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Role for NF-κB1 (p50) in an experimental model of rapid progressive glomerulonephritis

Dissertation

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1. INTRODUCTION

1.1 Glomerulonephritis

Glomerulonephritis (GN) is a term applied to a group of diseases characterized by inflammatory changes in glomerular capillaries and other compartments of the kidney and accompanying signs and symptoms of an acute nephritic syndrome. Symptoms are particularly hematuria, proteinuria and diminished renal function in some cases associated with fluid retention, hypertension and edema (Couser WG, 1999; Chadban SJ. and Atkins R.C., 2005).

In the USA and Europe glomerulonephritis is the third most common cause of end-stage renal disease (USRDS 2011 Annual Data Report). Worldwide, the prevalence of glomerulonephritis, as a consequence of various infectious agents in developing countries, makes it the commonest single cause of end-stage renal disease (Couser WG, 1999). The causative agents in most forms of human glomerulonephritis are unknown. Nevertheless, most evidence now suggests that infectious agents and other stimuli as well, induce glomerulonephritis by triggering an autoimmune response that results in formation of immune-complex deposits in glomeruli or elicits a cell-mediated immune response to antigens in, or of, the glomerulus (Couser WG, 1998). Goodpasture's syndrome induced by antibody to glomerular basement membrane (anti-GBM) is a classic example, but other disorders such as post-streptococcal glomerulonephritis, IgA nephropathy and membranoproliferative glomerulonephritis also show autoimmune features.

Glomerulonephritis may occur as a primary renal disease (renal manifestation of the disease) or as a manifestation of renal involvement in a systemic disease process, commonly vasculitis, collagenosis or endocarditis lenta. Some forms of glomerulonephritis need little or no disease specific therapy, whereas others are medical emergencies calling for urgent diagnosis and therapeutic intervention (Couser WG, 1999). Once glomerulonephritis is suspected by typical clinical presentation, serological markers (e.g. antibodies to streptococci, ANA, ANCA, anti-GBM-antibody) and

reduced renal function, the important information to obtain is the type of disease that is present, the severity of injury, and the potential reversibility or likelihood of response to therapy (Nachman PH and Glassock RJ, 2010; Markowitz GS, Barry Stockes, Kambham N, Herlitz LC and D'Agati VD, 2011).

Glomerular damage in glomerulonephritis occurs in two phases: acute and chronic. During the acute phase, as autoimmune reactions take place in glomeruli, a variety of mediators of tissue injury are activated: *complement*, which generates chemotactic factors that lead to leukocyte recruitment; *C5b-9*, which directly damages glomerular cells; *coagulation factors* that lead to fibrin deposition and crescent formation; *local and systemic release of growth factors and the sequential activation of pro-inflammatory signaling pathways*, which lead to the production of pro-inflammatory mediators such as cytokines and chemokines, direct the infiltration of monocytes/macrophages, dendritic cells (DCs) and T-lymphocytes into the kidney and play a crucial role in the pathogenesis of glomerulonephritis from the acute phase to eventual glomerulosclerosis (Johnson RJ, 1994; Kurts et al., 2007; Segerer et al., 2008). A secondary component of the acute phase of injury that leads to the chronic phase is the result of the response of glomerular cells themselves to these mediators. This involves cell proliferation with overproduction of oxidants and proteases, changes in phenotype and overproduction of extracellular matrix which results in sclerosis and permanently impaired renal function (Johnson RJ, 1994). Renal damage in the chronic-progressive phase of glomerulonephritis is mediated not by an acute inflammatory process but rather by non-immune mechanisms. It develops as a result of loss of filtering-surface with accompanying increase in glomerular pressure in remaining nephrons. These features lead to glomerular sclerosis as well as to chronic interstitial fibrosis which is a consequence of multiple injurious events including ischemia, glomerular cytokine release and toxic effects of increased protein filtration on tubules (Couser WG and Johnson RJ, 2012).

Despite the emerging burden of diabetic renal disease, glomerulonephritis remains a major cause of chronic renal disease and end-stage renal failure requiring dialysis and renal transplantation (Tipping PG and Kitching AR, 2005). Most Patients develop a

chronic kidney disease with the risk of associated cardiovascular diseases, such as hypertension, and progressive renal insufficiency (Kasiske BL, 2001; Mann et al., 2004). The major cause of death of patients with chronic kidney disease is based on the cardiovascular system (Hallan et al., 2007). The American National Kidney Foundation defined chronic kidney disease in their Kidney Disease Outcome Quality Initiative (KDOQI). It's defined by either *kidney damage* for ≥3 months or *abnormalities in imaging tests*. Kidney damage is defined by structural or functional abnormalities of the kidney with or without decreased glomerular filtration rate (GFR), manifest by either pathological abnormalities or markers of kidney damage including abnormalities in the composition of the blood or urine. A different criteria is GFR <60mL/min/1.73m^2 for ≥3 months with or without kidney damage. The GFR is traditionally considered the best overall index of renal function in health and disease. Because GFR is difficult to measure in clinical practice most clinicians estimate the GFR from the serum creatinine concentration. However, the accuracy of this estimation is limited because the serum creatinine concentration is affected by factors other than creatinine filtration (Levey AS., 1990; Perrone RD., 1992). Several formulas have been developed to estimate creatinine clearance from serum creatinine concentration, age, sex and body size. Most used formulas are the MDRD formula (Levey et al., 1999; Verhave JC et al., 2003; Stevens et al., 2007) and the Cockcroft-Gault formula (Cockcroft DW and Gault H, 1976). On February 2002, the American National Kidney Foundation introduced a five-stage classification system for chronic kidney disease based on an estimated glomerular filtration rate **(**eGFR**)** which is calculated from serum creatinine levels and levels of proteinuria. Adverse outcomes of chronic kidney disease including loss of kidney function, development of kidney failure and cardiovascular disease (CVD) can often be prevented or delayed through early detection and treatment.

In recent years numerous researches focused on the molecular signaling pathways of inflammatory diseases. Unfortunately, the underlying mechanisms which can efficiently down regulate pro-inflammatory mediators and switch from inflammation toward resolution are still largely unknown.

1.1.1 Rapid progressive Glomerulonephritis (RPGN)

The most aggressive form of glomerulonephritis is rapid progressive glomerulonephritis, also referred to as RPGN. This disease occurs in 2 to 5 % of nephropathies. It is one of the most disastrous of nephrologic conditions and consists clinically of sudden and severe deterioration in renal function and results in necrotizing and crescentic glomerulonephritis. Because of the urgent need for early and accurate diagnosis biopsy is required. Patients can progress from normal renal function to end-stage renal failure within days or a few weeks (Couser WG, 1999). The presence of crescents in Bowman's space is a pathognomic feature of RPGN. Crescents were first described by Langhans in 1879 defined as an aggregation of cells. This aggregation of cells is particularly macrophage origin (Hancock WW and Atkins RC, 1984; Atkins et al., 1980) and fibrous connective tissue that may encroach on and destroy the capillary tuft. Other pathologic findings are neutrophil infiltration into the glomerular capillaries and areas of necrosis (Kerr et al., 2007). Studies vary from 20% (Stilmant et al., 1979) to more than 80% (Whitworth et al, 1976) of glomeruli involved into crescentic formation. Patients with the most rapid progression and most severe renal failure have a greater number of glomerular crescents (Whitworth et al., 1976; Morrin et al., 1978). Nevertheless, there is no agreement on the extent of crescent formation required for the diagnosis of RPGN. Progressive tubulointerstitial injury is also a prominent feature of RPGN (Cameron JS, 1992) and may relate more to the outcome of the disease than do the glomerular changes (Hooke DH et al., 1987). Without therapy the GFR decline about 50% within 3 months and accordingly to terminal renal insufficiency in 6 months (Nachman PH and Glassock RJ, 2012).

RPGN may be followed by any form of glomerulonephritis and can be very heterogeneous (***Figure 2***). Overall, the entities can be divided into three groups (***Figure 1***). Type I RPGN is the anti-GBM glomerulonephritis which results from autoantibody-mediated injury by autoantibodies against the glomerular basement membrane. Immune-complex nephritis is also referred to as Type II RPGN and occurs often in combination with lupus erythematodes. Most frequently RPGN occurs as ANCA-associated vasculitis; however, it is referred to as Type III RPGN (Andres G et al.,

1978; Holdsworth S et al., 1983 and 1985; Jennette JC and Falk RJ, 1998; Fischer and Lager, 2006; Xiao et al., 2005).

Type of RPGN	Referred disease
I	anti-GBM glomerulonephritis • without pulmonary hemorrhage • and the pulmonary basement membrane (PBM) with pulmonary hemorrhage = Goodpasture's syndrome • and cross-reaction with the tubular basement membrane (TBM), which leads to a more severe interstitial affection
II	Immune-complex-nephritis
III	ANCA-associated vasculitis

Table 1: Types of rapid progressive glomerulonephritis

Although the actual antigens in immune-complex-RPGN are not known, the possibility of a viral etiology has been discussed (Graham AR et al., 1981). In many patients with RPGN significant immune deposits were not detected in the glomeruli (Stilmant et al., 1979), however, over 80% of these patients have circulating anti-neutrophil cytoplasmic antibodies (ANCA) and it appears that these patients have renal vasculitis with or without systemic clinical manifestations (Becker GJ et al., 1982; Salant DJ, 1987; Jennette JC and Falk RJ, 2011). ANCAs are associated with a distinctive group of necrotizing small vessel vasculitis that typically have a paucity of vascular deposition of immunoglobulin and complement which distinguishes ANCA disease from anti-GBM-disease and from the classic immune-complex disease. Both of them have conspicuous deposition of immunoglobulin in vessel walls (Jennette JC and Falk RJ, 2011). RPGN is considered as a medical

emergency since the success of therapy depends on how early the disorder is initiated (Couser WG, 1999).

Primary Renal Diseases	Systemic Diseases
• RPGN—anti-GBM without lung involvement • RPGN—immune complex deposition • RPGN—without immune deposits (>80% ANCA positive) • Membranoproliferative GN • Membranous nephropathy • IgA nephropathy • Hereditary nephritis	• Goodpasture's syndrome • Post-infectious • Post-streptococcal Endocarditis • Shunt nephritis • Abscess • Schoenlein-Henoch disease • Lupus nephritis • Polyarteriitis • Wegener's granulomatosis • Cryoglobulinemia • Scleroderma • Relapsing polychondritis • Malignancy • Malignant hypertension

Table 2: Types of glomerulonephritis that can be associated with rapid deterioration in renal function and glomerular crescent formation (Kerr et al, 2007).

1.1.2 Immune cells in Glomerulonephritis

Immune cells involved in acute glomerulonephritis are consisting of neutrophils, monocytes/macrophages, dendritic cells (DCs) and T-lymphocytes infiltrating the kidney at different time points after onset of inflammation. The sequential activation of pro-inflammatory signaling pathways lead to the production of pro-inflammatory mediators such as cytokines and chemokines which direct the infiltration of these cells into the kidney and play a pivotal role in the pathogenesis of glomerulonephritis (Kurts

et al., 2007; Segerer et al., 2008). While neutrophils are recruited within few hours after disease induction, monocytes are recruited rather more slowly. The recruitment of lymphocytes is even slower and occurs over several days (Stilmant et al., 1979; Yang et al., 1998; Wada et al., 2001).

Once neutrophils are activated they can release several inflammatory mediators including reactive oxygen species (ROS) and reactive nitrogen species (RNS), proteinases, cationic proteins, lipid mediators, cytokines (such as TNFα, IL-1β and IL-12) and chemokines (such as IL-8, CCL3/MIP-1α, MIP-1β, IP-10) and monokine (induced by IFNγ) which play major cytotoxic and regulatory roles in tissue injury (Scapini P et al., 2000; Nathan C, 2006; Mayadas TN et al., 2010). CCL3 (MIP-1α) promotes the chemotaxis of monocytes (Kasama T et al., 1993). IP-10 and IFNγ trigger adhesion of activated T-lymphocytes, whereas CCL19, CCL20 and β-defensins recruit dendritic cells (DCs) which are critical for the outcome of the specific T-cell response, tolerance or the induction of anergy (Gasperini S et al., 1999; Müller I et al., 2009; Boudaly S, 2009). The number of activated neutrophils in renal biopsies from patients with ANCA-associated vasculitis correlates with the ANCA titer and the renal damage (Brouwer E et al., 1994). ANCA, first described in 1982, is a marker for idiopathic glomerulonephritis such as microscopic polyangiitis (Davies et al., 1982; Andrassyet al., 1988; Walters et al., 1988; Jennette JC and Falk RJ, 2011).

Primarily in 1976 macrophages could be identified in glomerular cultures from patients with RPGN (Atkins RC et al., 1976). As mentioned above macrophages display the majority of cells found in crescentic glomeruli. The correlation between the degree of interstitial mononuclear cell infiltration and impairment of renal function is highly significant indicating the high importance of renal response in glomerular injury (Hooke DH et al., 1987; Nikolic-Paterson DJ and Atkins RC, 2001). Activation of the renal macrophage infiltration has been demonstrated by macrophage production of cytokines such as IL-1, TNFα and MIF (macrophage migration inhibitory factor), gene transcription of inducible nitric oxide (iNOS), matrix metalloproteinase (MMP-12) and expression of activation antigens MRP8/14 and CD86 which are capable of causing tissue damage (Kerr et al., 2007). Increased renal monocyte chemo-attractant protein-1

(MCP-1) production is associated with glomerular and interstitial macrophage accumulation in human RPGN (Rovin BH et al., 1996; Segerer S et al., 2000). MCP-1 and RANTES/CCL5 play an important role in the inflammatory phase of crescentic nephritis. Furthermore, MCP-1 is involved in crescent formation and interstitial fibrosis (Lloyd CM et al., 1997b; Krensky AM and Ahn YT, 2007). The importance of RANTES in renal disease was first apparent in a study of renal transplants undergoing rejection, actually its expression is involved in a variety of renal diseases such as renal injury, glomerulonephritis/nephritic syndrome, chronic kidney disease/progression, renal transplant rejection and in renal cancer. RANTES attracts immune cells from the peripheral blood to sites of inflammation (Pattison J et al., 1994; Krensky AM and Ahn YT, 2007). In contrast to most chemokines the expression of RANTES is upregulated 3-5 days after T-cell activation which is important for maintenance of inflammation and facilitating expansion of the inflammatory infiltrate in both space and time (Schall TJ et al., 1988; Krensky AM and Ahn YT, 2007). Key regulators of RANTES expression include Rel proteins, as well as the enhancesome complex comprising KLF13 (Krueppel-like factor 13), Rel proteins and scaffolding proteins (Krensky AM and Ahn YT, 2007).

Tumor necrosis factor (TNF), first isolated and cloned in 1984 (Pennica D et al., 1984), is a prototype molecule of a growing family of related proteins called the TNF superfamily (TNFSF) that share common features. TNFSF ligands bind to receptors of the TNF receptor superfamily (TNFRSF). The activation of TNFRSF members modulates cell proliferation, survival, differentiation and apoptosis, which are then a major participant in the pathogenesis of kidney injury. It then promotes inflammation, fibrosis, apoptosis and accumulation of extracellular matrix, reducing glomerular blood flow and damaging the glomerular permeability barrier with development of albuminuria (Ortiz A and Egido J, 1995; Sanchez-Nino et al., 2010). Many TNFSF cytokines activate NF-κB (Sanz AB, 2010). TNFα is a potent inflammatory cytokine, which plays an important role in rapidly progressive glomerulonephritis and causes inflammation in the produced tissue (Takemura et al., 1994). In the kidney it is expressed, synthesized and released by infiltrating macrophages and by endothelial, mesangial, glomerular and tubular epithelial cells (Ortiz A and Egido J, 1995; Sanchez-

Nino et al., 2010). Acute exposure to high doses of TNFα results in shock and tissue injury, whereas chronic TNFα exposure causes cachexia and tolerance (Tracey KJ and Cerami A, 1994). However, there are immunosuppressive functions of TNF as well. TNF receptors TNFR1 and TNFR2 induce different and possible opposing functions in inflammation and immunity (Sanchez-Nino et al., 2010).

The renal mononuclear phagocytic system plays a central role in health and disease of the kidney. This system is composed of diverse subsets of bone marrow-derived macrophages and dendritic cells (DC). Macrophages are defined as tissue-resident phagocytic cells which clear apoptotic material and produce growth factors during steady-state homeostasis and perform antimicrobial effector functions during infection (Nelson PJ et al., 2012). Dendritic cells reside in virtually all tissues and play a protective role in renal disease (Kurts C et al., 2007). They are defined primarily by the specialized functions of antigen presentation and regulation of immune effector cells. Kidney DCs have been characterized by expression of CD11b, CD11c, F4/80 and MHC-II. Their MHC-II expression might allow kidney DCs to interact with infiltrating $CD4^+$-T-lymphocytes and to modify their effects in glomerulonephritis (Kurts C et al., 2007; John R and Nelson PJ, 2007; Segerer S et al., 2008; Nelson PJ et al., 2012). However, macrophages and dendritic cells within the kidney exhibit additional and at times overlapping functional properties (Nelson PJ et al., 2012).

The role of T-lymphocytes in RPGN was first suggested by studies performed in 1970 in which lymphocytes from patients with RPGN showed in vitro delayed-type hypersensitivity responses in the presence of GBM (Rocklin et al., 1970). The number of interstitial T-lymphocytes, particularly activated T-lymphocytes, correlate significantly with the severity of proteinuria and renal impairment (Lan et al., 1991). Depending on activated cytokines and thus the cytokine-composition of the tissue, naive $CD4^+$-T-cells differentiate to Th1, Th2, Th17 or regulatory (T_{regs}) T-cells. Th1-predominant responses appear to be associated strongly with proliferative and crescentic forms of glomerulonephritis that result in severe renal injury (Tipping PG and Kitching AR, 2005). As a hypersensitivity reaction in autoantibody-mediated diseases, auto-reactive $CD4^+$-T-lymphocytes may perform the classical T-helper function and

stimulate auto-reactive B-lymphocytes to produce complement-fixing autoantibodies that target kidney auto-antigens, e.g., in Goodpasture's syndrome or in the anti-GBM glomerulonephritis model. $CD4^+$-Th1-lymphocytes can license dendritic cells to stimulate auto-reactive $CD8^+$-T-lymphocytes to cause cytotoxic damage, however, their exact role in immune-mediated kidney disease is unresolved (Kurts C et al., 2007; Bevan J, 2007).

Regulatory T-cells or T_{reg}-cells seem to be protective in renal disease and play a pivotal role in the maintenance of tolerance. They were first identified in 1995 when a subset of $CD4^+$-T-cells constitutively expressed high amounts of IL-2 receptor α-chain (CD25) (Sakaguchi S et al., 1995). T_{reg}-cells are characterized through the constitutive expression of the IL-2-receptor α-chain and of the transcription factor forkhead/winked-helixbox P3 (FoxP3). FoxP3 specifies the T_{reg} cell lineage and has a critical role in early life and throughout the life span for establishing immune homeostasis. Several lines of experimentation provide proof that the lack of T_{reg}-cells is the cause of fatal autoimmunity resulting from FoxP3-deficiency (Josefowicz SZ et al., 2012). Autoimmunity can arise from failure of T_{reg}-cells in controlling harmful T-cell activity. All types of T_{reg}-cells have been demonstrated to prevent autoimmunity affecting the gut, the lung and the skin (Kurts C et al., 2007).

Immune-activated T-lymphocytes are exclusively localized to the areas of histologic damage, appearing to play a role in Bowman's capsular rupture, glomerular crescent fibrosis, tubulointerstitial fibrosis, and pulmonary fibrosis (Lan et al., 1991a, 1991b, 1992, 1995). The number of interstitial T-lymphocytes correlates significantly with the severity of proteinuria and renal impairment (Lan et al., 1991). This is suggestive of a pathogenic role for T-lymphocytes in the mediation of the disease (Kerr et al., 2007).

1.2 NF-κB in inflammatory disease and induction of inflammation

The inflammatory response involves the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and release further mediators. This response is self-limiting and resolves through the

release of endogenous anti-inflammatory mediators and the clearance of inflammatory cells. Nuclear factor-κB (NF-κB), initially identified in 1986 as a protein specifically bound to an enhancer of κ immunoglobulin light-chain (Sen R and Baltimore D, 1986 and 2006), plays a central role in the expression of a large number of genes involved in the inflammatory and immune responses activated by a wide variety of pathogenic signals including cytokines, infectious agents, and radiation-induced DNA double-strand breaks, and functions as a potent and pleiotropic transcriptional activator (Baeuerle PA and Henkel T, 1994). It was first identified in murine B-cells but has subsequently been found in virtually every cell type (Sen R and Baltimore D, 1986). Activation of the NF-κB-pathway has been demonstrated in human and experimental glomerulonephritis (Sakurai H et al., 1996; Seto M et al., 1998; Hernández-Presa MA et al., 1999) and other inflammatory disorders (Barnes P and Karin M, 1997; Giuliani C et al., 2001; Saccani S et al., 2004; Pakala et al., 2010) which also results in inflammation, immune regulation, survival, and cell proliferation responses (Bonizzi G and Karin M, 2004). NF-κB activation often occurs in situations in which rapid and decisive action is required for cell survival, such as during activation of the innate immune response (Karin M et al., 2000). The persistent accumulation and activation of leukocytes is a hallmark of chronic inflammation (Lawrence T et al., 2001).

1.3 NF-κB family

NF-κB is a collection of dimers composed of various combinations of members of the NF-κB/Rel family. Five mammalian Rel proteins were identified: Nfkb1 (p50/p105), Nfkb2 (p52/p100), p65 (RelA), c-Rel and RelB. NF-κB-dimers are most frequently composed of the two subunits p65 (RelA) and Nfkb1 (p50) (Baeuerle PA and Henkel T, 1994). Hence, although NF-κB applies to all of the members of the family, it is often used to refer to the p65/p50-heterodimer. All NF-κB-proteins share a highly conserved 300-amino-acid Rel homology region (RHR), composed of two immunoglobulin (Ig)-like domains, which is located toward the N-Terminus and is responsible for dimerization, interaction with the inhibitors of NF-κB (IκB), binding to DNA and containing the nuclear localization sequence (NLS) present near the C-terminus of the RHR.

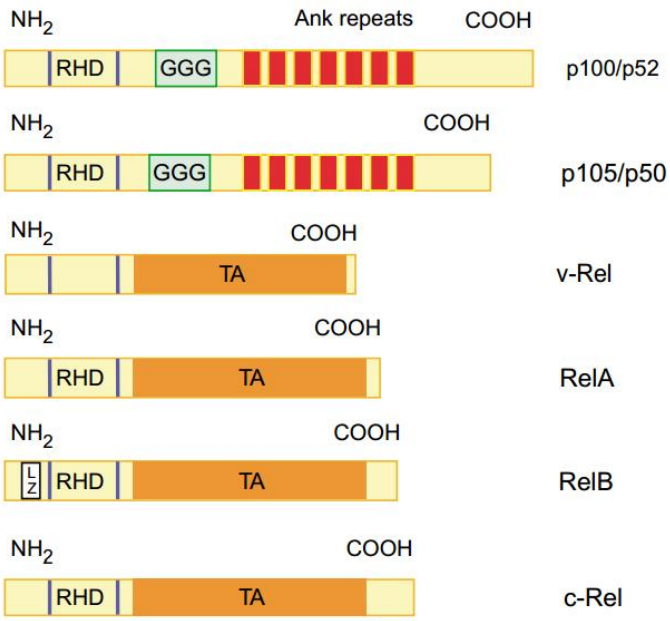


Figure 1: The NF-κB-family; NF-kB proteins contain a well conserved N-terminal ~300 amino acid Rel-homology domain (RHD), which includes DNA-binding and dimerization domains, and a nuclear localization signal (NLS). Both p105 and p100 contain a glycine-rich "hinge" region (GGG) followed by C-terminal ankyrin (Ank) repeats that are also present in the IkB family of proteins. Some proteins, such as c-Rel, RelB and RelA, contain transactivation (TA) domains in addition to the RHD (Bonizzi G and Karin M, 2004).

In mammalian cells this protein family can be divided into two classes. Class I includes Nfkb1 (p50, generated from p105) and Nfkb2 (p52, generated from p100) that are synthesized as precursor molecules (p105 and p100). Both remain in the cytoplasm upon proteolytic processing through phosphorylation and ubiquitin-dependent degradation of their C-terminal ankyrin-like motifs. The emerging DNA-binding subunits are p50 and p52. Containing ankyrin-like motifs at their C-termini p105 and p100 can serve an IκB-like function, retaining heterodimers formed with RelA, RelB, or c-Rel in the cytoplasm (Rice NR et al., 1992; Mercurio F et al., 1993; Naumann M et al., 1993). The Class II members RelA (p65), c-Rel and RelB do not undergo proteolytic processing (Attar RM et al., 1997). The Rel proteins differ in their abilities

to activate transcription, thus, only p65 (RelA) and c-Rel were found to contain potent transcriptional-activation (TA) domains among the mammalian family members. It is believed that dimers composed solely of Rel proteins that lack transcriptional activation domains bind to κB-consensus sites and mediate transcriptional repression, such as p50 (e.g. p50-p50-homodimers).

