MAP3K14 promotes acute kidney injury

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ABSTRACT

An improved understanding of pathogenic pathways may identify novel acute kidney injury (AKI) therapeutic approaches. Unbiased LC-MS/MS protein expression profiling combined with focused data mining identified MAP3K14 and non-canonical NFκB activation at the crossroads of the enriched pathways MAPK, ubiquitin-mediated proteolysis, chemokines, NFκB and apoptosis in the kidney cortex of experimental toxic AKI. In AKI the upstream kinase MAP3K14, the NFκB DNA binding heterodimer RelB/NFκB2, and proteins involved in NFκB2 p100 ubiquitination and proteasomal processing to p52, such as Ube2m and cullin1 were up-regulated. Immunohistochemistry localized MAP3K14 expression to tubular cells in experimental and human AKI. In vivo evidence of MAP3K14 activation in experimental folic acid-induced AKI consisted of NFκB2 p100 processing to p52, nuclear location and DNA binding of RelB and NFκB2. MAP3K14 activity-deficient aly/aly mice were protected from kidney dysfunction, inflammation and apoptosis in AKI induced by folic acid and from lethality in cisplatin-induced AKI. MAP3K14 siRNA targeting in cultured tubular cells decreased inflammation and cell death. Bone marrow transplantation experiments where consistent with a protective effect of renal cell MAP3K14 targeting. Cell culture and in vivo studies identified chemokines MCP-1, RANTES and CXCL10 as MAP3K14 targets in tubular cells, thus identifying potential mediators of the deleterious effect of MAP3K14 in kidney injury. In conclusion, MAP3K14 promotes kidney injury through promotion of inflammation and cell death and is a promising novel therapeutic target.

Key words: acute kidney injury, apoptosis, inflammation, MAP3K14, NIK, non-canonical NFκB, tissue proteomics.

INTRODUCTION

The incidence of acute kidney injury (AKI) is increasing [1]. However, there is currently no therapy that reliably prevents the progression to AKI or accelerates recovery of renal function [2;3]. Thus, reliable biomarkers and novel therapeutic approaches are needed [4]. AKI is characterized by kidney inflammation and tubular cell death, dedifferentiation and subsequent proliferation [5-9]. However, given the complexity and redundancies of the process, it is unlikely that targeting a single inflammatory molecule provides the kind of benefit that will be clinically relevant. Thus, attention has focused on upstream signaling pathways that may regulate the coordinated expression of an array of inflammatory molecules. The combination of unbiased protein expression profiling with focused data mining is a powerful tool to expand our knowledge of relevant pathways and key factors in disease. Liquid chromatography tandem mass spectrometry (LC-MS/MS) identified ~2,000 proteins in murine renal cortex [10]. However, its applications to the study of AKI has been limited and mainly concentrated in the analysis of biofluids such as urine or in the study of the metabolome rather than the proteome [11-14]. To identify novel pathways and mediator networks active in AKI in a comprehensive manner, we used tissue LC-MS/MS to assess changes in the renal proteome of experimental toxic AKI. Bioinformatics analysis of 41235 peptides in cortical kidney tissue by LC-MS/MS proteomics allowed the identification of 6516 unique proteins, of which 1480 were differentially expressed in samples from experimental nephrotoxic AKI as compared with controls [15]. On this previously reported raw dataset, we have now performed novel pathway analysis in search of cell death or inflammatory pathways that are activated in AKI. This analysis indicated enrichment of proteins from the non-canonical activation pathway for transcription factor nuclear factor kappa-B (NFκB). NFκB promotes inflammation by modulating gene transcription [16;17]. Canonical NFκB activation is rapidly initiated through degradation of IκB proteins by the proteasome, thus releasing complexes that translocate to the nucleus to promote transcription of pro-inflammatory genes and downregulate the expression of anti-inflammatory molecules such as Klotho [18;19]. By contrast, non-canonical NFκB activation is a delayed response that is engaged by a limited number of stimuli and involves activation of the mitogen-activated protein kinase kinase kinase 14 (MAP3K14)/NFκB-inducing kinase (NIK), proteasomal processing of NFκB p100 to p52 and nuclear translocation of p52/RelB complexes [16;20]. The role and regulation of MAP3K14 in AKI is poorly understood.

The combined proteomic and bioinformatics approach enabled identification of evidence for MAP3K14 activation and the upregulation of several proteins of the non-canonical NFκB activation pathway in AKI that were confirmed by Western blot and immunohistochemistry. Functional studies identified chemokine expression and cell death and proliferation as novel MAP3K14-regulated processes in tubular cells. Furthermore, MAP3K14 was overexpressed during human AKI and genetically modified mice confirmed the key role of MAP3K14 in AKI.

RESULTS

Kidney tissue proteomics bioinformatics analysis identifies upregulation of MAP3K14 and non-canonical NFκB components in AKI

Experimental AKI is characterized by increased serum creatinine (0.53±0.25 vs 0.10±0.02 mg/dl at 24h, $p<0.05$), tubular cell death and interstitial inflammation [15]. As previously described, unbiased proteomics combined with focused data analysis was conducted in 24h kidney cortex control and AKI samples [15]. LC-MS/MS identified 41235 peptides in the kidney cortex corresponding to 6516 unique non-redundant, proteins (Supplementary **Figure 1)** [15]. The present study represents a new complimentary analysis of this previously generated dataset. KEGG pathway analysis identified the enrichment of several pathways based on the up-regulation of key proteins in AKI samples (**Table 1**). NFκB was at the crossroads of several of these pathways. NFκB activation is regulated by MAPK, requires ubiquitination and proteasomal processing or degradation, and regulates apoptosis and chemokine secretion. Canonically activated NFκB signaling has long been implicated in kidney injury [16]. However, there is much less information on non-canonical NFκB activation and its components. A targeted data mining approach searched for components of the non-canonical NFκB pathway. A KEGG generated NFκB signaling pathway map (Supplementary **Figure 2)** summarizes the expression of non-canonical NFκB signaling pathway components and of NFκB2 (p100/p52) ubiquitination and proteasomal activation. Upregulation was observed for MAP3K14, the essential upstream kinase activating the non-canonical NFκB pathway [21;22], for proteins required for NFκB2 p100 ubiquitination and proteasomal processing to active NFκB2 p52, such as Ube2m/Ubc12 (E2) and cullin-1 (E3), and for the two main components of non-canonical NFκB DNA-binding heterodimers, NFκB2 and RelB (**Table 2**).

