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Lymphocyte-specific deletion of IKK2 or NEMO mediates an increase in intrarenal Th17 cells and accelerates renal damage in an ischemia-reperfusion injury mouse model

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Guo L, Lee HH, Noriega MM, Paust HJ, Zahner G, Thaiss F. Lymphocyte-specific deletion of IKK2 or NEMO mediates an increase in intrarenal Th17 cells and accelerates renal damage in an ischemia-reperfusion injury mouse model. *Am J Physiol Renal Physiol* 311: F1005–F1014, 2016. First published August 31, 2016; doi:10.1152/ajprenal.00242.2016.—Acute kidney injury (AKI) is associated with poor patient outcome and a global burden for end-stage renal disease. Ischemia-reperfusion injury (IRI) is one of the major causes of AKI, and experimental work has revealed many details of the inflammatory response in the kidney, such as activation of the NF-κB pathway. Here, we investigated whether deletion of the NF-κB kinases IKK2 or NEMO in lymphocytes or systemic inhibition of IKK2 would cause different kidney inflammatory responses after IRI induction. Serum creatinine, blood urea nitrogen (BUN) level, and renal tubular injury score were significantly increased in $CD4^{cre}IKK2^{f/f}$ ($CD4xIKK2^{\Delta}$) and $CD4^{cre}NEMO^{f/f}$ ($CD4xNEMO^{\Delta}$) mice compared with CD4cre mice after IRI induction. The frequency of Th17 cells infiltrating the kidneys of $CD4xIKK2^{\Delta}$ or $CD4xNEMO^{\Delta}$ mice was also significantly increased at all time points. CCL20, an important chemokine in Th17 cell recruitment, was significantly increased at early time points after the induction of IRI. IL-1β, TNF-α, and CCL2 were also significantly increased in different patterns. A specific IKK2 inhibitor, KINK-1, reduced BUN and serum creatinine compared with nontreated mice after IRI induction, but the frequency of kidney Th17 cells was also significantly increased. In conclusion, although systemic IKK2 inhibition improved kidney function, lymphocyte-specific deletion of IKK2 or NEMO aggravated kidney injury after IRI, and, in both conditions, the percentage of Th17 cells was increased. Our findings demonstrate the critical role of the NF-κB pathway in Th17 activation, which advises caution when using systemic IKK2 inhibitors in patients with kidney injury, since they might impair the T cell response and aggravate renal disease.

ischemia-reperfusion injury; nuclear factor-κB transcription factor; signalosome complex; Th17 cells; IκB kinase; nuclear factor-κB essential modulator; ischemia-reperfusion injury

ACUTE KIDNEY INJURY (AKI) is a global burden of renal diseases without effective therapy that significantly increases patient morbidity and mortality. Thus, AKI represents an important medical condition (1). Ischemia-reperfusion injury (IRI) is one of the major causes in the pathophysiology of AKI and occurs frequently due to interruption of the renal blood flow and undesirable hemodynamic changes during the perioperative period or sepsis (26). Currently, there is no specific therapy to either treat kidney IRI or improve healing. Experimental and clinical studies have shown that inflammation is a significant component in the etiology of renal IRI (31).

Both the innate and adaptive immune systems participate in kidney IRI (34). Blocking $CD4^+$ T cell activation protected renal function and attenuated local inflammation in a murine kidney IRI model (32). In particular, it has also been reported that, in the kidney, both functional and histological injury were dependent on the production of the cytokine IL17A, and both were attenuated by depleting IL17A (3), as also shown in myocardial (19) and lung ischemia-reperfusion injury (35). These experiments have demonstrated unconventional T cells or innate immune cells as the cellular source of IL-17 production (5, 37).

NF-κB functions as a homo- or heterodimer of five constitutive proteins [c-REL, RelA (p65), RelB, p50, and p52] and plays a key role in the regulation of proinflammatory genes (39). Under basal conditions, NF-κB dimers are retained in the cytoplasm in their inactive form by a family of inhibitory proteins, IκBs, which mask NF-κB nuclear localization sites, thereby blocking nuclear translocation. NF-κB activation occurs via the phosphorylation of these inhibitory IκB proteins by the IκB kinase (IKK) complex (21). The IKK complex, the so-called signalosome complex, contains three kinases: IKK1 and IKK2 (also known as IKKα and -β), and a regulatory protein termed NF-κB essential modulator (NEMO/IKKγ) (10). In our recently published paper, we demonstrated that systemic IKK2 inhibition after the induction of kidney disease increased renal injury in an experimental model of immune-mediated glomerulonephritis (11). NF-κB activation has been demonstrated after IRI by us and others (14, 16, 40), and the potential clinical importance of NF-κB as a therapeutic target in treating IRI has been derived from a number of experimental studies that demonstrated the amelioration of IRI after the inhibition of NF-κB pathway activation (2).

Based on these published data, we addressed the following hypotheses: *1*) that specific deletion of IKK2 or NEMO in lymphocytes influences kidney injury after IRI and *2*) that there is different renal outcome between systemic inhibition of IKK2 and lymphocyte-specific deletion of IKK2 after IRI induction. Therefore, IRI was induced in $CD4^{cre}IKK2^{f/f}$ ($CD4xIKK2\Delta$), $CD4^{cre}NEMO^{f/f}$ ($CD4xNEMO\Delta$), and CD4cre control mice as well as in mice injected with the specific IKK2 inhibitor KINK-1. Kidney function, morphology, cytokine production, and the phenotype of kidney-infiltrating lymphocytes and splenocytes were analyzed at 12 h and 2 and 7 days after the induction of IRI.

Our results demonstrated the following points: deletion of IKK2 or NEMO in lymphocytes aggravated kidney injury, and

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IKK2 or NEMO might play different roles by affecting cytokine and chemokine production, therefore activating different subsets of $CD4^+$ T cells at different time points after the induction of IRI. Systemic IKK2 inhibition improved renal function, but the frequency and the number of Th17 cells was also increased. Our results therefore direct attention to the different role of the NF-κB pathway in different cell types and suggest caution in using systemic NF-κB pathway inhibitors for the clinical treatment of patients with kidney injury.

MATERIALS AND METHODS

Animals and IKK2 inhibitor. Mice expressing Cre recombinase under the CD4 promoter were crossbred with floxed IKK2 or NEMO mice. The mice were generously provided by Dr. M. Karin (IKK2f/f mice), Dr. C. Wilson (CD4cre mice), and Dr. Manolis Pasparakis (NEMOf/f mice). The efficiency and specificity of the IKK2 or NEMO deletion were assessed by PCR from FACS-sorted $CD4^+$ and $CD4^-$ splenocytes (BD ARIAIII Cytometer; Becton Dickinson). All animals were housed in the animal facility of the University Medical Center Hamburg-Eppendorf. All experiments were approved by the local authorities (G13/058, G14/109). The IKK2 inhibitor KINK-1, also known as Bay 65–194, was kindly provided by Dr. K. Ziegelbauer (Bayer Health Care, Berlin, Germany) (43, 44). The inhibitor was dissolved in 10% Cremophor and intraperitoneally injected in an amount of 5 mg/kg body wt as described previously (11).

