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Role for IKK2-kinase mediated NF- κ B activation in ischemia reperfusion of the acute kidney injury (AKI) model in mice

Dissertation

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INDEX OF CONTENTS

1 INTRODUCTION

- 1.1 ACUTE KIDNEY INJURY
- 1.2 ISCHEMIA REPERFUSION INJURY
- 1.3 PATHOGENESIS OF IRI
- 1.4 NUCLEAR FACTOR-kB (NF-kB) AND IKK2
- 1.5 KINK-1

2 MATERIAL AND METHODS

2.1 MATERIAL

- 2.1.1 Animals
- 2.1.2 Inhibitor
- 2.1.3 Chemicals
- 2.1.4 Devices
- 2.1.5 Buffer
- 2.1.6 Primers for real-time PCR
- 2.1.7 Antibody

2.2 METHODS

- 2.2.1 Induction of IRI and administration of inhibitor
- 2.2.2 Parameters of kidney function
- 2.2.3 Morphological examinations
- 2.2.4 Quantification of CD3+ cells
- 2.2.5 Real Time PCR
- 2.2.6 FACS (Fluorescence-activated cell sorting)

2.2.7 Statistical analysis

3 RESULTS

3.1 CHARACTERIZATION OF THE IRI MODEL IN MICE

3.1.1 Functional analyzing of the IRI model in mice

3.1.2 Quantification of renal tissue damage in IRI mice

3.1.3 Quantification of renal infiltration of CD3+ cells in IRI mice

3.1.4 Time dependent chemokines mRNA expression in the IRI model

3.1.5 Renal and splenic T cells infiltration in IRI mice

3.2 THE ROLE OF IKK2 INHIBITOR KINK-1 IN IRI

3.2.1 KINK-1 and renal function in IRI mice

3.2.2 KINK-1 and renal tubular damage

3.2.3 KINK-1 changes renal infiltration of CD3+ cells

3.2.4 KINK-1 decreases cytokine and chemokine expression in IRI

3.2.5 KINK-1 changes the infiltration of T cells in kidney and spleen

4 DISCUSSION

5 ABSTRACT / ZUSAMMENFASSUNG

6 ABBREVIATIONS

7 REFERENCE

8 CURRICULUM VITAE

9 ACKNOWLEDGMENTS

10 EIDESSTATTLICHE VERSICHERUNG

1 INTRODUCTION

1.1 ACUTE KIDNEY INJURY

Acute kidney injury, previously called acute renal failure, refers to a clinical syndrome that includes reduced renal function of excretion in a short term (hours to days). It is clinically apparent by a decrease of urine output (not always existent), comes with the accumulation of products of nitrogen metabolism like creatinine, urea and other clinically unmeasured waste products.¹ It is now known as a common disease, which could often failed to be diagnosed, and it is closely connected with a high risk for mortality, development of chronic kidney disease and other multiple organ dysfunctions.²

Clinically, it is important to identify the underlying cause of AKI to improve its recovery. The causes of AKI are generally classified into prerenal, renal or postrenal.

Prerenal AKI is caused by decreased blood flow to the kidney as a result of impaired blood volume, low circulating volume to the kidneys for example in condition of impaired cardiac function, systemic vasodilatation or increased renal vascular resistance like during surgical processes.

Renal AKI means that the damage has taken to the renal parenchyma, such as glomeruli, renal tubules and intersitium. These occur under certain situation such as renal artery stenosis, artheroembolic disease or glomerular problem like immunglobulin A glomerulonephritis or Wegener's granulomatosis.

Postrenal AKI is due to the blockage of the urinary tract or bladder neck obstruction. The most common causes of AKI are usually associated with renal ischemia, infection and nephrotoxic drugs.^{3,4} Transplanted kidneys are liable to all forms of AKI seen in native organs, as well as medication toxicity, ischemia-reperfusion injury, and surgical complications.⁵

The Acute Kidney Injury Network (AKIN) is maintained as the most recent definition

of acute kidney injury and Risk, Injury, Failure, Loss, and End-stage kidney disease (RIFLE) staging criteria, and is suggested by the Kidney Diseases: Improving Global Outcomes (KDIGO) clinical practice guidelines workgroup. The rise in serum creatinine and decrease of urine output are current representative for recognizing acute kidney injury, which is the KDIGO criteria stage, rather than changes in glomerular filtration rates (GFR).^{6,7}

Recently, KDIGO suggest changing the staging for AKI. And this new standard regarding the criterion of time is important for medical practice. KDIGO covers both AKIN and RIFLE criteria, including changes in creatinine within 48 hours or decrease of the GFR over 7 days. Additionally, patients under 18 with a GFR <35mL/min and patients with a serum creatinine >4.0mg/dL (absolute value) were added to AKIN stage 3.⁸

Since the standardized criteria of diagnosis and staging of AKI has provided, prevalence of AKI has increased recently. Nowadays, it is reported that up to 45% patients of ICU and 20% of hospitalized patients have AKI.⁹

Some studies suggested another possibility of unexpected effects of AKI. AKI can induce the accumulation of uremic toxins, and that cause negative effect on immune systems. So that Dialysis required AKI patients, even though they have recovered from dialysis, have prominent higher chance to develop malignancy than the patients without AKI episode.¹⁰

Current understanding of acute kidney inflammation is based on preclinical (rodent mostly) models. The acute response in initial phase includes infiltration of leukocytes and their resident. Over several days, it develops into a stage of repairing and regeneration, regulated from reprogrammed leukocyte subsets. These could provide specific target for therapy, through blocking or inhibiting of particular elements. However, those interventions could disrupt the natural repair process, if it performed in improper period.¹¹

1.2 ISCHEMIA REPERFUSION INJURY

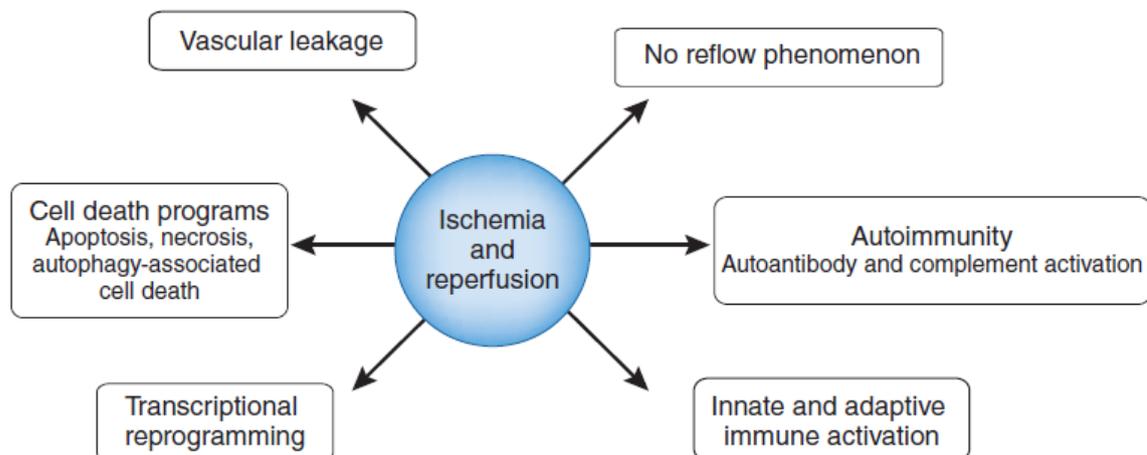
Ischemia reperfusion injury (IRI) is a major cause of acute renal failure, which is common kidney disorder with still high mortality. Its strong inflammatory and oxidative stress reaction of hypoxia and reperfusion frequently occurs in medical state of shock, sepsis or transplantation. During IR Phase, renal proximal tubules are seemed to be the most vulnerable. Subsequently, involve various cellular factor in the post IRI events and these lead to acute kidney injury.¹² The important parts of cell biological outcome of IRI have been researched, but still just few therapies are available.¹³

Ischemia reperfusion is a pathological state defined as an initial interruption of blood supply the organ and ensuing restoration of blood perfusion, which also means reoxygenation of the organ. Blockage of the arterial blood supply causes critical metabolic imbalance of supply and demand and consequentially leads to hypoxia. It is possible that the recirculation of blood supply and reoxygenation is usually related with an aggravation of tissue injury and exacerbate inflammation of organ (called 'reperfusion injury').¹⁴

1.3 PATHOGENESIS OF IRI

Various factors are involved in biological process of IRI [Figure 1]. Vascular leakage caused by damaged endothelium during hypoxia due to cAMP increase.¹⁵ Blood perfusion in an ischemic organ may not initially sufficient without delay (no reflow phenomenon). It is also including autoimmune responses and activation of the complement system (autoimmunity)¹⁶ with innate-/adaptive immune activation occurs in a sterile environment¹⁷. There are two major pathways of renal IRI, inflammation and apoptosis, related with a critical regulator NF- κ B¹⁸ (Transcriptional reprogramming) and therefore further complicated by endothelial cell damage,

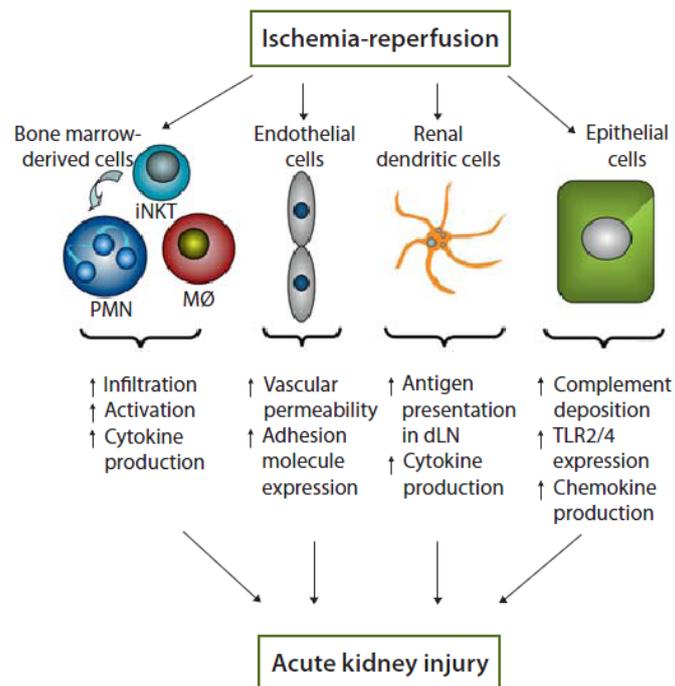
tubular apoptosis, tubular necrosis, inflammation and tubular cell proliferation. Many studies have shown that cell death through apoptosis critically contribute to the pathophysiology of renal IRI.¹⁹



[Figure 1] Pathological process in IRI (Cited from¹⁴)

Immune/Inflammatory cellular response

After ischemia in kidney innate and adaptive immune responses occur. Immune response begins with the rapid inflow of immune cells through disorganized endothelium. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on endothelium and TLRs (mainly TLR2 and TLR4) on the surface of the renal tubular epithelium are more expressed, which trigger the immune response.²⁰ Important cells for innate immune system like macrophages, NK cells, neutrophils and dendritic cells are contributed in pathogenesis of renal IRI. T cells, the major effector cells of adaptive immune system, are also involved in the renal IRI.



[Figure 2] Cellular responses in IRI (Cited from²¹)

Infiltration of macrophages occurs shortly after IRI. It enhances the cascade of inflammation while producing proinflammatory cytokines such as TNF α , IL-1 β , MCP-1 or IL-6. Systemic monocyte-macrophage depletion effects significant reduction of expression of those cytokine and chemokine genes, which results diminished inflammation, less tubular necrosis and apoptosis of epithelial cells. And this implies that M1 has its role in the early phase of IRI.²²

Other studies also suggest that macrophages (M2 macrophage)²³ play pivotal role for repair process and fibrosis in the later phase of IRI, showing that the depletion of macrophages blocking the tubular epithelial cell (TEC) regeneration. After injection of macrophage, regeneration of epithelial cell is recovered.²⁴

Natural killer (NK) cells destroy directly TEC rapid crossing the basement of the membrane in the early phase of IR. Its infiltration stimulates TECs to produce CXCR2 chemokines and inducing neutrophil migration.²⁵

As early as 3 hours after IRI, Natural killer T (NKT) cells infiltrate into postischemic kidney and decrease 24h after IRI. NKT cells, having both T cell receptors (TCRs) and natural killer cell receptor play important role in regulating autoimmune disease and allogenic immune response.²⁶ Recent studies showing that NKT cells mediate neutrophil INF-r production and that contribute the induction of early renal injury.²⁷

There have been studies that CD4 knocked out mice preserved significantly from renal IRI, not in CD8 knockout mice. CD4 T cell can be functionally differentiated to Th1 (producing IFN-r) or TH2 (IL-4) phenotype. And the enzymes STAT (signal transducers and activators of transcription) 4 and STAT6 are responsible for Th1 or Th2 differentiation. Studies with murine renal IRI model suggesting that CD4 T cells of the Th1 phenotype contribute to pathogenic process while CD4 T cells of the Th2 phenotype to protective process. The lack of STAT 4 (regulating Th1) showed an improved renal function after IRI. In contrast, STAT 6 (regulating Th2) deficiency aggravated renal injury.²⁸

B cells have both protective and pathogenic roles during AKI process. It has pathogenic function while mediating natural Ab IgM binding to the mesangium cells after IR. Peritoneum B-1 cells depletion showed reduced mesangial IgM and protective effect. And probably through producing of the anti-inflammatory cytokine IL-10, could B cells protect the kidney after IR.²⁹

After IRI, the number of MHC (major histocompatibility complex) class II antigen expressed renal dendritic cells (DC) found increased.³⁰ Dendritic cells have their function in early antigen-independent inflammatory response after reperfusion. Through IL-17/IL-23 signaling pathway and activation of NKT cells, they can contribute to produce cytokines or chemokines that induce the infiltration of neutrophil. On the other hand, DCs can also play role in inducing tolerance by insufficient positive or enhanced negative co-stimulatory signals and reduce pro-inflammatory cytokines. They can generate immune tolerance via inducing/deletion of T cell anergy or induction/expansion of regulatory T cell.³¹

Soluble components (Complement, cytokines and chemokines)

The complement system contributes considerably to the inflammatory response to IRI. It was believed at the first time that the complement system has their reaction to non-self-antigens. But in recent studies have proved that complement system play also complex role in sterile immune response to injury and following repairing tissue. After IRI, the three main pathways of complement system (classical, alternative and mannose-binding lectin) could be activated through releasing of danger-associated molecular patterns (DAMPs), neo-antigen formation and immune complex formation.³² It has been reported that C5a and C5b-9 complex (membrane attack complex) are connected to promoting renal injury after IRI.³³

Cytokines like IL-1, IL-6 or TNF- α are important here in the initiation phase of renal IRI and a few cytokines are supposed to be related to the repair process of kidney IRI.³⁰ Various strategies are on the development stage to prevent IRI, none are particularly satisfactory so far.³⁴

Chemokines modulate the recruitment of leukocyte to the inflammation sites in all steps. CXC chemokines, like CXCL9 (a monokine induced by interferon gamma), CXCL10 (IFN- γ -induced protein-10) or CXCL11 (IFN-inducible T-cell alpha-chemo-attractant) bind to CXCR and CC chemokines such as CCL3 (macrophage inflammatory protein-1 α), CCL4 (macrophage inflammatory protein 1 β) and CCL5 (regulated on activation, normal T cells expressed and secreted) bind to CCR5. Those findings signify that blocking the chemokines like CXCR3 or CCR5 could reduce T cells and NKT cells infiltrating and could contribute to protective effect for kidneys in IRI.³⁵

Cells for renal protection or repair of IRI

Along with Th2-CD4 T cells, regulatory T cells (Tregs) also considered to be involved in renal protection in IRI with their anti-inflammatory immune responses. Tregs, which have immunosuppressive function, could be identified with their expression of CD4 protein and CD25 protein on the cell surface and up-regulation of the transcription factor FoxP3. The performance of Tregs can be mediated for example by anti-inflammatory cytokines such as IL 10 or extracellular adenosine.