Validation of non-canonical NFκB pathway activation in AKI

The proteomics findings of increased MAP3K14, RelB and NFκB2 p100/p52 were validated by Western blot and immunohistochemistry and the mechanisms by which the system is up-regulated were explored by assessing mRNA expression. Kidney MAP3K14, RelB and NFκB2 mRNA expression was increased in AKI, suggesting transcriptional up-regulation (**Figure 1.A, 2.A, 2.C**). Western blot confirmed increased kidney MAP3K14, RelB, NFκB2 p100 and NFκB2 p52 in AKI (**Figure 1.B, 2.B, 2.D, 2.E**). Immunohistochemistry localized the increased expression of these proteins to tubular cells **(Figure 1.C**). In addition, NFκB2 p52 and RelB DNA-binding activity was increased in nuclear extracts from AKI kidneys (**Figure 2.F**). Thus, evidence for increased activation of MAP3K14 includes processing of NFκB2 p100 to NFκB2 p52 and nuclear translocation and increased DNA binding activity of the RelB/NFκB2 p52 transcription factor. The expression of the ubiquitination pathway Cullin-1 protein was also confirmed to be increased in AKI **(Supplementary Figure 3).**

Given the poor understanding of its role in kidney injury and its upstream situation in the pathway, we further explored the role of MAP3K14 in AKI. In this regard, extensive MAP3K14 immunostaining was also observed in kidney tubules in human AKI (**Figure 3**).

MAP3K14 deficient mice were protected from AKI

To explore the role of MAP3K14 in AKI, we used MAP3K14 activity-deficient alymphoplasia ($MAP3K14^{aly/aly}$) mice, which carry a point mutation causing an amino acid substitution in the carboxy-terminal interaction domain of MAP3K14 [23;24]. $MAP3K14^{+/aly}$ heterozygote mice and $MAP3K14^{+/+}$ mice were used as controls.

$MAP3K14^{+/+}$ or $MAP3K14^{+/aly}$ heterozygote mice developed AKI characterized by increased serum creatinine and urea levels (**Figure 4.A,B and supplementary figure 4**) and increased kidney NFκB2 activation (**Figures 4.C,D and supplementary figure 4),** expression of chemokines (**Figure 4.E-G and supplementary figure 4**) and interstitial macrophage.

MAP3K14 deficient mice were protected from AKI. Serum creatinine and urea (**Figure 4.A,B and supplementary figure 4**) and kidney expression of NFκB2 p100/52 protein and mRNA (**Figure 4.C,D and supplementary figure 4**), MCP-1 (**Figure 4.E and supplementary figure 4**), RANTES (**Figure 4.F and supplementary figure 4**), CXCL10 (**Figure 4.G and supplementary figure 4**) and

CCL21a mRNA expression (**Figure 4.H and supplementary figure 4**) were lower than in control MAP3K14$^{+/+}$ mice or MAP3K14$^{+/aly}$ heterozygote mice with AKI.

Immunohistochemistry confirmed the lack of RelB **(Fig 5.A)** and NFκB2 p52 expression in MAP3K14 deficient mice with AKI **(Fig 5.B)** and disclosed decreased F4/80 macrophages and CD3 T lymphocytes **(Fig. 6.A and B)** and TUNEL positive tubular cells representing dying cells (**Fig 6.C**) in MAP3K14 deficient mice than in heterozygous controls with AKI.

We next induced AKI in bone marrow chimeras to test whether MAP3K14 deficiency in kidney cells or in bone marrow derived cells was responsible for nephroprotection. Mice on a MAP3K14alyaly background were protected from AKI-induced death when compared to MAP3K14+/+ mice and this was independent of the bone marrow genotype **(Supplementary figure 5).**

Finally, we tested a different model, cisplatin-induced AKI. MAP3K14 deficient mice were protected from mortality associated with cisplatin-induced AKI: 0/5 (0%) survival in MAP3K14$^{+/+}$ AKI mice vs 5/5 (100%) survival in MAP3K14 deficient AKI mice at day 3.

MAP3K14 function in tubular cells

Following the findings of MAP3K14 upregulation and of evidence for MAP3K14 activation (NFκB2 p100 processing to p52) in tubular cells in vivo, and a beneficial effect of MAP3K14 deficiency in vivo, the function of MAP3K14 was explored in cultured murine tubular epithelial cells by siRNA targeting (**Figure 7.A,B**). Since KEGG pathway analysis had identified chemokine signaling and apoptosis as enriched pathways and MAP3K14 deficiency indeed resulted in lower inflammation and tubular cell death in vivo, we explored the potential regulation of chemokine secretion and cell death by MAP3K14 in tubular cells. For this we took advantage of TWEAK, the only cytokine characterized to date to activate the non-canonical NFκB pathway in tubular cells [25]. In order to assess for further functions of MAP3K14 we explored canonical NFκB targets, including CXCL10, whose expression was recently related to MAP3K14 polymorphisms in human lymphoblastoid cells, but that had not previously been linked to MAP3K14 by functional studies [26]. MAP3K14 silencing by specific siRNAs prevented TWEAK-induced upregulation of CXCL10 mRNA (**Figure 7.C**) and protein (**Figure 7.D**) as well as of canonical NFκB targets such as MCP1 and RANTES [27] (**Figure 7.E, F, Suppl fig 6**). Differences in MCP-1 expression, which peaks earlier than RANTES were more evident at earlier time points (**Suppl fig 6**). In this regard some genes are targeted by both canonical and non-canonical NFκB, while others such

as CCL21 are specifically targeted by non-canonical NFκB activation in tubular and extrarenal cells [25;28;29].