Induction and assessment of IRI. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. After animals were placed on a heated operation table, the left and right renal pedicles were identified through an abdominal midline incision and occluded with atraumatic microvascular clamps for 55 min. Next, the clamps were released and the kidneys allowed reperfusion. After visible reperfusion was completed, the abdomen was closed with continuous sutures, as previously described (14). Sham-operated an-

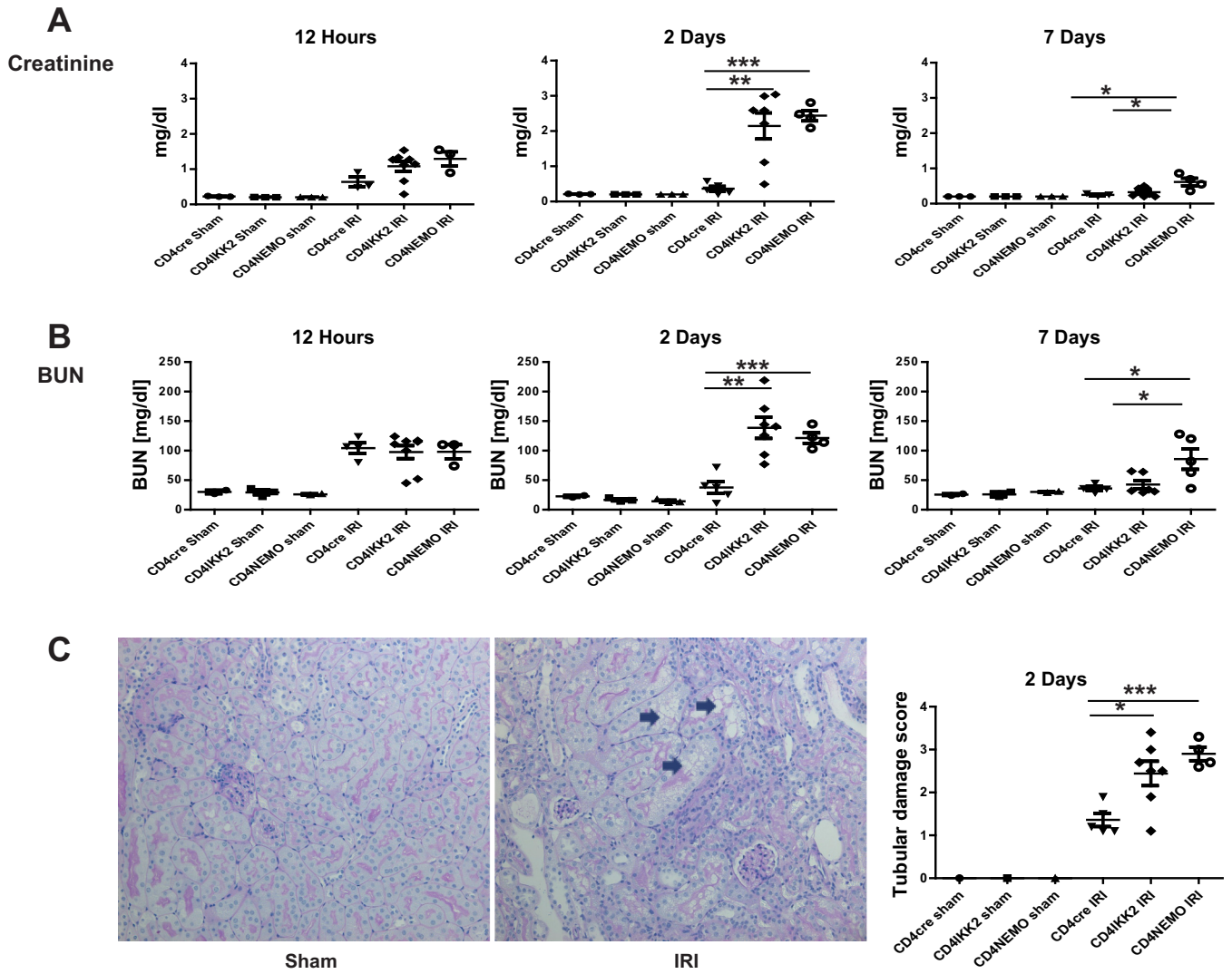

Fig. 1. Exacerbation of renal functional impairment after ischemia-reperfusion injury (IRI) in mice with cell-specific deletion of IκB kinase (IKK) 2 or NF-κB essential modulator (NEMO) in lymphocytes. At 12 h after IRI, there was no significant difference in serum creatinine (*A*) or blood urea nitrogen (BUN, *B*) among the three groups; at 2 days, creatinine and BUN were significantly increased in $CD4xIKK2^{\Delta}$ and $CD4xNEMO^{\Delta}$ mice compared with CD4cre mice; at 7 days, creatinine and BUN were significantly increased only in $CD4xNEMO^{\Delta}$ mice compared with the two other groups. *C*: representative images of sham-operated mice and of outer medulla tubular necrosis (arrows) taken from $CD4xNEMO^{\Delta}$ mice at 2 days after IRI [periodic acid-Schiff (PAS) staining, magnification: ×200]. Histological scoring of tubular injury at 2 days was significantly increased in $CD4xIKK2^{\Delta}$ and $CD4xNEMO^{\Delta}$ mice compared with CD4cre mice. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, CD4IKK2 ($CD4crexIKK2^{\Delta}$), CD4NEMO ($CD4crexNEMO^{\Delta}$).

imals were subjected to the same operating procedure without clamping. During the procedure, the animals were hydrated with saline. The animals were examined 12 h and 2 and 7 days after IRI. At each time point and in each group, at least five animals were examined, and the experiments were repeated three times.

Renal functional studies and histology. Blood urea nitrogen (BUN) and serum creatinine were quantified using standard laboratory methods. Kidney sections were fixed in buffered formalin for 24 h, processed, and embedded in paraffin wax. Tubular injury was assessed on periodic acid-Schiff-stained sections. The tubular injury score, determined by assessing tubular epithelial cell loss, tubular necrosis, accumulation of cellular debris, and tubular cast formation, was scored according to the percentage of affected tubules under high-power microscopy. The percentage of tubules affected was assigned a score: 0, normal; 1, 10–25%; 2, 26–50%; 3, 51–75%; and 4, 75%, according to Chan et al. (3). To determine the number of $CD3^+$ cells infiltrating the kidneys, immunohistology was used for CD3 staining as described previously (29).

Flow cytometry. Cell isolation and staining were performed as recently published (11, 29). For intracellular and intranuclear staining, samples were processed using a commercial Foxp3 staining kit (Ebioscience). Fluorochrome-labeled antibodies against IFNγ, IL17A, and Foxp3 (BD Biosciences, Heidelberg, Germany) were used. For intracellular cytokine staining of IFNγ and IL17A to