Recent studies have proved that Tregs can traffic to the areas of inflammation to reduce immune reaction and can suppress the innate immune response via prevent effector T cell proliferation and function.³⁶

Anti-inflammatory cytokines

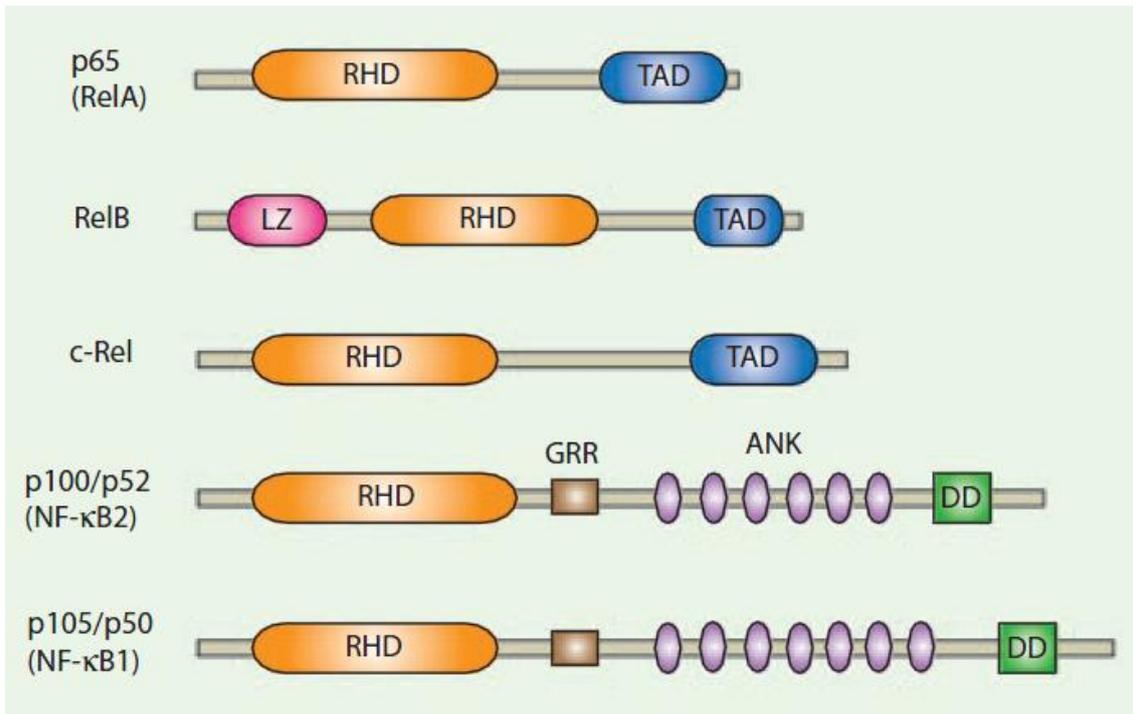
IL-4, IL-10 and IL-13 are considered to be protective cytokines reducing renal injury from IRI.³⁰ One recent study suggested that in the later phase of IRI, ischemic preconditioning could protect kidneys via enhancing mobilization and recruitment of endothelial progenitor cells and this protective effect is provided from vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1a (SDF-1a).³⁷

1.4 NUCLEAR FACTOR-kB (NF-kB) AND IKK2

During renal IRI, pathways of inflammation and apoptosis are activated. NF-kB is considered to be a critical regulator of both processes. NF-kB involves in induction and resolution of inflammation. Inflammatory events in IRI (with NF-kB activation) are considered as potential targets for treatment and prevention of the injury.¹⁸

The nuclear factor-kB (NF-kB) has been studied intensively since their finding by Sen and Baltimore in 1986. NF-kB transcription factors family has important role in adaptive immunity and innate immune responses, regulating anti-apoptotic genes and cell survival³⁸ or contributing regulation of various aspects of development and differentiation of T-cell.^{39,40}

Five Rel family members: RelA (p65), RelB, c-Rel, NF- κ B1(p50), NF- κ B2(p52) are composing NF- κ B in various homo-dimeric and hetero-dimeric form.⁴¹



[Figure 3] Members of NF- κ B/Rel members (Cited from³⁹)

RHD: Rel homology domain, TAD: trans-activation domain, LZ: leucin zipper domain, GRR: glycine-rich region, DD: death domain, ANK: ankyrin repeats

NF- κ B is in the cytoplasm in resting cells inactive binding with I κ B inhibitors (I κ Ba, I κ Bb and I κ Be).⁴²

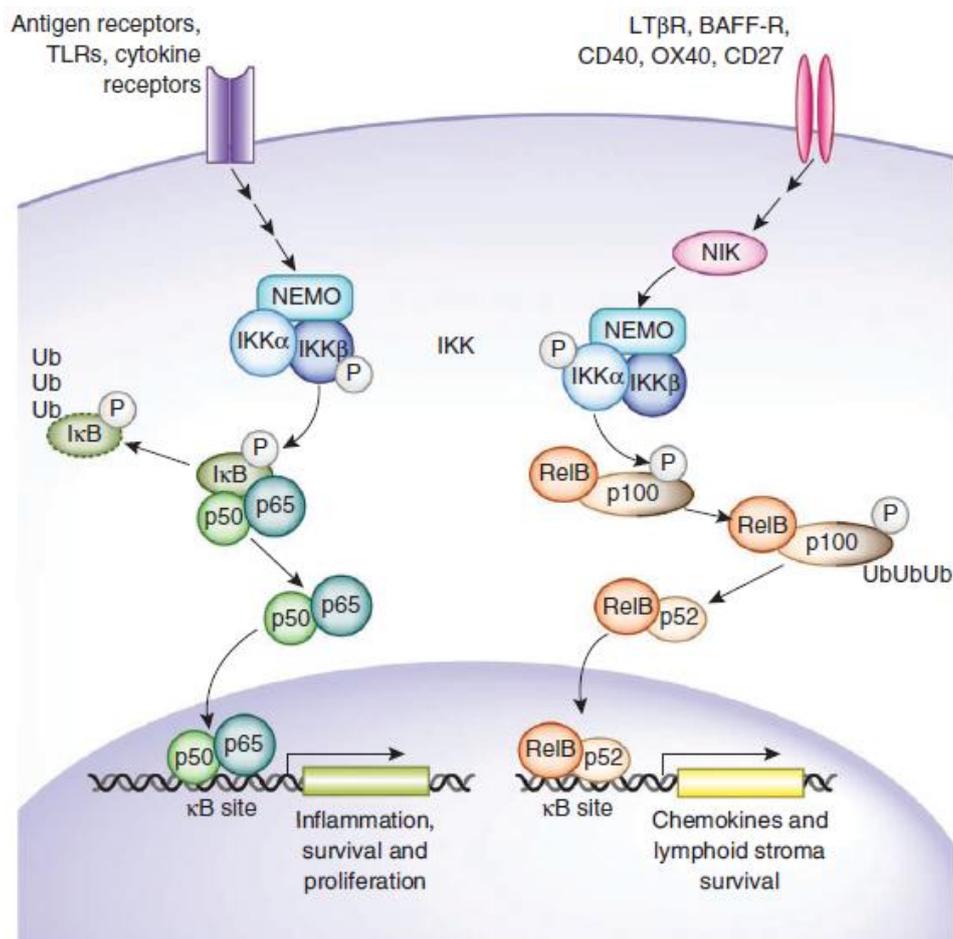
And the kinase complex which capable of phosphorylates I κ Bs composed of IKKa, IKK β and NEMO. The serine/threonine kinases IKKa (86 kDa; known as IKK1 or CHUK) and IKK β (87 kDa; known as IKK2) are in dimers form, proved as catalytic components of the IKK complex and be able to phosphorylate I κ Bs in vitro. The third component NEMO (48 kDa; known as IKKc, IKKAP1 or Fip-3) is a regulatory non-enzymatic scaffold protein.⁴³ The canonical I κ B kinase (IKK) complex contributes to

many cellular processes under various physiological and pathological stimuli.⁴⁴

There are two different pathways of NF- κ B activation: canonical and non-canonical.

In the canonical pathway, the IKK complex (IKK α , IKK β and NEMO) phosphorylates I κ B under a variety of stimuli like hypoxia or inflammatory cytokines. And this leads to ubiquitination and proteosomal degradation of I κ B. NF- κ B dimer (p50, p65, or c-Rel) is then released from I κ B. NF- κ B translocates into the nucleus, binds to a κ B-binding site.

In the non-canonical pathway IKK α phosphorylates p100. This leads to its ubiquitination and partial processing into p52. P52 and RelB heterodimer translocates into the nucleus.^{18,39,45}



[Figure 4] The canonical (left) and non-canonical (right) NF- κ B signal transduction

pathway (Cited from⁴⁶)

TLR: Toll-like receptor, LT β R: lymphotoxin β receptor, Ub: ubiquitination

NF- κ B has variety of inducers; include pro-inflammatory cytokines, tumor necrosis factor (TNF) and interleukin 1, which induce fibroblasts and macrophages, antigens for B cells or T cells, even DNA-damaging chemicals. Each of them are recognized by different receptors on the surface or inside cells and activated by different pathways through signal transduction process.⁴⁵

Activation of NF- κ B may also resulted from activation of TLR and leads to release of pro-inflammatory cytokines, for example IL-6 and TNF- α . TLRs are higher expressed in circumstance of acute kidney injury, ischemic kidney damage. And also have connection with end-stage renal failure, acute renal transplant rejection and even delayed allograft function. In recent study, TLR2 is detected to be up-regulated in both human and rodent renal allografts.⁴⁷

Intervention in IRI with NF- κ B inhibition

Generally, there are two major strategies for experimental mouse models of NF- κ B inhibition; Targeting IKK and blocking the activation of NF- κ B by through over-expression of a non-degradable inhibitor. But only the way of IKK inhibition is considered to be available for human studies, while yet just a few IKK inhibitors have been developed. They are also shown to have anti-tumor and anti-inflammatory effects, but still not clinically licensed. So the alternative blockers are also in research such as inhibitors of the ubiquitin-proteasome system.⁴⁸

Targeting IKK β with local injection of small interfering RNA (siRNA) showed that they have protective effect against renal ischemia-reperfusion injury in rats. The results imply that silencing of IKK β using siRNA induce inhibition of renal IKK β gene expression, so also NF- κ B/DNA binding activity and expression of IL-18 and NGAL, showing reduced Kidney injury from IR.⁴⁹

In vitro, both IKK1 and IKK2 phosphorylate I- κ B, but IKK2 has a higher kinase potential and affinity than IKK1.⁵⁰ Therefore, inhibition of IKK2 could be a considerable new therapeutic intervention in IRI induced AKI.

1.5 KINK-1

Several compounds, which are synthetic or plant-derived, have proved to inhibit NF- κ B activation either via directly inhibiting phosphoinositide-3-kinase (PI3K) or specific IKK inhibition or blockade of proteasome pathway.^{51,52}

KINK-1 (kinase inhibitor of NF- κ B-1, known as Compound A, CpdA) is the first compound of small-molecule substances which selectively targets IKK2-Kinase activity as aATP-competitive inhibitor.⁵³

We used in our study this KINK-1 (7-[2-(cyclopropyl-ethoxy) -6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-yrido[2,3-d][1,3]oxazin-2-one hydrochloric) whether it has protective effect in renal IRI.

2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Animals

9 to 11-weeks-old wild-type male C57BL/6 mice were used for these experiments. All animals were raised under sterile flow conditions. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (G13/058).

2.1.2 Inhibitor

Bayer HealthCare supplied KINK-1 for these experiments the specific IKK2-inhibitor (Dr. K. Ziegelbauer). Stock solution was prepared by dissolving the compound in cremophore 10%, diluted with PBS. Animals were injected intraperitoneally with 5mg/kg bw.

2.1.3 Chemicals

Chemicals used for following experiments.