Deprivation of the survival factors from serum is a classical inducer of apoptosis [30]. MAP3K14 targeting decreased apoptosis in tubular cells cultured in the absence of survival factors **(Figure 8).**

Discussion

For the first time we have uncovered evidence that MAP3K14 is a therapeutic target in kidney injury. A non-biased proteomics characterization of AKI kidneys disclosed enrichment for components of the non-canonical NFκB activation, chemokine and apoptosis pathways. Further studies evidenced activation of the apical kinase of the non-canonical NFκB pathway, and showed that in kidney tubular cells MAP3K14 regulates the expression of chemokines not previously associated with non-canonical NFκB, such as CXCL10, and cell survival. In vivo MAP3K14 targeting protected from AKI, improving renal function and decreasing inflammation and tubular cell death.

NFκB is a family of structurally homologous proteins, including NFκB1, NFκB2, RelA, RelB, and c-Rel, which form homo- or hetero-dimers that bind to κB enhancers in DNA to promote or inhibit gene transcription [16]. Two main pathways for NFκB activation are known. Canonical NFκB activation is usually a rapid, protein synthesis-independent and transient response to a wide range of stimuli that involves proteasomal degradation of cytosolic IκB inhibitory proteins leading to the release of RelA/p50 and other dimers that then migrate to the nucleus. NFκB-driven IκBα re-synthesis contributes to a fast turn-off of the response. By contrast, non-canonical NFκB activation requires MAP3K14 activation and NFκB2 p100 processing to p52 by the proteasome, resulting in a delayed nuclear translocation of RelB/p52 heterodimers and in prolonged activation of NFκB target genes [31-33]. Increased transcription of NFκB2 and RelB may contribute to persistence of the response [34]. By contrast to the canonical pathway, only a limited number of stimuli are known to activate the non-canonical NFκB pathway. These include advanced glycosylation end-products [33] and TNF receptor superfamily members such as lymphotoxin-β receptor, B-cell activating factor (BAFFR), CD40, receptor activator for NFκB (RANK), CD27 and the TWEAK receptor Fn14 [20;25;35]. None of these receptors or their ligands was identified in the proteomic analysis of kidney cortex. However, some of them had been previously shown to contribute to AKI. A literature search revealed a role in AKI for CD27 and TWEAK/Fn14 [36-38]. CD27 was localized to sloughed cells in tubular lumens post-ischemia and CD27-deficient mice were protected from AKI and

tubular cell apoptosis [36;37]. By contrast to this single report, multiple studies from several institutions have provided evidence for a role of TWEAK/FN14 in kidney injury [39]. Furthermore, TWEAK targeting decreased the expression of the non-canonical NFκB target CCL21 in tubular cells [25]. Thus for cell culture studies we chose TWEAK as a pathophysiologically relevant activator of the non-canonical NFκB pathway.

There is evidence for a role of canonical activation of NFκB in kidney injury [16]. However, no therapeutic approach specifically targeting NFκB systemically is undergoing clinical trials, suggesting a fundamental lack of understanding of the system. In this regard, the role of MAP3K14 and non-canonical NFκB activation in AKI has not been well characterized. There is very little and scattered information on activation of this pathway in kidney disease. MAP3K14 was phosphorylated in tubular cells during kidney ischemia-reperfusion [40] and levels were increased in experimental diabetic nephropathy and human delayed graft function [40;41]. TWEAK-dependent nuclear translocation of RelB and p52 was observed in tubular cells in nephrotoxic AKI [25]. In the present report, kidney tissue proteomics identified upregulation of several proteins in the non-canonical NFκB pathway, upregulation of these proteins was confirmed and localized to tubular cells, the contribution of transcriptional regulation was identified and the role of MAP3K14 in tubular cell injury was characterized. In this regard, MAP3K14 activity deficient mice were protected from AKI. Thus, MAP3K14 represents a key regulated step promoting AKI that may potentially be subject to therapeutic manipulation, although at present there are no satisfactory MAP3K14 inhibitors [42].

MAP3K14 is the essential upstream serine/threonine kinase of the non-canonical NFκB pathway that binds to TRAF2 and participates in NFκB signaling in response to the TNF superfamily and interleukin 1 receptors.[22]. MAP3K14 protein concentrations are low in quiescent cells as a result of rapid degradation. Cytokines and oxidative stress may increase MAP3K14 protein stability, leading to MAP3K14 activation [20]. In addition to this universal mechanism of MAP3K14 regulation, we now found increased steady-state MAP3K14 mRNA levels as an additional regulatory mechanism of MAP3K14 expression in tubular epithelium that also takes place in vivo during AKI. MAP3K14 induces IκB kinase-α (IKK-α)-mediated phosphorylation of NFκB2 p100, a prerequisite for p100 ubiquitination and subsequent proteasomal processing to active NFκB2 p52 [32].