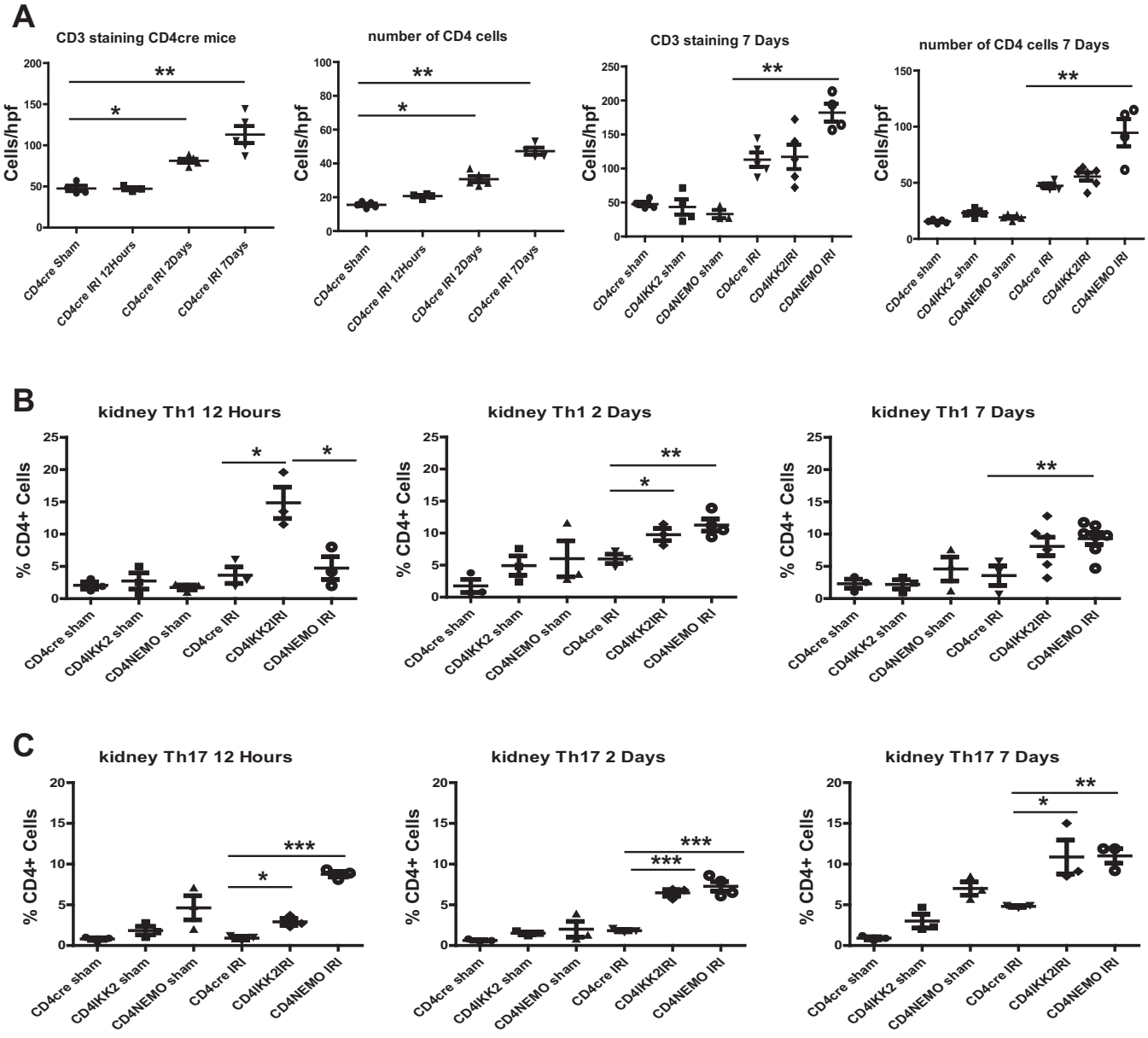

Fig. 2. Different subsets of T cells infiltrating IRI kidneys. *A*: significantly more $CD3^+$ cells, as determined by immunohistology, infiltrated the kidneys 2 and 7 days after induction of IRI in CD4cre mice. The number of $CD4^+$ T cells as recalculated by combining FACS and the number of $CD3^+$ cells were also significantly increased 2 and 7 days after IRI induction. Significantly more $CD3^+$ cells infiltrated the kidneys on *day 7* in all groups examined after IRI induction compared with sham-operated mice. Furthermore, $CD3^+$ and $CD4^+$ T cells in CD4xNEMO$^{\Delta}$ mice were significantly increased compared with CD4xIKK2$^{\Delta}$ and CD4cre mice on *day 7* after IRI induction. *B*: IFNγ-producing $CD4^+$ T cells (Th1) were significantly increased only in CD4xIKK2$^{\Delta}$ mice at 12 h, at 2 days in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice, and at 7 days only in CD4xNEMO$^{\Delta}$ animals compared with CD4cre mice after IRI. *C*: percentage of Th17 cells infiltrating the kidneys was significantly increased in both CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice at all time points examined compared with CD4cre mice after IRI induction. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, CD4IKK2 (CD4crexIKK2$^{\Delta}$), CD4NEMO (CD4crexNEMO$^{\Delta}$).

determine the percentage of Th1 and Th17 cells, the cells were activated by incubation for 3 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck). After 30 min of incubation, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. After exclusion of doublets, live/dead staining (Invitrogen Molecular Probes, Eugene, OR) was used to exclude dead cells. Cells in all FACS plots were pregated on CD45 expression. Experiments were performed on a BD LSRII Cytometer.

Quantitative real-time PCR analysis. Total RNA of the renal cortex was prepared, and real-time PCR was performed according to standard laboratory methods and as described in detail previously (11). The primer sequences are as follows: 18S, forward: CAC GGC CGG TAC AGT GAA AC, reverse: AGA GGA GCG AGC GAC CAA A; TNF-α, forward: AAA TGG CCT CCC TCT CAT CAG T, reverse: GCT TGT CAC TCG AAT TTT GAG AAG; IL-1β, forward: CCT TCC AGG ATG AGG ACA TGA, reverse: TCA TCC CAT GAG TCA CAG AGG AT; CCL2, forward: GGC TCA GCC AGA TGC AGT TAA, reverse: CCT ACT CAT TGG GAT CAT CTT GCT; and CCL20, forward: GGA TAC ACA ATA GGA GTA TG, reverse: CCT CAG GAT GTA GGA GGA AG.

Statistical analyses. The results are expressed as means ± SM. Groups were compared by Student's *t*-test, and a P value <0.05 was considered statistically significant.

RESULTS

IKK2 or NEMO deletion in lymphocytes aggravates kidney damage after IRI. $CD4^+$ T cells are important mediators of kidney damage in the renal ischemia-reperfusion injury model (30, 42). We and others have shown previously that NF-κB is activated during IRI (14), and therefore we hypothesized that inhibition of NF-κB activation by deletion of IKK2 or NEMO in lymphocytes would reduce kidney damage and improve renal function after IRI induction. With the use of $CD4xIKK2^{\Delta}$, $CD4xNEMO^{\Delta}$, and CD4cre control mice, our results demonstrated impaired kidney function, as determined by significantly increased serum creatinine and BUN after IRI induction compared with sham-operated mice (Fig. 1, *A* and *B*). However, at the early time point, 12 h after IRI, there was no significant difference among the three groups of mice. Surprisingly, in $CD4xIKK2^{\Delta}$ and $CD4xNEMO^{\Delta}$ mice, the serum creatinine and BUN levels were significantly increased on *day 2* after IRI induction compared with CD4cre mice (Fig. 1, *A* and *B*). At 7 days after reperfusion, the creatinine and BUN levels were significantly increased only in $CD4xNEMO^{\Delta}$, but not in $CD4xIKK2^{\Delta}$, mice compared with

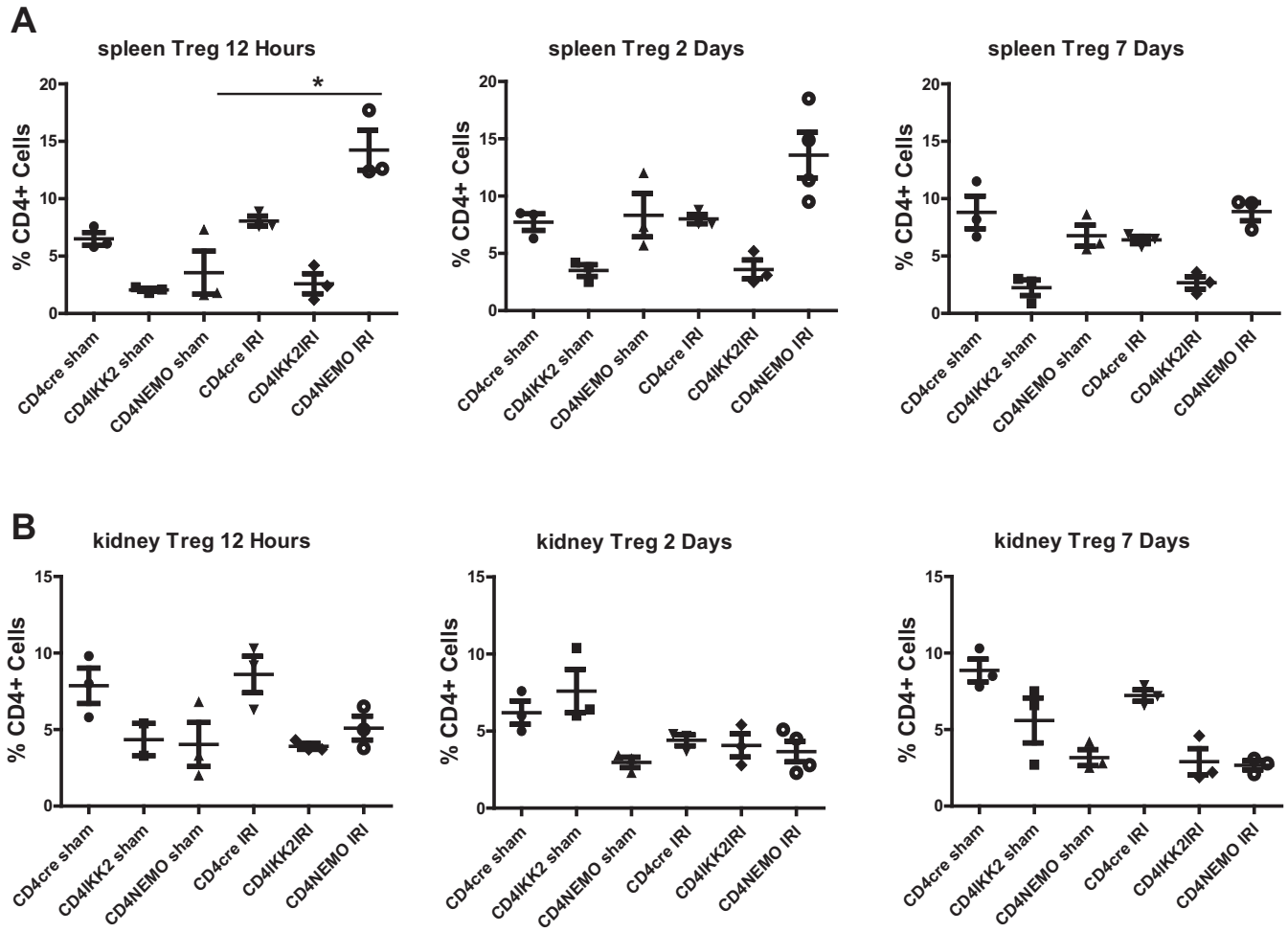

Fig. 3. Regulatory T cells (Tregs) in the kidney and spleen. *A*: the frequency of Tregs in the spleen was significantly increased in $CD4xNEMO^{\Delta}$ mice compared with CD4cre controls at 12 h but not at the other time points after IRI. *B*: percentage of Tregs in the kidneys was not changed significantly after induction of IRI; however, it was significantly reduced in both $CD4xIKK2^{\Delta}$ and $CD4xNEMO^{\Delta}$ mice compared with CD4cre mice. $^*P < 0.05$, CD4IKK2 ($CD4crexIKK2^{\Delta}$), CD4NEMO ($CD4crexNEMO^{\Delta}$).