1.4 Activation of NF-κB

1.4.1 Upon stimulation

In unstimulated cells NF-κB is mainly detected in the cytoplasm as an inactive complex through interaction with IκB. IκBα, IκBβ, p100 (IκB-γ activity), IκBε and Bcl-3 are members of the IκB-family and have been identified in mammals. They regulate nuclear translocation and DNA binding of NF-κB and contain 6–7 ankyrin repeats (ANK) at their C-terminus that mediate binding to the RHR to interfere functions of the NLS. These repeats are also present in the C-terminal halves of the Nfkb1- and Nfkb2-precursors, which retain their Rel proteins in the cytoplasm. IκB-α, IκB-β, IκB-γ and IκB-ε present amino-terminal regulatory regions required for stimulus-induced degradation. Both, p50/p50- and p52/p52-homodimers can interact with Bcl-3, an IκB-like protein which functions not as an inhibitor but rather as co-activator (Perkins ND, 2007).

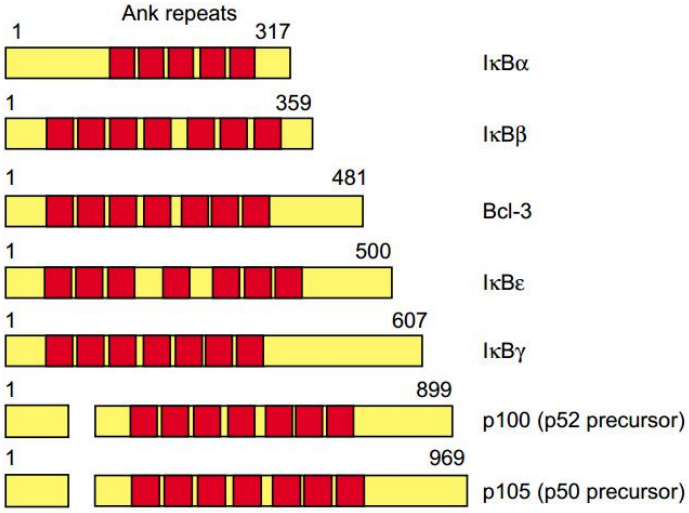


Figure 2: The IκB-family; IκB proteins contain conserved ankyrin (Ank) repeat motifs that mediate binding to the RHD. The number of repeats varies from five (IκBα) to seven (Bcl-3, IκBβ, IκBε, IκBγ, p100 and p105). p105 and p100 are the precursors for p50 and p52, respectively (Bonizzi G and Karin M, 2004).

1.4.2 The canonical (classical) and non-canonical (alternative) NF-κB-pathway

Two major signaling pathways lead to the translocation of NF-κB dimers from the cytoplasm to the nucleus. In response to several stimuli, pro-inflammatory cytokines and pathogen-associated molecular patterns (PAMPs) cause activation of the IκB-kinase, namely IKK. The most common form of this complex consists of the IKKα / IKK1 (85 kDa) and IKKβ / IKK2 (87 kDa) catalytic subunits and the IKKγ / NEMO (48 kDa) regulatory subunit. 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, whereas IKKγ lacks a kinase domain and exists as a trimolecular complex with IKKα and IKKβ. In the canonical or rather IKKβ-dependent IκB degradation pathway, the activated IKK-complex acts predominantly through IKKβ in an IKKγ-dependent manner and catalyzes the phosphorylation of two conserved serine (Ser) residues (Ser32 and Ser36) in the N-terminus of IκB. This is the most critical regulatory event in NF-κB activation. That in

turn 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 of two lysine (Lys) residues (Lys21 and Lys22), and subsequent degradation of IκB (α. β. ε) by the 26S proteasome (Shirane M et al., 1999; Karin M and Delhase M, 2000; Karin M and Ben-Neriah Y, 2000). The more recently discovered non-canonical or rather IKKα-dependent pathway leads to processing of p100 to p52 through polyubiquitination and proteasome degradation of its inhibitory C-terminal half. As the RHR of p100 is mostly associated with RelB, activation of this pathway results in nuclear translocation of p52/RelB-heterodimers (Senftleben U and Karin M, 2001 and 2002).

Whereas the canonical pathway, is most important for rapid degradation of NF-κB–bound IκBs (within minutes) and essential for a rapid and transient response or rather innate immunity whose main effector is the p65/p50-heterodimer, the non-canonical pathway is a more delayed response to a smaller range of stimuli resulting in processing of p100 and subsequent DNA binding of RelB/p52-heterodimers that is substantially slower (requires several hours) than the activation of IκB-bound dimers but essential for adaptive immunity (Bonizzi G and Karin M, 2004).

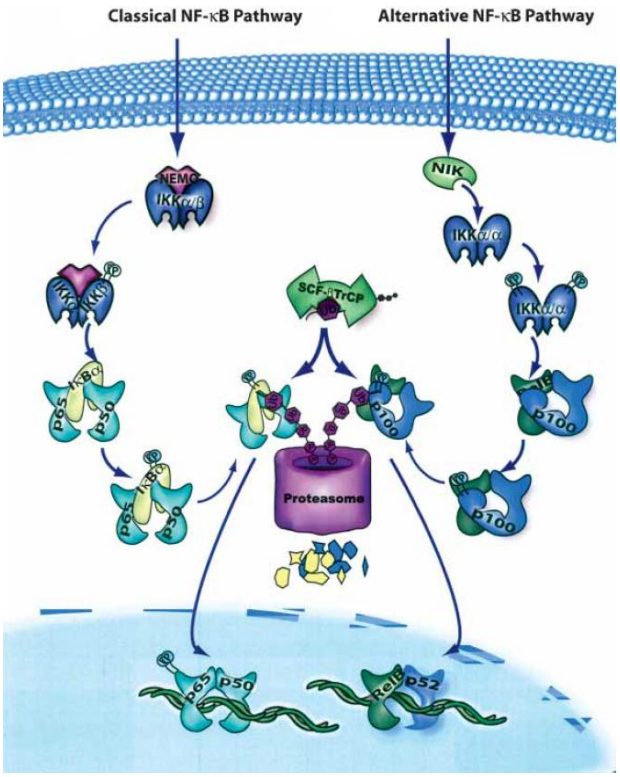


Figure 3: The classical and alternative NF-κB-pathway (Hayden MS and Ghosh S, 2004).

After degradation, IκB dissociates from NF-κB and frees its NLS, so that NF-κB is able to translocate into the nucleus in order to bind κB sites on promoters or enhancers of target genes (Miyamoto S and Verma IM, 1995; Baldwin AS, 1996; Siebenlist U et al, 1997; Gosh S et al., 1998; Karin M et al., 2000). Target genes include cytokines such as IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNFα), monocyte chemo-attractant protein-1 (MCP-1), cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and inducible nitric oxide synthase (iNOS). Nevertheless, further research is needed to elucidate other target genes. On the basis of gene expression examined within a few hours of induction, most NF-κB target genes are regulated by the p65/p50-heterodimer (Baeuerle PA and Henkel T, 1994). However, the RelB/p52-heterodimer might have an even broader range of activity on gene expression manifest at later time points (Fusco AJ et al., 2009). There are a group of early genes whose transcription is guaranteed by short stimulations of NF-κB regardless of stimulus concentration, including negative regulators of NF-κB activity

(IκBα, IκBε, and A20) and inflammatory cytokines (IL-6, IL-8, MCP-1, IP10). Thus, NF-κB promotes a negative regulatory loop through synthesis of IκB and A20 and, consecutively, cytoplasmic re-sequestration of NF-κB, which leads to the termination of the signal and contributes to activation dynamics (Senftleben U and Karin M, 2002; Sanz et al., 2010). In turn, there are late genes that are transcribed only when NF-κB activation lasts for at least 1 hour, such as cell surface receptors, adhesion molecules and signal adapters, and some chemokines such as RANTES/CCL5 that even need 3-5 days (Krensky AM and Ahn YT, 2007; Sanz et al., 2010).

1.5 NF-κB in Glomerulonephritis

Activation of the NF-κB-pathway has been demonstrated in human (in vivo) and experimental (in vitro) glomerulonephritis supporting the critical role of NF-κB in renal inflammation and the immune response (Sakurai et al., 1996; Guijarro C and Egido J, 2001). NF-κB is activated in podocytes and mesangial cells during glomerular injury as well as in tubular cells during the course of proteinuria or primary tubulointerstitial diseases, including ischemia reperfusion, obstruction, and septic or toxic acute kidney injury (Sanz et al., 2010). NF-κB- activation is involved in both induction and resolution of inflammation (Panzer et al., 2009). Several studies have shown the activation of NF-κB in the induction phase of several experimental models of glomerulonephritis (Sakurai et al., 1996; Ruiz-Ortega M et al., 1998; Tomita N et al., 2000; Panzer et al., 2002; Pocock J et al., 2003). The predominant NF-κB dimer during this early phase of inflammation appears to be p65/p50-heterodimer (Sen and Baltimore, 1986; Baeuerle and Henkel, 1994; Sha et al., 1995), which regulates neutrophil, macrophage, lymphocyte and dendritic cell biology and the expression of pro-inflammatory genes, including cytokines, chemokines and adhesion-molecules, whose actions are dynamic and essential for both the innate and the adaptive immune response (Sakurai H et al., 1996; Seto M et al., 1998; Hernández-Presa MA et al., 1999; Ghosh and Karin, 2002; Bonizzi and Karin, 2004; Fusco AJ et al., 2009). This correlates with parameters of severity of disease such as proteinuria and inflammation (Sakai et al. 2002). The TNF superfamily (TNFSF) cytokines (TWEAK and TNF) and Angiotensin II (AngII) are key activators of NF-κB in renal disease, whereas TNF lead to transient activation of the canonical NF-κB pathway and TWEAK promotes both, an early

canonical and a prolonged non-canonical pathway activation, resulting in the induction of early (MCP-1/CCL2) and more delayed (RANTES/CCL5) chemokines, but only TWEAK induces the NIK-sensitive, parthenolide-insensitive expression of CCL21 and CCL19 (Sanz et al., 2008 and 2010). Expression of NF-κB-dependent genes, such as the chemokines MCP-1/CCL2 and RANTES/CCL5, has been demonstrated both *in vivo* and *in vitro* cultured glomerular cells (Haberstroh U et al., 2002). MCP-1/CCL2 and RANTES/CCL5 play an important role in the inflammatory phase of crescentic glomerulonephritis (Lloyd CM et al., 1997). MCP-1 mediates glomerular monocyte/macrophage infiltration in anti-GBM-antibody- and anti-thymocyte-antibody-induced glomerulonephritis and is involved in the progression to fibrosis in murine crescentic glomerulonephritis (Stahl et al., 1993; Tang W et al., 1996; Wenzel U et al., 1997; Lloyd CM et al., 1997). Furthermore, early NF-κB blockade in an experimental model of glomerulonephritis markedly reduced proteinuria, glomerular lesions and monocyte/macrophage infiltration, which therefore represents an interesting new target for the treatment of renal inflammation (López-Franco O et al., 2002). However, this is highly dependent on the time course of inflammation. Blocking of NF-κB during the resolution phase of pleural inflammation was shown to have severe adverse effects (Lawrence et al., 2001).

NF-κB may also influence the inflammatory response in renal injury by actions beyond regulation of the expression of inflammatory mediators (Sanz et al., 2010). Furthermore, NF-κB activation may also promote cell proliferation and regulate cell survival, as it has pro- and anti-apoptotic properties.

1.6 Resolution of inflammation

The resolution of an inflammatory response is fundamental to health and differs from inhibiting inflammation and requires the termination of pro-inflammatory signaling pathways and clearance of inflammatory cells, allowing the restoration of normal tissue function. Although much attention has focused on pro-inflammatory pathways that initiate inflammation, relatively little is known about the mechanisms that switch off inflammation and resolve the inflammatory response. Similarly to the initiation of

inflammation the resolution of inflammation can be regarded as an intricate and active process requiring activation of endogenous signaling pathways that suppress pro-inflammatory gene-expression, leukocyte migration and activation followed by inflammatory-cell clearance by apoptosis and phagocytosis, thus leading to inhibition of inflammation and promotion of resolution (Lawrence T et al., 2002; Serhan CS, 2007; Buckley CD et al., 2013). Steroids, nitric oxide, adenosine, IL-10 and T_{reg}-cells have been shown to have anti-inflammatory functions by inhibiting immune responses and inflammation (Buckley CD et al., 2013). Furthermore, Eicosanoids and other lipid-mediators have pro-resolving or reparatory function. Cyclopentenone prostaglandins (cyPGs) may be endogenous anti-inflammatory mediators and promote the resolution of inflammation in vivo (Gilroy DW et al., 1999). First-phase eicosanoids promote a shift to anti-inflammatory lipids during the resolution of inflammation (Levy BD et al., 2001).

While the onset of inflammation is associated with expression of COX2, iNOS, production of the pro-inflammatory mediator prostaglandin E2 (PGE2) and nitric oxide (NO), the resolution of inflammation is associated with a further increase in COX2 expression, without PGE2 production or iNOS expression but accompanied by the production of the anti-inflammatory 15dPGJ2 and its precursor PGD2 (Tomlinson A et al., 1994; Gilroy DW et al., 1999; Murray PJ and Smale ST, 2012; Buckley CD et al., 2013).

Excessive and prolonged expression of pro-inflammatory mediators could be harmful to the host. Therefore, a variety of negative regulatory mechanisms have evolved to prevent prolonged inflammation such as negative feedback control of STAT signaling by inducible suppressors of cytokine signaling (SOCS) (Alexander W and Hilton DJ, 2004) and negative feedback control of both Toll-like receptors and IL-1 signaling by MyD88s (Janssens et al., 2002; Janssens et al., 2003). A20, a direct target gene for NF-κB signaling can also function as a negative regulator of TLR and TNFR signaling (Boone DL et al., 2004; Heyninck K and Beyaert R, 1999). These data suggest the negative feedback control as an important mechanism during resolution of inflammation.

In recent years, apoptosis has been identified as an important mechanism for the resolution of inflammation, required to clear inflammatory cells and the phagocytosis of apoptotic cells. This leads to switch off macrophage activation (Fadok et al., 1998). Apoptosis is likely to represent an injury limiting tissue removal process for granulocytes which would tend to promote resolution processes (Haslett C, 1997). Externalization of membrane phospholipids, which is a key marker of apoptotic cells, triggers recognition of phosphatidylserine (PS) by macrophages and facilitates phagocytosis (Fadok et al., 2001).

1.7 Role for Nfkb1 (p50) in the resolution of inflammation

Previous studies have shown a biphasic activation of NF-κB with a first peak during the induction phase and a second peak during the resolution phase. Despite the role of inducing pro-inflammatory genes including cytokines, chemokines and adhesion molecules early in inflammation recent research has revealed a role of NF-κB in the resolution of inflammation at later time points. Increased expression of chemokines, cytokines and infiltration of immune cells with subsequent tissue injury and increased mortality has been shown in Nfkb1$^{-/-}$ mice (Snapper CM et al., 1986; Mizgerd et al., 2003; Oakley F et al., 2005). Thus, NF-κB is involved in both, induction and resolution of acute inflammation (Panzer et al. 2009).

As mentioned above NF-κB transcription factors regulate genes involved in many aspects of the inflammatory response. NF-κB-activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis.

Different combinations of NF-κB dimers have distinct roles in the immune response. During resolution of glomerulonephritis the main NF-κB dimers switch to p50/p50-homodimers which lack transactivation (TA) domains and thus repress expression of NF-κB target genes (Kastenbauer S et al., 1999; Panzer U et al., 2009). Increased p50 expression suppresses TNFα production in LPS tolerance (Kastenbauer S et al., 1999).

p50/p50-homodimers were also found in resting T-lymphocytes and reduced p50 expression was observed after T-cell activation, furthermore, overexpression of p50 repress pro-inflammatory IL-2 expression (Kang S et al., 1992). These data suggest an important role of p50/p50-homodimers during the resolution of inflammation that leads to the expression of anti-inflammatory genes and the induction of apoptosis. Mice with a targeted IKKβ deletion in myeloid cells are more susceptible to endotoxin-induced shock than controls. This in turn is associated with elevated plasma IL-1β, suggesting an anti-inflammatory role for IKKβ (Greten et al., 2007).

The NF-κB-pathway regulates both pro- and anti-apoptotic pathways (Lin B et al., 1999). Inhibition of NF-κB activation decreases Fas (CD95) ligand expression on T-lymphocytes which is required for activation-induced cell death (AICD) (Kasibhatla et al., 1999). Overexpression of IκBα, specifically in T-lymphocytes, suggests a pro-apoptotic role for NF-κB in double positive thymocytes (Hettmann et al., 1999). However, the anti-apoptotic role of NF-κB relies on the expression of Bcl-x_L, TRAF1, TRAF2, c-IAP1 and c-IAP2 (Wang CY et al., 1998). Noteworthy, in most situations NF-κB has anti-apoptotic properties. Thus, $RelA^{-/-}$-mice display massive TNF-mediated liver apoptosis (Beg AA et al., 1995). TNF or TNF-related apoptosis-induced ligand (TRAIL) activates simultaneous death and NF-κB-dependent survival signals in renal cells (Lorz C et al., 2008). Inhibition of NF-κB during the resolution of inflammation protracts the inflammatory response and prevents clearance and apoptosis of leukocytes (Lawrence T et al., 2001).

1.8 Examination of NTN-induced Glomerulonephritis in $Nfkb1^{-/-}$-mice

Knockout mice for all of the NF-κB genes have been obtained, indicating specific roles for each NF-κB protein and a certain degree of redundancy among some of the family members, which is explained by their ability to form different homo- and heterodimers that can recognize the κB site (Staudt L et al., 1986). However, there are also clear examples for specific roles of individual Rel proteins. Interestingly, only the p65 (RelA) knockout is lethal, suggesting some functional redundancy among other members of the family (Sha WC et al., 1995).

This thesis examines the role of NF-κB subunit Nfkb1 (p50) in an experimental mice model of nephrotoxic nephritis (NTN). The observed mice model was the C57BL/6 wild type and its corresponding Nfkb1 knockout ($Nfkb1^{-/-}$). A sheep anti-mouse-glomerular basement membrane-(GBM)-antibody containing serum, injected in both wild type- and $Nfkb1^{-/-}$-mice, attacks proteins of the glomerular basement membrane and induces glomerulonephritis imitating rapidly progressive glomerulonephritis in humans. As mentioned above, it is known that the induction of glomerulonephritis due to anti-GBM-antibodies leads to the activation of the pro-inflammatory transcription factor NF-κB or rather NF-κB p65/p50-heterodimers. During the resolution of inflammation the dimer subunit composition switches to p50/p50-homodimers. However, p50 is required for both initiation and resolution of inflammation. Due to the absence of p50 other members of the NF-κB-family are required to form obligatory dimers during the inflammatory process in $Nfkb1^{-/-}$ mice. Mice lacking Nfkb1 (p50) develop normally and do not show any histopathological changes. However, these mutant animals do exhibit multifocal defects in immune responses mediated by B-lymphocytes and in non-specific responses to infection (Sha WC et al., 1995). Nfkb1 is essential for some but not all pathways leading to B-cell activation (Attar RM et al., 1997). $Nfkb1^{-/-}$ mice show defective clearance of the intracellular bacterium Listeria monocytogenes and an increased susceptibility with the extracellular gram-positive bacterium Streptococcus pneumonia (Sha et al., 1995), however, these mice do respond normally to challenges with the extracellular gram-negative bacteria Haemophilus influenzae and Escherichia coli K1 (Attar RM et al., 1997). Interestingly, $Nfkb1^{-/-}$ mice are more resistant to murine encephalomyocarditis virus (EMC) (Attar RM et al., 1997). The development of the B-cell lineage is not affected, but proliferation in response to stimulation with lipopolysaccharide (LPS) and soluble CD40 ligand (CD40L) is impaired (Sha WC et al., 1995; Snapper CM et al., 1996). In contrast, proliferative responses to stimulation by membrane-bound CD40L, anti-IgM or anti-IgD-dextran antibodies are normal (Snapper CM et al., 1996). Expression of the germ-line Ig constant heavy-chain gene (C_H) in stimulated $Nfkb1^{-/-}$ B-lymphocytes is also reduced specifically for $C_H\gamma 3$ and $C_H\varepsilon$. Class switching to IgG1 is normal but switching to IgG3,

IgE and IgA is down regulated. Taken together, these data define a specific role for Nfkb1 in B-cell proliferation and maturation (Attar RM et al., 1997).

We assessed the time-dependent NF-κB-activation and the functional role of Nfkb1 (p50). Mice have been observed for a maximum of 21 days after intraperitoneal injection of the nephrotoxic nephritis serum. Renal damage was measured by quantifying the loss of renal function, histopathological changes, infiltration of monocytes/macrophages and lymphocytes, and chemokine/cytokine expression. To further determine the role of NF-κB and especially the role of NF-κB subunit Nfkb1 (p50) electrophoretic mobility shift assay (EMSA), Supershift analysis and Western Blot experiments have been performed.

2. MATERIAL AND METHODS

2.1. Experimental animal models of nephritis

Immune-mediated glomerular cell injury was induced in C57BL/6J wild type and Nfkb1$^{-/-}$-mice by intraperitoneal injection (i.p.) of 1.5ml of a nephrotoxic nephritis serum (NTN). Urine was collected in a metabolic cage (MC) on days 3, 6, 9, 14 and 20 to determine renal function. Animals were killed by final anesthesia on the time points 2, 4, 7, 10, 14 and 21 days after i.p.-injection of NTN. At least, 3-5 animals were examined at each time point. (***Figure 6***)

All animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local ethical committees.

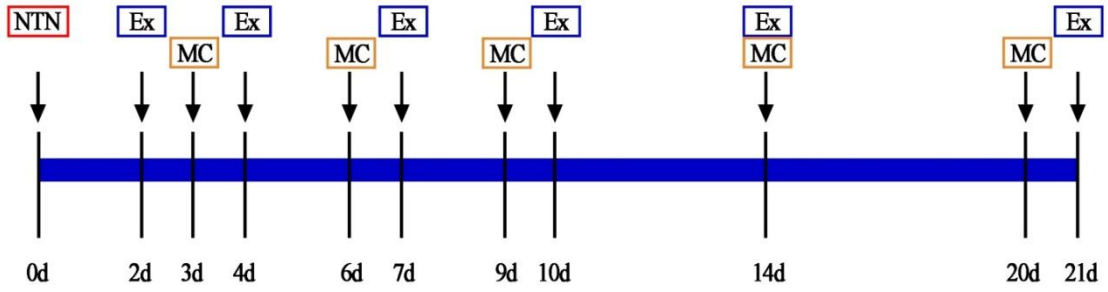


Figure 4: Timeline of experiments; NTN = injection of nephrotoxic nephritis serum; MC = Metabolic cage; Ex = Organ explantation.

2.2. Determination of Proteinuria

On the time points 3, 6, 9, 14 and 20 after disease induction the mice have been put into metabolic cages (MC) for a maximum of 6 hours to gain their urine. The protein concentration from the collected urine was semi-quantitative measured with urine stick to determine its dilution for further quantitative analyze of albumin by the Albumin-ELISA. Also, urine stick displayed the severity of kidney injury by measuring semi-quantitative protein concentration in the urine. The concentration of urea (serum) and

creatinine (serum and urine) was determined by the central laboratory of the University Medical Center Hamburg-Eppendorf (UKE). The Albumin/Creatinine-ratio was calculated from the concentration of albumin and creatinine in the urine.
In healthy glomeruli no or only a sign of albumin can be detected in the urine. Increased albumin concentration in the urine indicates a defective glomerular basement membrane and thus glomerulonephritis or kidney injury.