Ubiquitination is required for targeting of specific proteins to the proteasome. F-box proteins provide specificity for substrate recognition in the S-phase kinase associated protein 1 (SKP1)-cullin 1

(CUL1)-F-box protein (SCF) family of the Cullin-RING ligases (CRL) E3 ubiquitin ligase superfamily [43]. The F-box protein β-transducin repeat containing (β-TrCP; FBW1A) provides substrate specificity for MAP3K14-phosphorylated p100, allowing ubiquitination by $SCF^{\beta\text{-}TrCP}$ [44-47]. Efficient NFκB2 p100 ubiquitination requires Uba3 (ube1c) and Ube2m (UBC12), ube2d3 (UBCH5c) and intact Cullin1 in $SCF^{\beta\text{-}TrCP}$ [45]. Interestingly, the Ube2m E2 ubiquitin conjugating enzyme and cullin-1 of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase were found to be upregulated in AKI.

Evidence for MAP3K14 activation in vivo in AKI included increased MAP3K14 levels, NFκB p100 processing to NFκB p52, increased nuclear localization and DNA binding activity of p52/RelB and decreased kidney inflammation and cell death and preserved renal function in MAP3K14 activity deficient mice. Protection from AKI may depend on systemic MAP3K14 deficiency (e.g. leukocyte MAP3K14 deficiency) and/or kidney MAP3K14 deficiency. Bone marrow transplantation experiments results are consistent with the hypothesis that renal cell MAP3K14 targeting is important for nephroprotection. In this regard the fact that non-renal cells also express MAP3K14 may result in undesired side effects when targeting MAP3K14 with small molecules. MAP3K14 and non-canonical NFκB gene targets have been characterized in the immune system, but there is little information on kidney cells [16]. We now provide evidence of a role of MAP3K14 in the regulation of the inflammatory and cell death/proliferation responses in tubular cells that together with the upregulation of MAP3K14 in tubular cells in AKI suggest at least a partial contribution of MAP3K14 targeting in tubular cells to the therapeutic responses.

The p52/RelB heterodimers characteristic of MAP3K14-initiated non-canonical NFκB activation share a number of gene targets with RelA-containing, classically activated NFκB complexes [16;29]. Since canonical NFκB activation is an early transient response peaking at around 1-3h while and non-canonical NFκB activation is delayed and peaks at around 24h, non-canonical NFκB activation by contribute to sustained NFκB-dependent gene expression [16;28;48]. In this regard, the CC genotype of the MAP3K14 SNP rs7222094 was recently associated with increased mortality and renal dysfunction in septic shock patients [26]. CXCL10 was the gene with the greatest difference in expression between major and minor MAP3K14 genotypes. The rs7222094 genotype strongly associated with decreased CXCL10 levels in lymphoblastoid cell lines and in septic shock patients [26]. Urinary CXCL10 is increased in AKI patients [49;50] and, as shown here, in AKI kidney tissue. We now provide for the first time direct functional evidence that persistent CXCL10 expression in response to TWEAK is regulated by MAP3K14. CXCL10

(IP-10) had long been associated to kidney injury in animals and humans [51;52]. MCP-1 and RANTES, which are targets of canonical NFκB activation [53] were also found to be MAP3K14-dependent in tubular cells. CCL21a was previously shown to be MAP3K14-dependent in this cell system [27]. Thus, a wide spectrum of chemokines, both commonly considered as canonical NFκB targets or non-canonical NFκB targets, is regulated by MAP3K14 in cultured tubular cells and during AKI.

KEGG pathway analysis also disclosed apoptosis pathways as overrepresented in the AKI proteome. MAP3K14 had been identified as a cell death regulator in cancer cells. Indeed, MAP3K14 siRNA targeting reduced serum deprivation-induced death in tubular cells. These results were consistent with decreased tubular cell apoptosis in vivo in MAP3K14 activity-deficient mice during AKI. These results are also consistent with observations targeting another component of the non-canonical NFκB pathway, RelB. Thus, RelB targeting by siRNA protected mice against lethal kidney ischemia [54] and in cultured proximal tubular cells, knockdown of RelB abrogated the excess apoptosis induced by TNF in combination with cisplatin [55].

In conclusion, preclinical functional studies in cell culture and in vivo identified MAP3K14 as a promising therapeutic target in kidney injury. In this regard MAP3K14 was upregulated during human kidney injury, suggesting that experimental findings may be applicable to the clinical settings. This information sets the stage for the exploration of the potential of MAP3K14 as a therapeutic target in humans.

Materials and methods

Animal model

Studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Folic acid nephropathy is a classical model of AKI that shares several features with human AKI, including tubular cell death, compensatory tubular cell proliferation, activation of an inflammatory response and eventual progression to mild fibrosis [27;56-58]. Indeed, folic acid nephropathy has been reported in humans [59]. C57/BL6 female mice (12- to 14-week-old) from the IIS-Fundacion Jimenez Diaz animal facilities received a single i.p. injection of folic acid (Sigma) 250 mg/kg in 0.3 mol/L sodium bicarbonate or vehicle and were sacrificed 24 h or 72 h after injection (n=6 per group). The kidneys were perfused in situ with cold saline before removal. Half-kidney from each mouse was fixed in buffered formalin, embedded in paraffin and used for immunohistochemistry and the other half was snap-frozen in

liquid nitrogen for RNA and protein studies. The cortex from one kidney obtained 24h after folic acid or vehicle injection was carefully separated and snap-frozen for proteomics analysis.

To assess the role of MAP3K14 in AKI, MAP3K14 $^{aly/aly}$ mice deficient in MAP3K14 and MAP3K14 $^{+/aly}$ heterozygote or MAP3K14 $^{+/+}$ mice used as controls received a single intraperitoneal injection of folic acid (Sigma) 250 mg/kg in 0.3 mol/L sodium bicarbonate or vehicle and were sacrificed 72 h after injection (n=5 per group). MAP3K14 $^{aly/aly}$ mice deficient in MAP3K14 were from the CBM, Madrid, Spain animal facilities [23].