CD4cre mice (Fig. 1, *A* and *B*). Morphological examinations have shown flattening of the tubular epithelial cells and increased tubular necrosis after IRI induction (Fig. 1*C*), as described previously by us and others (14). The tubular damage score was significantly increased in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre control mice 2 days after IRI induction (Fig. 1*C*) but not, however, at 12 h and 7 days (data not shown).

More T lymphocytes infiltrate the kidneys of CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice after the induction of IRI. To further understand the mechanisms relevant to the reduced renal function in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre control mice, the lymphocytes infiltrating the kidneys, which have been shown previously by others to play a pivotal role after induction of IRI, were examined (15).

The total number of CD3^{+} cells infiltrating the kidneys was significantly increased 2 and 7 days after IRI induction, as determined by immunohistology (Fig. 2*A*). With the use of the absolute number of CD3^{+} cells counted from immunohistology and the percentage of CD4^{+} T cells, as determined by FACS, the absolute number of CD4^{+} T cells infiltrating the kidneys was calculated. The results showed a significant increase in ischemic CD4cre mice compared with sham-operated animals at all of the examined time points (Fig. 2*A*). More importantly, there were significantly more CD3^{+} and CD4^{+} T cells in CD4xNEMO$^{\Delta}$ mice compared with CD4xIKK2$^{\Delta}$ and CD4cre mice on *day 7* after IRI induction (Fig. 2*A*).

To understand whether CD4^{+} T cell subpopulations were affected after the induction of IRI, the time course of different subsets of renal CD4^{+} T cells was examined. The frequency of Th1 cells, which was determined as IFNγ-producing CD4^{+} T cells, was increased after IRI induction in the kidneys of all groups examined compared with the sham-operated mice. At

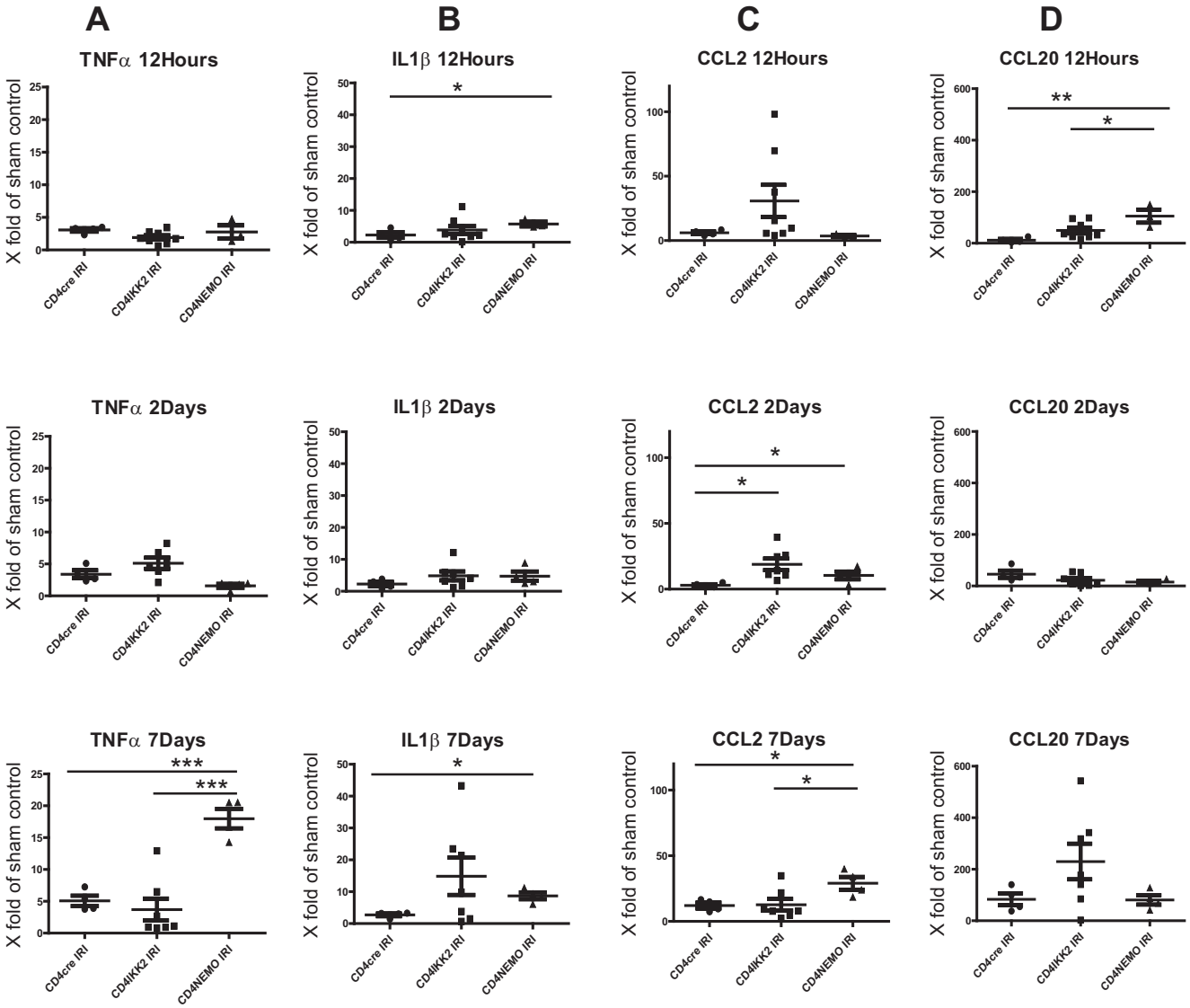

Fig. 4. Renal cytokine profiles showed different expression patterns after IRI. *A*: TNF-α expression: in CD4xNEMO$^{\Delta}$ mice, TNF-α expression was significantly increased at 7 days after reperfusion, but not at 12 h and 2 days, compared with CD4cre and CD4crexIKK2$^{\Delta}$ animals. *B*: IL-1β expression was significantly increased at 12 h and 7 days in CD4xNEMO$^{\Delta}$ mice compared with CD4cre mice. *C*: CCL2 expression was significantly increased at 2 and 7 days in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre mice. *D*: CCL20 expression was significantly increased 12 h after IRI in CD4xNEMO$^{\Delta}$ animals compared with CD4cre mice. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, CD4IKK2 (CD4crexIKK2$^{\Delta}$), CD4NEMO (CD4crexNEMO$^{\Delta}$).

the early time point, 12 h after IRI induction, the frequency of Th1 cells was significantly increased in CD4xIKK2$^{\Delta}$ mice, and, at 2 days, the frequency of Th1 cells was significantly increased in both CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice. However, at the later time point, 7 days, the frequency of Th1 cells was significantly increased in CD4xNEMO$^{\Delta}$ mice only compared with CD4cre mice (Fig. 2*B*). The frequency of Th17 cells, which was determined as IL17A-producing CD4^{+} T cells, was significantly increased in all groups at 2 and 7 days compared with sham-operated animals, whereas no difference was observed at 12 h. In CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice, the frequency of Th17 cells was also significantly increased compared with the CD4cre controls at all time points examined after IRI induction (Fig. 2*C*).