2.2.1. Albumin- ELISA

2.2.1.1. Solutions and Reagents

ELISA-Kit	ELISA Starter Accessory Kit (E101)
Coating Buffer	0.05 M Carbonate-Bicarbonate, pH 9.6
Wash Solution	50 mM Tris, 0.14 M NaCl, 0.05% Tween20, pH 8.0
Blocking (Post-coat) Solution	50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
Sample/ Conjugate Diluent	50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0
Enzyme Substrate	TMB
Stopping Solution	3 M H_3PO_4
Coating Antibody	A90-134A, Goat anti-Mouse Albumin-affinity purified, 1mg/ml, amount: 1ml, Working Dilution: 1:1.000
Calibrator	RS10-101, Mouse Reference Serum, amount: 0.1 ml, working range: 500-7.8 ng/ml
HRP Detection Antibody	A90-134P, Goat anti-Mouse Albumin-HRP conjugate, 1 mg/ml, amount: 0.1 ml, working dilution: 1:10.000 – 1:200.000

Table 3: Solutions and reagents for Albumin-ELISA

2.2.1.2. Procedure

1µl of the capture antibody has been diluted to 100 µl coating buffer for each well of the microtiter plate to be coated; then, it has been incubated over night in the freezer at 4 °C. After incubation, the capture antibody solution was aspirated from each well. These wells have been washed three times with 200 µl wash solution. The free binding sites of the plate which has not been occupied by the capture antibody, has been blocked with 150 µl/well poast-coat buffer and incubated 30 minutes at room temperature. After incubation, the blocking solution (poastcoat) was removed and each well washed three times with 200 µl wash solution. In the next step, the protein concentration of the samples has been detected by Multistix®10SG (Siemens, Ref: 01526748, LOT: 0D20CA). These test strips can discriminate the protein concentration by changing in color: the legend of the color is having no protein (negative), a sign (SPUR), 30 mg/dl (+), 100 mg/dl (++), 300 mg/dl (+++), >2.000 mg/dl (++++). The samples have been diluted with different amounts of sample diluent depending on the protein concentration, to fall within the concentration range of the standards:

Concentration	**Dilution**
SPUR	1 : 100
+	1 : 1.000
++	1 : 10.000
+++	1 : 50.000
++++	1 : 200.000

Table 4: Define the dilution for the samples

The standards have been generated from a reference mouse serum. The standard is diluted in sample diluent according to the chart below:

Step	ng/ml	Calibrator RS10-101-5	Sample Diluent
0	10.000	2 µl	9 ml
1	1.000	100 µl from Step 0	900 µl
2	500	500 µl from Step 1	500 µl
3	250	500 µl from Step 2	500 µl
4	125	500 µl from Step 3	500 µl
5	62,5	500 µl from Step 4	500 µl
6	31,25	500 µl from Step 5	500 µl
7	15,625	500 µl from Step 6	500 µl
8	7,8	500 µl from Step 7	500 µl

Table 5: Standard scheme for Albumin-ELISA

Then, 100 µl of standard and sample have been transferred to assigned wells, and incubated 60 minutes at room temperature. After incubation, the samples and standards have been removed, and each well washed 5 times with 200 µl wash solution. In the next step, 100 µl/well of HRP-conjugated detection antibody (A90-134P) [1:50.000] was added and incubated 60 minutes at room temperature. After incubation, the HRP-conjugated detection antibody has been removed, and each well has been washed 5 times with 200 µl wash solution. For the following step, enzyme substrate reaction, the substrate solution had to be mixed by mixing equal volumes of TMB and peroxidase. Then, 100 µl of substrate solution was transferred to each well, and incubated 15 minutes at room temperature at a dark place. Finally, the enzyme substrate reaction has been stopped with 100 µl/well H_3PO_4 (3 M), and the Albumin concentration was quantified in a photometer at 450 nm.

2.3. Organ extraction and preparation

2.3.1. Organ extraction

The mice have been applied with isofluran anesthesia. Then, the mouse was fixed on a pad and the abdomen or rather the peritoneum was opened from the xiphoid to the symphysis. Organs were pushed to the site to display the aorta, A. renalis and both kidneys. Finally, the A. renalis was cut through and the kidneys have been explanted.
Directly after extraction, the kidney was divided in three equal parts and each of them was stored in different tubes. The first part has been stored in a tube filled with Formalin in order to fixation for histology staining. The second part has been stored in a tube filled with Trizol in order to isolate RNA. The last part has been stored in an empty tube in order to isolate cytoplasmic and nuclear proteins.

2.3.1.1. Solution and reagents

2.3.1.1.1. Formalin

Sorensen's Buffer	3.03 g NaH_2PO_4 x H_2O 14.14 g Na_2HPO_4 x 2 H_2O add 1 L H_2O pH 7.2- 7.4
Formalin- solution (4%)	4% Formalin 0.4% Eosin in Sorensen's Buffer

Table 6: Solutions and reagents for organ fixation

2.3.2. Cytoplasmic protein isolation and Nuclear protein Isolation

For nuclear protein preparation we, adapted a method described by Sakurai et al. to prepare the cytoplasmic and nuclear mini-extracts. Cytoplasmic and nuclear proteins were isolated from whole kidney extracts and protein concentration was determined

(DC protein assay: Bio-Rad Laboratories). Isolated cytoplasmic and nuclear proteins can be used for electrophoretic mobility shift assay (EMSA) and Western blotting.

2.3.2.1. Solutions and Reagents

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, 1 mM Protease inhibitor, 1 mM Na-Vanadate; diluted 1:100
Hypotonic Buffer B	20 mM HEPES pH 7,9 400 mN NaCl 1 mM EDTA 1 mM EGTA *Add freshly:* 1 mM DTT, 1 mM Protease inhibitor, 1 mM Na-Vanadate; diluted 1:100, 10% Nonidet P-40 (NP-40)

Table 7: Solutions and reagents for protein and nuclear protein isolation

2.3.2.2. Procedure

1ml hypotonic buffer A was added to one third of a mice kidney and homogenized in a douncer on ice, and incubated 15 minutes. Then, 100 µl 10% NP40 was added, mixed thoroughly for about 15 seconds/sample, and immediately centrifuged at 13.000 rpm for 30 seconds at 4 °C. The supernatant was transferred to a new cup as cytoplasmic fraction and stored at -80 °C.

To isolate the nuclear proteins, further steps were required. The pellet was re-suspended in 200 µl of hypertonic buffer B, mixed, and incubated or 15 minutes on a rotator at 4 °C. After, the samples have been centrifuged for 5 minutes at 13.000 rpm at 4 °C. Finally, the supernatants were aliquot in 50 µl portions into new cups, and stored at -80 °C.

2.3.3. RNA-Isolation

The RNA has been isolated with the TRIzol®-Method. TRIzol® reagent is a complete, ready-to-use reagent for the isolation of high-quality total RNA or the simultaneous isolation of RNA, DNA, and protein from a variety of biological samples. This monophasic solution of phenol and guanidine isothiocyanate is designed to isolate separate fractions of RNA, DNA, and proteins from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour.

2.3.3.1. Solutions and Reagents

TRIzol®
Chloroform
2-Propanol
80% Ethanol/DEPC water

Table 8: Solutions and reagents for RNA-Isolation

2.3.3.2. Procedure

200 µl TRIzol® was added to one third of a mice kidney and homogenized by using a pistil. TRIzol® lyses the cells and inactivates RNase's and other enzymes. Phenols in TRIzol® separate proteins. After homogenization, further 800 µl TRIzol® was added to the samples, mixed thoroughly and incubated 10 minutes at room temperature. Then, 200 µl of chloroform was added and mixed thoroughly 15 seconds, and incubated 3 minutes at room temperature. This was centrifuged at 10.000 rcf for 10 minutes at 4 °C. The aqueous phase has been transferred to a new cup and 500 µl of 2-Propanol was added, mixed thoroughly 30 seconds and precipitated 30 minutes at room temperature. After incubation, the samples have been centrifuged at 10.000 rcf for 15 minutes at 4 °C. All further steps have been performed on ice. After centrifugation the supernatant was taken away. Finally, the pellet was washed three times with 500 µl 80%-Ethanol/DEPC-water. Between the washes, the samples were centrifuged 5 minutes at

10.000 rcf at 4 °C. After two washing steps the samples has been frozen at -80 °C in 80% Ethanol/DEPC water.

2.3.4. RNA-Purification

For purification of RNA, the Macherey&Nagel-Kit was used.

2.3.4.1. Solutions and Reagents

80% EtOH-DEPC H_20	
SpeedVac®	
β-Mercaptoethanol	
Macherey&Nagel-Kit	RA1-Buffer RA2-Buffer RA3-Buffer RNase-free water Mix of RA1-Buffer and β-Mercaptoethanol

Table 9: Solutions and reagents for RNA-Conditioning

2.3.4.2. Procedure

The tubes with the isolated RNA and 80% EtOH+DEPC have to be centrifuged at 11.000 g for 15 minutes at 4 °C. Then, the supernatant was thrown away and 500 µl 80% EtOH+DEPC H_2O was added and vortexed. This was centrifuged again at 11.000 g for 10 minutes at 4 °C. After centrifugation all liquid has to be removed. In the next step the pellets have been dried in the SpeedVac® for 5 minutes. The dried pellets have been incubated with 50 µl DEPC H_2O for 30 minutes on ice at room temperature.

2.4. Reverse Transcription Real-Time Polymerase-chain-reaction (RT-PCR)

The RNA-concentration of the probes was determined at 320 nm. Then, 400 ng of RNA was initiated for the transcription of cDNA with the following reaction mixture I:

Reaction mixture I
2 µl Hex Primer (100 ng/µl, Invitrogen)
2 µl dNTPs (10 mmol/l)
16 µl H_2O

Table 10: Transcription of cDNA: Reaction mixture I

This was incubated at 65 °C for 5 minutes and mixed with the reaction mixture II

Reaction mixture II
8 µl 5xBuffer + DTT (Invitrogen)
6 µl RNAse out (Invitrogen)
2 µl MMLV-Reverse Transcriptase (Invitrogen)

Table 11: Transcription of cDNA: Reaction mixture II

The incubation was performed referred to the following protocol:

Incubation protocol
10 minutes at 25 °C
60 minutes at 42 °C
10 minutes at 70 °C

Table 12: Transcription of cDNA: Incubation protocol

To determine the mRNA expression RT-PCR has been performed. This method is based on the concept of polymerase chain reaction (PCR) and duplicates the cDNA of the observed probes. For quantification of cDNA-concentration the fluorescence of the DNA-intercalating dye SYBR-Green has been measured.
RT-PCR has been performed with 1.5 µl of cDNA samples in the presence of 2.5 µl (0.9 µM) specific murine primers (forward/reverse primer) and 6.25 µl of 2xPlatinum SYBRGreen qPCR Supermix using StepOnePlus™ v2.0 Software. Prior to that cDNA has been compounded from purified RNA.

1.25 µl Forward primer (0.9 mmol/l)
1.25 µl Reverse primer (0.9 mmol/l)
2.25 µl H_2O
6.25 µl SYBR Green
1.5 µl cDNA

Table 13: Scale for quantitative PCR or rather RT-PCR

All PCR reagents and primers were obtained from Applied Biosystems and primer IDs were as follows:

18S	Fw	CAC GGC CGG TAC AGT GAA AC
	Rev	AGA GGA GCG AGC GAC CAA A
TNFα	Fw	AAA TGG CCT CCC TCT CAT CAG T
	Rev	GCT TGT CAC TCG AAT TTT GAG AAG
MCP-1 (CCL2)	Fw	GGC TCA GCC AGA TGC AGT TAA
	Rev	CCT ACT CAT TGG GAT CAT CTT GCT
RANTES (CCL5)	Fw	GCA AGT GCT CCA ATC TTG CA
	Rev	CTT CTC TGG GTT GGC ACA CA
CCL20	Fw	TGG GTG AAA AGG GCT GTG A
	Rev	AGC ATC AGT TTT TTA CAT CTT CTT GAC
IL-1β	Fw	CCT TCC AGG ATG AGG ACA TGA
	Rev	TCA TCC CAT GAG TCA CAG AGG AT

Table 14: Primer sequences

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 the housekeeping gen 18S rRNA to account for small RNA and cDNA variability. For determination of 18S-expression the cDNA was diluted 1:500. The probes were transferred to a 96-well plate in repeat determination. Then, the plate was incubated 40 cycles of the following qPCR or rather RT-PCR program:

1. Polymerase activation	95 °C	10 minutes
2. Denaturation	95 °C	15 seconds
3. Elongation	60 °C	1 minute
4. Denaturation	95 °C	15 seconds
5. Melt curve	60 °C	1 minute
	95 °C	15 seconds

Table 15: qPCR / RT-PCR program

Relative quantification of gene expression was calculated using the ΔΔCT method. CT- or rather threshold-value defines the point when fluorescence of SYBR-Green significantly increases. The arithmetic mean of the repeat determination of the 18S-housekeeping gen's CT-value was subtracted from the arithmetic mean of the probes CT-value (ΔCT). Finally, the ΔCT-value of non-nephritic controls was subtracted from the ΔCT-value of nephritic mice (ΔΔCT).

2.5. Electrophoretic mobility shift assay (EMSA) and Supershift

2.5.1. Solutions and reagents

2.5.1.1. Gel shift experiments

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 Polynucleotide Kinase	Fermentas
Poly(dl-dc) · (dl-dc)	Sigma

Table 16: Solutions and reagents for gel shift experiments

2.5.1.2. Buffer for Gel shift experiments

5-fold Binding Buffer	90 mM HEPES pH 7.9 1 mM EDTA pH 8.0 0.5 mM EGTA pH 8.0 200 mM NaCl 1 mM DTT 0.5 mM PMSF 15% Glycerol
10-fold TBE Buffer: 5 liter	540 g Tris 275 g Boric acid 200 ml 0.5 M EDTA (pH 8.0)

Table 17: Ingredients for gel shift buffer

2.5.2. EMSA

Electrophoretic mobility shift assay (EMSA) is a technique examining DNA- and RNA-binding protein interactions and thus is a method analyzing transcription factors. Furthermore, this method measures the protein concentration in the nucleus. EMSA were performed using NF-κB consensus oligonucleotide probes radiolabeled with ^{32}P-γ-ATP. The NF-κB oligonucleotides used in our experiments preferentially bind p65/p50-heterodimers and complex other NF-κB dimers with much less affinity (Baeuerle PA and Henkel T, 1994). Nuclear protein/oligonucleotide complexes were separated in a Polyacrylamidgel by electrophoresis. Bounded NF-κB dimers migrate slower through the gel, because of the increased molecular weight and can be illustrated by exposing gels to films. Exposed EMSA films were quantified using a phosphor imager Bio-Rad-GS-363 (multi-analyst software) and corrected to density of the probe.

2.5.3. Protein measurement

The Lowry protein assay is a reliable method for determining the total level of protein in a solution (Lowry et al., 1951). The total protein concentration is exhibited by a color change of the sample in proportion to protein concentration, which can be measured using colorimetric techniques. We used the Bio-Rad DC assay, which has some improvements in the Lowry assay. Initially, a standard range must be determined. For this, BSA is diluted from 10 mg/ml until 0.625 mg/ml in protein solution buffer (Buffer B). First, 5 µl from the standard range (S1 to S5) was pipetted in a 96-Well-plate. In the 6th well, 5 µl of Buffer B was pipetted as a blank or rather control. The remaining wells were filled with 5 µl of the examined proteins in a double determination. 25 µl of reagent A was added to each well, followed by 200 µl of reagent B. Then, the plate was incubated 10 minutes at room temperature and the solutions changed in color to blue. This blue coloration was then measured quantitative by ELISA-reader at 550 nm.

2.5.4. Labeling

NF-κB (*5'-AGTTGAGGGGACTTTCCCAGGC-3'*) consensus sequences were used for radiolabeling on their 5'-terminus to ^{32}P-γ-ATP using T4-Polynucleotidkinase. They were incubated 30 minutes at 37 °C, followed by an inactivation of T4-Polynucleotidkinase in a water bath for 10 minutes at 70 °C. Adjacent, radiolabeled

oligonucleotides were filtrated with Spin Columns G-50. Specific probe activity was measured by β-counter in the 32-P-channel for 1 minute.

2.5.5. Supershift experiments

Identifying NF-κB subunits, Supershift experiments were performed using NF-κB subunits p65 (RelA) and Nfkb1 (p50) consensus oligonucleotide probes radiolabeled with ^{32}P-γ-ATP and mixed with a Mastermix as mentioned above (2.5.3). The probes were incubated for 30 minutes and subsequent ~800 ng till 4µg of the antibody was added and incubated at 4 °C over night. On the following day, electrophoresis was performed.

2.5.6. Polyacrylamide gel electrophoresis (PAGE)

2 µl of corresponding nuclear protein dilution (=10 g protein) and 18 µl of a Shift-Mastermix were added into a tube and incubated 30 minutes at room temperature. Meanwhile, the gel was activated in a 1x Tris-Borat-EDTA-Buffer (TBE) for 30 minutes at 200 V. Thereafter, the activated gel was loaded with the probes and the electrophoresis was performed for approximately 2 hours at 200/250 V. Afterwards, the gel was dried in the extractor hood over night and subsequent exposed to an X-ray-film. Exposing to the X-ray-film lasted 2 weeks.

2.6. Nuclear protein Western Blotting

To further determine the composition of nuclear NF-κB subunits, Western blotting experiments on nuclear protein extracts were performed using an anti-p65 antibody. To determine nuclear p65 translocation, Western blotting experiments on cytoplasmic protein extracts were performed using an anti-IκBα-antibody.

Protein concentration was determined with the DC protein assay reagent (Bio-Rad Laboratories) as described above (2.5.3). 100 µg of protein was loaded onto a 12.5% SDS-PAGE and blotted semi-dry onto a polyvinylidene difluoride membrane. The blots were blocked in 5% nonfat dry milk in PBS and 0.1% Tween20 before incubating the blots for 1 h in a 1:1000 dilution of antibodies directed against p65 or rather IκBα. After

washes in PBS and 0.1% Tween20, the blots were incubated for another hour in a 1:2000 dilution of goat anti-rabbit HRP-linked IgG as the secondary antibody. The antibody-labeled proteins were detected with ECL according to the manufacturer's description. Blots were washed and re-probed with a mouse antibody against β-actin to control for small variation in protein loading and transfer.

2.6.1. Solutions and Reagents

Reagent / Material	**GmbH / Composition**
anti-p65-antibody	sc-109; Santa-Cruz Biotechnology and Biolabs (Heidelberg, Germany)
anti-IκBα-antibody	Santa-Cruz Biotechnology and Biolabs (Heidelberg, Germany)
goat anti-rabbit HRP-linked IgG	dilution: 1:2000; Southern Biotechnology Associates (Birmingham, AL)
β-actin	Sigma (Taufkirchen, Germany)
DC protein assay reagent	Bio-Rad Laboratories
Polyvinylidene diflouride (Hybond-P)	Amersham Pharmacia Biotech
12.5% SDS-PAGE	
Blocking solution	5% nonfat dry milk PBS 0.1% Tween20
Wash solution	PBS 0.1% Tween20

Table 18: Solutions, reagents and material for nuclear protein Western blotting

2.7. Histology

2.7.1. Morphological examinations

Renal tissue injury was assessed in paraffin-embedded and paraformaldehyde (4%)-fixed tissue sections stained by the periodic acid-Schiff (PAS) reaction. A semi-quantitative score (glomerulosclerosis index) for tubular injury and for acute tubular necrosis was calculated for each animal.

2.7.1.1. Glomerulosclerosis-Index

The percentage of tubules that displayed cellular necrosis, loss of brush border, interstitial edema, vacuolization, and tubule dilation were scored as described by el Nahas et al., 1991:

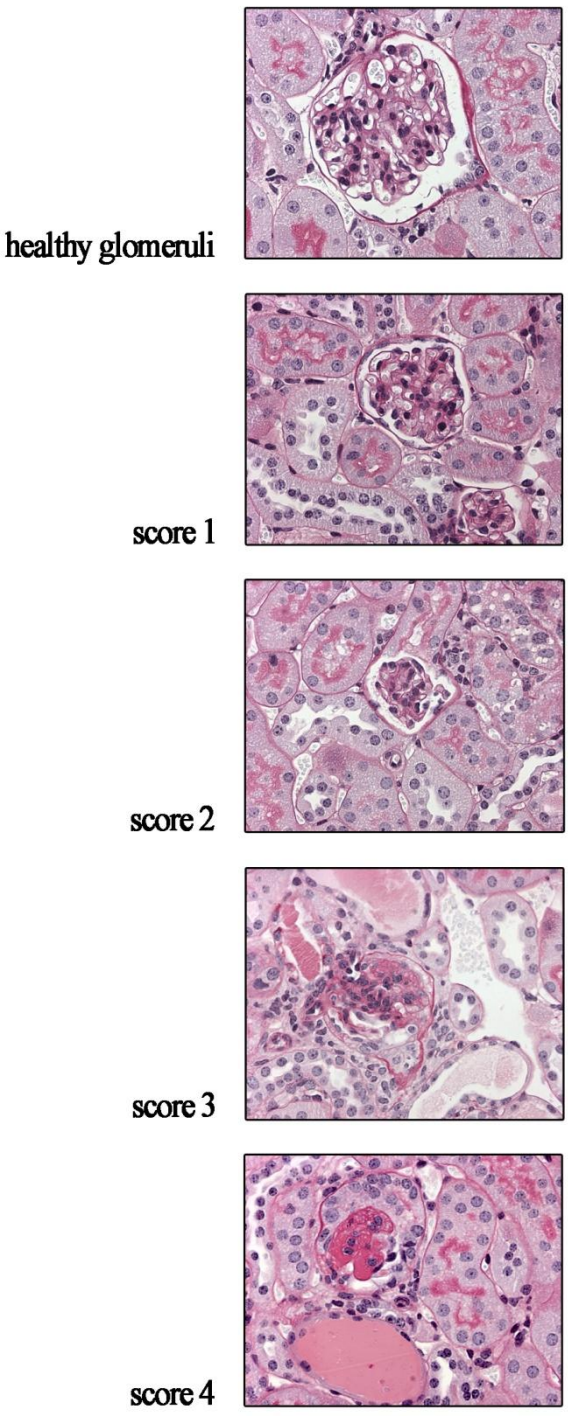


Figure 5: Histological changes after induction of glomerulonephritis. Glomeruli are scored from 0 (=healthy) to 4 (=severe damage) due to examined histological changes.

Stadium	Histological changes	Percentage of the convolute
0	None	0%
1	Mesangial thickening with/without mesangial proliferation. No involvement of capillaries	≤25%
2	Mesangial proliferation with partial involvement of capillaries. Segmental sclerosis	≤50%
3	Obliteration of capillaries due to mesangial proliferation or crescentic formation. Diffuse sclerosis	≤75%
4	Total obliteration of capillaries with/without capillary thrombosis; global sclerosis with capillary collapse	≤100%

Table 19: Glomerulosclerosis index

GLOMERULOSCLEROSIS-INDEX (GSI) per kidney was calculated with following formula:

$$GSI = \frac{(0 * nx0) + (1 * nx1) + (2 * nx2) + (3 * nx3) + (4 * nx4)}{n(= 15)}$$

nx0-nx4: number of glomeruli in the stages 1-4; n= number of examined glomeruli.

At least 15 glomeruli per kidney were evaluated under a light microscope (Axioskop; magnification = x40) and photographed by a connected camera (Axiocam).

2.7.2. Immunohistochemistry

For examining the monocyte/macrophage and lymphocyte infiltration, immunohistochemistry of the kidneys were performed. The pan-T-cell marker CD3 and the monocyte and renal dendritic cell marker F4/80 were used as primary antibodies. Immunohistochemical staining of the kidneys was performed by the pathology institute of the University Medical Hospital Hamburg-Eppendorf (UKE).

$F4/80^+$- and $CD3^+$- cells in at least 15 high-power fields (hpf) per kidney cross sections were counted by light microscopy (Axioskop) and photographed by a connected camera (Axiocam). $FoxP3^+$-cells were counted in at least 15 low-power fields (lpf) per kidney cross section.

2.8. Statistical Analysis

Results are expressed as means ± SD. Differences between the individual groups were compared by Kruskal-Wallis test with post hoc analysis by Mann-Whitney test. Statistical significance was defined as $P < 0.05$. Mouse survival data were plotted as Kaplan-Meier curves and analyzed for significant differences among the two groups by the log-rank test.

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. We assessed the renal function and performed morphological detailed examinations and chemokine expression analysis. To characterize the role of Nfkb1 (p50) in the nephrotoxic serum nephritis, the time-dependent activation and function of NF-κB was determined in wild type mice (C57BL6/N) as well as in Nfkb1$^{-/-}$ (C57BL6/N) mice. At each time point determined at least 3-5 animals were examined.