A different model of AKI was induced by the intraperitoneal injection of a single dose of 25 mg/kg cisplatin (Sigma) dissolved in 0.9% saline solution. The cisplatin dose was based on literature analysis and results of preliminary experiments, showing renal function impairment at day 3 after cisplatin injection. MAP3K14 $^{aly/aly}$ mice (n=5) and MAP3K14 $^{+/+}$ mice (n=5) were used in these experiments and sacrificed at 72h.

Generation of bone marrow chimera

Recipient MAP3K14$^{+/+}$ mice and MAP3K14 $^{aly/aly}$ mice at age 6 weeks were γ-irradiated with 2 doses of 5 Gy for ablation of endogenous bone marrow cells. For bone marrow transplantation, bone marrow cells were isolated (donor) by flushing the femurs and tibias using a 25G needle with Dulbecco's modified Eagle medium (DMEM; Invitrogen). After resuspension, bone marrow cells were centrifuged (300 × g, 5 min, 4 °C). After resuspension with ice-cold DMEM, bone marrow cells were filtered through a 35-µm filter. Irradiated recipient MAP3K14$^{+/+}$ and MAP3K14 $^{aly/aly}$ mice were injected intravenously with 4×10^6 donor bone marrow cells (in 100 µL per recipient) within 4 h after the last irradiation dose. 8 weeks after bone marrow transplantation, bone marrow chimeric mice (4 groups of 5 mice: recipient MAP3K14$^{+/+}$ with donor MAP3K14$^{+/+}$, recipient MAP3K14$^{+/+}$ with donor MAP3K14$^{aly/aly}$, recipient MAP3K14$^{aly/aly}$ with donor MAP3K14$^{+/+}$ and recipient MAP3K14$^{aly/aly}$ with donor MAP3K14$^{aly/aly}$) were subjected to folic acid nephropathy and killed at 72 h.

Sample preparation and mass spectrometry analysis

Tissue samples were weighed out and extracted using the Filter Aided Sample Preparation (FASP) method [60], as described previously [15]. Briefly, tissue samples were homogenised in SDS-lysis

buffer (1:10 sample to buffer ratio) (0.1 M Tris-HCl pH 7.6 supplemented with 4% SDS and 0.1 M DTT) using an Ultra-Turrax T 25 (IKA, Staufen, Germany), incubated at 95 ºC for 3 minutes and clarified by centrifugation at 16,000 g for 5 min at room temperature. An aliquot of the supernatant was taken and placed in a Micron YM-30 filter device (Millipore, Watford UK). 8 M Urea buffer (UA) was added to the protein extract and then centrifuged at 14,000 g for 15 minutes and then repeated. The protein extract was then mixed gently for 1 minute with 0.05 M iodoacetamide buffer (IAA) and incubated for a further 20 minutes prior to centrifugation. UA buffer was again added and centrifuged (twice). Ammonium bicarbonate buffer (50 mM NH_4HCO_3, pH 8) (ABC) was added and centrifuged (twice) before incubating overnight with trypsin. The trypsin homogenate was centrifuged and washed with ABC buffer prior to acidification with 10% formic acid. Sample volumes were adjusted to match final concentration of protein prior to analysis by LC-MS/MS.

Tissue extracts were separated on a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK). A 5 µl sample was loaded in 0.1% formic acid and acetonitrile (98:2) onto a Dionex 100 µm x 2 cm, 5 µm C18 nano trap column at a flow rate of 5µl/min. Elution was performed on an Acclaim PepMap C18 nano column 75 µm x 50 cm, 2 µm, 100 Å with a linear gradient of solvent A, 0.1% formic acid and acetonitrile (98:2) against solvent B, 0.1% formic acid and acetonitrile (20:80) starting at 1% B for 5 minutes rising to 30% at 400 minutes then to 50% B at 480 minutes. The sample was ionized in positive ion mode using a Proxeon nano spray ESI source (Thermo Fisher, Hemel, UK) and analyzed in an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). The MS was operated in a data-dependent mode (top 40) to switch between MS and MS/MS acquisition and parent ions were fragmented by collision-induced dissociation (CID). Data files were searched against the IPI mouse non-redundant database using SEQUEST with enzyme specified as trypsin. A fixed modification of carbamidomethylation was set and oxidation of methionine and proline as variable modifications were selected. Mass error windows of 20 ppm and 0.8 Da were allowed for MS and MS/MS, respectively. In SEQUEST, only peptides that showed mass deviation of less than 10 ppm were passed, the peptide data were extracted using high peptide confidence and top one peptide rank filters. Statistical p-value analysis was performed using the Wilcoxon Mann Whitney test.

Bioinformatics analysis

Protein identification and a significant dataset of 1480 entries with p-values <0.05 and fold changes of >2 have been previously described [15]. This dataset was used for metabolic and signaling pathway analysis using the KEGG web-resource (www.genome.jp/kegg-bin/) or with PathVisio (www.pathvisio.org). Focused data mining was then amplified to all molecules with a p-value≤0.05.

Cells and reagents

MCT cells are a cultured line of proximal tubular epithelial cells harvested originally from the renal cortex of SJL mice and have been extensively characterized [61]. MCT cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA), 10% decomplemented fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in 5% CO2 at 37 ºC [61]. Recombinant human soluble TWEAK (Millipore, Billerica, MA) was used at 100 ng/ml.

