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Activation of NF-κB via the canonical pathway in nephrotoxic serum nephritis in mice: *possible therapeutic applications with specific IKKß / IKK2 – inhibition*

Dissertation
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1 INTRODUCTION

1.1 Glomerulonephritis (GN)

Glomerulonephritis (GN) is an immune mediated inflammatory renal disease, which remains the major worldwide cause of chronic renal insufficiency and end-stage renal failure requiring dialysis and renal transplantation[1]. In glomerulonephritis, the sequential activation of proinflammatory signaling pathways lead to the production of proinflammatory mediators such as cytokines and chemokines, which direct the infiltration of monocytes/macrophages, dentritic cells (DCs) and T-cells into the kidney and play a pivotal role in the pathogenesis of glomerulonephritis from the acute phase to eventual glomerulosclerosis[2 3]. In recent years, there are numerous researchs focus on the molecular signaling pathways of proinflammation. Unfortunately, the underlying mechanisms which can efficiently down regulate proinflammatory mediators and switch from inflammation toward resolution are still largely unknown.

1.1.1 The immune competent cells in glomerulonephritis

The main immune competent cells actively involved in glomerulonephritis are consisting of neutrophils, monocytes/ macrophages and lymphocytes. Once

glomerular injury is evoked, neutrophils are recruited within a few hours to reach a peak as early as 24 hours after disease induction; monocytes are recruited rather more slowly, the maximum number typically being reached at 48 hours when maturation into macrophages is already well advanced. The kinetics of lymphocytes recruitment is still slower and occurs over several days[4 5].

There are now many evidences for the central role for T cells and DCs in directing cellular immune mechanisms in glomerulonephritis. Differential activation of T helper cell subsets has been proposed and the ability of T helper cell subsets to influence immune effector pathways has been demonstrated in experimental models[1 6 7 8].

The principal DCs function is the induction of adaptive immune response, in particular those executed by T-cells. DCs reside in virtually all tissues, including the kidney. Kidney DCs have been characterized by expression of CD11b, CD11c, F4/80 and MHCII. Their MHCII expression might allow kidney DCs to interact with infiltrating $CD4^+$T cells and to modify their effects in glomerulonephritis. The role DCs might play in glomerulonephritis has come into focus recently[2-3 9] .

Recently, $CD4^+CD25^+$regulatory T cells (Treg) were described to play a pivotal role in the maintenance of tolerance. The mechanism by which Treg regulate the immune response remains controversial. Immunosuppression mediated by Treg is known to be strictly dependent on cell-to-cell contact. Transfer of Treg protected mice from the development of glomerulonephritis[10 11].

1.1.2 The role of chemokines in the acute phase of glomerulonephritis

Detailed molecular mechanisms involved in leukocyte migration to the kidney via chemokines have shed light on the pathogenesis of glomerulonephritis. Chemokines expressed on the surface of glomerular endothelial cells interact with their cognate receptors on specific infiltrates. Proinflammatory chemokines and cytokines produced by both resident cells and immune competent cells exert a wide range of biological activities at inflammatory sites. Selective expression of chemokine receptors and adhesion molecules on specific cell populations may determine the specific phenotypes of infiltrating cells in inflamed glomeruli.

The acute phase of glomerulonephritis is pathologically characterized by infiltration of inflammatory cells into glomeruli, and proliferation of mesangial cells in the glomeruli. In vitro studies revealed that proinflammatory stimuli such as interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFN-γ), immune complexes and growth factors are able to induced monocyte chemoattractant protein-1 (MCP-1)/ macrophage chemotactic and activating factor (MCAF)/CCL2, interferon-inducibleprotein-10 (IP-10)/ CXCL10, regulated upon activation of normal T cells that express and secrete (RANTES)/CCL5 from glomerular resident cells[12] .

1.2 Nuclear factor-κB (NF-κB)

NF-κB was initially identified as a nuclear factor bound to the enhancer of the immunoglobulin (Ig)k light chain gene of B lymphocytes[13]. NF-κB is a family of pleiotropic transcription factors that integrate an intricate network of extracellular signaling pathways, resulting in the transcriptional regulation of numerous of genes related to inflammation, including chemokines, cytokines, adhesion molecules and growth factors. Clinical and experimental data are confirming the presence of activited NF-κB in a variety of inflammatory disorders [14 15 16 17] . NF-κB has also been found in renal cells and activated upon stimulation, both *in vivo* and *in vitro*. Although the evidence linking NF-κB activation to human renal disease is limited, recent data suggest that NF-κB plays a pivotal role in many nephropathies[18 19 20 21]. Therefore, selective intervention of NF-κB signaling pathway may shed light on treatment of glomerulonephritis. A large number of research has increased our understanding on the structure, ways of activation, and the regulation of NF-κB [22 23].

1.2.1 NF-κB family members

In mammalian cells, there are five NF-κB family members, RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), and different NF-κB complexes are formed from their homo- and heterodimers. The transcription activation domain necessary for target gene expression is present only in RelA, c-Rel, and RelB subunits [24].

All NF-κB family members contain a highly conserved N-terminal domain of approximately 300 amino acids called the Rel-homology domain (RHD), which contains a nuclear localization sequence (NLS) and mediates dimerization, DNA binding and interaction with inhibitory IκB proteins. Despite obvious structural similarities and their ability to bind related DNA sequences (κB site: GGGRNNYYCC, where R is purine, Y is pyrimidine, and N is any base), genetic studies have shown that all NF-κB subunits have distinct and non-overlapping functions. For instance, p50 and p52 lack the transactivation domain, are unable to promote transcriptional activity, and are considered to mediate transcriptional repression. Conversely, p65/RelA and c-Rel are potent transcriptional activators. Finally, RelB produces transcriptional activation in certain cell types. Knockout mice for all of the NF-κB genes have been obtained, indicating specific roles for each NF-κB protein. Interestingly, only the p65 knockout is lethal, suggesting some functional redundancy among other members of the family. By far the most abundant dimer in most cell types, and the most widely studied is composed of subunits p50 and p65. Hence, although NF-κB applies to all of the members of the family, it is often used to refer to the p50-p65 dimer.

Both p50 and p52 are synthesized as unprocessed, full-length precursor proteins, p105 and p100, that contain ankyrin-repeat motifs in their C terminus, similar to those found in IκB proteins. p100 and p105 also function as IκB-like proteins and retain their NF-κB-subunit dimeric partners in the cytoplasm, inhibiting their activity. Processing of p100 and p105 can occur through several mechanisms and is required before p50 and p52 can function as nuclear transcription factors [22 23 25 26] .(***Figure 1.2.1***)

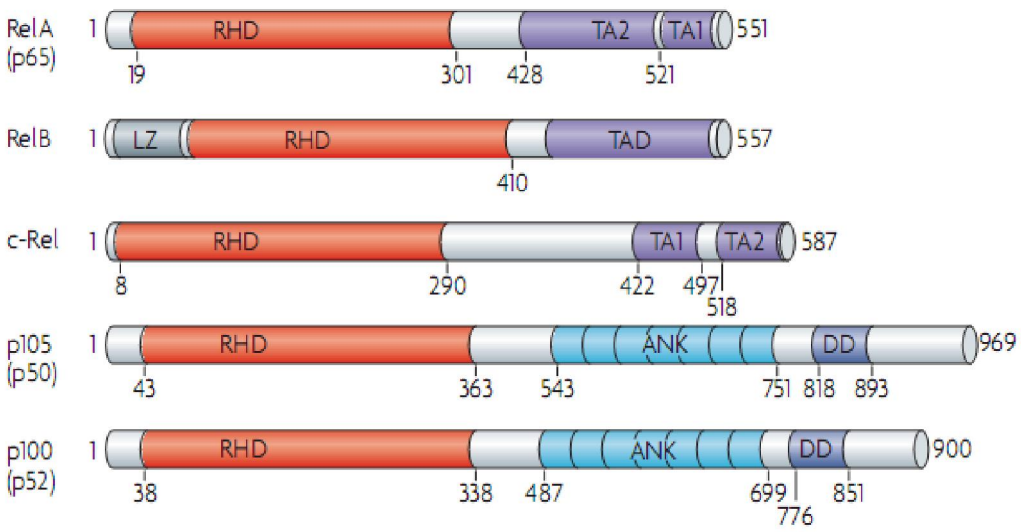


Figure 1.2.1 | The mammalian members of the NF-κB families (Cited from [23]).

1.2.2 IκB family members

Several members of the IkB family have been identified in mammals: IkBα, IkBβ, IkBε and Bcl-3, and the precursors of NF-κB 1 (p105) and NF-κB 2 (p100) (***Figure 1.2.2***). All of them contain six or seven ankyrin repeats motifs (ANK) in their C terminal, which function in part by masking a conserved NLS that is found in the RHD of the NF-κB subunits. In most non-diseased mammalian cells, the majority of NF-κB are bound to inhibitory IκB proteins and are thereby sequestered in the cytoplasm[25,27]. IkBα, IkBβ, and IkBε present amino-terminal regulatory regions required for stimulus-induced degradation. Most of the knowledge of IKK-dependent

IκB degradation is based on studies of IκBα. Phosphorylation of two conserved serine (S) residues in its N terminus, S32 and S36, leads to recognition by the β-TrCP F-box–containing component of a Skp1-Cullin-F-box (SCF)–type E3 ubiquitin-protein ligase complex, called SCFβTrCP, resulting in polyubiquitination and degradation of IκBα by the 26S proteasome[28]. IκBβ and IκBε are also regulated by N-terminal, site-specific phosphorylation, but the kinetics of their phosphorylation and degradation are much slower than that of IκBα and may reflect different substrate specificities of the IKK complex[29]. BCL3 is IκB-like proteins.Both p50 and p52 homodimers can interact with BCL-3, which functions not as NF-κB inhibitors, but rather as co-activator[22-23].

By far, IκBα is the best characterized member of the group, whose gene expression is under the control of NF-κB. By inducing IkBα expression, active NF-κB promotes a negative regulatory loop able to terminate its own activity. In fact, newly synthesized IκBα binds to active DNA-bound NF-κB, and the trimer is exported to the cytoplasm, ending the NF-κB dependent transactivation [30]. (***Figure 1.2.2***)

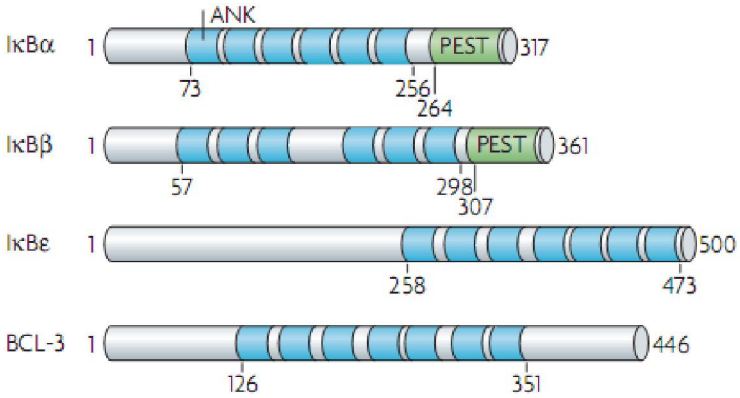


Figure 2 | The mammalian members of the IκB families (Cited from [23]).

1.2.3 IκB kinase (IKK) complex

The most critical regulatory event in NF-κB activation is site-specific phosphorylation of IκB (α, β, and ε) by IκB kinase (IKK), which leads to polyubiquitination and pro-teasomal degradation[22]. IKK is a complex composed of three subunits: IKKα or IKK1 (85 kDa), IKKβ or IKK2 (87 kDa), and IKKγ or NEMO/IKKAP (48 kDa)[31]. IKKα and IKKβ are highly homologous proteins (50% sequence identity, 70% protein similarity) and contain N-terminal protein kinase domains as well as C-terminally located leucine zipper (LZ) and helix-loop-helix (HLH) motifs. Both proteins exhibit kinase activity toward IκB. The third component of this complex is a 48-kD regulatory subunit, named IKKγ or NEMO (NF-κB essential modulator). IKKγ lacks a kinase domain and exists as a trimolecular complex with IKKα and IKKβ. The precise mechanistic role of IKKγ is still enigmatic. Despite their similarities and presence in a common

protein complex, IKKα and IKKβ have largely nonover-lapping functions, due to different substrate specificities and other factors.

IKKβ is most important for rapid degradation of NF-κB–bound IκBs, IKKα controls processing of p100, leading to activation of p52:RelB dimers, a response that is substantially slower than the activation of IκB-bound dimers[32]. IKKβ-dependent degradation of IκBα occurs within minutes, IKKα-dependent processing of p100 requires several hours[33]. (***Figure 1.2.3***)

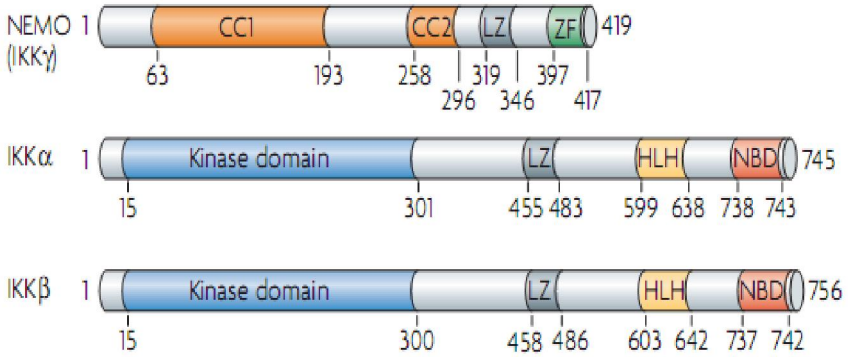


Figure 3 | The mammalian members of the IKK families (Cited from [23]).