3.2. Kaplan-Meier Survival

After intraperitoneal injection of 1.5µl nephrotoxic nephritis serum different outcomes could be observed. On the site of wild type mice 1 subject died, whereas on the site of Nfkb1$^{-/-}$ mice 19 subjects died. All deaths within the Nfkb1$^{-/-}$ group happened within the first six days after disease induction. Subjects surviving the sixth day did not die within the 21-day observation period. (***Figure 6***)

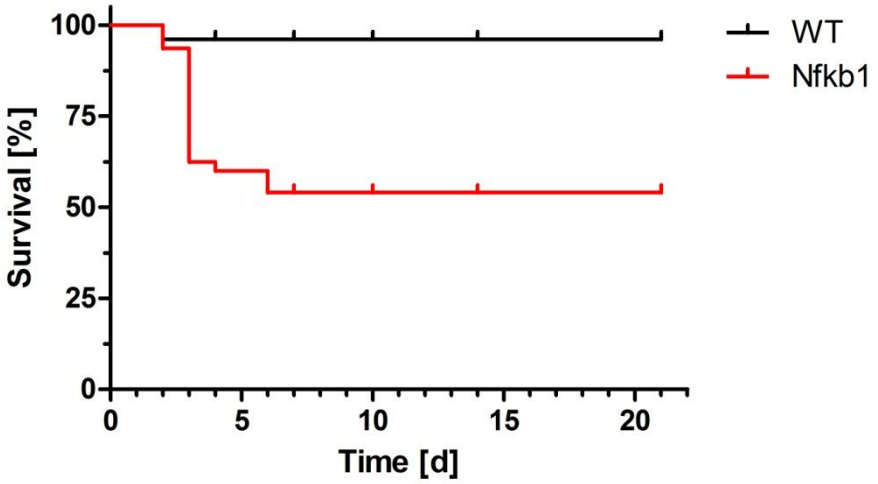


Figure 6: Kaplan-Meier Survival; number of deaths: WT = 1, Nfkb1$^{-/-}$ = 19; median survival: undefined; p<0.001.

3.3. 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 at 3, 6, 9, 14, and 20 days after disease induction to prove the illness and examine proteinuria.

3.3.1. Blood urea nitrogen (BUN) levels

3.3.1.1. Wild type mice

After NTN-Induction blood urea nitrogen levels (BUN; mg/dl) in nephritic wild type mice (WT) were significantly elevated on day 2 and 4 when compared with non-nephritic controls (*Con*: 37.8 ± 3.0; *NTN 2d*: 256.8 ± 7.7; *NTN 4d*: 62.8 ± 15.5; *NTN 7d*: 50.2 ± 10.9; *NTN 10d*: 51.3 ± 10.3; *NTN 14d*: 39.3 ± 5.4; *NTN 21d*: 40.4 ± 2.3; p at least <0,05 for time points 2, 4, 10 and p>0,05 for time points 14, 21 after NTN-induction vs. non-nephritic controls). Within the first two days BUN increased rapidly and

reached its peak, then decreased but was still at a high level on day 4. The BUN decreased to the level of non-nephritic controls after the fourth day (***Figure 7 A, B***).

3.3.1.2. Nfkb1$^{-/-}$-mice

In Nfkb1$^{-/-}$-mice blood urea nitrogen levels (BUN; mg/dl) were significantly elevated on day 2, 4, 7, 10, and 21 (*Con*: 30.6 ± 6.8; *NTN 2d*: 193.3 ± 30.6; *NTN 4d*: 95 ± 36.3; *NTN 7d*: 61.7 ± 8.6; *NTN 10d*: 42.4 ± 1.1; *NTN 14d*: 33.8 ± 2.8; *NTN 21d*: 42.0 ± 4.5; p at least <0.05 for each time point after NTN-induction vs. non-nephritic controls). Within the first two days BUN increased rapidly and reached its maximum on day 2 and 4. Then, it decreased but was still at a high level on day 7, 10 and 21 when compared to non-nephritic controls (***Figure 7 A, B***).

3.3.2. Albumin-to-creatinine ratio

3.3.2.1. Wild type mice

The albumin-to-creatinine ratio in nephritic wild type mice was markedly increased on all time points when compared to non-nephritic controls (*Con: 0.2 ± 0.03; NTN 3d: 225.1 ± 112.0; NTN 6d: 57.5 ± 32.1; NTN 9d: 18.3 ± 10.5; NTN 20d: 1.5 ± 0.3*; p at least <0.05 for each time point). The albumin-to-creatinine ratio reached its peak on the third day and declined thereafter, but was significantly high when compared to non-nephritic animals. (***Figure 7 C, D***)

3.3.2.2. Nfkb1$^{-/-}$-mice

The albumin-to-creatinine ratio in nephritic Nfkb1$^{-/-}$-mice was markedly increased on all time points when compared to non-nephritic controls (*Con*: 0.4 ± 0.02; *NTN 2d*: 179.0 ± 0.0; *NTN 3d*: 230 ± 103.7; *NTN 6d*: 194.3 ± 43.8; *NTN 9d*: 5.6 ± 0.8; *NTN 14d*: 4.5 ± 0.2; *NTN 20d*: 1.7 ± 0.3; p at least <0,05 on all time points). The albumin-to-creatinine ratio reached its maximum on day 2 and 6, and declined thereafter, but was still significantly high when compared to non-nephritic mice (***Figure 7 C, D***).

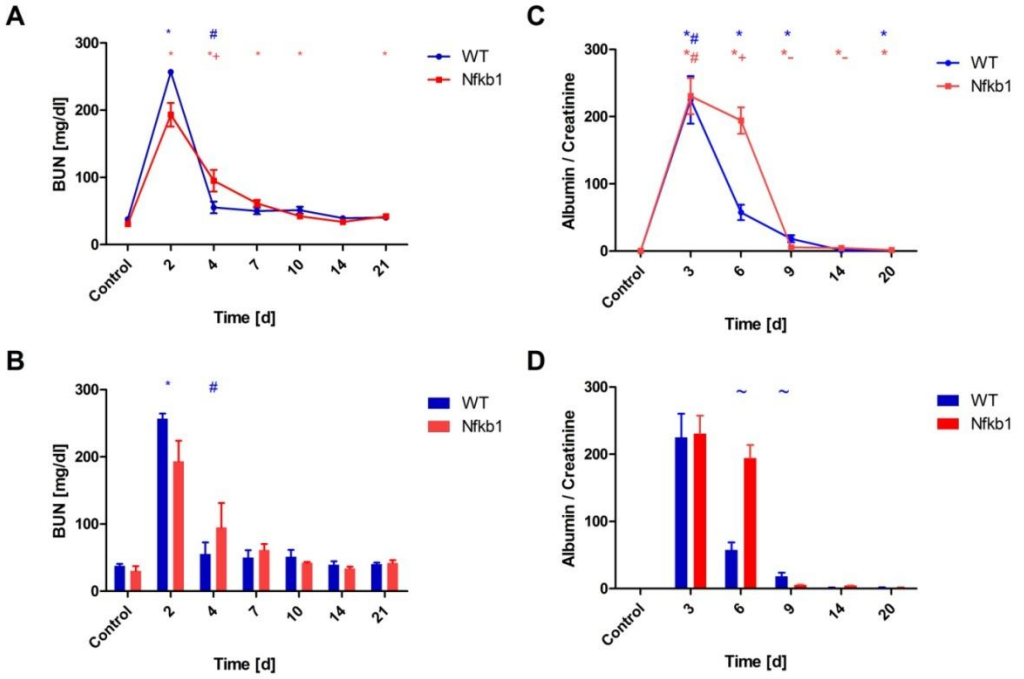


Figure 7 A, B, C, D: Renal function of NTN-mice. **A)** BUN of WT-NTN-mice significantly increased after disease induction with a maximum at day 2, thereafter decrease within the 21-days observation period to non-nephritic-level (n>3 for each time point; * p<0.03, # p<0.05 when compared with non-nephritic WT-mice). BUN of Nfkb1$^{-/-}$-NTN-mice significantly increased after disease induction and remained significantly elevated up to day 7 after induction of disease high (n>3 for each time point; * p<0.05 when compared with non-nephritic Nfkb1$^{-/-}$-mice, + p<0.05 compared with time points 7, 10, 14, 21). **B)** BUN of WT-mice at day 2 is significantly higher (* p<0.03) and at day 4 significantly lower (# p<0.05) than in Nfkb1$^{-/-}$-NTN-mice of the same time point **C)** The albumin-to-creatinine ratio was significantly elevated in WT-NTN-mice on all time points (n>3 for each time point, * p<0.05 when compared with WT-non-nephritic-mice, # p<0,05 when compared with the time points 6, 9, 20). The albumin-to-creatinine ratio was also significantly elevated in Nfkb1$^{-/-}$-NTN-mice on all time points examined, but decreased after its peak at day 3 and 6 (n>3 for each time point, * p<0.05 when compared with Nfkb1$^{-/-}$-non-nephritic-mice, # p<0.05 when compared with day 6, + p<0.05 when compared with day 9, - p<0.001 when compared with day 20). **D)** The albumin-to-creatinine ratio of nephritic WT-mice at day 6 is significantly lower and at day 9 significantly higher than in nephritic Nfkb1$^{-/-}$-mice of the same time point (~ p<0.02).

3.4. Light microscopic evaluation

3.4.1. Quantification of renal tissue damage in NTN mice of wild type- and Nfkb1$^{-/-}$-Animals

PAS stained kidney sections were evaluated by light microscopy for quantification of renal tissue damage in the NTN model. 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 hyper cellularity, formation of cellular crescents, and intraglomerular deposition of PAS-positive material. The tubulointerstitial compartment showed protein casts within the tubules, tubular dilation, and loss of brush border, interstitial edema, vacuolization, necrosis and atrophy.

A semi-quantitative 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 a dramatically increased histology score of the kidneys in nephritic wild type mice when compared to non-nephritic controls on all time points (*$p<0.02$). In wild type mice kidney injury was most severe at day 7 (*1.52±0.10*) and day 21 (*1.95±0.05*). Kidney injury improved between day 10 and 14(*1.0±0.08*), however, afterwards it increased to a maximum on day 21 (*1.95±0.05*). (***Figure 8 A***)

In Nfkb1$^{-/-}$-mice the renal histology score significantly increased at day 4, 7, 14 and 21 when compared to non-nephritic controls (***Figure 8 B***). Furthermore there have been two peaks of kidney injury. The first peak was around the fourth (*1.33±0.05*) and seventh (*1.16±0.06*) day which was followed by a second peak around the twenty-first day (*1.95±0.2*).

A significant difference between wild type and Nfkb1$^{-/-}$ mice could be demonstrated on the second (* $p<0.05$), 7 (* $p<0.05$) and the tenth day (# $p<0.03$) (***Figure 8 C***).

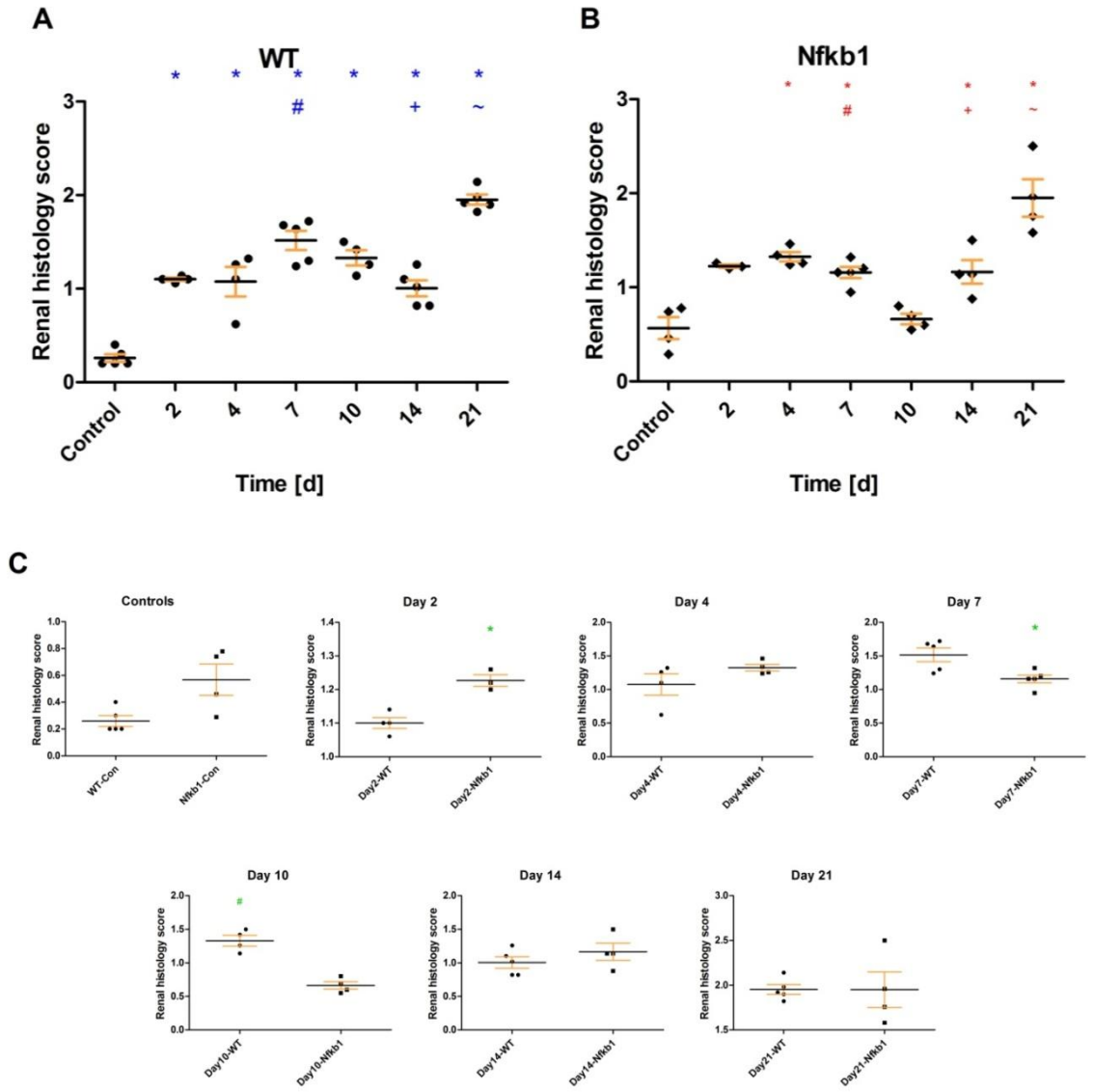


Figure 8 A, B, C: Renal Histology score (RHS) of NTN-mice. **A)** RHS of WT-mice significantly increased after disease induction and remained constant in all time points with a first peak around day 7 and a second peak at day 21 (n>3 on all time points; * p<0.02 when compared to non-nephritic-mice, # p<0.02 when compared to day 2 and day 14, + p<0.05 when compared to day 10, ~ p<0.02 when compared to day 14). **B)** RHS of Nfkb1$^{-/-}$-mice was significantly higher at day 4, 7, 14 and 21 compared to non-nephritic controls (n>3 on all time points; * p<0.03 when compared to non-nephritic-mice, # p<0.02 when compared to day 10, + p<0.03 when compared to day 10, ~ p<0.03 when compared to day 14). **C)** A significant difference in the RHS of WT- and Nfkb1-mice can be shown at day 2, 7 and 10 (n>3 on all time points; * p<0.05, # p<0.03 when compared to WT-mice)

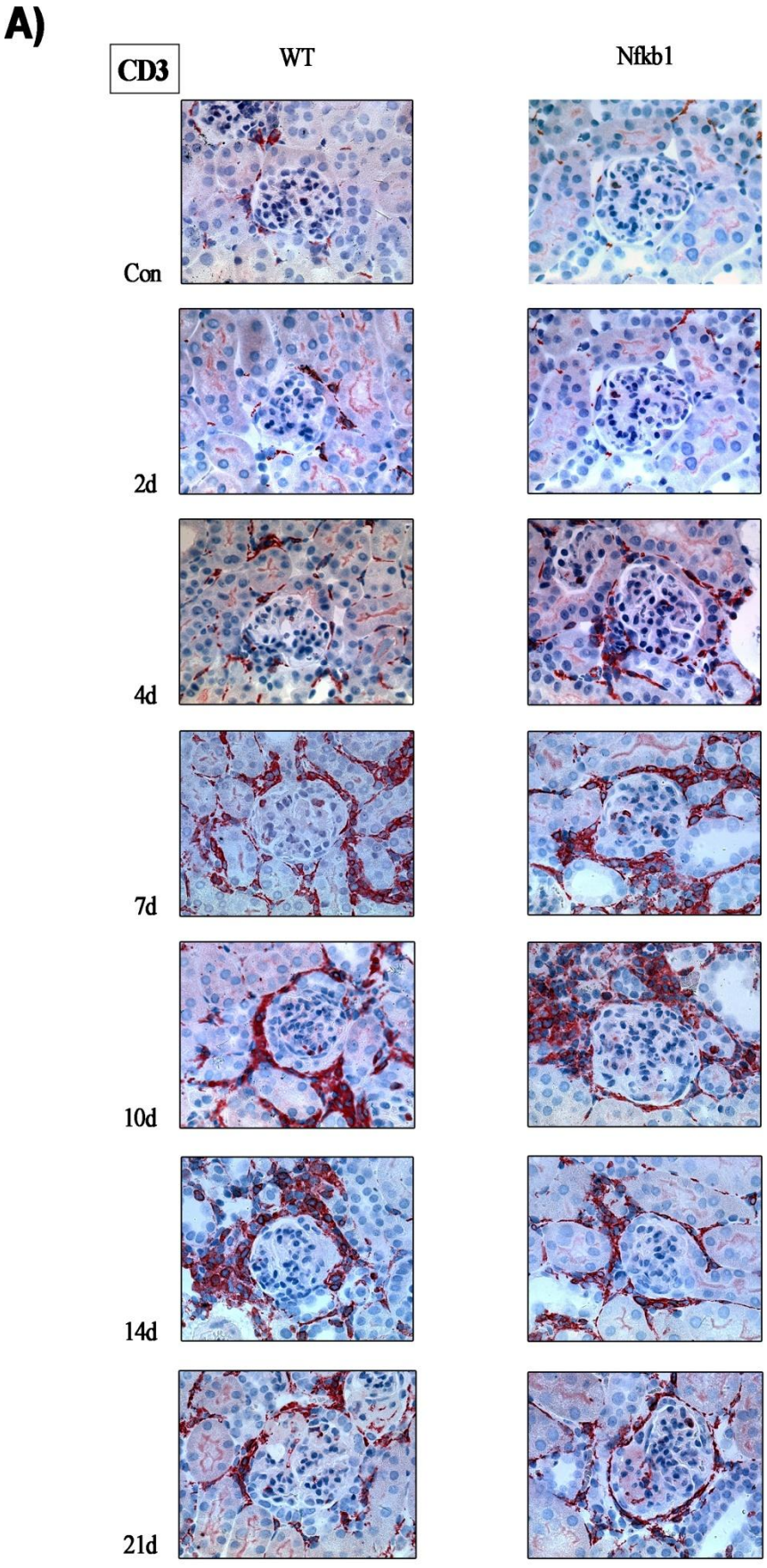
3.4.2. Quantification of $CD3^+$- and $F4/80^+$-cells in NTN-mice of WT- and $Nfkb1^{-/-}$-mice

For quantification of renal cell infiltration in the NTN model F4/80, CD3 and FoxP3 stained kidney sections were evaluated by light microscopy. A quantitative score for the presence of $CD3^+$-, $F4/80^+$- and $FoxP3^+$-cells was calculated for each animal in a double-blinded counting procedure.

The results demonstrated varying degrees of cell infiltration in the NTN mice at different time points, when compared to the healthy control group.

Interestingly, the infiltration of $CD3^+$-cells in wild type mice significantly decreased at day 2 after induction of disease (* $p<0.05$, when compared to non-nephritic control mice). Afterwards it increased until day 7, 10 and 14 (# $p<0.02$, when compared to non-nephritic controls) with its maximum at day 7 (*106.3±26.15 cells/ visual field;* ~ $p<0.04$, when compared to day 2, 14 and 21). On day 21 the $CD3^+$-infiltration significantly decreased ($p<0.04$, when compared to day 7). In summary, there was a significant decrease of infiltration after disease induction followed by an increase to a high maximum and a final decrease (***Figure 9 A***).

The infiltration of $CD3^+$-T-cells in $Nfkb1^{-/-}$-mice increased from day 4 (*39±5.23;* + $p<0.04$ when compared to day 2 and 7) to its maximum at day 7 (*100.5±14.1*) and was constant until day 21 (*85.25±14.85;* * $p<0.02$). On day 2 there was an indicated but no significant decrease of $CD3^+$-T-cell-Infiltration (*16±0.58*) (***Figure 9 B***).


A)
CD3
WT
Nfkb1
Con
2d
4d
7d
10d
14d
21d

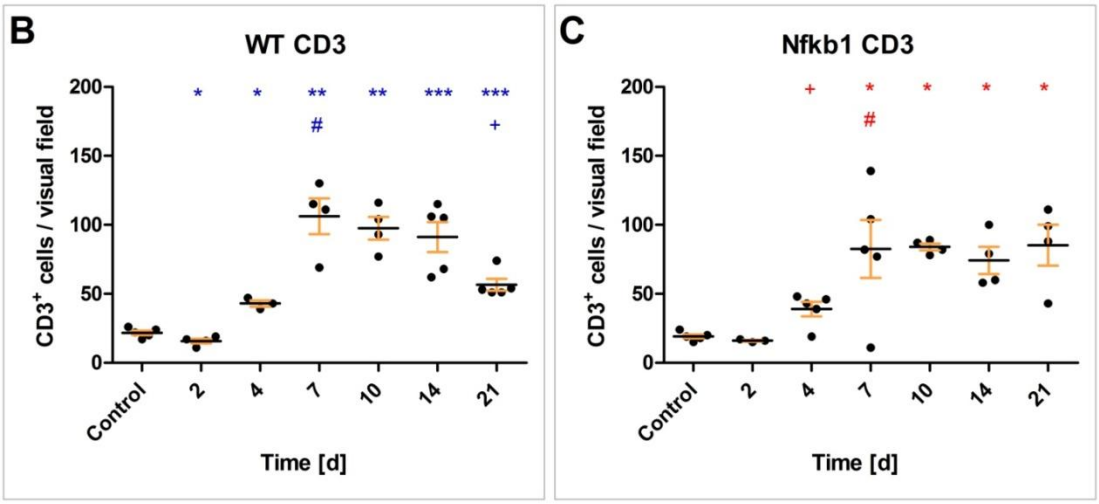
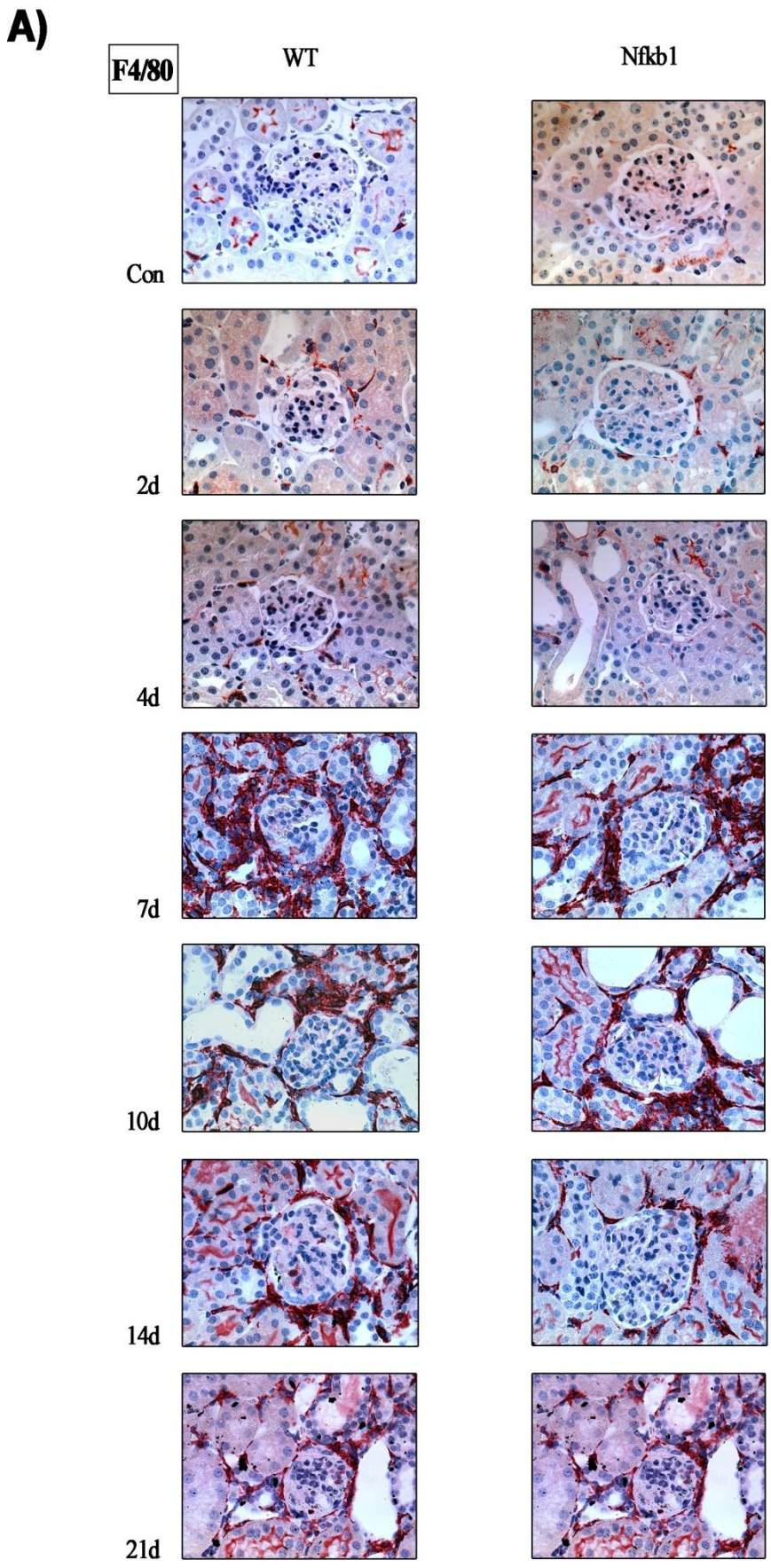


Figure 9 A, B, C: Counting of $CD3^+$-cells in immunohistochemical staining; cells were counted in high power fields (HPF/ visual field); magnification= x200. **A)** Demonstrated are morphological differences between wild type and $Nfkb1^{-/-}$ mice concerning infiltration of $CD3^+$-cells. $CD3^+$-cells appear red (magnification: x200). **B)** After disease induction the infiltration of $CD3^+$-cells in WT-mice decreased until day 2, but increased afterwards and was higher on all time points compared to non-nephritic controls (* $p<0.05$; ** $p<0.02$, *** $p<0.01$ when compared to non-nephritic controls; # $p<0.03$ when compared to day 2; + $p<0.04$ when compared to day 7 and 14. **C)** The Infiltration of $CD3^+$-cells in $Nfkb1^{-/-}$-mice significantly increased at day 7, 10, 14 and 21 after disease induction (* $p<0.02$ when compared to non-nephritic controls; # $p<0.02$ when compared to day 4).