To further analyze whether the increased frequency of Th17 cells correlated with reduced regulatory T cells (Tregs), the frequency of Tregs was also determined in the spleens and kidneys. In general, the percentage of Tregs was not significantly increased in the spleens of CD4xNEMO$^{\Delta}$ mice compared with those of the sham-operated mice, except at 12 h (Fig. 3*A*). The percentage of Tregs in the kidneys as determined by FACS was reduced, albeit not significantly, in both CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre mice at all time points examined after IRI induction (Fig. 3*B*). Foxp3 staining by immunohistochemistry also showed that the number of Tregs after IRI induction in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice was very low both in the interstitium of the renal cortex and the outer medulla in CD4cre mice; however, more Tregs were present after IRI induction compared with sham-operated animals, which is consistent with the results of our FACS analysis (data not shown).

Renal cytokine profiles change after kidney IRI. To further investigate the nature of the renal inflammatory immune response in mice with IKK2 or NEMO deletion in lymphocytes, the mRNA expression of cytokines and chemokines was determined in the renal samples.

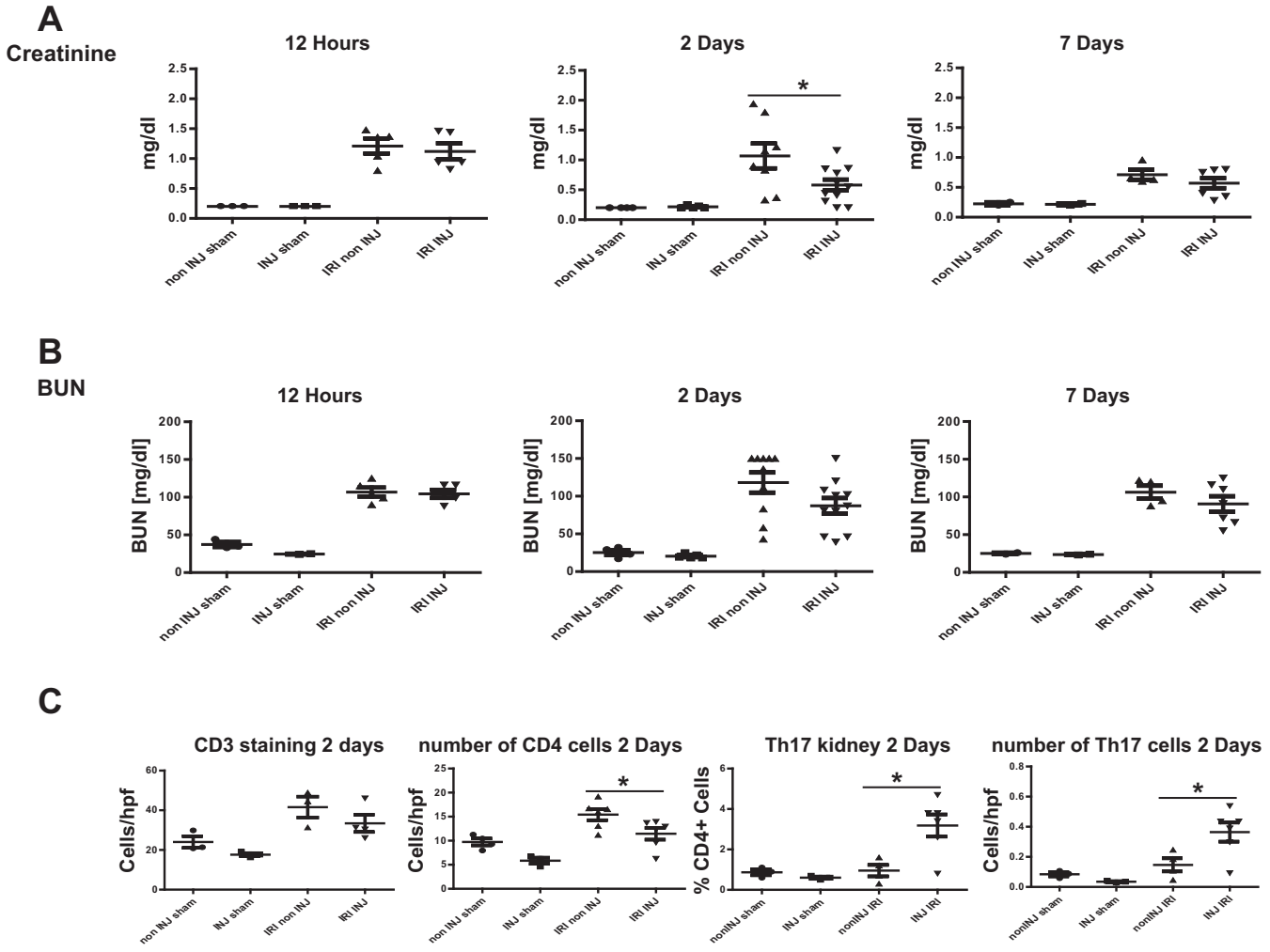

Fig. 5. Outcome of injection of the systematic IKK2 inhibitor KINK-1 differs from transgenic mice. *A*: serum creatinine; *B*: BUN. At 2 days after IRI creatinine was significantly reduced, whereas BUN was not significantly reduced in KINK-1-pretreated mice compared with nontreated animals, not, however, at 12 h and 7 days after IRI induction. *C*: number of CD3^{+} cells, as determined by immune histology, significantly increased after induction of IRI compared with sham-operated mice; however, no significant difference was found between the KINK-1-treated and nontreated groups. The number of CD4^{+} T cells as recalculated by combining FACS data and the number of CD3^{+} cells were significantly decreased in ischemic mice with KINK-1 pretreatment at 2 days. The number and percentage of Th17 cells were significantly increased in the mice treated with KINK-1 at 2 days after the induction of IRI. *$P < 0.05$. Four groups of animals were examined as follows: *1*) sham-operated animals without KINK-1 treatment (non-INJ sham), *2*) sham-operated animals with KINK-1 pretreatment (INJ sham), *3*) ischemic mice without KINK-1 treatment (IRI non-INJ), and *4*) ischemic mice with KINK-1 pretreatment (IRI INJ).

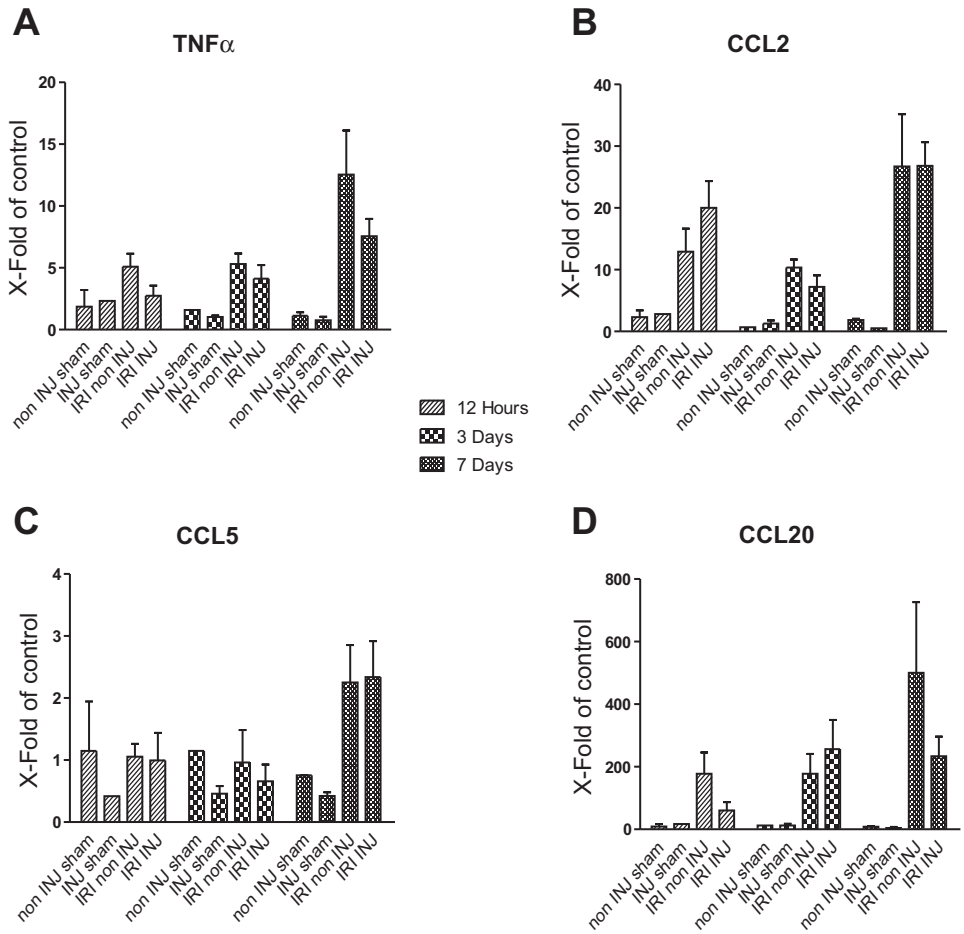

Fig. 6. Renal cytokine profiles showed different expression patterns after IRI. TNF-α (*A*), CCL2 (*B*), and CCL20 (*C*) expression was significantly increased after IRI induction compared with sham-operated mice at all time points examined, and CCL5 (*D*) expression significantly increased at later time points only. KINK-1 pretreatment before induction of IRI did in general not change renal cytokine or chemokine expression significantly after IRI induction.