1.2.4 NF-κB activation pathway

There are several distinct signaling pathways, which lead to the

translocation of NF-κB dimers from the cytoplasm to the nucleus. The most frequently observed is the canonical (or classical) pathway (***Figure 1.2.4***), which is induced in response to various inflammatory stimuli, including the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), engagement of the T-cell receptor (TCR) or exposure to bacterial products such as lipopolysaccharide (LPS) and is dependent on activation of IKK β. The activated IKK complex (predominantly in an IKKβ and IKKγ dependent manner) catalyzes the phosphorylation of IκB protein (at sites equivalent to Ser32 and Ser36 of IκBα), polyubiquitination (at sites equivalent to Lys21 and Lys22 of IκBα), and subsequent rapid degradation by the 26S proteasome. In many cell types, IκBβ and IκBε are also subject to phosphorylation and degradation, but with slower kinetics[34]. The released NF-κB dimers (mainly p50/RelA heterodimers) translocate to the nucleus, bind DNA, and activate the transcription of κB target genes. Genetic experiments have shown that IKKβ is the predominant IκB kinase in the canonical pathway.

An non-canonical (alternative) pathway for NF-κB activation has been identified (***Figure 1.2.4***), which is activated by NF-κB-inducing kinase (NIK), such as stimulation of the CD40 and lymphotoxin-β receptors, B-cell-activating factor of the TNF family (BAFF), LPS and latent membrane protein-1 (LMP1) of Epstein–Barr virus[35] [36]. The substrate of NIK-IKKα in this pathway is NF-κB2/p100, which can be phosphorylated at two C-terminal serine residues. Phosphorylation of these sites results in the formation of p52 from p100, which further undergoes polyubiquitination and proteasomes degradation by the 26S proteasome. p52–RelB heterodimers, which are frequently activated as a consequence of non-canonical pathway activation, have a higher affinity for

distinct κB elements and might therefore regulate a distinct subset of NF-κB target genes. Thus, p100 functions as a specific and potent inhibitor of RelB. It has been suggested that the non-canonical pathway is regulated by IKKα homodimers that function independently of the larger IKK complex[37 38].

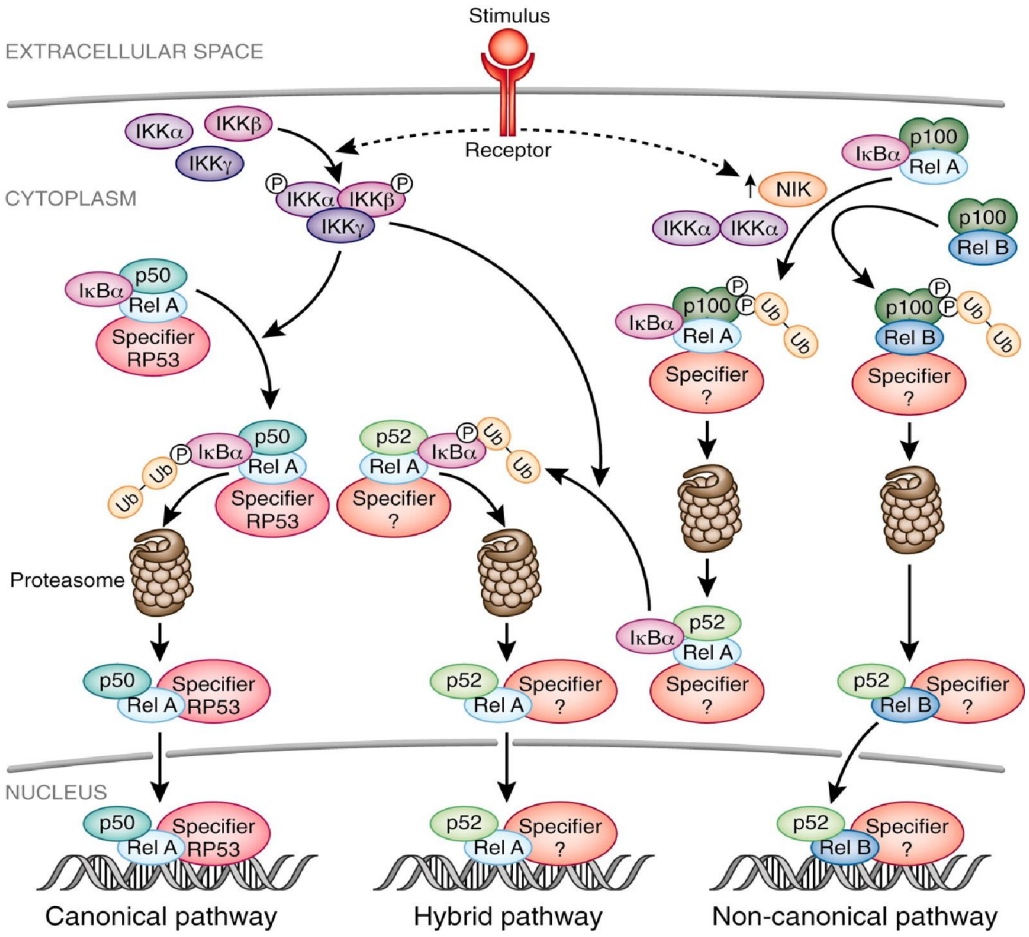


Figure 1.2.4 | NF-κB activation pathway(Cited from [38]).

1.2.5 The consequences of activation.

The IKKβ-dependent pathway leading to rapid degradation of IκBα, IκBβ, and IκBε is commonly referred to as the canonical NF-κB pathway. Activation of IKKβ stimulates anti-apoptotic, pro-inflammatory and proliferative pathways. As well as activating the canonical NF-κB-signalling pathway through phosphorylation (P) of the IκB proteins, IKKβ phosphorylates several other substrates, including NF-κB subunits. For example, phosphorylation of RelA at Ser536 can result in nuclear localization and stimulation of its transactivation functions by promoting interactions with co-activator proteins. (***Figure 1.2.5***).

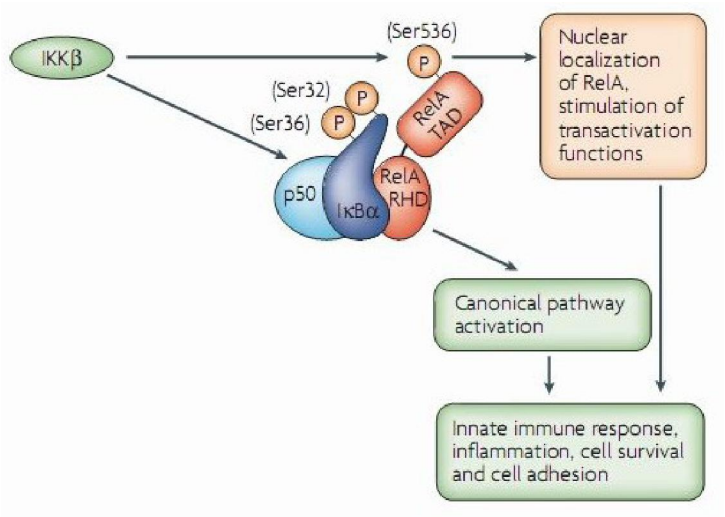


Figure 5 | The consequences of IKKβ activation (Adapted from [23]).

1.3 Nephrotoxic serum nephritis (NTN).

The most widely used model of glomerulonephritis is nephrotoxic serum nephritis (NTN), in which heterologous anti-glomerular basement membrane (GBM) antibodies act as a stimulating antigen in the glomeruli.

Increased evidence has implied that the NTN nephritis model is a Th1/Th17 cell-mediated disease induced in mice serves as a model of human endogenous rapid progressive glomerulonephritis[39]. The glomerular changes included hypercellularity and formation of cellular crescents, capillary aneurysms, and intraglomerular deposition of PAS-positive material. In addition to massive leukocyte infiltrates, the tubulointerstitial compartment showed tubular dilation, necrosis and atrophy, and protein casts and tubular protein reuptake due to proteinuria[21 40 41 42].

1.4 Compound A

The IKKβ / IKK2 inhibitor COMPOUND A (CpdA) ｛7-[2-(cyclopropyl-ethoxy) -6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-di-hydro -2H-yrido[2,3-d][1,3]oxazin-2-one hydrochlorid｝ also known as Bay 65-194, were synthesized in the laboratories of Bayer Yakuhin, Ltd. CpdA is an ATP-competitive inhibitor that selectively targets IKK2 kinase activity. For administration to animals, compounds were solved in 10% cremophor, which

was used as the vehicle in all experiments. Previous pharmacokinetic testing of CpdA demonstrated that dosages in mice of 2 mg/kg intravenously and 10 mg/kg orally had moderate clearance CpdA is a potent and selective IKKβ inhibitor fulfilling the expectation of being antinflammatory in vitro and in vivo, and might pave the way or the development of IKKβ inhibitors as a novel class of anti-inflammatory drugs[43 44]. We investigated whether specific inhibition of the CpdA subunit would resolve glomerulonephritis.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Animals

Eight-weeks-old wild-type C57BL/6 mice (20-25 g b.w.) were obtained from Charles River (Sulzfeld,Germany). All animals were raised under specific pathogen-free conditions. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (FI 16/05 and 17/10).

2.1.2 Animals model

The experimental animal model of nephrotoxic serum nephritis (NTN) was induced in eight-weeks-old male C57BL/6 wild-type mice by intraperitoneal injection of 2.5 mg of sheep-anti-mouse GBM antiserum per gram of mouse body weight, as described previously[45].

2.1.3 Inhibitor

Compound A is a specific IKK2-inhibitor described previously by Ziegelbauer et al.[46] [47]. Bayer Schering Pharma AG supplied Compound A for

these experiments.

This compound was used throughout the experiments. Stock solution was prepared by dissolving the compound in cremaphore 10% (Sigma, Munich, Germany, dilution with PBS).

Animals were injected three times a week subcutaneously with 5 mg/ kg bw of the compound.

2.1.4 Radiochemical

[32-γ-P]-dATP: (3,000 Ci/mmol; GE Healthcare, Germany)

2.1.5 Chemicals

Reagent	Manufacturer
Acrylamide-solution(30%)	Applichem
Alexa Fluor 568 Phalloidin	Invitrogen
Alkaline phosphatase	Merck
Ammoniumperoxodisulfate	Merck
Aqua ad injectabilia	Braun
ApoTagPeroxidase	Millipore
Caliculin A	Merck
Complete 25x	Merck
Cremaphor EL	Flukaas
DAPI Vectashield Hard Set Mount	Vector
Dimethylsulfoxid	Sigma
DTT	Invitrogen
Elisa Starter Accessory package	Bethyl
Ethanol 100%	J.T. Baker
Formalin	Merck
Gel Code Blue Stain Reagent	Pierce
Glycerol	Biochemika
Glycin	Roth
HCl 1N	Merck
Hematoxylin	Merck
H_2O_2	Sigma-Aldrich
Isoflurane	Abbot
Isopropanol	Fluka
Methanol	Roth
Na_2HPO4 x 2 H20	Merck
Naphtol- AS- Bisphosphat	Fluka
Sodium	Merck
Sodium acetate 3M	Merck
Sodium azide	Serva
Sodium chloride	Serva
Sodium citrate x 2 H2O	Merck
Sodium dodecyl sulfate (SDS)	Merck

Sodium fluoride	Merck
Sodium iodate	Merck
Sodium nitrite (NaNo2)	Merck
Sodium orthovanadate	sigma
Sodium vanadate	Merck
M-MLP reverse transcriptase	Invitrogen
Mice-Albumin Kit	Bethyl
New Fuchsin	Serva
NP-40	Sigma
Paraformaldehyde (16%)	Electron Microscopy Sciences
PBS	Biowhittaker
Periodic Acid	Merck
Protease XIV	Sigma
Protein ladder	Invitrogen
Random hexamer	Invitrogen
Reagent A + B, Protein Assay	BioRad
Schiff's reagent	Sigma
Skimmed milk powder	Spinnrad
Super Block blocking reagent	Pierce
TEMED	Sigma
Tris	Sigma
Tween 20	Serva
Hydrogen peroxide (H2O2)	Sigma
Xylene	Th. Geyer GmbH & CoKG
5xfirst strand Buffer	Invitrogen
100mM dNTP-set	Invitrogen
2xPlatinum SYBRGreen qPCR Supermix	Invitrogen

2.1.6 Primers for real-time PCR

All primers were synthesized by Invitrogen. Primers with melting temperature (Tm) of 79°C were designed using Stepone Software v2.0 from Applied Biosystem.