The infiltration of $F4/80^+$-cells in wild type mice were significantly high from day 7 (*109.5±24.3 cells/visual field; * $p<0.02$* when compared to non-nephritic controls), 10 (*96.75±26.5 cells/visual field; * $p<0.02$* when compared to non-nephritic controls), 14 (*113.2±42.3 cells/visual field; + $p<0.01$* when compared to non-nephritic controls) and 21 (*74.4±20.9 cells/visual field; + $p<0.01$* when compared to non-nephritic controls) after disease induction. On day 2 there was a significant decrease in $F4/80^+$-cell-infiltration (*16.75±3.3 cells/visual field; # $p<0.04$*). In summary, there was a significant decrease of infiltration after disease induction followed by an increase to a high maximum without final decrease over the observed period (***Figure 10 B***).

The infiltration of F4/80^{+}-cells in Nfkb1$^{-/-}$-mice significantly increased from day 7 (*88.25±11.8 cells/visual field; * p<0.03* when compared to non-nephritic controls) with a maximum on day 10 (*122±11.1 cells/visual field; # p<0.05* when compared to non-nephritic controls), and was constant until day 21 (*65±12.6 cells/visual field; * p<0.03* when compared to non-nephritic controls). On day 2 (*12.4±4.7 cells/visual field*) the infiltration had not significantly changed when compared to control animals (*14±1.4 cells/visual field*). On day 4 (*29.8±9.6 cells/visual field*) there was a light but no significant increase of F4/80^{+}-cell-infiltration. This light increase was significantly lower when compared to day 7 (*+ p<0.04* when compared to days 2 and 4). In summary there was a significant increase in F4/80^{+}-cell-infiltration beginning from day 7 and a maximum of infiltration on day 10 after induction of disease. There was no final decrease over the observed period (***Figure 10 C***).


A)
F4/80
WT
Nfkb1
Con
2d
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7d
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14d
21d

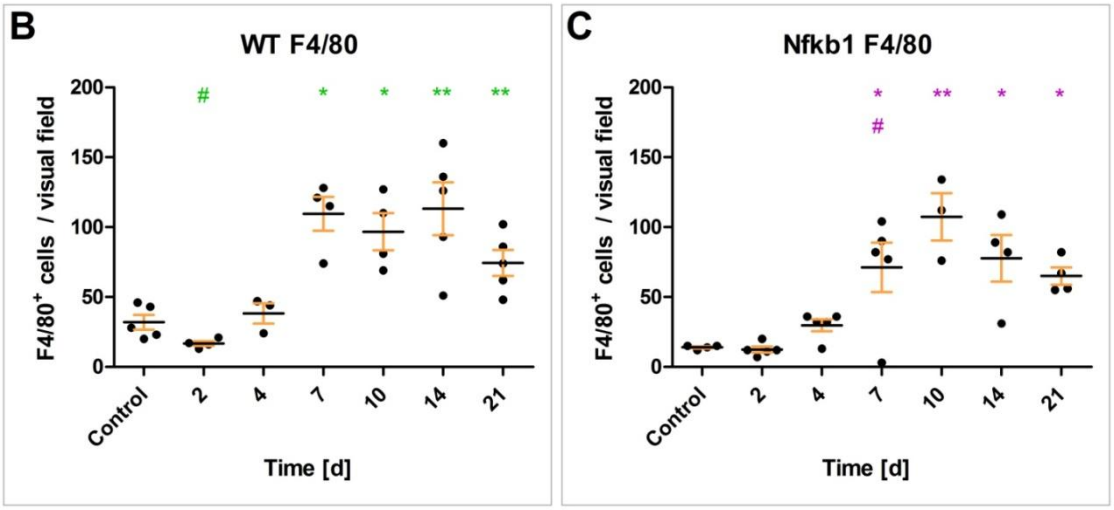
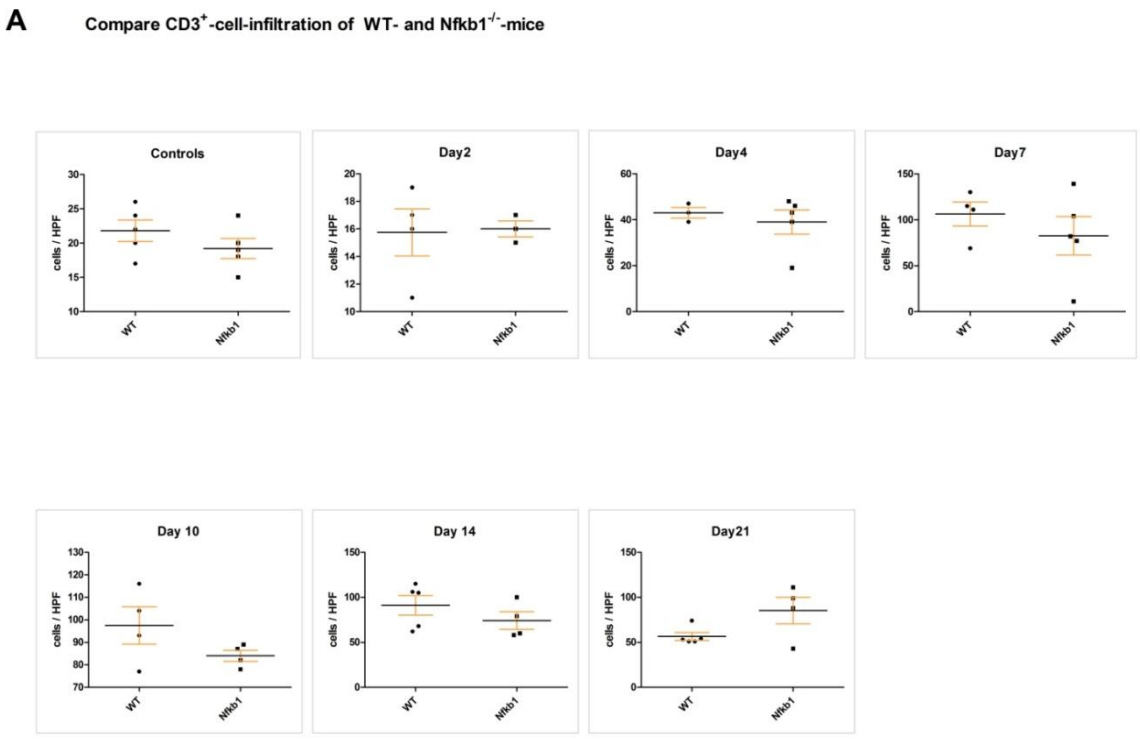


Figure 10 A, B, C: Counting of F4/80^{+}-cells in immunohistochemical staining; cells were counted in high power fields (HPF/visual field); magnification= x200. **A)** Demonstrated are differences between wild type and Nfkb1$^{-/-}$ mice concerning infiltration of F4/80^{+}-cells. F4/80^{+}-cells appear red (magnification: x200). **B)** After disease induction the Infiltration with F4/80^{+}-cells in WT-mice significantly decreased until day 2 (# $p<0.04$), thereafter it significantly increased at day 7, 10, 14 and 21 (* $p<0.02$, ** $p<0.01$ when compared to non-nephritic controls). **C)** The Infiltration of F4/80^{+}-cells in Nfkb1$^{-/-}$-mice significantly increased at day 7, 10, 14 and 21 after disease induction (* $p<0.03$, ** $p<0.05$ when compared to non-nephritic controls); # $p<0.03$ when compared to day 2).

To further determine the differences between wild type and Nfkb1$^{-/-}$ mice concerning CD3^{+}- and F4/80^{+}-cell infiltration statistical analysis have been performed. Altogether the infiltration of cells was identical between wild type and Nfkb1$^{-/-}$ mice. Concerning infiltration of CD3^{+}-cells there was a decrease on the second day in both wild type- and Nfkb1$^{-/-}$-mice. Both had their maximum on the seventh day. In contrast to Nfkb1$^{-/-}$ mice the infiltration of CD3^{+}-cells decreased in wild type mice until the end of the observation period. Nevertheless, this was an indicated but no significant difference. Concerning F4/80^{+}-cells the infiltration was significantly lower in non-nephritic Nfkb1$^{-/-}$ mice ($p<0.02$). After induction of nephrotoxic nephritis there were no significant differences in in F4/80 cell infiltration in both wild type- and Nfkb1$^{-/-}$-mice. Both had

no significant final decrease until the end of the observation period, respectively, the infiltration of F4/80^{+}-cells remained high (***Figure 11 A, B***).



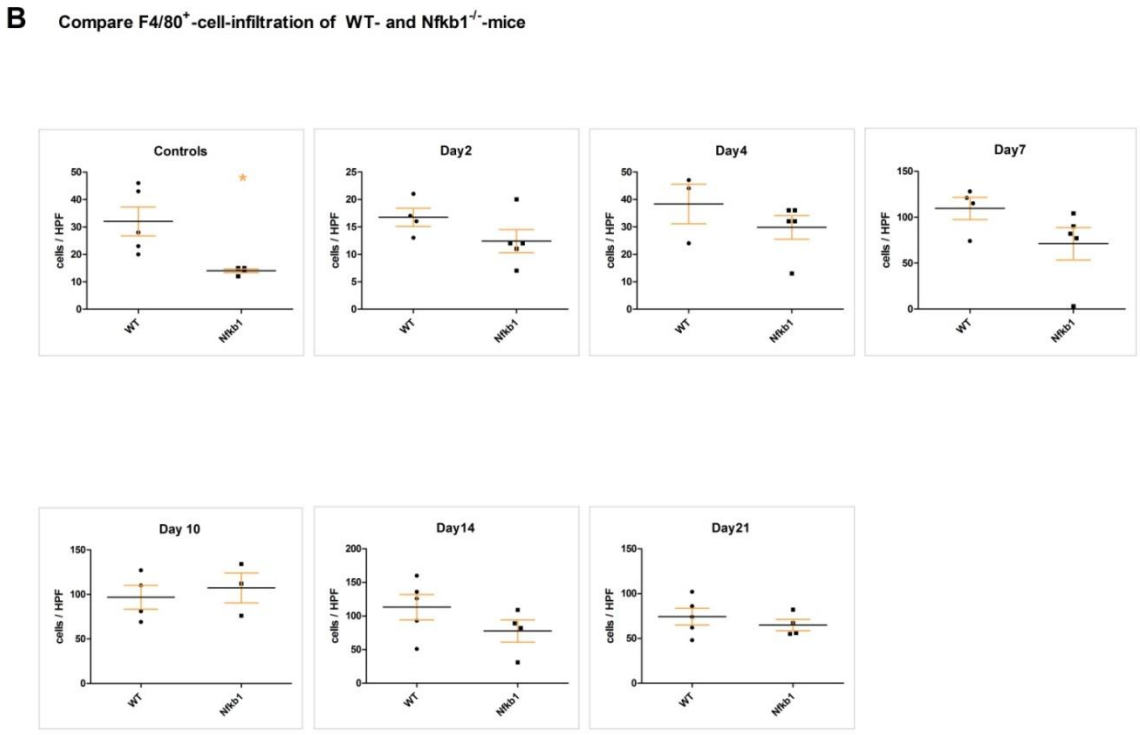


Figure 11 A, B: Comparison of $CD3^+$- and $F4/80^+$-cell-infiltration between wild type and $Nfkb1^{-/-}$ mice. **A)** There was a comparable infiltration of $CD3^+$-cells between WT- and $Nfkb1^{-/-}$-mice. **B)** During the nephritic 21-day observation period there was a comparable infiltration of $F4/80^+$-cells between WT- and $Nfkb1^{-/-}$-mice. But, in the non-nephritic controls, the infiltration was significantly higher in $Nfkb1^{-/-}$-mice (* $p<0.02$ when compared to non-nephritic WT-mice).

There was no significant infiltration of $FoxP3^+$-cells. Nevertheless, the results indicate a slight increase of FoxP3 infiltration after induction of nephrotoxic nephritis in both wild type- and $Nfkb1^{-/-}$-mice (***Figure 12 A, B***). There was no difference between wild type and $Nfkb1^{-/-}$ mice (***Figure 12 C***). To show a difference between the examined mice more subjects are needed.

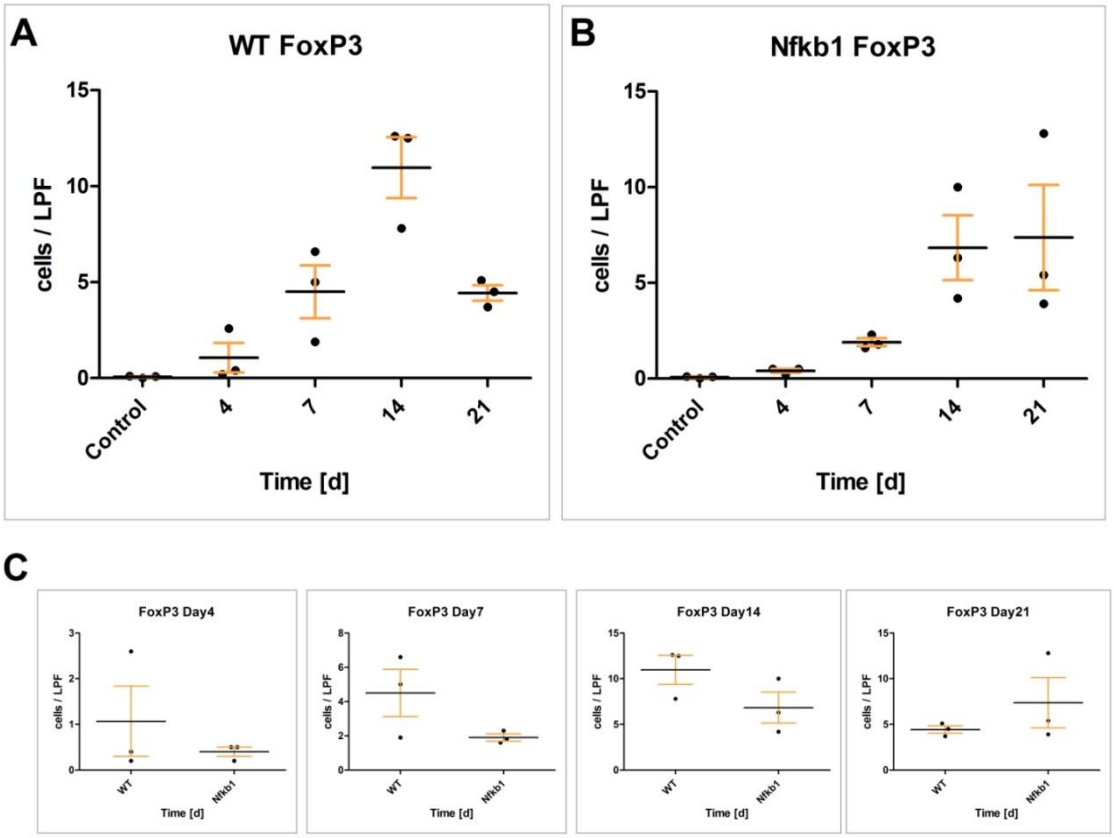


Figure 12 A, B, C: Counting of $FoxP3^+$-cells in immunohistochemical staining; cells were counted in 15 low power fields (LPF) per kidney cross section; magnification= x200. **A)** The infiltration of FoxP3-cells

in WT-mice slightly increased after disease induction to an indicated maximum on day 14 and decreased afterwards (n=3, for each examined time point) **B)** The infiltration of $FoxP3^{+}$-cells in $Nfkb1^{-/-}$ mice slightly increased after disease induction. C) There was no significant difference between wild type and $Nfkb1^{-/-}$ mice concerning $FoxP3^{+}$-infiltration.

3.5. Time dependent chemokines mRNA expression

At 2, 4, 7, 10, 14 and 21 days after intraperitoneal injection of NTN-serum, RNA was isolated from kidney tissues of control and nephritic mice. Out of this RNA we compounded cDNA and performed real-time PCR (RT-PCR) to measure the expression of the cytokines TNFα, MCP-1/CCL2, RANTES/CCL5, CCL20 and IL-1β.

3.5.1. Cytokine expression in wild type mice

3.5.1.1. Expression of MCP-1/CCL2

The expression of MCP-1/CCL2 significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 62.8 ± 22.0 –fold*) and reached its maximum on day 4 (*4286.0 ± 222.4 –fold*). Afterwards, the expression significantly decreased until day 10 (*day 7: 29.0 ± 1.8 –fold; day 10: 14.3 ± 6.8*) before it increased again and had a second peak on day 14 (*585.1 ± 120.6-fold*). In summary, the expression of MCP-1/CCL2 was significantly high during the whole observation period when compared to non-nephritic controls and it presented a biphasic trend with two maxima and a lower expression between the peaks. ***(Figure 13 A CCL2)***

3.5.1.2. Expression of RANTES/CCL5

The expression of RANTES/CCL5 significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 2.1 ± 0.2 –fold*) and reached its maximum on day 4 (*88.7 ± 5.3 –fold*) which was followed by a decrease until day 10 (*day 7: 21.68 ± 2.4 –fold; day 10: 5.3 ± 2.1 –fold*) and a second increase on day 14 (*15.0 ± 1.1 –fold*). In summary, the expression of RANTES/CCL5 was significantly high during the whole observation period when compared to non-nephritic controls and it presented a biphasic trend with two maxima and a lower expression between the peaks. ***(Figure 13 A CCL5)***

3.5.1.3. Expression of CCL20

The expression of CCL20 significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 2.1 ± 0.3 -fold*) and reached its maximum on day 4 (*183.7 ± 14.8 -fold*) which was followed by a decrease until day 14 (day 7: 3.1 ± 0.1 –fold; day 10: 8.5 ± 4.6 –fold; day 14: 2.5 ± 0.5 –fold). After that it increased again until day 21 (*31.0 ± 14.2 -fold*). In summary, the expression of CCL20 was significantly high during the whole observation period when compared to non-nephritic controls and it presented a biphasic trend with two maxima and a lower expression between the peaks. ***(Figure 13 A CCL20)***

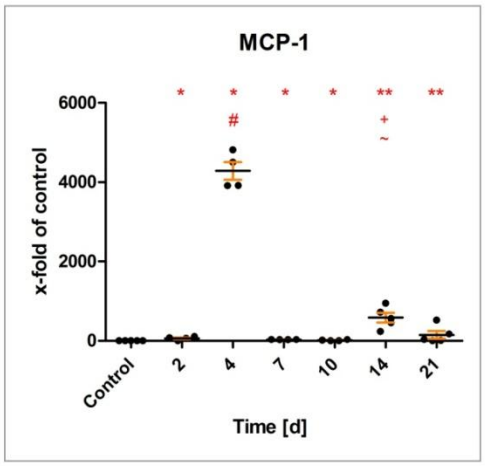
3.5.1.4. Expression of TNFα

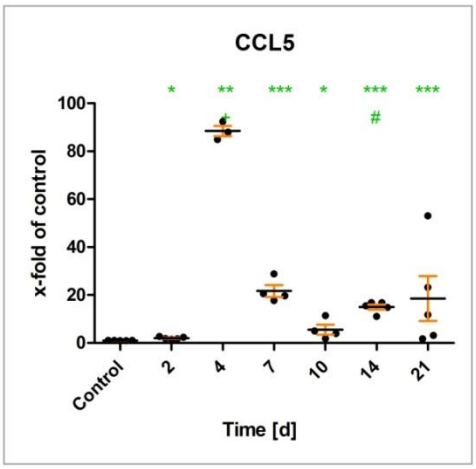
The expression of TNFα significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 75.6 ± 11.2 –fold; day 4: 48.0 ± 6.1 –fold; day 7: 108.6 ± 26.0 –fold*). On day 10 the infiltration decreased (*5.1 ± 1.6 –fold*) which was followed by a second increase to reach its maximum on day 14 (*235.3 ± 39.2 –fold*) and a final decrease afterwards (*day 21: 16.3 ± 7.3 –fold*). In summary, the expression of TNFα was significantly high during the whole observation period when compared to non-nephritic controls and it presented a biphasic trend with a first peak (day 2 until 7) and a second higher peak (day 14) which was followed by a decrease. ***(Figure 13 A TNFα)***

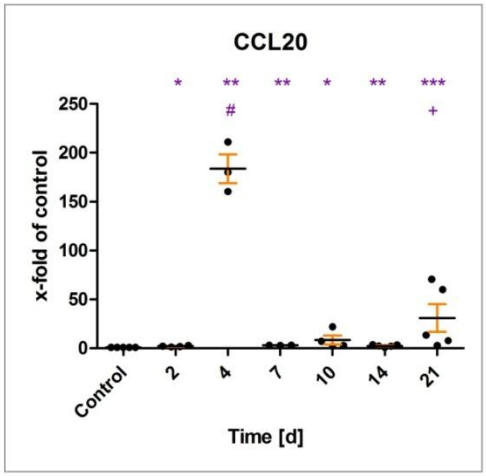
3.5.1.5. Expression of IL-1β

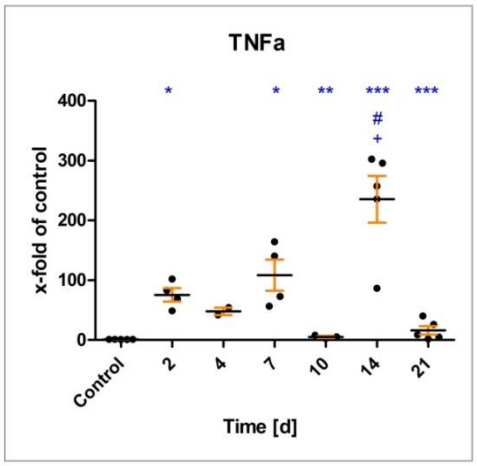
The expression of IL-1β increased after intraperitoneal injection of the NTN-serum and was significantly high from day 4 until day 21 when compared to non-nephritic controls (*day 4: 20.1 ± 0.3 –fold; day 7: 7.3 ± 4.1 –fold; day 10: 6.1 ± 2.8 –fold; day 14: 13.6 ± 2.1 –fold; day 21: 17.7 ± 10.5 -fold*). In summary, the expression of IL-1β increased and remained high until the end of observation. ***(Figure 13 A IL-1β)***

A


MCP-1
x-fold of control
6000
4000
2000
0
Control
2
4
7
10
14
21
Time [d]


CCL5
x-fold of control
100
80
60
40
20
0
Control
2
4
7
10
14
21
Time [d]


CCL20
x-fold of control
250
200
150
100
50
0
Control
2
4
7
10
14
21
Time [d]


TNFa
x-fold of control
400
300
200
100
0
Control
2
4
7
10
14
21
Time [d]

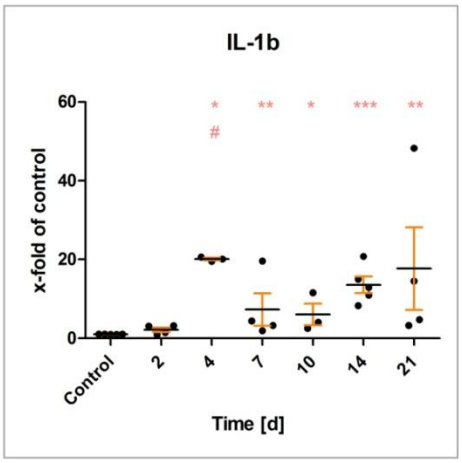

IL-1b
x-fold of control
60
40
20
0
Control
2
4
7
10
14
21
Time [d]

Figure 13 A: RT-PCR analysis of renal chemokine mRNA expression in nephritic and non-nephritic wild type mice. mRNA expression is indicated as x-fold increase when compared to non-nephritic controls. The horizontal lines indicate mean value. **A) MCP1:** expression is significantly high on all examined time points with a first maximum on day 4 and a lower second peak on day 14 (* $p<0.02$, ** $p<0.01$ when compared to non-nephritic controls; # $p<0.03$ when compared to day 2 and 7; + $p<0.02$ when compared to day 10; ~ $p<0.04$ when compared to day 21); **CCL5:** expression significantly increased after disease induction and reached its maximum on day 4, followed by a decrease until day 10 and an increase to a second peak on day 14 (* $p<0.02$, ** $p<0.04$, *** $p<0.01$ when compared to non-nephritic controls; + $p<0.04$ when compared to day 2 and 7; # $p<0.04$ when compared to day 4 and 10); **CCL20:** expression significantly increased after disease induction and reached its maximum on day 4, followed by a decrease until day 14 and an increase to a second peak on day 21 (* $p<0.02$, ** $p<0.05$, *** $p<0.01$ when compared with non-nephritic controls; # $p<0.05$ when compared to day 2, 14 and 21; + $p<0.04$ when compared to day 14); **TNFα**: expression of TNFα is significantly high on all time points with a maximum on day 14 (* $p<0.02$, ** $p<0.04$, *** $p<0.01$ when compared to non-nephritic controls; # $p<0.04$ when compared to day 10; + $p<0.01$ when compared to day 21); **IL1β:** expression is significantly high on all time points with a maximum on day 4 (* $p<0.04$, ** $p<0.02$, *** $p<0.01$ when compared with non-nephritic controls; # $p<0.05$ when compared to day 2).

