete Aggravation der Nephritis in den KO-Tieren verhindern konnte, war dies ein aussichtsreicher Ansatz für weitere Experimente (Yang, Kim et al. 2008; Mesnard, Keller et al. 2009). Wir postulierten, dass der protektive Effekt unreifer renaler DCs im NTN-Modell durch die Rekrutierung von iNKT Zellen in die Nieren zu erklären sei.

Die *in vivo* Mechanismen, welche die Rekrutierung von iNKT Zellen in die Niere ermöglichen, sind bisher nicht bekannt, jedoch ist in der Literatur beschrieben, dass diese Zellen den Chemokinrezeptor CXCR6 in großem Maße exprimieren (Matloubian, David et al. 2000; Johnston, Kim et al. 2003; Germanov, Veinotte et al. 2008). In den entzündeten Nieren zeigten die Analysen, dass die iNKT Zellen diesen Rezeptor zu über 90% exprimierten und in unreifen renalen DCs wurden in der Frühphase der Entzündung große Mengen an mRNA für das Chemokin CXCL16 gefunden, den einzig bekannten Liganden für CXCR6. Hiermit wurde neben den schon bekannten Tubuluszellen und Podozyten eine weitere zelluläre Quelle für dieses Chemokin identifiziert (Schramme, Abdel-Bakky et al. 2008; Gutwein, Abdel-Bakky et al. 2009). Darüber hinaus zeigte sich ein periglomeruläres Muster der CXCL16 Expression, die dem periglomerulären Verteilungsmuster renaler DCs in humanen und murinen Glomerulonephritiden stark ähnelte (Segerer, Heller et al. 2008; Heymann, Meyer-Schwesinger et al. 2009). In der Literatur wurde die CXCR6-CXCL16 Achse nach ihrer Erstbeschreibung hauptsächlich als wesentlich für die Rekrutierung proinflammatorischer CD4- und CD8-positiver Effektor-T-Zellen in Entzündungsgeschehen von Transplantaten, Blutgefäßen und Gelenken beschrieben (van der Voort, van Lieshout et al. 2005; Galkina, Harry et al. 2007; Jiang, Sun et al. 2010). Erste Untersuchungen in einem Modell der anti-GBM-Nephritis der Ratte zeigte eine abgeschwächte Entzündung nach Blockade von CXCL16 durch einen polyklonalen Antikörper, wesentlich erklärbar durch eine Verminderung der Einwanderung von Makrophagen (Garcia, Truong et al. 2007). Im Modell der unilateralen Uretherobstruktion in CXCL16-Knockout Mäusen zeigte sich eine verringerte Fibrosierung durch Verminderung der Einwanderung von CXCR6-positiven Fibroblastenvorläuferzellen (Chen, Lin et al. 2011). Jedoch wurde z.B. in einem murinen Herztransplantationsmodell eine wesentliche Rolle der CXCL16-CXCR6

Achse für die Infiltration von NKT-Zellen und nachfolgende Aufrechterhaltung der Transplantat-Toleranz beschrieben (Jiang, Shimaoka et al. 2005).

Die verstärkte Ausprägung der rasch progredienten GN in CXCR6-KO Mäusen stützt diese Theorie; möglicherweise vermittelt die CXCL16-CXCR6 Achse die Migration von iNKT-Zellen in ihrer Funktion als Vermittler von Immunhomeostasis im akuten Entzündungsgeschehen, während proinflammatorische Effekte im Stadium einer Chronifizierung überwiegen, beispielsweise durch Vermittlung der Einwanderung von Effektor-T-Zellen.