Experiment	Reagent	Manufacturer
Induction of IRI	Isofluran	Abbvie
	Ketamine	Pfizer
	Xylazine	Bayer
	NaCl	Braun
	Tramal	Grünenthal

	Cremophore	Sigma
Blood Nitrogen	Heparin	Braun
Histology	NaH ₂ PO ₄	Merck
	Na ₂ HPO ₄	Merck
	Formalin	Merck
	PBS	Biowhittaker
RNA Isolation	In NucleoSpin kit	Macherey-Nagel
cDNA	Aqua	Braun
	Hex-Primer	Invitrogen
	dNTPs	Invitrogen
	5xBuffer RT	Invitrogen
	RnaseOutH ₂ O	Invitrogen
	Reverted-RT	Invitrogen
PCR	Sybr green	Invitrogen
Cell Staining	RPMI Medien 1640 1x	Gibco
	Heat Inactivated FBS	Gibco
	DPBS	Gibco
	HBSS	Gibco
	Collagenase D	Roche Diagnostics, Indianapolis, IN
	DNase	Invitrogen
	Tyran blue Stain	Gibco life technology
	Mouse-Serum	Invitrogen
	Cellwash	BD Biosciences
	Fixation concentrate	eBioscience

	Fixation Diluent	eBioscience
	10x Permeab. Buffer	eBioscience
FACS	Clean (for machine)	BD Biosciences
	Rinse (for machine)	BD Biosciences

2.1.4 Devices

Devices used for following experiments.

Experiment	Devices	Manufacturer
Induction of IRI	Microvascular Clamps (Straight, MORIA MC43, 53 mm)	FST
	Syringe 1ml	Braun
	Syringe 10 ml	Braun
	Injection needle 20G	Braun
	Suture material (Vicryl Ethicon 3-0)	Johnson & Johnson
Histology	Light microscope	Zeiss
RNA Isolation	NucleoSpin kit	Macherey-Nagel
	Biological safety cabinet	Nu Aire
cDNA	GeneQuant	GE Healthcare
	Thermocycler	Biometra
PCR	StepOne	Applied Biosystems
Cell Staining	Laboratory fume hoods	Biological safty Cabinets
	Heat bath	GFL
	GentleMACS Dissociator	MACS Miltenyl Biotec
	TC20 Cell counter	BioRad
FACS	LSR II	BD Biosciences
Scale		Sartorius, Scaltec
Shaker (Rocky-3D)		LTF Labortechnik

Centrifuge Biofuge fresco		Heraeus
Centrifuge Multifuge 1S-R		Heraeus
Pipette		Eppendorf, Gilson, Hirschmann Laborgeräte
Vortex		Janke & Kunkel, B.Braun Biotech
Fridge 4°		LIEBHERR
Fridge -20°		LIEBHERR
Fridge -80°		Heraeus
Computer		Hp, Dell
Software	Windows 7, XP	Microsoft
	Excel	Microsoft
	Power Point	Microsoft
	Word	Microsoft
	Microscope software	AxioVisionLE
	StepOne Software	Applied Biosystems
	GraphPad prism 5	GraphPad
	KC Junior	Bio-Tek
	Image J	Fiji
	FACSDiva Software	BD Biosciences

2.1.5 Buffer

Buffers for histological examination:

Sorensen's Buffer:

3.03 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$

14.14 g $\text{Na}_2\text{HPO}_4 \times \text{H}_2\text{O}$

Add 1L H_2O

pH 7.2 – 7.4

Formalin-solution (4%):

4% Formalin

0.4% Eosin

In Sorensen's Buffer

Buffer for cell staining (1:9)

Lysis Buffer 1ml:

Tris HCl 170 mmol/L (pH7.6) 8.5 ml + 41.5ml H_2O

Lysis Buffer 9ml:

NH_4Cl 160 mmol/L 4,27g in 500ml

2.1.6 Primers for real-time PCR

Primers were synthesized by Invitrogen and designed for using StepOne from Applied Biosystem.

Sequences of Primer	
18s	Fw: CAC GGC CGG TAC AGT GAA AC Rev: AGA GGA GCG AGC GAC CAA A
TNF α	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	Fw: GGC TCA GCC AGA TGC AGT TAA Rev: CCT ACT CAT TGG GAT CAT CTT GCT
CCL5	Fw: GCA AGT GCT CCA ATC TTG CA Rev: CTT CTC TGG GTT GGC ACA CA
CCL20	Fw: GGA TAC ACA ATA GGA GTA TG Rev: CCT CAG GAT GTA GGA GGA AG

Antibodies used for cell staining.

Antibody	Conjugate	Manufacturer
CD3	AF700	BD Biosciences
CD25	APC	BD Biosciences
CD4	APC H7	BD Biosciences
CD45	PerCP	BD Biosciences
CD8a	V500	BD Biosciences
FoxP3	PE	BD Biosciences
IFN γ (Th1)	BV710	BD Biosciences
IL17 (Th17)	V450	BD Biosciences

2.2 METHODS

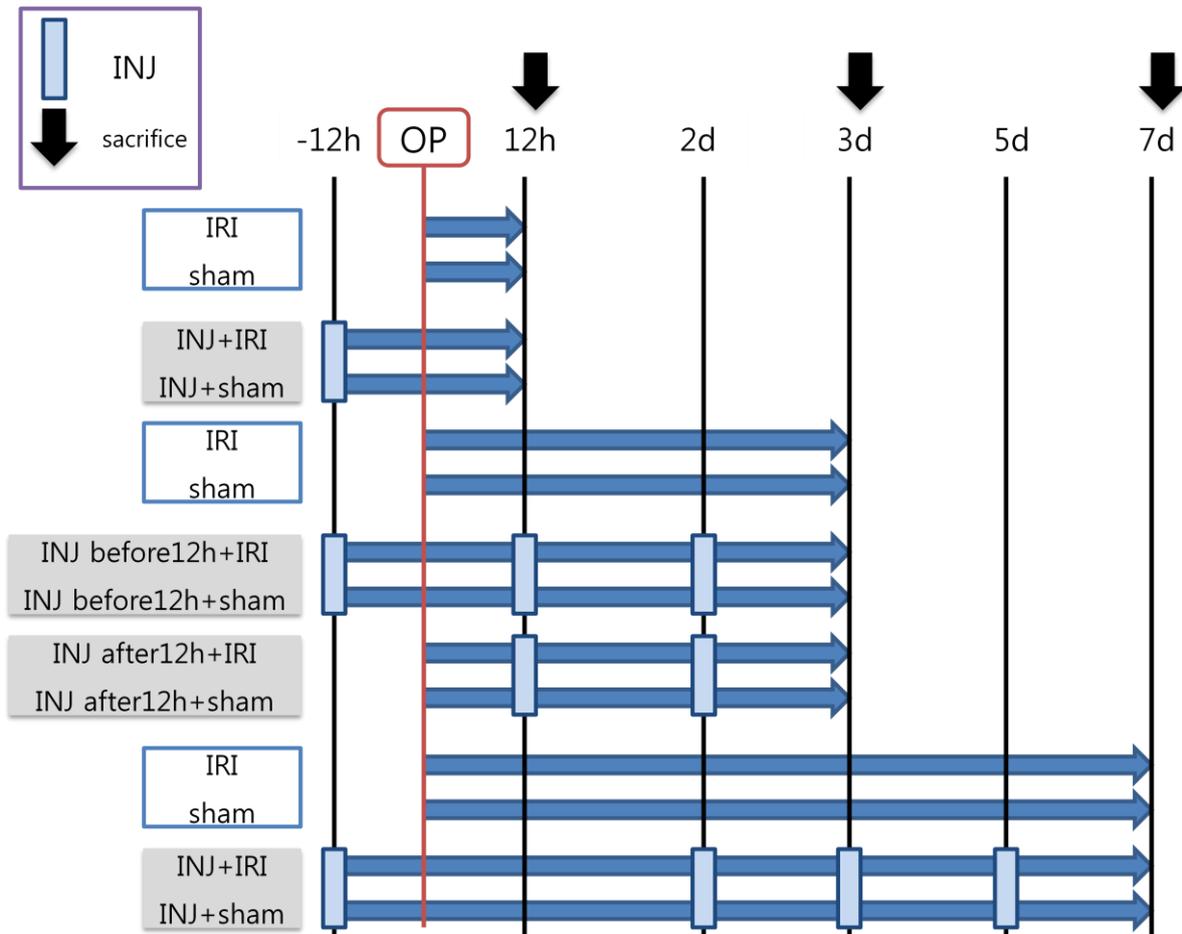
2.2.1 Induction of IRI and administration of inhibitor

Mice were anesthetized by inhalation of isofluran as well as intraperitoneal injection of a mixture of ketamine (120 mg/kg bw) and xylazine (10 mg/kg bw) during the surgical process. After a midline incision, the left and right renal pedicles were occluded with microvascular clamps for 55 minutes, and the mice were placed on a heated operation table. Afterward, the clamps were removed and the abdomen was closed. Sham-operated mice had the same operative procedure except the placement of clamps.

Mice were sacrificed by cervical neck dislocation at 12 hours, 3 days, and 7 days after IRI induction. The mice were either treated or non-treated with KINK-1. In every experimental group at least 3-5 animals with IRI and 3 sham-operated animals were examined at each time point. KINK-1 injection started 12 hours before IRI-induction and was repeated every two days thereafter. The 3-days-reperfusion group also included one additional experimental group in which animals received the first injection of KINK-1 12 hours after IRI induction or sham operation. [Figure 5]

Spleen and kidneys were harvested after the observation period. Left kidneys were used for FACS analysis, right kidneys were divided into three pieces for morphological examination and for RNA and protein isolation.

Blood samples were obtained at the time of sacrifice via puncture of cava inferior.



[Figure 5] Animals were sacrificed at the following time points after operation (OP): 12h, 3d and 7d after induction of IRI. KINK-1 was injected intraperitoneally in every 48 hours intervals 5mg/kg bw. **Group 12h:** with IRI and sham (without IRI) / injected 12h before IRI and same injected sham. **Group 3d:** with IRI and sham / injected 12h before IRI and same injected sham / injected 3h after IRI and same injected sham **Group 7d:** with IRI and sham / injected 12h before IRI and same injected sham.

2.2.2 Parameters of kidney function

Blood urea nitrogen levels (BUN, mg/dl) and serum creatinine were determined by standard methods using an auto analyzer in the Department of Clinical Chemistry at the University Hospital Hamburg.

2.2.3 Morphological examinations

Renal tissue was fixed with para-formaldehyde (4%), embedded in paraffin and

stained with periodic acid-Schiff (PAS) reaction.

The percentage of tubular damage was assessed by loss of brush border, tubule dilatation and vacuolization and scored as: 0 = none, 1= 0-25%, 2 = 25-50%, 3 = 51-75%, 4 >76%.

For each sample, at least 15 fields were examined (x200)

2.2.4 Quantification of CD3+ cells

Renal infiltration of CD3+ was examined with staining the cells in paraffin embedded preparation.

At least 15 fields were examined (x200) and calculated the average.

2.2.5 Real Time PCR

Total RNA was prepared from 1/3 of right kidney slice and reverse-transcribed (Invitrogen) by using random hexamer primers (Invitrogen). Real-time quantitative PCR was performed with an Applied Biosystems StepOne using SYBR green.

The PCR primers were:

See above

All samples were run in duplicate and normalized to 18s rRNA to correct for small variations in RNA quality. $\Delta\Delta CT$ method was used for calculating the relative quantification of gene expression.

2.2.6 FACS (Fluorescence-activated cell sorting)

Complete kidneys and spleens were digested with collagenase and DNase-1. Single-cell suspensions were stained with fluorochrome-conjugated antibodies in PBS containing 10% FCS.⁵⁴ The following antibodies from BD Pharmingen or eBioscience (San Diego, CA) were used: CD3, CD25, CD4, CD45, CD8a, Foxp3.

And also additional experiment was performed to detect the populations of Th1 and Th17 cells.

Cells were analyzed with a Becton & Dickinson LSRII System using Diva and FlowJo software.

First, we selected CD45+ cells from the whole population, and from them selected CD3+ cells. Then again from them we selected CD4+ cells population and CD8+, and Tregs from it.⁵⁵

2.2.7 Statistical analysis

All data were expressed as mean values \pm SE; n refers to the number of animals or individual measurements in separate samples. Statistical significance was performed with t-test, defined as $p < 0.05$.

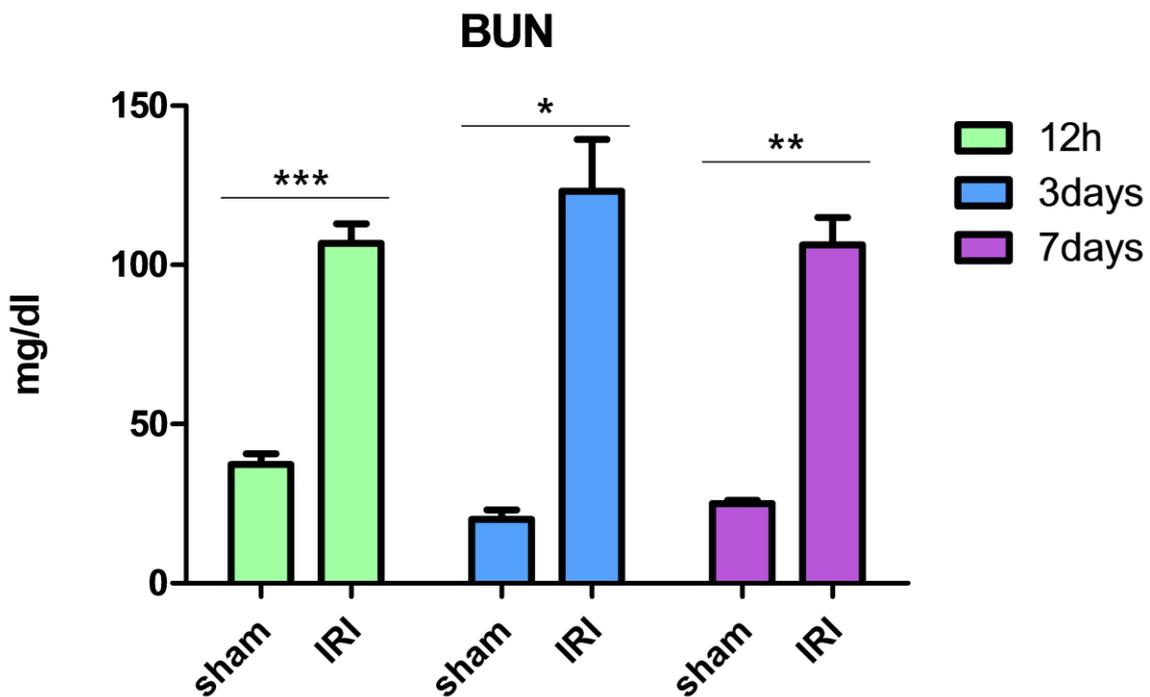
3 RESULTS

3.1 CHARACTERIZATION OF THE IRI MODEL IN MICE

To ensure the validity of this IRI model, we collected blood and kidney of mice at 12 hours, 3 days and 7 days after induction of IRI and assessed the renal function and morphological examinations and chemokine expression. To examine the NF- κ B activation we used western blotting in this model.