3.5.2. Cytokine expression in Nfkb1$^{-/-}$-mice

3.5.2.1. Expression of MCP-1/CCL2

The expression of MCP-1/CCL2 significantly increased after intraperitoneal injection of the NTN-serum to its maximum on day 4 (*7698.0 ± 699.9 –fold*), but decreased thereafter (*day 7: 2038 ± 102.5 –fold; day 10: 2406.0 ± 494.1 –fold; day 14: 431.7 ± 93.6 –fold; day 21: 145.5 ± 92.8 –fold*). At the end of the observation period the expression remained significantly high when compared to non-nephritic controls. In summary, the expression of MCP-1/CCL2 reached a maximum and decreased thereafter but remained high until the end of the observation period. ***(Figure 14 A CCL2)***

3.5.2.2. Expression of RANTES/CCL5

The RANTES/CCL5 expression increased after intraperitoneal injection of the NTN-serum (*day 2: 6.6 ± 3.9 –fold*) and was significant from day 4 on (*186.0 ± 53.8 –fold*) to reach its maximum on day 7 (*2986.0 ± 1194.0 –fold*) and 10 (*3921.0 ± 1775.0 –fold*). After its maximum, the expression decreased but remained high when compared to non-nephritic controls (*day 14: 1067 ± 53.3 –fold; day 21: 41.4 ± 13.5 –fold*). In summary, the expression increased to its maximum and decreased thereafter but remained high until the end of the observation period. ***(Figure 14 A CCL5)***

3.5.2.3. Expression of CCL20

The expression of CCL20 significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 15.9 ± 1.5 –fold*) and reached its maximum on day 4 (*459.4 ± 83.5 –fold*), thereafter it decreased until day 21, but remained high when compared to non-nephritic controls (*day 7: 46.6 ± 3.9 –fold; day 10: 15.5 ± 2.6 –fold; day 14: 5.5 ± 0.9 –fold; day 21: 52.4 ± 37.5 –fold*). In summary, the expression of CCL20 increased to a maximum on day 4 and decreased thereafter but remained high until the end of the observation period. ***(Figure 14 A CCL20)***

3.5.2.4. Expression of TNFα

The expression of TNFα significantly increased after intraperitoneal injection of the NTN-serum (*day 2: 301.1 ± 17.2 –fold; day 4: 96.0 ± 9.0 –fold*) to reach its maximum around day 7 (*915.6 ± 204.2 –fold*) and 10 (*1948.0 ± 744.2 –fold*). Afterwards the expression decreased but remained significantly high when compared to non-nephritic controls (*day 14: 295.9 ± 57.7 –fold; day 21: 63.9 ± 33.2 –fold*). In summary, the expression of TNFα significantly increased to a maximum (days 7 and 10) which was followed by a decrease. ***(Figure 14 A TNFα)***

3.5.2.5. Expression of IL-1β

The expression of IL-1β increased after intraperitoneal injection of the NTN-serum and was significantly high from day 4 on (*14.6 ± 2.0 –fold*) and remained high until the end of the observation period when compared to non-nephritic controls (*day 7: 26.2 ± 4.9 –fold; day 10: 27.9 ± 5.7 –fold; day 14: 11.8 ± 1.0 –fold; day 21: 14.4 ± 11.1 –fold*). The

discrepancy or rather the standard error of IL-1β-expression was relatively high on day 21. In summary, the expression of IL-1β significantly increased after disease induction and remained high until the end of the observation. ***(Figure 14 A IL-1β)***

A

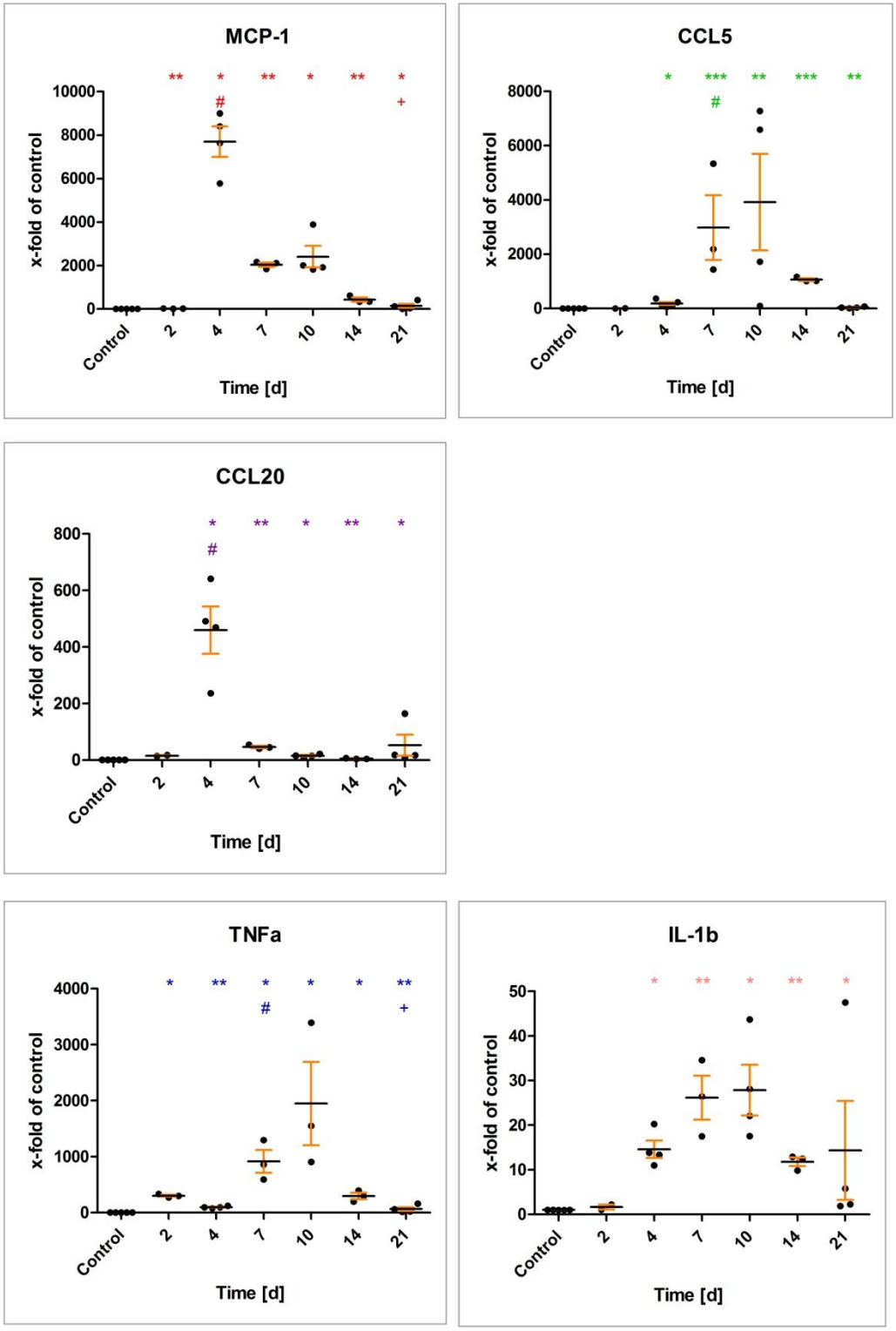

MCP-1
x-fold of control
Time [d]
Control
2
4
7
10
14
21
CCL5
CCL20
TNFa
IL-1b

Figure 14 A: RT-PCR analysis of renal chemokine mRNA expression in nephritic and non-nephritic Nfkb1$^{-/-}$ mice. mRNA expression is indicated as x-fold increase when compared to non-nephritic controls. The horizontal lines indicate mean value. **A) MCP1:** expression significantly increased to its maximum on day 4, thereafter decreased until day 21 but remained on high levels (* p<0.02, ** p<0.04 when compared to non-nephritic-controls; # p<0.03 when compared to day 10; + p<0.03 when compared to day 10); **CCL5:** expression increased after disease induction, then was significant from day 4 on to reach its maximum on day 7 until day 10, thereafter it began to decrease. On day 21, the expression remained on a significant high level compared to non-nephritic controls (* p<0.01, ** p<0.02, *** p<0.04 when compared with non-nephritic-controls; # p<0.05 when compared to day 4 and 21); **CCL20:** expression significantly increased to its maximum on day 4, thereafter the expression decreased until day 21 but remained on a high level (* p<0.02, ** p<0.04 when compared with non-nephritic controls; # p<0.03 when compared with day 10 and 21); **TNF*α*:** expression significantly increased after disease induction to its maximum on day 7 and 10, followed by a decrease until day 21. The expression was significantly high over the whole observation period (* p<0.04, ** p<0.02 when compared to non-nephritic-controls; # p<0.05 when compared to day 4; + p<0.05 when compared to day 7, 10 and 14); **IL1b**: expression significantly increased and remained high over the whole observation period (* p<0.02, ** p<0.04 when compared with non-nephritic controls)

3.5.3. Compare the cytokine expression of wild type and Nfkb1$^{-/-}$ mice

3.5.3.1. Compare the expression of MCP-1/CCL2

The expression of MCP-1/CCL2 was significantly higher in Nfkb1$^{-/-}$ mice on the days 4 (*1.8 –fold*), 7 (*70.2–fold*) and 10 (*168.4–fold*) when compared to wild type mice of the same time point. In Nfkb1$^{-/-}$-mice the expression increased to its maximum on day 4 and decreased thereafter. In contrast, the expression in wild type mice was biphasic with a first peak on day 4, a second peak on day 14 and a lower expression between the peaks. In both nephritic-mice the expression was significantly high over the whole observation period and remained high until the end of the study (*WT-mice: 149.0 ± 98.7 –fold; Nfkb1$^{-/-}$-mice: 145.5 ± 92.8 –fold*) when compared with non-nephritic controls. ***(Figure 15 A, B MCP-1)***

3.5.3.2. Compare the expression of RANTES/CCL5

The expression of RANTES/CCL5 was significantly higher in Nfkb1$^{-/-}$-mice on the days 7 (*169.0–fold*), 10 (*709.6–fold*) and 14 (*71.1–fold*) when compared to wild type mice of the same time point. In Nfkb1$^{-/-}$-mice the expression increased to its maximum (days 7 and 10) and decreased thereafter. In contrast, the expression in wild type mice was biphasic with a first peak on day 4, a second lower peak on day 14 and a reduced expression between the peaks. In both nephritic-mice the expression was significantly high over the whole observation period and remained high until the end of the study (*WT-mice: 18.6 ± 9.4 –fold; Nfkb1$^{-/-}$-mice: 41.4 ± 13.5 –fold*) when compared with non-nephritic controls. ***(Figure 15 A, B CCL5)***

3.5.3.3. Compare the expression of CCL20

The expression of CCL20 was significantly higher in Nfkb1$^{-/-}$-mice on the days 4 (*2.5–fold*), 7 (*15.2–fold*) and 14 (*2.2–fold*) when compared to wild type mice of the same time point. In Nfkb1$^{-/-}$ mice the expression increased to its maximum on day 4 and decreased thereafter. On day 21 there was an indicated increase of the expression, however, this was not significant (*p=0.0571*). In contrast, the expression in wild type mice was biphasic with a first peak on day 4, a lower second peak on day 21 and a reduced expression between the peaks. In both nephritic-mice the expression was significantly high over the whole observation period and remained high until the end of the study (*WT-mice: 31.0 ± 14.2 –fold; Nfkb1$^{-/-}$-mice: 52.4 ± 37.5 –fold*) when compared with non-nephritic controls. ***(Figure 15 A, B CCL20)***

3.5.3.4. Compare the expression of TNFα

The expression of TNFα was significantly higher in Nfkb1$^{-/-}$-mice on the days 2 (*4.0–fold*), 4 (*2.0–fold*), 7 (*8.4–fold*) and 10 (*378.2–fold*) when compared to wild type mice of the same time point. In Nfkb1$^{-/-}$ mice the expression increased to its maximum (days 7 and 10) and decreased thereafter. In contrast to this the expression in wild type mice was biphasic with a first peak around the days 2, 4 and 7 and a maximum on day 14 which was then followed by a decrease. In both the expression of nephritic-mice was significantly high over the entire observation period and remained high until the end of

the study (*WT-mice: 16.3 ± 7.3 –fold; Nfkb1$^{-/-}$-mice: 63.9 ± 33.2 –fold*) when compared with non-nephritic controls. ***(Figure 15 A, B TNFα)***

3.5.3.5. Compare the expression of IL-1β

The expression of IL-1β was significantly higher in Nfkb1$^{-/-}$-mice on day 10 (*4.6-fold*) when compared to wild type mice of the same time point. On day 7 there was an indicated higher expression in Nfkb1$^{-/-}$-mice. However, this was not significant. In both the expression increased after intraperitoneal injection of the NTN-serum and remained high until the end of the observation. ***(Figure 15 A, B IL-1β)***

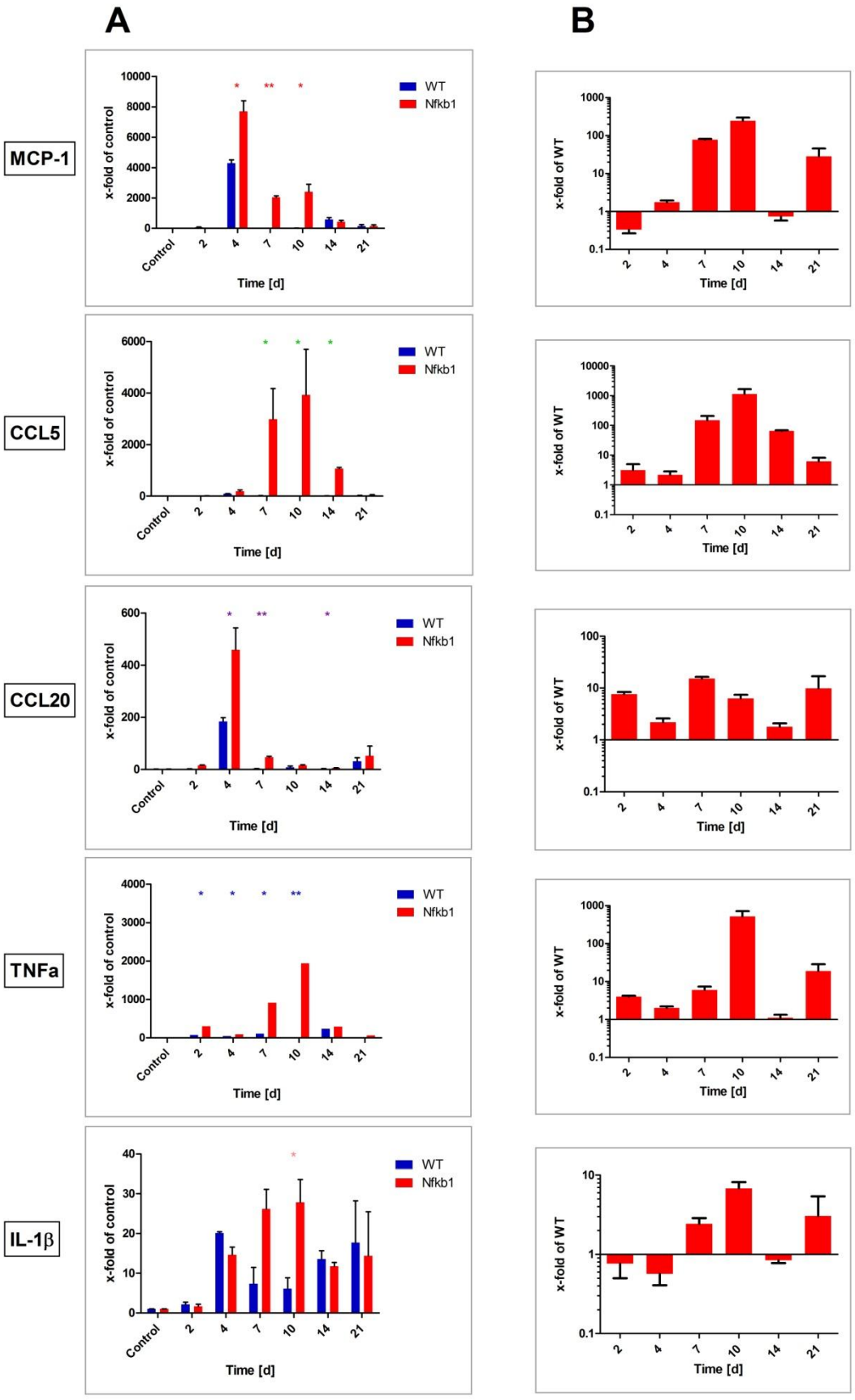

A
B
MCP-1
CCL5
CCL20
TNFa
IL-1β
x-fold of control
x-fold of WT
WT
Nfkb1
Time [d]
Control
2
4
7
10
14
21

Figure 15 A, B: Compare renal chemokine mRNA expression between wild type and Nfkb1$^{-/-}$ mice. mRNA-expression is indicated as x-fold increase when compared to non-nephritic controls [*A)*] and to wild type mice [*B)*]. The horizontal lines indicate mean value. **A and B) TNFα:** expression in Nfkb1$^{-/-}$-mice is significantly high on day 2, 4, 7 and 10 (* p<0.03, ** p<0.05 when compared to WT-mice of the same time point); **MCP-1:** expression in Nfkb1$^{-/-}$-mice is significantly high on day 4, 7 and 10 (* p<0.02, ** p<0.03 when compared to WT-mice of the same time point); **CCL5:** expression in Nfkb1$^{-/-}$-mice is significantly high on day 7, 10 and 14 (* p<0.02 when compared to WT-mice of the same time point); **CCL20:** expression in Nfkb1$^{-/-}$-mice is significantly high on day 4, 7 and 14 (* p<0.03, ** p<0.05 when compared to WT-mice of the same time point); **IL-1b:** expression in Nfkb1$^{-/-}$-mice is significantly high on day 10 (* p<0.03 when compared to WT-mice of the same time point).

3.5.4. Expression of iNOS in wild type and Nfkb1$^{-/-}$ mice

The expression of iNOS was increased on day 4 as well as on day 21 in both wild-type and Nfkb1$^{-/-}$ mice (*WT day 4:* 64.1 ± 16.1; *WT day 21:* 3.8 ± 3.3; *Nfkb1$^{-/-}$ day 4:* 1300 ± 1802; *Nfkb1$^{-/-}$ day 21:* 54.94 ± 48.05). The maximum of expression was early (day 4) in the course of the disease and decreased until the end of the observation period (day 21). (***Figure 16 A, B***)

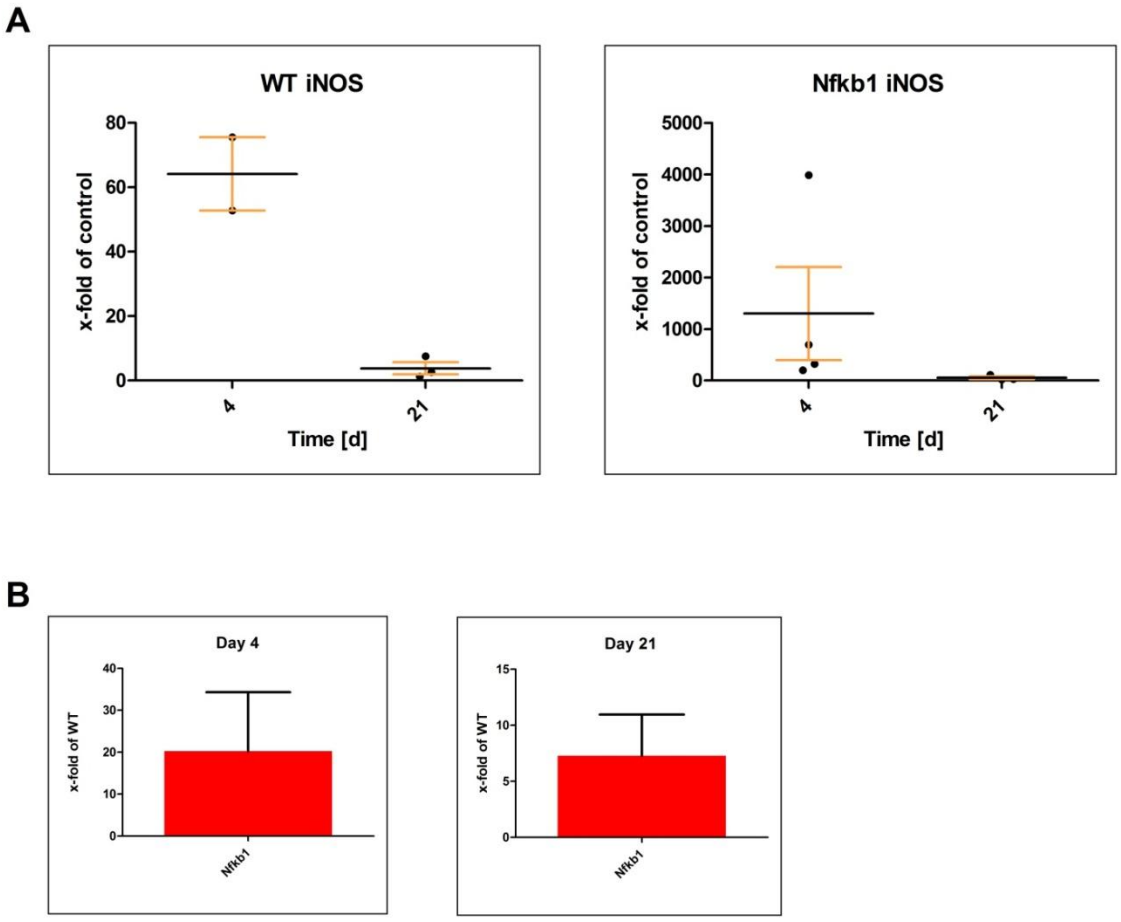


Figure 16 A, B: RT-PCR analysis of iNOS mRNA expression in nephritic and non-nephritic Nfkb1$^{-/-}$ mice. Expression of iNOS is indicated as x-fold increase when compared to non-nephritic controls. The horizontal lines indicate mean value. ***A)*** There is an indicated higher expression of iNOS early after intraperitoneal injection of the NTN-serum in both wild-type and Nfkb1$^{-/-}$ mice. ***B)*** iNOS expression is indicated higher in Nfkb1$^{-/-}$ mice when compared to wild type mice.