Western blot analysis

Tissue and cell samples were homogenized in lysis buffer [62] then separated by 10% or 12% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Millipore, Bedford, MA, USA), blocked with 5% skimmed milk in PBS/0.5% v/v Tween 20 for 1 h, and washed with PBS/Tween. Primary antibodies were rabbit polyclonal anti-p100/52 (1:500, Cell Signaling, Danvers, MA), anti-RelB (1:500, Santa Cruz, CA, USA), anti-MAP3K14 (1:1000, Cell Signaling), anti-Cyclin D1 (1:1000, Cell Signaling) and anti-cullin-1 (1:500 Santa Cruz, CA, USA). Antibodies were diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare/Amersham, Aylesbury, UK). After washing, the blots were developed with the chemiluminescence method (ECL). Blots were then re-probed with monoclonal anti- mouse α-tubulin antibody (1:2000, Sigma, St. Louis, MO, USA) and levels of expression were corrected for minor differences in loading.

Quantitative reverse transcription-polymerase chain reaction

One μg RNA isolated by Trizol (Invitrogen, Paisley, UK) was reverse transcribed with High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method [63]. Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays were from Applied Biosystems, Foster City,

CA.

Immunohistochemistry

Immunohistochemistry was carried out as previously described on paraffin-embedded 5 µm thick tissue sections [62]. Primary antibodies were rabbit polyclonal anti-RelB (1:50, Santa Cruz, CA, USA), anti-NFκB2 p100/p52 (1:20, Santa Cruz, CA, USA), anti-MAP3K14 (1:100, Cell Signaling), rat polyclonal anti-F4/80 antigen (1:50; Serotec, Oxford, UK), rabbit monoclonal anti-CD3 (1:100, Dako, Denmark) and anti-Cullin-1 (1:80, Santa Cruz, CA, USA). Sections were counterstained with Carazzi`s hematoxylin. Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody.

Apoptosis was assayed by deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) (In Situ Cell Death Detection Kit; Roche) according to the manufacturer's instructions [63].

For human kidney immunohistochemistry, control kidney tissue from nephrectomy specimens (n=4) and AKI tissue (n=7) diagnosed as "acute tubular necrosis" was studied. Mean age was 36-4±18.6 years, four patients were females and serum creatinine ranged from 1.7 to 10.0 mg/dl (5.7±3.5 mg/dl). Immunohistochemistry was performed as described above by using anti-human MAP3K14 from Abcam.

Transfection with small interfering RNA

Cells were grown in six-well plates (Costar, Cambridge, MA) and transfected with a mixture of 20 nmol/mL MAP3K14 siRNA (Santa Cruz, CA, USA), Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 (Invitrogen) [64]. After 18 hours, cells were washed and cultured for 6 hours in complete medium, and serum-depleted for 24 h before addition of stimulus. This time point was selected from a time-course of decreasing MAP3K14 protein expression in response to siRNA. A negative control scrambled siRNA provided by the manufacturer did not reduce MAP3K14 protein.

Cell death and apoptosis

Cells were cultured to subconfluence in six-well plates and transfected with MAP3K14 siRNA as previously described [65]. Apoptosis was assessed by flow cytometry of DNA content. For assessment of the cell cycle and apoptosis, adherent cells were pooled with spontaneously detached cells, and stained in 100 µg/mL propidium iodide, 0.05% NP-40, 10 µg/mL RNAse A in PBS at 4°C for >1 hour. This assay

permeabilizes the cells. Permeabilization allows entry of propidium iodide into all cells, dead or alive. Apoptotic cells are characterized by a lower DNA content (hypodiploid cells) because of nuclear fragmentation. Thus, this assay is not based on the known ability of propidium iodide to enter dead cells. The percentage of apoptotic cells with decreased DNA content (A_o) was counted [30].

ELISA

Cells were transfected with MAP3K14 siRNA and stimulated with 100 ng/ml TWEAK. Murine CxCL10 in the supernatants was determined by ELISA (BD Pharmingen, San Diego, CA).

NFκB DNA-binding activity

RelB and NFκB2 p52 subunits in nuclear extracts from kidney tissue were assessed by their binding to an oligonucleotide containing the NFκB consensus site using TransAM NFκB Family Kit (Active Motif, Carlsbad, CA).

Statistics

Statistical analysis was performed using SPSS 11.0 statistical software (IBM, NY, USA). Results are expressed as mean ± SD. Significance at the $p<0.05$ level was assessed by Student´s t test for two groups of data and ANOVA for three of more groups.

Conflict of Interest: H. Mischak is the co-founder and co-owner of Mosaiques Diagnostics.

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Figure legends

Figure 1. Increased kidney mRNA and protein expression of MAP3K14 in experimental AKI. Kidney mRNA levels were assessed by quantitative RT-PCR and protein levels by Western blot. **A)** MAP3K14 mRNA *p<0.009 vs vehicle. **B)** MAP3K14 protein *p<0.005 vs vehicle. **C)** MAP3K14 immunohistochemistry. Increased MAP3K14 expression was localized to tubular cells in AKI samples from wild type mice at 24 h. Original magnification 40. N= 6 animals per group.

Figure 2. Increased kidney RelB and NFκB2 expression and evidence for non-canonical NFκB activation in experimental AKI. Kidney mRNA levels (A;C) were assessed by quantitative RT-PCR and protein levels by Western blot (B;D). **A)** RelB mRNA, *p<0.009 vs vehicle. **B)** RelB protein, *p<0.03 vs vehicle. **C)** NFκB2 mRNA, *p<0.006 vs vehicle. **D)** NFκB2 p100 and p52 proteins, representative Western blot. **E)** NFκB2 p100 and p52 protein quantification, *p<0.03 and **p<0.05 vs vehicle. NFκB2 p100 is processed to NFκB p52 by the proteasome. **F)** Increased nuclear DNA-binding activity of NFκB2 p52 and RelB in experimental AKI. A DNA-binding ELISA was used to quantify DNA-binding activity of NFκB2 p52 and RelB in nuclei obtained from kidneys 24 h following induction of AKI or vehicle administration. *p<0.009 vs vehicle. N= 6 animals per group.