Primer sequences	
TNFa	Fw: AAA TGG CCT CCC TCT CAT CAG T Rev: GCT TGT CAC TCG AAT TTT GAG AAG
IL-1 β	Fw: CCT TCC AGG ATG AGG ACA TGA Rev: TCA TCC CAT GAG TCA CAG AGG AT
CCL2/ MCP-1	Fw: GGC TCA GCC AGA TGC AGT TAA Rev: CCT ACT CAT TGG GAT CAT CTT GCT
CCL5/ Rantes	Fw: GCA AGT GCT CCA ATC TTG CA Rev: CTT CTC TGG GTT GGC ACA CA
CXCL10/IP-10	Fw: GCC GTC ATT TTC TGC CTC AT Rev: TGC AGC GGA CCG TCC TT

2.1.7 Antibody

Primary Antibody	Species	Use	Dilution	Manufacturer
Anti-CD3	rabbit	Immunohistochemistry	1:1000	Dakocytomation
Anti-Mac-2	rat	Immunohistochemistry	1:1000	Technically
Anti-F4/80	rat	Immunohistochemistry	1:2000	Dianova
Secondary Antibody	**Species**	**Use**	**Dilution**	**Manufacturer**
Anti- Rabbit	donkey	HRP	1: 5 000	Jackson
Anti- Rabbit	goat	Biotin	1: 200	Vector
Anti- Rat	donkey	Biotin	1: 200	Jackson

2.1.8 Gel shift experiment

Reagent	GmbH
Oligonucleotide Sequence of NF-κB 5´-AGT TGA GGG GAC TTT CCC AGG C-3´ 3´-TCA ACT CCC CTG AAA GGG TCC G-5´	Promega
10x Reaction Buffer A	Fermentas
T4 Polynuleotide Kinase	Fermentas
Poly(dl-dc)· (dl-dc)	Sigma

2.1.9 Devices

Apparatus	GmbH
Autoanalyzer	Hitachi 717; Roche
AbiPrism Sequence Detection System 7000	Applied Biosystems
ELISA- Reader	Severin
Heating block (Neoblock 1)	Bosch
Fridge 4°C	Nanodrop Technologies
Fridge -20°C	LG
Fridge -80°C	WTW
Magnetic stirrer (Variomag Maxi)	Sarstedt
Microwave (Microwave 800)	Brand
Microwave	Agfa
Nanodrop spectrophotometer (ND 1000)	Eppendorf
PC	Fröbel Labortechnik
pH- Meter (Inolab)	Biometra
Pipette	Eppendorf
Pipette (Accu- jet)	Eppendorf
X-ray film developer (CP 1000)	Intas
Shaker (Thermo Mixer comfort)	Janke & Kunkel
Shaker (Rocky)	Scaltec
Thermocycler	Köttermann
Table centrifuge (Typ 5415 R)	Heraeus
Table centrifuge (Mini Spin)	Heraeus
UV-table and documentation system	Heraeus

Vortex	Severin
Libra (SBA 41)	Bosch
Heat bath	Nanodrop Technologies
Workbench (Hera Safe)	LG
Centrifuge (Biofuge primo R)	WTW
Centrifuge (Megafuge 1.0 R)	Sarstedt
Microscope	
Lichtmicroscop (Axioscop)	Zeiss
Lichtmicroscop (Axioobserver)	Zeiss
Lichtmicroscop (Axiovert 25)	Zeiss
Lichtmicroscop (IM 35)	Zeiss
Lichtmicroscop (LSM 510 beta)	Zeiss
Software	
Adobe Illustrator	Adobe
Adobe Photoshop	Adobe
Excel	Microsoft
Stepone Software v2.0	Applied Biosystems
Windows XP	Microsoft

2.1.10 Buffer

Buffer for Gel shift experiment

5-fold Binding Buffer:

90mM HEPES pH 7,9
1mM EDTA pH 8,0
0,5mM EGTA pH 8,0
200mM NaCl
1mM DTT
0,5mM PMSF
15% Glycerol

10-fold TBE Buffer:5 liter

540g Tris
275g Boric acid
200ml 0.5M EDTA(pH8.0)

Buffers for isolation of nuclear proteins from tissue:

Hypotonic Buffer A:

10 mM HEPES pH 7.9
10 mM KCl
0.1 mM EDTA pH 8.0
0.1 mM EGTA pH 8.0
Add freshly: 1 mM DTT and protease inhibitor mix, 1 mM Na-Vanadate, diluted 1 : 100

Hypertonic Buffer B:

20 mM HEPES pH 7.9
400 mN NaCl
1 mM EDTA
1 mM EGTA
Add freshly: 1 mM DTT and protease inhibitor mix, 1 mM Na-Vanadate, diluted 1 : 100

Formalin:

Sorensen's Buffer

3.03 g $NaH_2PO4 \times H_2O$
14.14 g $Na_2HPO4 \times 2\ H_2O$
ad 1L H_2O
pH 7.2- 7.4

Formalin- solution (4%)
4% Formalin
0.4 % Eosin
in Sorensen's Buffer

2.2 Methods

2.2.1 Preparation of sheep anti-mouse GBM serum

Sheep anti-mouse GBM antiserum was prepared as described previously[45]. Briefly, glomeruli of C57BL/6 mice were isolated from the renal cortex through a series of sieves of decreasing pore size (250-, 150- and 75-mm mesh), and disrupted by sonication. The GBM fractions were then collected by centrifugation. For immunization, 1 mg GBM protein was emulsified with 1 ml Freund's complete adjuvant and was administered to the sheep by subcutaneous (s.c.) injection. Anti-mouse GBM serum were raised in goat by repeated immunization.

2.2.2 Induction of nephrotoxic serum nephritis (NTN)

For induction of the NTN mouse model eight-week-old wild-type C57BL/6 mice (20-25 g b.w.) were injected intraperitoneally (i.p.) with 0.6 ml/ 20 g b.w. NTN serum (n=4 animals per time point examined, experiments were repeated 3 times). Animals were sacrificed at the following time points after induction of NTN: 2d, 4d, 7d, 10d ,14d and 21d after induction of the disease. In addition control animals were examined. Control animals were not further treated as previous experiments of our group demonstrated that the i.p. injection of normal

sheep IgG did not induce renal injury.

2.2.3 Treatment with Compound A (CpdA)

Experiments using the specific IKK2-inhibitor CpdA focused on time point 10 days after induction of NTN.

Compound A (5 mg/kg b.w) was administered three times a week by subcutaneous injection. According to the time point of CpdA injection, 4 groups of animals were examined (n=4 per group, experiment were repeated 3 times): ***Group 1:*** NTN mice without any further treatment [NTN-10d]. ***Group 2:*** the injection of CpdA started 24 hours before disease induction until 10 days at the time of sacrifice [-1/+10]. ***Group 3:*** the injection of CpdA started 24 hours prior to disease induction up to day 4; the animals were followed up until day 10 after induction of NTN without any further treatment [-1/+4]. ***Group 4:*** the injection of CpdA started 4 days after disease induction until 10 days at the time of sacrifice [+4/+10].

In additional experiments C57/BL6 control mice were injected with the IKKβ inhibitor for the 10 days observation period for comparison with control non-treated mice.

The end point of the study was day 10 after NTN injection. All animals

were sacrificed at this time point.

2.2.4 Parameters of kidney function

For urine sample collection, mice were housed in metabolic cages for 6 h the day before the end of the observation period.

Blood samples for BUN measurement were obtained at the time of sacrifice.

BUN and urine creatinine were determined by standard methods using an autoanalyzer in the Department of Clinical Chemistry at the University Hospital Hamburg. Albuminuria was determined by standard ELISA analysis.

2.2.5 Morphological examinations

Renal tissue injury was assessed in paraffin-embedded and paraformaldehyde (4%)-fixed tissue sections stained by the periodic acid-Schiff reaction. A semiquantitative score for tubular injury and for acute tubular necrosis was calculated for each animal by a double-blinded observer as described before[21]. The percentage of tubules that displayed cellular necrosis, loss of brush border, interstitial edema, vacuolization, and tubule dilation were

scored as follows: ***0*** = none, ***1*** ≤25%, ***2*** = 26-50%, ***3*** =51%-75%, ***4*** ≥76%. For each animal, at least 10 high-power fields (hpf) were examined (x200).

Immuno-histochemistry was performed as described in detail previously[21]. The following primary antibodies were used: the pan-T cell marker CD3, the monocyte and renal dendritic cell marker F4/80 and the monocyte-specific marker MAC-2. For immuno-histochemistry, 1- to 3-µm thin paraffin sections were baked overnight at 40°C, deparaffinized, and rehydrated. Antigen retrieval was performed by microwave boiling and protease digestion. Unspecific binding was blocked with 5% normal horse or goat serum (Vector, Burlingame, VT) for 30 min at room temperature. Primary antibody incubations were performed in 5% normal horse or goat serum overnight at 4°C, followed by incubation with biotinylated affinity purified donkey secondary antibodies. Color development was performed with the ABC-AP kit (Vector) according to the manufacturer's instructions. Neufuchsin was used as substrate for alkaline phosphatase, and nuclei were counter stained with hematoxylin for light microscopic evaluation. Stainings were evaluated under an Axioskop and photographed with an Axiocam HRc using the Axiostar software.

F4/80- and CD3-positive cells in 30 tubulo-interstitial hpf per kidney and MAC-2-positive cells in 30 glomerular cross sections were counted by light microscopy in a double-blinded observation manner.

2.2.6 Real time RT-PCR

Total RNA of renal cortex of each mouse was prepared according to standard laboratory methods as described previously[48] and reverse-transcribed by the use of random hexamer primers. mRNA expression was performed with 1.5µl of cDNA samples in the presence of 2.5µl (0.9µM) of specific murine primers (primer sequences are available upon request) and 12.5µl of 2xPlatinum SYBRGreen qPCR Supermix using an AbiPrism Sequence Detection System 7000.

The following primers were used:

TNFα: *Fw:* AAA TGG CCT CCC TCT CAT CAG T; *Rev:* GCT TGT CAC TCG AAT TTT GAG AAG

IL-1β: *Fw:* CCT TCC AGG ATG AGG ACA TGA; *Rev:* TCA TCC CAT GAG TCA CAG AGG AT

CCL2 / MCP-1: *Fw:* GGC TCA GCC AGA TGC AGT TAA; *Rev:* CCT ACT CAT TGG GAT CAT CTT GCT

CCL5 / Rantes: *Fw:* GCA AGT GCT CCA ATC TTG CA; *Rev:* CTT CTC TGG GTT GGC ACA CA

CXCL10 / IP-10: *Fw:* GCC GTC ATT TTC TGC CTC AT; *Rev:* TGC AGC GGA CCG TCC TT

The primers were used as an internal control to correct for small variations in RNA quality and cDNA synthesis essentially as described by AbiPrism. All samples were run in duplicate and normalized to 18S rRNA to account for small RNA and cDNA variability. Relative quantification of gene expression was calculated using the △△CT method[49 50].

2.2.7 Nuclear fractionation

Kidneys were harvested after in situ perfusion with 2 ml of cold (4°C), sterile phosphate buffered saline (PBS). For nuclear protein preparation, we adapted a method described by Sakurai et al. to prepare the nuclear mini-extracts and run the electrophoretic mobility shift assay (EMSA) as described previously in detail[51]. Nuclear proteins were isolated from whole kidney extracts and protein concentration was determined (DC protein assay; Bio-Rad Laboratories).

2.2.8 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA, gel shift experiments) were performed using NF-kappa B, consensus oligonucleotide probes end-labeled with ^{32}P-γ- ATP[19 52]. The NF-kappa B oligonucleotides used in our experiments preferentially bind p65/p50 heterodimers and complex other NF-kappa B dimers with much less affinity. Exposed EMSA films were quantified using a phosphor imager Bio-Rad-GS-363 (multi-analyst software) and corrected to the density of the probe.

2.2.9 Statistical analysis

Results are expressed as mean ± SE; n refers to the number of animals or individual measurements in separate samples. Differences between the multiple experimental groups were compared by Kruskal Wallis test with post hoc analysis by Mann-Whitney test. Paired Student's t-test was used to compare mean values within one experimental series. Statistical significance was defined as $p<0.05$. Experiments yielding insufficient independent data for statistical analysis due to the experimental setup were repeated at least three times.