3.6. NF-κB activation in the NTN-WT- and -Nfkb1$^{-/-}$-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 and of its subunits p65 and p50 in nephritic wild type and nephritic Nfkb1$^{-/-}$ mice as well as in their non-nephritic controls.

3.6.1. EMSA and Supershift

We assessed the DNA-binding-activity of NF-κB and its subunits p65 and p50 in nephritic wild type and Nfkb1$^{-/-}$ mice as well as in their non-nephritic controls with electrophoretic mobility shift assays (EMSA) and Supershift experiments. Nuclear proteins were isolated from whole kidney nuclear extracts. We performed n=3 experiments for each displayed picture and show the picture with the best quality.

3.6.1.1. EMSA of wild type mice

Gel shift experiments of whole kidney nuclear extracts from nephritic wild type mice demonstrated an oscillating NF-κB-activation with a first peak on day 4 and a second peak on day 14. There was a high activation between the peaks on day 7 and 10, too. On the beginning (day 2) and the end (day 21) of the experiment the activation was comparable to non-nephritic controls. (***Figure 17 A***)

3.6.1.2. Supershift of wild type mice with anti-p65-antibody

In these nephritic wild type mice the binding activity of the NF-κB-subunit p65 was high on the time point day 2, 7, 10, 14 and 21. After a rapid increase on day 2 the activation decreased on day 4 and increased again to reach its maximum on day 14. (***Figure 17 B***)

3.6.1.3. Supershift of wild type mice with anti-p50-antibody

The NF-κB subunit p50 was active in non-nephritic controls but decreased after disease induction until day 10 (*day 2: 0.9–fold; day 4: 0.9–fold; day 7: 0.7–fold; day 10: 0.6–fold*). On day 14 the activity of p50 increased to the 1.26–fold of non-nephritic controls but decreased again to the 0.3-fold of non-nephritic controls until day 21. (***Figure 17 C***)

3.6.1.4. EMSA of Nfkb1$^{-/-}$-mice

In gel shift experiments of whole kidney nuclear extracts from nephritic and non-nephritic Nfkb1$^{-/-}$ mice there was no specific NF-κB-band. The densitometry demonstrated only the background radiation which is not a sign of NF-κB-activation. (***Figure 17 D***)

3.6.1.5. Supershift of Nfkb1$^{-/-}$ mice with anti-p65-antibody

Supershift experiments of Nfkb1$^{-/-}$ mice with anti-p65-antibody demonstrated an oscillating binding activity of the NF-κB-p65-subunit with a first peak on day 2, a second peak on day 7 and a third peak on day 14. Between the peaks, the binding activity of the NF-κB subunit p65 decreased to the background activity. A slight binding activity of the NF-κB subunit p65 could be measured in non-nephritic controls as well. (***Figure 17 E***)

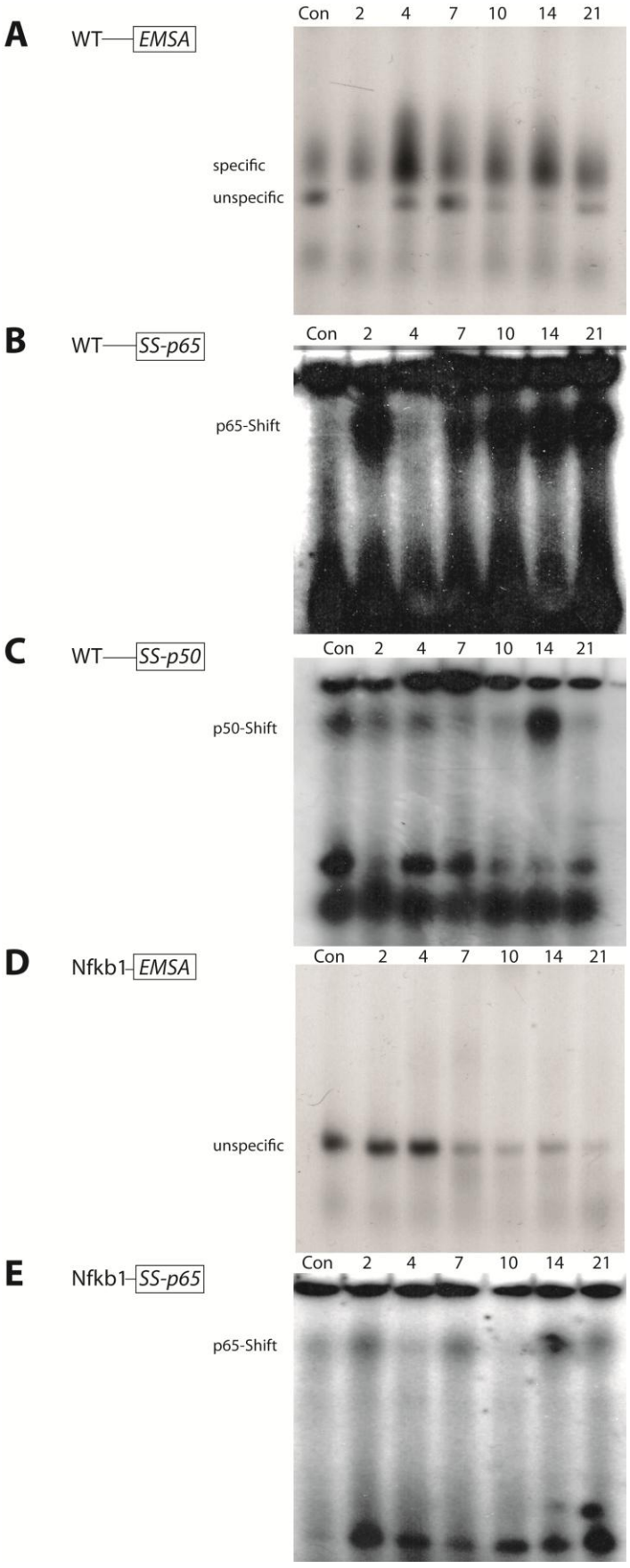

A
WT—EMSA
Con 2 4 7 10 14 21
specific
unspecific
B
WT—SS-p65
Con 2 4 7 10 14 21
p65-Shift
C
WT—SS-p50
Con 2 4 7 10 14 21
p50-Shift
D
Nfkb1–EMSA
Con 2 4 7 10 14 21
unspecific
E
Nfkb1–SS-p65
Con 2 4 7 10 14 21
p65-Shift

Figure 17 A, B, C, D, E: Time dependent NF-κB-Activation in nephritic and non-nephritic wild type and Nfkb1$^{-/-}$ mice. Experiments n=3, there are shown pictures with best quality. **A)** Gel shift experiments of WT-mice for NF-κB showed an oscillating binding activity with a first peak on day 4 and a second peak on day 14 and a high activation between the peaks on day 7 and 10. **B)** Supershift experiments of WT-mice with anti-p65-antibody showed a high activation of the NF-κB-subunit p65 on day 2, 7, 10, 14 and 21; on day 4 there is a p65-shift, too, but lower compared to the other days. **C)** Supershift experiments of WT-mice with anti-p50-antibody showed a decrease of the p50-shift after disease induction, but it increased on day 14. **D)** Gel shift experiments from Nfkb1$^{-/-}$-mice for NF-κB have no specific NF-κB-shift **E)** Supershift experiments of Nfkb1$^{-/-}$-mice with anti-p65-antibody showed an oscillating binding activity with a first peak on day 2, a second peak on day 7 and a third peak on day 14. Also, there was a binding activity in non-nephritic control mice. Between the peaks, the p65-shift decreased to lower activation as in non-nephritic-mice.

3.6.2. Western Blot

We next assessed the DNA-binding-activity of the NF-κB-subunit p65 and of the IκB-family member IκBα in nephritic wild type and nephritic Nfkb1$^{-/-}$ mice as well as in their non-nephritic controls with Western Blots.

3.6.2.1. Western Blot of wild type mice with anti-p65-antibody

Western Blot analysis of wild type mice with anti-p65-antibody showed a binding activity of the NF-κB-subunit p65 on day 2, 7, 10 and 14. (***Figure 18 A***)

3.6.2.2. Western Blot of Nfkb1$^{-/-}$ mice with anti-p65-antibody

Western Blot analysis of Nfkb1$^{-/-}$ mice with anti-p65-antibody showed a high binding activity of the NF-κB-subunit p65 in non-nephritic controls as well as on day 2 and 21 after intraperitoneal injection of the NTN-serum. On day 10 and 14 there was an indicated band in the Western Blot as a sign of low binding activity. (***Figure 18 B***)

3.6.2.3. Western Blot of wild type mice with anti-IκBα-antibody

Western Blot analysis of wild type mice with anti-IκBα-antibody showed an oscillating binding activity of IκBα. When compared to non-nephritic controls IκBα decreased after disease induction. Until day 4 and 7 IκBα decreased to the 0.7–fold of non-nephritic controls which was followed by an increase until day 10 and a second decrease on day 14. In summary, the concentration of IκBα was lower in nephritic mice when compared to the non-nephritic control group and it presented a biphasic de-activation of IκBα in wild type mice. (***Figure 18 C***)

3.6.2.4. Western Blot of Nfkb1$^{-/-}$ mice with anti-IκBα-antibody

Western Blot analysis of Nfkb1$^{-/-}$ mice with anti-IκBα-antibody showed a monophasic de-activation of IκBα on day 4. On the other examined time points IκBα is active but lower as in non-nephritic controls. (***Figure 18 D***)

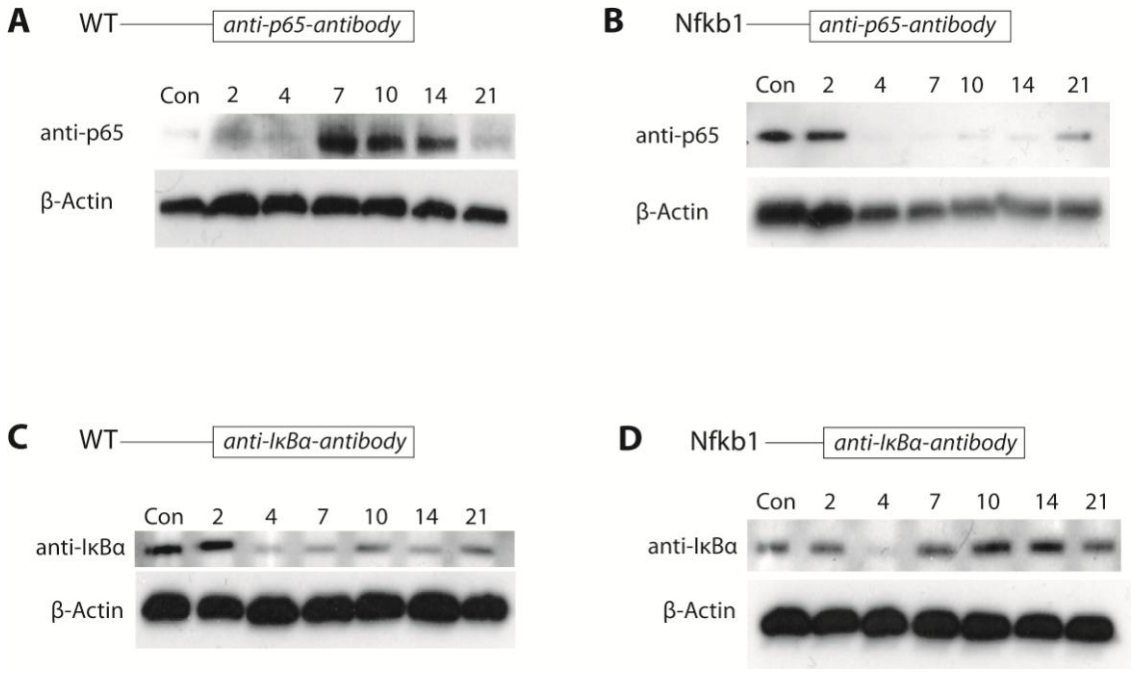


Figure 18 A, B, C, D: Western Blot analysis of glomerular nuclear protein extracts from nephritic and non-nephritic wild type and Nfkb1$^{-/-}$ mice. **A)** Analysis from WT-mice for anti-p65-antibody showed a

binding activity on day 7, 10 and 14. **B)** Analysis from Nfkb1$^{-/-}$-mice for anti-p65-antibody showed a binding activity in no-nephritic controls as well as on day 2 and 21. **C)** The IκBα-band of WT-mice showed an oscillating binding activity with activation in non-nephritic-controls as well as on day 2 and 10 after disease induction. **D)** The IκBα-band of Nfkb1$^{-/-}$mice decreased on day 4 after disease induction but was active on all other time points as well as in non-nephritic controls.

4. DISCUSSION

The aim of this study was to characterize the role of the NF-κB-subunit Nfkb1 (p50) during renal inflammation in a mice model of nephrotoxic nephritis (NTN) mediated kidney injury. We have previously shown that the nephrotoxic nephritis model induced by a sheep-anti-mouse-GBM-antiserum is an effective model of glomerulonephritis, which resembles the human rapidly progressive glomerulonephritis (RPGN).

After intraperitoneal (i.p.) injection of the NTN-serum, it was resorbed into the blood circulatory system. Clinical symptoms like fatigue, retardation and tremor within the first hours after injection have been observed more frequently in Nfkb1$^{-/-}$ mice than in wild type mice. 93.6% of Nfkb1$^{-/-}$ mice survived until day 2 but after the third day only 62.4% of Nfkb1$^{-/-}$ mice where alive; hence, more than one third of mice lacking Nfkb1 died 3 days after disease induction. In contrast, 96.2% of the wild type mice survived until the third day. According to this, the early phase after disease induction is critical for mice lacking Nfkb1. When passing the critical phase on the one hand the death rate decreased, but on the other hand renal damage, cell infiltration and cytokine expression increased. Around the 4th and 6th day another 8% of mice lacking Nfkb1 died, which determines this period as a second critical phase. After the 6th day no further animals died. Therefore, the first 6 days after intraperitoneal injection of the nephrotoxic nephritis serum reflect the early critical phase. Nevertheless, the high mortality could be affected by non-renal or rather systemic factors such as natural antibodies or involvement of other organs. Acute inflammatory responses could be affected by natural antibodies circulating before infection, which could differ in wild type and Nfkb1$^{-/-}$ mice. One of the most conserved functions of the NF-κB signaling pathway is the regulation of the immune system; indeed, NF-κB is even the primary regulator of innate immunity in insects such as Drosophila and mosquitoes (Minakhina and Steward, 2006). However, beside the lack of mice handling with the acute immune process, the rapid aggravation could be a result of allergic reaction to the serum or an overdose of the serum. Other possible explanations could be cross reactions to the pulmonary basement membrane (PBM) and accordingly pulmonary involvement with a more

severe course of the disease. The glomerular and pulmonary basement membrane is identical in their α3-chain of the collagen IV, thus, in Goodpasture's syndrome anti-GBM-antibodies attacks the glomerular and pulmonary basement membrane (Saus J et al., 1988). Therefore, a pulmonary involvement after induction of anti-GBM nephritis with the nephrotoxic nephritis serum is not unlikely. Also, the development of a systemic inflammatory response syndrome (SIRS) is a legitimate explanation. It is most likely that all these factors are important, although we didn't follow parameters for an allergic reaction or pulmonary involvement. A large population based study, the HUNT II study, has documented that impaired kidney function and urinary albumin excretion were strongly associated with cardiovascular mortality (Kasiske and Bertram, 2001; Hallan S et al., 2007). This suggests an increasing risk for cardiovascular complications in the long term for mice surviving the acute glomerulonephritis. Similarly to other studies we haven't found any histopathological differences between non-nephritic wild type and Nfkb1$^{-/-}$ mice (Sha et al. 1995). Furthermore, we haven't found differences in cytokine expression in non-nephritic mice. Therefore we can assume that the lack of Nfkb1 doesn't affect the state of renal health before disease induction but it has a critical role in the immune response after disease induction. Data from other studies support the role of Nfkb1 as a vital transcription factor for both, specific and non-specific immune responses (Sha et al. 1995). Our experiments indicate a protective role of F4/80^{+}-cells as infiltration of these cells was lower in non-nephritic Nfkb1$^{-/-}$ mice and did not increase sufficiently after disease induction. F4/80^{+}-cells are monocytes/macrophages as well as dendritic cells. It has been previously shown that dendritic cells are protective during renal inflammation (Tadagavadi RK and Reeves WB, 2010). Nevertheless, to proof this assumption other tissues needs to be evaluated since other organs could be involved in the course of the disease as mentioned above.

We characterized the effect of the nephrotoxic nephritis model on renal function, renal damage, cell infiltration, expression of chemokines/cytokines and NF-κB-activation.

A functioning glomerular basement membrane (GBM) is the basis for a proper filtration of obligatory urinary excreted substances like blood urea nitrogen (BUN) and do not allow the filtration of blood components like albumin. Hence, an increased BUN is a

sign of defective glomerular function and albumin in the urine, also referred to as albuminuria, is a sign of a defective GBM. After induction of nephrotoxic nephritis the BUN and albuminuria significantly increased when compared to non-nephritic controls. In both wild type- and Nfkb1$^{-/-}$ -mice BUN reached its maximum shortly after disease induction. At its maximum the BUN was significantly high in wild type mice when compared to Nfkb1$^{-/-}$ mice, but decreased to levels of non-nephritic controls afterwards. In contrast, the BUN of Nfkb1$^{-/-}$ mice was lower at its maximum on day 4 but it remained significantly high until the end of the observation period. The albumin/creatinine-ratio of both wild type- and Nfkb1$^{-/-}$-mice reached their maximum shortly after disease induction. After passing its maximum it declined immediately in wild type mice, whereas in Nfkb1$^{-/-}$ mice it persisted high until day 6 before it declined. In both, the albumin/creatinine-ratio remained high when compared to non-nephritic controls until the end of the observation period. In summary, the maximum of the albumin/creatinine-ratio in Nfkb1$^{-/-}$ mice was extended but acted similar to the wild type mice after its decrease. Nevertheless, BUN and albumin/creatinine-ratio are relatively weak parameters to characterize glomerular function and damage.

Light microscopy illustrates histological changes and gives a direct insight into the kidney. In the morphological examination both wild type- and Nfkb1$^{-/}$-mice demonstrated severe renal damage after disease induction (***Figure 8***). The results demonstrated varying degrees of focal glomerular and tubular damage in nephritic mice at different time points when compared to non-nephritic controls. Glomerular changes included hyper-cellularity, formation of cellular crescents and intraglomerular deposition of PAS-positive material. The tubulointerstitial compartment showed protein casts within the tubules, tubular dilation and loss of brush border, interstitial edema, vacuolization, necrosis and atrophy. While the renal damage was more severe in Nfkb1$^{-/-}$ mice at the beginning, it became more severe in wild type mice in the middle of the observation period. At the end, the severity of renal damage increased and was severe in both but there was no significant difference between nephritic wild type- and Nfkb1$^{-/-}$-mice. The serious damages in the early period were associated with a high mortality (***Figure 6***) and indicate a critical role of Nfkb1 during the early phase of inflammation as mentioned above. Moreover, if Nfkb1$^{-/-}$ mice pass the early phase of inflammation

they have a good chance of survival and remission since mortality and histopathological changes decrease. The high difference between wild type and Nfkb1$^{-/-}$ mice concerning renal damage and mortality during the early period suggests a lack or deficiency of accurate immune response and demonstrates the critical role of Nfkb1 during the early immune response in inflammation.

The results for the quantification of renal cell infiltration demonstrated varying degrees of cell infiltration in nephritic mice when compared to non-nephritic controls (***Figure 9 - 12***). In both wild type- and Nfkb1$^{-/-}$-mice monocyte/macrophage (F4/80^{+}-cells) and lymphocyte (CD3^{+}-cells) infiltration increased to a maximum around day 7 after intraperitoneal injection of nephrotoxic nephritis serum and were significantly high until the end of the observation period. Wada et al have shown that macrophages in the absence of select T-cell populations are incapable of promoting kidney disease (Wada T et al., 2001). Hence, the simultaneous increase of macrophage and T-cell infiltration into the kidneys show the induction of kidney disease and the ongoing inflammation process. The identical kinetics implies the mutual necessity of macrophages and T-cells for induction of inflammation as well as resolution of inflammation or rather establishing a homeostatic situation. In contrast to Nfkb1$^{-/-}$-mice, in wild type-mice both monocyte/macrophage and lymphocyte infiltration decreased on the second day after disease induction. Interestingly, non-nephritic Nfkb1$^{-/-}$ mice presented a significantly lower monocyte/macrophage infiltration when compared to non-nephritic wild type mice. It has been previously shown that Nfkb1 makes essential contributions to neutrophil accumulation elicited by LPS in the lungs and that this Nfkb1-dependent pathway for neutrophil accumulation can be overcome by bacterial products other than LPS (Mizgerd et al, 2004). In contrast, our results presented a similar trend of cell infiltration after disease induction in both wild type- and Nfkb1$^{-/-}$-mice (***Figure 11***). In consolidation to the study of Mizgerd et al we assume that the Nfkb1-dependent pathway for neutrophil accumulation can be overcome by bacterial products but not antibodies like anti-GBM-antibody. These findings are suggestive for diverging functions of Nfkb1 in different tissues. The critical role of Nfkb1 in inflammation has already been shown in other organs. The absence of Nfkb1 is associated with more profound hepatic inflammation and development of a more severe fibrogenic response

(Oakley et al. 2005). When challenged by pulmonary infection with Escherichia coli, Nfkb1$^{-/-}$ mice are more susceptible to severe lung inflammation and damage and have in addition increased gene expression and neutrophil recruitment suggesting that Nfkb1 normally limits these innate immune responses (Mizgerd et al. 2003). Peritoneal infection with Streptococcus pneumonia leads to an increased mortality of Nfkb1$^{-/-}$ mice when compared to wild type mice (Sha et al., 1995). During gastrointestinal Helicobacter hepaticus infection Nfkb1$^{-/-}$ mice developed worse lesions with elevated cytokine expression (Erdmann et al., 2001). These mentioned studies demonstrate the role of Nfkb1 during infection with organisms. In our experiments we assessed Nfkb1's role in non-infectious disease and demonstrated a similar progression of inflammation suggesting a critical role of Nfkb1 during infectious- and non-infectious-related inflammation. Taken together, these observations indicate that Nfkb1 is an important negative regulator of tissue inflammation and protective in multiple organs responding to injury and infections.

Interestingly, morphological changes occurred quickly after disease induction whereas cell infiltration rose with latency (***Figure 8 - 12***). Until day 4, morphological changes were light; the renal histology score increased not over 1.2 which indicates slight changes. Nevertheless, this slight increase in renal damage was significant and must be affected by a systemic inflammation process or cells which do not express F4/80 and CD3 since these cells increased not until day 7. A possible explanation for the early damage could be the infiltration of neutrophils. Neutrophils are the first leukocyte subset to arrive at a site of inflammation and are recruited via a multistep process. Previous studies have already shown glomerular accumulation of neutrophils early in the course of disease and recognized it as the effector cell responsible for the observed proteinuria (Mayadas TN et al., 2010). Moreover it has been shown, that neutrophil influx precedes the accumulation of macrophages, which play a key role in development of crescentic glomerulonephritis (Mayadas TN et al., 2010). In a rat model of mesangial proliferative glomerulonephritis, iNOS strongly associates with neutrophil infiltration. Our experiments have shown an increased expression of iNOS early in disease (day 4) in wild type as well as in Nfkb1$^{-/-}$ mice (***Figure 16 A, B***). Deductive iNOS expression or rather neutrophil influx could be the reason for the early renal damage. Nevertheless, the role of iNOS in glomerulonephritis has not been concluding evaluated.

Studies of the past few years have highlighted the role of chemokines and their receptors as main regulators of leukocyte recruitment in renal inflammation (Panzer et al., 2006; Turner et al., 2007). There have been some important distinctions in the cytokine expression and its interaction with NF-κB-activation regarding nephritic mice.