The expression of all examined cytokines and chemokines was increased compared with sham-operated mice at all time points examined. The TNF-α levels were significantly higher in CD4xNEMO$^{\Delta}$ mice on *day 7* but not at 12 h or 2 days after IRI induction (Fig. 4*A*). The expression of IL-1β, known to be important for promoting IL-17 production in CD4$^+$ T cells, was significantly increased in CD4xNEMO$^{\Delta}$ mice 12 h and 7 days after reperfusion but not, however, at 2 days (Fig. 4*B*). The expression of the chemokine CCL2 (MCP-1), a chemoattractant mainly for T lymphocytes and monocytes, was significantly elevated 2 days after IRI in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice but only at 7 days in CD4xNEMO$^{\Delta}$ mice compared with CD4cre controls (Fig. 4*C*). CCL20 expression, an important chemokine for Th17 cell recruitment, was significantly increased at 12 h in both CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre mice but not, however, at later time points (Fig. 4*D*). These data suggested that the loss of IKK2 or NEMO in lymphocytes significantly altered the inflammatory response of the total renal tissue after IRI.

The IKK2-specific inhibitor KINK-1 improves renal function after IRI. Cell-specific deletion of IKK2 or NEMO in lymphocytes did not improve kidney function in IRI. Therefore, to understand whether systemic IKK2 inhibition functions differently, KINK-1, a specific IKK2 inhibitor, was given before and after the induction of IRI. Because of practical issues to obtain NEMO inhibitors, systemic inhibition of NEMO was not examined.

In mice treated with KINK-1 before the induction of IRI, serum creatinine was significantly reduced at 2 days compared with nontreated mice but not at 12 h and 7 days after the induction of injury (Fig. 5*A*). BUN level in KINK-1-treated animals was also lower than in nontreated animals at 2 days not, however, at 12 h and 7 days after the induction of injury (Fig. 5*B*).

To understand whether there was a difference between KINK-1 treatment and specific deletion of IKK2 regarding renal tissue infiltrating lymphocytes, the number of CD3$^+$ cells was counted, and the subsets were determined (Fig. 5*C*). Our results showed a significant increase of CD3$^+$ cells in kidneys compared with sham-operated mice; however, no significant difference was found between KINK-1-treated and nontreated mice. Nevertheless, KINK-1 treatment significantly reduced the number of renal CD4$^+$ T cells (Fig. 5*C*). More important, frequency and numbers of IL17A-producing CD4$^+$ T cells infiltrating the kidney after IRI were significantly increased in KINK-1-pretreated animals compared with nontreated animals at 2 days after IRI induction (Fig. 5*C*).

TNF-α, CCL2, and CCL20 expression was significantly increased after IRI induction compared with sham-operated mice at all time points examined, and CCL5 expression significantly increased at later time points only. KINK-1 treatment before induction of IRI did in general not change renal cytokine or chemokine expression significantly after IRI induction (Fig. 6, *A–D*).

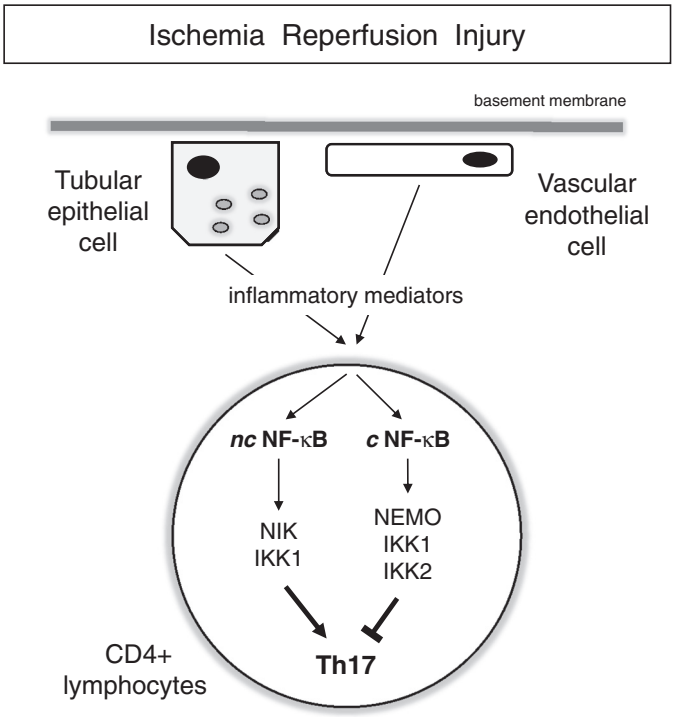

Fig. 7. Summary of the role of IKK2 and NEMO in Th17 induction. Induction of IRI in the kidney induces the release of inflammatory mediators such as cytokines and chemokines from tubular epithelial and vascular endothelial cells to attract inflammatory cells such as $CD4^+$ lymphocytes in the kidney. The canonical (c) and noncanonical (nc) NF-κB pathways are activated in the intrarenal $CD4^+$ lymphocytes and induce Th17 cell differentiation. Via NF-κB cross talk, the canonical pathway inhibits noncanonical pathway-induced Th17 cell differentiation.

DISCUSSION

The NF-κB pathway is activated during IRI, as shown previously by us and others (14), and it has been demonstrated that $CD4^+$ T cells play a pivotal role in this process (41, 42). We therefore examined the role of IKK2 and NEMO in $CD4^+$ lymphocytes after the induction of IRI. Our data demonstrated impaired kidney function, increased tubular damage, and increased renal inflammatory cytokine and chemokine expression in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre controls. Our results are in contrast to recently published data that demonstrated that tubular epithelial-specific NF-κB inhibition attenuated renal damage after ischemic AKI (24). Because it is well known that the NF-κB pathway plays an important role in proinflammatory and anti-inflammatory processes (17), inhibition of NF-κB activation exclusively in renal tubular epithelial cells reduced proinflammatory cytokine and chemokine expression and induced less inflammation in the kidneys, as shown by Marko et al. (24). However, in our experiments, NF-κB activation was inhibited only in $CD4^+$ lymphocytes, which resulted in the increased expression of proinflammatory cytokines and chemokines in the renal tissue after IRI induction. Systemic IKK2 inhibition also significantly reduced serum creatinine and BUN in ischemic mice treated with the IKK2 inhibitor. Our data in part confirm the data published by Marko et al. (24) that indicate potential differences between cell-specific and systemic NF-κB inhibition, as has been addressed previously in the gut and other tissues (4, 28). Therefore, systemic KINK-1 application has more beneficial effects on tubular epithelial cells than on infiltrating lymphocytes.