3.1.1 Functional analyzing of the IRI model in mice

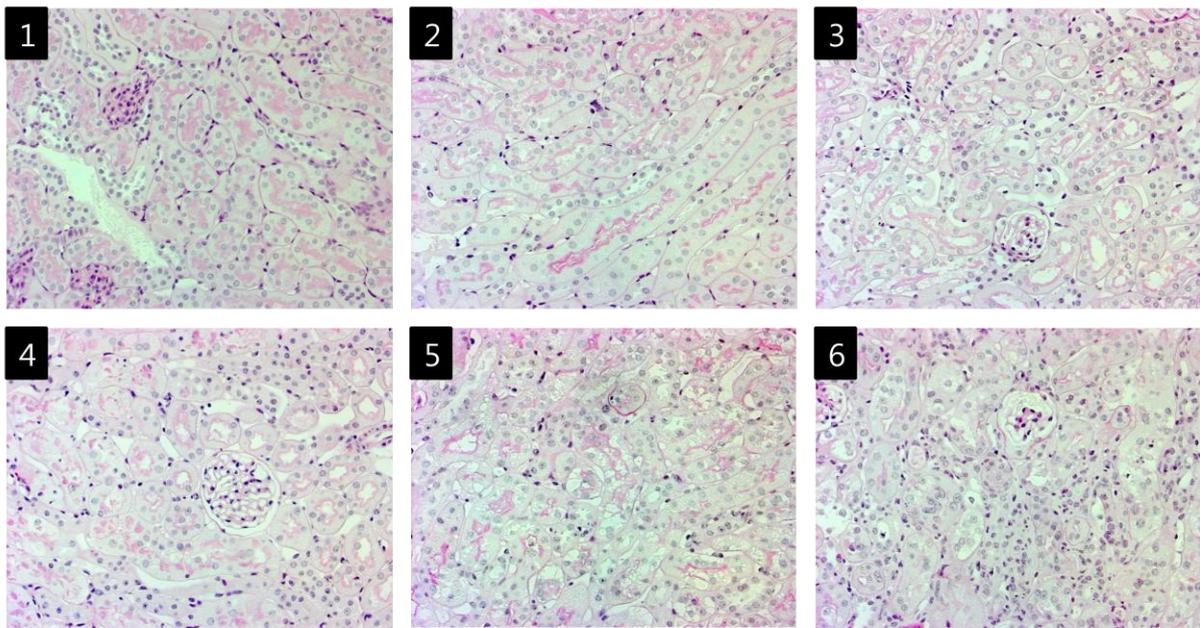
Blood of mice was collected at 12 hours, 3 days and 7 days after induction of IRI at sacrifice via vena cava puncture. After IRI induction, blood urea nitrogen levels (BUN; mg/dl) were significantly elevated at all time points in IRI mice when compared to sham operated mice (IRI 12h: 106.8 ± 6.102 ; sham 12h: 37.333 ± 3.383 ; IRI 3d: 123.143 ± 16.246 ; sham 3d: 20 ± 3 ; IRI 7d: 106.25 ± 8.645 ; sham 7d: 25 ± 1). The maximal BUN level was shown in the group of IRI 3d, but this was not significantly different from the other time points after IRI (Figure 6).



[Figure 6] Renal function: after IRI induction at different time points. The BUN increases significantly after disease induction. (n \geq 4 for each IRI group, n \geq 2 for each sham group; * p<0.05; ** p<0.01; *** p<0.001 compared with sham mice)

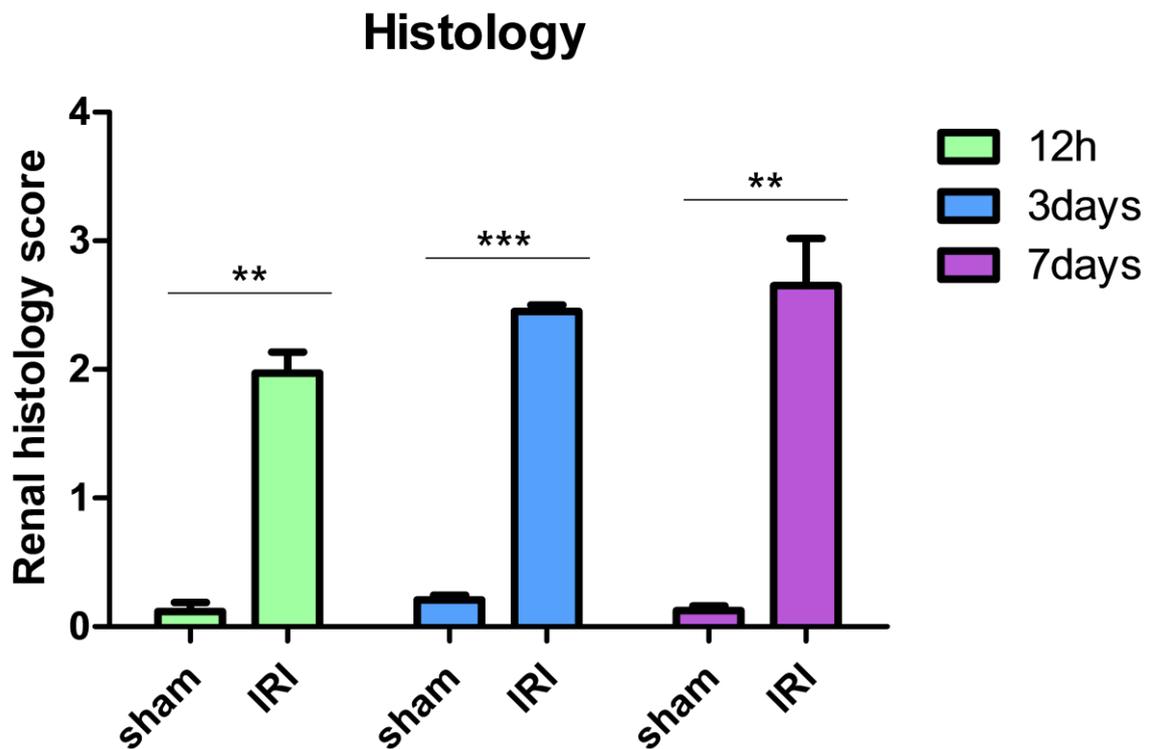
3.1.2 Quantification of renal tissue damage in IRI mice

To quantify renal tissue damage in the IRI model, PAS, CD3 stained kidney sections were evaluated by light microscopy. The results show different degrees of tubular damage in IRI mice at the time points examined in comparison to the sham operated mice. Tubular cells have mainly damaged after induction of IRI, showing vacuolization, loss of brush border and tubular dilatation [Figure 7].



[Figure 7] Characterization of renal tissue damage in IRI mice. Representative Periodic acid-Schiff (PAS) photographs of each time point are shown. ① Sham 12h, ② Sham 3d, ③ Sham 7d, ④ IRI 12h: little loss of brush border, ⑤ IRI 3d: loss of brush border with vacuolization, ⑥ IRI 7d: loss of brush border, vacuolization with tubular dilatation (Original magnification x200)

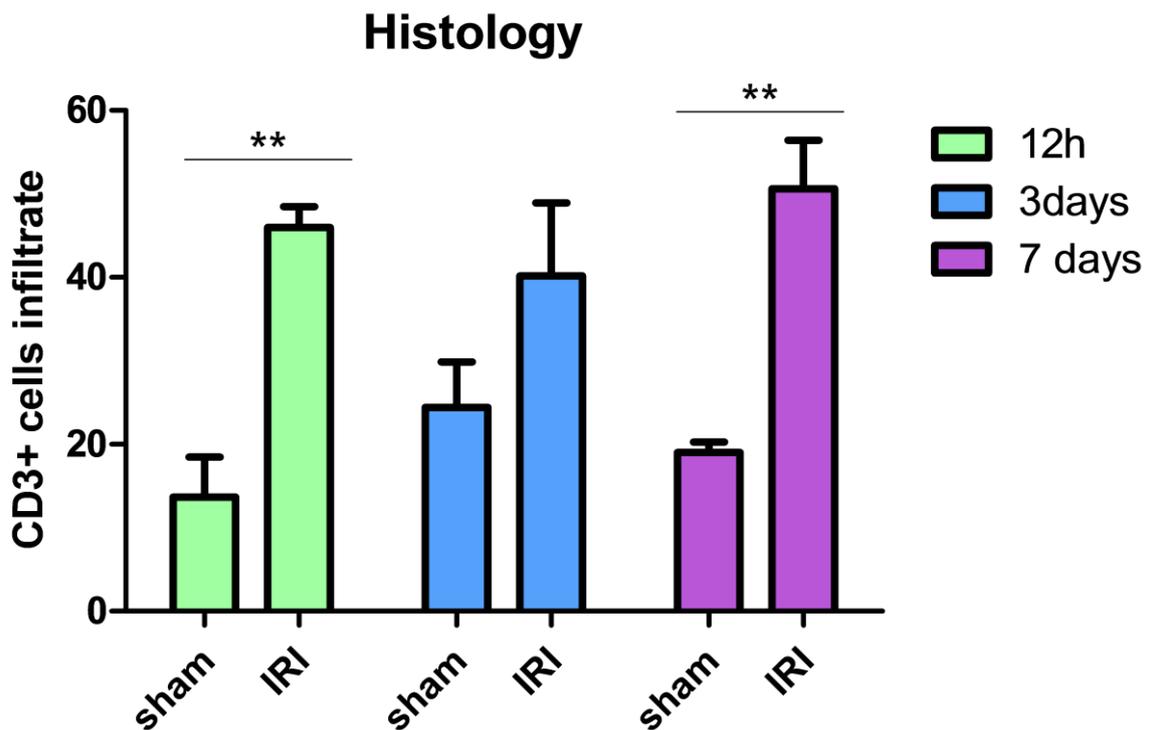
A semi-quantitative score for the presence of tubular damage was calculated for each animal in a double-blinded observation procedure. The histology score of kidney after IRI significantly increased when compared to sham operated controls at all time points ($P < 0.05$) (IRI 12h: 1.97 ± 0.1648 ; sham 12h: 0.12 ± 0.07 ; IRI 3d: 2.45 ± 0.05 ; sham 3d: 0.2 ± 0.038 ; IRI 7d: 2.65 ± 0.3669 ; sham 7d: 0.1233 ± 0.037). [Figure 8]. Kidney injury was observed to be most severe at day 7 and the progress of damaged tissue recovery was not completed at this time point.



[Figure 8] Histology score of IRI mice were significantly increased in all time points of examination. (n= 2-5 for each group; ** p<0.01; *** p<0.001 when compared with sham) Observation was performed in original magnification x200.

3.1.3 Quantification of renal infiltration of CD3+ cells in IRI mice

The infiltration of CD3+ cells was also calculated in a double-blinded procedure. CD3+ cells were increased in all groups at all time points examined after reperfusion. Most prominent at the time points 12h and 7d (IRI 12h: 45.99 ± 2.48 ; sham 12h: 13.69 ± 4.76 ; IRI 3d: 40.16 ± 8.775 ; sham 3d: 24.43 ± 5.43 ; IRI 7d: 50.63 ± 5.791 ; sham 7d: 19.03 ± 1.23). [Figure 9]



[Figure 9] Renal infiltration of CD3+cells was significantly increased in 12h and 7d groups of examination. (n= 2-5 for each group; ** p<0.01 when compared with sham) Observation was performed in original magnification x200.

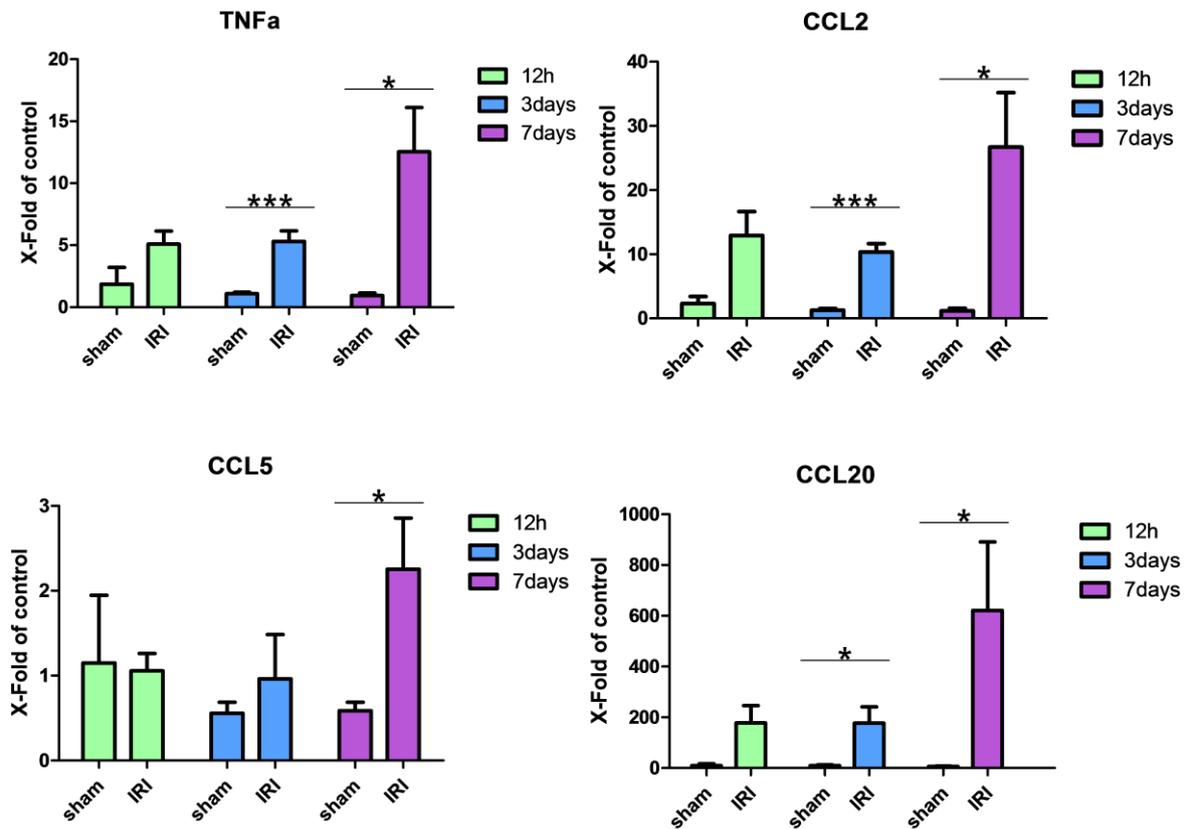
3.1.4 Time dependent chemokines mRNA expression in the IRI model

Real-time PCR was performed after isolation of RNA from right kidney tissue from IRI and sham operated mice at 12 postoperative hours, 3 and 7 postoperative days of reperfusion.