2.3 Experimental outline

2.3.1 Time kinetic experiments up to day 21 after NTN injection

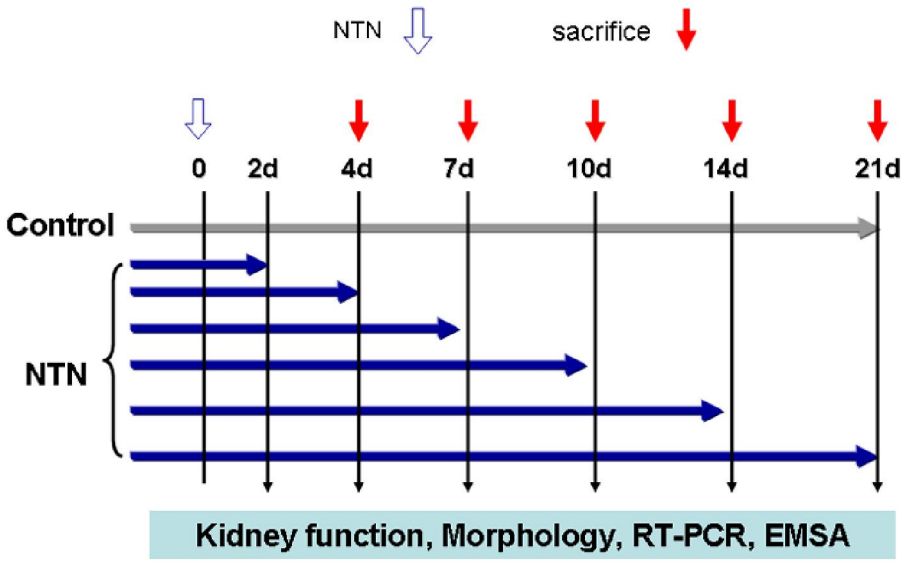


Figure 2.3.1 Mice were injected intraperitoneally with 0.6 ml/20 g b.w. NTN serum (n=4 animals per time point examined; experiments were repeated 3 times). Animals were sacrificed at the following time points after induction of NTN: 2d, 4d, 7d, 10d, 14d and 21d after induction of the disease. In addition control animals were examined. Control animals were not further treated as previous experiments of our group demonstrated that the i.p. injection of normal sheep IgG did not induce renal injury.

2.3.2 Experiments using the specific IKK2-inhibitor(CpdA) up to day 10 after NTN-injection

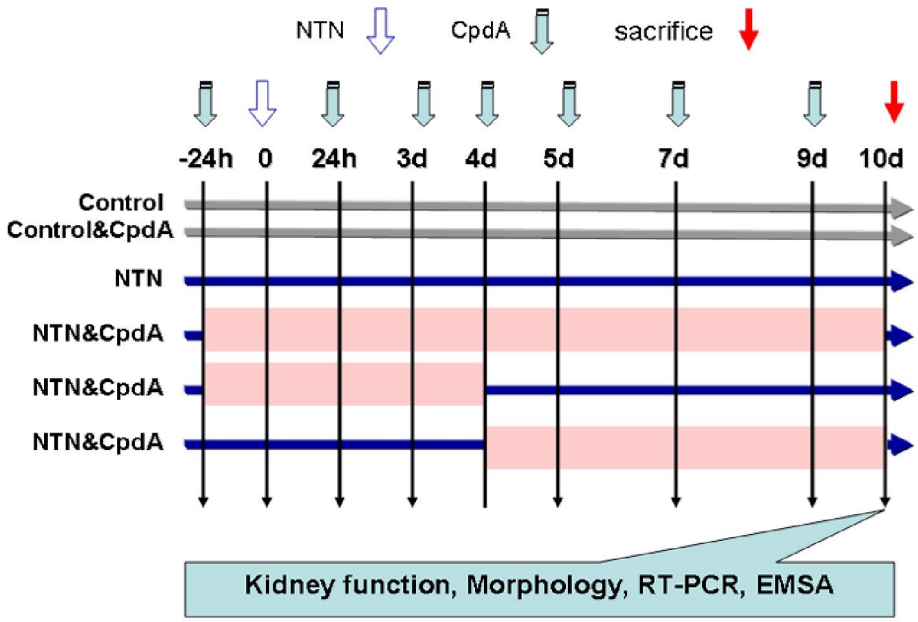


Figure 2.3.2 Compound A (CpdA) was administered in 48 hours intervals by subcutaneous injection. According to the time points of CpdA injection, 4 groups of animals were examined (n=4 per group, experiment were repeated 3 times): ***Group1:*** NTN mice without any further treatment [NTN-10d]. ***Group2:*** NTN mice treated with CpdA; the injection of CpdA started 24 hours before disease induction until 10 days at the time of sacrifice [-1/+10]. ***Group 3:*** NTN mice in which the injection of CpdA started 24 hours prior to disease induction up to day 4; the animals were followed up until day 10 after induction of NTN without any further treatment [-1/+4]. ***Group 4:*** NTN mice in which the injection of CpdA started 4 days after disease induction until 10 days at the time of sacrifice [+4/+10].

In additional experiments C57/BL6 control mice were injected with the IKKβ inhibitor for the 10 days observation period for comparison with control non-treated mice. The end point of the study was day 10 after NTN injection. All animals were sacrificed at this time point.

3 RESULTS

3.1 Characterization of the NTN model in mice

The nephrotoxic serum nephritis (NTN) is a model of Th1/Th17 cell-mediated disease. To ensure the validity of this nephritis model, we collected urine, blood and kidneys of mice at 2, 4, 7, 10, 14 and 21 days after disease induction and assessed the renal function and performed morphological detailed examinations and chemokine expression analysis. To characterize the role of NF-κB in the nephrotoxic serum nephritis, the time-dependent activation and function of NF-κB was determined in this NTN model.

3.1.1 Functional assay of the NTN model in mice.

Blood of mice was collected at 2, 4, 7, 10, 14 and 21 days after disease induction at sacrifice via inferior vena cava puncture. Urine was collected the day before the mice were sacrificed by metabolic cages.

After NTN induction, blood urea nitrogen levels (BUN; mg/dL) were significantly elevated at all time points examined in NTN mice when compared with non-nephritic controls (*Con*: 24.50±2.63; *NTN 2d*: 87.60±16.97; *NTN 4d*: 122.50±26.58; *NTN 7d*: 88.00±8.00; *NTN 10d*: 97.18±24.34; *NTN 14d*: 42.57±1.822; *NTN 21d*: 49.00±9.00; p at least < 0.05 for each time point after NTN induction vs non-nephritic controls). Within the first 4 days BUN increased rapidly, at day 4 the value reached to the peak, but from day 10 a resolution period started, and the BUN levels decreased at day 14 but was still significantly increased when compared with controls (***Figure 3.1.1A***).

The albumin-to-creatinine ratio at all time points examined was markedly increased NTN mice compared with non-nephritic controls (*Con*: 3.667±1.59; *NTN 2d*: 21.50±5.06; *NTN 4d*: 28.00±5.73; *NTN 7d*: 13.00±1.00; *NTN 10d*: 29.44±10.83; *NTN 14d*: 15.00±2.00; *NTN 21d*: 16.00±1.00; p at least < 0.05 for each time points of NTN mice vs non-nephritic controls). The albumin-to-creatinine ratio was maximal at day 10 and declined thereafter (***Figure 3.1.1B***).

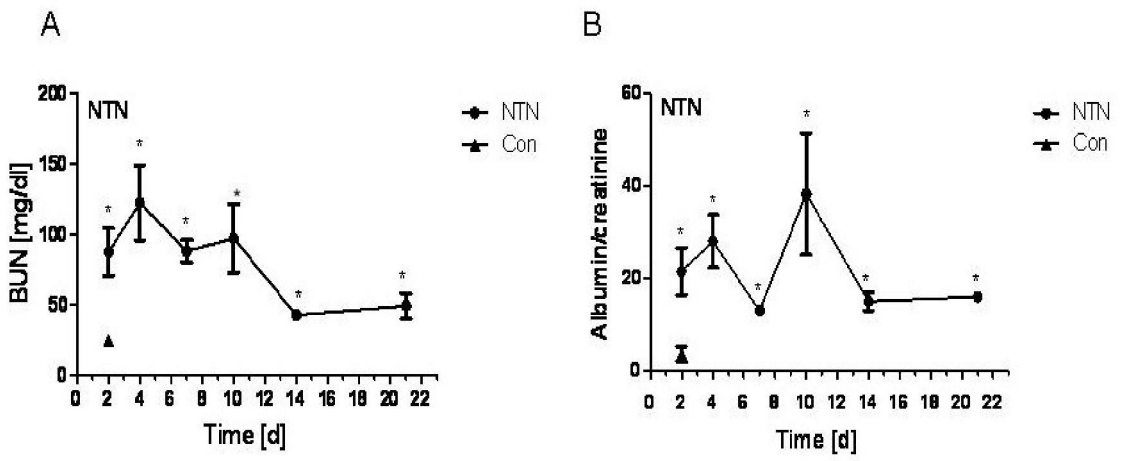


Figure 3.1.1 A, B: Renal function of NTN mice at different time points. ***A)*** The BUN of NTN mice increases significantly after disease induction ($n \geq 3$ for each group; * $p<0.05$ compared with non-nephritis mice). ***B)*** The albumin-to-creatinine ratio is also elevated markedly at different time points ($n \geq 3$ for each group; * $p<0.05$ compared with non-nephritis mice).

3.1.2 Quantification of renal tissue damage in NTN mice.

For quantification of renal tissue damage in the NTN model, PAS stained kidney sections were evaluated by light microscopy. The results demonstrated varying degrees of focal glomerular and tubular damage in NTN mice at different time points when compared to the healthy control group. Glomerular changes included hypercellularity, formation of cellular crescents, and intraglomerular deposition of PAS-positive material. The tubulointerstitial compartment showed protein casts within the tubuli, tubular dilation, loss of brush border, interstitial edema , vacuolization, necrosis and atrophy (***Figure 3.1.2 A***).

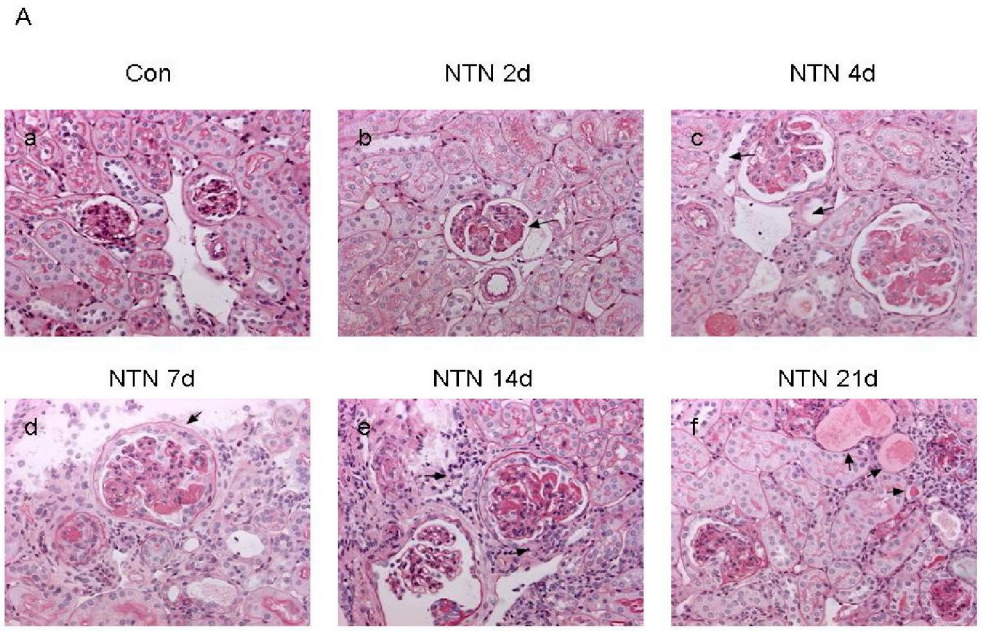


Figure 3.1.2: ***A)*** Characterization of NTN model of glomerulonephritis. Representative Periodic acid-Schiff (PAS) photographs of each time point are shown. ***a:*** non-nephritis control mice. ***b:*** The glomeruli hyalinosis and sclerosis. ***c:*** prominent glomerular sclerosis and loss of brush border, interstitial edema and vacuolization. ***d:*** glomeruli show crescentformation. *e:* glomerular hypercellularity and sclerosis and tubulo-interstitial cellular infiltrate. ***f:*** The tubulointerstitial compartment shows a lot of protein casts in the tubular lumen and cellular necrosis.(original magnification x 200).

A semiquantitative score for the presence of glomerular crescents, glomerular sclerosis and tubulo-interstitial injury was calculated for each animal in an double-blinded observation procedure and demonstrated that the histology score of kidneys at all time points after NTN induction dramatically increased

when compared with non-nephritic controls (P<0.05). Kidney injury was most severe at days 4 (3.07±0.15 vs control; p<0.01) and 7 (3.23±0.23 vs control; p<0.05). After day 7, kidney glomerular and tubulo-interstitial injury improved, however, did not completely resolve (***Figure 3.1.2 B***).

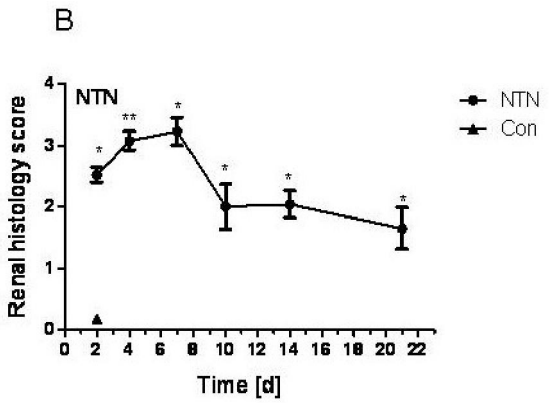


Figure 3.1.2 B: Histology score of NTN mice dramatically increased in all time points examined (n≥3 for each group; * p<0.05; ** p<0.01 when compared with non-nephritis mice). The histology score peaked at around day 4 to 7,declined thereafter, however, did not resolve completely.