To further examine the relevant mechanisms responsible for deteriorated kidney function in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre controls, infiltrating lymphocytes in the kidneys were analyzed because IKK2- and NEMO-mediated NF-κB pathway activation has been demonstrated to be important for T cell development and maturation (9, 25, 36). Our data showed significant differences in total renal $CD3^+$ and $CD4^+$ T cells after IRI induction in the three groups examined compared with sham-operated animals, whereas more $CD4^+$ T cells were present in the kidneys of CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre controls. However, we could not show a significant increase in Tregs in CD4cre control mice after IRI induction, in contrast to the data published by Gandolfo et al. (8). One possible reason is that the model is different; we did observe that the frequency of Tregs in the spleen was significantly increased compared with sham-operated mice, so it is likely that our observation period was too short, and it could be possible that more Tregs would infiltrate in the kidneys at later time points after 7 days. The major finding in our model was that Tregs were significantly reduced in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with sham-operated mice and also in mice after IRI induction compared with CD4cre animals, which demonstrates that IKK2 and NEMO activity in $CD4^+$ T cells plays a pivotal role in Treg induction. It has recently been demonstrated by us and others that peripheral Treg proliferation and maturation critically depends on IKK2 activity and NF-κB pathway activation (13). Because the frequency of Tregs in the spleens of CD4xNEMO$^{\Delta}$ mice was significantly increased compared with CD4xIKK2$^{\Delta}$ and CD4cre mice after IRI induction and also compared with sham-operated mice, we assume that Tregs might be differentially regulated by IKK2 and NEMO activity in the spleens and kidneys of mice with $CD4^+$ T cell-specific deletion of IKK2 and NEMO.

Next, to further understand the relevant mechanisms of decreased renal function in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice compared with CD4cre controls, the frequency of Th17 cells in the kidneys was determined, which was significantly increased at 2 and 7 days after IRI in mice with IKK2 and NEMO deletion in $CD4^+$ T cells compared with CD4cre mice. Surprisingly, in the mice systemically treated with KINK-1 also a significantly higher frequency of renal Th17 cells than nontreated mice was found at 2 days after IRI induction. Our experiments therefore demonstrated that IKK2 and NEMO have negative regulatory effects on Th17 cell activation in the IRI model. NF-κB pathway activation in Th17 cell induction has been described recently. These studies have suggested that c-Rel controls Th17 cell differentiation by inducing RORγ and RORγt expression (7, 33). However, an anti-inflammatory role for IKK2 through the inhibition of "classical" pathway activation has also been described recently (6, 23). Because inhibition of the IKK2 or NEMO kinases of the canonical NF-κB pathway induces Th17 cell activation in our IRI model, we reason that activation of IKK2 or NEMO in lymphocytes inhibits Th17 cell differentiation, and noncanonical NF-κB activation must mediate Th17 cell induction in this model in addition to other factors not addressed in our experiments.

The NF-κB-inducing kinase (NIK), regulating the noncanonical NF-κB pathway, has been shown to be activated in an

experimental IRI model and also in patients with delayed graft function after kidney transplantation (20). NIK-induced IKK1 activity has been demonstrated in T cells expressing a nonactivatable form of IKK1 to increase Th17 cell numbers (18), and nuclear IKK1 maintains the Th17 phenotype by activating the Il17a gene (13). Cross talk between noncanonical and canonical NF-κB activation has been described recently (12), and canonical NF-κB signaling enhances RelB, which in turn inhibits noncanonical NF-κB-dependent gene expression (22). Therefore, these findings demonstrated that the classical pathway negatively regulates noncanonical NF-κB-dependent gene expression and further supported our results in the IRI model indicating that, at 7 days, increased numbers of Th17 cells in CD4xIKK2$^{\Delta}$ and CD4xNEMO$^{\Delta}$ mice might be due to additional IKK2- and NEMO-mediated regulation of the IKK1 pathway, as has been demonstrated recently (27, 38).

The new concept of necroinflammation has been introduced recently as a unifying theory of bidirectional causality between kidney injury and inflammation to integrate tissue injury, necrosis, and inflammation conceptually (16, 25). Dying tubular epithelial cells or T cells initiate adaptive immunity by providing both antigens and inflammatory stimuli for dendritic cells, and it has been demonstrated recently that coordinated inflammatory and cell death signaling pathways within dying cells orchestrate adaptive immunity, linking both events to NF-κB activation (31). However, in RIPK1 kinase-dependent apoptosis or necroptosis, an unexpected NF-κB-independent role for the IKK complex in protecting cells from RIPK1-dependent death downstream of TNFR1 has also been described recently (3, 11). These recent findings demonstrate that the newly described concept of necroinflammation plays a pivotal role in renal injury models such as ischemia-reperfusion injury and will help to explain different outcomes of renal injury in which NF-κB activation is exclusively inhibited in renal tubular cells, as described by Marko et al. (24), or selectively in T cells, as described in our study. However, the exact cellular mechanisms and the role the NF-κB pathway might play in tubular epithelial cells or inflammatory cells, such as Th17 cells infiltrating the kidney, in inducing necroptosis warrants further investigation.

In conclusion, based on our data, we propose the following model of NF-κB pathway-induced Th17 cell differentiation in the IRI model of sterile inflammation (Fig. 7): *1*) renal tubular epithelial cells and vascular endothelial cells release cytokines and chemokines after reperfusion that attract and stimulate CD4^{+} lymphocytes, and *2*) noncanonical NF-κB activation directs CD4^{+} lymphocytes toward a Th17 cell immune response that is inhibited by the IKK2- and NEMO-mediated canonical NF-κB pathway. Thus, distinct mechanisms via the canonical and noncanonical NF-κB pathways regulate Th17 generation in the IRI model, and, therefore, IKK2 and NEMO inhibitors act on multiple layers of the signaling pathway and thereby function as positive and negative regulators of Th17 cell activation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.G. and F.T. conception and design of research; L.G., H.H.L., and F.T. performed experiments; L.G., H.H.L., M.d.l.M.N., H.-J.P., G.Z., and F.T. analyzed data; L.G., M.d.l.M.N., H.-J.P., G.Z., and F.T. interpreted results of experiments; L.G. prepared figures; L.G., H.-J.P., G.Z., and F.T. drafted manuscript; L.G. and F.T. edited and revised manuscript; F.T. approved final version of manuscript.

REFERENCES

1. **Basile DP, Bonventre JV, Mehta R, Nangaku M, Unwin R, Rosner MH, Kellum JA, Ronco C, Group AXW.** Progression after AKI: understanding maladaptive repair processes to predict and identify therapeutic treatments. *J Am Soc Nephrol* 27: 687–697, 2016.
2. **Cao CC, Ding XQ, Ou ZL, Liu CF, Li P, Wang L, Zhu CF.** In vivo transfection of NF-kappaB decoy oligodeoxynucleotides attenuate renal ischemia/reperfusion injury in rats. *Kidney Int* 65: 834–845, 2004.
3. **Chan AJ, Alikhan MA, Odobasic D, Gan PY, Khouri MB, Steinmetz OM, Mansell AS, Kitching AR, Holdsworth SR, Summers SA.** Innate IL-17A-producing leukocytes promote acute kidney injury via inflammasome and Toll-like receptor activation. *Am J Pathol* 184: 1411–1418, 2014.
4. **Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M.** The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med* 9: 575–581, 2003.
5. **Feng M, Li G, Qian X, Fan Y, Huang X, Zhang F, Lu L.** IL-17A-producing NK cells were implicated in liver injury induced by ischemia and reperfusion. *Int Immunopharmacol* 13: 135–140, 2012.
6. **Fong CH, Bebien M, Didierlaurent A, Nebauer R, Hussell T, Broide D, Karin M, Lawrence T.** An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation. *J Exp Med* 205: 1269–1276, 2008.
7. **Gaffen SL, Jain R, Garg AV, Cua DJ.** The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol* 14: 585–600, 2014.
8. **Gandolfo MT, Jang HR, Bagnasco SM, Ko GJ, Agreda P, Satpute SR, Crow MT, King LS, Rabb H.** Foxp3+ regulatory T cells participate in repair of ischemic acute kidney injury. *Kidney Int* 76: 717–729, 2009.
9. **Gerondakis S, Fulford TS, Messina NL, Grumont RJ.** NF-kappaB control of T cell development. *Nat Immunol* 15: 15–25, 2014.
10. **Ghosh S, Hayden MS.** New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* 8: 837–848, 2008.
11. **Gotot J, Piotrowski E, Otte MS, Tittel AP, Linlin G, Yao C, Ziegelbauer K, Panzer U, Garbi N, Kurts C, Thaiss F.** Inhibitor of NFkappaB kinase subunit 2 blockade hinders the initiation but aggravates the progression of crescentic GN. *J Am Soc Nephrol* 27: 1917–1924, 2016.
12. **Gray CM, Remouchamps C, McCorkell KA, Solt LA, Dejardin E, Orange JS, May MJ.** Noncanonical NF-kappaB signaling is limited by classical NF-kappaB activity. *Sci Signal* 7: ra13, 2014.
13. **Guckel E, Frey S, Zaiss MM, Schett G, Ghosh S, Voll RE.** Cell-intrinsic NF-kappaB activation is critical for the development of natural regulatory T cells in mice. *PLoS One* 6: e20003, 2011.
14. **Kezic A, Becker JU, Thaiss F.** The effect of mTOR-inhibition on NF-kappaB activity in kidney ischemia-reperfusion injury in mice. *Transplant Proc* 45: 1708–1714, 2013.
15. **Kinsey GR, Okusa MD.** Expanding role of T cells in acute kidney injury. *Curr Opin Nephrol Hypertens* 23: 9–16, 2014.
16. **Kusch A, Hoff U, Bubalo G, Zhu Y, Fechner M, Schmidt-Ullrich R, Marko L, Muller DN, Schmidt-Ott KM, Gurgen D, Blum M, Schunck WH, Dragun D.** Novel signalling mechanisms and targets in renal ischaemia and reperfusion injury. *Acta Physiol (Oxf)* 208: 25–40, 2013.
17. **Lawrence T.** The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 1: a001651, 2009.
18. **Li L, Ruan Q, Hilliard B, Devirgiliis J, Karin M, Chen YH.** Transcriptional regulation of the Th17 immune response by IKK(alpha). *J Exp Med* 208: 787–796, 2011.
19. **Liao YH, Xia N, Zhou SF, Tang TT, Yan XX, Lv BJ, Nie SF, Wang J, Iwakura Y, Xiao H, Yuan J, Jevallee H, Wei F, Shi GP, Cheng X.** Interleukin-17A contributes to myocardial ischemia/reperfusion injury by