Wir konnten zeigen, dass die Einwanderung von iNKT Zellen nach Induktion der NTN in CXCR6-Knockout Mäusen signifikant reduziert war und, was von großer Bedeutung ist, dass sich die Erkrankung in den KO-Tieren aggraviert zeigte. Ein kausaler Zusammenhang zwischen diesen Befunden wird gestützt von der Tatsache, dass sich die Erkrankung in den KO-Tieren nach Repopulation mit CXCR6-exprimierenden NKT Zellen wieder abgeschwächt auf dem Niveau der Wildtyptiere zeigte. Eine entwicklungs- oder anlagebedingte Beeinträchtigung der renalen Struktur der KO-Tiere oder eine überschießende zelluläre oder humorale Immunantwort in diesen Tieren konnte ausgeschlossen werde. Somit beschreiben wir mit dieser Arbeit einen neuen Mechanismus, mit welchem unreife DCs über die Rekrutierung von regulatorischen iNKT Zellen den Verlauf von Autoimmunerkrankungen abschwächen können. Die Mechanismen des suppressiven Potentials der iNKT-Zellen sind bislang nicht ausreichend untersucht, jedoch scheint immunmodulierenden Molekülen wie z.B. IL-4, IL-10 und TGFβ, für die eine suppressive Funktion in der NTN beschrieben wurde, entscheidende Bedeutung zuzukommen (Kitching, Tipping et al. 1997; Scholz, Lukacs-Kornek et al. 2008). mRNA-Analysen zeigten eine erhöhte Expression dieser Zytokine in sortierten iNKT-Zellen aus Nieren nephritischer Tiere im Vergleich zur Kontrollgruppe. Gleichzeitig war die Anzahl von iNKT-Zellen in den Nieren der kranken Tiere signifikant reduziert und auch das Potential der verbleibenden Zellen, die o.g. antiinflammatorischen Zytokine zu produzieren, signifikant reduziert.

Die genauen Mechanismen weiter aufzuklären bedarf jedoch weiterer Studien. Vorstellbar wären weitergehende Transferexperimente, beispielsweise von zytokindefizienten iNKT Zellen, oder durch die Charakterisierung der NTN in Mäusen mit spezifischem CXCR6-Knockout in iNKT-Zellen, welche derzeit jedoch noch nicht entwickelt wurden. Somit können wir in der vorliegende Studie nicht ausschließen, dass andere durch den Pan-Knockout CXCR6-defiziente Zellen zu der beobachteten

Aggravation der Erkrankung in den KO-Tieren führten. Zukünftig wäre der sichere Nachweis der Migration von intravenös transferierten Zellen in die entzündeten Nieren, so wie es angedeutet in einer vorausgegangenen Studie gezeigt wurde (Yang, Kim et al. 2008), ein wichtiger Hinweis für eine direkte, immunsuppressive Funktion von iNKT-Zellen im erkranken Endorgan; darüber hinaus stellt die Migration der transferierten Zellen in sekundäre lymphatische Organe und eine so stattfindende Modulation der Immunantwort eine weitere mögliche Erklärung der beobachteten Effekte durch den Zelltransfer dar.

iNKT-Zellen stellen somit, zusätzlich zu dem anerkannten Konzept der regulatorischen T-Zellen, eine weitere distinkte Zellpopulation dar, welche den renalen Gewebeschaden im Model der experimentellen Glomerulonephritis vermindern kann und welche wesentlich unter der Kontrolle der CXCL16/CXCR6 Achse zu stehen scheint. Die Übertragbarkeit dieser Ergebnisse auf die Immunopathogenese der humanen rasch progredienten Glomerulonephritis bedarf allerdings weiterer Untersuchung, da die humanen iNKT Zellen einige phänotypische Unterschieden zu ihren murinen Pendants aufweisen. Die große Menge an Medikamenten und Interventionen, die heutzutage am Menschen Verwendung finden und auf Erfahrungen im Tiermodell beruhen, zeigen, dass das Tiermodell in der medizinischen Forschung etabliert ist. Ein besseres Verständnis der Eigenschaften und Funktionen der humanen NKT Zellen in Autoimmunerkrankungen wie den rasch progredienten Glomerulonephritiden ist jedoch vonnöten bevor der Einsatz oder die Manipulation von iNKT Zellen eine potentiell neue Therapieoption darstellen kann.

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4 Erklärung des Eigenanteils

Das Konzept und das experimentelles Design der hier vorliegenden Arbeit wurden unter Federführung von Prof. Dr. Ulf Panzer in engem Austausch mit mir und anderen Mitautoren erstellt.