We examined TNF α (Tumor necrosis factor alpha), a cytokine which plays a major role in acute phase of inflammation. CCL2, CCL5 and CCL20 are also examined which are chemotactic for monocytes and lymphocytes in severe inflammation.

All of the four cytokines and chemokines are up-regulated after IRI Induction when compared to sham. TNF α is expressed at early time point and is highest at day 7 (12.54 \pm 3.565, sham: 0.9425 \pm 0.1966). CCL2 also reaches its peak expression at day 7 (26.72 \pm 8.468, sham: 1.18 \pm 0.388). CCL5 doesn't change that much before day 3 and is highest at day 7 (2.253 \pm 0.604, sham: 0.5875 \pm 0.09978). Increase of CCL20 is

significantly different at day 3 (177.4 ± 63.63) and reaches highest point at day 7 (621.6 ± 269.7) when compared to sham (6.403 ± 1.929).



[Figure 10] RT-PCR analysis of renal chemokine mRNA expression after IRI induction. mRNA expression is indicated as x-fold increase when compared to sham mice. (n= 3-6 in each group. * p<0.05; *** p<0.001 when compared with sham mice) **TNF α** , **CCL2** and **CCL20** expression was significantly up regulated after 3d, **CCL5** in 7d.

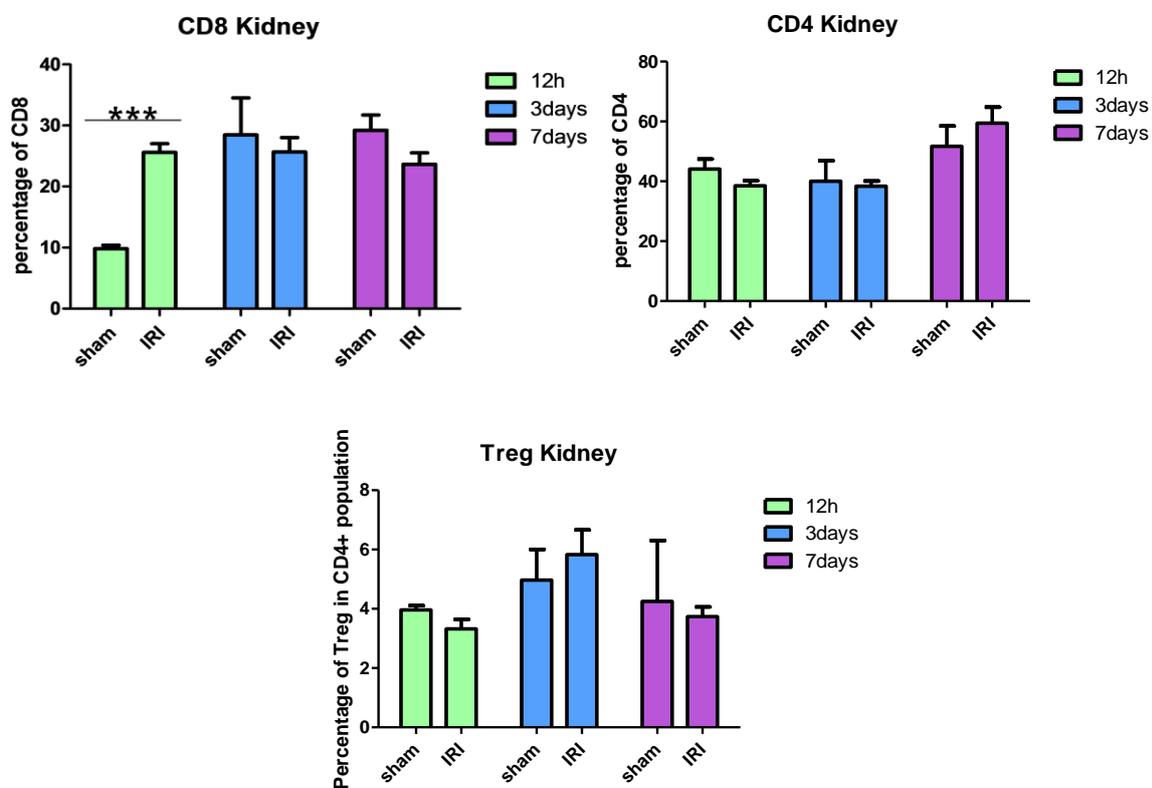
3.1.5 Renal and splenic T cells infiltration in IRI mice

The percentage of T cells including CD8+, CD4+, CD3+ and Treg was quantified with FACS (Fluorescence-activated cell sorting) from kidney and spleen tissue.

The increase of renal CD8+ cells, which play important role in acute inflammatory reaction, is significantly different at 12 hours of reperfusion when compared to its sham (IRI 12h: 25.58 ± 1.422 ; sham 12h: 9.8 ± 0.5859) not, however, at 3 days and 7

days of reperfusion.

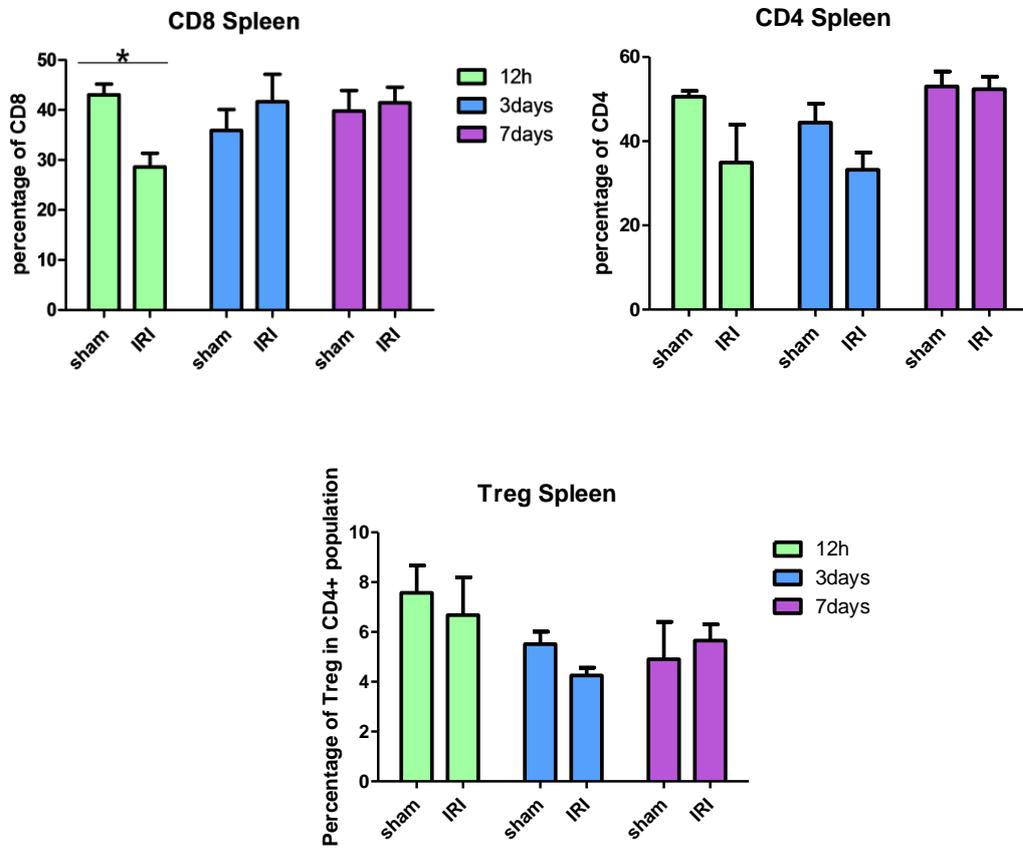
The percentage of renal CD4+ cells also showed not that much difference in between IRI groups and sham operated groups. Generally it was increased slightly in 7 days in compare to other reperfusion time groups (IRI 12h: 38.52 ± 1.626 ; sham 12h: 44.1 ± 3.305 ; IRI 3d: 38.35 ± 1.769 ; sham 3d: 40.05 ± 6.85 ; IRI 7d: 59.43 ± 5.327 ; sham 7d: 51.7 ± 6.8). The Treg population in kidneys was also not significantly different in all IRI groups.



[Figure 11] Renal T cell infiltration in IRI model. The renal infiltration of CD8+ cell was significantly increased in 12h of IRI (n= 2-11 in each group. *** $p < 0.001$ compared with sham mice).

The relative amounts of CD8, CD4 positive T cells and Tregs were also examined in spleens. We could prove that it has significant difference after 12h reperfusion in CD8+ lymphocytes groups (IRI 12h: 43.03 ± 2.154 ; sham 12h: 28.62 ± 2.745). But there are no significant differences in other reperfusion groups in CD4+ and Treg

lymphocytes in spleens.



[Figure 12] Splenic T cell infiltration in IRI model. The splenic infiltration of CD8+ cell was significantly decreased in 12h of IRI (n= 2-11 in each group. * p<0.05 compared with sham mice). Differences for the percentage of CD4+ cells and Tregs were, however, not significant at 3 and 7 days after IRI.

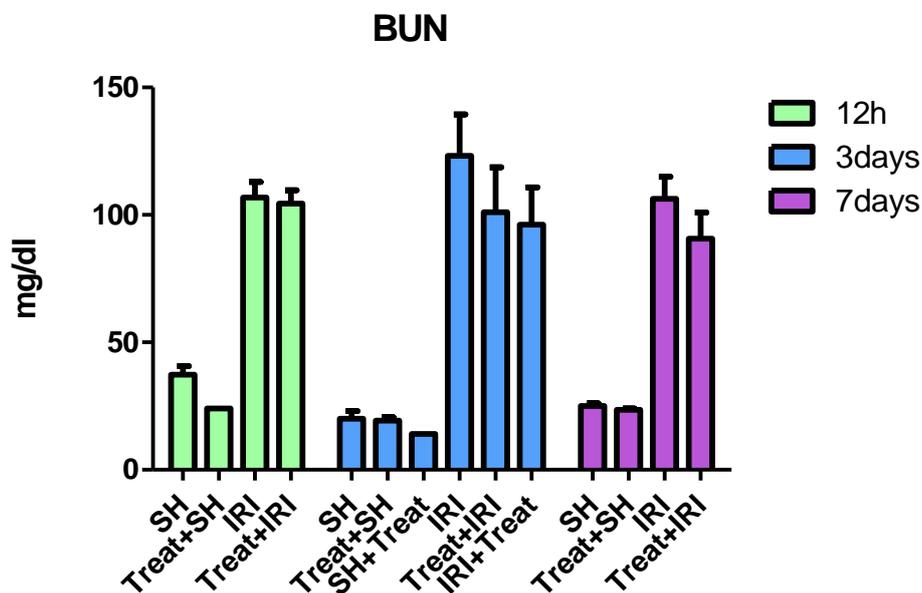
3.2 THE ROLE OF IKK2 INHIBITOR KINK-1 IN IRI

3.2.1 KINK-1 and renal function in IRI mice

As shown previously the dose and route of KINK-1 application used significantly reduced NF- κ B activation in renal tissue.^{54,56}

KINK-1 treated mice showed slightly improved renal function when compared to non-treated mice after IRI-induction. Treated and non-treated mice have most difference at 3 days of IRI (IRI 3d: 123.1 ± 16.25 , Treat+IRI 3d: 101 ± 17.66 , IRI+Treat: 96.14 ± 14.66) but those differences were not significant.

12h RT have almost no difference (IRI 12h: 106.8 ± 6.102 ; Treat+IRI 12h: 104.4 ± 5.335) and the renal function of 7d RTs are both improved when compared with 3d RT (IRI 7d: 106.3 ± 8.645 ; Treat+IRI 7d: 90.71 ± 10.26).