regulating cardiomyocyte apoptosis and neutrophil infiltration. *J Am Coll Cardiol* 59: 420–429, 2012.

20. **Loverre A, Ditonno P, Crovace A, Gesualdo L, Ranieri E, Pontrelli P, Stallone G, Infante B, Schena A, Di Paolo S, Capobianco C, Ursi M, Palazzo S, Battaglia M, Selvaggi FP, Schena FP, Grandaliano G.** Ischemia-reperfusion induces glomerular and tubular activation of proinflammatory and antiapoptotic pathways: differential modulation by rapamycin. *J Am Soc Nephrol* 15: 2675–2686, 2004.
21. **Luo JL, Kamata H, Karin M.** IKK/NF-kappaB signaling: balancing life and death–a new approach to cancer therapy. *J Clin Invest* 115: 2625–2632, 2005.
22. **Madge LA, May MJ.** Classical NF-kappaB activation negatively regulates noncanonical NF-kappaB-dependent CXCL12 expression. *J Biol Chem* 285: 38069–38077, 2010.
23. **Mankan AK, Canli O, Schwitalla S, Ziegler P, Tschopp J, Korn T, Greten FR.** TNF-alpha-dependent loss of IKKbeta-deficient myeloid progenitors triggers a cytokine loop culminating in granulocytosis. *Proc Natl Acad Sci USA* 108: 6567–6572, 2011.
24. **Marko L, Vigolo E, Hinze C, Park JK, Roel G, Balogh A, Choi M, Wubken A, Cording J, Blasig IE, Luft FC, Scheidereit C, Schmidt-Ott KM, Schmidt-Ullrich R, Muller DN.** Tubular epithelial NF-kappaB activity regulates ischemic AKI. *J Am Soc Nephrol* 27: 2658–2669, 2016.
25. **Miller ML, Mashayekhi M, Chen L, Zhou P, Liu X, Michelotti M, Tramontini Gunn N, Powers S, Zhu X, Evaristo C, Alegre ML, Molinero LL.** Basal NF-kappaB controls IL-7 responsiveness of quiescent naive T cells. *Proc Natl Acad Sci USA* 111: 7397–7402, 2014.
26. **Molitoris BA.** Actin cytoskeleton in ischemic acute renal failure. *Kidney Int* 66: 871–883, 2004.
27. **Oeckinghaus A, Hayden MS, Ghosh S.** Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* 12: 695–708, 2011.
28. **Pasparakis M.** Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. *Nat Rev Immunol* 9: 778–788, 2009.
29. **Paust HJ, Turner JE, Riedel JH, Disteldorf E, Peters A, Schmidt T, Krebs C, Velden J, Mittrucker HW, Steinmetz OM, Stahl RA, Panzer U.** Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis. *Kidney Int* 82: 72–83, 2012.
30. **Pinheiro HS, Camara NO, Noronha IL, Maugeri IL, Franco MF, Medina JO, Pacheco-Silva A.** Contribution of CD4+ T cells to the early mechanisms of ischemia-reperfusion injury in a mouse model of acute renal failure. *Braz J Med Biol Res* 40: 557–568, 2007.
31. **Rabb H, Griffin MD, McKay DB, Swaminathan S, Pickkers P, Rosner MH, Kellum JA, Ronco C, Acute Dialysis Quality Initiative Consensus.** Inflammation in AKI: current understanding, key questions, and knowledge gaps. *J Am Soc Nephrol* 27: 371–379, 2016.
32. **Rong S, Park JK, Kirsch T, Yagita H, Akiba H, Boenisch O, Haller H, Najafian N, Habicht A.** The TIM-1:TIM-4 pathway enhances renal ischemia-reperfusion injury. *J Am Soc Nephrol* 22: 484–495, 2011.
33. **Ruan Q, Kameswaran V, Zhang Y, Zheng S, Sun J, Wang J, DeVirgiliis J, Liou HC, Beg AA, Chen YH.** The Th17 immune response is controlled by the Rel-RORgamma-RORgamma T transcriptional axis. *J Exp Med* 208: 2321–2333, 2011.
34. **Sharfuddin AA, Molitoris BA.** Pathophysiology of ischemic acute kidney injury. *Nat Rev Nephrol* 7: 189–200, 2011.
35. **Sharma AK, LaPar DJ, Zhao Y, Li L, Lau CL, Kron IL, Iwakura Y, Okusa MD, Laubach VE.** Natural killer T cell-derived IL-17 mediates lung ischemia-reperfusion injury. *Am J Respir Crit Care Med* 183: 1539–1549, 2011.
36. **Silva A, Cornish G, Ley SC, Seddon B.** NF-kappaB signaling mediates homeostatic maturation of new T cells. *Proc Natl Acad Sci USA* 111: E846–E855, 2014.
37. **Tan Z, Jiang R, Wang X, Wang Y, Lu L, Liu Q, Zheng SG, Sun B, Ryffel B.** RORgammat+IL-17+ neutrophils play a critical role in hepatic ischemia-reperfusion injury. *J Mol Cell Biol* 5: 143–146, 2013.
38. **Temmerman ST, Ma CA, Zhao Y, Keenan J, Aksentijevich I, Fessler M, Brown MR, Knutsen A, Shapiro R, Jain A.** Defective nuclear IKKalpha function in patients with ectodermal dysplasia with immune deficiency. *J Clin Invest* 122: 315–326, 2012.
39. **Wang JM, Wang XY, Hussain S, Zheng Y, Sanjabi S, Ouaaz F, Beg AA.** Distinct roles of different NF-kappa B subunits in regulating inflammatory and T cell stimulatory gene expression in dendritic cells. *J Immunol* 178: 6777–6788, 2007.
40. **Yang L, Brooks CR, Xiao S, Sabbisetti V, Yeung MY, Hsiao LL, Ichimura T, Kuchroo V, Bonventre JV.** KIM-1-mediated phagocytosis reduces acute injury to the kidney. *J Clin Invest* 125: 1620–1636, 2015.
41. **Yang Z, Sharma AK, Linden J, Kron IL, Laubach VE.** CD4+ T lymphocytes mediate acute pulmonary ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 137: 695–702, 2009.
42. **Ysebaert DK, De Greef KE, De Beuf A, Van Rompay AR, Vercauteren S, Persy VP, De Broe ME.** T cells as mediators in renal ischemia/reperfusion injury. *Kidney Int* 66: 491–496, 2004.
43. **Ziegelbauer K.** A dual labelling method for measuring uptake of low molecular weight compounds into the pathogenic yeast Candida albicans. *Med Mycol* 36: 323–330, 1998.
44. **Ziegelbauer K, Babczinski P, Schonfeld W.** Molecular mode of action of the antifungal beta-amino acid BAY 10–8888. *Antimicrob Agents Chemother* 42: 2197–2205, 1998.