3.1.3 Renal T cell and monocytes/ dendritic cells recruitment in NTN mice.

The number of $CD3^+$ T cells and $F4/80^+$ monocytes / macrophages were quantified by immuno-histochemistry. $CD3^+$ T cells and $F4/80^+$ monocytes / macrophages dramatically increased in the tubulointerstitial compartment after induction of NTN (***Figure3.1.3A***).

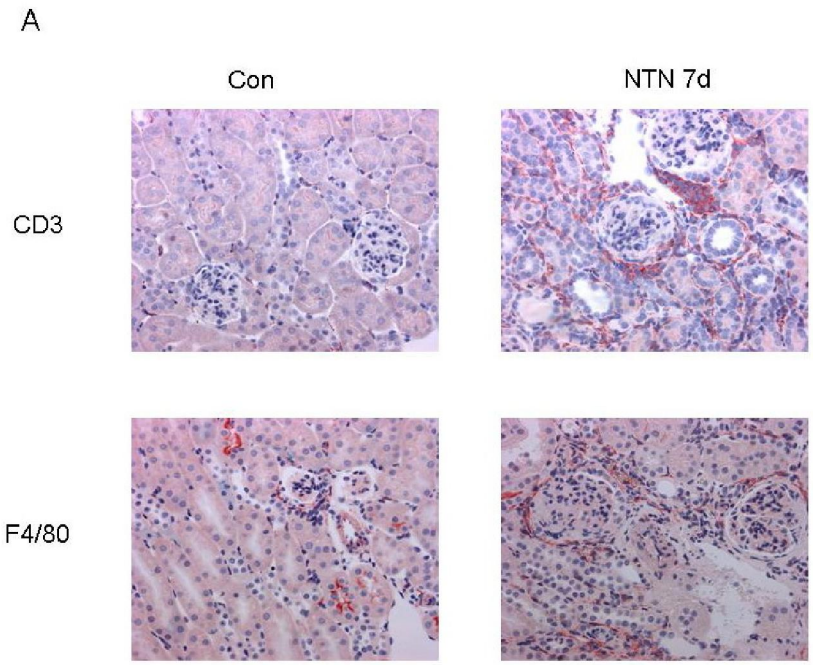


Figure 3.1.3 A: Representative photographs of immunohistological stained kidney section are shown. CD3 antibody and F4/80 antibody reveal a marked increased infiltration of $CD3^+$ cells and F4/80+ cells into the tubulo-interstitial compartment after NTN induction as shown at day 7 when compared with controls (Original magnification x 200).

The recruitment of $CD3^+$ and $F4/80^+$ cells reached a maximum at day 7 (***$CD3^+$ cells***: NTN 7d 33.65±3.19 vs control mice 13.55±1.60, $p<0.05$; ***$F4/80^+$ cells***: NTN 7d mice 3.23±0.23 vs control mice 0.18±0.05, $p<0.05$). At the following time points the number of cells began to decline, however, did not completely resolve and did not reach control values (***Figure 3.1.3 B*** and ***C***).

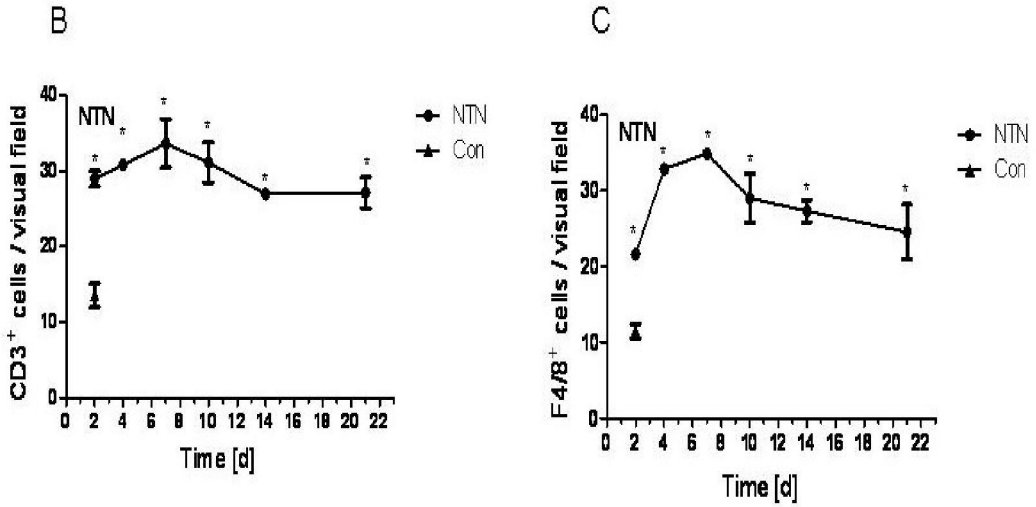


Figure 3.1.3 ***B)*** The number of $CD3^+$ cells was significantly elevate at all time points after disease induction ($n\geq3$ in each group; compared with non-nephritic control mice). ***C)*** the number of $F4/80^+$ cells significantly increase at all time points after disease induction ($n\geq3$ for each group; $*p<0.05$ compared with non-nephritic mice). After day 7, both the number of $CD3^+$ cells and $F4/80^+$ cells declined, however, did not reach control values.

3.1.4 Time dependent chemokines mRNA expression in the NTN model.

RNA was isolated from kidney tissues of control animals and nephritic mice at 2, 4, 7, 10, 14 and 21 days after disease induction and real-time PCR was performed.

MCP-1/CCL2 which acts via CCR2 mainly on monocytes and RANTES/CCL5 via CCR5 on monocytes and T cells were examined. MCP-1/CCL2 expression was significantly upregulated after disease induction and peaked at day 7 (92.09-fold). RANTES/CCL5 expression was markedly upregulated in nephritic animals at days 4 and 7 (2946.94 and 2740.08-fold) when compared to the control group. After 7 days MCP-1/CCL2 and RANTES/CCL5 expression decreased, but was still significantly increased when compared with control animals (***Figure 3.1.4*** A and B).

CXCL10/IP-10 which attracts T cells also showed a strong increase to maximum at day 4 (7231.10-fold) (***Figure 3.1.4*** C).

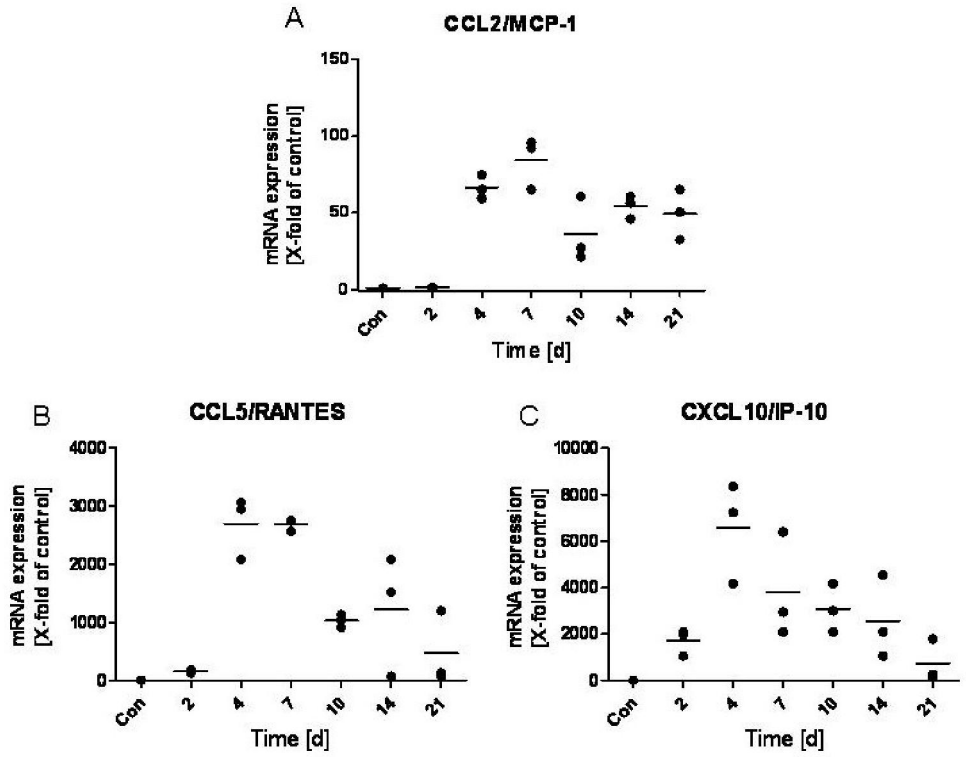


Figure 3.1.4 RT-PCR analysis of renal chemokine mRNA expression in NTN mice (n=3) and non-nephritic control mice. mRNA expression is indicated as x-fold increase when compared to non-nephritic controls. The horizontal lines indicate mean values. ***A), B)*** MCP-1/CCL2 and RANTES/CCL5 expression reach a maximum expression at 7 days, and declined afterwards. ***C)*** The expression of CXCL10/IP10 increased to maximum at day 4, and then began to decrease.

3.1.5 NF-κB activation in the NTN mice model

Having characterized different periods after the induction of the NTN model in terms of renal function, histological changes, leukocytes recruitment and chemokine expression, we next assessed the DNA binding activity of NF-κB in this model.

The gel shift experiments from whole kidney nuclear extracts demonstrated an oscillating activation of NF-κB with a first peak during days 2 and 4, a second peak at around day 10 day and a further peak at day 21 after NTN induction (***Figure 3.1.5***). A nadir of NF-κB binding activity was seen at days 7 and 14 after NTN-induction.

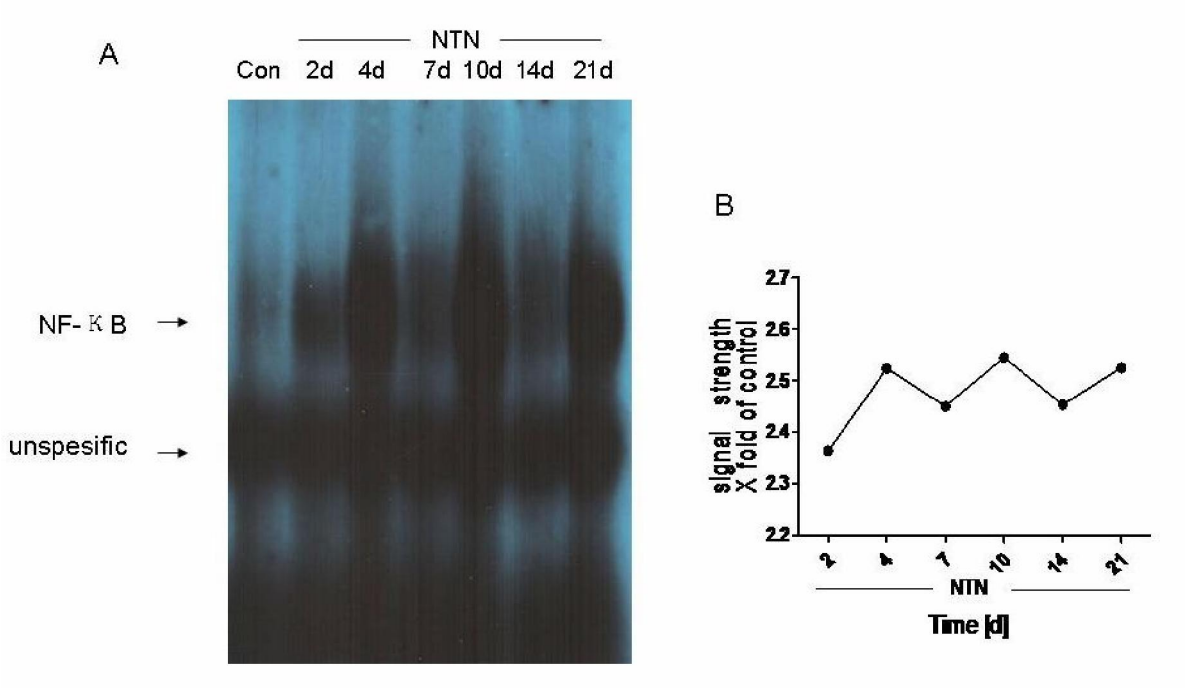


Figure 3.1.5 Time dependent NF-κB activation. ***A)*** Gel shift experiments for NF-κB show an oscillating binding activity with a first peat at day 4 and further peaks at days 10 and 21 after NTN induction. ***B)*** The densitometry of a band at different time points is indicated as X-fold increase when compared with non-nephritis control mice (p at least < 0.05 at all time points examined).

3.2 The role of IKK2 inhibitor CpdA on renal inflammation

To characterize the role IKK2 activation might play in this NTN model, NTN mice were treated with the specific IKK2 inhibitor Compound A (CpdA) at different intervals and periods. All animals were killed at day 10 and compared with non-nephritis mice treated or not treated with the inhibitor with respect to renal function, morphology, expression of chemokines and activation of NF-κB.