Figure 3. MAP3K14 expression in human kidney tissue. Immunohistochemistry was performed in human control and AKI tissue. Increased tubular cell immunostaining for MAP3K14 was observed in AKI. Original magnification x20, detail x100.

Figure 4. MAP3K14 deficient mice were protected from experimental AKI. A) Serum creatinine. *p<0.015 vs heterozygous mice. **B)** Serum urea. *p<0.0001 vs heterozygous mice. **C)** NFκB2 p100 and p52 proteins (representative Western blot). **D)** NFκB2 mRNA. *p<0.01 vs heterozygous AKI mice. **E)** Decreased whole kidney MCP-1, **F)** RANTES and **G)** CXCL10 mRNA expression in MAP3K14 deficient mice with AKI compared to heterozygous mice. *p<0.02 vs heterozygous AKI mice. **H)** CCL21a mRNA expression. Mean±SD of 6 mice per group at the 72 h time-point. *p<0.03 vs heterozygous AKI mice.

Figure 5. MAP3K14 deficient mice are protected from tubular non-canonical NFκB pathway activation in AKI. A) RelB and **B)** p100/52 immunohistochemistry. Nuclear p52 is observed in renal tubules from heterozygous mice with AKI (arrows) while no staining was observed in MAP3K14 deficient mice with AKI. Immunohistochemistry does not discriminate between NFκB2 p100 and NFκB2 p52. However, Western blot shown in figure 4.C shows the presence of the active NFκB2 p52 protein.

Images representative of 6 animals per group at the 72 h time-point. Original magnification x40. Detail x400. N= 6 animals per group.

Figure 6. MAP3K14 deficient mice were protected from experimental AKI-induced inflammation and cell death. A) F4/80 macrophage and **B)** CD3 immunohistochemistry. Macrophage infiltration is milder in MAP3K14 deficient mice with AKI than in heterozygous mice with AKI. * $p<0.001$, ** $p<0.02$. Original magnification ×20. **C)** TUNEL for fragmented DNA characteristic of apoptosis was frequently positive in tubular cells in heterozygous mice with AKI. The rate of apoptosis was lower in MAP3K14 deficient mice with AKI. * $p<0.03$. Original magnification x20. Mean±SD of 6 mice per group at the 72 h time-point.

Figure 7. Functional characterization of MAP3K14 actions on cultured proximal tubular cells: chemokine expression. A) MAP3K14 siRNA silencing in cultured murine proximal tubular cells suppressed MAP3K14 protein expression. Representative Western blot. **B)** MAP3K14 siRNA silencing in cultured murine proximal tubular cells suppressed MAP3K14 mRNA expression. **C)** MAP3K14 siRNA silencing prevents CXCL10 mRNA upregulation induced by a 24h stimulation by the non-canonical NFκB activator TWEAK (100 ng/ml). qRT-PCR. *$p<0.005$ vs control, **$p<0.005$ vs TWEAK alone. **D)** MAP3K14 siRNA silencing prevents the increase in culture supernatants of the CXCL10 chemokine induced by exposure for 24h to 100 ng/ml TWEAK (ELISA) *$p<0.001$ vs control, **$p<0.01$ vs TWEAK alone. **E)** MAP3K14 siRNA silencing prevent MCP1 mRNA upregulation induced by the non-canonical NFκB activator TWEAK. qRT-PCR. *$p<0.0001$ vs scrambled, **$p<0.0001$ vs TWEAK alone. **F)** MAP3K14 siRNA silencing prevents RANTES mRNA upregulation induced by TWEAK. qRT-PCR. *$p<0.002$ vs scrambled, **$p<0.003$ vs TWEAK alone. Cells were treated with scramble or MAP3K14 siRNA prior to addition of 100 ng/ml TWEAK for 24h. Mean±SD of 3 independent experiments.

Figure 8. Functional characterization of MAP3K14 actions on cultured proximal tubular cells: cell death. A) MAP3K14 siRNA silencing decreases spontaneous apoptosis of serum-deprived tubular cellsγ. Representative flow cytometry diagrams of cell DNA content. Hypodiploid cells consistent with apoptosis are indicated by a horizontal bar. **B)** Quantification of hypodiploid apoptotic cells. *$p<0.05$ vs control, **$p<0.03$ vs TWEAK/TNFα/INFγ alone. Mean±SD of 3 independent experiments.

Supplementary figure 1. Proteomics and bioinformatics approaches. LC/MS-MS proteomics of kidney cortex from 6 AKI or 6 control samples identified 41235 peptides belonging to 6516 unique proteins, of which 1480 were significantly differentially expressed. A bioinformatics analysis of this dataset identified several processes and protein functions enriched in upregulated proteins in AKI. NFκB activation was found at the crossroads of several of these processes, including MAPK, ubiquitin-mediated proteolysis, chemokines, NFκB and apoptosis. Since canonical NFκB activation in kidney injury has been studied in depth, we focused on non-canonical NFκB activation and validated changes in the expression of components of the pathways identified by proteomics. For functional studies we used key target cells in AKI, tubular cells, to explore the function of the apical kinase of the non-canonical NFκB pathway (MAP3K14) in processes that are known to be involved in AKI pathogenesis and that were identified as overrepresented in AKI sample proteomics by KEGG database searching, that is, chemokines and apoptosis. The function of MAP3K14 in AKI was validated in vivo in MAP3K1 activity-deficient mice.