In gel shift experiments we could demonstrate an oscillation of NF-κB activation in wild type mice (***Figure 17 A***). Subsequent Supershift analysis demonstrated the presence of the NF-κB-subunit p65 on day 2, 7, 10, 14 and 21 after disease induction (***Figure 17 B***). Corresponding Western Blot analysis demonstrated a similar presence of the NF-κB-subunit p65 (***Figure 17 A***). The NF-κB-subunit p50 was activated in non-nephritic controls and decreased after disease induction but was still detectable (***Figure 17 C***). The NF-κB subunits p65 and p50 or rather p65/p50-heterodimers were activated on the 2nd day after disease induction which was accompanied by a high expression of the pro-inflammatory cytokines TNFα, MCP-1, RANTES and CCL20. On day 4 the solely presence of the NF-κB subunit p50 or rather p50/p50-homodimers was linked to a resolved expression of TNFα. On day 7 and 10 there was a high activation of NF-κB subunit p65 and a relatively low activation of p50, which was associated with a steady expression of pro-inflammatory cytokines. On day 14 high activation of NF-κB subunit p65 as well as p50 or rather p65/p50-heterodimers was associated with a maximal expression of TNFα as well as of MCP-1 and a high expression of RANTES, CCL20 and IL-1β. In summary, the presence of p65/p50-heterodimers leads to the transactivation of pro-inflammatory genes, which results in excessive expression of the pro-inflammatory cytokines TNFα, MCP-1 (CCL2), RANTES (CCL5) and CCL20 (***Figure 13 - 15***). The increased expression of MCP-1 leads to the infiltration of F4/80^{+}-cells such as monocytes and macrophages. These cells as well as T-cells lead to the observed renal damage (***Figure 8***). The activation of p50 on day 4 indicates the initiation of resolution as cytokine expression decreased. Nevertheless, the concurrent activation of p65/p50-heterodimers and p50/p50-homodimers on day 7 and 10 and the associated steady cytokine expression, reflect the dynamic inflammatory process and a dynamic NF-κB subunit composition. Thus, different subunit compositions of NF-κB can act simultaneously. Different concentrations of the acting NF-κB-dimers are

causing the resulting pro- or anti-inflammatory gene transcription, and thus the cytokine expression which displays the state of inflammation.

The increased expression of pro-inflammatory cytokines and chemokines such as TNFα, MCP-1, RANTES, CCL20 and IL-1β in the course of glomerulonephritis has already been observed in recent studies (Baer et al., 1998; Stahl et al., 1993; Tang et al., 1996; Wenzel et al., 1997; Lloyd et al., 1997; Xie et al., 2010;). In wild type mice as well as in Nfkb1$^{-/-}$ mice the examined chemokines were significantly increased over the entire observation period when compared to non-nephritic controls (***Figure 13 and 14***). In wild type mice the expression of the chemokines TNFα, MCP-1, RANTES and CCL20 presented a *biphasic trend* with a *first peak* between day 2 (TNFα) and 4 (MCP-1, RANTES, CCL20) and a *second peak* between day 14 (TNFα, MCP-1, RANTES) and 21 (CCL20) (***Figure 13***). The expression of MCP-1, RANTES, CCL20 and IL-1β exceeded to their maximums on time points next to that of p65/p50-heterodimer activation (day 4 and 21), whereas the expression of TNFα reaches its maximum on the same time points as NF-κB-activation. The absence of NF-κB-subunit p65 and at the same time the presence of NF-κB-subunit p50 or rather the presence of p50/p50-homodimers on day 4 was consistent with a nearly resolved expression of pro-inflammatory TNFα; hence, a situation of limiting or better controlling the acute inflammatory process occurs.

As expected, gel shift experiments of Nfkb1$^{-/-}$ mice didn't show specific NF-κB-bands, since the used NF-κB-antibody binds specifically p65/p50-heterodimers (***Figure 17 D***). Subsequent Supershift analysis demonstrated the presence of the NF-κB-subunit p65 on day 2, 7, 14 and 21 after disease induction (***Figure 17 E***). Corresponding Western Blot analysis demonstrated a similar presence of the NF-κB-subunit p65 (***Figure 18 B***). This activation of the NF-κB subunit p65 in Nfkb1$^{-/-}$-mice is similar to the activation in wild type mice except of one time point. The presence of NF-κB-subunits p65 is consistent with a high renal damage (***Figure 8***) and excessive expression of the pro-inflammatory cytokines TNFα, MCP-1, RANTES and CCL20 (***Figure 13 and 14***). The absolute expression of the examined cytokines exceeded in Nfkb1$^{-/-}$ mice after induction of disease. In contrast to the expression kinetics of wild type mice, in Nfkb1$^{-/-}$ mice the expression of all examined chemokines presented a *monophasic trend* with a maximum on day 4 (MCP-1, CCL20, IL-1β) or 7 (TNFα, RANTES) and a high expression until

the end of the observation period (***Figure 14***). In Nfkb1$^{-/-}$ mice the expression of TNFα was higher from day 2 to day 10, the expression of MCP-1 was higher from day 4 to day 10, the expression of RANTES was higher from day 7 to day 14, the expression of CCL20 was higher from day 4 to day 14 and the expression of IL-1β was tended higher from day 7 to day 10 when comparing to wild type mice. Interestingly the expression of IL-1β presented a *monophasic trend* in wild type as well as in Nfkb1$^{-/-}$ mice. Mizgerd et al have shown similar trends of IL-1β in the bronchoalveolar fluid (BALF) of Nfkb1$^{-/-}$ mice indicating that there is no significant effect of p50-deficiency for the expression of IL-1β (Mizgerd et al., 2004).

The increase of the pro-inflammatory cytokine MCP-1 was associated with an increased monocyte/macrophage influx (F4/80^{+}-cell infiltration) into the kidneys of nephritic mice. MCP-1 is known as a mediator of monocytes/macrophages and it is involved in the progression of fibrosis in the NTN-model (Stahl et al., 1993; Tang et al., 1996; Wenzel et al., 1997; Lloyd et al., 1997). From day 4 to day 10, F4/80^{+}-cell infiltration increased and renal function became worse (***Figure 7 and 10***). This infiltration equalized to wild type mice until the end of the observation period but was still significantly increased when compared to non-nephritic controls. Hence, the higher expression of MCP-1 in nephritic Nfkb1$^{-/-}$ mice from day 4 to day 10 leads to monocyte/macrophage infiltration into the kidneys and induces an increased progression to fibrosis due to worse renal function. Lloyd et al. have demonstrated that both MCP-1 and RANTES play an important role in the initial development of inflammation during crescentic nephritis (Lloyd et al., 1997). The similar renal damage in both makes clear that the lack of Nfkb1 leads to higher expression of MCP-1 from day 4 to day 10 and of RANTES from day 7 to day 14. This leads to a worse renal function and severe damage and could play an important role for the high mortality during this phase through a heavy and uncoordinated development of inflammation. Therefore, Nfkb1 seems to control the expression of MCP-1 and RANTES and thus monocyte/macrophage infiltration as well as crescentic formation during the acute inflammatory phase.

Interestingly, except of TNFα none of the examined cytokines is so much higher expressed in Nfkb1$^{-/-}$ mice when compared to wild type mice (***Figure 14 and 15***). Previously it has been shown that overexpression of Nfkb1 blocked LPS-induced transcription from a TNF-α promoter reporter construct (Baer et al., 1998; Kastenbauer

and Ziegler-Heitbrock, 1999) and p50/p50-homodimers which lack transactivation domains have been shown to repress expression of NF-κB target genes including TNFα. These indicate that Nfkb1 is an inhibitor of the TNF-α gene (Kastenbauer and Ziegler-Heitbrock, 1999). A homo-dimeric complex of p50 was also found in resting T-lymphocytes and reduced p50 expression was observed after T-cell activation. Furthermore overexpression of p50 was shown to repress IL-2 expression (Kang et al., 1992). Accordingly it appears obvious that the lack of Nfkb1 was the reason for the excessive expression of TNFα in Nfkb1$^{-/-}$ mice in our experiments and subsequent the reason for the overexpression of pro-inflammatory cytokines. Acute exposure to high doses of TNFα results in shock and tissue injury (Tracey K and Cerami A, 1994) so that the high mortality in Nfkb1$^{-/-}$ mice could be affected by the overexpression of TNFα. Hence, we think that Nfkb1 is essential to limit inflammation and tissue damage by decreasing and coordinating the cytokine expression especially of TNFα.

Summing up, the cytokine expression in Nfkb1$^{-/-}$ mice was significantly high when compared to wild type mice. The cytokine expression of Nfkb1$^{-/-}$ mice exceeded when wild type mice passed their first maximum. It then, equalized when wild type mice reached their second maximum (***Figure 15***). Therefore the absence of Nfkb1 leads to excessive pro-inflammatory cytokine expression and disastrous renal damage. We assume that the *monophasic trend* of cytokine expression in Nfkb1$^{-/-}$ mice is a result of disability to decrease the cytokine expression after reaching the maximum, or it is a result of a severe damage which induces an ongoing cytokine expression. The ability of wild type mice to limit cytokine expression after day 4 was associated with an activation of NF-κB subunit p50 in corresponding Supershift analysis. This sustains the role of Nfkb1 or rather p50/p50-homodimers to induce the resolution of inflammation.

NF-κB activation and its subunit composition demonstrated a dynamic process as we could measure in Supershift and Western Blot analysis. Although mortality and pro-inflammatory cytokine expression was significantly high in Nfkb1$^{-/-}$ mice, especially in the early phase, obviously it could be formed heterodimers which had the ability to limit the inflammation as renal function, renal damage, cell infiltration and cytokine expression equalized to wild type mice until the end of the observation period. Due to the lack of Nfkb1 necessarily other members of the NF-κB-family formed heterodimers

with p65. Functional compensation within the NFκB family has been demonstrated in various NF-κB null cell lines; thus p52 compensated for p50 in Nfkb1$^{-/-}$ mice (Hoffmann A, 2003). However, in Nfkb1$^{-/-}$ fibroblasts p52 formed heterodimers with p65; whereas in Nfkb1$^{-/-}$ mammary endothelial cells RelB complexed with p52. These data suggest that the compensation between NF-κB proteins is a common but cell type-specific event (Hoffmann A, 2003). Concerning resolution of inflammation, applicable NF-κB family members need to lack the transactivation (TA) domain to repress the transactivation of pro-inflammatory genes. Besides Nfkb1 (p50), only Nfkb2 (p52) or rather p52/p52-homodimers complies this requirement. Mizgerd et al. suggested that no homodimer or heterodimer of p65 or p50 is absolutely essential for the early gene induction by gram-negative bacterial stimuli in the lungs but transcription factors other than NF-κB may induce this gene expression (Mizgerd et al., 2003). Different combinations of NF-κB subunits have distinct roles in the immune response. Targeted disruption of NF-κB subunit p50 leads to multifocal defects in immune responses (Sha et al., 1995). This is consistent with the high mortality of mice lacking Nfkb1 by failing to show an adequate immune response (***Figure 6***).

In view of our results we assume that Nfkb1 has a critical role or a higher ability to limit tissue damage and cytokine expression, as well as having the ability of their immune system to show an adequate response to the acute process in the early phase of inflammation, when compared to other NF-κB-heterodimers. Other transcription factors like IFN may induce or influence the gene expression instead or rather together with NF-κB. It is likely that limiting the inflammation is based on coordinating the inflammation process as well as actively inducing the resolution of inflammation by Nfkb1. It has been previously shown that NF-κB has a role in both induction and resolution of inflammation (Lawrence T et al., 2001; Panzer et al., 2009). We suggest that resolution of inflammation is an active process and Nfkb1 has a critical role during this process, since Nfkb1 or rather p50/p50-homodimers are activated during acute inflammation in wild type mice which is associated with a better renal function, a steady renal damage, less cell infiltration and lower cytokine expression. This assumption is supported by others who have also shown that distinct biochemical pathways are turned on when inflammation is induced and lead to the production of eicosanoids and other

lipid-mediators that have pro-resolving or reparatory functions (Buckley CD et al., 2012). Moreover, anti-inflammatory agents such as steroids, nitric oxide, adenosine, IL-10 and T_{reg}-cells are activated during the inflammation process and thus inhibit actively the immune response (Buckley CD et al. 2012). As the lack of Nfkb1 is not lethal and does not affect healthy mice as we have shown, it is likely that other subunit compositions can substitute Nfkb1 even if they are less competent in regulating the inflammation process.

The key to successful therapy and long-term prognosis is early diagnosis. In several examinations, standard treatment of anti-GBM-antibody disease is a combination of plasmapheresis, prednisolone (glucocorticoids) and cyclophosphamide or alternatively mycophenolate mofetil (Lahmer T and Heemann U, 2012). Progression to end-stage renal failure requires either dialysis or renal transplantation. NF-κB is an optimal target of anti-inflammatory and immunosuppressant therapies. One of the beneficial effects of glucocorticoids may derive from their ability to inhibit signaling through the NF-κB and JNK signaling pathways, resulting in inhibition of inflammatory cytokine production (Kerr et al., 2007).
Nevertheless, current therapeutic options are quite rare and unspecific. Many of the used therapies have the potential to induce serious complications including sepsis, hemorrhage, sterility and malignancy (Kerr et al., 2007).

Due to its disastrous effects and acting as key communication systems between cells of the immune system, TNF-α and its receptors, TNFR1 and TNFR2, play a crucial role in the pathogenesis of renal disease. Hence, it needs to be evaluated whether blocking of TNF-α is a useful therapeutic target. Anti-TNF-α-therapy interferes with the pathogenic processes at multiple levels inhibiting inflammatory cell recruitment, inducing cell death in inflammatory cells and lowering the cytokine production (van Hauwermeiren F et al., 2011). *Etanercept*, a soluble p75 TNF receptor, and *Infliximab*, a humanized anti-TNF chimeric antibody, has been used with limited success in vasculitis, since there have been reports of high relapse-rate especially for *Etanercept* (Kerr et al., 2007). However, due to the pleiotropic functions of TNF it is not surprising that long-term use of TNF-α-blockers can cause serious side effects, such as increased incidence of

infection, induction of cancer and induction of other auto-immune diseases such as vasculitis. More specific targeting of pathological TNF is possible at the ligand and at the receptor level. Autoimmune disease models in mice have illustrated that TNFR2 might be the preferred target as its expression is induced during renal inflammation and TNFR2-deficiency in mice confers significant protection from renal tissue (Speeckaert MM et al., 2012). TNFR2-dependent diseases such as glomerulonephritis might profit from more specific TNFR2-targeted therapies.
On molecular studies regarding the NF-κB pathway it is important to understand the pathogenesis of inflammatory and autoimmune diseases, and to identify new drugs that inhibit NF-κB activation.

Regarding anti glomerular basement membrane glomerulonephritis (anti-GBM-GN) or rather rapidly progressive glomerulonephritis in humans, the extension of this disease is wide-ranging from very harmless to very severe. Beside age, sex, pre-existing conditions and genetics in general a main factor might be polymorphisms of NF-κB and its subunits. Polymorphisms with a less functioning NF-κB subunit Nfkb1 (p50) may be the reason for a more severe course. Although complete loss of Nfkb1 is rarely seen clinically, reduced Nfkb1 expression is not uncommon and has been associated with an Nfkb1 promoter polymorphism. A 4-base pair deletion in the promoter region of Nfkb1 (-94 insertion/deletion ATTG, rs28362491) whose presence results in loss of binding to nuclear proteins and reduced promoter activity of Nfkb1 promoter-luciferase, was discovered in 2004 (Karban AS et al., 2004). A study of Gao et al indicates that this polymorphism plays a pivotal role for the initiation and progression of systemic lupus erythematodes (SLE) in the Chinese population; hence, the $ATTG_1/ATTG_2$ genotype but not the $ATTG_1$ allele was associated with a significantly decreased risk of SLE (Gao M et al., 2012). Studies suggest an association between the Nfkb1 -94ins/delATTG polymorphism and certain autoimmune and inflammatory diseases in Asian populations but not in Caucasian populations (Zou et al., 2011). Also, mutations of other NF-κB subunits resulting in preferred binding to p65 and subsequent composition of different heterodimers could be possible, especially during the early period of inflammation. Udalova et al have shown that a single base change at nucleotide -863 in the human TNF-promoter region (TNF-863A allele) leads to a specifically inhibition of p50/p50-

homodimer binding and this results in a reduction of lipopolysaccharide-inducible gene expression in primary human monocytes (Udalova et al., 2000).

It has to be investigated whether patients with glomerulonephritis undergo more often inflammatory diseases or if the course of disease is more severe than the generality. This would be an evidence for a defective immune system and maybe even a defective NF-κB-system. Moreover, it is interesting whether these patients have a higher risk to develop a chronic kidney disease. Recognition of polymorphisms or mutations would have epidemiologic and prognostic meaning and may be a justification for a more radical treatment; since the therapy needs to be started quickly but recognizing a polymorphism could take a relatively long time and would not change the initial therapeutic strategy. Nevertheless, knowing the polymorphism or the mutation in the patient, the disease can be treated with drugs which interferers more specifically in the NF-κB-pathway. Current options are quite rare and non-specific, resulting in unsatisfying outcome and hazardous side effects.

5. CONCLUSION

Rapid progressive glomerulonephritis is one of the main causes for end-stage renal disease. The fundamental characteristic and morphological hallmark of glomerular inflammatory lesions are infiltrating macrophages and T-cells into the kidney. These cells produce cytokines and attract other immune cells by the secretion of chemokines which promotes glomerular tissue injury and damage. Therapeutic strategies so far are toxic and unspecific and therefore specific therapies with fewer side effects are needed.

In renal tissue injury activation of the transcription factor NF-κB plays a pivotal role in the induction of proinflammatory gene expression, which is involved in the development of progressive renal inflammatory disease. The function of NF-κB during the switch from the inflammatory process toward resolution, however, is largely unknown. NF-κB acts as a pro-inflammatory as well as an anti-inflammatory transcription factor depending on its subunit composition. NF-κB subunit Nfkb1 (p50) or rather p50/p50-homodimers play a decisive role during the switch to resolution of inflammation. This thesis therefore analyzes the time-dependent activation and function of NF-κB in wild type as well as in $Nfkb1^{-/-}$ mice in a model of experimental nephrotoxic nephritis (NTN).

After induction of nephrotoxic serum nephritis both strains of mice developed a nephritic syndrome and inflammatory renal disease with reduced renal function and severe renal damage during the 21 days observation period. Renal damage was accompanied by an infiltration of $CD3^{+}$-T- and $F4/80^{+}$-cells. When compared to wild type mice, mice lacking Nfkb1 had a much higher pro-inflammatory cytokine and chemokine expression and a higher mortality rate. Therefore, Nfkb1 is essential to limit inflammation and tissue damage by reducing cytokine and chemokine expression after NTN induction. As activation of NF-κB was oscillatory after induction of disease Nfkb1 and p50/p50-homodimers in wild type mice was associated with better renal function, reduced renal damage, less infiltrating inflammatory cells and reduced cytokine expression.

Therefore, Nfkb1 plays a critical role to limit tissue damage and cytokine expression after induction of an acute inflammatory glomerular disease. It seems obvious that resolution of inflammation is an active process and Nfkb1 has a critical role during this process.

6. FIGURE

7. TABLE

8. LITERATURE

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9. LIST OF ABBREVIATIONS

A

AICD	activation-induced cell death
ANA	anti-nuclear antibody
ANCA	anti-neutrophil cytoplasmic antibody
Ank	ankyrin

B

Bcl-3	B-cell lymphoma 3-encoded protein
BSA	Bovine Serum Albumin

C

CCL	Chemokine (C-C motif) ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
c-IAP	cellular inhibitor of apoptosis
COX	cyclooxygenase
cpm	counts per minute
CT	cycle threshold
CVD	cardiovascular disease
cyPG	cyclopentenone prostaglandine

D

d	day(s)
DC	dendritic cell
DEPC	diethyl dicarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DTT	Dithiothreitol

E

EDTA	Ethylendiamintetraacetat
EGTA	Ethylenglycoltetraacetat
eGFR	estimated glomerular filtration rate
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay

F

FoxP3	forkhead/winked-helixbox P3
FW	Forward

G

°C	grade Celsius
g	Gramm

GBM	glomerular basement membrane
GFR	glomerular filtration rate
GN	glomerulonephritis

H

H_2O	water
HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLH	helix loop helix
HRP	Horseradish Peroxidase
h	hours

I

ICAM	intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible nitric oxide synthase
IκB	Inhibitor of NF-κB
IKK	IκB-kinase
IP	Interferon-gamma induced protein
i.p.	intra-peritoneal

K

KDOQI	Kidney Disease Outcome Quality Initiative
KLF	Krueppel-like factor

L

L / l	liter
LPS	lipopolysaccharide
Lys	lysine
LZ	leucine zipper

M

MC	metabolic cage
MCP	monocyte chemo-attractant protein
MDRD	Modification of Diet in Renal Disease
mg	milligram (Gramm x 10^{-3})
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
min	minute(s)
Mio.	million
ml	milliliter (Liter x 10^{-3})
MMLV	Moloney murine leukemia virus

MMP	matrix metalloproteinase
mol	Mol
mRNA	messenger ribonucleic acid

N

NaCl	sodium chloride
NF-κB	nuclear factor-κ-light-chain-enhancer of activated B cells
ng	nanogramm (Gramm x 10^{-9})
NLS	nuclear localization sequence
NO	nitric oxide
NP-40	nonyl-phenoxypolyethoxylethanol
NTN	nephrotoxic nephritis

P

P	phosphor
PAMP	pathogen-associated molecular patterns
PAS	Periodic Acid Schiff stain
PBM	pulmonary basement membrane
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGE	pro-inflammatory mediator prostaglandin E

PNK	polynucleotide kinase
PS	phophatidylserine

R

RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RHR	Rel homology region
RNA	ribonucleic acid
RNAse	ribonuclease
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPGN	rapid progressive glomerulonephritis
rpm	rounds per minute
RV	Reverse

S

s	second(s)
SEM	standard error of the mean
Ser	serine
SOCS	suppressors of cytokine signaling

T

TA	transcriptional activation
TAE	Tris-Acetate-EDTA

TBE	Tris-Borat-EDTA
TBM	tubular basement membrane
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFRSF	TNF receptor super family
TNFSF	TNF super family
TRAF	TNF receptor-associated factor
TRAIL	TNF related apoptosis-induced ligand
T_{reg}	T regulatory cell ($CD4^+CD25^+Foxp3^+$)
Tris	tris-(hydroxymethyl)-aminomethane
TWEAK	TNF-like weak inducer of apoptosis

U

µg	microgram (Gramm x 10^{-6})

V

VCAM	vascular cell adhesion molecule

W

WT	wild type

9. APPENDIX

CURRICULUM VITAE

MARTIN NAUROZ

GEBOREN AM 4. MAI 1987 IN HAMBURG

VERHEIRATET

SCHULE, WEHRDIENST, STUDIUM

Gesamtschule Blankenese in Hamburg	
Abschluss: Abitur [Note: 1.6]	1997 – 2006
Zivildienst im Asklepios Westklinikum Hamburg	2006 – 2007
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FAMULATUREN

Allgemeinchirurgie	Asklepios Klinik St. Georg	08/10 – 09/10
Kardiologie	Universitätsklinikum Hamburg-Eppendorf	06/11 – 07/11
Allgemeinmedizin	Praxis für Allgemeinmedizin	01/11 – 02/11
		07/11 – 08/11

PRAKTISCHES JAHR

Innere Medizin	Universitätsklinikum Hamburg-Eppendorf	08/12 – 12/12
Chirurgie	Universitätsklinikum Hamburg-Eppendorf	12/12 – 03/13
Pädiatrie	Universitätsklinikum Hamburg-Eppendorf	seit 04/2013

BERUFLICHE ERFAHRUNGEN

Wissenschaftlicher Mitarbeiter am Institut für Anatomie und experimentelle Morphologie (UKE); entscheidende Mitarbeit an der Neukonzeption des ZNS-Seminars für das 4. Semester Humanmedizin	01/09 – 10/11
Freiberuflicher chirurgischer Assistent (OP-Rufdienst)	seit 12/2010
Blutentnahmetätigkeit am Wochenende in der Schön Klinik Eilbek	seit 11/2011
Chirurgischer Assistent in der HELIOS Endo-Klinik Hamburg	11/10 – 06/12

AUSLANDSERFAHRUNGEN

3-wöchiger Schüleraustausch in die USA (St. Louis/Missouri)	2003
Nicaragua – Aufbau einer solargestützten Feldbewässerungsanlage	2004

ZUSATZINFORMATIONEN

Jugend forscht, 2. Platz (Thema: *„Entfernung toxischer Bestandteile aus ätherischen Ölen“*)	2006

FREMDSPRACHENKENNTNISSE

Englisch (fließend)

Französisch (Grundlagen)

Dari (Muttersprache)

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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 mit dem Titel

Role for NF-κB1 (p50) in an experimental model of rapid progressive glomerulonephritis

bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Hamburg, den 12.06.2013

Martin Nauroz