3.2.1 CpdA improved renal function in NTN mice.

The BUN level and the albumin-to-creatinine ratio in nephritic animals, which were treated with CpdA strating before disease induction at day -1 until 10 days were markedly reduced compared with non-treated NTN mice (*BUN level*: non-treated NTN mice, 97.18±24.34 mg/dL; NTN&CpdA(-1/+10) mice, 48.58±21.54 mg/dL; $p<0.01$; *the albumin-to-creatinine ratio*: non-treated NTN mice, 29.44±10.83; NTN&CpdA(-1/+10) mice, 5.33±2.05; $p<0.01$) (***Figure 3.2.1***).

However, in the mice pretreated by CpdA starting at day -1 before NTN induction until 4 days [CpdA(-1/+4)] and in mice in which treatment with CpdA was delayed until day 4 after NTN induction and continued until 10 days [CpdA(+4/+10)], BUN level and the albumin-to-creatinine ratio were not significantly improved when compared with non-treated NTN animals ($p>0.05$) (***Figure 3.2.1***).

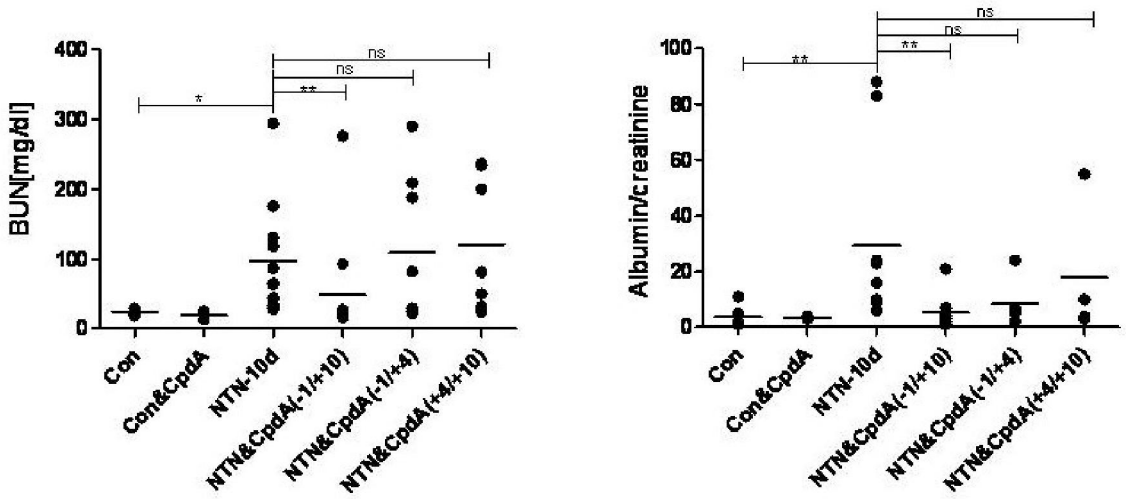


Figure 3.2.1 Renal function was assessed by determination of serum BUN levels and albumin-to-creatinine ratio in CpdA treated groups and non-treated animals. Non-treated NTN mice (NTN-10d: n=11). NTN mice treated with CpdA starting before disease induction at day -1 until 10 days [NTN&CpdA(-1/+10d): n=12]; mice pretreated with CpdA before NTN induction until 4 days [NTN&CpdA(-1/+4d): n=8]; mice posttreated by CpdA after NTN induction from day 4 until day 10 [NTN&CpdA(+4/+10d): n=9]. Symbols represent individual data points, and the horizontal lines indicate mean values (*p<0.05, **p<0.01, ns= non significant).

3.2.2 CpdA decreases renal tissue damage indices

Glomerular and tubulo-interstitial tissue damage score were significantly reduced in the NTN-group pretreated with CpdA until 10 days [CpdA(-1/+10)] when compared with non-treated NTN mice (*renal histology score*: non-treated NTN mice, 1.87±0.36; NTN&CpdA(-1/+10) mice, 0.82±0.24; p <0.05;). But renal injury scores were not significantly reduced in the group pretreated with CpdA before induction of NTN until 4 days [CpdA(-1/+4)] and the group with the delyed CpdA-treatment protocol starting after NTN induction at day 4 until

10 days [CpdA(+4/+10)] when compared with non-treated mice (p>0.05) (***Figure 3.2.2***).

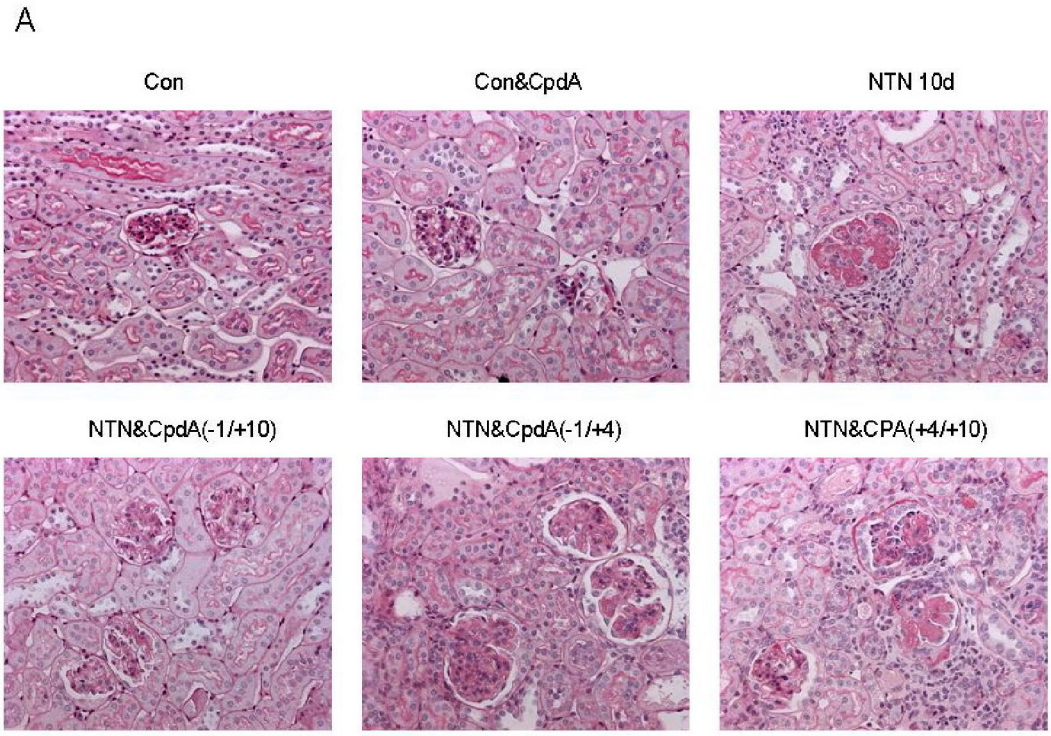


Figure 3.2.2 A) Representative Periodic acid-Schiff (PAS) photographs of non-nephritis mice (control), non-treated NTN mice (NTN-10d); NTN mice treated with CpdA starting before disease induction day -1 until 10 days [NTN&CpdA(-1/+10d)]; mice pretreated with CpdA before NTN induction until 4 days [NTN&CpdA(-1/+4d)]; mice with a delayed treatment protocol with CpdA starting at day 4 after NTN induction until 10 days [NTN&CpdA(+4/+10d)] (original magnification 200x).

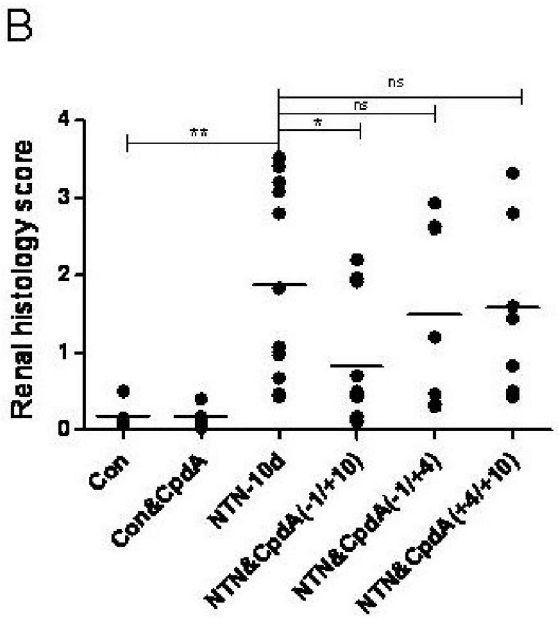


Figure 3.2.2 ***B)*** The renal tissue histology score of NTN&CpdA (-1/+10d) mice was significantly reduced (n=11; *p<0.05) when compared with non-treated NTN mice (n=12). The other two CpdA treatment protocols did not show significant improvement when compared with non-treated NTN mice (*p<0.05, **p<0.01, ns= non significant).

3.2.3 CpdA inhibits the infiltration of T cells, monocytes/dendritic cells and monocytes/macrophages in nephritic animals.

To investigate the effects of IKK2 inhibitor CpdA on renal T-cell and monocyte recruitment, tubulointerstitial T-cells ($CD3^+$), tubulointerstitial monocytes/dendritic cells ($F4/80^+$) and glomerular monocytes/macrophage ($MAC\text{-}2^+$) were assessed by immuno-histochemistry in kidney sections. Representative staining patterns of control animals, non-treated NTN animals and CpdA treated NTN mice are shown in ***Figure 3.2.3*** A.

The influx of T-cells, dendritic cells and macrophages was significantly reduced in NTN mice pretreated with CpdA starting day -1 before disease induction until 10 days [CpdA(-1/+10)] versus non-treated NTN mice (*p<0.05). The numbers of T-cells, dendritic cells and macrophages in the group pretreated with CpdA until 4 days [CpdA(-1/+4)] and the group with delayed CpdA-treatment until 10 days [CpdA(+4/+10)] were, however, not significantly changed when compared with non-treated nephritis mice (p>0.05) (***Figure 3.2.3***).

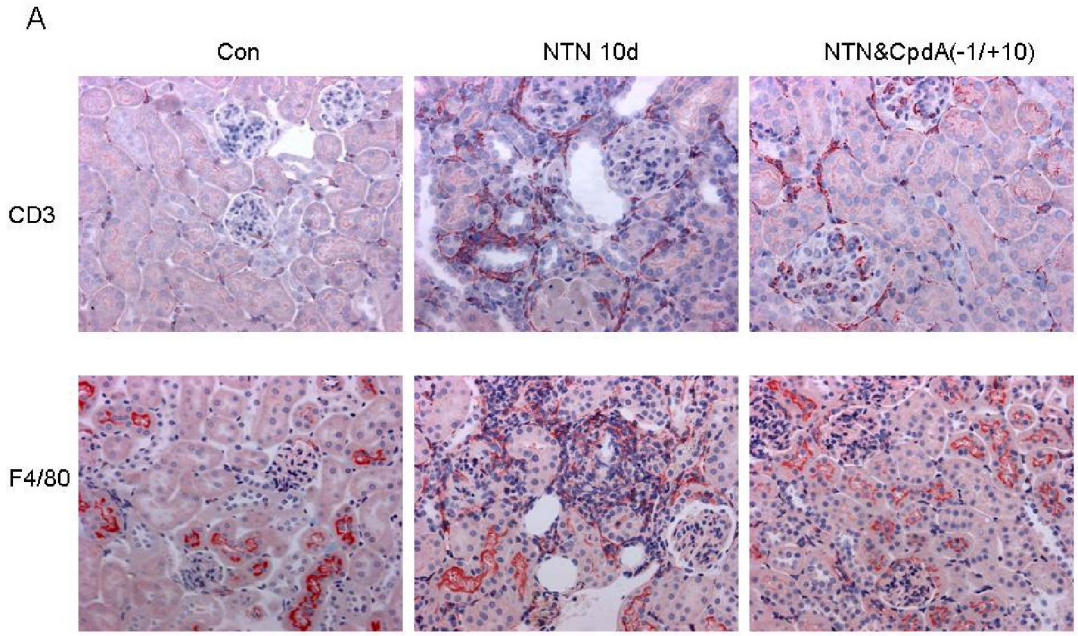


Figure 3.2.3 A) Representative photographs of kidney sections immuno-histochemically stained by CD3 and F4/80 in group of non-nephritis mice (control) ,non-treated NTN mice (NTN-10d) and the NTN mice treated with CpdA before disease induction until 10 days [NTN&CpdA(-1/+10d)] (magnification 200x).