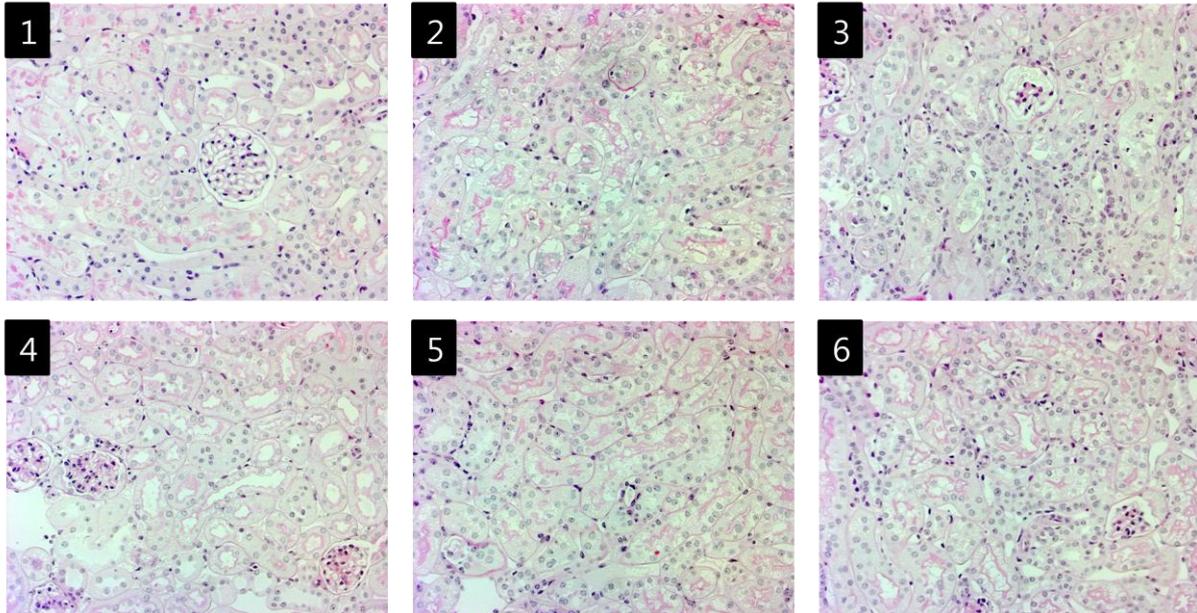
[Figure 13] Renal function: after IRI induction at different time points. BUN was not significantly reduced in KINK-1 treated vs. non-treated mice after disease induction. (n= 3-7 for each IRI or treated IRI group, n= 1-3 for each sham group)

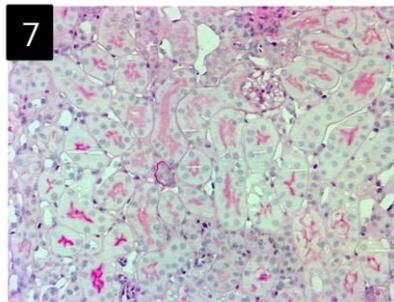
3.2.2 *KINK-1 and renal tubular damage*

Renal histology scores are generally only little reduced in all KINK-1 treated groups when compared to non-treated groups. The difference between the groups is lowest in 12h of RT (IRI 12h: 1.97 ± 0.1648 ; Treat+IRI 12h: 1.65 ± 0.1351), which also correlate with the results of BUN.

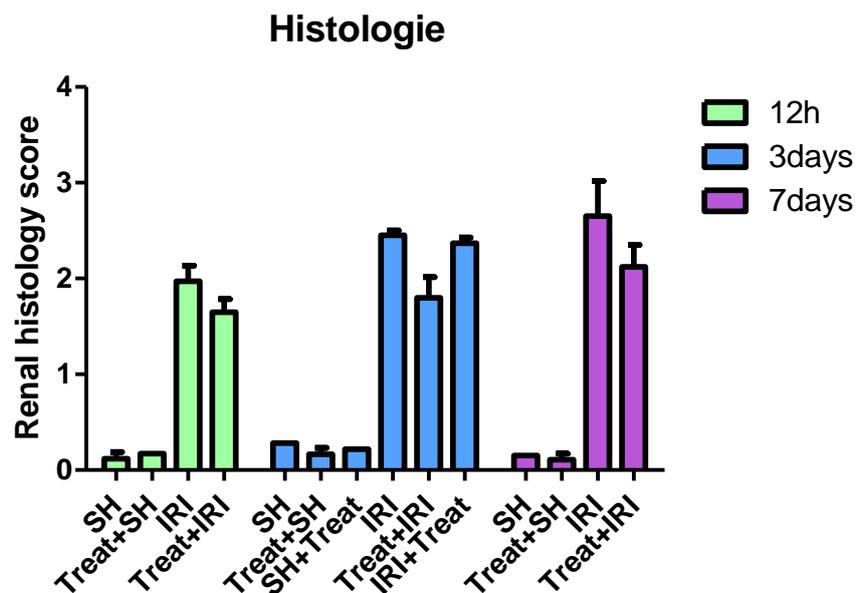
In 3d RT groups show the most difference than any other groups between treated and non-treated samples (IRI 3d: 2.45 ± 0.05 ; Treat+IRI 3d: 1.95 ± 0.1969), however, there was barely any difference in the group treated afterward IRI in compare to non-treated (IRI+Treat 3d: 2.367 ± 0.0628).

There were also little difference in 7d RT groups, but also not remarkably changed (IRI 7d: 2.65 ± 0.3669 ; Treat+IRI 7d: 2.121 ± 0.2288).





[Figure 14] Representative Periodic acid-Schiff (PAS) photographs of IRI and treated+IRI mice. KINK-1 was injected before or after disease induction. ① IRI 12h, ② IRI 3d, ③ IRI 7d, ④ IRI+treat 12h, ⑤ Treat+IRI 3d, ⑥ IRI+treat 7d, ⑦ IRI+treat 3d (Original magnification x200)



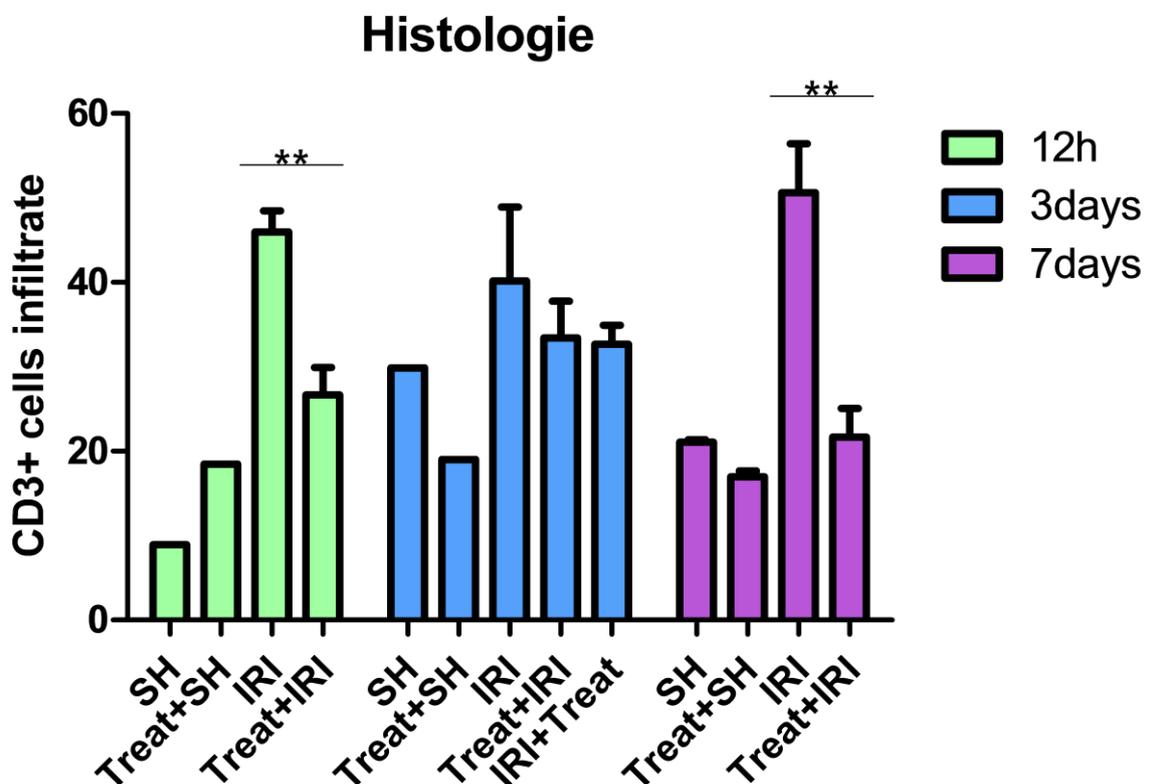
[Figure 15] The renal tissue histology score of IRI and treated groups. There was no significant difference between IRI treated and non-treated mice ($n = 2-7$ for each IRI or treated IRI group. $n = 1-2$ for each sham group).

3.2.3 KINK-1 changes renal infiltration of CD3+ cells

The infiltration of CD3+ cells into the kidney tissue showing certain tendency. CD3+ cells in non-treated groups remain almost in same level in during the reperfusion time.

In contrast, treated animals show remarkably reduced of CD3+ cells in kidney already at early time point (IRI 12h: 45.99 ± 2.48 ; Treat+IRI 12h: 26.69 ± 3.25). That difference is decrease in 3d IRI (IRI 3d: 40.16 ± 8.775 ; Treat+IRI 3d: 33.42 ± 4.351), but reaches its maximum at 7d of IRI (IRI 7d: 50.63 ± 5.791 ; Treat+IRI 7d: 21.67 ± 3.407).

In this test, we couldn't find much difference between KINKI-1 treated afterward and before IRI in 3d RT (IRI+Treat 3d: 32.66 ± 2.261).



[Figure 16] Renal infiltration of CD3+ cells with or without KINK-1 treating in IRI. CD3+ cells were significantly reduced in pretreated animals compare to non-treated at 12h and 7d (n= 2-5 for each IRI or treated IRI group, n= 1-2 for each sham group, ** p<0.01 when compared with treated mice).

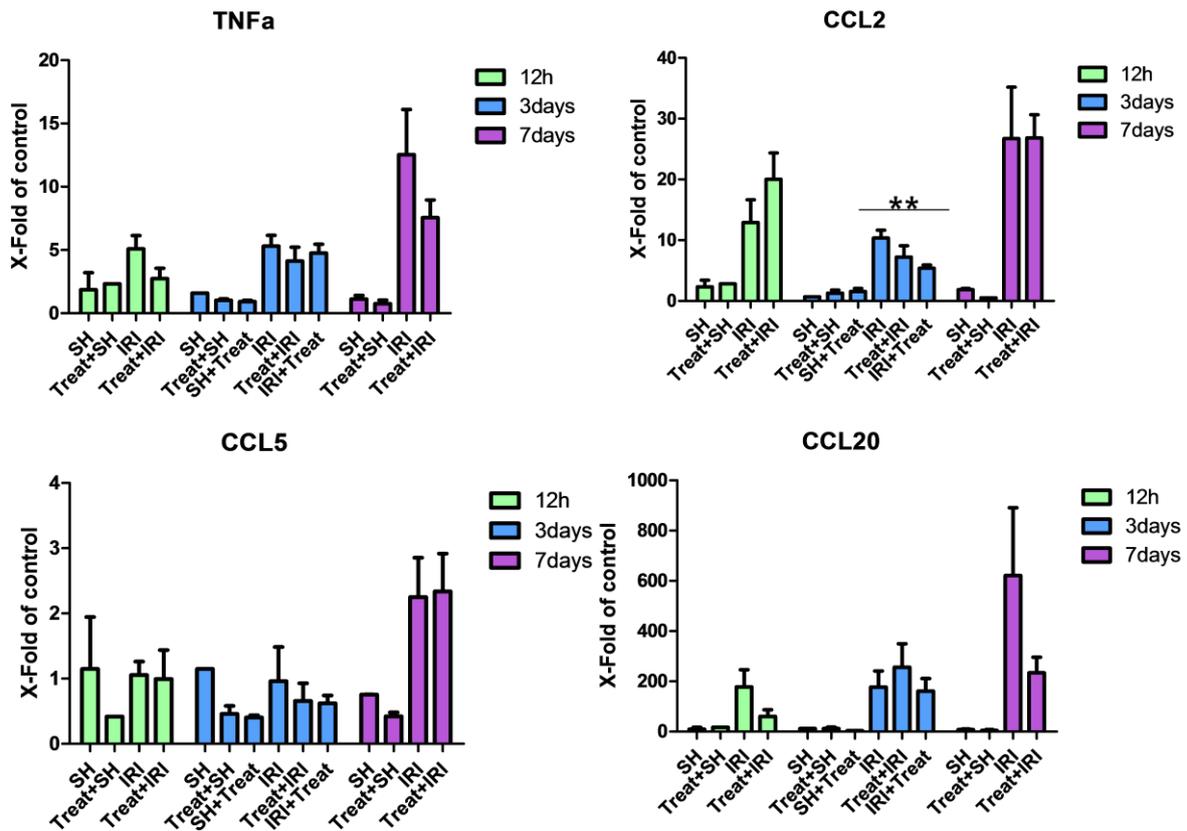
3.2.4 *KINK-1 decreases cytokine and chemokine expression in IRI*

Treated group of TNFa hasn't remarkably changed than non-treated group. But it reaches its maximum gap in 7d RT (IRI 7d: 12.54 ± 3.565 ; Treat+IRI 7d: 7.559 ± 1.392).

CCL2 of treated animals tends to initially increase (IRI 12h: 21.93 ± 3.71 ; Treat+IRI 12h: 20 ± 4.366). And decrease in 3d RT (IRI 3d: 10.34 ± 1.325 ; Treat+IRI 3d: 7.217 ± 1.882), showing especially significant in IRI+Treat group compare to IRI (IRI+Treat 3d: 5.378 ± 0.5319). But it comes to almost in same level in 7d (IRI 7d: 26.72 ± 8.468 ; Treat+IRI 7d: 26.81 ± 3.853).

CCL5 levels showed no significant differences through the RT (IRI 12h: 1.056 ± 0.2057 ; Treat+IRI 12h: 0.996 ± 0.4426 ; IRI 3d: 0.96 ± 0.5236 ; Treat+IRI 3d: 0.6583 ± 0.27 ; IRI+Treat 3d: 0.6213 ± 0.1192 ; IRI 7d: 2.253 ± 0.604 ; Treat+IRI 7d: 2.339 ± 0.5784).

Decrease at the early time point of CCL20 was not remarkable (IRI 12h: 177.7 ± 68.19 ; Treat+IRI 12h: 60.16 ± 27.09), reaches almost the same level in 3d RT (IRI 3d: 177.4 ± 63.63 ; Treat+IRI 3d: 256.1 ± 93.56 ; IRI+Treat 3d: 161.6 ± 49.77). Although we could see a major reduction of CCL20 in 7d RT, the t-test calculated as not significant (IRI 7d: 621.6 ± 269.7 ; Treat+IRI 7d: 233.9 ± 62.12).