Größtenteils alleine durchgeführt und/oder ausgewertet habe ich die Experimente auf der Basis folgender Methoden: Tierexperimentelles Arbeiten (Immunisierung, Stoffwechselkäfige, Organentnahme) – Auswertung (Immun)-Histochemie (PAS-, CD3-, F4/80-, MAC2-, FoxP3-, Schaf-IgG-, Maus-IgG-Färbungen) – Leukozytenisolation aus Gewebe (Niere, Leber, Milz) – *in situ* Hybridisierung – *in vitro* Stimulationsassays – ELISA (Albumin, Immunglobuline, Zytokine) – realtime RT-PCR – Statistische Analysen. An der Durchführung und Auswertung der durchflusszytometrischen Färbungen, Messungen und Zellsortierung (FACS), die Federführung von Dr. H.-J. Paust erfolgten, war ich wesentlich beteiligt. Darüber hinaus habe ich Methoden, Materialien und Know-how aus anderen Laboren organisiert und in unserer Arbeitsgruppe etabliert.

Alle Figuren, einschließlich der *Supplemental Figures*, wurden von mir aus den erhobenen Daten erstellt. Die Fotos von histologischen Färbungen wurden teilweise von anderen Autoren, teilweise von mir erstellt. Das Figurendesign entstand in Diskussion mit den Mitarbeitern unserer Arbeitsgruppe.

In Zusammenarbeit mit anderen Autoren habe ich das endgültige Manuskript entworfen und mit Dr. H.-J. Paust, Prof. Dr. C. Kurts und Prof. Dr. U. Panzer geschrieben.

5 Danksagung

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6 Wissenschaftliche Beiträge

Publikationen:

HJ Paust, JE Turner, JH Riedel, E Disteldorf, A Peters, T Schmidt, C Krebs, J Velden, HW Mittrücker, OM Steinmetz, RA Stahl, U Panzer:
"Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis"
Kidney Int. 2012 Jul; 82(1):72-83. doi: 10.1038/ki.2012.101. Epub 2012 Apr 11.

JE Turner, HJ Paust, OM Steinmetz, A Peters, JH Riedel, A Erhardt, C Wegscheid, J Velden, S Fehr, HW Mittrücker, G Tiegs, RAK Stahl, U Panzer:
"CCR6 recruits regulatory T cells and Th17 cells to the kidney in glomerulonephritis"
J Am Soc Nephrol. 2010 Jun; 21(6):974-85.

Vorträge:

JH Riedel, HJ Paust, JE Turner, E Disteldorf, J Velden, HW Mittrücker, RA Stahl, OM Steinmetz, U Panzer:
"*Critical Role of the Chemokine Receptor CXCR6 for Renal NKT Cell Localization and Function in Murine Crescentic Glomerulonephritis"*
American Society of Nephrology - Kidney Week, 08. - 13.11.2011, Philadelphia, Pensylvania, USA
Assigned "Top oral presentation"

JH Riedel, HJ Paust, JE Turner, OM Steinmetz, A Peters, HW Mittrücker, RAK Stahl, U Panzer:
„*Funktion des Chemokinrezeptors CXCR6 bei der experimentellen Glomerulonephritis*"
Forum Junge Niere, 19. - 20.06.2009, München, Deutschland

Poster:

JH Riedel, HJ Paust, JE Turner, OM Steinmetz, A Peters, HW Mittrücker, RAK Stahl, U Panzer:
"*CXCR6 deficiency aggravates crescentic glomerulonephritis in mice*"
ISN Forefronts Meeting "Induction and Resolution of Renal Inflammation", 06. - 09.05.2010, Rantum/Sylt, Deutschland

HJ Paust, JH Riedel, JE Turner, A Peters, J Velden, OM Steinmetz, HW Mittrücker, RAK Stahl, U Panzer:
"*Interaction of Th1 and Th17 immune response in experimental glomerulonephritis*"
ISN Forefronts Meeting "Induction and Resolution of Renal Inflammation", 06. - 09.05.2010, Rantum/Sylt, Deutschland

HJ Paust, JH Riedel, JE Turner, A Peters, OM Steinmetz, HW Mittrücker, RAK Stahl, U Panzer:
„*Regulation of renal Th1- and Th17-cell attracting chemokines by IFNγ and IL-17* "
American Society of Nephrology – Renal Week, 27. 10. - 01.11.2009, San Diego, California, USA

7 Lebenslauf

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8 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....

Hamburg, Januar 2013