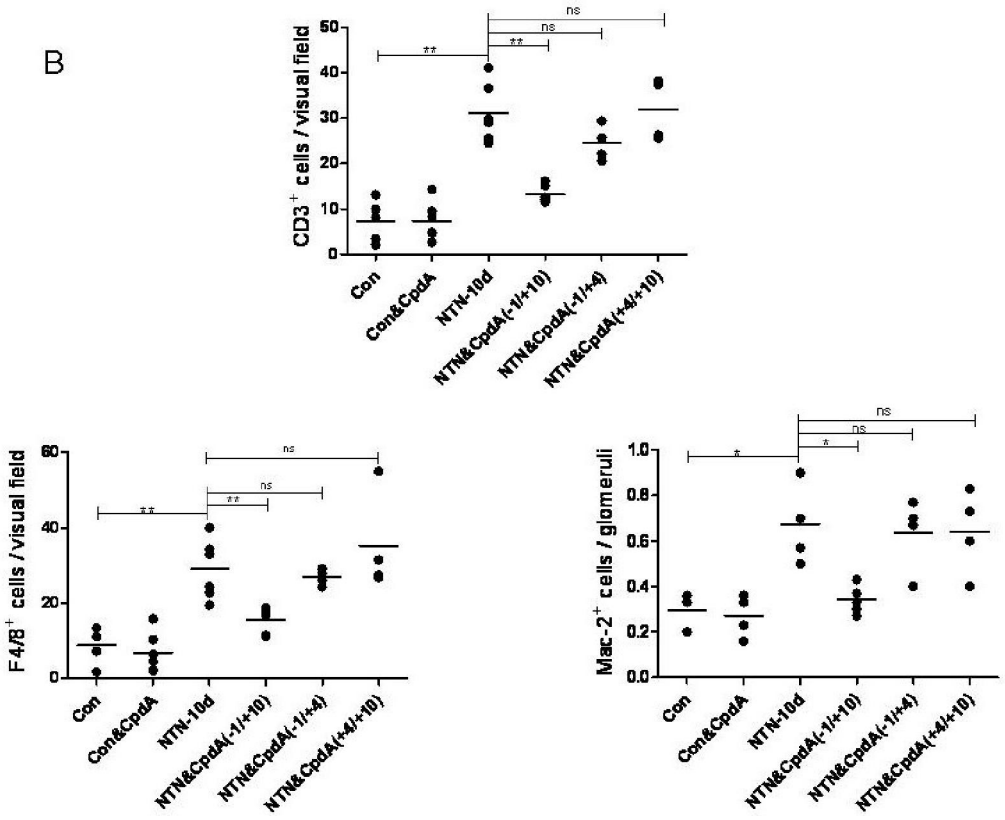


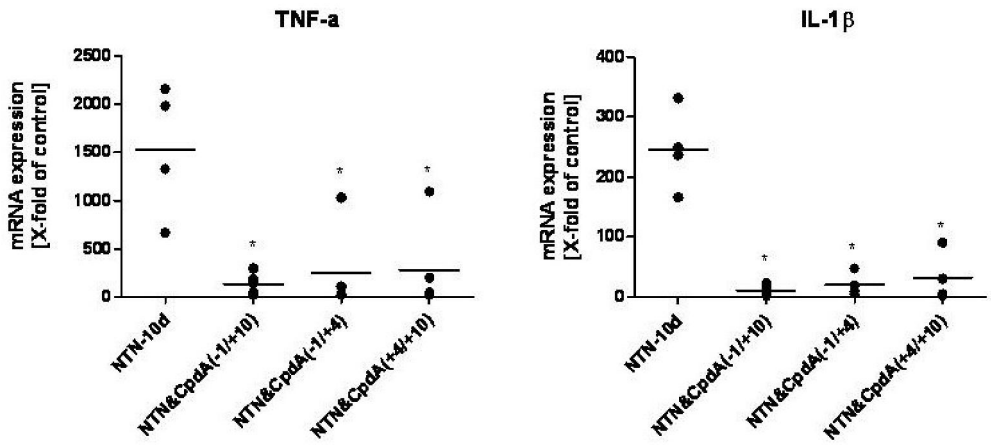
Figure 3.2.3 *B)* Quantification of tubulointerstitial CD3+-T cells and F4/80+ monocytes in non-nephritis mice (control), non-treated NTN mice (NTN-10d), the NTN mice treated with CpdA starting before disease induction until 10 days [NTN&CpdA(-1/+10d)], mice pretreated with CpdA starting before NTN induction until 4 days [NTN&CpdA(-1/+4d)], mice posttreated with CpdA after NTN induction 4 days until 10 days [NTN&CpdA(+4/+10d)]. Symbols represent individual data points, and the horizontal lines indicate mean values (*p<0.05, **p<0.01, ns= non significant).

3.2.4 CpdA decreases cytokine and chemokine expression in the NTN model

TNFα and IL-1β are NF-κB-dependent cytokines activated in response to the induction of glomerulonephritis. Expression of TNFα and IL-1β were significantly reduced in all three groups of mice which were treated with CpdA

when compared with nephritic animals ($p<0.05$).

The expression of MCP-1/CCL2, RANTES/CCL5 and CXCL10/IP-10 was only significantly decreased in mice pretreated with CpdA until 10 days (*MCP-1/CCL2:* NTN mice, 143.79 fold; NTN&CpdA mice, 1.90 fold; $p<0.01$; *RANTES/CCL5:* NTN mice, 1338.44 fold; NTN&CpdA mice, 38.64 fold; $p<0.05$; *CXCL10/IP-10:* NTN mice, 1626 fold; NTN&CpdA mice, 193.90 fold; $p<0.05$). However, the expression was not different in CpdA pretreated until day 4 treated mice [CpdA(-1/+4)] and posttreated mice [CpdA(+4/+10)] versus non-treated NTN mice ($p>0.05$).



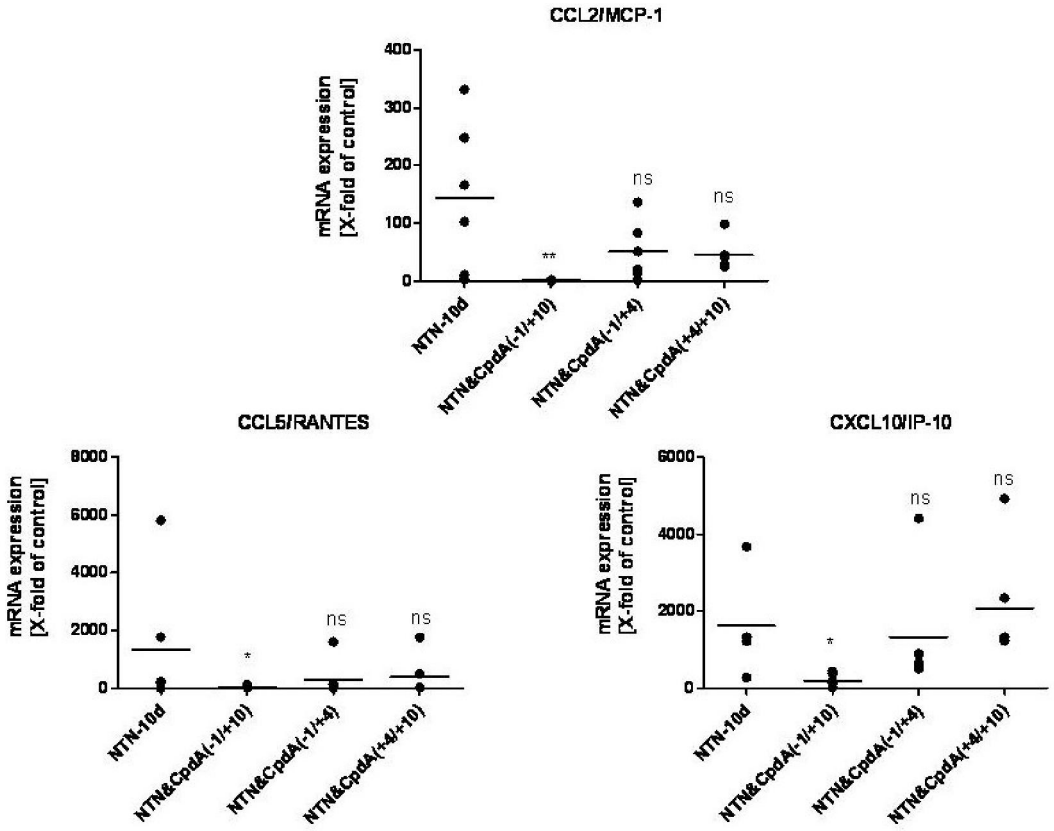


Figure 3.2.4 RT-PCR analysis of renal cytokine and chemokine mRNA expression in non-treated NTN mice (NTN-10d: n=4 for each group), NTN mice treated with CpdA starting before disease induction until 10 days [NTN&CpdA(-1/+10d): n=4 for each group], mice pretreated with CpdA until 4 days [NTN&CpdA(-1/+4d): n=4 for each group], and mice with delayed CpdA-treatment after NTN induction starting day 4 until 10 days [NTN&CpdA(+4/+10d): n=4 for each group]. Symbols represent individual data points, and the horizontal lines indicate mean values (*p<0.05. **p<0.01, ns= non significant when compared with non-treated nephritic NTN mice).

3.2.5 IKKβ inhibitor CpdA blocks translocation of NF-κB

In order to understand the molecular mechanisms of the above mentioned

observations, the NF-κB DNA-binding activity was examined by electrophoretic mobility shift assay in animals treated or not-treated with the specific IKK2 inhibitor CpdA.

Treating control animals with CpdA did not influence NF-κB binding activity [lane *b* vs lane *a*]. The signal of NF-κB DNA-binding activity was significantly reduced in the mice pretreated with CpdA starting before disease induction and continued until 10 days after induction of nephritis [CpdA(-1/+10)] versus non-treated NTN mice ($p<0.05$) [lane *d* vs lane *c*]. NF-κB DNA-binding activity was also significantly reduced ($p<0.05$) in NTN mice treated during the induction period of the disease [CpdA(-1/+4)] when examined at day 10 after nephritis was established [lane *e* vs lane *c*]. The NF-κB DNA-binding activity was, however, not blocked by CpdA in nephritic animals at day 10 when start of treatment was delayed until day 4 after the induction of disease [CpdA(+4/+10)] [lane *f* vs lane *c*].

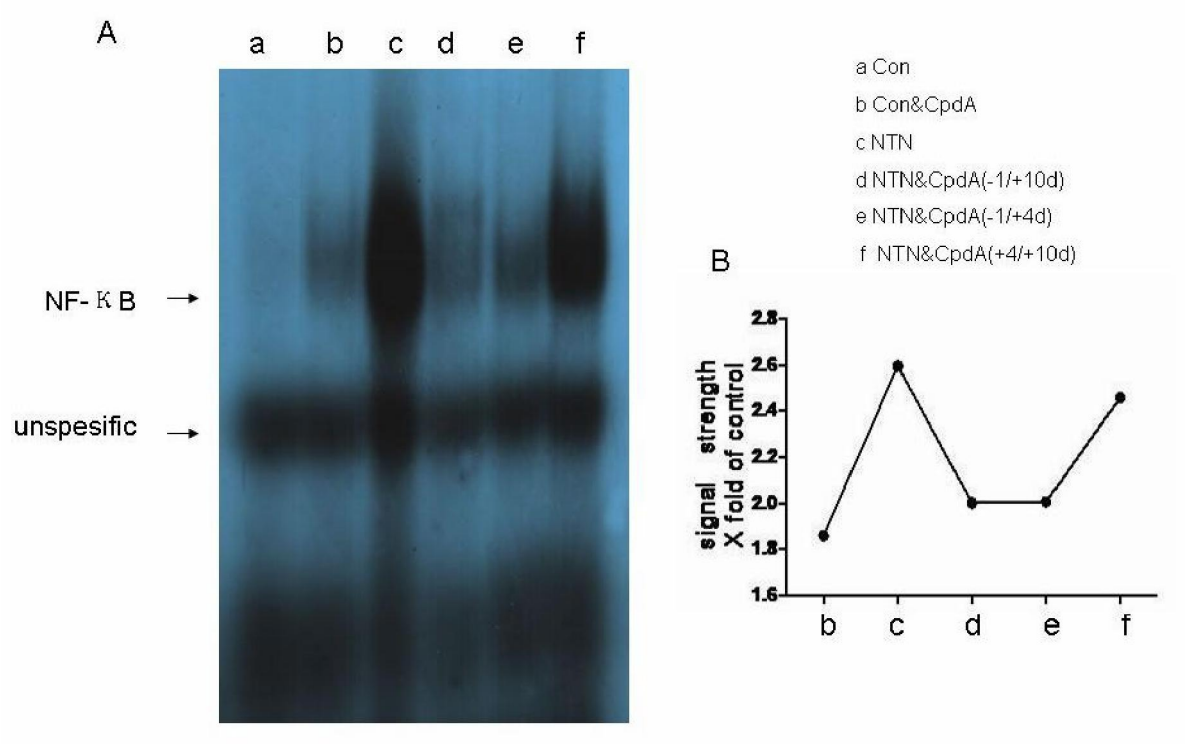


Figure 3.2.5 Characterization of NF-κB activation in the four groups of NTN animals treated or not-treated with CpdA. ***A)*** The NF-κB activation was definitely blocked in the NTN mice treated by CpdA starting before disease induction at day -1 until 10 days [NTN&CpdA(-1/+10d)], also significantly reduced in NTN animals treated during the induction period of the disease [NTN&CpdA(-1/+4d)] but in the CpdA-delayed treated group [NTN&CpdA(+4/+10d)] NF-κB binding activity was prominent and not reduced when compared with the NF-κB binding activity of non-treated NTN mice. ***B)*** The intensity of NF-κB binding as determined by densitometry of the different groups is indicated x-fold when compared with the non-nephritic control group.

4 DISCUSSION

Glomerulonephritis plays a pivotal role in the cause of chronic renal disease and end-stage renal failure requiring dialysis and renal transplantation. Inflammatory renal disease such as glomerulonephritis is induced by the sequential activation of proinflammatory signaling pathways leading to the production of proinflammatory mediators such as cytokines and chemokines. The increased renal expression of chemoattractants is followed by the recruitment of monocytes and T-cells into the kidney where they can exert their deleterious effector function. In the last decades the molecular signaling pathways that initiate these inflammatory reactions have been extensively studied.