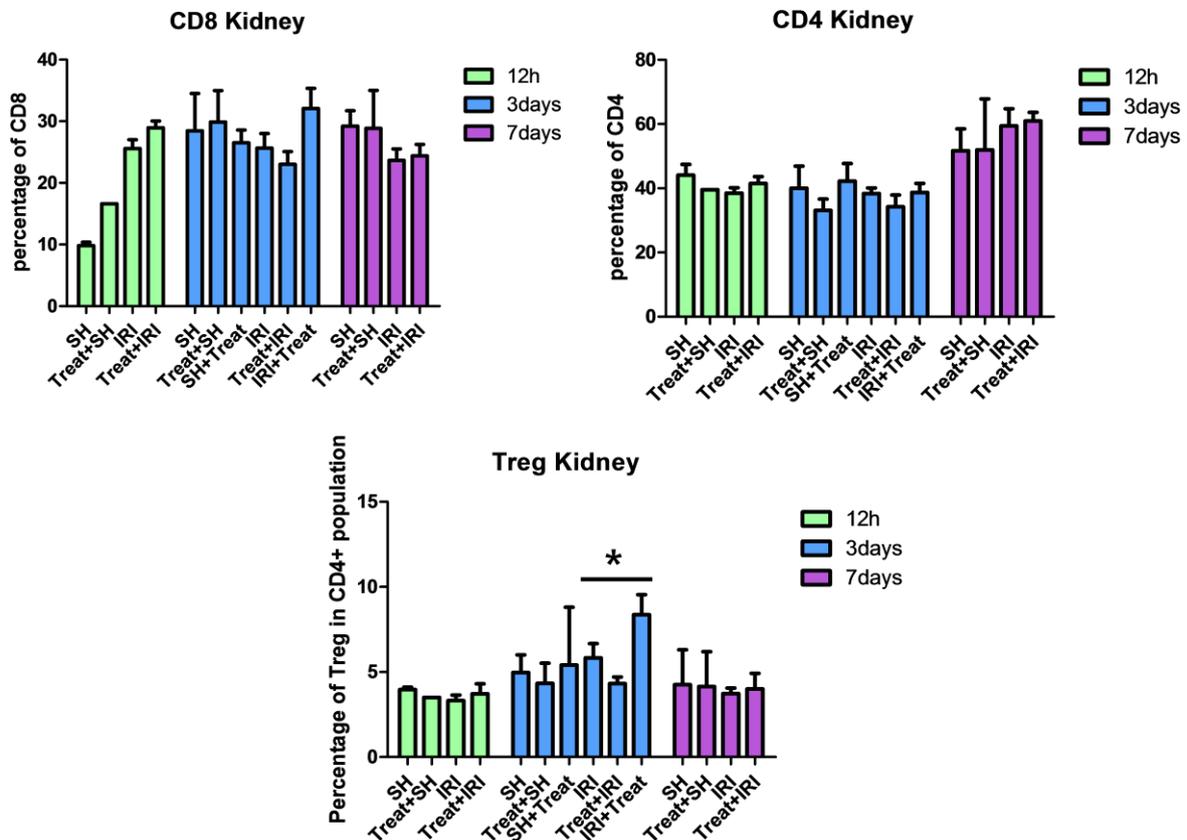
[Figure 17] RT-PCR analysis of renal chemokine mRNA expression after IRI induction. mRNA expression is indicated as x-fold increase when compared to sham mice. The expression of **CCL2** was significantly down regulated after 3d in IRI+Treat group compare to non treated IRI (n= 4-8 for each IRI or treated IRI group, n= 1-3 for each sham group, ** p<0.01 when compared with treated mice).

3.2.5 KINK-1 changes the infiltration of T cells in kidney and spleen

Percentage of CD8+ cells in kidney changed not that much (IRI 12h: 25.58 \pm 1.422; Treat+IRI 12h: 28.96 \pm 1.078; IRI 3d: 25.64 \pm 2.39; Treat+IRI 3d: 23.02 \pm 2.063; IRI+Treat 3d: 32.1 \pm 3.26; IRI 7d: \pm ; Treat+IRI 7d: 23.65 \pm 1.87). And CD4+ cells remained also almost same level (IRI 12h: 38.52 \pm 1.626; Treat+IRI 12h: 41.52 \pm 2.098; IRI 3d: 38.35 \pm 1.769; Treat+IRI 3d: 34.25 \pm 3.649; IRI+Treat 3d: 38.68 \pm 3.824; IRI 7d: 59.43 \pm 5.327; Treat+IRI 7d: 60.93 \pm 2.673).

However, we could see changes in the percentage of Treg at 3d RT when

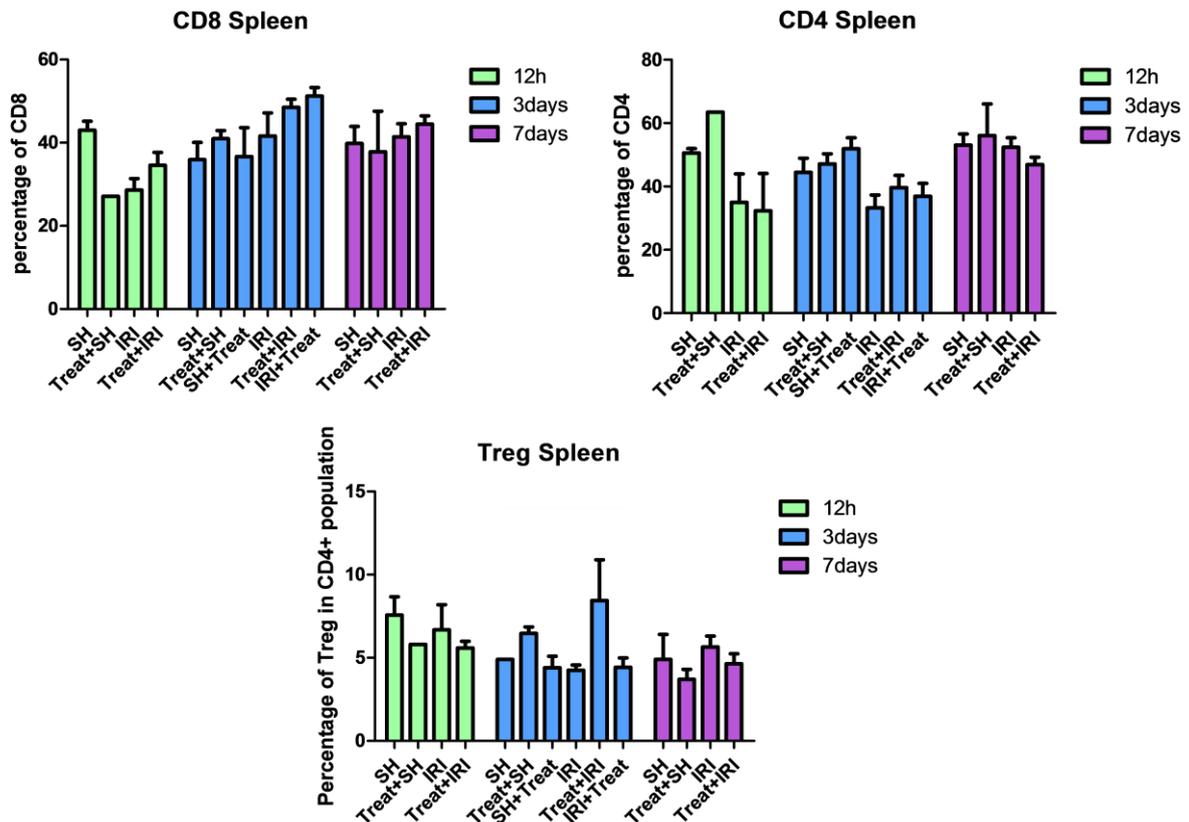
compared with before and afterward treated groups (IRI 3d: 5.829 ± 0.834 ; Treat+IRI 3d: 4.317 ± 0.3902 ; IRI+Treat 3d: 8.363 ± 1.187 , $p < 0.01$).



[Figure 18] Percentage of renal infiltrating T cells. There are some changes in Treg at 3d RT when compared with before and afterward treated groups ($n = 4-11$ in each for each IRI or treated IRI group, $n = 1-3$ for each sham group, * $p < 0.05$ compared between the groups).

CD8+ T cells in spleen remained also in same level (IRI 12h: 28.62 ± 2.745 ; Treat+IRI 12h: 34.6 ± 3.064 ; IRI 3d: 41.63 ± 5.537 ; Treat+IRI 3d: 48.53 ± 1.921 ; IRI+Treat 3d: 51.23 ± 2.031 ; IRI 7d: 41.43 ± 3.145 ; Treat+IRI 7d: 44.47 ± 1.991), similar to those of kidney, as well as CD4+ T cells (IRI 12h: 34.94 ± 8.995 ; Treat+IRI 12h: 32.32 ± 11.81 ; IRI 3d: 33.23 ± 4.094 ; Treat+IRI 3d: 39.62 ± 3.878 ; IRI+Treat 3d: 36.9 ± 4.041 ; IRI 7d: 52.38 ± 2.971 ; Treat+IRI 7d: 46.91 ± 2.324).

And the percentage of Treg cells in spleen showing little difference in 3d RT comparing IRI and Treat+IRI (IRI 3d: 4.257 ± 0.3085 ; Treat+IRI 3d: 8.45 ± 2.448 ; IRI+Treat 3d: 4.425 ± 0.5688).

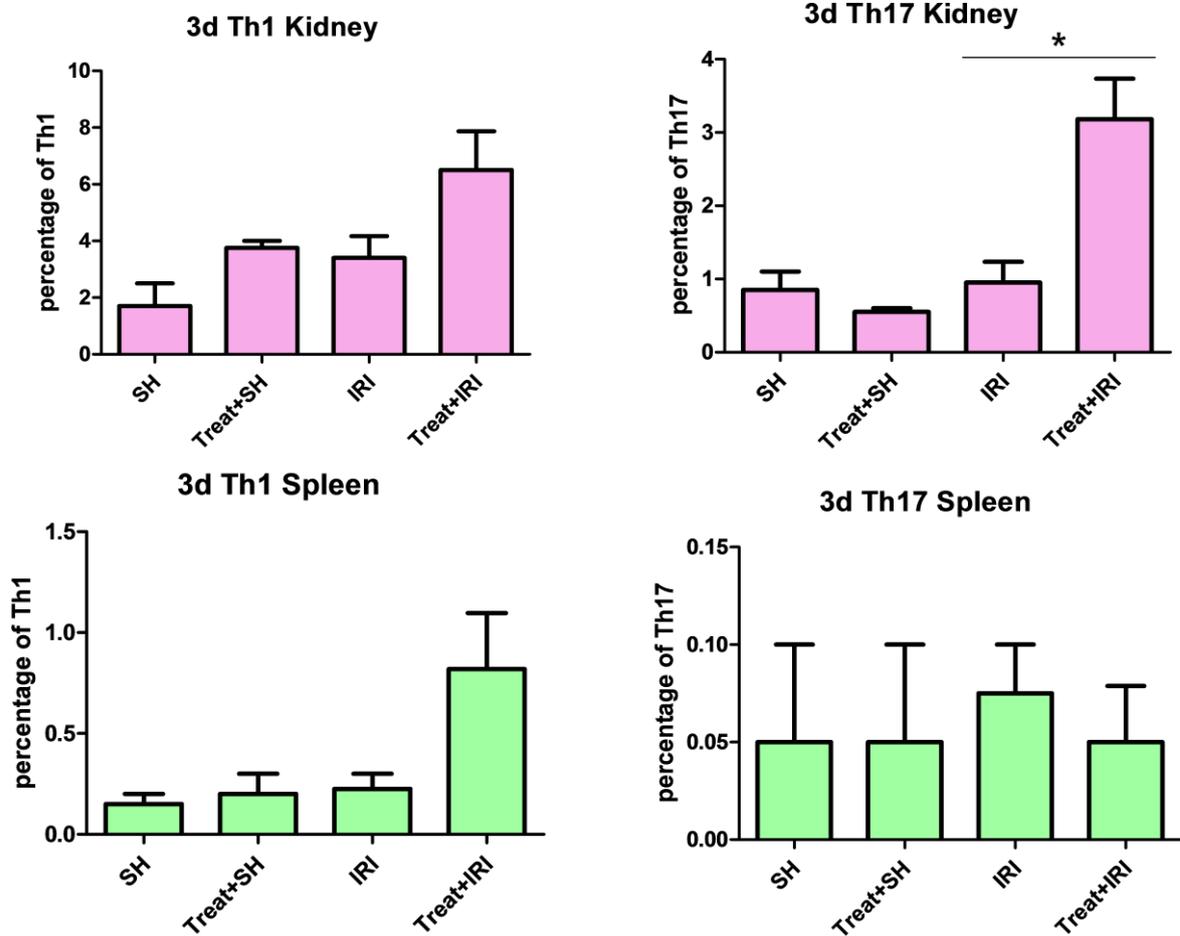


[Figure 19] Percentage of splenic infiltrated T cells. The percentage of Treg cells in spleen showing little difference in 3d RT comparing IRI and Treat+IRI ($n = 4-11$ in each for each IRI or treated IRI group, $n = 1-3$ for each sham group).

According to the results of FACS, KINK-1 was most effective at 3d of IRI. So we repeated the experiments to examine the Th1+ and Th17+ cells infiltrating kidney and spleen after 3d of RT.

As a result, it showed a significant increase in the percentage of Th17+ cells in kidney (IRI 3d: $0.950 \pm$; Treat+IRI 3d: 3.183 ± 0.5516 , $p < 0.05$). Th1+ cells in spleen tends to increase after 3d of RT but was not remarkable (IRI 3d: 0.225 ± 0.075 ;

Treat+IRI 3d: 0.82 ± 0.2764).



[Figure 20] The percentage of Th1+cells and Th17+ cells in kidney and spleen. The renal Th17+ cells was significantly increased in pretreated mice ($n= 4-6$ in each for each IRI or treated IRI group, $n= 2$ for each sham group, * $p<0.05$ when compared with IRI mice).

4 DISCUSSION

Clinical importance of acute kidney injury is more increasing with its incidence and complications. Ischemia reperfusion injury, which causes severe inflammatory reaction in kidney, occurs in situation such as trauma or cardiac arrest and it is also one of the main causes of AKI. It's complicated post-ischemic processes controlled by cytokines, chemokines and cellular responses. And those factors contribute to the clinical outcome. Nowadays there are various studies on to prevent IRI but without thoroughly satisfying.