Supplementary figure 2. NFκB signaling and ubiquination pathway map. Map shows an integration of KEGG-generated NFκB signaling and ubiquitination proteasome pathways. TWEAK, a known activator of non-canonical NFκB signaling was added manually. Non-canonical NFκB is elicited by a limited set of extracellular ligands and requires MAP3K14 activation. MAP3K14 induces IκB kinase-α (IKK-α)-mediated phosphorylation of NFκB2 p100. F-box proteins provide specificity for substrate recognition in the S-phase kinase associated protein 1 (SKP1)-cullin 1 (CUL1)-F-box protein (SCF) family of the Cullin-RING ligases (CRL) E3 ubiquitin ligase superfamily. IKK-α-phosphorylated NFκB2 p100 is recognized by the F-box protein β-transducin repeat containing (β-TrCP; FBW1A) allowing ubiquitination by $SCF^{\beta\text{-}TrCP}$. Efficient NFκB2 p100 ubiquitination requires Ube2m (UBC12) and Cullin1 in $SCF^{\beta\text{-}TrCP}$. $SCF^{\beta\text{-}TrCP}$-ubiquitinated NFκB2 p100 is processed by the proteasome to active NFκB2 p52. NFκB2 p52/RelB heterodimers migrate to the nucleus to regulate transcription. Red: over-expressed >2, orange: over-expressed >1 and <2 ($p<0.05$), grey: identified in the sample, but not statistically significant differences.

Supplementary figure 3. Increased mRNA and protein expression of Cullin-1 in experimental AKI. **A)** Quantification and representative Western blot of Cullin-1 protein *$p<0.02$ vs

vehicle. **B)** Cullin-1 immunostaining in 24 hours AKI and vehicle samples. Note increased Cullin-1 expression in tubular cells from AKI samples. Images representative of 6 animals per group.

Supplementary figure 4. MAP3K14 deficient mice were protected from experimental AKI. **A)** Serum creatinine. *p<0.015 vs MAP3K14$^{+/+}$ mice. **B)** Serum urea. *p<0.0001 vs MAP3K14$^{+/+}$ mice. **C)** NFκB2 mRNA. *p<0.01 vs MAP3K14$^{+/+}$ AKI mice. **D)** MCP-1, **E)** RANTES and **F)** CXCL10 mRNA expression in MAP3K14 deficient mice with AKI compared to MAP3K14$^{+/+}$ mice. *p<0.02 vs MAP3K14$^{+/+}$ AKI mice. **G)** CCL21a mRNA expression. Mean±SD of 6 mice per group at the 72 h time-point. *p<0.03 vs MAP3K14$^{+/+}$ AKI mice. In F-G vehicle injected mice were considered to have 100% mRNA expression levels and data are presented as percentage change over those values.

Supplementary figure 5. Renal cell MAP3K14$^{aly/aly}$ were protected from folic acid-induced AKI. Four groups of 5 chimeric mice were studied: MAP3K14$^{aly/aly}$ mice with MAP3K14$^{aly/aly}$ bone marrow (BM), MAP3K14$^{aly/aly}$ mice with MAP3K14$^{+/+}$ BM, MAP3K14$^{+/+}$ mice with MAP3K14$^{aly/aly}$ BM and MAP3K14$^{+/+}$ mice with MAP3K14$^{+/+}$ BM. **A)** Mouse survival: 40% of MAP3K14$^{+/+}$ mice with either MAP3K14$^{+/+}$ or MAP3K14$^{aly/aly}$ BM died. No deaths were recorded in MAP3K14$^{aly/aly}$ mice, independently of the BM characteristics. **B)** Among surviving mice no differences in serum creatinine were observed. However, the most severely affected mice had died.

Supplementary figure 6. Functional characterization of MAP3K14 actions on cultured proximal tubular cells: regulation of MCP-1 mRNA expression. Cells were treated with scramble or MAP3K14 siRNA prior to addition of 100 ng/ml TWEAK for 3h. *p<0.0001 vs scrambled, **p<0.007 vs MAP3K14 siRNA alone.

Table 1. Signaling pathways modulated in AKI samples and identified by pathway analysis using KEGG database searching. NFκB activation is at the crossroads of the pathways marked in bold.

KEGG map	number of hits	Name
mmu04151	46	PI3K-Akt signaling pathway
mmu04010	29	**MAPK signaling pathway**
mmu04910	27	Insulin signaling pathway
mmu04020	22	Calcium signaling pathway
mmu04120	21	**Ubiquitin mediated proteolysis**
mmu04062	19	**Chemokine signaling pathway**
mmu04310	17	Wnt signaling pathway
mmu04630	16	Jak-STAT signaling pathway
mmu04660	15	T cell receptor signaling pathway
mmu03320	13	PPAR signaling pathway
mmu04064	13	**NF-kappa B signaling pathway**
mmu04370	11	VEGF signaling pathway
mmu04912	11	GnRH signaling pathway
mmu04210	11	**Apoptosis**
mmu04722	10	Neurotrophin signaling pathway

Table 2. Non-canonical NFκB signaling pathway and ubiquitination and proteasomal degradation proteins significantly modulated in AKI. Data represent focused data mining following non-biased analysis of the significant dataset.

Name	gene	fold change	p-value
Non-canonical NFkappaB pathway			
Nuclear factor NF-kappa-B p100 subunit	Nfkb2	1.46	0.03
Mitogen-activated protein kinase kinase kinase 14 (NIK)	Map3k14	1000	0.05
Transcription factor RelB	Relb	3.33	0.01
Ubiquitination system			
Cullin-1	Cul1	1000	0.02
NEDD8-conjugating enzyme Ubc12	Ube2m	3.01	0.01