We and others have described the activation of NF-κB in the induction phase of several experimental models of glomerulonephritis and confirmed that NF-κB activation plays a role in the pathogenesis of glomerulonephritis. Moreover, it was shown recently that early NF-κB blockade in an experimental model of glomerulonephritis markedly reduced proteinuria, glomerular lesions and monocyte infiltration[19 52 53]. However, the role of IKK2 on activation of NF-κB and the effects on glomerulonephritis was not examined in detail.

The aim of this study therefore was to characterized the role of NF-κB in NTN mediated kidney injury and to examine the functional role IKKβ-inhibition

might play on activation of NF-κB and functional parameters in NTN mice.

We first characterized the effect of the NTN model on renal function, proteinuria, expression of chemokines, and monocytes and T-cells infiltration. During the 21 days observation period after induction of NTN, BUN and albuminuria significantly increased, the expression of chemokines were markedly upregulated, and T cells and monocyte/dendritic cells renal infiltration was significant in NTN mice when compared with non-nephritis control mice.

By morphology, we could demonstrate severe renal damage after disease induction. The glomerular changes included hypercellularity, formation of cellular crescents, and intraglomerular deposition of PAS-positive material. The tubulointerstitial compartment showed protein casts, tubular dilation, necrosis and atrophy. The results suggest that the nephrotoxic serum nephritis model induced by a sheep-anti-mouse GBM antiserum is an effective glomerulonephritis model which resembles human rapid progressive glomerulonephritis.

The functional parameters examined demonstrate a dynamic behavior with an increase within the induction period of the NTN disease during the first 4 to 7 days and a decline thereafter with an incomplete resolution, however, within the 21 days observation period.

To search for possible relevant mechanisms to explain this dynamic process we examined the activation of the transcription factor NF-κB. Gel shift experiments demonstrated an oscillation of NF-kappa B activation with a first

peak during the induction phase at days 2 to 4 and additional peaks at days 10 and 21 after NTN induction.

These are the first *in vivo* data demonstrating NF-κB oscillation after the induction of an inflammatory renal disease in mice. In the Thy-1.1 nephritis model in rats and the LPS-nephritis model in mice we have recently demonstrated a biphasic NF-κB activation[51]. In these models we could demonstrate that the activation of NF-κB during the early period plays a role in the induction of the disease, during the later period of the disease, however, NF-κB activation plays a pivotal role in the resolution process of the nephritis[54] [55]. The idea of NF-κB oscillatory dynamics having a key functional role in stress-response systems has been described recently at the single cellular level.

NF-κB is one of the major families of transcription factors activated during the inflammatory response in renal inflammatory diseases. Inhibitory factor-κB kinase 2 (IKK-2) has been shown to play a pivotal role in cytokine-induced NF-κB activation in glomerular mesangial or tubular epithelial cells and also in disease-relevant infiltrating inflammatory cells. Nevertheless, the potential effect of specific IKK-2 inhibitor has not been described in detail in glomerular inflammatory diseases. Based on the own results of the above described NF-κB oscillation during inflammatory renal disease, the effects of NF-κB blockade are probably highly dependent on the time course of the disease[56] [51,57]. Therefore, blocking of NF-κB at different phases of renal inflammation might have profound effects on the outcome of the disease. Next, intervention with a specific IKK2-inhibitor to block NF-κB activation via the classical pathway was performed to establish NF-κB as a possible new target for the treatment of renal

inflammatory disease.

NF-κB is an important regulator of inflammation of immune responses by mediating proinflammatory gene expression. Under basal conditions NF-κB dimers remain in the cytoplasm as an inactive form. A family of inhibitory proteins, IκBs, bind NF-κB and mask nuclear localization signal, thereby blocking nuclear translocation. Exposure of cells to a varity of proinflammatory stimuli leads to the rapid phosphorylation, ubiquitinylation, and ultimately proteolytic degradation of IκB by IκB kinase (IKKα and IKKβ), which allows nuclear translocation of NF-κB and subsequent gene transcripton[22]. In the two IKK subunits, IKKα and IKKβ, IKKβ is most important for rapid degradation of NF-κB–bound IκBs[32 33].The affinity of Compound A for IKKβ over IKKα is greater than 50-fold despite the 50% sequence homology between the two IKK subunits. As such, it has a significant advantage over other NF-κB inhibitors in its specific suppression of one kinase critical to the classical activation of the NF-κB pathway. IKKβ plays a pivotal role in the nuclear factor-κB-regulated production of proinflammatory molecules by stimuli such as TNFα and IL-1β. Multiple studies have demonstrated that IKKβ inhibition could reduce proinflammatory mediators and prevent antigen-induced T cell proliferation in vitro and vivo[43 58].We therefore applied the IKKβ inhibitor CpdA to block the classical pathway of NF-κB activation at the initiation and later phase of nephritis in the NTN model and get further insight into the functional role of IKKβ.

Our experiments focused on the first 10 days after NTN induction. During this period we have demonstrated two peaks of NF-κB activation, one at day 4

and the second at day 10. The NTN mice were treated with CpdA, a specific IKK2-inhibitor, at different time intervals after the induction of the disease. Nuclear translocation of NF-κB was complete suppressed by CpdA in animals pretreated with CpdA starting before disease induction at day -1 until day 10 when compared with non-treated NTN mice. The signal of NF-κB DNA-binding was also significantly reduced at day 10 in mice pretreated with CpdA before NTN induction until 4 days, however, was not significantly reduced in animals in which CpdA treatment was delayed until day 4 when compared with non-treated nephritic mice.

To determine the possible therapeutic role of IKKβ inhibition with CpdA in NTN glomerulonephritis renal function was examined in the different CpdA treated groups of NTN mice. BUN significantly decreased in the mice pretreated until 10 days. But there was no significant change in the mice with the interrupted and delayed treatment protocols when compared with non-treated nephritis mice. Albuminuria was also significantly reduced in the pretreated group not, however, in the other treatment groups versus non-treated NTN mice. To assess histological changes, PAS staining of kidneys from CpdA treated NTN mice and non-treated NTN mice was examined in detail. The glomerular damage score showed no significant differences between nephritic animals with interrupted and delayed CpdA treatment protocols when compared with non-treated NTN mice. However, kidney morphology of mice pretreated with CpdA and continued treatment until 10 days after NTN-induction was almost completely normal. These data demonstrated that the IKKβ inhibitor CpdA when applicated during the whole observation period of NTN-glomerulonephritis could almost completely abolish NTN induction in mice and preserve kidney

function.

To further address the pathophysiological role of IKKβ-inhibition infiltration of tubulointersitial $CD3^+$ T cells, $F4/80^+$ monocytes/dendritic cells and glomerular MAC-2^+ monocytes/macrophages was examined. Inflammatory cell infiltration was significantly reduced in pretreated animals not, however, in animals in which CpdA-treatment was interrupted or delayed.

Correlating, quantitative PCR analyses demonstrated reduced chemokine expression of MCP-1/CCL2, RANTES/CCL5 (both attract mostly neutrophils and monocytes) and CXCL10/IP-10 (attracts T cells) in the group pretreated with CpdA until 10 days. There was no significant change between CpdA pretreated until 4 days mice, CpdA posttreated mice and CpdA non-treated NTN mice. The expression of TNFα and IL-1β was, however, significantly downregulated in all of the CpdA treated groups when compared with CpdA non-treated NTN mice. In the regulation of TNFα and IL-1β cytokines NF-κB activation via the canonical pathway is the master transcription factor which regulates their expression. Therefore these data demonstrate that the activation of NF-κB has been effectively blocked in all CpdA treated groups.

These differences in the expression profiles described may be due to differential regulation of expression of NF-κB induced genes by the IKKß -inhibitor. Cell specific differences in the activation of IKKß and NF-κB have been recognized recently. It has been demonstrated that IKKß plays a tissue-specific role in inflammation. In resident tissue cells, IKKß-mediated NF-κB activation drives cytokine and chemokine production required to initiate the

inflammatory response. However, NF-κB activation in resident macrophages or leukocytes recruited during inflammation has an antiinflamatory role[59 60].

In conclusion, our experiments demonstrate an oscillation of NF-κB activation during a 21 days observation period in the NTN model, with a first peak during the induction phase at days 2 and 4 and further peaks at days 10 and 21 after the induction of the disease. IKKβ inhibition significantly down regulated renal inflammatory cells infiltration and chemokine expression at day 10 when treatment was started before induction of the disease not, however, when treatment was interrupted or delayed. The selective IKKβ inhibitor reduced NF-κB DNA-binding activity at day 10 in pretreated animals and also in animals in which treatment was interrupted not, however, in the group in which treatment was delayed. These results demonstrate that NF-κB is a master transcriptional regulator in experimental glomerulonephritis. However, other transcription factors might become major players during the later course of the disease. An interaction between the protein kinases JNK1 and IKK2 or the IKK/NF-κB and STAT3 pathways have been described recently to serve as critical molecular links in different inflammatory diseases and carcinogenesis. The precise mechanisms of these linkages are still being investigated. Also, NF-κB might even be activated by non-canonical pathways in situations where IKKβ-inhibition is delayed or inconsistent. Thus NF-κB might be differentially activated in renal cells and infiltrating inflammatory cells depending not only on the cell types but also on the progression state of the disease.

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ABSTRACT

Glomerular diseases are often immune-mediated and the renal infiltration of leukocytes and activation of chemokines play a pivotal role in the pathogenesis of glomeruonephritis. Recent studies have uncovered that the transcription factor nuclear factor-κB (NF-κB) is a key mediator in induction of proinflammatory chemokine and cytokines gene expression in many inflammatory renal diseases. Activation of NF-κB is dependent upon the phosphorylation of its inhibitor, IκBα, by the specific inhibitory κB kinase (IKK) subunit IKKβ. We hypothesized that specific antagonism of the NF-κB inflammatory pathway through IKKβ inhibition attenuates glomerulonephritis.

Experimental nephrotoxic serum nephritis (NTN) in mice was served as a model of human rapid progressive glomerulonephritis. In the present study we first assessed the characteristics of NF-κB activation in this NTN model and found an oscillation of NF-κB activation with a first peak at day 4 after disease induction and further peaks at days 10 and 21.

To further describe the role for IKKβ-activation on the course of glomerulonephritis after NTN induction in mice a selective inhibitor of IKKβ, COMPOUND A (CpdA) was administered. After 10 days kidney function, glomerular morphology, inflammatory cells infiltration and chemokine expression were determined. Compared with untreated NTN animals the NTN mice pretreated with the IKKβ inhibitor had significant reduction in renal tissue injury. Gel shift experiment demonstrated that the translocation of NF-κB was

blocked by the IKKβ inhibitor. Interrupted or delayed IKKβ-inhibition, however, did not influence renal injury after the induction of NTN. These results therefore suggest that inhibition of IKKβ might be a potential novel therapeutic approach for patients with glomerulonephritis when applicated during the early induction period of the disease not, however, during later stages of the disease. These findings need to be confirmed in additional experimental models of renal inflammatory diseases.

ABBREVIATIONS

Ab(s)	antibody
Ag	antigen
ANK	ankyrin repeats motifs
BAFF	B-cell-activating factor of the TNF family
bp	base pair
cDNA	complementary DNA
Ci	curie
CpdA	COMPOUND A
cpm	counts per minute
Cre	cyclization recombination
DCs	dendritic cells
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine-N, N-tetraacetate
EGTA	ethylenguanidine- N, N-tetraacetate
et al.	Lat. et alii and others
g	gram
GN	glomerulonephritis
h	hour (s)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HLH	helix-loop-helix
HR	homologous recombination
IFN-γ	interferon-gamma
Ig	Immunoglobulin
IL	interleukin
IκB	inhibitor of NF-κB
IKK	IκB kinase
LMP1	latent membrane protein-1
IP-10	interferon-inducible protein-10
kDa	kilodalton
KO	knockout

LPS	lipopolysaccharide
LZ	leucine zipper
M	molar
m	milli
mAb	monoclonal Ab
MCAF	macrophage chemotactic and activating factor
MCP-1	monocyte chemoattractant protein-1
mg	milligram
MHC	major histocompatibility Complex
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger RNA
m	micro
mg	microgram
ml	microliter
mM	micromolar
MZ	marginal zone
n	nano
N	number
NEMO	NF-κB essential modulator (IKKγ)
NF-κB	the transcription factor nuclear factor-κB
ng	nanogram
NIK	NF-κB inducing kinase
NLS	nuclear localization signal
NTN	nephrotoxic serum nephritis
P	phosphorylation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p- IκBα	phospho- IκBα
p-p65	phospho-p65
RHD	Rel-homology domain
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase PCR
SE	standard error
Ser/S	serine
SCFβTrCP	Skp1-Cullin-F-box(SCF)–type
TBE	Tris-boric acid-EDTA

TCR	T cell receptor
TEMED	N, N, N’, N’ tetramethylene-diamine
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR-associated factors
U	unit(s)
µl	microliter
wt	wild-type

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