Variety of experiments for AKI performed in small animal models. Previously, there have been attempt to describe the function of NF-kB in IRI and turned out that it contributes to regulation of the process of pathogenesis.⁵⁷ Histology data provide the evidence of NF-kB activation in diabetic nephropathy, glomerular disease and AKI in human. Correlates with severity of disease (such as proteinuria or inflammation), NF-kB activates in glomerular, tubular parenchymal cells and macrophages. These are supportive data, suggesting that NF-kB promotes inflammation, but particular role of the various NF-kB complexes are still unclear in human kidney injury.⁵⁸

The aim of our study was therefore to characterize the role of NF-kB during ischemia reperfusion injury in kidney by inhibiting IKK2, and examine its function in their outcomes.

First, we characterized the effect of our IRI model in C57BL/6 mice by examine the BUN, morphology, infiltration of lymphocytes and the expression of chemokines. And each of these examinations has proven remarkable differences between injury induced groups and the sham operated control groups.

To analyze the renal function we examined blood urinary nitrogen. During the whole

observed time points we could see significantly increased BUN. With calculated histology score we could also prove the severe damage induced by IRI. Tubular damage with loss of brush border and vacuoles in tubular cells could be seen in all IRI groups in increasing tendency along the reperfusion time.

Significant increase of renal CD3+ lymphocytes infiltration is also seen at 12 hours and 7 days of reperfusion groups. Expression of chemokines are also remarkably up regulated after 3 days of reperfusion, however CCL5 first in 7d of RT. It is also proved that infiltration of CD8+ lymphocytes into the renal tissue is significantly increased in an early time point in our FACS results, correlating with decreasing of spleen percentage of CD8+ cells in the same point of RT.

These results suggest that our mouse model of acute kidney injury in ischemia reperfusion designed and performed effective. In another IRI model in rat left renal pedicle was exposed and clamped with a non-traumatic vascular clamp for 45 min after right kidney nephrectomy and followed up to 12 weeks. Serum blood urea nitrogen (BUN) and creatinine (Cr) levels were measured in these 12 weeks.⁵⁹ An experiment IRI with atorvastatin treatment took bilateral flank incisions of rats. Right kidney was also nephrectomized and left renal pedicle was occluded for 60 min.⁶⁰ Compare to those studies, we have chosen 55min of RT with midline incision in our mouse model, the outcome of BUN was successfully increased.

NF- κ B is a transcription factor, which considered playing a pivotal role in IRI process, involving the pathways of inflammation and apoptosis. During IRI, NF- κ B affects to various aspects of T-cell regulation such as its development, activation, differentiation, and survival. They are in inactive dimer form binding with I κ B inhibitors (I κ B α , I κ B β and I κ B ϵ). Under inflammatory stimuli, the IKK complex phosphorylates I κ B via the canonical pathway. According to this reaction, I κ Bs are ubiquitinated and degraded. NF- κ B dimer could be released and transfer into the nucleus. It comes to activation of NF- κ B. In non-canonical pathway, just IKK1 phosphorylates p100 so that RelB with p52 could be translocated into the nucleus.¹⁸

NF- κ B is also known to have important role in pathogenesis of lung IRI after transplantation, and its inhibition expected to improve lung function with decreased lung injury and apoptotic cell death. Animal model was developed to investigate the effects of liquid endobronchial perfluorocarbon (PFC) in IRI, which reduce the nuclear translocation of NF- κ B and therefore attenuate inflammatory and apoptotic responses. PFC-treated group resulted in down regulated NF- κ B activation and reduced expression of caspase 3 and iNOS, showing anti-inflammatory and anti-apoptotic effects.⁶¹

Study model, designed ischemia reperfusion injury of superior mesenteric artery in mice, demonstrated also the organ protective effect through the inhibition of NF- κ B. In these experiments the early activation of NF- κ B after reperfusion was blocked with MOL-294, a thioredoxin inhibitor, which resulted in decrease neutrophil influx and production of TNF α and therefore prevented injury of tissue and lethality.⁶²

Middle cerebral artery occlusion (MCAO) is used for develop brain IRI models. Using mesenchymal stem cell (MSC), proved that transplantation of a human MSC line (B10 cells) can reduce the expression of proinflammatory factors in rat transient cerebral ischemic model. The group also found that MSC transplantation can regulate proinflammatory genes expression by modulating NF- κ B pathway in the pathology of cerebral ischemia.⁶³ Therefore the regulation of NF- κ B may also have protective effect in renal Ischemia reperfusion injury for its clinical outcome.

The function of IKK2 (and IKK1) is required for rapid NF- κ B activation, under certain stimuli like tumor necrosis factor α (TNF α) or lipopolysaccharide (LPS).⁶⁴ Several studies showing that inhibition of IKK2 could results to reduction of proinflammatory reaction.⁵⁶ KINK-1, which inhibits the activity of IKK-kinase, has greater affinity against IKK2 than IKK1. Therefore, we used KINK-1 for effective inhibition of IKK2 to block the canonical pathway of NF- κ B activation and examined whether KINK-1 also as consequence reduces of IRI induced damage and decrease of T cell proliferation.

Our experiments were performed in 3 groups of different reperfusion time after induction of IRI. Mice were treated with KINK-1 to inhibit IKK2 sufficiently, 12h before

IRI induction and every two days afterward. And additional group was examined parallel to 3 days of RT, in which, however, treatment was delayed until 12 hours after IRI induction.

First for the clinical parameter, we examined the BUN level. Significant difference could not be seen between treated and non-treated groups after IRI-induction, but there are little difference the biggest in the 3d RT group. That difference reduces afterwards at day 7. Interestingly, there are also slight differences between the sham and treated-sham animals with treated groups showing less damaged. Histological examination in PAS staining proved not significant difference between the treated- and non-treated groups. Correlate with the BUN, influence of KINK-1 was the most in 3d of RT especially in the pre-treated group. Post-treated group has barely changed than non-treated group. Recovering from the damage could not be seen in our experiment, due to the short time of observation period (7days).

CD3+ cells were stained in histological tissue to examine the cell infiltration at different time points of RT. We could see significant differences between treated- and non-treated groups at 12h and 7d of RT. Infiltration of CD3+ cells is significantly reduced in treated-animals, but not at 3d of RT.

Producing chemokines and cytokines are not changed that much generally. Expression of TNFa was down regulated at 12h and 7d after disease induction. Significant difference showed in expression of CCL2, which interestingly are reduced in post-treated animals compare to non-treated group. CCL5 are not much affected from KINK-1 administration. However, expression of CCL20 is remarkably reduced in 7d of RT, but not significant. Data of non-treated mice were differs a lot in this examination, maybe we could also see a significant difference between treated and non-treated animals if overall kidney damage would have been less severe by a reduction of the total ischemia time.

To examine the precise cell population after disease induction, we extracted the

kidney and stained multiple proteins on the cell surfaces in FACS analysis. Population of CD8 cells and CD4 cells showed not much difference from treated- and non-treated groups. The population of Tregs cells in kidney, on the other hand, showed significant difference between pre-treated and post treated groups in 3d of RT. Tregs cell population was increased in post-treated kidney. However, we could also see the remarkable difference in Treg population in spleen tissue, which has their most population in pre-treated animals in spleen tissue.

Additional FACS examination was performed to investigate pathophysiological process. Since we confirmed that the most difference was seen in 3d of RT, we stained Th1 and Th17 cells in kidney and spleen tissue. We could see a significant increase of Th17+ cells in 3d pre-treated mice compare to non treated mice. However, the population of Th1+ cells was increased in spleen tissue, but not in significant way.

In conclusion, our experimental model demonstrates that the function of NF- κ B in IRI has their most effect at 3 days after disease induction and during the whole 7 days of observation, correlating with the results of CCL2 expression and the lymphocyte population. Especially, the population of Tregs is significantly different between KINK-1 pre and post treated animals, showing that the role of NF- κ B as a regulator may have time dependent different functions. Also our results of Th17 cells population demonstrate the importance of NF- κ B in that their canonical pathway activation functions rather by inhibiting of Th17 recruitment.

However, we could prove significant reduction of CD3+ cells in renal tissue at 12h and 7d of reperfusion after IKK2 inhibition, which indicates that NF- κ B is not only of importance at 3d but also in a different way at 7d after injury.

There are still many open questions to explain the critical role of NF- κ B in immune-associated renal disease especially how T cell differentiation is controlled.⁴⁶ It will be important in future to identify for example the T cell receptors which play a role for activating NF- κ B–dependent induction of certain cytokine in vivo such as IL-7R α . And also whether their ligands are sufficient for induction those cytokine or other

receptors activate NF- κ B involved.⁶⁵ To understand the kinetic mechanisms in various networks combining to control the reaction of multiple NF- κ B dimers will be also important.⁶⁶ Generation of conditional mouse mutants could help understanding the pathophysiological functions of IKK subunits and NF- κ B activation.⁶⁷

5 ABSTRACT

Ischemia reperfusion injury, a main cause of acute kidney injury, remains with still high mortality rate with not much therapeutic option. Recent studies suggest that the transcription factor nuclear factor κ B (NF- κ B) could play a pivotal role in renal disease process by regulating the expression of cytokines and T cells infiltration.

The aim of our study is to demonstrate the function of NF- κ B during ischemia reperfusion injury, by blocking the canonical NF- κ B activation with a specific IKK2 inhibitor.

Our experimental model was surgically induced ischemia reperfusion injury in mice. Animals were treated or non-treated with KINK-1 to inhibit IKK2 and examined at different reperfusion time points 12h and 3d and 7d after disease induction.

Kidney function, morphology, chemokine expression and population of cell infiltration were determined. KINKI-1 treatment could not change the function of kidney significantly, according to the BUN results. But the animals treated with KINK-1 showed significant reduction of CD3+ cell infiltration at early and late time points. Also, Treg cells population was increased especially in post-treated animals at 3d of RT, and increased Th17 cells in pre-treated 3d of RT.

The results suggest that NF- κ B has different functions at various time points after IR, and could play a pivotal role in the regulation of T cells infiltration. Moreover, inhibiting IKK2 might regulate the function of NF- κ B differentially during or after disease and therefore could provide a therapeutic approach but these still needs to be precisely described for its practical clinical use.

5 ZUSAMMENFASSUNG

Die Ischämie- Reperfusionstörung ist eine der Hauptursachen des akuten Nierenversagens mit hoher Mortalitätsrate und leider nur wenigen therapeutischen Optionen. Transkriptionsfaktoren, wie Nuclear Factor kappa B (NF- κ B) spielen, durch die Regulierung der Expression von Zytokinen und der T-Zellen Infiltration, eine zentrale Rolle in der Pathogenese der durch ischämie- Reperfusion ausgelösten Nierenerkrankung.

Das Ziel der vorgelegten experimentellen Studie war daher, die Funktion von NF- κ B bei der Ischämie- Reperfusionstörung nachzuweisen und durch die Blockierung der kanonischen NF- κ B Aktivierungskaskade mittels eines IKK2-spezifischen Inhibitors (KINK-1) die mögliche therapeutische Relevanz der NF- κ B-Aktivierung nachzuweisen.

Das experimentelle Modell der Ischämie- Reperfusionstörung wurde durch Obliteration und Desobliteration der Nierenarterien bei Mäusen induziert. Die Tiere waren entweder mit KINK-1 behandelt oder nicht behandelt und wurden zu verschiedenen Zeitpunkten nach Reperfusion der Nieren untersucht, nämlich 12 Stunden, 3 Tagen und 7 Tagen.

Die Nierenfunktion, Morphologie, Chemokinexpression und entzündliche Zellinfiltrationen in die Niere wurden am Ende des Beobachtungszeitraumes bestimmt.

Die Ischämie- Reperfusionstörung führt zu einer passageren Nierenfunktionsverschlechterung, morphologisch einem akuten Nierenversagen, einem gesteigerten entzündlichen Zellinfiltrat in die Niere und geht einher mit einer Aktivierung von NF- κ B.

Die Applikation von KINK-1 konnte zwar die Funktion der Niere nicht wesentlich verbessern, jedoch war bei den mit KINK-1 behandelten Tieren die Infiltration von CD3+ Zellen in die Niere zu den frühen und späten Zeitpunkten signifikant reduziert.

Bei mit KINK-1 *vor* Induktion der Ischämie- Reperfusionstörung behandelten Mäusen waren vermehrt Th17 Zellen in der Niere, bei mit KINK-1 *nach* Induktion der Ischämie- Reperfusionstörung behandelten Mäusen waren Tregs vermehrt in der Niere nachweisbar.

Die Ergebnisse zeigen, dass NF- κ B verschiedene Funktionen in unterschiedlichen Zellpopulationen der Niere im Modell des akuten Nierenversagens durch ischämie- / Reperfusionstörung hat und legen nahe, dass NF- κ B eine zentrale Rolle in der Regulation der T-Zellen Infiltration spielt. Darüber hinaus zeigen die Daten, dass eine systemische, spezifische IKK2 Inhibition den Verlauf der Ischämie-/ Reperfusionstörung unterschiedlich beeinflusst, abhängig vom Zeitpunkt der ersten Applikation. Inwiefern sich daraus ein therapeutischer Ansatz entwickeln könnte, muss noch genauer vor einem eventuellen klinischen Einsatz charakterisiert werden.

6 ABBREVIATIONS

AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
D	days
DC	dendritic cells
BUN	Blood urea nitrogen levels
Bw	Body weight
dL	Deciliter
FACS	Fluorescence-activated cell sorting
h	hours
GFR	glomerular filtration rates
ICU	Intensive care unit
ICAM-1	intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunglobulin
IKK2	inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRI	Ischemia reperfusion injury
KDIGO	Kidney Diseases: Improving Global Outcomes
kg	Kilogram
KINK-1	kinase inhibitor of NF-kB-1
TCRs	T cell receptors
TEC	tubular epithelial cell

TLR	Toll-like receptor
MHC	major histocompatibility complex
mg	Miligram
min	Minute
mL	Mililiter
mM	Milimol
Na	Natrium
NF-kB	Nuclear factor kappa-B kinase
NK	Natural killer
PBS	phosphate buffer saline
RT	Reperfusion time
STAT	signal transducers and activators of transcription
Th17	T helper 17 cell
Treg	regulatory T cells

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8 CURRICULUM VITAE

For reasons of data protection, the Curriculum vitae is not published in the online version.

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10 EIDESSTATTLICHE VERSICHERUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Hamburg, den 12.06.2016

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